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Ralf Bartenschlager *Editor*

Hepatitis C Virus: From Molecular Virology to Antiviral Therapy

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Hepatitis C Virus: From Molecular Virology to Antiviral Therapy

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Preface

Hepatitis C has long been regarded as the ‘silent epidemic’. Although there is some truth in this statement that reflects the mostly asymptomatic course of infection and the low perception of the disease in the general public, the situation has changed profoundly in the last couple of years. On one hand this is due to the increasing awareness of chronic hepatitis C, especially as improved therapy options become available and are announced in press releases; on the other hand enormous progress has been made in our understanding of the molecular and cellular biology of the hepatitis C virus (HCV), which starts to put HCV into the position of a role model for many other, especially plus-strand RNA viruses. In these respects, the history of hepatitis C is a success story, which started in 1989 with the first molecular clone of the HCV genome and culminated in the approval of the first in class selective antiviral therapy in 2011, which increased cure rates substantially; and this is not the end of the story as further improved antiviral therapeutics will become available soon, most likely curing the majority of HCV-infected individuals.

Advances in HCV-specific therapy are tightly linked to progress made in basic research that lead to the establishment of robust and easy to handle cell culture systems and unravelled the basic principles of the HCV replication cycle. Given this tremendous advancement, the time was ripe to put together a book dealing with the multiple aspects of the viral replication cycle, the interaction of HCV with the host cell, the viral countermeasures to overcome innate and adaptive immune responses, the development of antiviral therapies and their implementation into daily clinical practice. Moreover, the recent identification of HCV-related genomes in dogs and horses raises speculations on the possible origin of HCV and its penetration into the human population.

To cover these different aspects, I had the privilege to gather a group of distinguished colleagues and leaders in the HCV field, who inspite of their busy schedule accepted the invitation to contribute book chapters. In this way it became possible to generate a comprehensive and very timely overview of the multiple facets of HCV and hepatitis C. Clearly, the focus of this book is on the molecular and cellular principles underlying the viral replication cycle. Nevertheless, the book would have been incomplete without proper description of the cell biology of virus–host interaction, which includes immune responses as well as

HCV-associated pathogenesis. Finally, most of these discoveries were inevitably linked to the development of cell culture and animal models that also provided essential tools for drug discovery.

I want to thank all the authors for their excellent contributions, their tremendous efforts, and great support. In addition, I want to thank the members in my laboratory, who provided valuable help in reading and editing individual chapters. Without this support, the book would not have become reality. On a final note, I very much hope that the reader will find this book a valuable source of information about HCV and enjoys reading the chapters as much as I did.

Ralf Bartenschlager

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The Origin of Hepatitis C Virus

Peter Simmonds

Abstract The origin of hepatitis C virus (HCV) can be conceptualised at several levels. Firstly, origins might refer to its dramatic spread throughout the Western world and developing countries throughout the twentieth century. As a blood-borne virus, this epidemic was fuelled by new parenteral transmission routes associated with medical treatments, immunisation, blood transfusion and more recently injecting drug use. At another level, however, origins might refer to the immediate sources of HCV associated with its pandemic spread, now identified as areas in Central and West sub-Saharan Africa and South and South East Asia where genetically diverse variants of HCV appear to have circulated for hundreds of years. Going back a final step to the actual source of HCV infection in these endemic areas, non-human primates have been long suspected as harbouring viruses related to HCV with potential cross-species transmission of variants corresponding to the 7 main genotypes into humans. Although there is tempting analogy between this and the clearly zoonotic origin of HIV-1 from chimpanzees in Central Africa, no published evidence to date has been obtained for infection of HCV-like viruses in either apes or Old World monkey species. Indeed, a radical rethink of both the host range and host-specificity of hepaciviruses is now required following the very recent findings of a non-primate hepacivirus (NPHV) in horses

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and potentially in dogs. Further research on a much wider range of mammals is needed to better understand the true genetic diversity of HCV-like viruses and their host ranges in the search for the ultimate origin of HCV in humans.

This review is written at a highly significant time in evolutionary studies of HCV and its origins. The discovery of closely related viruses to human HCV in horses and possibly dogs termed non-primate hepacivirus (NPHV) (Burbelo et al. 2012; Kapoor et al. 2011) throws an entirely new light on the species distribution of hepaciviruses and their host range. Despite the significance of these very recent discoveries, however, in many ways it is a particularly difficult time to write a review of HCV origins and evolutionary history. Frequent infection of horses worldwide with a virus reasonably similar to HCV breaks a key assumption of much previous research that the closest relative of HCV would be found in non-human primates. In its place we now have total uncertainty; domestic horses seem an incongruous host species and the suspicion must be that hepaciviruses are much more widely distributed in other mammals. At present, however, we simply do not know what these are. More importantly, we do not know whether viruses more similar to human HCV than NPHV exist and what species these may infect. Discovering a zoonotic source for the epidemic of HCV infection that has swept through the human population in the last century would be a truly important step in our understanding of host relationships, adaptation and pathogenicity.

This review of HCV origins therefore concentrates initially on the better characterised recent epidemic transmission of HCV in the twentieth century and the existence of suspected source areas for infection in sub-Saharan Africa and South-East Area. Some aspects of the much less well understood history of HCV before this recent spread will be speculatively discussed, as will the existence of HCV-like viruses in non-human species. Inevitably any comments made about the latter will, through further research, be revealed as either hopelessly cautious or naively overstated in a very short space of time, errors for which I apologise in advance. However, the findings cannot be simply omitted from a review with this title and I hope that they spur a greater interest in the wider group of hepaciviruses and whether the attributes of HCV that make it such an important human pathogen (persistence and hepatotropism) are shared with other members of the genus.

1 HCV Genetic Diversity and Genotype Classification

HCV is classified as the type member of the genus *Hepacivirus* within the virus family *Flaviviridae* (Fig. 1) (Bukh 1995; Simmonds et al. 1993, 2005). Although variants of HCV show substantial genetic diversity from each other, the 7 currently classified genotypes are all classified as one species under current ICTV rules notwithstanding their considerably antigenic variability and geographical differences in distribution (Simmonds et al. 2011). Until recently, the only other virus classified as a hepacivirus was GBV-B, a virus recovered from a laboratory

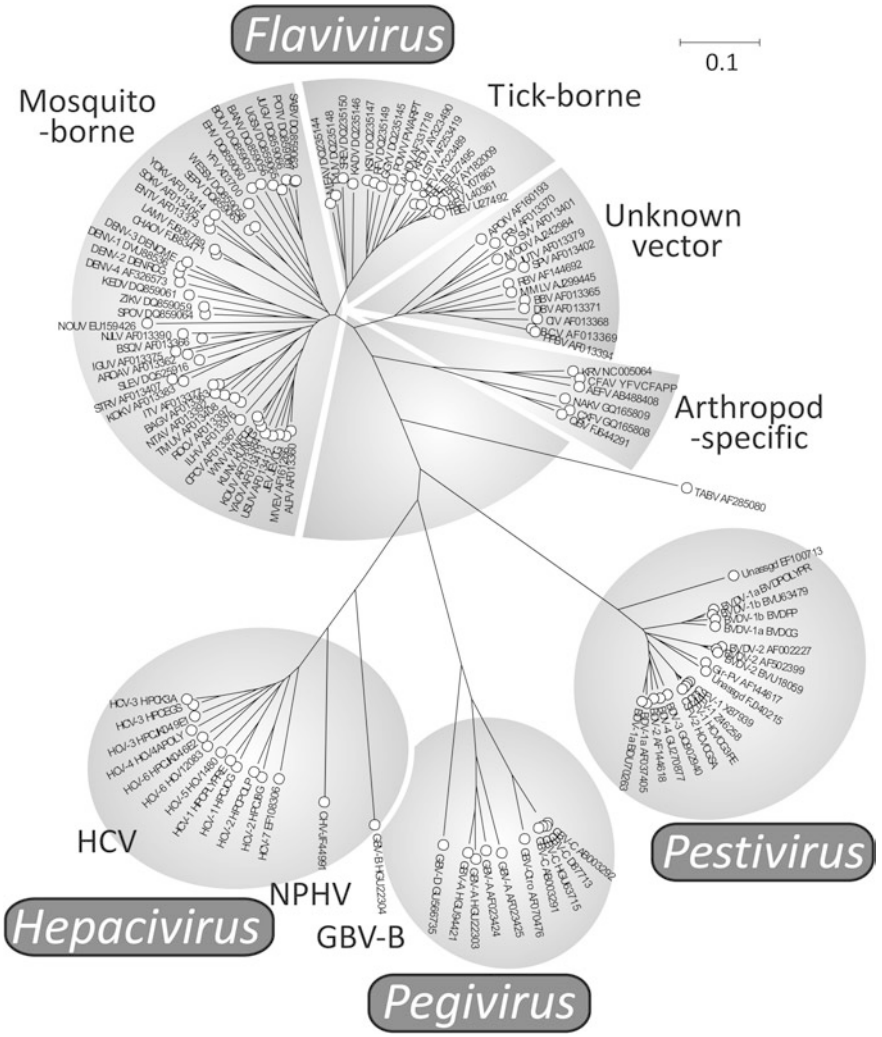

Fig. 1 Phylogenetic tree of members of the family *Flaviviridae* showing its primary division into four genera. The tree was based on comparison of conserved regions of the RNA polymerase sequences (positions 7704–8550 numbered as in the HCV-1 genome, AF011751) from representative variants within each genus and species. The unrooted phylogenetic tree was constructed by neighbour-joining of (uncorrected) amino acid p-distances. A divergence of 0.1 (10 % amino acid sequence divergence) is depicted on the scale bar. Variants variously described as GBV-A, GBV-C and hepatitis G virus have been assigned to the new proposed genus, *Pegivirus* as recently proposed (Stapleton et al. 2011)

housed tamarind [New World primate; (Simons et al. 1995; Muerhoff et al. 1995)]. Only the one isolate of GBV-B has been identified to date and its ultimate origin (primate or non-primate) remains unclear.

Members of the *Hepacivirus* genus are distinct genetically and in genome organisation from members of the recently assigned *Pegivirus* genus (Stapleton et al. 2011) (Fig. 1). This group comprises a number of non-pathogenic viruses infecting humans apes (Adams et al. 1998; Birkenmeyer et al. 1998), non-human primates (Simons et al. 1995) and more recently, bats (Epstein et al. 2010). The recent proposal to re-designate these viruses as human, simian and bat pegiviruses (Stapleton et al. 2011) was designed to dispel the confusion in their original nomenclature (terms such as GB virus C and hepatitis G virus have both been applied to pegiviruses infecting humans) and to differentiate these viruses clearly from GBV-B, a member of the *Hepacivirus* genus.

HCV genotypes are substantially divergent in sequence from each other and fall into 7 phylogenetic clades, designated as genotypes (Fig. 2). Within these, a variable number of sub-groupings are apparent. HCV variants circulating in Western countries have been designated as subtypes, of which 1a, 1b, 2a, 2b, 3a, 4a and 6a are the most frequently identified. HCV subtypes are epidemiologically distinct, with differences in risk group targeting and geographical distributions that reflect their recent epidemic spread. As examples, genotype 3a (along with 1a) typically infects injecting drug users in Northern Europe and 4a in most frequently found in the Middle East. Genotypes

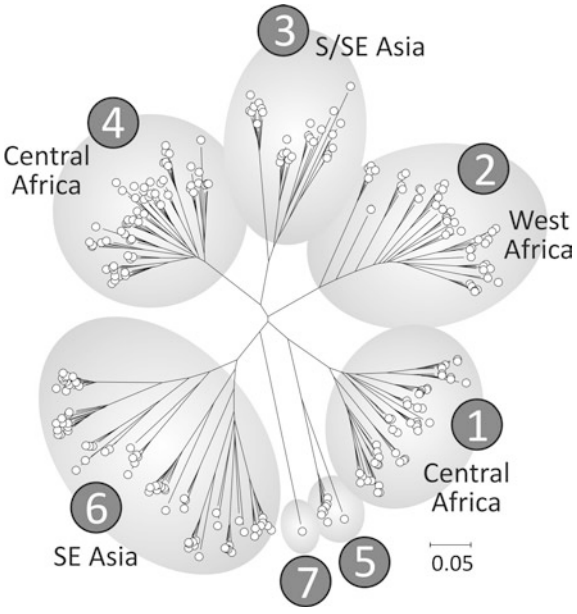

Fig. 2 Evolutionary tree of NS5B sequences of HCV genotypes 1–7 (positions 8276–8615 as numbered as in the H77 reference sequence). High diversity areas in sub-Saharan Africa and South East Asia contain a large number of variants additional to subtypes such as 1a, 1b and 3a found in Western countries, displaying an endemic pattern of diversity. The tree was constructed by neighbour-joining using maximum composite likelihood distances as implemented in the MEGA 4 program (Tamura et al. 2007). The scale bar depicts an evolutionary distance of 0.05

1b, 2a and 2b infections are in contrast most prevalent in older population groups throughout Europe and Asia and are most frequently linked to past blood transfusions.

A distinct pattern of viral diversity is observed in areas such as sub-Saharan Africa and South East Asia, where infections with individual genotype predominate over large geographical areas (such as genotype 1 in Central Africa, genotype 2 in West Africa and genotype 6 in South East Asia), within which there is substantial genetic diversity. The pattern of diversity observed within HCV is thus both the consequence of its very recent epidemic spread into new risk groups, overlaid on top of the much older "endemic" circulation of HCV in sub-Saharan Africa and South East Asia. These different ways to conceptualise "origins" of HCV are discussed in the next two sections.

2 The Recent Spread of HCV

The discovery of HCV in 1989 (Choo et al. 1989) was a remarkable achievement that heralded the use of molecular methods for virus aetiological studies refractory to previously used virus isolation methods. The very active research programme throughout the 1970s and 1980s that culminated in the discovery of HCV was primarily driven by pressing concerns of clinicians and epidemiologists who increasingly recognised chronic non-A, non-B hepatitis associated with blood transfusion and therapy with plasma-derived blood products (Prince et al. 1974; Feinstone et al. 1975; Alter et al. 1975). Since the development of effective diagnostic tests for HCV, the full scale of the spread of HCV became rapidly apparent. It is currently thought that HCV chronically infects 170 million people worldwide, 3 % of the world's population and creates a huge disease burden from chronic progressive liver disease (Pawlotsky 2003; Hoofnagle 2002; Seeff 2002). In addition to recipients of blood transfusion and medical treatment with unsterilised needles, diagnostic screening has identified the extensive spread of infection through needle-sharing drug abuse, an epidemic starting in the 1960s or earlier in Western countries and the primary route of ongoing transmission of infection following the introduction of effective blood donor screening and blood product inactivation steps in the 1990s (Nelson et al. 2011).

Both the time of initial spread of HCV into Western countries and the population dynamics of the epidemic can only be indirectly inferred. However, available evidence is consistent with relatively recent dates for its worldwide spread although it likely preceded the AIDS epidemic by some decades. A lack of samples available for screening collected before the Second World War has prevented a direct demonstration of this hypothesis and reconstruction of the HCV epidemic has been largely based on modelling evolutionary histories of currently circulating variants and by identifying historical factors such as widespread use of blood transfusion and other parenterally delivered treatments and vaccinations that facilitated HCV transmission.

In epidemiological terms, transmission of HCV through sexual contact or from mother to child is inefficient and infrequent (Wasley and Alter 2000; Pradat and Trepo

2000; Thomas 2000). The restriction of HCV transmission through primarily parenteral routes therefore implicates medical treatment with unsterilised needles (including large-scale vaccination programmes), blood transfusion and more recently injecting drug use as routes as the principal means of HCV spread and a relatively recent timescale (Drucker et al. 2001). None of these risk factors were common before the Second World War and supports the current model for the spread of genotypes 1b and type 2 subtypes from the 1940s–1950s, overlaid by more recent transmission among IDUs from the 1960s onwards (Pybus et al. 2001; Cochrane et al. 2002).

This scenario is strongly supported by genetic analysis of HCV genotypes and subtypes most frequently detected among IDUs and those infected previously through medical treatment. A recent large-scale coalescent analysis of 1a and 1b subtypes demonstrated relatively small and constant population sizes for both subtypes from the early twentieth century followed by an exponential period of population growth between the 1940s and 1980s in the USA (Magiorkinis et al. 2009). The slowing of population growth thereafter is additionally consistent with reductions in blood transfusion risk through HIV-1 followed by HCV screening and the expansion of needle exchange programmes that have led to significant falls in HCV incidence among IDUs. Emphasising the global nature of the recent spread of HCV, parallel phylogeographic analyses have revealed similar demographic histories of these subtypes in Brazil, Indonesia and Japan (Nakano et al. 2004). A detailed analysis of reconstructed population sizes of HCV and the emergence of parenteral routes of exposure in Japan Egypt and the USA further strengthens these conclusions (Mizokami et al. 2006), including the close links between HCV emergence and parenteral antischistosomal therapy in Japan and subsequently in Egypt. In the latter, the extremely high population prevalence of HCV is dominated by genotype 4a, whose spread can be reconstructed to have occurred between the 1930s–1950s, a period that coincides with targeted extensive antischistosomal injection campaigns using largely unsterilised injection equipment (Pybus et al. 2003).

Collectively, these and several further combined phylogenetic and epidemiological reconstructions provide a convincing narrative for the spread of HCV worldwide. Although earlier by some decades, its spread is paralleled by the explosive worldwide spread of HIV-1 from Africa from the 1980s onwards leading to the current AIDS pandemic. In one sense, the question of the origins of HCV has likely already been answered. However, where HCV was before then and what factors led to its emergence are much less well understood and are discussed in the next section.

3 Endemic Circulation of HCV

While the epidemic spread of HCV is associated with specific, very prevalent subtypes such as 1a, 1b, 3a and 4a, these represent a small part of the diversity existing with HCV. In sub-Saharan Africa and South East Asia, a quite distinct pattern of genetic diversity exists (Fig. 2). Discounting recent introductions, infections in large, geographically contiguous areas among several countries in Central Africa or the South East Asian peninsula are dominated by individual genotypes

(genotypes 1 and 6 respectively in these examples). Individual variants within these genotypes show striking genetic diversity from each other matching the genetic divergence observed between subtypes such as 1a and 1b found in Western countries. For example, sequence characterisation of genotype 2 variants infecting 23 blood donors in Ghana (West Africa) revealed the presence of 20 highly diverse variants that would merit their assignment as new subtypes, as divergent from each other as 2a is from 2b (≈25 % nucleotide sequence divergence) (Candotti et al. 2003). Although far from fully mapped systematically, infections throughout Western Africa are predominantly by genotype 2 (Candotti et al. 2003; Jeannel et al. 1998; Mellor et al. 1995; WansbroughJones et al. 1998; Ruggieri et al. 1996), while those in Central Africa, such as the Congo, Cameroon and Gabon are by genotypes 1 and 4 (Mellor et al. 1995; Bukh et al. 1993; Fretz et al. 1995; Stuyver et al. 1993; Menendez et al. 1999; Xu et al. 1994; Ndjomou et al. 2003; Li et al. 2009, 2012). Genotype 3 and 6 are typically found in the Indian sub-continent and South East Asia (Mellor et al. 1995; Tokita et al. 1994, 1994, 1995; Lu et al. 2008). It is further suspected, although with very limited data that genotypes 5 and 7 are concentrated in Central/Southern Africa.

The extensive genetic heterogeneity of HCV in these regions has been described as an "endemic" pattern of diversity and is consistent with its long-term presence and diversification in these populations. As such, it is currently hypothesised that they represent source areas fuelling the worldwide spread of HCV in the last 100–200 years. Indeed, the distinct subtypes that have been described in Western countries such as 1a, 1b and 3a might simply represent the explosive expansion of certain variants within new risk groups for infection. Although we do not know and may never be able to reconstruct their ultimate origins and initial transmission pathways, 1a, 1b, 3a and others may simply happen to be the most successful of variants that entered previously unexposed and highly susceptible individuals exposed parenterally. In the same way that HIV-1 subtype B entered and spread within male homosexuals and IDUs in the USA and subsequently in Europe (Gao et al. 1999), our current collection of classified subtype might similarly represent founder viruses that were among the first to spread epidemically in the last century in Western countries where HCV was first genetically characterised.

Supporting this model are the more recently described examples of introductions and varying degrees of local spread of a range of otherwise undescribed "subtypes" of HCV. As examples, substantial diversity and restricted distributions of genotype 2 variants infecting have been described in Europe (Thomas et al. 2007), Indonesia (Utama et al. 2010) and throughout the Caribbean (Sulbaran et al. 2010; Martial et al. 2004), the latter examples in particular perhaps representing the shipment of infected West Africans through the slave trade in the eighteenth and nineteenth centuries (Markov et al. 2009). The more recent spread of genotype 4 variants within Cameron and Egypt through medical treatment (Pybus et al. 2003; Pepin and Labbe 2012), into Mediterranean countries and the recent rapid spread of genotype 4 variants among IDUs in Southern Europe (Nicot et al. 2005; de Bruijne et al. 2009) provide further examples of this model (Ndjomou et al. 2003).

What remains unexplained is the nature of the "endemic" circulation of HCV in these implicated source areas and in particular the transmission routes that have

sustained long-term circulation of HCV in what have been until recently relatively frequently highly isolated human communities. As discussed, transmission by either sexual contact or from mother to child is inefficient at least in areas where it has been studied (Wasley and Alter 2000; Pradat and Trepo 2000; Thomas 2000) and various factors that may enhance transmission have been proposed. Examples include sexually transmitted infections (STIs), circumcision, excision and scarification practices (Shepard et al. 2005) which at least in Central Africa show associations with HCV infection and more remote possibilities such as mosquito or other arthropod vectors (Pybus et al. 2007). These various hypotheses are yet to be resolved.

The time depth of "endemic" circulation of HCV remains similarly uncertain. Molecular evolutionary reconstructions of the recent spread of HCV have produced robust and reproducible estimates of its substitution rate (see previous section). Substitution rates extrapolated to the much larger sequence distances that exist within genotypes (such as between subtypes 1a and 1b) have been used to provide some kind of estimate of the minimum period over which the observed "endemic" diversity developed (Pybus et al. 2001; Markov et al. 2009; Smith et al. 1997; Pybus et al. 2009). Reconstructed dates for the common ancestor of different genotypes vary but are estimated to be several hundred years ago for genotype 2 and even longer for genotype 6. In the opinion of the author of this review, such estimates should be treated with extreme caution and minimum estimates at best. Extrapolating substitution rates measured over short observation intervals to the much longer periods of subtype and genotype diversification makes assumptions about the evolutionary process that are not self-evidently justified. Factors such as extreme rate variation between sites, large-scale RNA secondary structure, greater selective constraints and fitness optimisation of viruses association with large population sizes may create substantial underestimates of the real period of virus diversification [reviewed in (Sharp and Simmonds 2011)]. While it is beyond the scope of the current article to discuss this in detail, what can be said is that the subtype diversification in HCV and thus the likely period of endemic circulation in sub-Saharan Africa and Southern Asia is prolonged and likely long before long distance travel and interactions with colonial powers. These genotypes are therefore likely to be truly indigenous to areas where they currently endemically circulate. This takes us a step further back to the question of the ultimate source of HCV. This much more speculative area will be reviewed in the next section.

4 Origins of Human Infections and HCV Homologues in Other Mammals

A compelling scenario which has driven much research endeavour in the last decade and a half is the hypothesis for a non-human primate source for HCV infections in humans. The theory makes epidemiological sense in that high diversity areas of endemic circulation in humans are those where human, ape and Old World monkey populations overlap. Before long range travel and the means for

wider dissemination, human infections acquired zoonotically from non-human primates may have remained geographically focussed and thus account for the specific association of each of the genotypes in defined areas of sub-Saharan Africa and Southern Asia. The idea of a non-human primate source for humans is additionally consistent with the observation of its poor transmissibility between humans, largely confined to parenteral routes and a reflection perhaps of its lack of host adaptation as might also be its severe, immune-mediated liver pathology.

This model is, of course, also driven by the tempting analogy with the origin of HIV-1, which similarly exploded worldwide out of Central Africa in the twentieth century through infections directly or indirectly from chimpanzees (Gao et al. 1999). As might be imagined for HCV, HIV-1 infections acquired through contact with Central African chimpanzees (*Pan troglodytes*) may have been occurring for centuries or millennia, but only in the last 50–70 years were demographic and societal changes suitable for its wider pandemic spread. Important differences from the HIV-1 model of origins would be the earlier spread of HCV worldwide and the existence of multiple potential source areas and possibly different primate species. These would be necessary to account for the distinct endemic distributions of HCV genotypes in different parts of sub-Saharan Africa and also South East Asia. Finally, into this model would come GBV-B, which might perhaps represent a much more divergent homologue of HCV in a New World primate species.

Despite the elegance, plausibility and potential medical relevance of the primate origin hypothesis, the fundamental problem has always been that HCV or homologues cannot be found in ape or monkey species, at least to the author's knowledge. Extensive screening programmes both published (Makuwa et al. 2003, 2006) and unpublished have failed to document either seropositivity or viral sequences in literally hundreds or thousands of plasma samples collected from different ape and monkey species. As a possibly related problem, GBV-B has to date never been recovered nor serological evidence for past infections obtained from any tamarind or other New World primate among wild populations in South America.

Without an obvious primate source for infection and the genetic evidence for circulation of HCV in what would have been largely isolated human populations in distinct parts of the world for centuries or more likely millennia, studies of the ultimate origins of HCV have reached something of a frustrating impasse. As with many other virus discoveries, however, its resolution is likely to be considerably stranger than could have been imagined even as recently as last year. By pure serendipity, attempts by Kapoor and colleagues to identify viral causes of respiratory disease in dog held in kennels by deep sequencing revealed the existence of an RNA virus extraordinarily similar to HCV (Fig. 3) but with suspected biological and epidemiological properties quite different from what had been previously described for both HCV and GBV-B (Kapoor et al. 2011).

The virus, initially termed canine hepacivirus (CHV) showed approximately 50 % nucleotide sequence divergence from HCV. Data presented in that study demonstrated high viral loads in respiratory samples and an implied respiratory route of transmission and association with respiratory disease, none of which have been observed in HCV (or GBV-B) infections. Infections were found in dogs from

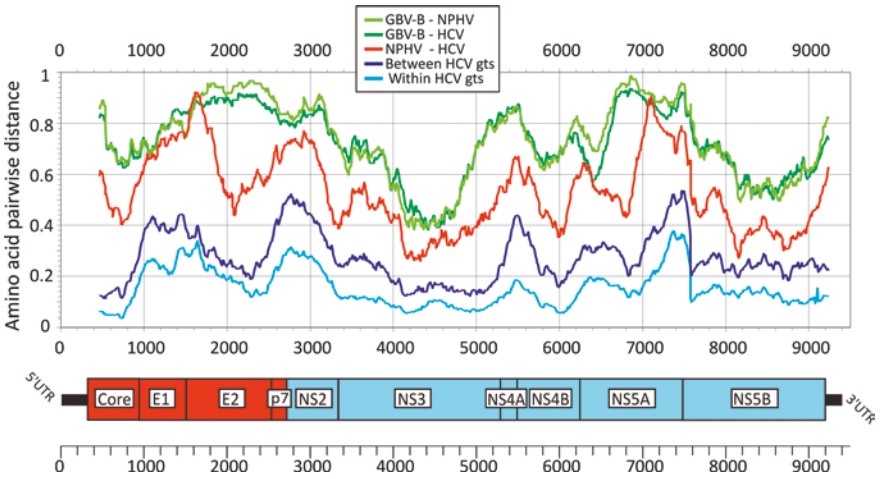

Fig. 3 Amino acid sequence divergence scan of members of the *Hepacivirus* genus, with genome diagram drawn to scale underneath plot. NPHV is more similar to HCV throughout the genome (*red line*) than GBV-B (*dark green line*). However, NPHV/HCV divergence is substantially greater than between genotypes (*dark and light blue lines* respectively). This figure has been adapted from Fig. 2 in (Kapoor et al. 2011). For details related to the HCV polyprotein and the cleavage products see chapter "Hepatitis C virus Proteins From Structure to Function" by Moradpour and Penin, this volume

different regions of the USA but partial genome characterisation demonstrated a virtual absence of genetic diversity that would be expected for an RNA virus like HCV. Whether the virus spread systemically or persisted was not demonstrated although imaging of viral RNA in liver by in situ hybridisation was presented.

More recently, further studies of the host range of hepaciviruses in a range of mammalian species were performed by the same group using a serological assay for antibodies to a peptide expressed from the NS3 region of the CHV genome (Burbelo et al. 2012). This produced further unexpected findings. From the 80 dogs, 81 deer, 84 cows, 103 horses and 14 rabbits screened, only horses showed frequent seropositivity (35 %) with one weak positive sample from a cow while, remarkably, all 80 dogs were seronegative. Of the 103 horse samples, 8 were PCR-positive (all seropositive) and from each of these near-complete genome sequences were obtained. Sequences showed moderate sequence diversity from each other (6.4–17.2 % nucleotide sequence divergence) with the CHV sequence grouping with horse-derived variants. As viruses similar to CHV were frequently found in horses, the investigators coined the name NPHV to describe this group.

The diversity of NPHV variants was somewhere between inter-subtype and within subtype divergence of HCV, certainly not the equivalent of HCV genotypes (Fig. 3). The high degree of amino acid sequence conservation did, however, contrast markedly with the degree of sequence variability at synonymous (non-coding) sites in the genome. The extraordinarily low ratio between synonymous to non-synonymous

substitutions (0.03–0.06) indicates however that its evolution has been more severely constrained and/or less subject to positive selection pressures than HCV. These low sequence distances are therefore not necessarily an indication of their recent divergence.

There was no information available on the clinical features of infection with NPHV in horses. To address this we have recently surveyed horses in Scotland by PCR and identified 3 viraemic horses from 136 screened (Lyons et al. 2012). Using veterinary records and further sampling, these have been evaluated for evidence of hepatitis or other systemic disease manifestations. Positive horses were originally referred for reasons such as lameness, foot abscess or respiratory infections with no evidence of the ill-health that might be associated with severe systemic infections. Although most liver indices were in the normal range, gamma glutamyl transferase (GGT) levels, a sensitive marker of liver inflammation were marginally or significantly elevated along with elevation in bile acids, perhaps providing some tentative evidence for an aetiological role of NPHV in hepatitis. Repeated sampling from one of the study horses demonstrated persistence over at least a 6-month period and viral loads comparable to those observed in HCV infections (7×10^4–5×10^7 RNA copies/ml). Respiratory samples and peripheral blood mononuclear cells from the infected horse have proven uniformly negative although no opportunity to perform a liver biopsy of the horse has yet presented itself. Overall, these more recent findings provide some reassurance that hepacivirus infections in horses are both persistent and potentially associated with mild liver disease rather than the respiratory disease and viral secretion found originally in dogs. However, large-scale PCR-based screening of other mammalian species using primers conserved between NPHV and HCV failed to detect hepaciviruses in dogs (nearly 200 screened), cats, pigs and rodents (Lyons et al. 2012), very much as found in the previous serology-based study (Burbelo et al. 2012).

This, to date, represents current published knowledge of non-human hepaciviruses, a series of findings that present several conflicting interpretations and difficulties. This author believes that, despite the negative results from screening so far, domestic horses are most unlikely to be the only mammalian species (other than human or tamarins) infected with hepaciviruses and there is clearly much to be learned in short term from more extensive screening.

5 Concluding Thoughts

Our understanding of the ultimate origins of HCV infection in humans will doubtless be hugely enhanced once proper mammalian screening for other hepaciviruses has been performed and the genetic diversity and, more importantly, the specificity of different hepaciviruses to individual host species is more clearly established. From such studies, it may well turn out that hepaciviruses are highly catholic in their host range perhaps capable of jumping between horses and dogs as suggested by the published screening data (Burbelo et al. 2012; Kapoor et al. 2011) and perhaps all species in-between. An ability of hepaciviruses to jump species is consistent

with the observation that the NPHV protease is able to cleave human MAVS and TRIF (Parera et al. 2012); this ability to prevent interferon signalling is essential for HCV replication (Foy et al. 2005) and may therefore function across species barriers and potentially favour zoonotic transmission. A wide mammalian host range is also characteristic of vector-borne flaviviruses and pestiviruses, the latter at least within ruminant species. In this scenario, HCV infections in humans may well have a zoonotic origin consistent with its relatively recent emergence (at least in Western countries). While being still relatively poorly adapted for infecting its new host, this may further account for its peculiar, inefficient transmission routes.

Alternatively, it may be that each hepacivirus species is uniquely adapted to one target species, HCV in humans, NPHV in horses and perhaps further hepaciviruses in other mammalian species waiting to be discovered. The ability of HCV to persist lifelong in humans, an attribute that greatly enhances its transmissibility and evidence for subtle virus/host interactions such as the enhancing role of human micro RNA, miR-122 expressed in liver on virus replication (Jopling et al. 2005) certainly hints at long-term virus/host co-adaptation. HCV may always have infected humans throughout their evolution and it is only through greater life expectancy, scope for epidemic transmission and better surveillance and understanding of causes of hepatitis that it has come to current medical attention. In that sense, HCV does not have an "origin", it is just one of those viruses like herpesviruses that have always infected humans and before them hominoids, proto-apes and potentially right back to the ancestor of mammals themselves.

Future research will be truly important in resolving these two diametrically opposed possibilities.

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Cell Culture Systems for Hepatitis C Virus

Eike Steinmann and Thomas Pietschmann

Abstract Due to the obligatory intracellular lifestyle of viruses, cell culture systems for efficient viral propagation are crucial to obtain a detailed understanding of the virus–host cell interaction. For hepatitis C virus (HCV) the development of permissive and authentic culture models continues to be a challenging task. The first efforts to culture HCV had limited success and range back to before the virus was molecularly cloned in 1989. Since then several major breakthroughs have gradually overcome limitations in culturing the virus and sequentially permitted analysis of viral RNA replication, cell entry, and ultimately the complete replication cycle in cultured cells in 2005. Until today, basic and applied HCV research greatly benefit from these tremendous efforts which spurred multiple complementary cell-based model systems for distinct steps of the HCV replication cycle. When used in combination they now permit deep insights into the fascinating biology of HCV and its interplay with the host cell. In fact, drug development has been much facilitated and our understanding of the molecular determinants of HCV replication has grown in parallel to these advances. Building on this groundwork and further refining our cellular models to better mimic the architecture, polarization and differentiation of natural hepatocytes should reveal novel unique aspects of HCV replication. Ultimately, models to culture primary HCV isolates across all genotypes may teach us important new lessons about viral functional adaptations that have evolved in exchange with its human host and that may explain the variable natural course of hepatitis C.

Abbreviations

BMEC	Brain microvascular endothelial cells
CNS	Central nervous system
Con1	Consensus genome 1
DAA	Direct acting antiviral

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DC-SIGN	Dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin
EGFR	Epidermal growth factor receptor
EMCV	Encephalomyocarditis virus
EphA2	Ephrin receptor A2
GFP	Green fluorescent protein
HCV	Hepatitis C virus
HCV_{TCP}	Hepatitis C virus trans-complemented particles
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
iPSC	Induced pluripotent stem cells
IRES	Internal ribosomal entry site
JFH1	Japanese fulminant hepatitis
LDL-R	Low-density lipoprotein receptor
MEF	Mouse embryonic fibroblasts
MPCC	Micropattern co-cultures
mL	Milliliter
MLV	Murine leukemia virus
NPC1L1	Niemann-Pick C1-like cholesterol adsorption receptor
PBMC	Peripheral blood mononuclear cells
PHH	Primary human hepatocytes
REM	Replication enhancing mutations
RIG-I	Retinoic acid-inducible gene I
SEAP	Secreted embryonic alkaline phosphatase
siRNA	small interfering RNAs
$TCID_{50}$	Tissue culture infectious dose 50
VSV	Vesicular stomatitis virus

Contents

1 Introduction

When HCV, the causative agent of hepatitis C, was first cloned in 1989 many attempts to culture the elusive infectious agent in vitro had already failed. These efforts were a prelude to the hurdles ahead to push the doors open for model systems fully permissive to cell culture replication of HCV. Some of these early limitations, like for instance the inefficient replication of primary HCV isolates, prevail until today. However, during the past decades several breakthrough developments have much improved our repertoire to study this virus in vitro. In fact, our increasing knowledge of molecular replication mechanisms may help to overcome the remaining roadblocks that prevent us from analyzing the complex interplay of HCV with its host cells in a yet further refined fashion.

The development of HCV-permissive cell culture models was a step-wise process. The establishment of subgenomic replicons that autonomously amplify in cultured human hepatoma cells was a first major breakthrough. Another important achievement was the generation of infectious retroviral pseudotypes displaying functional HCV glycoproteins for the study of HCV entry. Finally, the identification of a novel HCV isolate, termed JFH1, paved the way for the production of infectious virions to investigate all steps of the viral life cycle. Recently, remarkable advances were also made with regard to measuring HCV infection and replication in primary cell cultures. In this chapter, we will highlight essential components of HCV cell culture models and provide an overview of viral adaptation to replication in cell culture. In addition, we attempt to provide a perspective on future developments that may help to unravel new features of the HCV host cell interaction.

Historically, three key achievements build the foundation of the most widely used HCV tissue culture systems. Besides these models described below in greater detail, during the past years a variety of additional cell-based systems to monitor HCV cell entry and receptor interactions have been reported. The interested reader is referred to a recent review for a detailed description of these systems (Vieyres and Pietschmann 2012). Details on the HCV entry pathway are reviewed in the chapter "Hepatitis C Virus Entry" by Zeisel et al., this volume.

2 HCV Replicon System

During the 1990s, numerous attempts were made to initiate robust HCV infection and replication in cultured cells after inoculation with patient sera or transfection with cloned viral RNA. Although in the past long-term productive HCV replication was reported, these experimental systems suffered from low replication efficiency (Bartenschlager and Lohmann 2000). Highly sensitive, but also error-prone techniques, like RT-PCR were necessary to document HCV replication and only small amounts of viral proteins or infectious virus were produced precluding molecular dissection of HCV replication mechanisms. In fact, until today replication of the vast majority of cloned HCV genomes is poor in cultured cells (see also below Sect. 4).

Encouraged by reports that subgenomic RNA molecules of other plus strand RNA viruses readily replicate in transfected cells (Khromykh and Westaway 1997; Mittelholzer et al. 1997), similar approaches were attempted for HCV. By trimming the HCV genome to those components essential for RNA replication (see chapter "Hepatitis C Virus RNA Replication" by Lohmann, this volume), so-called replicons were created (Lohmann et al. 1999). Due to deletion of viral structural genes (core, envelope 1 and 2), p7 and NS2 these RNA molecules were much smaller than the authentic viral genome. This provided the freedom to insert a heterologous dominant selectable marker (e.g., neomycin phosphotransferase, neo) without exceeding the natural length of the HCV genome. The prototype replicon was a bicistronic RNA of genotype 1b (Con1 isolate) encoding a neomycin resistance gene under the control of the HCV internal ribosomal entry site (IRES), followed by a second IRES from encephalomyocarditis virus (EMCV) that controlled expression of the genes for NS3-NS5B. Upon transfection of synthetic RNAs derived from such a construct into the human hepatoma cell line Huh-7 and G418 selection, cell lines containing high amounts of self-replicating HCV RNAs could be obtained (Lohmann et al. 1999). Based on quantification by Northern hybridization, an average copy number of 1,000–5,000 positive-strand RNA molecules per cell was determined. Minus-strand RNA was present in about tenfold lower amounts in comparison with plus-strand RNA and HCV protein expression was readily detected by metabolic radiolabeling and immunoprecipitation (Lohmann et al. 1999). Replicon cell clones continuously passaged under selective pressure maintain the viral RNA for many years. After the introduction of the replicon system in 1999 this cell culture system has been widely applied in HCV research. During the following years an increasing number of replicon constructs with varying reporter genes including luciferases and fluorescent proteins were developed to tailor the system to the needs of the researcher and to facilitate exploration of the mechanisms of HCV RNA replication. A detailed summary of replicon constructs currently in use was compiled by Bartenschlager et al. (2006). While initially replicons were developed for the genotype 1b (GT1b) consensus genome Con1 (Lohmann et al. 1999), meanwhile replicons are available for GT1a, various GT1b isolates, GT2a and GT4a strains (Table 1), thus increasing the versatility of this important model tremendously.

2.1 Replication Enhancing Mutations

The HCV RNA-dependent RNA polymerase NS5B lacks a proof-reading activity and as observed for many other viruses HCV replicates with a high mutation rate (see chapter "Hepatitis C Virus RNA Replication" by Lohmann, this volume). Initially, genotype 1b replicons showed a low G418 transduction efficiency despite high level of RNA replication within the surviving cell clones. It turned out that the reason for this was twofold. First, during the selection procedure replicons acquired so-called replication enhancing mutations (REMs) permitting more efficient RNA replication in transfected Huh-7 cells. Second, the selection process

Table 1 Molecular HCV clones

Strain	Genotype	Replicon		HCVcc	
		Wild type	Adapted	Wild type	Chimeric/adapted
H77	1a	–	Blight et al. (2003), Grobler et al. (2003), Tscherne et al. (2006)	–	Pietschmann et al. (2006), McMullan et al. (2007)
H77C	1a	–	Yi et al. (2004)	–	Russell et al. (2009), Yi et al. (2007), Scheel et al. (2008), Yi et al. (2006)
HCV-1	1a	–	Lanford et al. (2006)	–	–
Con1	1b	Lohmann et al. (1999)	Blight et al. 2000), Guo et al. (2001), Krieger et al. (2001), Lohman et al. (2003)	Pietschmann et al. (2009)	Gottwein et al. (2009), Kaul et al. (2007), Pietschmann et al. (2006)
HCV-N	1b	Guo et al. (2001), Ikeda et al. (2002), Yi et al. (2002)	–	–	–
HCV-BK	1b	–	Grobler et al. (2003)	–	–
HC-J4	1b	–	Maekawa et al. (2004)	–	–
O	1b	–	Abe et al. (2007), Ikeda et al. (2005), Kato et al. (2003a)	–	
AH1	1b	–	Mori et al. (2008)	–	–
NC1	1b	–	Date et al. (2012)	–	Date et al. (2012)
BHCV1	1b	–	–	–	Koutsoudakis et al. (2011)
JFH1	2a	Kato et al. (2003b)	–	Wakita et al. (2005), Zhong et al. (2005)	Delgrange et al. (2007), Kang et al. (2009), Kaul et al. (2007), Russell et al. (2008), Zhong et al. (2006)

(continued)

Table 1 (continued)

Strain	Genotype	Replicon		HCVcc	
		Wild type	Adapted	Wild type	Chimeric/adapted
J6/JFHI, Jc1	2a	–	–	Lindenbach et al. (2005), Pietschmann et al. (2006)	Bungyoku et al. (2009)
HC-J8	2b	–	–	–	Gottwein et al. (2007)
S52	3a	–	–	–	Gottwein et al. (2007), Gottwein et al. (2009)
452	3a	–	–	–	Kaul et al. (2007), Pietschmann et al. (2006)
ED43	4a	Peng et al. (2012)	Peng et al. (2012)	–	Scheel et al. (2008)
SA13	5a	–	–	–	Gottwein et al. (2007), Jensen et al. (2008)
HK6a	6a	–	–	–	Gottwein et al. (2007)
QC69	7a	–	–	–	Gottwein et al. (2007)

enriched for those few host cells in the total population of transfected cells that were more permissive to HCV replication than "standard" Huh-7 cells. Evidence for this second mechanism was elegantly provided by transfection of replicons into individual Huh-7 clones that had been obtained after transfection and selection with selectable replicons and subsequent purging of the replicon by inhibitor or IFN-treatment (Blight et al. 2002; Friebe et al. 2005). In fact, the most widely used host cells for HCV research—Huh-7.5, Huh-7.5.1, and Huh7-Lunet—were all obtained by this strategy (Blight et al. 2002; Friebe et al. 2005; Zhong et al. 2005).

While for most of these highly permissive cells it remains elusive why they are so amenable for HCV replication, viral adaptation permitting increased replication has been linked to distinct mutations within individual non-structural proteins. These mutations have originally been designated 'cell culture adaptive mutations', but should be renamed as 'replication enhancing mutations' (REMs) in order to discriminate them from cell culture adaptive mutations that increase virus titers without affecting replication (Pietschmann et al. 2009). Sequence analysis of replicons within selected cell clones identified numerous conserved changes within the coding region of the viral non-structural proteins. Introduction of these mutations back into the parental genome and transfection of in vitro transcribed RNA revealed an enhancement of RNA replication to various degrees as determined by the number of G418-resistant colonies (Blight et al. 2000; Guo et al. 2001; Lohmann et al. 2001; Ikeda et al. 2002; Kishine et al. 2002; Grobler et al. 2003; Gu et al. 2003; Kato et al. 2003a; Lohmann et al. 2003).

REMs were mainly located in the N-terminus of NS3, at two distinct amino acids in NS4B and in the central domain of NS5A. Several of the most potent REMs in NS5A change phosphorylation sites within the protein suggesting that replication efficiency may be regulated via phosphorylation. Interestingly, Evans et al. (2004) observed an interaction between HCV NS5A and human vesicle-associated membrane protein-associated protein A (hVAP-A) which is modulated by NS5A phosphorylation. Their findings support a model where NS5A hyperphosphorylation disrupts the interaction with h-VAP-A which negatively regulates viral RNA replication (Evans et al. 2004). With the generation of replicons from other HCV isolates it could be shown that Con1 adaptive mutations also enhanced replication efficiency of other genotype 1b strains, including HCV-O (Kato et al. 2003a; Ikeda et al. 2005; Abe et al. 2007), HCV-BK (Grobler et al. 2003), J4 (Maekawa et al. 2004) and AH1 (Mori et al. 2008). In case of the HCV N-isolate adaptive mutations were not required for efficient replication due to a unique four amino acid insertion naturally present in NS5A (Guo et al. 2001; Ikeda et al. 2002; Yi et al. 2002).

The establishment of genotype 1a replicons turned out to be more difficult as even the introduction of genotype 1b-specific mutations did not result in high levels of RNA replication (Blight et al. 2000; Guo et al. 2001; Lanford et al. 2003; Yi and Lemon 2004; Liang et al. 2005). However, with passage of genotype 1a replicon RNA in highly permissive cell lines REMs could be identified in NS3, NS4B, or NS5A (Blight et al. 2003; Grobler et al. 2003; Yi and Lemon 2004; Lanford et al. 2006). Generation of non-genotype 1 replicons have not been described so far except for a genotype 4a (Peng et al. 2012) and genotype 2a isolate that replicates with high efficiency without the requirement of adaptive mutations (Kato

et al. 2003b). The latter genome was cloned from a Japanese patient suffering a fulminant course of hepatitis and thus designated "Japanese fulminant hepatitis 1" (JFH1). This genome has become the basis of the most widely used HCV cell culture system which will be described in more detail below (see Sect. 4).

With the identifications of REMs further improvements of the original replicon system could be developed. These include alternative drug resistance genes (Frese et al. 2002; Evans et al. 2004; Appel et al. 2005; Liang et al. 2005), monocistronic replicons (Blight et al. 2003), and transient replication assays that are based on the detection of reporter genes like luciferase, ß-lactamase, green fluorescent protein (GFP), and secreted alkaline phosphatase (Krieger et al. 2001; Yi et al. 2002; Lohmann et al. 2003; Murray et al. 2003; Ikeda et al. 2005). The exact mode of action of cell culture REMs is still not fully understood. Generally, these mutations have not been observed in natural HCV isolates suggesting that the stimulatory effect on HCV RNA replication in vitro does not increase viral fitness in vivo. In fact, at least for Con1 evidence has been provided that REMs interfere with production of infection virus and viral spread in vitro and in vivo (Bukh et al. 2002; Pietschmann et al. 2009) and see also below Sect. 4.

3 Retroviral Pseudoparticles

In the absence of an efficient cell culture system encompassing the entire life cycle of the virus, surrogate models were developed that were useful to study the role of HCV glycoproteins in virus entry (see chapter "Hepatitis C Virus Entry" by Zeisel et al., this volume). The most successful among the models to investigate early steps of HCV infection was the establishment of retroviral pseudotypes bearing unmodified HCV glycoproteins (HCVpp) (Bartosch et al. 2003; Hsu et al. 2003). This system is based on the co-transfection of 293T cells with expression vectors encoding HCV E1 and E2, the gag-pol proteins of either murine leukemia virus (MLV) or human immunodeficiency virus (HIV) and a retroviral genome encoding a reporter gene. Entry of these particles leads to the delivery of the retroviral capsid into the cytoplasm of the target cell following reverse transcription and integration of the viral genome into the host cell genome. The reporter gene is expressed by the integrated provirus to detect productive entry events in a rapid manner. Importantly, attachment and receptor interaction of these retroviral pseudotypes is governed by the functional HCV E1-E2 protein complex incorporated into the envelope of these particles. Therefore, HCVpp's can be neutralized with antibodies targeting the viral glycoproteins E1, E2, and with sera of infected patients (Hsu et al. 2003; Cai et al. 2005). The incorporation of patient-derived glycoproteins has also been described for HCVpp and can be used to study cross-neutralizing antibodies (Bartosch et al. 2003; Owsianka et al. 2005; Tarr et al. 2007). Moreover, utilization of HCVpp is an elegant way of analyzing HCV cell entry independent of the other parts of the viral life cycle. Consequently, this system offers the freedom to investigate cell entry into cells that are not permissive to HCV RNA replication. Various viral entry attachment factors and receptors have been identified or verified using this system

including glycosaminoglycans, low-density lipoprotein receptor (LDL-R), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), claudin-1, claudin-6, claudin-9, occludin, and recently also epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2), and the Niemann-Pick C1-like cholesterol adsorption receptor (NPC1L1) [for review see (Ploss and Evans 2012)]. A limitation of the HCVpp system is that these particles are produced in a non-liver cell line (293T) and that they assemble in post-Golgi compartments and/or the plasma membrane as retroviruses do. Therefore, the close association of HCV particles with lipoproteins cannot be reproduced which may affect studies including antibody neutralization assays and entry studies with lipid receptors LDL-R, SR-BI, and NPC1L1. However, this "limitation" offers the exciting opportunity to learn about the relevance of these host-derived modifications of HCV particles by directly comparing cell entry properties of HCVpp with natural HCV particles.

In summary, although not covered with lipoproteins, HCVpps can be used to study viral entry events of HCV independent of RNA replication and assembly and have been a valuable tool to identify cellular entry molecules.

3.1 Other Models to Study Individual Steps of HCV Cell Entry

In addition to HCVpp, additional model systems have been developed to study HCV entry. These include the most widely used cell culture model systems HCVcc and HCV_{TCP} that are covered in separate sections. For the identification of cellular receptors C-terminally truncated secreted forms or cell surface expressed versions of the glycoprotein E2 have been described (Flint et al. 1999; Flint et al. 2000). One of the first tools used to study HCV cell entry and to discover receptors involved in this pathway was a soluble and truncated form of the E2 glycoprotein ($sE2_{661}$), in which the last 85 amino acids, encompassing the hydrophobic transmembrane domain, are deleted (Spaete et al. 1992; Michalak et al. 1997). It is not clear to which degree this truncated form reflects the proper folding of E2 in the context of the E1/E2 complexes within the HCV envelope (see also chapter "Hepatitis C Virus Proteins: From Structure to Function" Moradpour and Penin, this volume). However, the discovery of CD81 and SR-BI as part of the HCV receptor complex was achieved using $sE2_{661}$ (Pileri et al. 1998; Scarselli et al. 2002). Current approaches to solve the structure of the E2 glycoprotein are based on differently truncated forms of the E2 protein (Krey et al. 2010; McCaffrey et al. 2011). Further surrogate models to study glycoprotein and receptor interaction as well as early entry events include E1-E2 liposomes (Lambot et al. 2002), virus-like particles generated in insect cells (Baumert et al. 1998; Triyatni et al. 2002; Wellnitz et al. 2002), and vesicular stomatitis virus (VSV) pseudotyped with chimeric glycoproteins consisting of the ectodomains of HCV E1 and E2 fused to the transmembrane domain of the VSV-G glycoprotein (Lagging et al. 1998; Matsuura et al. 2001; Buonocore et al. 2002). More recently, a soluble form of E2 was reported that blocks HCVcc entry and is produced in mammalian or insect cells (Whidby et al. 2009).

Virus binding to the cellular surface can be measured by quantification of cell-bound RNA copy numbers (Vieyres et al. 2009; Calland et al. 2012) or by radioactive labeling of HCV virions (Ciesek et al. 2011a). After cell binding, receptor interactions and conformational changes in the glycoproteins the virus is taken up by endocytosis (Ploss and Evans 2012). Molecular inhibitors like small interfering RNAs (siRNAs) and dominant-negative constructs have been applied in addition to the use of chemical inhibitors in specifically blocking distinct stages of endocytosis (Sieczkarski and Whittaker 2002). With respect to HCV, trafficking, acidification, and clathrin-mediated endocytosis has been studied with different inhibitors (Blanchard et al. 2006; Meertens et al. 2006; Tscherne et al. 2006). Recently, Coller et al. (2009) developed infectious fluorescent particles to visualize the association of HCV virions with the endocytosis machinery. They labeled particles with membrane-permeable lipophilic dyes, called DiD (1,1′dioctadecyl-3,3,3′,3′tetramethyl-indodicarbo-cyanine 4-chlorobenzenesulfonate salt) and DiI (1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate), that can be used to study HCV cell interactions in the HCV entry process (Coller et al. 2009). Uptake of HCV particles can also be analyzed by protease treatment. Internalized viral particles are resistant to proteolysis, whereas viruses remaining on the cell surface are inactivated (Meertens et al. 2006; Aizaki et al. 2008; Schwarz et al. 2009; Vieyres et al. 2009).

As structural information about the HCV glycoproteins is lacking, the fusion process is not fully understood at a molecular level. Several different fusion assays have been designed that rely on cell-to-cell fusion or fusion between HCVpp/HCVcc and liposomes or target cells. The ‘cell-to-cell’ fusion assay is based on 293T cells that ectopically express the HCV glycoproteins and that encode a T7-polymerase-dependent GFP gene. These cells are co-cultured with Huh-7 cells expressing the T7-polymerase and successful fusion results in multinucleated cells expressing the GFP reporter gene (Kobayashi et al. 2006). Modifications of this system for example with luciferase as reporter gene have been developed (Evans et al. 2007; Lavillette et al. 2007). Productive fusion in vitro can be also monitored with fluorescent probes that are incorporated into either the virus particles or liposomes and upon fusion membrane mixing results in fluorescence dequenching and emission (Lavillette et al. 2006; Haid et al. 2009). Recently, another fluorescence-based fusion assay was developed in which HCVcc viruses were labeled with the hydrophobic DiD fluorophore that inserts into the membrane at self-quenching doses. After fusion of viral and target membranes the DiD fluorophores diffuse away causing dequenching which can be monitored in real time (Sainz et al. 2012).

It has been reported that HCV can also be transmitted via cell-to-cell spread. This mode of transmission may be particularly relevant in vivo in the context of infected liver tissue. It was reported that infection via cell-to-cell spread was refractory to neutralization by E2 monoclonal antibodies and that it may occur in a CD81-independent manner (Timpe et al. 2008; Witteveldt et al. 2009). Cell-to-cell spread of HCV can be studied by co-culturing HCV-positive donor cells with target cells that can be monitored by fluorescent markers. Cell-free spread can be blocked by the presence of neutralizing antibodies or an agarose overlay (Timpe et al. 2008; Witteveldt et al. 2009;

Brimacombe et al. 2011). A recently described cell-based reporter system that is based on NS3/4A-mediated cleavage of a fluorescent substrate can also be applied to investigate this route of transmission at a single cell level (Jones et al. 2010). Combination of this system with an agarose overlay facilitates this assay setup (Ciesek et al. 2011a; Ciesek et al. 2011b). Instead of using two distinct cell populations, quantification of the number of infected cells per infection focus under an agarose overlay similarly allows to study cell-to-cell spread (Baldick et al. 2010; Calland et al. 2012).

4 Cell Culture Infectious HCV Genomes and Host Cells

Initial attempts of transfecting genomic in vitro transcripts of HCV derived from cloned viral genomes into human liver cells were unsuccessful, due to non-functional sequences or mutations introduced by RT-PCR. To circumvent these problems, consensus genomes were constructed which were based on a master sequence that is representative of the dominant nucleotide sequences at each position of the genome. The consensus sequence was established by sequencing of multiple clones of a single isolate which then guided construction a consensus genome based on this sequence information. The first constructs generated by this procedure were derived from a patient designated "H77" who had been infected with a genotype 1a virus (Kolykhalov et al. 1997; Yanagi et al. 1997). Importantly, intrahepatic inoculation of these consensus RNA genomes into chimpanzees initiated a productive infection of the animals (for detailed description of HCV animal models see chapter "Animal Models for Hepatitis C" by Billerbeck et al., this volume). This evidence provided formal proof that indeed these H77 consensus genomes are functional and infectious in vivo (Kolykhalov et al. 1997; Yanagi et al. 1997). However, despite the availability of these and a few other consensus genomes with proven infectivity in vivo, attempts to initiate robust replication and production of infectious progeny with these genomes were initially not fruitful (Fig. 1).

After the successful construction of autonomously replicating selectable subgenomic Con1-derived replicons, we added the viral structural genes and thus created selectable full-length genomes expressing the complete HCV open reading frame of this isolate. Adaptive mutations initially identified with the subgenomic replicons were added to increase RNA replication and virus protein expression. While these selectable genomes as well as authentic genomes with only the adaptive mutations but no selectable marker replicated relatively efficiently, neither stable cell lines harboring selectable full-length replicons of Con1, nor the transiently replicating Con1 full-length RNAs gave rise to infectious HCV progeny (Pietschmann et al. 2002). Likewise full-length RNA of the HCV N strain (GT1b) did not support the production of infectious particles (Ikeda et al. 2002). It was suggested that either the host cells lack factors important for particle formation or that REMs interfere with the production of infectious particles. In line with the second hypothesis, replication-promoting mutations selected for tissue culture experiments are rarely found in HCV sequences from human or chimpanzee (Grobler et al. 2003; Sarrazin et al. 2005).

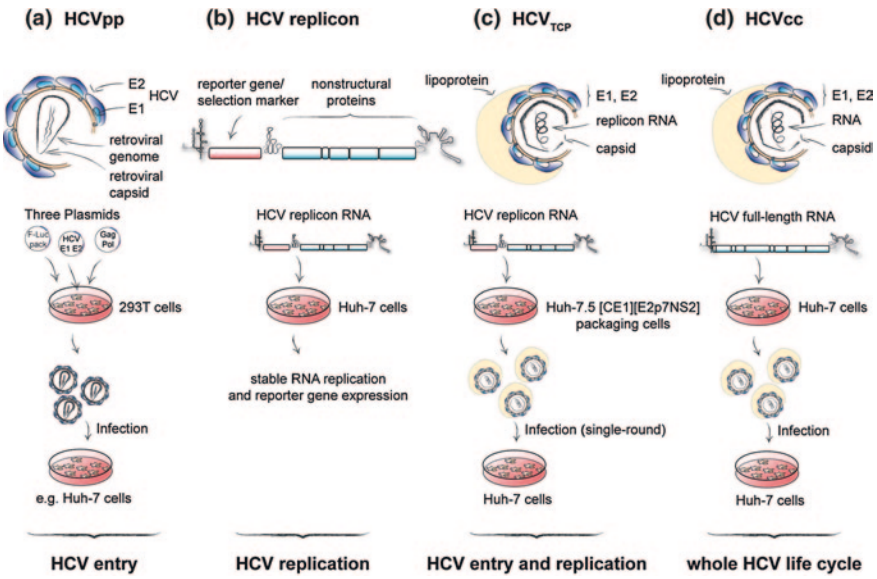


Fig. 1 Key HCV cell culture systems to investigate different steps of the viral replication cycle. **a** For dissection of the entry process HCV pseudoparticles (HCVpp) can be utilized. HCVpp are produced by transfection of 293T cells with three plasmids encoding for (1) retroviral gag and pol genes, (2) a retroviral vector harboring a reporter gene, and (3) the HCV glycoproteins E1, E2. These retroviral particles contain a vector that encodes the reporter gene and display the HCV glycoproteins in their envelope and thus, enter cells in an HCV-dependent manner. **b** HCV RNA replication can be quantified using subgenomic replicons. Those self-replicating HCV RNAs are based on a selectable marker or reporter gene replacing the coding region from core to NS2 upstream of a second IRES from EMCV that allows translation of the non-structural proteins NS3 to NS5B. After transfection, the viral RNA is directly translated and replication can be monitored for example by reporter gene expression. **c** HCV trans-complemented particles (HCV_{TCP}) are authentic viral particles that contain a replicon RNA instead of the full-length genome. They are produced by transfection of replicon RNA into so-called packaging cell lines that stably express the lacking structural proteins and thus, provide them in *trans*. Infection of naïve cells with HCV_{TCP} results in a single round infection with only viral entry and RNA replication taking place since the structural proteins necessary for virus production are missing. **d** Production of cell culture-derived HCV particles (HCVcc) are based on the genotype 2a isolate JFH1 and derivatives thereof. Full-length viral genomes are transfected into permissive human hepatocytes which leads to translation and RNA replication giving rise to the production of viral particles that are able to infect new target cells, thereby completing the whole viral life cycle of HCV

Pietschmann et al. (2009) recently reported that those very same REMs which were used to increase the replication capacity of Con1 genomes actually interfered with production of infectious virus. In fact most of the Con1-replication enhancing changes within the viral non-structural proteins stimulated replication at the expense of production of infectious particles. This observation was a first hint suggesting that the non-structural proteins contribute to production of infectious HCV, an observation which was much refined and extended using the infectious JFH1 system (Jones et al. 2007; Steinmann et al. 2007; Yi et al. 2007; Appel et al. 2008; Ma et al. 2008;

Jones et al. 2009; Phan et al. 2009). Besides this, the observation that REMs can interfere with virus production in Con1 genomes provided a simple explanation why the adapted Con1 genomes unlike the wild type were noninfectious in Chimpanzee or reverted back to the wild-type sequence (Bukh et al. 2002). It is, however, important to stress here that REMs do not necessarily lead to inactivation of virus production. This is exemplified by one of the most potent adaptive changes within the Con1 replicase (i.e., the K1846T exchange within the NS4B protein of Con1) that does not interfere with production of infectious particles (Pietschmann et al. 2009). Even more striking, an adapted H77 genome designated H77S has been generated that carries multiple mutations which substantially enhance virus production and at the same time permit production of infectious virus (Yi et al. 2006; Yi and Lemon 2009). Thus, there are apparently different modes of increasing replication fitness of HCV consensus genomes in tissue culture, some of which interfere with production of virus particles. Certainly, gaining a deeper understanding of how REMs enhance replication in tissue culture is an important challenge for future research.

When Kato and colleagues in 2001 reported construction of a subgenomic replicon termed JFH1 which replicated with very high efficiency and without the requirement of adaptive mutations (Kato et al. 2001; Kato et al. 2003b; Date et al. 2004), this observation was a major finding itself. However, it turned out to be the prelude to a yet more important major breakthrough in HCV research: A few years later, three groups reported that the complete wild-type JFH1 genome or chimeras consisting of the JFH1 replicase genes NS3-NS5B and Core to NS2 regions of alternative HCV genomes replicated efficiently in Huh-7 cells and produced infectious viral progeny both in tissue culture and in animal models (Lindenbach et al. 2005; Wakita et al. 2005; Zhong et al. 2005). These particles were designated cell culture-derived HCV (HCVcc) and they are now routinely used in many laboratories. Each step of the viral life cycle can be studied with this system including viral entry, replication, and also the late events like genome packaging, virion assembly, maturation, and release. Immunoelectron microscopy with E2-specific antibodies demonstrated the presence of spherical particles with 50–65 diameter and cell culture viruses had a density profile comparable to serum-derived viruses (Lindenbach et al. 2005; Wakita et al. 2005; Zhong et al. 2005) (for detailed description of HCV particles see chapter by "Virion Assembly and Release" Lindenbach, this volume). The authenticity of recombinant virus particles was confirmed by demonstrating HCVcc infectivity in chimpanzees and in mice containing human liver xenografts (Lindenbach et al. 2005; Wakita et al. 2005; see also chapter "Animal Models for Hepatitis C" by Billerbeck et al., this volume). Interestingly, the specific infectivity of HCVcc recovered after passage in vivo was increased in comparison to cell culture produced viruses (Lindenbach et al. 2006). The highly infectious nature of the animal-derived viruses correlated with a lower buoyant density compared to cell culture-derived HCV. Interestingly, these features (lower density and higher infectivity) were lost after a single round of passaging in cell culture, suggesting that modifications that were not fixed within the viral genome were responsible for these alterations. Infection could be neutralized with patient sera or E2 and CD81-specific antibodies. Importantly, HCVcc were sensitive

to inhibitors targeting the viral protease and polymerase as well as to interferon-α (Lindenbach et al. 2005; Wakita et al. 2005; Zhong et al. 2005).

As the JFH1-based infection system belongs to genotype 2a, a major challenge is still the generation of molecular clones from other genotype supporting production of infectious virus in cell culture. As alluded to above, Yi et al. (2006) reported in 2006 an infectious clone of the genotype 1a designated H77S that contains five REMs. These mutations were selected through a tedious and iterative process of adaptation of subgenomic H77 replicons and are located therefore in the non-structural genes (Yi and Lemon 2004). However, virus titers that were recovered from transfected Huh-7.5 cells were about 100–1,000-fold lower compared to the JFH1 system. Passaging of the full-length H77S genome did not result in increased viral titers as observed for JFH1, probably due to a lower replication efficiency of H77S (Yi et al. 2006).

As mentioned above, transfection of a full-length wild-type Con1 clone resulted in a transient release of virus particles which was blocked by cell culture adaptive mutations in NS5A or NS3 (Pietschmann, Zayas et al. Pietschmann et al. 2009). Recently, another genotype 1b isolate (NC1) from a patient with acute severe hepatitis was described that shares 91 % nucleotide and 94 % amino acid sequence homology with the Con1 isolate (Date et al. 2012). The replication efficiency of a NC1 subgenomic replicon was lower compared to JFH1 but could be enhanced with the introduction of REMs. After transfection of full-length RNA only cells harboring genomes with NS5A mutations S2197 or S2204G showed significant amounts of core protein secreted into the cell culture supernatant. Next, the authors combined the identified REMs with previously described mutations in NS3 (Krieger et al. 2001) and NS4B (Lohmann et al. 2003) to increase replication and virus production. Enhanced production of infectious particles was observed, however, the efficiency was not sufficient for autonomous virus propagation in cell culture and for infectivity studies in vivo (Date et al. 2012).

4.1 JFH1 and Chimeric Genomes

Although these infection models described so far are an important achievement permitting studies of the complete HCV replication cycle in cell culture, these systems are restricted to specific isolates and limit comparable studies of all HCV genotypes. To overcome this restraint, a comprehensive panel of chimeric genomes was constructed by combining the JFH1 isolate with heterologous strains of all major HCV genotypes (Table 1). In most cases, the replicase proteins necessary for generating the membrane-bound replicase complex and nontranslated regions are derived from the highly efficient JFH1 strain. The proteins Core to NS2 which are required for viral morphogenesis (chapter "Virion Assembly and Release", this volume) are derived from another genotype. With this strategy the yield of HCVcc particles was subsequently enhanced by creating an intragenotypic chimera using the C-NS2 part of a different genotype 2a isolate, J6 (Yanagi et al. 1997; Lindenbach et al. 2005).

To analyze whether it is also possible to generate intergenotypic chimeras, an analogous chimeric genome that carried the Core to NS2 part of the GT 1b Con1 isolate was constructed (Pietschmann et al. 2006). However, although this genome produced infectious HCV, virus titers were very low probably due to incompatibilities between the Con1 (GT1b) and JFH1 (GT2a) proteins. Therefore, a series of intergenotypic reporter chimeras were generated with different cross-over sites varying from the C-terminus of E2 to the NS2-NS3 cleavage site. The results of this mapping analysis identified a cross-over site located right after the first trans-membrane domain of NS2 as the best choice for construction of infectious JFH1-Con1 chimeras JFH-J6 and JFH1-H77 chimeras (Pietschmann et al. 2006). Similar studies describing the generation of chimeric genomes of genotype 3a, 4a, 5a and finally all major seven genotypes have been reported (Pietschmann et al. 2006; Gottwein et al. 2007; McMullan et al. 2007; Yi et al. 2007; Jensen et al. 2008; Scheel et al. 2008; Gottwein et al. 2009; Gottwein et al. 2011a; Scheel et al. 2011a). These chimeric genomes were shown to be highly useful to study entry, neutralization, and virus assembly of all seven known HCV genotypes. They have been further validated to be infectious in vivo as human liver-chimeric mice developed high-titer infections after inoculation with HCV of genotypes 1–6 (Bukh et al. 2010). Highest virus titers could be achieved with a J6-JFH1 chimera designated Jc1 that allows the production of virus particles of about 10^6 infectious units per ml (Pietschmann et al. 2006).

Further improvements were the construction of reporter genomes of different HCV chimeric genomes (luciferase or GFP) for rapid and sensitive detection of replication or infection (Koutsoudakis et al. 2006; Tscherne et al. 2006; Schaller et al. 2007; Gottwein et al. 2011a; Reiss et al. 2011) (recently summarized in (Vieyres and Pietschmann 2012).

The idea of chimeric genomes was further expanded to also include non-structural proteins. As mentioned above, HCV replicons have only been described for genotypes 1 and 2 and efficiency or resistance of direct acting antiviral agents (DAAs) targeting non-structural proteins could not be tested for all genotypes. Recently, the construction of viable JFH1-based chimeras in which sequences encoding NS3/4A or NS5A were replaced with homologous sequences of other genotypes were described (Gottwein et al. 2011b; Scheel et al. 2011a, b). These technical developments that are based on adaptation approaches allow analyzing effects of antiviral compounds against NS3/4A and NS5A and antiviral resistance for all HCV genotypes in the context of infectious full-length HCV RNAs.

4.2 Adaptation of Infectious HCV Genomes to Cell Culture

The generation of chimeric genomes as discussed in the previous section is one way to increase viral yields in cell culture. However, genetic incompatibility between JFH1 and the alternative HCV genome segment fused to it often limits production of infectious virus This restriction can be overcome by serial passage of the chimeras in cell culture which over time results in the accumulation of adaptive changes

compensating the genetic differences between the fused genomes and thus increases virus yields (Abe et al. 2007; Gottwein et al. 2007; McMullan et al. 2007; Yi et al. 2007; Jensen et al. 2008; Scheel et al. 2008; Bungyoku et al. 2009; Gottwein et al. 2009; Gottwein et al. 2011a; Koutsoudakis et al. 2011; Chan et al. 2012). For instance, Yi and colleagues demonstrated that mutations in E1, p7, NS2, and NS3 contribute to the ability of a H77/JFH1 chimeric genome to assemble and release high amounts of virus particles (Yi et al. 2007). These mutations act independent of any detectable effect on viral RNA replication or polyprotein processing indicating a crucial role of these proteins in virus assembly and relase (Yi et al. 2007).

It is important to realize that the process of adapting inter- or intra-genotypic chimeric genomes is fundamentally different from the selection process which yields REM in replicons. In the former, functional incompatibility between JFH1 and the fused non-JFH1 derived proteins is overcome. In other words, the selection process "shapes" these specific partners to better cooperate in the viral replication cycle. In the latter, however, the monogenetic replicon is modified to better fit to the host cell environment (e.g., Huh-7). Likely as a consequence, most of the changes adapting a given non-JFH1 strain to the JFH1-derived NS3 to NS5B replicase in full-length chimeras are strictly chimera-specific and cannot be transferred to other chimeras. In contrast, there is a certain degree of flexibility with REMs which can be successfully transferred from Con1 to H77 and even to genotype 2a replicons (Grobler et al. 2003; Kato et al. 2003a; Maekawa et al. 2004; Ikeda et al. 2005; Abe et al. 2007; Mori et al. 2008).

Cell culture adaptations of JFH1-chimeras were mostly conducted in the highly permissive cell line Huh-7.5 and are based on the passage of JFH1 infected cells or by serial passages of viral supernatants. Due to this strategy, the selection process optimizes viral fitness of the chimera across the entire replication cycle and not only the processes of RNA translation and RNA replication are in the selection scheme of subgenomic replicons. Over time, viral variants emerge that harbor adaptive mutations leading to increased viral titers up to 100–1,000-fold over the parental genome.

Interestingly, several groups found that also the JFH1 wild-type genome can be efficiently adapted in cell culture with an increase in viral titers from 10^3 tissue culture infectious doses ($TCID_{50}$) per milliliter (mL) to 10^5–10^6 $TCID_{50}$/mL (Zhong et al. 2005; Delgrange et al. 2007; Russell et al. 2008; Kang et al. 2009; Kaul et al. 2007). This indicates that also JFH1 *per se* is not optimally suited for replication and propagation in Huh-7 cells. However, compared to all other known HCV isolates the degree of replication competence of this particular isolate in these cells is certainly unprecedented.

Titer-enhancing mutations were identified throughout the HCV genome (Core, E2, p7, NS2, NS3, NS5A, NS5B) and interestingly, repetition of an adaptation process showed that none of the mutations identified in the first experiment reappeared in the second selection (Zhong et al. 2005; Delgrange et al. 2007; Russell et al. 2008; Kang et al. 2009; Kaul et al. 2007). Thus, there are likely varying independent options to adapt JFH1 to cell culture replication in Huh-7-derived cells. Notably, adaptation of JFH1 itself yielded one of the few adaptive changes

(V2440L within domain III of NS5A) which boosts virus production not only of the construct it was selected with but also of other JFH1-based chimeras including GT1a, 1b, and 3a (Kaul et al. 2007). Therefore, this adaptive change likely optimize JFH1-based virus assembly in a manner that is compatible with divergent viral structural proteins—possibly by acting on properties of the viral replicase that generally favor assembly of infectious progeny. Notably, this mutation which is located at the P3 position of the NS5A-NS5B cleavage site was shown to delay polyprotein processing at this junction (Kaul et al. 2007). Although this alteration did not measurably change RNA replication, a subtle change in polyprotein processing could modulate interaction of the replicase with viral structural proteins and in turn efficiency of virus production. Interestingly, the very same mutation was later shown to confer partial resistance against drugs inhibiting cyclophilin A, a crucial replication co-factor of HCV (Kaul et al. 2009). Therefore, it is possible that not only RNA replication but also the efficiency of HCV assembly is modulated by cyclophilin A, possibly via modulation of polyprotein processing and folding. In many cases, however, the underlying mechanisms by which adaptive mutations facilitate production of infectious JFH1 or JFH1-chimeras are the beginning to emerge. First evidence suggests that enhanced physical interactions between the structural and non-structural proteins as well as within non-structural proteins during virus morphogenesis may be in part responsible for increased virus yields (Murray et al. 2008a; Jiang and Luo 2012). Alternatively, mutations were described which reduced cytotoxicity JFH1 (Kang et al. 2009) or increased specific infectivity of released particles likely by altered recognition of CD81 (Zhong et al. 2005; Russell et al. 2008). Notably, the G451R mutation within E2 of JFH1 falls into the latter category of mutations: First described as adaptive mutation for JFH1 by Zhong et al. (2006) additional work by Grove and Bitzegeio revealed that this mutation reduces dependence on SR-BI and increases exposure of the CD81 binding site on the virus particle (Grove et al. 2008; Bitzegeio et al. 2010). While these changes optimize cell entry in cell culture they are unlikely to confer a gain of fitness in vivo as these modification increase virus neutralization through a number of neutralizing antibodies (Grove et al. 2008; Bitzegeio et al. 2010). This example illustrates how cell culture adaptive changes increase viral fitness in tissue culture but at the same time skew viability in vivo. While such subtle changes facilitate in vitro experimentation and can give important and unique clues to the function and interplay of viral factors among themselves as well as with host determinants, this example also highlights the urgent need for better replication models based on primary HCV isolates.

4.3 HCV Trans-Complemented Particles

It is known from other plus-strand RNA viruses that assembly of progeny virus can be achieved when structural proteins are expressed in *trans* and independently from the RNA molecule that encodes the replicase proteins. This flexibility

has been widely used to generate viral vectors for gene delivery and immunization approaches (Lundstrom 2004). Subgenomic replicons of HCV contain all genetic elements needed for replication in human liver cells, while lacking the coding region of the viral structural proteins, p7 and NS2 (Lohmann et al. 1999). Consequently, these RNAs replicate in cells but are unable to produce infectious progeny virus. It was reported by several groups that this defect can be rescued by expression of the HCV structural proteins in *trans* via helper viruses or varying DNA-based expression systems in so-called in packaging cell lines (Ishii et al. 2008; Steinmann et al. 2008; Adair et al. 2009). With the production of these *trans*-complemented JFH1 particles (referred to as HCV_{TCP}) virus entry and replication can be studied independently from the late steps of the viral life cycle and thus from secondary rounds of infection. Moreover, due to the inability of HCV trans-complemented particles (HCV_{TCP}) to spread, this model has improved biosafety (Steinmann et al. 2008). Naturally occurring HCV subgenomic RNAs with different deletions in the structural proteins have been found in several patients and could also be *trans*-complemented in vitro (Pacini et al. 2009) highlighting that HCV_{TCP} can circulate in vivo and may modulate disease progression and outcome.

4.4 Permissive Host Cells

HCV replicates primarily in human hepatocytes, but multiple reports suggest that also extrahepatic reservoirs exist which may include the lymphatic system, gut, and the brain (Zignego et al. 2007; Weissenborn et al. 2009). Besides mutations that affect viral fitness in cell culture, also the host cell plays a crucial role in HCV replication. The most permissive cell line for efficient RNA replication in vitro is the human hepatoma cell line Huh-7 and its clonal descendants. Studies with HCV replicons demonstrated that only a subpopulation of Huh-7 cells allowed high levels of replication and efficiency was dependent on the cell passage number (Lohmann et al. 2003) and cell density (Guo et al. 2001; Pietschmann et al. 2001; Miyamoto et al. 2006). In fact the observation that during the replicon selection process a cell population which sustains elevated replication fitness emerges was utilized to create the most HCV-permissive cell lines currently available. Using IFN-α or a selective HCV inhibitor numerous highly permissive Huh-7-descendant cell clones were established including Huh-7.5 (Blight et al. 2002; Murray et al. 2003) and Huh-7-Lunet cells (Friebe et al. 2005) as well as other derivatives (Murray et al. 2003; Maekawa et al. 2004; Ikeda et al. 2005; Lanford et al. 2006). (Blight et al. 2002; Lohmann et al. 2003). The often dramatic differences between the permissiveness of individual Huh-7-derived cell clones including individual cell passages of the same polyclonal Huh-7 cell population highlights the strong host factor dependence of HCV the high variability of these cultured cells. As mentioned above, the molecular mechanisms that govern differential permissiveness of these cells are poorly defined. However, it is assumed that the abundance of crucial

host cell factors critical for replication plays an important role (Blight et al. 2002; Murray et al. 2003). Interestingly, in case of Huh-7.5 cells a lesion in the innate antiviral defence signaling pathway caused by a mutation in RIG-I has been implicated in the phenotype of high permissiveness of these cells (Sumpter et al. 2005). Although another group has not been able confirm a tight correlation between HCV-permissiveness and RIG-I status in these cells (Binder et al. 2007), this observation nevertheless illustrates that also lack antiviral restrictions may substantially increase permissiveness for HCV (for further details see chapter "Innate Immune Responses to Hepatitis C Virus" by Schoggins and Rice, this volume).

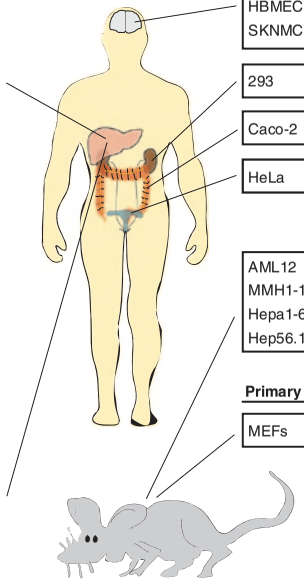
Initial approaches to establish robust HCV replication in cells other than Huh-7 were difficult. A first report that HCV replication is possible in human non-liver cells and even in non-human, mouse liver cells was published by Zhu et al. (2003) who described moderate replication of subgenomic replicons of the HCV N-isolate in Hela and the murine hepatoma cells Hepa1-6 cells. Interestingly, these cell lines were transfected with RNA isolated from stable Huh-7 cells instead of in vitro transcribed RNA to have a higher genetic variability (Zhu et al. 2003). However, unlike with selection of replicons in Huh-7 cells, in the mouse context no conserved mutations were identified that increase viral replication fitness in these non-human cells. Although the reason for this remains unclear, it is possible that the genetic barrier to adapt HCV proteins to murine replication co-factors was too high to permit selection of adaptive changes. Importantly, these findings provided formal proof that the essential host factors needed for HCV RNA replication can be found outside of human liver cells. Meanwhile numerous authors have described various alternative human liver-derived HCV-permissive cells including HuH-6 (Windisch et al. 2005), HepG2, IMY-N9 (Date et al. 2004), and LH86 (Zhu et al. 2007). Moreover, several human cells of non-liver origin are well established (Ali et al. 2004; Kato et al. 2005; Mee et al. 2009). Finally, a spectrum of non-human cells have been reported that sustain HCV replication (Zhu et al. 2003; Chang et al. 2006; Uprichard et al. 2006; Long et al. 2011) (Fig. 2).

However, it is important to note that RNA replication is in general lower in these cells, particularly in transient replication assays, compared to Huh-7-derived cell clones. HCV RNA replication has also been demonstrated in mouse embryonic fibroblast (MEFs) using JFH1 replicons (Chang et al. 2006). This study could be confirmed and extended by Lin and colleagues who showed that expression of the liver-specific miR-122 in MEFs stimulated the synthesis of HCV replicons in the rodent fibroblasts and that the combined effects of miR-122 expression and deletion of IRF-3 lead to cooperative stimulation of HCV subgenome replication (Lin et al. 2010). Therefore, MEFs now provide an important opportunity to utilize the powerful mouse genetic systems and the available mouse strains to unravel host factors that determine or preclude efficient HCV replication in these animals.

Similar to what has been observed in the replicon system, the identification of highly permissive Huh-7 cell lines is a prerequisite of a robust infection system. Huh-7.5 and Huh-7-Lunet/CD81 cells are two examples that support high levels of RNA replication and infection (Blight et al. 2002; Koutsoudakis et al. 2007). It was shown that some Huh-7 cell clones express low levels of the important HCV

Cell line	Origin	HCV isolate
Huh7-derived cell lines:		
Huh7.5 *	Hepatocellular carcinoma	Con1, H77, HCV-N, IB-1, IB-2, HCV-BK, JFH1
Huh7.5.1 *	Hepatocellular carcinoma	like in Huh7.5
Huh7-Lunet *	Hepatocellular carcinoma	Con1, JFH1
DMSO-treated Huh7 *	Hepatocellular carcinoma	JFH1
3D Huh7 culture *	Hepatocellular carcinoma	JFH1
Other human hepatoma cell lines:		
HuH6	Hepatocellular carcinoma	Con1, JFH1
HuH6-CLDN1 *	Hepatocellular carcinoma	JFH1
HepG2	Hepatocellular carcinoma	JFH1
HepG2-CD81-miR122 *	Hepatocellular carcinoma	JFH1
IMY-N9	Hepatocellular carcinoma	JFH1
LH86	Hepatocellular carcinoma	JFH1

Primary cells	Origin	HCV isolate
Fetal hepatocytes	Fetal liver	JFH1
Adult hepatocytes	Human adult liver	JFH1
Ault hepatoctes (MPCC)	Human adult liver	JFH1
Induced hepatocyte-like cells	Pluripotent stem cells	JFH1
Induced hepatocyte-like cells	Embryonic stem cells	JFH1
Human liver slices	Human adult liver	JFH1



Cell line	Origin	HCV isolate
hCMEC/D3 *	Brain microvascular endothelium	JFH1
HBMEC *	Brain microvascular endothelium	JFH1
SKNMC	Peripheral neuroblastom	JFH1
293	Embryonic kidney cells	Con1, JFH1
Caco-2	Colon adenocarcinoma	JFH1
HeLa	Cervical carcinoma	Con1, JFH1
AML12	Hepatoma cell line	JFH1
MMH1-1	Hepatoma cell line	JFH1
Hepa1-6	Hepatoma cell line	Con1, JFH1, HCV-N
Hep56.1D	Hepatocellular carcinoma	JFH1

Primary cells	Origin	HCV isolate
MEFs	Embryonic fibroblast	JFH1

Fig. 2 Primary cells and cell lines supporting HCV replication. The origin of human and murine cell lines permissive for HCV replication is given. Cells reported to support the complete HCV replication cycle including cell entry, RNA replication, and de novo production of infectious viral progeny are marked by an asterisk. Note that permissiveness between cells varies greatly and is generally highest in Huh-7-derived cell clones. References to the individual reported cell lines are given in the text

entry factor CD81 and that ectopic expression of this tetraspanin leads to much higher viral spread and infection events (Akazawa et al. 2007; Koutsoudakis et al. 2007). Conversely, selection of Huh-Lunet cell clones essentially lacking CD81 expression permitted receptor complementation studies for this important entry factor and the analysis of HCV cell-to-cell spread in the presence or absence of CD81. In a similar fashion, we have now HCV-permissive cell lines available permitting receptor complementation assays for SR-BI (Dreux et al. 2009; Catanese et al. 2010), CLDN1 (Haid et al. 2010), and OCLN (Ciesek et al. 2011b), thus greatly facilitating HCV cell entry studies with HCVcc. In parallel, a novel human hepatoma cell line, named LH86, was demonstrated to be permissive and susceptible to HCVcc (Zhu et al. 2007) and overexpression of CD81 and miR122 rendered HepG2 cells which were initially refractory to HCVcc infection fully permissive to HCV propagation (Narbus et al. 2011). Since the latter cells are known to polarize in cell culture HepG2-CD81-miR122 cells provide a unique opportunity to assess HCV infection and replication in polarized cells.

Instead of construction of reporter viruses, host cells can be modified for rapid and sensitive scoring of HCV infection events especially in a high-throughput format. One reported assay is based on a reporter cell line stably expressing the enhanced green fluorescent protein (EGFP) fused in-frame to the secreted alkaline phosphatase (SEAP) via a recognition sequence of the viral NS3/4A serine protease (Iro et al. 2009). Upon HCV infection and cleavage of the NS3/4A protease SEAP is released into the cell culture supernatant. Cell lines were also engineered to express the pro-apoptotic factor n4mBid, where NS3-dependent cleavage and activation led to an easily measurable cytopathic effect (Chockalingam et al. 2010).

Although HCV infects mainly hepatocytes, there is evidence for the existence of non-hepatic reservoirs suggesting that the virus might have a broader cell tropism. Genomic viral RNA could be detected in peripheral blood mononuclear cells (PBMCs) and negative-strand RNA and HCV were reported in brain autopsies of HCV-infected patients with neuropathological abnormalities [reviewed in (Morgello 2005; Weissenborn et al. 2009)]. Additionally, microscope techniques and strand-specific detection of HCV showed that microglia and macrophages are the dominant brain cell population positive for HCV (Wilkinson et al. 2009). Interestingly, there seemed to be differences between HCV sequences in the brain and those circulating in plasma (Radkowski et al. 2002; Fishman et al. 2008; Murray et al. 2008b) strengthening the possibility of HCV replication in cells of CNS origin. Direct detection of HCV antigens in the brain remains technically challenging as reported for the liver probably due to low HCV replication. However, it could recently be shown by in vitro studies that two neuroepithelioma cell lines express all HCV receptors essential for viral entry (Fletcher et al. 2010; Burgel et al. 2011) and support RNA replication (Fletcher et al. 2010). These cell lines were the first extrahepatic cells that sustain HCV infection without ectopic expression of cellular factor required for viral entry (Lindenbach 2010). HCV tropism for the brain is further supported by a recent study by Fletcher et al. (2012) demonstrating productive HCV infection of brain microvascular endothelial cells (BMEC), a major component of the blood/brain barrier. In this study, two independent-derived brain microvascular

endothelial cell lines were described to express all HCV receptor molecules and could be infected with HCVpp and HCVcc.

Collectively, the cell tropism of HCV could be expanded to several other human liver cell lines plus murine hepatoma cells and non-liver cell lines. With the discovery of novel HCV-specific dependency and restriction factors and genetic modifications of host cells further in vitro systems that sustain the entire HCV life cycle in cell culture are in development.

5 HCV Replication Models in Primary Cells and Patient Isolates

Studies on virus–host interactions have been hampered by limited in vivo and ex vivo models that mimic the natural environment of the liver. Due to the narrow host tropism of HCV small animal models are challenging and primary HCV isolates show a poor ability to replicate in tissue culture.

One drawback of human hepatoma cell lines like Huh-7 and its derivatives is that they do not polarize or express markers of mature hepatocytes and therefore may not fully recapitulate the polarized status of human hepatocytes in vivo (Decaens et al. 2008). However, chemical treatment or ectopic expression of host factors required for viral propagation may overcome some of these hurdles. Interestingly, treatment of human hepatoma cells with 1 % dimethyl sulfoxide was shown to differentiate cells in culture, induce the expression of hepatocyte-specific genes, and arrest cell growth (Sainz and Chisari 2006). These more hepatic-like cell cultures were still highly permissive for HCV infection and represent a more physiological relevant system compared to dividing Huh-7 cells and allow studies, e.g., of HCV persistence (Bauhofer et al. 2012) HepG2 cells can also grow in a polarized manner that mimics the bile canalicular configuration of hepatocytes. After ectopic expression of CD81 these cells have been used as a model of polarized culture to study HCV, however, these cells weakly support HCV RNA replication (Lindenbach et al. 2005; Flint et al. 2006; Mee et al. 2009). Overcoming this bottleneck, it was recently reported that the ectopic expression of miR122 in HepG2 cells permitted efficient RNA replication and support the entire HCV life cycle (Narbus et al. 2011). Furthermore, the growth of hepatoma cells in 3D cultures can also resemble the natural host cells of HCV in vivo and can be used to study HCV infections (Sainz et al. 2009; Molina-Jimenez et al. 2012). A human liver progenitor cell line, named HepaRG, can be differentiated into hepatocytes with specific cell culture conditions (Parent et al. 2004) and has been widely used for HBV infection experiments. It could now be shown that these cells are also susceptible to serum-derived HCV particles and support long-term production of viral particles, albeit at very low levels (Ndongo-Thiam et al. 2011).

Primary human hepatocytes (PHH) provide the closest in vitro model for the natural host cell of HCV. However, their use in HCV research is limited as PHH are difficult to obtain. In addition to limited availability, these cells have high donor variability, and the rapid loss of their differentiation status complicates tissue culture

experiments. Nevertheless, several groups reported infection of cultured PHH using sera from HCV-infected patients and could demonstrate CD81- and LDL receptor-dependent entry of serum-derived particles or inhibition of HCV replication by interferon. However, in general low-level replication was observed and results were difficult to reproduce (Carloni et al. 1993; Iacovacci et al. 1993; Fournier et al. 1998; Rumin et al. 1999; Lazaro et al. 2007; Buck 2008; Molina et al. 2008). Recently, a study by Podevin et al. (2010) described a method of culturing PHH with hepatocyte-specific gene expression for up to 2 weeks. Importantly, under these conditions PHH supported the complete infectious cycle of HCV, including production of new progeny virus, termed primary culture-derived HCV (HCVpc). The authors further could show that HCVpc had a lower average buoyant density and a higher specific infectivity than HCVcc particles produced in Huh-7 cells (Podevin et al. 2010). A limitation of this study is still that this model is restricted to cell culture-derived viruses and it is unclear if also patient-derived viruses can be propagated in this system. A very recent study which is based on ex vivo human adult liver slices demonstrated a productive infection using human primary isolates of genotype 1b as well as JFH1 viruses and genotype 1 JFH1 chimeric genomes (Lagaye et al. 2012). This new experimental model system in which viral titers above 10^5 ffu/ml were achieved allows in addition the validation of antiviral drugs.

Improvements of PHH cultivation were also made with the addition of non-parenchymal feeder cells to hepatocytes in micopatterned co-cultures (MPCC) (Ploss et al. 2010). MPCCs displayed hepatic function for several weeks, could be adapted to high-throughput format and were susceptible to HCV infection with limited virus spread for cell culture- and patient-derived viruses (Ploss et al. 2010). HCV detection techniques in PHH such as RNA quantification turned out to be sensitive but at the same time limited due to high background of input RNA. Detecting HCV infection events can be facilitated with a novel cell-based reporter system, in which the NS3/4A protease cleaves a fluorescent substrate that then relocated the reporter signal from a mitochondrial localization to the nucleus (Jones et al. 2010). This method permits visualizing HCV infection events in Huh-7 cells as well as PHH and could be extended to several other cell culture systems (Jones et al. 2010; Ploss et al. 2010).

Robust experimental model systems to study the role of host genetics like *IL-28B* polymorphism are restricted to needle biopsies, surgical resections, and organ donation. Two recent studies by Schwartz et al. (2012) and Wu et al. (2012) reported that hepatocyte-like cells derived from induced pluripotent stem cells (iPSC) allowed the productive infection with HCV, including inflammatory responses to infection. This novel development of an iPSC model can have the potential to study the impact of host genetics on hepatitis viral pathogenesis (Schwartz et al. 2012; Wu et al. 2012). Furthermore, these pluripotent stem cells can be genetically modified prior to differentiation and used to generate, e.g., HCV-resistant hepatocytes. Importantly, these hepatic-like cells also permitted direct infection by patient sera. Similar findings were also recently reported with human embryonic stem cells (hESC)-derived hepatocytes demonstrating that these cells could be infected with JFH1 viruses and supported the complete HCV replication cycle (Roelandt et al. 2012).

In conclusion, recent advances in the development of more physiologically relevant infection systems will advance our understanding of host–pathogen interactions in the liver.

6 Future Perspectives and Conclusions

After the molecular cloning of the HCV genome, it took more than a decade to establish functional cell culture systems for this human pathogen. Since then, step-by-step improvements were achieved that finally led to an infection system covering every step of the viral life cycle. Further improvements allow now to apply all virological techniques to study viral replication facilitating drug discovery. Chimeric genomes were created in which the structural proteins of all genotypes were fused to the JFH1 replicase, however, the challenge remains to propagate additional genotypes in vitro. Moreover, development of cell-based models to culture primary HCV isolates across different genotypes would open novel and unique perspectives to investigate viral determinants responsible for the different natural course and treatment outcome of hepatitis C. As Huh-7-derived cell lines do not recapitulate the functional liver tissue including differentiation and polarization more relevant host cell system are needed. Along these lines, a robust supply of primary human cells with differentiated hepatocyte function and morphology would greatly facilitate our ability to study the relevance of host factors for replication and pathogenesis of HCV. The recent exciting reports about HCV replication in human stem cell-derived cells raise hopes that these models are within reach and will permit robust molecular studies of pathogenesis and replication mechanisms. These achievements will further close the gap between in vitro studies and the clinical situation of HCV infections.

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Animal Models for Hepatitis C

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Abstract Hepatitis C remains a global epidemic. Approximately 3 % of the world's population suffers from chronic hepatitis C, which is caused by hepatitis C virus (HCV)—a positive sense, single-stranded RNA virus of the *Flaviviridae* family. HCV has a high propensity for establishing a chronic infection. If untreated chronic HCV carriers can develop severe liver disease including fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Antiviral treatment is only partially effective, costly, and poorly tolerated. A prophylactic or therapeutic vaccine for HCV does not exist. Mechanistic studies of virus-host interactions, HCV immunity, and pathogenesis as well as the development of more effective therapies have been hampered by the lack of a suitable small animal model. Besides humans, chimpanzees are the only species that is naturally susceptible to HCV infection. While experimentation in these large primates has yielded valuable insights, ethical considerations, limited availability, genetic heterogeneity, and cost limit their utility. In search for more tractable small animal models, numerous experimental approaches have been taken to recapitulate parts of the viral life cycle and/or aspects of viral pathogenesis that will be discussed in this review. Exciting new models and improvements in established models hold promise to further elucidate our understanding of chronic HCV infection.

Abbreviations

AAT	Alpha-1 antitrypsin
ALT	Alanine transaminase
CD	Cluster of differentiation
CHV	Canine hepacivirus

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CLDN1	Claudin 1
CTL	Cytotoxic T lymphocyte
DAA	Directly acting antiviral
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
ES cell	Embryonic stem cell
FAH	Fumaryl acetoacetate hydrolase
Flt3-L	Fms-like tyrosine kinase receptor-3 ligand
GBV	George Baker virus
GM-CSF	Granulocyte–macrophage colony-stimulating factor
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIS	Human immune system
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSV-TK	Herpes simplex virus thymidine kinase
HVR1	Hypervariable region 1
IFN	Interferon
IL	Interleukin
IL-2Rγ^{null}	Interleukin 2 receptor gamma deficient
iPSC	Induced pluripotent stem cell
JFH	Japanese patient with fulminant hepatitis
IRF	Interferon regulatory factor
LDL-R	Low density lipoprotein receptor
MAVS	Mitochondrial antiviral signal protein
MHC	Major histocompatibility complex
MUP	Major urinary protein
NK	Natural killer cell
NANB	Non-A/non-B hepatitis
NOD	Non-obese diabetic
NS	Non-structural protein
NPHV	Non-primate hepacivirus
NPC1L1	Niemann-Pick C1-like 1
NTBC	2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione
OCLN	Occludin
PI4IIIα	Phosphatidylinositol 4 kinase III alpha
PKR	Protein kinase R
Rag	Recombinase activating gene
SCARB1	Scavenger recepter type B class I
SCID	Severe combined immunodeficiency
SCF	Stem cell factor
SIRP-α	Signal regulatory protein alpha
SVR	Sustained virologic response
TNF	Tumor necrosis factor

TPO	Thrombopoietin
uPA	Urokinase plasminogen activator
UTR	Untranslated region
VLDL	Very low-density lipoprotein

Contents

1 Introduction

At least 130 million individuals are chronically infected with hepatitis C virus (HCV). Chronic HCV carriers are at risk of developing severe liver disease including fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Epidemiological data, however, are incomplete and the actual number in the United States alone may be twice as high as currently estimated (Edlin 2011).

Since the discovery of HCV as the etiologic agent for non-A, non-B (NANB) viral hepatitis in 1989 (Choo et al. 1989) numerous experimental tools have been developed enabling a detailed analysis of various aspects of the viral life cycle and interaction of the virus with its human host. Human liver cancer cell lines, in particular Huh7 cells and its derivatives, have been the workhorse of in vitro HCV research (see chapter "Cell Culture Systems for Hepatitis C Virus" by Steinmann and Pietschmann, this volume). The development of robust primary hepatocyte culture platforms has facilitated the study of the virus in a more physiological environment (reviewed in Sheahan et al. 2010). The complexity of the host responses and clinically relevant disease phenotypes, however, cannot be readily recapitulated in cell culture. At the organism level, analysis of patient samples has provided valuable insights into the natural history of infection and has helped to characterize host responses and pathogenesis. Clinical research, however, is inherently hampered by the heterogeneity of human study cohorts, restricted access to relevant tissue compartments—the liver—and the limited control over critical experimental parameters including the infection time and dose. HCV has a very narrow host range limited to

humans and chimpanzees. The ability to experimentally infect chimpanzees has shed light on the host immune response and aided the preclinical assessment of drug and vaccine candidates (reviewed in Bukh 2004). However, studies in these large apes are hampered by limited availability, high costs, and ethical concerns, which have led to a ban on the use of these animals for biomedical research in most countries.

Despite substantial efforts to develop antivirals directly blocking replication, treatment options remain limited. The current standard of care, consisting of pegylated interferon (IFN) alpha, ribavirin, and one of two protease inhibitors (telaprevir or boceprevir), has significant side effects and is not effective in all individuals (see chapter "Treatment of Chronic Hepatitis C: Current and Future" by Pawlotsky, this volume). Numerous compounds that have the potential to improve treatment response rates and achieve a cure with fewer side effects are in development. Although most candidates that are currently filling the drug pipeline have been advanced into clinical trials without prior efficacy testing in animal models, preclinical testing of new compounds and/or drug combinations in predictive models is likely to increase the chances of successfully bringing a product to market. However, it remains unclear how highly potent antiviral drug cocktails will affect the global HCV disease burden. High costs, lack of adequate infrastructure for distribution, and medical supervision may lessen the impact of future therapies in resource poor environments. Thus, the development of a potent, pan-genotypic cost-effective therapeutic and/or prophylactic vaccine remains a priority to conquer the global HCV health problem. The development of vaccines would benefit from the availability of suitable HCV animal models. For instance, due to the complexity of the immune system, responses to vaccines are difficult to predict and can hardly be modeled in vitro. Vaccine vectors, dosing regimen, and vaccination schedules often are needed to be empirically optimized, not only to achieve maximal immunogenicity, but to also to capture vaccine-induced protection to viral challenge.

Undoubtedly, mechanistic studies of HCV biology as well as the development of therapeutics and/or preventative vaccines would likely benefit from readily accessible animal models. In this review, we summarize the existing animal models for HCV (Table 1), discuss different approaches toward more tractable system and highlight the areas of current and future improvements that are needed to adequately mimic human disease in experimental in vivo systems.

2 Non-human Primate Models for Hepatitis C

2.1 Chimpanzees

Chimpanzees (*Pan troglodytes*) are the closest living relative to humans with more than 98 % genetic identity. Not surprisingly, chimpanzees are the only species besides humans that is readily susceptible to HCV infection. Chimpanzees have played a pivotal role in the discovery of HCV as the etiological agent of NANB viral hepatitis (reviewed in Houghton 2009). Until the advent of the infectious cell

Table 1 Current and future models for the study of HCV infection

	Human	Chimpanzee	Tree shrew	Humanized mice xenotransplantation	Humanized mice genetic humanization	Mice viral adaptation
						
HCV infection	Yes	Yes	Yes	Yes	Entry only	Unknown
HCV pathogenesis	Fibrosis cirrhosis HCC	Milder than in humans	Evidence for hepatitis, fibrosis cirrhosis	Evidence for fibrosis	No	Unknown
Immune system	Human	Chimp	Tupaia	Human	Mouse	Mouse
Costs	High	High	Medium	Medium	Low	Low
Throughput	Low	Low	Low	Low	High	High
Genetic manipulation	No	No	No	Yes (limited)	Yes	Yes
Drug/Vaccine development	Yes	Yes	Unkown	Yes (inhibitors)	Yes (entry inhibitors)	Unknown

culture system, the chimpanzee was a central experimental model faithfully recapitulating the entire HCV life cycle. Infection can be initiated by intravenous inoculation with clinical isolates (Alter et al. 1978; Tabor et al. 1978), tissue culture derived virus (Lindenbach et al. 2006; Wakita et al. 2005), or intrahepatic injection of in vitro transcribed RNA of infectious HCV cDNA clones (Kolykhalov et al. 1997). The ability to test infectivity of molecular HCV clones in chimpanzees has permitted for the first time reverse genetic studies to determine the significance of viral genetic elements for virus infectivity (reviewed in Murray and Rice 2011).

The clinical course of HCV infection in chimpanzees has been characterized in detail (reviewed in Bukh 2004). Usually, within 1 week post infection viremia becomes detectable in circulation reaching peak titers of 10^5–10^7 genome copies per ml. Infection induces significant innate and adaptive immune responses measurable in peripheral blood and the liver (Bigger et al. 2001; Cooper et al. 1999; Major et al. 2004; Thimme et al. 2002) resulting frequently in hepatitis as evidenced by elevated concentrations of liver enzyme in serum and inflammatory infiltrates in the liver (see chapters "Innate Immune Responses to Hepatitis C Virus", by Schoggins and Rice and "Adaptive Immune Responses in Hepatitis C Virus Infection" by Neumann-Haefelin and Thimme, this volume). While acute hepatitis is somewhat milder in chimpanzees than in humans, experimentally infected animals also frequently progress to chronicity (Abe et al. 1992; Bassett et al. 1998; Forns et al. 2002). The chronic phase of disease in chimpanzees is characterized by persistent viremia, the emergence of viral escape mutations in T cell epitopes and mild hepatitis (Erickson et al. 2001; Major et al. 2004; Thomson et al. 2001). Chimpanzees rarely develop more severe disease including fibrosis, cirrhosis, and hepatocellular carcinoma, which are major clinical complications of hepatitis C in humans. Subclinical hepatitis, after many years of chronicity, however is also common in humans without risk factors for rapid progression, such as older age or alcohol intake. The chimpanzee model has been of critical importance for defining the nature of protective immunity following re-infection. Experiments in chimpanzees demonstrated that clearance of a primary infection with HCV does not provide complete protective immunity against challenges with homologous or heterologous viruses (Farci et al. 1992; Prince et al. 1992). However, HCV serum viremia was often significantly shorter following re-infection and hepatitis was rarely observed. Depletion of CD4+ or CD8+ T cells with specific antibodies prolonged HCV viremia after rechallenge (Grakoui et al. 2003; Shoukry et al. 2003), thus highlighting the critical role of both lymphocyte subsets in containing HCV infection.

Proof-of-concept for numerous novel therapeutic modalities against HCV has been shown in chimpanzees. For example, first evidence that interferon-free control of HCV infection is possible with combinations of directly acting antivirals (DAAs; Olsen et al. 2011) and that interference with the liver specific microRNA122 (miR122) can prevent viral replication was shown in the chimpanzee model (Lanford et al. 2010). Due to their ability to mount virus-specific immune responses, chimpanzees have been particularly useful to assess the preclinical efficacy of vaccine candidates (reviewed in Houghton 2011). Vaccination with adjuvanted recombinant envelope E1/E2 glycoproteins (Choo et al. 1994) or prime/boost immunization regimens using defective adenoviruses expressing

non-structural genes (Folgori et al. 2006) has not led to induction of sterilizing immunity, but has prevented chronicity following experimental infection in chimpanzees.

Overall, the chimpanzee has been an important tool for HCV research and remains the gold standard for all other animal models. However, high costs and growing ethical concerns will limit access to the chimpanzee model, and thus development of suitable alternatives is critical.

2.2 Constraints of Other Non-human Primate Models

To determine the natural host range and to identify other experimental models various species have been challenged with HCV. Woodchucks, old- and new world monkeys, including Cynomolgus monkeys, Rhesus monkeys, Japanese monkeys, Green monkeys, Doguera (Abe et al. 1993), Chacma Baboons (Sithebe et al. 2002), Cottontop tamarins (Garson et al. 1997), and marmosets, which have been tested to date, appear to be mostly resistant to HCV infection. The barriers in transmission of HCV to other monkey species are poorly defined. Most simian orthologs of HCV entry factors share a high degree of sequence similarity with humans and chimpanzees. Some evidence has been presented that these orthologs can support viral entry. For example, it was previously demonstrated that CD81 of African Green monkeys can bind soluble HCV E2 efficiently (Flint et al. 2006). Furthermore, orangutan and Rhesus macaque occludin (OCLN) can mediate HCV glycoprotein specific uptake (Michta et al. 2010). While HCV may be capable of entering simian hepatocytes, different kinetics, and magnitudes of antiviral defenses in monkey cells may antagonize viral RNA replication. In support of this hypothesis, it was recently shown that the mitochondrial antiviral signal protein (MAVS, also known as IPS1, VISA or Cardiff) from multiple primates is resistant to inhibition by the HCV NS3/4a protease (Patel et al. 2012). This resistance maps to single changes within the protease cleavage site in MAVS, which protect MAVS from getting cleaved by the HCV protease. While these data are intriguing a more comprehensive analysis of species-specific barriers limiting HCV infection in closely related non-human primates is still needed to fully explain their resistance phenotype.

3 Tree Shrews

The tree shrew (*Tupaia belangeri*) has shown to be susceptible to HCV infection, and is considered a putative candidate for a small animal model of HCV infection. Tree shrews are small squirrel-like mammals, belonging to the family of *Tupiidae*, which are indigenous in Southeast Asia. It was demonstrated that Tupaia orthologs of critical HCV entry factors support viral uptake (Jia et al. 2008; Tian et al. 2009; Tong et al. 2011), and consequently HCV is capable of entering primary Tupaia hepatocytes (Barth et al. 2005; Guitart et al. 2005; Zhao et al. 2002). In a challenge study,

intermittent and transient HCV viremia was documented in 2/10 animals challenged with a serum mixture of HCV genotypes 1a, 1b, and 3 and in 8/23 animals infected with genotype 1b (Xie et al. 1998). Immunosuppression of animals by whole body X-ray irradiation resulted in increased infection frequency of 50 % (2/4 animals), suggesting that anti-viral immune responses may suppress infection. In two follow up reports, infection rates of more than 80 % were observed in tree shrews challenged with patient or cell-culture derived HCV (Amako et al. 2010; Xu et al. 2007) without the need for immunosuppression. Infected animals frequently progressed to low level, intermittent viremia reaching peak viral loads between 10^2 and 10^5 international units per ml serum. Acute infection was associated in all three studies with mild hepatitis. Interestingly, some animals developed severe liver disease including steatosis, fibrosis, and cirrhosis after 3 years following initial infection (Amako et al. 2010).

These data are encouraging but the natural history of infection in this species needs to be validated in larger cohorts. Tree shrews are more accessible than chimpanzees and can be subjected to terminal experimentation. However, their use for a mechanistic analysis of HCV pathogenesis will be challenging, as they cannot easily be genetically manipulated, the limited availability of tupaia-specific reagents and as an outbred species, they are genetically heterogeneous.

4 HCV Orthologs

4.1 GB Virus B

Genetically, closely related viruses have been proposed as potential surrogate models for the study of HCV infection (see chapter "The Origin of Hepatitis C Virus" by Simmonds, this volume). GB viruses are named after the surgeon George Barker (GB) who was diagnosed with acute hepatitis in 1966 (Deinhardt et al. 1967). Blood from this patient was used to inoculate marmosets (*Callithrix jacchus*) intravenously. The monkeys developed acute hepatitis and infection was transmissible from monkey to monkey. Approximately 25 years later, two viral genomes, GBV-A and GBV-B, were identified in sera taken from acutely infected marmosets (Simons et al. 1995). While both viruses were able to replicate in marmosets, only GBV-B caused hepatitis. Besides marmosets, (Lanford et al. 2003) several other species of New World monkeys, including tamarins (*Saguinus sp.*) (Karayiannis et al. 1989; Schaluder et al. 1995) and owl monkeys (*Aotus trivirgatus*) (Bukh et al. 2001) were shown to be susceptible to GBV-B.

GBV-B belongs to the closely related *Flaviviridae* family. The genomes of HCV and GBV-B are identically organized. The entire genome of both viruses contains a single, long open reading frame that is flanked by 5′ and 3′ untranslated regions (UTR); the structural proteins are located toward the N terminus and the non-structural proteins toward the C terminus of the resulting polyprotein (Muerhoff et al. 1995). Despite this overall similarity, GBV-B and HCV share only 28 % amino acid identity in their polyproteins and even less in the 5′ and 3′ non-coding regions. This

makes the potential usage of GBV-B for drug testing problematic as therapeutics are usually highly virus specific. Similarly, antigenic variation across these related, but nonetheless genetically distinct, viruses poses challenges for vaccine development. To address these shortcomings GBV-B/HCV chimeras have been constructed. These constructs may help to examine specific functions of HCV genes within the GBV-B backbone. For example, it was demonstrated that chimeric GBV-B genomes containing sequences of the HCV 5′UTR (Rijnbrand et al. 2005), p7 (Griffin et al. 2008) or hypervariable region 1 (HVR1) (Haqshenas et al. 2007) are infectious in vivo.

The natural course of GBV-B also differs significantly from that of HCV. In contrast to HCV, GBV-B causes primarily acute, self-limiting infection in marmoset and tamarins, but usually does not become chronic. Although a case of long-term persistence of GBV-B in a tamarin infected by intrahepatic inoculation of synthetic viral RNA has been reported (Martin et al. 2003). Taken together, important differences at the molecular level and overall in pathogenesis lessen the utility of GBV-B as a surrogate for HCV.

4.2 Canine/Non-primate Hepaciviruses

More recently, canine hepacivirus (CHV) was isolated from the respiratory tract of dogs (Kapoor et al. 2011), which is phylogenetically the closest homolog of HCV (see chapter "The Origin of Hepatitis C Virus" by Simmonds, this volume). The natural host appears to be broader, as highly similar non-primate hepaciviruses (NPHV) have been identified in horses (Burbelo et al. 2012). Experimental evidence that CHV causes hepatitis has yet to be shown. Genetic and biological characterization of these new hepaciviruses may contribute to our understanding of the origins of HCV infection in humans.

5 Rodent Models

Mice have a long-standing track record in biomedical research. A plethora of tools for the analysis of biological processes in mice of genetically defined backgrounds are available. However, mice are not readily susceptible to HCV infection. Taking advantage of sophisticated mouse genomic manipulation technologies, various approaches have been explored to model HCV infection and pathogenesis in rodents (Fig. 1), which will be discussed in the subsequent sections.

5.1 Transgenic Animals for the Study of Hepatitis C

In the absence of a permissive mouse model, numerous transgenic strains expressing individual or combinations of HCV gene products (Table 2 and reviewed in Kremsdorf and Brezillon 2007) were developed to study HCV-induced liver

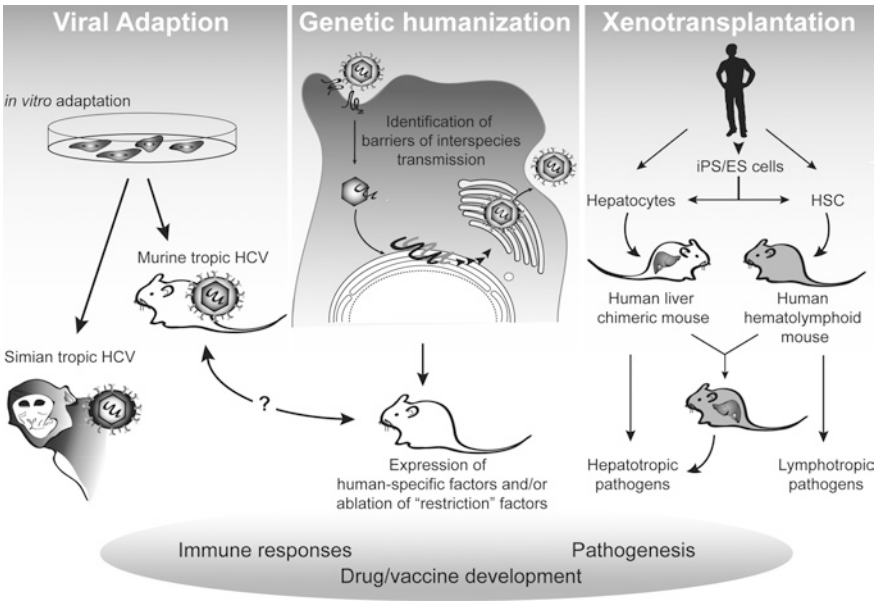

Fig. 1 Different approaches to create new mouse models for the study of hepatitis C: viral adaptation, genetic humanization and xenotransplantation

disease. Reports on the histopathology in HCV protein transgenic mice differ vastly between mouse strains depending on the expressed HCV gene product, mouse background or promoter differences used for the expression of viral proteins. For example, expression of HCV core driven by a HBV promoter was reported to result in severe liver disease, including the development of hepatocellular carcinoma (HCC) (Moriya et al. 1997, 1998). However, in other mice expressing core and/or E1/E2 under the control of the major urinary protein (MUP) or CMV promoter, evidence for no to various degrees of liver disease became apparent (Benali-Furet et al. 2005; Chang et al. 2008; Chang et al. 2009; Chiyo et al. 2011; Jeannot et al. 2012; Kamegaya et al. 2005; Lerat et al. 2009; Naas et al. 2005; Pasquinelli et al. 1997; Satoh et al. 2010; Tanaka et al. 2008). Similarly, in some instances NS5A expression induced a range of liver pathologies in transgenic mice (Wang et al. 2009), while in others NS5A expression under the control of an apoE or MUP promoter was neither directly cytopathic nor oncogenic (Majumder et al. 2003). Transgenic expression of NS3/NS4A or NS4B has not been associated with liver pathology in any mouse model so far (Desai et al. 2011; Frelin et al. 2006; Wang et al. 2006).

Expression of HCV proteins has proven to be useful for studying intrahepatic, virus-specific adaptive immune responses against HCV (Alonzi et al. 2004; Disson et al. 2004; Ernst et al. 2007; Furutani et al. 2006; Kanda et al. 2009; Kriegs et al. 2009; Naas et al. 2010; Takaku et al. 2003; Tsukiyama-Kohara et al. 2011; Tumurbaatar et al. 2007; Wegert et al. 2009). The murine immune system is

Table 2 HCV transgenic mouse models

Transgene	Phenotype	References
Core	Hepatic steatosis, HCC, hepatocyte apoptosis, lipogenesis, cell cycle perturbation, and ER stress	Benali-Furet et al. (2005), Chang et al. (2008), Chang et al. (2009), Lerat et al. (2009), Moriya et al. (1997, 1998), Tanaka et al. (2008)
Core-E2	No evidence for liver disease	Pasquinelli et al. (1997)
Core-E1-E2	Contradicting findings ranging from lacking liver pathology to development of HCC	Kamegaya et al. (2005), Naas et al. (2005), Naas et al. 2010
Core-E1-E2-NS2	Reduced liver inflammation in transgenic mice compared to controls	Chiyo et al. (2011), Satoh et al. (2010)
E1-E2-P7	Induction of liver tumors by aflatoxin B1	Jeannot et al. (2012)
E1-E2-NS2	Liver injury due to induction of CTL responses	Takaku et al. (2003)
NS3/4A	Resistance to TNFa-induced liver disease, differential IFN-induced autophagy	Ahlen et al. (2009), Desai et al. (2011), Frelin et al. (2006)
NS4B	No evidence for liver disease	Wang et al. (2006)
NS5A	Contradicting findings regarding the occurrence of liver pathology, inhibition of IFN-γ induction	Kanda et al. (2009), Kriegs et al. (2009), Majumder et al. (2003), Wang et al. (2009)
HCV polyprotein	Impaired clearance of HCV transgene-positive hepatocytes, hepatic steatosis and lymphocyte infiltrates, lymphomagenesis, interruption of type 1 IFN production, and ER stress and hepatocyte apoptosis	Alonzi et al. (2004), Disson et al. (2004), Ernst et al. (2007), Furutani et al. (2006), Tsukiyama-Kohara et al. (2011); Tumurbaatar et al. (2007), Wegert et al. (2009)

usually tolerized against HCV proteins, which are prenatally expressed in transgenic mice. However, DNA vaccination can break tolerance and cytotoxic T cells were shown to clear NS3/4A-expressing hepatocytes (Ahlen et al. 2009).

However, transgenic models have numerous caveats that lessen their utility. Often several copies of the transgene cassette integrate randomly throughout the mouse genome. High transgene copy numbers along with the use of strong viral or cellular promoters results in HCV protein expression levels that massively exceed the amount of viral proteins present during normal infection. Transgenic HCV protein expression also does not properly mimic the inflammatory milieu, which is established in the liver during acute and chronic viral infection. Consequently, while some of the histopathology observed in HCV protein transgenic mice is reminiscent of clinical features of hepatitis C, the pathways leading to the respective phenotypes may vastly differ. In summary, despite contradictory results HCV transgenic mouse models have helped to gain a better understanding of the mechanisms involved in HCV-induced pathogenesis. However, interpretation of liver disease phenotypes must take into account variations in the genetic background of mice, constitutive transgene expression and the development of histopathology in the absence of chronic liver inflammation.

5.2 Toward a Genetically Humanized Mouse Model for HCV Infection

Genetic host humanization, i.e. expression of human host factors and/or inactivation of inhibitory murine molecules has been proven successful in various infectious diseases, e.g. Polio (Ren et al. 1990), *Listeria monocytogenes* (Lecuit et al. 1999), and HIV (Browning et al. 1997). However, this approach requires an in-depth knowledge of the barriers that restrict species tropism. For HCV, the narrow host range is incompletely understood and the viral life cycle is blocked or insufficiently supported at multiple steps in murine cells. HCV utilizes numerous host proteins to enter hepatocytes including glycosaminoglycans (GAGs) (Barth et al. 2003; Koutsoudakis et al. 2006), the low density lipoprotein receptor (LDL-R) (Agnello et al. 1999; Molina et al. 2007; Monazahian et al. 1999; Owen et al. 2009), the high density lipoprotein receptor scavenger receptor class B type I (SCARB1; Scarselli et al. 2002), tetraspanin CD81 (Pileri et al. 1998), and two tight junction (TJ) proteins, claudin-1 (CLDN1; Evans et al. 2007) and occludin (OCLN; (Liu et al. 2009; Ploss et al. 2009)) (see chapter "Hepatitis C Virus Entry" by Zeisel et al., this volume). CD81, SCARB1, CLDN1, and OCLN constitute the minimal set of host factors required for uptake into mouse cell lines, but CD81 and OCLN need to be of human origin to overcome the species barrier (Ploss et al. 2009). While the overall sequence similarity between mouse and human orthologs of both molecules is high, differences in critical residues in the second extracellular loops of CD81 (Flint et al. 2006; Higginbottom et al. 2000) and OCLN (Michta et al. 2010) likely account for the entry block into rodent cells. More recently, additional host factors including the cholesterol absorption receptor Niemann-Pick C1-like 1 (NPC1L1; Sainz et al. 2012) and two receptor tyrosine kinases, epidermal growth factor receptor (EGFR; Lupberger et al. 2011), and EphrinA2 (Lupberger et al. 2011) have been implicated in the viral uptake pathway into human cells. However, it is unclear whether they contribute to limiting HCV species tropism into murine cells.

Building on the previous observation that CD81 and OCLN comprise the minimal set of human factors required to render mouse cells permissive for HCV entry in vitro, a genetically humanized mouse supporting HCV entry was constructed (Dorner et al. 2011). It was shown that expression of human CD81 and OCLN appears to be sufficient to allow HCV infection of fully immunocompetent inbred mice. This model allowed for the study of HCV entry in vivo and to assess the efficacy of entry inhibitors and vaccine candidates (Dorner et al. 2011; Giang et al. 2012). The transient adenoviral delivery approach is high throughput and allows rapid evaluation of mutant genes. However, to limit variability and prevent vector-mediated immune activation, stable expression of HCV entry factors in transgenic and/or knock-in mice would be desirable. The first entry factor-transgenic animal, which was generated before OCLN had been implicated in the HCV entry process, expressed only CD81 in murine hepatocytes (Masciopinto et al. 2002), and consequently did not support viral uptake. Recently, mice harboring CD81, SCARB1, CLDN1, and OCLN transgenes were constructed, but did not appear to be permissive

to HCV infection in vivo (Hikosaka et al. 2011). This apparent discrepancy between the transient adenoviral and the stable transgenic expression approach may be attributed to the lower level of entry factor expression in the transgenic mice and the need for a very sensitive reporter system to quantify viral entry (Dorner et al. 2011).

Following uptake into murine cells, HCV RNA is translated (Dorner et al. 2011; McCaffrey et al. 2002) but does not seem to accumulate, suggesting that viral RNA replication is impaired in mouse cells (see chapter "Hepatitis C Virus RNA Translation" by Niepmann). Selectable HCV RNA genomes, so-called HCV replicons (chapter "Cell Culture Systems for Hepatitis C Virus" by Steinmann and Pietschmann), can replicate in murine cell lines (Frentzen et al. 2011; Uprichard et al. 2006; Zhu et al. 2003), demonstrating that interfering dominant negative inhibitors do not appear to exist and that murine orthologs of host factors critical for HCV replication cooperate sufficiently with the viral replication machinery. These functional data are corroborated by the high degree of sequence identity between mouse and human miR122, cyclophilin A, and phosphatidylinositol 4 kinase IIIα (PI4KIIIα); host molecules for which strong experimental evidence exists that confirms their role in the HCV replication cycle (reviewed in Bartenschlager et al. 2010). Although numerous other host factors have been implicated in promoting or restricting viral replication their relevance to HCV biology remains to be demonstrated as independent studies are often minimally overlapping (see chapter "Hepatitis C Virus RNA Replication" by Lohmann, this volume).

What has emerged from several reports is that antiviral cellular defenses limit HCV replication (see chapter "Innate Immune Responses to Hepatitis C Virus" by Schoggins and Rice, this volume). Human hepatoma cells as well as human primary hepatocytes impaired in innate immunity are more conducive to viral replication (Andrus et al. 2011; Marukian et al. 2011). Although HCV has devised ways to blunt innate immunity signaling, e.g. by cleavage of MAVS (Meylan et al. 2005) or Toll/L-1 receptor domain-containing adaptor inducing IFN-beta (TRIF or TICAM; Li et al. 2005), these mechanisms may not work with equal efficiency in all cell types and in particular, murine cells. The NS3/4A protease cleavage motifs of mouse MAVS and TRIF (or TICAM) are conserved, but it has not been formally proven that the viral protease actually cleaves the murine ortholog and that this targeted proteolysis translates into increased RNA replication. Thus, it is conceivable that different kinetics and/or a greater magnitude of virally induced innate defenses prevent induction or maintenance of HCV RNA replication in mouse cells. In support of this hypothesis, it was previously demonstrated that mouse embryonic fibroblasts with targeted disruptions in protein kinase R (PKR; Chang et al. 2006) or interferon regulatory factor 3 (IRF3; Lin et al. 2010) more efficiently support HCV RNA replication. Whether disruption of these or other innate signaling pathways were sufficient to render mice expressing human HCV entry factors permissive to HCV infections has yet to be shown. Later stages in the HCV life cycle do not seem to be a bottleneck in mouse cells as it was recently demonstrated that infectious HCV particles can assemble and be released if sufficient apolipoprotein E is present (Long et al. 2011). Taken together, these data further raise the hope that an inbred mouse model for HCV infection can be achieved.

5.3 Expanding the Host Range of HCV by Viral Adaptation

A complementary approach to genetic host humanization is the adaptation of HCV to efficiently utilize entry factor orthologs of non-human origin, and to replicate and produce progeny virus in non-human host cells. This approach takes advantage of the remarkable mutational plasticity of HCV. Proof-of-concept that the host range of normally human-tropic pathogens can be expanded has been provided for HIV (Luciw et al. 1995), Ebola (Bray et al. 1998), and Dengue virus (Hotta and Evans 1956). Recently, using an iterative selection approach, adaptive mutations in HCV E1 and E2 have been identified that resulted in a gain of function enabling the resulting murine-tropic HCV (mtHCV) to efficiently engage mouse CD81 and OCLN (Bitzegeio et al. 2010). The mutations in E1 and E2 likely resulted in conformational changes of the viral envelope since the affinities for neutralizing antibodies targeting conformational epitopes were altered. Furthermore, adaptive mutations within the viral glycoproteins altered usage of human SCARB1 and OCLN. Indeed, blocking antibodies against human SCARB1 and silencing of human OCLN had a significantly less pronounced effect on entry of the mutant virus as on the parental strain (Bitzegeio et al. 2010). This exciting breakthrough suggests that species barriers may be overcome with a few adaptive mutations. However, it also raises the concern whether such a mutant virus is suitable for testing of entry therapeutics. Conformational differences in the adapted E1/E2 complex may not accurately assess the efficacy of neutralizing antibodies. Furthermore, therapeutics generated to interfere with viral entry may be human specific, and thus difficult to test. Whether mtHCV is actually capable of entering murine hepatocytes in vivo has yet to be demonstrated. Most likely, additional adaptations are needed to overcome the block of HCV replication in murine hepatocytes. Whether this goal can be achieved in an evolutionary vastly divergent species, such as the mouse, is unclear.

Thus, it may be more tangible to fully adapt HCV to a species that is more closely related to humans. Small non-human primates, such as Rhesus monkeys are more similar to humans but resistant to HCV infection (Abe et al. 1993) possibly due to the inability of HCV to counteract antiviral innate immune responses (Patel et al. 2012). Nonetheless, adaptation of HCV to small non-human primates offers several considerable advantages. Given the greater similarity to humans it may potentially be easier to overcome putative incompatibilities between virally encoded proteins and host factors. Studies conducted in monkeys may translate more readily into clinical results. Macaques may also offer a better platform for pharmacokinetic studies of HCV vaccines and drug candidates. Additionally, when assessing HCV vaccine candidates it should be considered that a large fraction of HCV carriers is co-infected with HIV. Thus, a macaque model for HIV/HCV co-infection is of interest and significant clinical relevance, especially since a simian-tropic HIV-1 (stHIV) is already available (Hatziioannou et al. 2006, 2009).

5.4 Xenotransplantation Models

Humanization of the mouse liver by xenoengraftment of permissive human cells, such as hepatoma cell lines or primary hepatocytes, has been explored as a way to study HCV infection in rodents. Most of the commonly used xenorecipients share common features: they need to be immunodeficient to prevent xenograft rejection and often suffer from an endogenous liver injury to promote hepatocyte proliferation and provide human donor cells a competitive growth advantage over mouse hepatocytes. Donor cells are usually injected intrasplenically from where they rapidly migrate through the portal venous system into the liver. In contrast to localized intrahepatic injections this results in a more even distribution of donor cells throughout all liver lobes.

Here, we will highlight distinct approaches to repopulate rodent livers with human hepatocytes and the applications of these models for the study of HCV infection (Table 3).

5.4.1 Hepatoma Transplantation Models

The human Huh7 hepatoma cell line and derivatives have been widely used in HCV research (chapter "Cell Culture Systems for Hepatitis C Virus" by Steinmann and Pietschmann, this volume). In these cell lines, selectable subgenomic or full-length HCV genomes can robustly replicate in cell culture (Blight et al. 2000; Lohmann et al. 1999). The development of HCV replicons constituted a significant milestone as it enabled for the first time study of HCV RNA replication in a tractable tissue culture platform. To adopt this platform for in vivo studies, a Huh7 subclone stably harboring a luciferase expressing replicon was selected for efficient growth in severe combined immunodeficient (SCID)/beige mice (Zhu et al. 2006). This reporter system enabled quantification of HCV RNA replication and responses to antiviral treatment such as interferon-α (IFN-α) by whole-body bioluminescence imaging (Zhu et al. 2006). While the in vivo replicon-containing mouse model gains utility in its simplicity and reproducibility, it does not recapitulate the entire viral life cycle and study of the antiviral host response is hampered by the immunodeficiency of the xenorecipient.

In an attempt to overcome these shortcomings, immunocompetent fetal rats were tolerized to Huh7 cells by intra-uterine injection, and subsequently engrafted post-birth by intrahepatic injection with a larger number of the same cells (Wu et al. 2005). Shortly, after the second transplantation rats were infected with a patient-derived HCV genotype 1a isolate that replicated and produced virus at low levels for over 3 months. These observations were remarkable as these human hepatoma cells in culture are not readily permissive to infection with most patient isolates, except the unusual JFH1 isolate. This suggests that properties of Huh7 cells change in the hepatic environment in vivo and—at least in terms of susceptibility to HCV infection—acquire characteristics of primary hepatocytes. However, this tolerized rat model has not found traction because of the complexity of the experimental set up,

Table 3 Xenotransplantation models for the study of HCV infection

		FRG mice	Alb-uPA mice	AFC8 mice
Liver injury	Mutation	Fumaryl acetoacetate hydrolase deficiency	Urokinase plasminogen activator overexpression	Inducible caspase 8 activation
	Occurance	At birth, inducible upon NTBC withdrawl	At birth	Upon FK506 administration
Transplantation	Age hepatocytes	Any (adult) adult, fetal not tested	7–21 days post birth adult	Any (adult) adult not tested, fetal (low level)
	ES/iPS	Not tested	Low level	Not tested
Human chimerism		50–80 %	Up to 100 %	Up to 30 %
Throughput		Medium-high	Low	Medium-high
Additional challenges		Murine HCC after long-term NTBC withdrawal	Hypofertility of homozygous transgenic mice	Unknown
Susceptibility to	HCV	HCVcc and patient isolates (only in highly engrafted mice)	HCVcc and patient isolates (only in highly engrafted mice)	Patient isolates
	HBV	Yes	Yes	Not tested
	Plasmodium	Not tested	Yes	Not tested
HCV or HBV induced human liver disease		No	No	Fibrosis
Human immune system		No	No	Yes

the low dynamic range of viremia and concerns about the ability of rat T cells to recognize virally derived antigens in the context of human HLA on infected donor cells.

5.4.2 Ectopic Liver Implant Models

In an effort to generate more physiologically relevant animal models for HCV, numerous approaches have been undertaken to engraft human hepatocytes instead of hepatoma cell lines into suitable xenorecipients. It was shown that small pieces of human liver could be maintained for several weeks in ectopic sites, such as the kidney capsule or the ear of irradiated SCID mice. Because of concurrent transplantation of mouse tissues this model was termed the 'Trimera mouse' (Ilan et al. 2002). When human livers were infected before transplantation, HCV genotype 1b viremia could be followed for several weeks, and this model has shown some utility for assessing the efficacy of neutralizing antibodies (Eren et al. 2006). However, these extrahepatic tissues suffered from architectural distortion and necrosis, and to date, have shown only low levels of HCV viremia. Therefore, these caveats have limited widespread use of this model.

More robust hepatocyte engraftment in an ectopic site was recently demonstrated in an elegant tissue-engineering approach (Chen et al. 2011). Here, the function of cryopreserved primary human hepatocytes was stabilized through juxtacrine and paracrine signaling in polymeric scaffolds of human ectopic artificial livers (HEALs). Mice transplanted with HEALs exhibit humanized liver functions for weeks, including the synthesis of human proteins, human drug metabolism, drug–drug interactions, and drug-induced liver injury (Chen et al. 2011). While their susceptible to hepatotropic pathogens, including HCV, has yet to be shown, HEAL implanted mice have several considerable advantages over other hepatocyte transplantation models. HEALs are relatively easy to prepare enabling the generation of larger cohorts of animals, and human liver functions can be assayed just days after implantation. Furthermore, addition of other non-parenchymal cell types to the tissue organoid may allow for assessing the impact of specific cell populations to liver (patho-) physiology.

5.4.3 Human Liver Chimeric Mice

The liver is normally a quiescent organ with minimal cell turnover. In response to liver injury, hepatocytes have a remarkable capacity to proliferate and regenerate injured liver tissue. This phenomenon is the basis for many xenotransplantation models in which human hepatocytes are injected into immunodeficient recipients suffering from liver injury. Engraftment can be facilitated by several kinds of treatments to give human hepatocytes an advantage over mouse liver cells. These include partial hepatectomy, treatment with hepatotoxic chemicals, such as retrorsine or carbon tetrachloride, or genetic approaches. The latter have been explored most extensively as they provide a more selective control over the severity of the

liver injury and hepatotoxicity is usually limited to mouse hepatocytes. In the following sections, various liver injury models that have been tested for human hepatocyte engraftment will be discussed.

Urokinase Plasminogen Activator Transgenic Mice

Originally developed as a model to study bleeding disorders, transgenic overexpression of Urokinase Plasminogen Activator (uPA) under the albumin promoter (alb-uPA) led to sustained liver injury and failure (Sandgren et al. 1991). Transplantation of normal mouse hepatocytes could rescue alb-uPA mice from liver failure (Rhim et al. 1994), and after backcrossing these mice to various immunodeficient backgrounds they were shown to be good recipients for a variety of xenografts. As shown by Sandgren et al., hepatocytes in hemizygous transgene recipients are able to inactivate the transgene, and subsequently repopulate the liver (Sandgren et al. 1991). Therefore, early studies with human hepatocytes into hemizygous alb-uPA recipients lead to low human chimerism, but in 2001, Mercer and colleagues were able to successfully engraft homozygous recipients with human hepatocytes and showed that these mice can support sustained HCV infection with high viremia (Mercer et al. 2001). These alb-uPA mice on a variety of immunodeficient backgrounds have contributed to our understanding of many aspects of HCV biology, e.g. viral entry (Lacek et al. 2012; Meuleman et al. 2012); the role of anti-HCV antibodies (Vanwolleghem et al. 2008); and infectious particle composition (Lindenbach et al. 2006; Steenbergen et al. 2010). One additional area in which alb-uPA mice have shown their advantage over other mouse models is the pre-clinical evaluation of antiviral compounds. After over a decade of poorly effective IFNα and ribavirin-based treatment regimens, a plethora of DAAs are currently under clinical evaluation. Similar to humans treated with IFN-α monotherapy for HCV-1 infection, IFNα therapy alone in the alb-uPA mice only showed a modest reduction in viremia (Kneteman et al. 2009). However, several DAAs have been shown to decrease viremia by several logs in this model, similar to phase 1 trials in humans (Kamiya et al. 2010; Kneteman et al. 2009; Vanwolleghem et al. 2008). More impressively, combination treatment was recently reported to eradicate HCV from alb-uPA mice with stable high-level viremia (Ohara et al. 2011). This is in agreement with recent phase 2 clinical trials in which DAA combination therapy lead to sustained virologic response (SVR), the clinical correlate for eradication (Chayama et al. 2012). These findings support the utility of the alb-uPA model for studying several aspects of the HCV life cycle and therapy.

However, there are several drawbacks to the alb-uPA model, most notably the infertility of homozygous mice requiring large breeder colonies and the pre-weaning mortality with the need to transplant recipients in the first 2 weeks of life. These drawbacks, combined with their bleeding tendency, have made generating highly engrafted mice laborious and costly. Some of these caveats, in particular the hypofertility, can be overcome by engraftment of murine wild-type hepatocytes into homozygous alb-uPA breeding pairs (Brezillon et al. 2008). Nonetheless, several

groups have generated variations of the original alb-uPA model to overcome some of the remaining issues. In one approach, uPA transgene expression under the control by the MUP promoter was generated. This promoter becomes active later in post-natal liver development, and consequently delays the need for hepatocyte transplantation into adulthood when the recipient is more resilient. Such immunodeficient MUP-uPA mice can be engrafted with human hepatocytes (Heo et al. 2006), but infection with HCV has not yet been reported. More recently, a uPA cDNA transgene was expressed under an albumin promoter in the highly immunodeficient non-obese diabetic (NOD) SCID interleukin 2 receptor gamma deficient (IL-2Rγ^{null}) mice (Suemizu et al. 2008), which were termed uPA-NOG mice and hold several advantages over the original alb-uPA mice; they can be bred as homozygous transgenic animals; can be transplanted later in life as young adults and possibly have lower spontaneous mortality (Suemizu et al. 2008). Interestingly, only male mice supported high level hepatocyte engraftment. Whether the liver chimeric uPA-NOG mice are equally susceptible to HCV infection as the original alb-uPA model remains to be shown. Lastly, a promising variation has been reported using the inducible tet-on uPA transgenic system, in which recipient hepatocytes can be ablated at will by administering doxycycline in adult mice (Song et al. 2009). It remains to be reported if these mice allow for efficient human xenoengraftment and HCV infection.

In summary, uPA transgenic mice, in particular the original alb-uPA model, have greatly contributed to studying HCV biology. Modifications to these models that improve their robustness will likely accelerate their widespread use.

Fumaryl Acetoacetate Hydrolase Deficiency

A different mouse liver injury model is based on the deficiency of fumaryl acetoacetate hydrolase (FAH), an enzyme in the tyrosine catabolic pathway. The lack of FAH leads to accumulation of metabolites that are hepatotoxic, and FAH$^{-/-}$ mice die from liver failure (Grompe et al. 1993). However, the drug, NTBC, can block a different enzyme in this tyrosine breakdown pathway upstream of FAH and when mice are maintained on NTBC they were fertile and viable (Grompe et al. 1995). Crossing FAH mice to an immunodeficient RAG2$^{-/-}$ IL-2Rγ^{null} background (FRG mice) allowed for the engraftment of human hepatocytes. After transplantation of human hepatocytes, mice are cycled off the protective NTBC drug, which induces mouse hepatocyte death and drives proliferation of the human cells (Azuma et al. 2007; Bissig et al. 2007). It was subsequently demonstrated that high hepatic chimerism correlates with susceptibility to HCV infection from both cell culture-derived JFH1 and patient-derived HCV-1 (Bissig et al. 2010). Similarly to studies with antivirals in the alb-uPA model, treatment with IFN-α and a cyclophilin inhibitor transiently lowered viremia in this model.

Advantages of the FRG over the original alb-uPA model are the ability to breed mutant mice on the drug NTBC, induce liver injury at any age by withdrawing NTBC, and perform surgeries on healthy mice. One disadvantage is the requirement in FRG mice for large numbers of human hepatocytes to achieve the high chimerism needed for HCV infection.

Other Liver Injury Models for Human Xenotransplantation

Herpes simplex virus thymidine kinase (HSV-TK) can convert non-toxic prodrugs, such as acyclovir or ganciclovir via phosphorylation into highly toxic triphosphates. This "suicide system" has been widely used for cell type specific ablation in vivo (Clark et al. 1997; Drabek et al. 1997; Felmer et al. 2002) Recently, HSV-TK was transgenically expressed in the livers of immunodeficient NOD-SCID IL2Rγ^{NULL} (TK-NOG) allowing for selective ablation of mouse hepatocytes with ganciclovir (Hasegawa et al. 2011). Remarkably, only a single dose of the prodrug was needed to attain high-level human hepatic chimerism exceeding 1 mg/ml of human albumin in mouse serum, this level of hepatocyte engraftment was maintained for almost 9 months, without the requirement for further ganciclovir administration.

It was previously observed that accumulation of a mutant form of α-1 antitrypsin (AAT-Z) could lead to liver injury in patients. The transgenically expressed mutant, AAT-Z, results in liver injury in so-called PiZ mice, thereby mimicking the patient phenotype. PiZ mice can be robustly engrafted with wild-type mouse hepatocytes (Ding et al. 2011) but has yet to be shown to also support human hepatic engraftment when bred to an immunocompromised background. Both HSK-TK and PiZ transgenic mice hold promise as an alternative liver injury recipient for human hepatocyte engraftment, but data to demonstrate susceptibility to HCV infection are still lacking.

In a recently developed immunodeficient liver injury strain, transgenically expressing active caspase 8 fused with a FK506 binding domain (AFC8) in the liver (Washburn et al. 2011), injection of the FK506 dimerizer AP20187 induced selective apoptosis in mouse hepatocytes. In contrast to most other liver injury strains, fetal hepatoblasts and non-parenchymal cell injections into AFC8 mice resulted in a higher frequency of albumin positive cells, which did not translate into previously observed levels of albumin in circulation. Presumably, due to the limited human hepatic engraftment, HCV RNA was only detectable in the chimeric liver but not in circulation. This model is of particularly interest, as it allowed for dual engraftment of fetal hepatoblasts and autologous human hematopoietic stem cells giving rise to a liver chimeric animal harboring components of a matching human immune system, which will be discussed in the following section.

5.4.4 Toward a Xenotransplantation Model for the Study of HCV Pathogenesis and Immunity

Human liver chimeric mice can serve as useful models for the study of basic HCV virology or the development and evaluation of antiviral therapies. HCV pathogenesis, human adaptive immune responses or vaccine development, however can only be studied in a mouse model harboring both, a human liver graft and a functional human immune system. Here, we will provide an overview of the current human immune system (HIS) mouse models and discuss their potential application for the development of HCV pathogenesis models.

Humanized mice, specifically mice reconstituted with human immune system, have emerged as a promising model to study human immunity in vivo (reviewed in Shultz et al. 2007). Indeed, the development of immunodeficient mouse strains suitable for the engraftment of a human hemato-lymphoid system has made significant progress during the last decade. Currently, the most commonly used mouse strains include the Balb/c recombinase activating gene (Rag) $2^{-/-}$ IL2Rγ^{NULL} (BRG) strain (Gimeno et al. 2004; Traggiai et al. 2004), the NOD-SCID IL2Rγ^{NULL} (NSG) strain (Ishikawa et al. 2005; Shultz et al. 2005) and the NOD Rag $1^{-/-}$ IL2Rγ^{NULL} (NRG) strain (Pearson et al. 2008). These mice do not develop functional T and B cells due to mutations in the *Prkdc* gene (protein kinase, DNA activated, catalytic polypeptide), which cause the SCID phenotype, or deletion of the *Rag* gene. Furthermore, mutations in *Il2Rg*, the common IL-2 receptor γ chain, result in the absence of NK cells and functional impairment in myeloid cells. Human immune system engraftment in these highly immunodeficient BRG, NSG, or NRG mice can be achieved by transplantation of human fetal liver or cord blood derived CD34+ hematopoietic stem cells (HSC) into sublethally irradiated newborn recipients. Using this engraftment protocol leads to the substantial development of all major human hematopoietic cell lineages including T cells, B cells, NK cells, and myeloid cells (Ishikawa et al. 2005; Shultz et al. 2005). The engraftment of a human immune system can also be achieved by co-transplantation of small pieces of human fetal liver and thymus together with the injection of CD34+ HSC into sublethally irradiated adult recipients. In these so-called bone marrow/liver/thymus ("BLT") mice T cell selection occurs in the context of a human thymic environment (Melkus et al. 2006).

Consequently, human immune system mice provide a new platform to study human hematopoiesis and immune responses in vivo. Furthermore, they are susceptible to many lymphotropic pathogens exhibiting an almost exclusive human tropism, such as HIV, EBV, dengue virus, or *Salmonella typhi* (Denton and Garcia 2011; Jaiswal et al. 2009; Libby et al. 2010; Strowig et al. 2009). Thus, they allow for studying the pathogenesis and immune responses of these infections. For example, it has been shown that HIS mice infected with EBV develop virus-associated tumors and mount EBV-specific CD8+ T cell responses (Strowig et al. 2009). Virus-specific immune responses are also detectable in dengue virus infected HIS mice (Jaiswal et al. 2009). Studies of HIV infection have mostly been performed in the BLT mouse model. These mice show a better reconstitution of human immune cells in the gastrointestinal and reproductive tract and more extensive HIV-specific immune responses compared to other HIS mouse models (reviewed in Denton and Garcia 2011).

An HCV-permissive HIS mouse model would need to combine a human liver graft with a human immune system in one mouse. Creating such a mouse model is technically challenging, because the immunodeficient mouse background of choice must be suitable for efficient engraftment of human hepatocytes as well as human immune cells. In addition, the transplanted hepatocytes and HSC should be derived from the same human donor to avoid a MHC mismatch and human allo-immune responses. One recent study demonstrated the successful generation of such a dually engrafted mouse model (Washburn et al. 2011). Intrahepatic injection of human hepatocytes and CD34+ HSC from the same fetal donor into

newborn AFC8 mice resulted in the establishment of humanized mice harboring both, human liver and immune cells (Washburn et al. 2011). Those mice were permissive for infection with HCV genotype 1a patient isolates as indicated by detectable HCV genomic RNA in the liver 1–4 months post infection (Washburn et al. 2011). HCV viremia in the blood however could not be detected. HCV infection in dually reconstituted mice correlated with the infiltration of human immune cells into the liver, the induction of HCV-specific T cell responses, elevated ALT levels, and the induction of liver fibrosis (Washburn et al. 2011). In conclusion, this study provides an encouraging first step toward the generation of a humanized mouse model that allows for the study of HCV pathogenesis and immune responses.

Nevertheless, open questions about the functionality of this model remain. It was demonstrated that HCV specific IFN-γ and TNF-α producing T cells are detectable in dually engrafted HCV-infected humanized mice (Washburn et al. 2011). It remains to be determined, however, whether these virus-specific T cells are able to control and eventually clear the infection. It is generally accepted that the human immune system of the current humanized mouse models are not fully functional and various factors contribute to this impairment. For instance, limited interspecies cross-reactivity of important hematopoietic cytokines, such as TPO (thrombopoietin), SCF (stem cell factor), Flt3-L, GM-CSF, IL-3, or IL-15, results in impaired development, maintenance and function of certain immune cell lineages (reviewed in Manz 2007; Willinger et al. 2011). The development and function of human T and B cells in humanized mice are likely impaired due to a lack of HLA expression in the mouse thymus and peripheral tissue and limited secondary lymphoid organ development (Manz 2007; Shultz et al. 2007). Due to these significant shortcomings, a major topic in current humanized mouse research is the improvement of these systems.

Several recent studies describe the improvement of the human immune cell development in humanized mice by cytokine delivery. For example, the transient delivery of human IL-15, Flt-3L, or IL-3/GM-CSF through injection of recombinant proteins or hydrodynamic delivery of DNA, led to increased numbers in human NK cells, dendritic cells, and monocytes (Chen et al. 2009; Huntington et al. 2009). Furthermore, humanized NSG mice transgenic for human SCF, GM-CSF, and IL-3 showed improved human myeloid cell development in the bone marrow (Billerbeck et al. 2011; Nicolini et al. 2004). Transient or transgenic overexpression of selective human cytokines, however, might influence human hematopoiesis in an unexpected way and should be considered with caution. Indeed, SCF, GM-CSF, and IL-3 transgenic humanized mice show an unexpected skewing toward the development of regulatory T cells (Billerbeck et al. 2011). To achieve physiologically relevant expression of human cytokines, a knock-in gene replacement approach was utilized. One study described the replacement of the mouse *tpo* gene by the human homolog in the BRG strain (Rongvaux et al. 2011). Engraftment of these mice with human CD34+ HSC resulted in improved maintenance of primary self-renewing human HSC in the bone marrow and stable high-level reconstitution of a human immune system for more than 6 months.

Another important step toward the improvement of long-term human immune system reconstitution and lineage development in HIS mice is the inhibition of human immune cell elimination by murine phagocytes. Phagocytes, such as macrophages and dendritic cells, express the receptor SIRP-α (signal regulatory protein alpha; Takenaka et al. 2007). Interactions of SIRP-α with its ligand CD47 negatively regulate phagocytosis, which prevents the elimination of CD47-expressing cells. Human CD47 does not properly interact with mouse SIRP-α resulting in transplanted human cells susceptible to phagocytosis (Takenaka et al. 2007). Two recent studies show that either the transgenic expression of human SIRP-α in the recipient mouse strain or the transduction of mouse CD47 into human HSC prior transplantation can inhibit phagocytosis of human cells and significantly improve long-term engraftment of a human immune system (Legrand et al. 2011; Strowig et al. 2011).

In regard to a humanized mouse model for the study of HCV a stable long-term engraftment of the human immune system would be highly desirable in order to analyze immune responses during the chronic phase of infection. The development of functional dendritic cells and NK cells is also of significant importance since those cells play a crucial role in antiviral immunity. Thus, the use of mouse strains that express human cytokines, such as TPO, IL-15, IL3/GM-CSF, and Flt3L, and those that express human SIRP-α should be considered for the future development of HCV-permissive HIS mice. The transgenic expression of human SIRP-α would, most likely, also enhance human hepatocyte engraftment efficiency in these mice. In line with that, one recent study reports improved engraftment of human hepatocytes that ectopically express murine CD47 (Waern et al. 2012).

Next to human cytokine or SIRP-α expression, HLA expression is thought to significantly improve the functionality of the human immune system in HIS mice. In current HIS mouse models, human T cells are educated on murine MHC molecules in the mouse thymus and it remains unclear how human thymocytes become selected in the absence of HLA (Manz 2007). A more physiological human T cell selection can be achieved in the BLT mouse model. Here, small pieces of human thymus are transplanted under the kidney capsule prior to injection of autologous HSCs (Melkus et al. 2006). These mice, however, still lack expression of HLA in the periphery, where continued interaction of naïve T cells with self-peptide has been shown to be important for maintenance. Mice transgenic for human HLA class I and/or class II molecules might help to overcome these issues. Indeed, it has recently been shown that humanized NSG mice transgenic for a human MHC class I molecule, HLA-A2, exhibit improved virus-specific and HLA-A2 restricted CD8+ T cell responses during EBV and dengue virus infection (Jaiswal et al. 2009; Shultz et al. 2010; Strowig et al. 2009). Furthermore, HIS mice transgenic for a human MHC class II, HLA-DR4, showed a generally improved development of human T and B cells (Danner et al. 2011). In order to analyze HLA-restricted HCV-specific T cells responses, the expression of specific HLA molecules in HCV-permissive humanized mice would be essential. Here it will be important to engraft mice with HLA matched HSC and hepatocytes, preferentially from the same donor. Advances in induced

pluripotent stem cell (iPSC) technology might overcome current limitations in the availability of HLA-matching cells in the future (Schwartz et al. 2012; Wu et al. 2012).

Taken together, recent, and future, improvements in the functionality of the human immune system (Fig. 2) will be advantageous for the development of a humanized mouse model for the study of HCV pathogenesis. Future improvement strategies include those that enhance human B cell functionality and neutralizing antibody production. This could be achieved through the expression of human Fc receptors. Further, the migration and homing capacity of human immune cells through murine endothelium and tissue should be investigated. Indeed, species-specific mismatches between mice and human might not only affect hematopoietic cytokines but chemokines and adhesion molecules as well. This would be of significant interest for the functionality of immune responses to tissue-specific infections, caused by HCV.

In conclusion, the development of a functional humanized mouse model for the study of HCV pathogenesis is challenging. Yet, the report about the first successful generation of HCV-permissive dually reconstituted mice and the rapid progress in

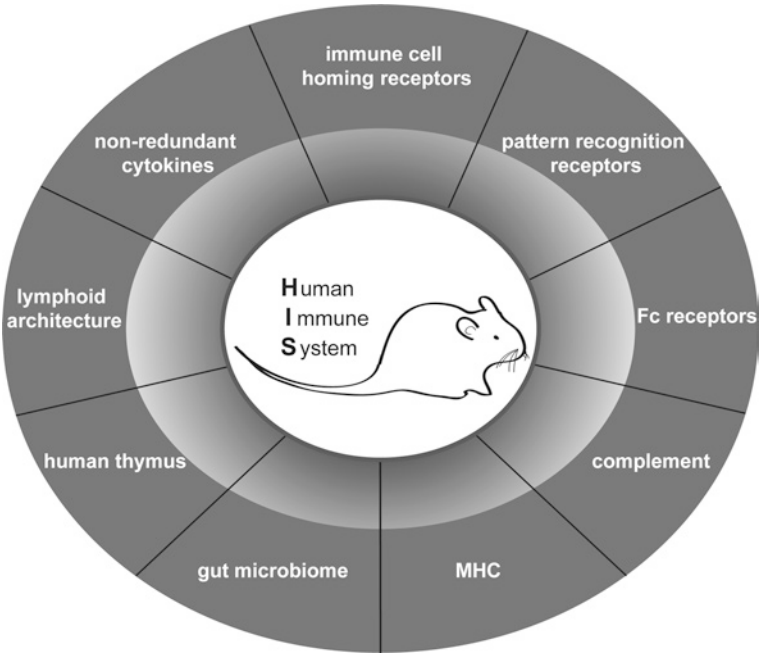

Fig. 2 Strategies for the improvement of human immune system functionality in humanized mice: expression of non-redundant cytokines, immune cell homing-, pattern recognition- or Fc receptors; expression of human MHC molecules; implantation of a human thymus; improvement of the lymphoid architecture, e.g. through implantation of artificial lymph nodes; administration of human complement or a human gut microbiome

the improvement of HIS mouse functionality hold promise for the development of more functional models in the near future.

5.4.5 Xenotransplantation Models: Accomplishments and Goals

Xenotransplantation models have greatly advanced our understanding of certain aspects of HCV infection. For example, with the exception of chimpanzees these mouse models have thus far been the only reproducible system for studying non-JFH based viral clones. Several pre-clinical lessons in regard to HCV infection have been learned: responses to antivirals in these models appear to mimic the response in patients; even in the absence of adaptive immunity HCV can be cleared using combination DAA treatment; passive immunization strategies allow for protection, possibly opening the way to protect liver allografts from reinfection. However, several poorly explained observations remain. With the exception of the caspase 8 transgenic model, only highly engrafted mice with human serum albumin levels greater than 1 mg/ml appear to be susceptible to HCV infection. This was observed in the alb-uPA model (Vanwolleghem et al. 2010), as well as the FRG model (Bissig et al. 2010). Two explanations, namely the requirement for secreted human serum factors to allow infection or clearance of low viremia below our limit of detection, remain possibilities. The presence of detectable viral RNA exclusively in the liver of the caspase 8 transgenic model (Washburn et al. 2011) supports the latter hypothesis. In addition, many viral patient isolates fail to establish infection. Whether this is mainly due to the presence of neutralizing antibodies in patient serum, low levels of infectious virions or other factors remains to be elucidated.

Furthermore, many challenges remain to advance these models, both to make them more robust and reproducible as well as to improve their biological complexity. In regard to improving throughput of these models, one current limitation is the shortage of high quality adult human hepatocytes. Improvements in repeated transplantations of human hepatocytes into new recipients (Azuma et al. 2007) has to be optimized so that secondary and tertiary recipients will engraft to >1 mg/ml human serum albumin levels. Elucidating why fetal hepatocytes fail to engraft to high levels would open a different source of human hepatocytes, which in theory would have high proliferation potential. Lastly, another obvious source of human hepatocytes is iPS cell derived hepatocyte-like cells (iHEPs), which would form a renewable source. Recently, these cells were shown to have differentiated enough to support HCV infection in vitro (Schwartz et al. 2012; Wu et al. 2012), but transplantation of iHEPs into liver injury models has not yet resulted in proliferation of these cells, nor sustained engraftment over many weeks. In regard to increasing their biological complexity, one of the biggest limitations is the lack of an adaptive immune response. Whether this partially explains the lack of fibrosis and HCC development remains to be proven. Generating models with both high human hepatocyte engraftment and functional human T and B cells should be a priority for the near future.

6 Summary and Outlook

In order to address the medical need for more effective therapeutic interventions, as well as to answer questions of basic virology, HCV pathogenesis, and correlates of protective immunity, more tractable in vivo platforms are needed. For the longest time it has only been possible to study hepatitis C in vivo in experimentally infected chimpanzees or patient volunteers. While the chimpanzee has been instrumental to analyze HCV infection, studies in this species are hampered by high costs, genetic heterogeneity, small cohort sizes, limited access to relevant tissue compartments, the inability to genetically manipulate large apes and growing ethical concern, and all of which preclude the mechanistic analysis of HCV immunity and pathogenesis. Numerous approaches are currently being pursued in parallel toward the development of small animal models for HCV infection (Fig. 1). New animal homologs of HCV such as non-primate hepaciviruses have been discovered, which may not only shed light on the evolutionary origin of HCV but also serve as surrogates for HCV in vivo. Our understanding of the host tropism of HCV has considerably improved and this knowledge can now be exploited to overcome barriers in interspecies transmission via viral adaptation and/or genetic host humanization. Recapitulation of the entire HCV life cycle in rodents seems tangible and would be greatly beneficial in opening unprecedented opportunities to genetically dissect HCV infection in vivo. More than a decade ago, the first landmark studies demonstrated that human liver chimeric mice are susceptible to HCV infection. Current xenotransplantation models have become more robust and can be generated in higher throughput. Recently, precedent has been established that mice engrafted with a human liver and immune system may be suitable to model viral hepatitis including relevant co-infections with HBV and/or HIV in vivo. Stem cell-derived tissue regeneration protocols are rapidly evolving and may soon create animals harboring patient specific cells, which would provide means to functionally assess genetic polymorphisms associated with HCV disease progression or clearance. To ensure relevance, improved and new HCV models should be correlated to aspects of HCV pathogenesis and its sequels in humans. Besides facilitating studies of basic questions of HCV biology, fully immunocompetent small animal models would be a valuable platform to prioritize vaccine candidates and define effective treatment regimens. In the light of the increased scrutiny of chimpanzee experiments, the development of small animal models that accurately reflects important hallmarks of HCV infection remains a high priority.

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Hepatitis C Virus Entry

Mirjam B. Zeisel, Daniel J. Felmlee and Thomas F. Baumert

Abstract Hepatitis C virus (HCV) is a hepatotropic virus and a major cause of chronic hepatitis and liver disease worldwide. Initial interactions between HCV virions and hepatocytes are required for productive viral infection and initiation of the viral life cycle. Furthermore, HCV entry contributes to the tissue tropism and species specificity of this virus. The elucidation of these interactions is critical, not only to understand the pathogenesis of HCV infection, but also to design efficient antiviral strategies and vaccines. This review summarizes our current knowledge of the host factors required for the HCV-host interactions during HCV binding and entry, our understanding of the molecular mechanisms underlying HCV entry into target cells, and the relevance of HCV entry for the pathogenesis of liver disease, antiviral therapy, and vaccine development.

Abbreviations

Apo	Apolipoprotein
CD81	Cluster of differentiation 81
CLDN1	Claudin 1
EGFR	Epidermal growth factor receptor
EphA2	Ephrin receptor A2
HCV	Hepatitis C virus
HCVcc	Cell culture-derived HCV
HCV-LPs	HCV-like particles
HCVpp	HCV pseudoparticles
HDL	High-density lipoprotein
IgG	Immunoglobulin G

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JFH1	Japanese fulminant hepatitis 1
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LT	Liver transplantation
mAb	Monoclonal antibody
NPC1L1	Niemann-Pick C1-like 1
OCLN	Occludin
PKA	Protein kinase A
RTKs	Receptor tyrosine kinases
SR-BI	Scavenger receptor class B type I (alias SCARBI)
TG	Triglyceride
VLDL	Very-low-density lipoprotein

Contents

1 Discovery and Characterization of Hepatitis C Virus Binding and Entry Factors

Hepatitis C virus (HCV) is a small, enveloped RNA virus that primarily targets human hepatocytes (reviewed in Lindenbach et al. 2007). The HCV envelope is composed of two virus-encoded glycoproteins, E1 and E2 (reviewed in Lavie et al. 2007). As with other enveloped viruses, the envelope glycoproteins largely define the interactions between HCV and the host cell. Moreover, HCV has been demonstrated to circulate in the blood of infected individuals in complexes with host lipoproteins and lipoprotein components (Andre et al. 2002; Miyamoto et al. 1992; Nielsen et al. 2006; Thomssen et al. 1992, 1993), which also contribute to HCV–host cell interactions. Since the discovery of HCV in 1989 (Choo et al. 1989), increasingly complex model systems have been designed to study HCV–host interactions that have allowed identification of key cell factors required for binding of HCV particles to the hepatocyte surface and entry into this cell (reviewed in Barth et al. 2006a; Bartosch and Cosset 2006a, b; Bukh 2012; Murray and Rice 2011; von Hahn and Rice 2008). Some of these factors have been demonstrated to be involved in direct HCV envelope glycoprotein binding and/or subsequent viral entry, and are thus likely to act directly as receptors and co-receptors while others act as indirect, albeit essential, entry factors (Fig. 1).

The first identified entry factors were discovered by their capacity to bind directly to HCV envelope glycoprotein E2. Pileri et al. knowing that purified E2 protein could bind to human hepatocellular carcinoma cell lines, but not mouse cell lines, transduced a cDNA expression library derived from HCV-binding cells into nonbinding cells and identifíed the tetraspanin CD81 as a direct binding partner of E2 (Pileri et al. 1998). Subsequently, it has been demonstrated that CD81 contributes to post-binding steps of the HCV entry process resulting in the internalization of the viral particle (Bertaux and Dragic 2006; Farquhar et al. 2012; Koutsoudakis et al. 2006). The observation that HCV RNA in patient serum was associated with lipoproteins prompted Agnello et al. to assess the role of low-density lipoprotein receptor (LDLR) as an HCV receptor. Indeed, by using in situ hybridization of HCV RNA in HepG2 hepatoma cells, that were exposed to HCV, the authors showed that LDLR is important for uptake of HCV (Agnello et al. 1999; Monazahian et al. 1999; Wunschmann et al. 2000). Recent evidence suggests that LDLR most likely is not an essential entry factor, but given its role in cholesterol metabolism it may play a role in HCV replication (Albecka et al. 2012).

Other factors that were determined to bind directly to HCV E2 were identified by observations garnered from human immunodeficiency virus (HIV) and other viruses. This strategy revealed that HCV E2 could bind the C type lectins dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), and liver/lymph node-specific intercellular adhesion molecule 3-grabbing integrin (L-SIGN) (Gardner et al. 2003; Lozach et al. 2003; Pohlmann et al. 2003). These findings are intriguing in the role that they play in hepatocyte entry, since these molecules are not on the surface of hepatocytes, but are rather on dendritic cells and liver endothelial cells for DC-SIGN and L-SIGN, respectively. It is thought that these molecules may play a similar role as DC-SIGN plays in HIV, as an avenue of transinfection, in which the virus passes through a different type of cell to capture and localize the virus for infection of the hepatocytes that can sustain replication (Cormier et al. 2004; Lozach et al. 2004).

Soon after the identification of CD81 as a host factor playing a role in HCV entry, experimental evidence suggested that other host factors most likely contribute to HCV entry. Thus, cross-linking studies using E2 and HepG2 cells, which lack CD81 expression, lead to the identification of scavenger receptor BI (SR-BI) as an additional direct binding partner of E2 (Scarselli et al. 2002). Interestingly, since SR-BI is also a receptor for lipoproteins, the HCV virion may interact with SR-BI through associated lipoproteins and/or E2 (Barth et al. 2005a, 2008; Bartosch et al. 2003b; Catanese et al. 2007, 2010; Dreux et al. 2009). Further, by using an *inter alia* mutant virus in the SR-BI binding region of E2 and a lipoprotein binding-deficient SR-BI mutant as well as monoclonal SR-BI-specific antibodies, two recent studies showed that SR-BI acts at different steps during the HCV entry process (Dao Thi et al. 2012; Zahid et al. 2012). Indeed, SR-BI might first interact with the lipoprotein component of the lipoviral particle in an E2 binding independent manner (Dao Thi et al. 2012; Maillard et al. 2006). However, later during entry the interaction between SR-BI and the lipoviral particle becomes E2-dependent, for which HVR1 appears to play a major role (Dao Thi et al. 2012). These attributes point to an interesting role of

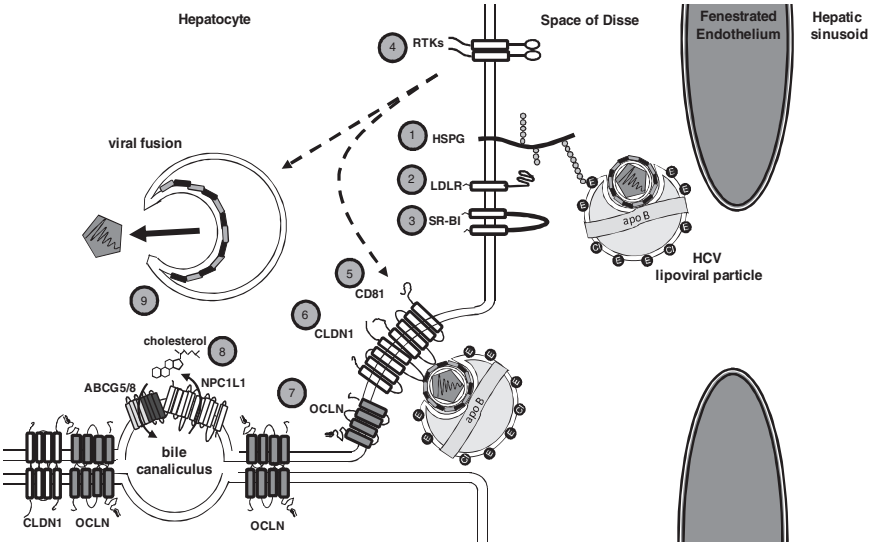

Fig. 1 HCV entry into hepatocytes. HCV particles circulate physically associated with lipoproteins such as VLDL, which contain protein components apolipoprotein B-100, and exchangeable apolipoproteins E and CI (shown as *black circles*). HCV gains access to hepatocytes by entering the space of Disse through fenestrae between hepatic endothelial cells. The first step of entry *1* is binding, when HCV envelope glycoproteins E1 and E2 (black and gray rectangles surrounding virion) and/or apoE on the lipoviral particle (LVP) binds virion) and/or apoE on the lipoviral particle (LVP) binds to heparan sulfate proteoglycan (HSPG), *2* LDL receptor (LDLR) is also a high-affinity binding partner of apoE which has been reported to be important for HCV entry, *3* SR-BI may also contribute to HCV binding via lipoproteins and/or HCV E2. Moreover, SR-BI also plays a role at post-binding steps of HCV entry, *4* Receptor tyrosine kinases (RTKs) including epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) mediate HCV entry through facilitating associations between *5* CD81 and, *6* claudin 1 (CLDN1), and through envelope glycoprotein-membrane fusion. CLDN1 is a component of tight junctions along with the entry factor, *7* occludin (OCLN) that contributes to post-binding steps of the HCV entry process. Recently identified entry factor, *8* Niemann Pick C-1 like 1 (NPC1L1) plays an important role in cholesterol reabsorption from biliary secretion of cholesterol by transporter ATP binding cassette (ABC) G5/8 heterodimeric protein, and acts as a cofactor for HCV entry during post-binding steps. Viral envelope fusion with host membranes *9* is the final step of HCV entry, as the nucleocapsid escapes the early endosome to begin translation and replication of its genome in the cytosol

SR-BI in HCV entry, as this protein acts at similar time points than CD81 forming part of the receptor complex required for HCV entry into the target cell (Kapadia et al. 2007; Zeisel et al. 2007). Interestingly, SR-BII, a splice variant of SR-BI, has also been demonstrated to promote HCV entry (Grove et al. 2007).

Subsequent to these advances, further investigations were conducted using surrogate HCV particle model systems such as HCV-like particles (HCV-LPs) isolated from insect cells (Baumert et al. 1998; Saunier et al. 2003; Triyatni et al. 2002) (see chapter "Cell Culture Systems for Hepatitis C Virus" by Steinmann and Pietschmann, this volume), and it was shown that E2 and HCV-LPs bind highly sulfated heparan

sulfate proteoglycans (HSPG) either purified or on liver-derived membranes (Barth et al. 2003, 2006b). The relevance of HSPG for HCV binding was then subsequently confirmed using HCV pseudoparticles (HCVpp) and cell culture-derived HCV (HCVcc) (Haberstroh et al. 2008; Jiang et al. 2012; Koutsoudakis et al. 2006; Morikawa et al. 2007) (for cell culture models see chapter "Cell Culture Systems for Hepatitis C Virus" by Steinmann and Pietschmann, this volume). Interestingly, it could be further shown that both E1 and apolipoprotein E (apoE) may also contribute to HCV attachment to HSPG (Barth et al. 2006a, b; Haberstroh et al. 2008; Jiang et al. 2012). To date, HSPG is the only known host factor that solely mediates HCV attachment to target cells without having any other described role in the viral life cycle.

The development of the HCVpp system (Bartosch et al. 2003a; Hsu et al. 2003), which consists of ectopically expressed HCV envelope glycoproteins on retrovirus particles containing vector RNAs that encode reporter genes (see chapter "Cell Culture Systems for Hepatitis C Virus" by Steinmann and Pietschmann, this volume), enabled new additional screening strategies. Using such a screening strategy with a cDNA library from the highly permissive Huh7.5 cell line in human kidney cell line 293T, Evans et al. discovered claudin 1 (CLDN1) as an important entry factor for HCV (Evans et al. 2007). Interestingly, the HCV envelope glycoproteins do not directly interact with CLDN1, but CLDN1 interacts with CD81 and thereby plays an important role during post-binding steps of the HCV entry process (Evans et al. 2007; Harris et al. 2008; Krieger et al. 2010). In the human liver as well as in polarized human hepatoblastoma HepG2 cells, CLDN1 is expressed at the basal and lateral membranes as well as in tight junctions (TJs) (Mee et al. 2009; Reynolds et al. 2008). Interestingly, CD81-CLDN1 co-receptor association could only be detected at the basal membranes but not in TJ-associated pools of CLDN1 and CD81 suggesting that the nonjunctional CLDN1 plays a role in HCV entry (Mee et al. 2009). This is in line with the fact that CLDN1 is more highly expressed at the TJs in polarized cells that demonstrate reduced viral entry as compared to nonpolarized cells (Mee et al. 2009). Noteworthy, in various nonpolarized cell lines, it has been shown that the subcellular localization of CLDN1 correlates with HCV entry permissivity. Indeed, in contrast to intracellular CLDN1, only cell surface expressed CLDN1 enriched at cell–cell contact sites promotes HCV entry (Yang et al. 2008) and mutations of CLDN1 that delocalize the protein outside cell–cell contacts do not support HCV entry (Cukierman et al. 2009; Liu et al. 2009). Mutagenesis studies of CLDN1 have revealed that the intracellular domains of this entry factor, including the C-terminal domain that is important for the transport of CLDN1 into TJs, are dispensable for infection, but domains within the first extracellular loop are critical (Cukierman et al. 2009; Evans et al. 2007). The mutations that particularly disrupted HCV entry were those that interrupted cell–cell contacts but did not affect lateral interactions within the plasma membrane between either CLDN1-CLDN1 or CLDN1-CD81 (Cukierman et al. 2009). Moreover, other members of the CLDN family of proteins, e. g., CLDN6 and CLDN9, may also promote HCV entry in 293T-derived cell lines (Meertens et al. 2008; Zheng et al. 2007). In contrast to other CLDNs, CLDN6 and CLDN9 are also able to form co-receptor associations with CD81, a process that is indispensable for HCV entry (Harris et al. 2010). Since

the expression of CLDN6 and 9 is very low in human hepatocytes and liver tissue (J. A. McKeating, personal communication), the relevance of these molecules for HCV entry in vivo needs to be demonstrated. Further use of similar screening strategies in mouse-derived cell lines identified occludin (OCLN) as a species-tropism defining entry factor, and it was determined that among the identified entry factors, CD81 and OCLN determine the tropism of HCV for human cells (Ploss et al. 2009). Interestingly, expression of human CD81 and human OCLN in mouse cells removes the species-specific restriction of HCV entry and allowed development of a mouse model for the early steps of HCV infection (Dorner et al. 2011). The important role of OCLN in the HCV entry process was further demonstrated by two other studies using HCVcc (Benedicto et al. 2009; Liu et al. 2009). However, whether OCLN is a true receptor that binds the HCV envelope or is an indirect, though indispensable, entry co-factor required for post-binding steps still remains an open question. Of note, HCV infection can modulate localization and expression level of CLDN1 and OCLN in the TJ and promote superinfection exclusion (Benedicto et al. 2008; Liu et al. 2009). In this way, HCV infection appears to downregulate protein amounts in TJs of the infected cell, which may contribute to pathologic symptoms such as cholestatic disorders.

Recent functional siRNA screens that specifically focused on host cell kinases, and using HCVpp as well as HCVcc, further identified two cell surface receptor tyrosine kinases. Epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) are important co-factors for HCV entry and infection (Lupberger et al. 2011). It should be noted that EGFR does not directly interact with the HCV particle, but EGFR-dependent signaling pathways lead to the formation of CD81-CLDN1 complexes required for HCV entry (Lupberger et al. 2011).

Finally, since the HCV virion is rich in cholesterol, the role of cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) was investigated recently and identified as an additional entry factor (Sainz et al. 2012), yet its exact role in HCV entry remains to be determined. Within the last years the functional in vivo relevance of most of the entry factors has been confirmed using mouse models such as the human liver chimeric upA-SCID mouse model (Lacek et al. 2012; Lupberger et al. 2011; Meuleman et al. 2008, 2012; Sainz et al. 2012) or a humanized mouse model expressing the human entry factors (Dorner et al. 2011) (see also Sect. 5).

2 The HCV Entry Pathway: From Viral Attachment to the Hepatocyte Basolateral Membrane to Fusion Within the Endosome

Viral entry plays an important role for hepatocyte tropism of HCV. Indeed, tissue tropism is at least partially defined by the interaction of the virus with a set of cell surface factors on the target cell as described above, ultimately leading to viral entry and initiation of the viral life cycle. As the virus circulates in the blood of

HCV-infected patients in association with lipoproteins, both the lipoprotein components and the viral envelope glycoproteins may contribute to the first interactions between the virion and the target cell. Experimental data indicate that HCV uses a single entry pathway for initiation of productive viral infection. The entry pathway consists of three key steps: (1) viral attachment to the hepatocyte, (2) receptor-mediated endocytosis of the viral particle, and (3) endosomal fusion (Fig. 1).

Binding studies using infectious viral particles suggested that HSPG, LDLR, and SR-BI contribute to viral attachment (Albecka et al. 2012; Dao Thi et al. 2012; Catanese et al. 2010; Cormier et al. 2004; Gardner et al. 2003; Koutsoudakis et al. 2006; Lozach et al. 2003; Ludwig et al. 2004; Maillard et al. 2006; Pohlmann et al. 2003). Docking of the virus to the target cell membrane is then believed to be followed by the interaction of the virus with several (other) cell surface factors leading to molecular rearrangements at the plasma membrane and subsequently resulting in viral internalization. Among the host factors contributing to these molecular rearrangements, host cell kinases have been demonstrated to play an important role in regulating HCV entry. Indeed, phosphatidylinositol 4-kinases type III alpha (PI4KIIIα) and beta (PI4KIIIβ) have been suggested to play a role in membrane remodeling and trafficking during HCV entry in a genotype-dependent manner (Trotard et al. 2009). However, the underlying molecular mechanisms have not yet been investigated. Using protein kinase inhibitors, two studies reported that host cell kinases are able to promote co-receptor association between CD81 and CLDN1, which is mandatory for HCV entry (Farquhar et al. 2008; Harris et al. 2008, 2010; Lupberger et al. 2011). Indeed, a protein kinase A (PKA) inhibitor disrupts CD81-CLDN1 complexes and leads to intracellular localization of CLDN1, thereby reducing HCV entry (Farquhar et al. 2008). Moreover, erlotinib and dasatinib, protein kinase inhibitors of EGFR and EphA2, respectively, also disrupt CD81-CLDN1 complexes and inhibit HCV entry (Lupberger et al. 2011). These data indicate that PKA, EGFR, and EphA2 promote the CD81-CLDN1 co-receptor association that is required for HCV entry. Moreover, these data suggest that kinase signaling pathways contribute to this process (Lupberger et al. 2011). SR-BI and OCLN have also been demonstrated to participate in post-binding steps of the HCV entry process (Benedicto et al. 2009; Zeisel et al. 2007). Intriguingly, CD81, SR-BI, and CLDN1 seem to act at very closely related time-points during HCV entry (Krieger et al. 2010; Zeisel et al. 2007). The exact role of OCLN and the interplay of the different receptors in this process are yet to be uncovered.

HCV is known to endocytose in a clathrin-dependent process (Blanchard et al. 2006; Codran et al. 2006; Coller et al. 2009; Meertens et al. 2006). Interestingly, it has been recently demonstrated that CD81-CLDN1 complexes are endocytosed in a clathrin- and dynamin-dependent manner (Farquhar et al. 2012) consistent with a previous imaging study reporting that entering HCV particles are associated with CD81 and CLDN1 (Coller et al. 2009). This mechanism involves the HCV envelope glycoproteins and the GTPase Rho, while EGFR does not appear to play a role in this process (Farquhar et al. 2012). Whether other HCV entry factors are concomitantly endocytosed along with HCV still remains to be

determined. Following internalization, HCV fusion has been reported to occur in early endosomes (Coller et al. 2009; Meertens et al. 2006). This process is dependent on low pH and involves both viral and host proteins (Bartosch et al. 2003a; Blanchard et al. 2006; Haid et al. 2009; Hsu et al. 2003; Kobayashi et al. 2006; Koutsoudakis et al. 2006; Lavillette et al. 2006; Meertens et al. 2006; Tscherne et al. 2006). Indeed, the HCV envelope glycoproteins E1 and E2 contain potential fusion domains (Drummer et al. 2007; Lavillette et al. 2007) and HCV E2 has been shown to be required for liposome/HCVcc fusion in vitro (Haid et al. 2009). Moreover, the importance of the HCV envelope in viral fusion is emphasized by the fact that patient-derived anti-HCV antibodies are able to inhibit cell–cell fusion and liposome/HCVpp fusion in vitro (Haberstroh et al. 2008; Kobayashi et al. 2006). Interestingly, the CD81 and CLDN1 proteins that co-endocytose with HCV (Farquhar et al. 2012), also play a role in HCV envelope glycoprotein-dependent cell–cell fusion (Evans et al. 2007; Kobayashi et al. 2006). Moreover, the protein kinase inhibitors erlotinib and dasatinib reduced HCV envelope glycoprotein-dependent cell–cell fusion suggesting that EGFR and EphA2 may contribute to the HCV fusion process (Lupberger et al. 2011).

3 Impact of Lipoproteins and Cholesterol for HCV Entry

Soon after the structure of the viral genome of HCV was discovered, characterization of the virus in serum of infected individuals revealed that HCV RNA distributed in a wide spectrum, due to HCV forming complexes with low-density lipoproteins (LDL), and very-low density lipoproteins (VLDL) (Hijikata et al. 1993; Kanto et al. 1995; Miyamoto et al. 1992; Nielsen et al. 2006; Prince et al. 1996; Thomssen et al. 1992, 1993) (for virus structure see chapter "Virion Assembly and Release" by Lindenbach, this volume). It was also demonstrated that HCV RNA in the higher density fractions could be immunoprecipitated with antibodies recognizing immunoglobulin G (IgG), indicating that these viruses were circulating bound with antibodies, while the lipoprotein-associated viruses had diminished antibody association (Andre et al. 2002; Thomssen et al. 1993). Virus particles that are associated with VLDL have been termed lipoviral particles (Andre et al. 2002), and their presence and composition is dependent on host factors that impact lipoprotein metabolism such as diet and lipoprotein profile (Bridge et al. 2011; Felmlee et al. 2010). Interestingly, HCV strains that were passaged through chimpanzees demonstrated a correlation between viral association with lipoproteins and infectivity: strains that had more RNA associated with lipoproteins tended to have higher infectivity (Bradley et al. 1991; Hijikata et al. 1993). This was also observed for HCVcc that were passaged in human liver transplanted mice as these HCVcc were characterized by a higher infectivity and a lower buoyant density (Lindenbach et al. 2006). These experimental findings suggested an association of infectious virions with lipoproteins in full agreement with early reports (Monazahian et al. 1999).

The liver is the major organ of lipid homeostasis, regulated in part by lipoproteins. VLDL particles are assembled in hepatocytes when the large 550 kDa monomeric apolipoprotein B (apoB) protein is translated in the endoplasmic reticulum (Shelness and Sellers 2001). This protein is co-translationally loaded with lipids by microsomal triglyceride transfer protein (MTP), the limiting enzyme for VLDL formation (Jamil et al. 1998). Further lipidation of the nascent VLDL may occur directly by fusion with other lipid droplets present in the lumen of the secretory pathway, where exchangeable apolipoproteins such as apoC and apoE can bind VLDL (Wang et al. 2007). Secreted VLDL undergoes significant remodeling while in circulation by docking onto lipoprotein lipase (LPL), which lines the endothelium and hydrolyzes the triglyceride (TG)-rich core for delivery of free fatty acids to skeletal muscle and adipocytes. This process will begin the conversion of a triglyceride-rich VLDL coated in exchangeable apolipoproteins, which steer the VLDL's lipolysis and clearance rate, into a smaller, cholesterol-rich LDL particle with fewer bound exchangeable apolipoproteins (Berneis and Krauss 2002; Packard and Shepherd 1997; Zheng et al. 2008). High-density lipoproteins (HDL) are produced by another mechanism and act as a reservoir for exchangeable apolipoproteins and are key in reverse cholesterol transport from peripheral tissues to the liver (Rothblat and Phillips 2010; Rye et al. 2009; Shachter 2001).

Like for other viruses, there is a tight relation between HCV morphogenesis (see chapter "Virion Assembly and Release" by Lindenbach, this volume) and HCV entry as the composition of the viral particle largely defines virus–host cell interactions and viral entry into target cells. In human hepatoma cells, HCV has been shown to assemble in close proximity to lipid droplets and endoplasmic reticulum (Blanchard et al. 2002; Boulant et al. 2007; Miyanari et al. 2007; Op De Beeck and Dubuisson 2003; Roingeard et al. 2008). HCV NS5A and apoE play a major role in this process (Appel et al. 2008; Benga et al. 2010; Cun et al. 2010). HCV assembly and release have been linked to VLDL biogenesis (Chang et al. 2007; Gastaminza et al. 2006, 2008; Huang et al. 2007). Using mass spectrometry, a recent study reported that the lipid composition of purified HCVcc resembles that of VLDL and LDL and that the majority of E2-containing particles also contain apoE on their surface (Merz et al. 2011). Moreover, HCV virions can be precipitated with antibodies directed against apoB, apoE, and apoCI suggesting that these apolipoproteins are part of the lipoviral particles (Andre et al. 2002; Chang et al. 2007; Meunier et al. 2008). The exact morphogenesis and morphology of HCV particles still remains elusive, but as HCV E1 is able to directly interact with both apoE and apoB (Mazumdar et al. 2011), this envelope glycoprotein may contribute to the association of the viral envelope with host-derived lipoproteins. The virus may take advantage of this association with host-derived lipoproteins in order to evade the host's immune response by masking its envelope glycoproteins and by including host-derived ligands on the virion surface for interaction with attachment factors such as HSPG, LDLR, and SR-BI (Bankwitz et al. 2010; Dreux et al. 2006; Grove et al. 2008; Prentoe et al. 2011).

While apoE on the viral particle is essential for HCV infectivity, the role of apoB for viral entry remains less well-defined (Gastaminza et al. 2008; Huang

et al. 2007; Jiang and Luo 2009; Maillard et al. 2006; Merz et al. 2011; Owen et al. 2009). Indeed, apoE on HCV particles may directly interact with HSPG, LDLR, and SR-BI (Andre et al. 2002; Hishiki et al. 2010; Jiang et al. 2012; Dao Thi et al. 2012; Maillard et al. 2006; Saito et al. 2003) and antibodies directed against apoE or human apoE-derived peptides inhibit HCVcc infection by blocking virus particle binding (Chang et al. 2007; Jiang et al. 2012; Liu et al. 2012; Merz et al. 2011; Owen et al. 2009). Interestingly, it has been suggested by one study that apoE isoforms may influence HCVcc infectivity (Hishiki et al. 2010). Moreover, functional apoE gene polymorphism has been suggested as a determinant of the outcome in HCV infection: the epsilon three allele is associated with persistent HCV infection while the epsilon two allele may protect against viral persistence (Price et al. 2006). It is thus tempting to speculate that there is a link between apoE polymorphism and HCV infection. However, it has to be pointed out that another in vitro study did not report differences in HCVcc assembly and infectivity using different apoE isoforms (Cun et al. 2010).

Given the association of HCV with lipoproteins, it is not surprising that exogenous addition of various lipoproteins has been reported to influence HCV infection. Interestingly, the results of such experiments were dependent on the HCV model system used. VLDL strongly inhibited the interaction of serum-derived HCV with hepatic cells, whereas HDL and LDL did not show a significant effect (Maillard et al. 2006). In contrast, HCVcc infection and HCVpp entry were both increased by HDL while LDL had no effect (Bartosch et al. 2005; Voisset et al. 2005; von Hahn et al. 2006). Moreover, HCVcc infection was inhibited by oxidized LDL (oxLDL) and to a lesser extent by oxidized HDL (oxHDL) (Bartosch et al. 2005; Voisset et al. 2005; von Hahn et al. 2006). The physiological SR-BI ligands HDL and oxLDL/oxHDL do not appear to act as receptor agonist/antagonists, but it has been suggested that the modulation of HCV entry may involve a ternary interplay between the virus, SR-BI, and the respective lipoprotein (Bartosch et al. 2005; von Hahn et al. 2006). Furthermore, apoCI has been reported to enhance HCVcc and HCVpp infectivity and to increase fusion between viral and target membranes (Dreux et al. 2007).

Changes in the nature of the HCV-associated lipoproteins have also been demonstrated to influence HCV infectivity. Although LPL treatment of cells is able to enhance HCV attachment to target cells, at the same time it reduces HCV infectivity (Andreo et al. 2007; Maillard et al. 2011). Furthermore, treatment of HCVcc with a high dose of LPL shifts HCV to higher densities, and decreases the amount of apoE-associated HCV (Shimizu et al. 2010). In addition, the cholesterol and sphingolipid content of both the HCV virion and the target cell membrane, is important for HCV infectivity (Aizaki et al. 2008; Kapadia et al. 2007; Voisset et al. 2008; Yamamoto et al. 2011). Indeed, depletion of cholesterol from HCV particles or hydrolysis of virion-associated sphingomyelin almost completely abolished HCV infectivity without reducing viral binding (Aizaki et al. 2008). Moreover, hydrolysis of target cell plasma membrane sphingomyelin resulted in the internalization of CD81 and reduced HCV infection (Voisset et al. 2008).

In this context, it is interesting to note that the physiological roles of three identified HCV entry factors, namely SR-BI, LDLR, and NPC1L1, are to be key cholesterol transporters. SR-BI primarily functions at the basolateral surface of hepatocytes by binding esterified-cholesterol enriched HDL particles and transferring this cholesterol to the cell (Out et al. 2004). LDLR binds avidly to apoE on the surface of TG-rich lipoprotein remnants and internalizes these particles via clathrin-coated pits. The physiological role of NPC1L1 is to reabsorb unesterified cholesterol secreted into the bile by ABCG5/8 transporters. NPC1L1 is primarily located on the apical (bile canalicular) surface of hepatocytes while the other entry factors are located at the basolateral side, or are part of the TJ complex. When cellular cholesterol content is low, NPC1L1 is present at the plasma membrane to bind extracellular cholesterol. Cholesterol-bound NPC1L1, rather than acting like a cholesterol channel, is endocytosed in a caveolin-1 independent manner (Skov et al. 2011; Valasek et al. 2005) into sorting endosomes followed by progression either to the endosome recycling center or to late endosomes followed by lysosomal degradation (Jia et al. 2011). Sainz et al. showed that silencing NPC1L1, or using chemical inhibitors (ezetimibe) or antibodies to the cholesterol binding domain of NPC1L1, diminish HCVcc infection (Sainz et al. 2012). Given the physiological role for NPC1L1 and its primary location on the bile canalicular surface, NPC1L1 may act as an indirect host entry factor by modulating cholesterol levels. However, enteric cholesterol absorption has been reported to involve cooperation between SR-BI and NPC1L1, raising the possibility that these proteins may cooperate also in HCV entry. Interestingly, while ezetimibe's primary inhibitory function is binding NPC1L1, it is intriguing that ezetimibe also binds to SR-BI (Altmann et al. 2002).

4 Viral Spread Through Cell-to-Cell Transmission

While initiation of HCV infection is dependent on cell-free infection of hepatocytes by virions entering the liver through the bloodstream, HCV dissemination within the liver and establishment of chronic HCV infection may be possible by different routes including direct viral cell-to-cell transmission between adjacent hepatocytes (Timpe et al. 2008). HCV cell-to-cell transmission appears to be more efficient than cell-free particle entry in vitro (Brimacombe et al. 2011; Timpe et al. 2008). Moreover, in contrast to cell-free HCV particle transmission, this process seems to be resistant to the majority of neutralizing antibodies thereby potentially contributing to evasion from the host humoral immune responses and establishment of chronic HCV infection in vivo (Brimacombe et al. 2011; Timpe et al. 2008). Furthermore, like cell-free particle entry, HCV cell-to-cell transmission has been described for all major genotypes, though genotype-dependent differences between the relative infection by cell-free versus cell-to-cell transmission have been reported (Brimacombe et al. 2011).

There are striking similarities between the molecular mechanisms of cell-free HCV entry and HCV cell-to-cell transmission. Indeed, HCV cell-to-cell transmission appears to require numerous host factors that also play a role during cell-free entry: CD81, SR-BI, CLDN1, OCLN, EGFR, EphA2, and potentially NPC1L1 (Brimacombe et al. 2011; Lupberger et al. 2011; Sainz et al. 2012; Timpe et al. 2008). The role of LDLR in HCV cell-to-cell transmission has not been investigated to date. Interestingly, in contrast to cell-free virus entry, CD81-independent cell-to-cell transmission pathways have been described (Jones et al. 2010; Witteveldt et al. 2009). Moreover, it is worth noting that SR-BI appears to have a relevant role in cell-to-cell transmission and thus, targeting SR-BI allows to inhibit HCV spread (Brimacombe et al. 2011; Meuleman et al. 2012; Zahid et al. 2012). Furthermore, it has been shown that SR-BI may be a limiting factor in HCV cell-to-cell transmission since overexpression of SR-BI in target cells increases virus spread (Brimacombe et al. 2011).

Although HCV cell-to-cell transmission had been defined to be resistant to neutralizing antibodies, certain monoclonal antibodies directed against HCV E2 (mAb 9/27 targeting HVR1; mAb 11/20 targeting aa 436–447) have been demonstrated to partially interfere with cell-to-cell transmission, although less efficiently than with cell-free infection (Brimacombe et al. 2011). These data indicate the intriguing possibility of developing potential therapeutic neutralizing antibodies that may inhibit both cell-free HCV entry and cell-to-cell transmission, thereby limiting both initiation of HCV infection and viral spread.

5 Impact of Hepatitis C Virus Entry for Pathogenesis of Liver Disease and Antiviral Therapy

HCV entry is an important target of the host's immune responses. Neutralizing antibodies have been reported to interfere with different steps of the HCV entry process in vitro including attachment, entry, and fusion (Fofana et al. 2012; Haberstroh et al. 2008; Jiang et al. 2012; Steinmann et al. 2004; Vieyres et al. 2011). Different anti-E2 antibodies, such as AP33 (directed against E2 aa 412–423) or 3E5-1 (directed against E2 aa 522–529), have been reported to reduce HCV attachment to target cells (Barth et al. 2005b), as well as other steps of the HCV entry process (Haberstroh et al. 2008), whereas human anti-E1 antibodies targeting aa 313–326 interfere with HCV entry during post-binding steps without blocking HCVpp or HCV-LP binding to human hepatoma cells in vitro (Haberstroh et al. 2008). Interestingly, the HCV post-binding steps targeted by neutralizing antibodies are closely related to the interaction of HCV with CD81, SR-BI, and/or CLDN1 (Haberstroh et al. 2008). Moreover a recent study demonstrated that viral escape from neutralizing antibodies also takes place during HCV post-binding steps (Fofana et al. 2012). These data highlight the impact of post-binding steps of HCV entry for neutralization and evasion of immune responses.

Viral entry has been shown to play an important role for the pathogenesis of HCV infection. Indeed, using patient-derived HCVpp and antibodies from a cohort of liver transplant patients, a recent study demonstrated that enhanced viral entry and escape from antibody-mediated neutralization play a key role for the selection of viral variants in the early phase of liver transplantation (LT) (Fafi-Kremer et al. 2010). This study suggested that the abrupt change of the host environment during LT leads to the selection of viral variants with an efficient entry phenotype (Fafi-Kremer et al. 2010). Furthermore, the analysis of HCV infection in the chimeric liver uPA-SCID mouse model corroborated this hypothesis (Fafi-Kremer et al. 2010). Using reverse genetics and chimeric HCVcc, it was then demonstrated that the enhanced entry of an escape variant isolated from a liver transplant patient is associated with an altered usage of entry factors such as CD81 and SR-BI (Fofana et al. 2012). An altered host entry factor usage associated with escape from neutralizing antibodies may thus contribute to the reinfection of the liver graft and establishment of persistent HCV infection (Fofana et al. 2012). In this context, it is interesting to point out that HCV receptor levels at the time of LT seem to modulate early HCV kinetics and that recurrence of HCV infection after LT was associated with increased levels of CLDN1 and OCLN (Mensa et al. 2011). Taken together, these data suggest a key role of virus–host interactions during HCV entry for virus spread, persistence, and liver graft infection in transplanted patients.

Given the importance of HCV entry into target cells for the initiation and maintenance of infection as well as the pathogenesis of liver disease, this multistep process offers several promising targets for antiviral strategies (see also chapter "Hepatitis C Virus-Specific Directly Acting Antiviral Drugs" by Delang et al. this volume). Indeed, although the introduction of novel direct acting antivirals (targeting the viral protease) in combination with interferon-alfa and ribavirin, the standard treatment for chronic HCV infection, has markedly improved the outcome of patients, resistance remains an important challenge (Chevaliez and Asselah 2011; Pawlotsky 2011) (see also chapter "Treatment of Chronic Hepatitis C: Current and Future" by Pawlotsky this volume). Furthermore, important adverse effects limit the use of standard treatment or direct acting antivirals in patients with advanced liver disease, co-morbidity, co-infection, or immunosuppression (Pawlotsky 2011).

In a significant proportion of HCV infected individuals, chronic infection ultimately leads to cirrhosis and hepatocellular carcinoma, requiring LT. Unfortunately, LT is characterized by a rapid reinfection of the liver graft and an accelerated progression of infection and to date no strategy to prevent HCV reinfection of the graft is available. For this purpose, HCV entry inhibitors, by targeting cell-free virus entry, may address this unmet medical need. Furthermore, this treatment can also be efficient for established HCV infection by targeting HCV cell-to-cell transmission in combination with direct acting antivirals. A variety of entry inhibitors are in preclinical and early clinical development (reviewed in Zeisel et al. 2011). These entry inhibitors can be categorized into (1) compounds targeting the lipoviral particle, e.g., anti-envelope antibodies, heparin, and lectins (Barth et al. 2003; Davis et al. 2005;

Helle et al. 2006; Koutsoudakis et al. 2006; Law et al. 2008), (2) compounds targeting essential host entry factors, e.g., antibodies blocking CD81, SR-BI, CLDN1, EGFR, EphA2, and NPC1L1 (Bartosch et al. 2003b; Catanese et al. 2007; Flint et al. 1999; Fofana et al. 2010; Lupberger et al. 2011; Sainz et al. 2012; Zeisel et al. 2007; Zahid et al. 2012) or small molecule compounds targeting CD81, SR-BI, EGFR, EphA2, and NPC1L1 (Lupberger et al. 2011; Sainz et al. 2012; Syder et al. 2011; VanCompernolle et al. 2003), and (3) compounds interfering with viral internalization and fusion, e.g., phosphorothioate oligonucleotides, arbidol, chloroquine, and silymarin (Blanchard et al. 2006; Boriskin et al. 2006, 2008; Matsumura et al. 2009; Polyak et al. 2007; Tscherne et al. 2007; Wagoner et al. 2010).

The development of liver humanized transgenic mice has allowed demonstrating proof-of-concept of entry inhibitors in vivo (for animal models see chapter "Animal Models for Hepatitis C" by Billerbeck et al. this volume). The first clinical trials with HCV entry inhibitors have been conducted (Davis et al. 2005; Hawke et al. 2010; Neumann et al. 2010; Rutter et al. 2011) or are currently being initiated. Indeed, several entry inhibitors targeting different steps of the HCV entry process have been demonstrated to prevent and/or delay HCV infection in vivo using the uPA-SCID mouse model. The first study investigated the ability of polyclonal patient-derived neutralizing antibodies to protect mice from HCV infection with a homologous viral strain (Vanwolleghem et al. 2008). This passive immunization strategy protected 5 out of 8 challenged mice. Although this study did not investigate cross-reactive neutralization of various viral strains, it provides promising evidence, which opens the perspective for potential neutralizing antibody-based immunotherapies to prevent HCV infection (Vanwolleghem et al. 2008). Recently, the ability of neutralizing antibodies to cross-neutralize different HCV genotypes in vivo was assessed (Meuleman et al. 2011b). Remarkably, protection against challenge with heterologous viral strains was achieved in a portion of the animals, though differences were observed between genotypes and depending on the viral load of the inoculum (Meuleman et al. 2011b). Importantly, although no sterilizing immunity was achieved, a delay in the establishment of HCV infection was observed and adaptive mutations in the HCV envelope glycoproteins were absent in the majority of nonprotected animals, indicating that the failure of cross-neutralization in vivo was most likely not directly linked to viral escape (Meuleman et al. 2011b). Furthermore, another study assessed the ability of human cross-neutralizing monoclonal anti-E2 antibodies derived from a chronic HCV patient and demonstrated that the majority of chimeric mice were protected from challenge with a heterologous virus (Law et al. 2008). Most recently, novel human cross-neutralizing anti-envelope antibodies targeting five novel antigenic regions (ARs) designated numerically have been described and these antibodies (particularly those that bind AR3A, AR4A, and AR5A) are also characterized by the ability to protect from heterologous virus challenge in genetically humanized mice (Giang et al. 2012). Future studies assessing the potential of potent broadly cross-neutralizing monoclonal antibodies (independently or in combination) will allow determination of whether neutralizing antibodies have the potential for clinical application in prevention of HCV infection. Interestingly, other compounds

targeting the HCV envelope and preventing attachment of the virion to target cells, such as the lectin Griffithsin, also demonstrated the ability to delay HCV infection in vivo (Meuleman et al. 2011a).

In addition to virus-neutralizing antibodies, antivirals that target host cell surface factors are being developed (reviewed in Zeisel et al. 2011). Antibodies directed against CD81 and SR-BI have been investigated in both protection and treatment studies. While both anti-CD81 and anti-SR-BI monoclonal antibodies protect mice from challenge with HCV, only anti-SR-BI monoclonal antibodies appear also able to reduce viral spread (Lacek et al. 2012; Meuleman et al. 2008, 2012). Anti-CLDN1 antibodies are currently being evaluated in the uPA-SCID mouse model to assess their antiviral activity against HCV infection. Furthermore, the clinically available kinase inhibitor erlotinib, targeting EGFR, and the cholesterol lowering drug ezetimibe, targeting NPC1L1, significantly impaired the establishment of HCV infection in the uPA-SCID mouse model (Lupberger et al. 2011; Sainz et al. 2012). Given the relevance of host cell kinases for HCV entry (Lupberger et al. 2011) and the number of kinase inhibitors being developed to treat a wide variety of human diseases, kinase inhibitors have been suggested as a novel class of antivirals for the prevention and treatment of HCV infection.

Since HCV entry is a major target of B cell responses, the virus–host interactions during viral entry are also relevant for HCV vaccine development. Indeed, B cell vaccines inducing cross-neutralizing antibodies are in preclinical and clinical development (Frey et al. 2010; Garrone et al. 2011; Halliday et al. 2011; Houghton 2012).

6 Conclusions and Perspectives

Since the discovery of HCV more than 20 years ago, the development of model systems has allowed investigators to decipher the molecular mechanisms of the different steps of the viral life cycle. In the past decade, several host cell surface factors contributing to HCV binding and/or entry have been identified. The functional relevance of most factors has been validated in cell culture and animal model systems. The thorough characterization of the role of each of these host factors enabled researchers to gain insights into the complex process of HCV entry. Given the complexity of virus–host interactions during this step of the viral life cycle, viral entry offers numerous potential targets for antiviral therapy. Since HCV entry is a major target of B cell responses, a detailed understanding of virus–host interactions during viral entry is also relevant for HCV vaccine development. Although highly orchestrated and complex, the mechanism of viral entry is conserved for all HCV genotypes and appears to be restricted to a single pathway. In contrast to the highly variable virus, host factors are less susceptible to mutations; therefore targeting host factors may allow increase of the genetic barrier to resistance (reviewed in Zeisel et al. 2012). Furthermore, the efficient in vitro and in vivo neutralization of patient-derived HCV isolates known to escape autologous neutralizing responses, by using monoclonal antibodies directed against the HCV envelope glycoproteins or

CD81, CLDN1, and SR-BI, suggests that viral entry is a viable target for prevention of HCV reinfection of the liver graft (Fafi-Kremer et al. 2010; Fofana et al. 2010; Lacek et al. 2012; Zahid et al. 2012). Given the essential role of host cell surface entry factors in HCV cell-to-cell transmission and thus for spread and maintenance of infection, it is not surprising that defined entry inhibitors have also been shown to prevent cell-to-cell transmission and viral spread in vivo (Lacek et al. 2012; Meuleman et al. 2012). Thus entry inhibitors may hold promise for the development of novel antiviral strategies for the prevention and treatment of HCV infection.

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Hepatitis C Virus Proteins: From Structure to Function

Darius Moradpour and François Penin

Abstract Great progress has been made over the past years in elucidating the structure and function of the hepatitis C virus (HCV) proteins, most of which are now actively being pursued as antiviral targets. The structural proteins, which form the viral particle, include the core protein and the envelope glycoproteins E1 and E2. The nonstructural proteins include the p7 viroporin, the NS2 protease, the NS3-4A complex harboring protease and NTPase/RNA helicase activities, the NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase. NS4B is a master organizer of replication complex formation while NS5A is a zinc-containing phosphoprotein involved in the regulation of HCV RNA replication versus particle production. Core to NS2 make up the assembly module while NS3 to NS5B represent the replication module (replicase). However, HCV proteins exert multiple functions during the viral life cycle, and these may be governed by different structural conformations and/or interactions with viral and/or cellular partners. Remarkably, each viral protein is anchored to intracellular membranes via specific determinants that are essential to protein function in the cell. This review summarizes current knowledge of the structure and function of the HCV proteins and highlights recent advances in the field.

Abbreviations

aa	Amino acid
AH	Amphipathic α-helix
CHV	Canine hepacivirus
CK	Casein kinase
CsA	Cyclosporin A

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CypA	Cyclophilin A
DHPC	1,2-diheptanol-sn-glycero-3-phosphocholine
E	Envelope glycoprotein
ER	Endoplasmic reticulum
GBV-B	GB virus B
HCV	Hepatitis C virus
HVR	Hypervariable region
igVR	Intergenotypic variable domain
IRES	Internal ribosome entry site
ISDR	Interferon sensitivity determining region
LCS	Low complexity sequence
LD	Lipid droplet
MD	Molecular dynamics
NCR	Noncoding region
NMR	Nuclear magnetic resonance
NS	Nonstructural protein
ORF	Open reading frame
PI4KIII	Phosphatidylinositol 4-kinase III
PI4P	Phosphatidylinositol 4-phosphate
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
RdRp	RNA-dependent RNA polymerase
SPP	Signal peptide peptidase
TMD	Transmembrane domain

Contents

1 Introduction

Hepatitis C virus (HCV) infection is now the leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma in many parts of the world, with a peak of the disease burden expected around 2020 (Nature Outlook 2011). On this

background, great progress has been made in elucidating the structure and function of the HCV proteins, most of which are now actively being pursued as antiviral targets (Moradpour et al. 2007; Lindenbach et al. 2013).

HCV as well as GB virus B (GBV-B) and canine hepacivirus (CHV) have been classified in the *Hepacivirus* genus within the *Flaviviridae* family, which also includes the genera *Flavivirus* (e.g., yellow fever virus and dengue virus), *Pestivirus* (e.g., bovine viral diarrhea virus), and *Pegivirus* (e.g., human pegivirus, also known as GB virus C) (Kapoor et al. 2011 and references therein) (see also chapter "The Origin of Hepatitis C Virus" by Simmonds, this volume). HCV contains a 9.6 kb positive-strand RNA genome composed of a 5′ noncoding region (NCR), which includes an internal ribosome entry site (IRES), an open reading frame (ORF) that encodes the structural and nonstructural proteins, and a 3′ NCR (Fig. 1). The structural proteins, which form the viral particle, include the core protein and the envelope glycoproteins E1 and E2. The nonstructural proteins include the p7 viroporin, the NS2 protease, the NS3-4A complex harboring protease and NTPase/RNA helicase activities, the NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase (RdRp).

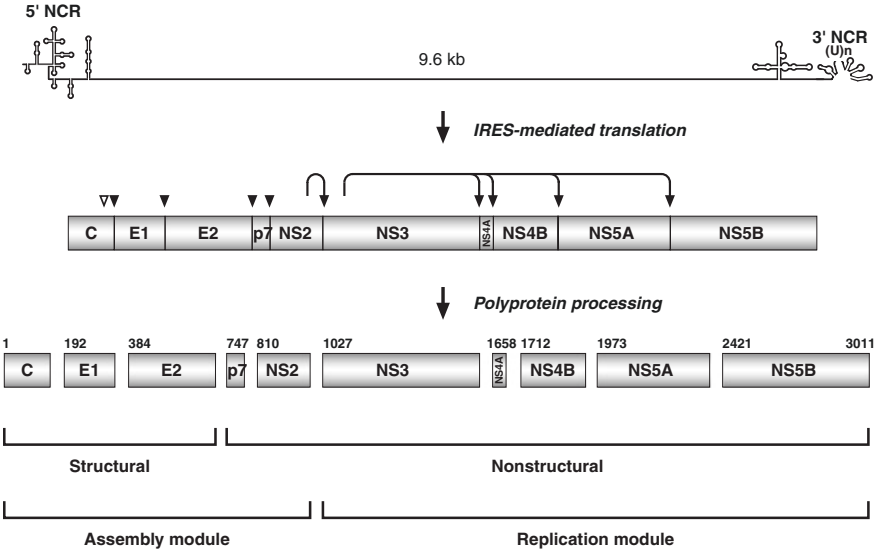
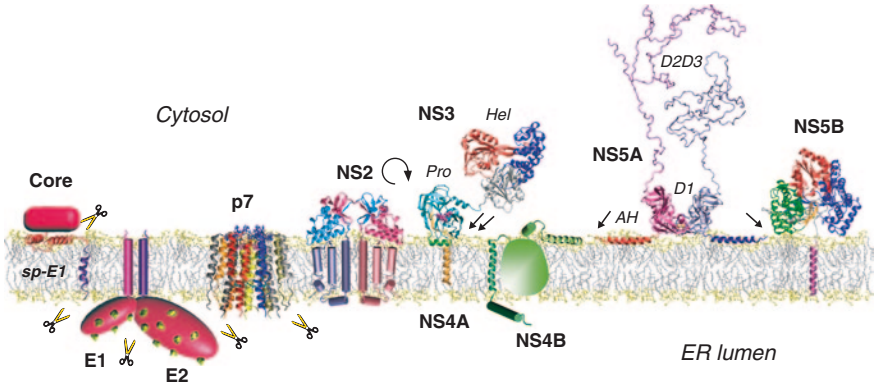


Fig. 1 Genetic organization and polyprotein processing of HCV. The 9.6 kb positive-strand RNA genome is schematically depicted at the *top*. Simplified RNA secondary structures in the 5′ and 3′ noncoding regions (*NCRs*) as well as the core and NS5B coding regions are shown. Internal ribosome entry site (*IRES*)-mediated translation yields a polyprotein precursor that is processed into the mature structural and nonstructural proteins. Amino acid numbers are shown above each protein (HCV H strain; genotype 1a; GenBank accession number AF009606). *Solid arrowheads* denote cleavages by the endoplasmic reticulum signal peptidase. The *open arrowhead* indicates further C-terminal processing of the core protein by signal peptide peptidase. *Arrows* indicate cleavages by the HCV NS2 and NS3-4A proteases. Note that polyprotein processing, illustrated here as a separate step for simplicity, occurs co- and posttranslationally. Also note that all components of the replication module (replicase) are also involved in assembly



IRES-mediated translation of the HCV ORF (see also chapter "Hepatitis C Virus RNA Translation" by Niepmann, this volume) yields a polyprotein precursor that is co- and posttranslationally processed by cellular and viral proteases into the mature structural and nonstructural proteins (Fig. 1). The structural proteins and the p7 polypeptide are processed by the endoplasmic reticulum (ER) signal peptidase and signal peptide peptidase (SPP) while the nonstructural proteins are processed by the viral NS2 and NS3-4A proteases. The nonstructural proteins are cleaved in a preferential order (Pietschmann et al. 2001 and references therein). The first cleavages by the viral proteases occur cotranslationally at the NS2/NS3 and NS3/NS4A sites and liberate NS3 from the remainder of the polyprotein. Subsequent NS3-4A protease-mediated processing events occur *in trans*, with rapid processing at the NS5A/NS5B site to produce an NS4A-5A intermediate, followed by cleavage between NS4A and NS4B, to produce a relatively stable NS4B-5A precursor, and subsequently between NS4B and NS5A. These kinetics appear to be important, and some precursors may have time-dependent functions (Herod et al. 2012 and references therein).

From a functional point of view, HCV proteins can be divided into an assembly module (core-NS2) and a replication module (NS3-NS5B, making up the replicase) (Fig. 1). However, it has become apparent that each of the replicase proteins is also involved in HCV assembly, although the precise mechanisms are elusive (Appel et al. 2008; Ma et al. 2008; Tellinghuisen et al. 2008; Jones et al. 2009; Gouklani et al. 2012; reviewed in Murray et al. 2008). Current evidence indicates that NS5A might function as a "molecular switch" between RNA replication and assembly, possibly by tethering the viral RNA to membranes and/or by providing a physical link between replication complexes and viral assembly sites on lipid droplets (LD) or the ER-LD interface and/or by engaging in interactions with host factors involved in replication and assembly (see below). However, the mechanisms governing HCV RNA translation and replication versus the assembly and release of newly formed viral particles are still poorly understood (see also chapter "Virion Assembly and Release" by Lindenbach, this volume).

◀ **Fig. 2** Structure and membrane association of HCV proteins. Scissors indicate cleavages by the endoplasmic reticulum (*ER*) signal peptidase, except on the cytosolic side where it indicates the processing of core by signal peptide peptidase (*SPP*). The *cyclic arrow* denotes cleavage by the NS2 protease. *Black arrows* indicate processing by the NS3-4A protease. Known protein structures are shown as ribbon diagrams. The structures and the membrane bilayer are shown at the same scale. Proteins or protein segments of unresolved structure are represented as colored *spheres* or *cylinders* with their approximate sizes. From *left* to *right* are shown: (1) Core protein (*red*) includes the N-terminal natively unfolded domain (D1) and two amphipathic α-helices connected by a hydrophobic loop (D2 domain; Boulant et al. 2006) as well as the core-E1 signal peptide (PDB entry 2KQI) (Oehler et al. 2012) cleaved by SPP. (2) E1-E2 glycoprotein heterodimer associated by the C-terminal transmembrane domains. *Green spots* denote glycosylation of the envelope proteins. (3) Oligomeric model of p7 based on the structure of the monomer solved by nuclear magnetic resonance (Montserret et al. 2010) and molecular dynamics simulations in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) phospholipid bilayer (Chandler et al. 2012). (4) NS2 catalytic domain (dimer subunits in *blue* and *magenta*; PDB entry 2HD0; Lorenz et al. 2006) connected to their N-terminal membrane domains constituted of three putative transmembrane segments (PDB entries 2JY0, 2KWT, and 2KWZ; Jirasko et al. 2008, 2010). The active site residues His 143, Glu 163, and Cys 184 are represented as spheres. (5) NS3 serine protease domain (*cyan*) associated with the central protease activation and the N-terminal transmembrane domains of NS4A (*yellow*). The catalytic triad of the NS3 serine protease (His 57, Asp 81, and Ser 139) is represented as *spheres* (*magenta*). NS3 helicase domains 1, 2, and 3 are colored in *silver*, *red* and *blue*, respectively. This representation of NS3 (derived from PDB entry 1CU1; Yao et al. 1999) indicates that the helicase domain can no longer interact with the NS3 protease domain when the latter is associated with the membrane through its amphipathic α-helix 11–21 (*green*) and the transmembrane domain of NS4A (BMRB entry 15580; Brass et al. 2008). (6) NS4B with the N-terminal part, including two amphipathic α-helices of which the second has the potential to traverse the membrane bilayer (PDB entry 2KDR; Gouttenoire et al. 2009a), the central part harboring multiple predicted transmembrane segments, and the C-terminal cytosolic part, including a predicted highly conserved α-helix and an amphipathic α-helix interacting in-plane with the membrane (PDB entry 2JXF; Gouttenoire et al. 2009b). (7) NS5A domain 1 dimer (D1; PDB entry 1ZH1 (Tellinghuisen et al. 2005); subunits colored in *magenta* and ice *blue*) as well as intrinsically unfolded domains 2 and 3 (D2D3; Liang et al. 2007; Hanoulle et al. 2009; Verdegem et al. 2011). The N-terminal amphipathic α-helix in-plane membrane anchor (PDB entry 1R7E; Penin et al. 2004; helices colored in *red* and *blue*) are shown in relative position to the phospholipid membrane (adapted from Tellinghuisen et al. 2005). (8) NS5B RNA-dependent RNA polymerase (RdRp) catalytic domain (PDB entry 1GX6; Bressanelli et al. 2002) associated with the membrane via its C-terminal transmembrane segment (F.P. et al. unpublished data). The fingers, palm, and thumb subdomains of the catalytic domain are colored *blue*, *red*, and *green*, respectively. The catalytic site of the RdRp lies within the *center* of the cytosolic domain and the RNA template-binding cleft is located vertically on the *right* along the thumb subdomain β-flap (*orange*) and the C-terminal part of the linker segment (*silver*), connecting the cytosolic domain to the transmembrane segment (*magenta*). The membrane is represented as a simulated model of a POPC bilayer (obtained from Tieleman, http://moose.bio.ucalgary.ca/). Polar heads and hydrophobic tails of phospholipids (stick structure) are colored *light yellow* and *light gray*, respectively. The positions of the NS5A in-plane membrane helices at the membrane interface as well as that of the transmembrane domain of NS5B were deduced from molecular dynamics simulations in POPC bilayer (F.P., D.M. et al. unpublished data). The positioning of the NS3-4A membrane segments and of the amphipathic α-helices in core and NS4B are tentative. The figure was generated from the structure coordinates deposited in the PDB using Visual Molecular Dynamics (*VMD*) (http://www.ks.uiuc.edu/Research/vmd/) and rendered with POV-Ray (http://www.povray.org/)

An important aspect of the HCV proteins is their multifunctionality, as exemplified by the multiple functions of the NS3-4A complex in the replication and pathogenesis of HCV (see below) or the involvement of replicase components in viral assembly. The diverse functions of HCV proteins may be governed by different structural conformations and/or interactions with viral and/or cellular partners (de Chassey et al. 2008). In addition, it is likely that some or most nonstructural proteins exert "structural" functions beyond their enzymatic activities, forming a specific microenvironment through protein cluster, array, or lattice formation on intracellular membranes. Indeed, as all positive-strand RNA viruses investigated thus far, HCV replicates its genome in a membrane-associated replication complex composed of viral proteins, replicating RNA, rearranged intracellular membranes, and additional host factors (Moradpour et al. 2007; Miller and Krijnse-Locker 2008; den Boon et al. 2010). In fact, each viral protein is anchored to intracellular membranes via specific determinants that are essential to protein function in the cell (Fig. 2).

In the following, we will summarize the current knowledge of the structure and function of the HCV proteins (Table 1) and highlight recent advances in the field.

2 Structural Proteins

2.1 Core

The first structural protein encoded by the HCV ORF is the core protein, which forms the viral nucleocapsid. An internal signal sequence located between core and E1 targets the nascent polypeptide to the translocon of the ER membrane, followed by translocation of the E1 ectodomain into the ER lumen (Santolini et al. 1994). Of note, the signal sequence at the C terminus of core is the only true signal sequence in the HCV polyprotein, while other transmembrane segments may act as stop transfer sequences or reinitiation of translocation signals. Cleavage of the core-E1 signal sequence by signal peptidase yields an immature 191-amino-acid (aa) core protein. Further C-terminal processing by the intramembrane cleaving protease SPP yields the mature 21 kDa core protein of ~177 aa (McLauchlan et al. 2002; Okamoto et al. 2008; Oehler et al. 2012 and references therein). Mutagenesis and *trans*-complementation studies suggest that at least 177 residues of core protein are needed for infectious particle production (Kopp et al. 2010). However, the precise C terminus of mature core has not yet been identified unequivocally. The recently determined nuclear magnetic resonance (NMR) structure of a synthetic core-E1 signal peptide provides the structural basis for further studies on the mechanism of SPP cleavage and its role in HCV infection (Oehler et al. 2012).

Mature core is a dimeric membrane protein stabilized through disulfide bond formation at Cys 128 (Boulant et al. 2005; Kushima et al. 2010). However, the signals and processes that mediate RNA packaging and the assembly of core into nucleocapsids are largely unknown.

Table 1 Structure and function of hepatitis C virus proteins

Protein	aa	MW (kDa)	Structure	Function
C	191	21	N-terminal basic, RNA-binding domain 1 (aa 1-117); hydrophobic, lipid droplet-binding domain 2 (aa 118 to ~177, harboring 2 amphipathic α-helices connected by a hydrophobic loop); C-terminal signal sequence. Maturation via signal peptide peptidase-mediated removal of the C-terminal signal sequence. Dimeric protein (stabilized by disulfide bond formation through Cys 128)	Nucleocapsid formation
E1	192	35	Highly glycosylated (up to 6 glycosylation sites), 4 potential disulfide bonds, C-terminal transmembrane domain	Envelope glycoprotein. Heterodimer formation with E2
E2	363	70	Highly glycosylated (up to 11 glycosylation sites), 9 potential disulfide bonds, C-terminal transmembrane domain. Hypervariable region 1 (aa 1–28). Binds directly to CD81 and scavenger receptor BI	Envelope glycoprotein. Heterodimer formation with E1
p7	67	7	Two transmembrane α-helices connected by a cytoplasmic loop. Forms oligomers (hexamer and heptamer)	Viroporin
NS2	217	23	N-terminal membrane domain with 3 predicted transmembrane passages. C-terminal catalytic domain (aa 94–217) forms a dimeric cysteine protease with two composite active sites	Membrane-associated dimeric cysteine protease
NS3	631	70	N-terminal serine protease domain (aa 1–180) with a chymotrypsin-like fold with two β-barrel subdomains. Includes a structural zinc binding site. NTPase/RNA helicase domain (aa 181–631) with 3 subdomains. Membrane association through helix α_0 and N-terminal transmembrane segment of NS4A	Serine protease and NTPase/RNA helicase activities. Forms a non-covalent complex with NS4A
NS4A	54	8	N-terminal transmembrane α-helix (aa 1–21); central part (aa 21–32) forms a β-sheet as part of the N-terminal β-barrel of NS3; C-terminal acidic portion (aa 40–54) interacts with other replicase components, including NS3 NTPase/RNA helicase domain	Cofactor for NS3
NS4B	261	27	Integral membrane protein comprising an N-terminal portion (aa 1 to ~69, including amphipathic α-helices AH1 and AH2, extending from aa 3–35 and aa 42–66, respectively), a central part harboring four predicted transmembrane passages (aa ~70 to ~190), and a C-terminal portion (aa ~191–261, including amiphipathic α-helices H1 and H2, extending from aa 201–213 and 229–253, respectively)	Induction of the membranous web. NTPase activity? RNA binding?

(continued)

Table 1 (continued)

Protein	aa	MW (kDa)	Structure	Function
NS5A	447[1]	56–58	N-terminal amphipathic α-helix as membrane anchor (aa 1-31); domain 1 (aa 36–213) includes a zinc binding site and forms either a 'claw-like' dimer with a basic groove or a side-by-side dimer; both natively unfolded domains 2 (aa 250–352) and 3 (aa 356–447) exhibit intrinsic α-helical propensity	Serine phosphoprotein; RNA binding; phosphorylation-dependent modulation of RNA replication (domains 1 and 2) and viral assembly (domain 3)
NS5B	591	68	Fingers, palm, and thumb subdomains. Interactions between fingers and thumb subdomains result in encircled active site. Membrane association mediated by C-terminal transmembrane α-helix (tail-anchored protein)	RNA-dependent RNA polymerase

[1]NS5A from genotype 2 isolates contains a 20-aa insertion in domain 3, close to the C terminus

HCV core is composed of two domains (Boulant et al. 2005) (Fig. 3). The N-terminal hydrophilic domain 1 (D1, aa 1–117) contains a high proportion of basic aa residues and has been implicated both in RNA binding and homooligomerization, thereby promoting nucleocapsid assembly. As a general feature of nucleocapsid proteins, core D1 exhibits RNA chaperone activity likely required for the structural remodeling and packaging of the RNA genome in the viral particle (Cristofari et al. 2004). D1 is also involved in interactions with numerous cellular factors (de Chassey et al. 2008) and may thereby contribute to alterations of host cell functions upon HCV infection. Core D1 behaves as an intrinsically unstructured protein when isolated (Boulant et al. 2005), but it includes several potential α-helices, notably a helix-loop-helix motif involving aa 15–41, constituting an immunodominant antigenic site (Jolivet et al. 1997) and harboring residues critical for core function (Angus et al. 2012). Moreover, D1 folds upon interaction with the C-terminal hydrophobic domain 2 (D2, aa 118 to ~177) (Boulant et al. 2005), which mediates association with LDs (Boulant et al. 2006). D2 consists of a central hydrophobic loop connecting two amphipathic α-helices (comprising aa 119–136 and 148–165) interacting in-plane with the LD phospholipid interface (Boulant et al. 2006).

Recent fluorescent tagging and live cell imaging studies have provided elegant insights into the trafficking of functional core to putative assembly sites and the interaction with viral nonstructural proteins (Counihan et al. 2011; Coller et al. 2012). The association of core with LDs (Boulant et al. 2007; Miyanari et al. 2007; Shavinskaya et al. 2007) and interaction with NS5A (Masaki et al. 2008) play central roles in nucleocapsid assembly (see also chapter "Virion Assembly and Release" by Lindenbach, this volume). NS5A is indeed thought to deliver the HCV genome RNA to core protein (see below), and as its membrane anchor is able to associate with the phospholipid monolayer of LDs, RNA transfer likely occurs on LDs or the ER-LD interface. Because of the particular features of the core membrane-binding domain, the resulting RNA-containing nucleocapsid is expected to be surrounded by a lipid monolayer, allowing its full immersion into the hydrophobic core of LDs and, ultimately, of lipo-viro-particles (reviewed in Bartenschlager et al. 2011).

Additional translation products from an alternative reading fame overlapping the core coding region (alternative reading frame protein, F protein, core +1; reviewed in Branch et al. 2005) as well as a family of minicores ranging in size from 8 to 14 kDa have been detected (Eng et al. 2009), but the role of these proteins, if any, in the life cycle and pathogenesis of HCV remains to be elucidated.

2.2 Envelope Glycoproteins

The envelope glycoproteins E1 and E2 play pivotal roles at different steps of the HCV life cycle, including the assembly of the infectious particle, virus entry, and fusion with the endosomal membrane (see also chapter by "Hepatitis C Virus Entry" Zeisel et al., this volume). To fulfill these functions, E1 and E2 likely adopt

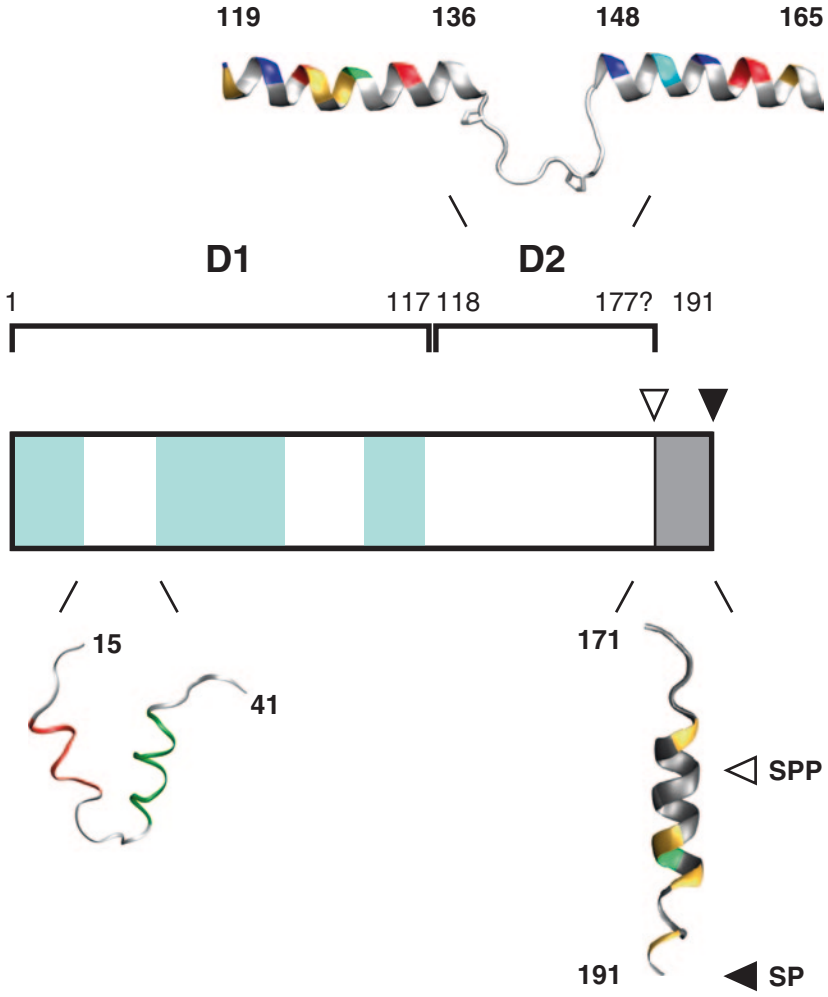


Fig. 3 Schematic representation of the HCV core protein. Core protein is composed of two domains (Boulant et al. 2005). The N-terminal hydrophilic domain 1 (D1, aa 1–117) contains three hydrophilic, highly basic segments (*blue boxes*) separated by two more hydrophobic segments. D1 is an intrinsically unstructured protein when isolated but includes several potential α-helices, notably a helix-loop-helix motif involving aa 15–41 (ribbon representation (PDB entry 1CWX; Jolivet et al. 1997; Angus et al. 2012). The α-helices 1 (aa 19–24) and 2 (aa 30–37) are *red* and *green*, respectively. The C-terminal hydrophobic domain 2 (D2, aa 118 to ~177) consists of a central hydrophobic loop connecting two amphipathic α-helices (comprising aa 119–136 and 148–165; ribbon representation; Boulant et al. 2006). At the C terminus of core is the core-E1 signal peptide (PDB entry 2KQI; Oehler et al. 2012), which is cleaved by signal peptidase (SP; after aa 191; *solid arrowhead*) and signal peptide peptidase (SPP; after aa ~177; *open arrowhead*) to yield mature core protein. Hydrophobic residues are *gray*, neutral residues (Gly and Ala) are *light gray*, and hydrophilic residues are colored accordingly to their physico-chemical properties: Ser, Thr, Asn, and Gln in *yellow*; Asp and Glu in *red*; Arg and Lys in *blue*; His in *cyan*; and Cys in *green*

markedly different conformations that should be tightly controlled to occur at the appropriate moment during the viral life cycle.

E1 and E2 are type I transmembrane proteins with an N-terminal ectodomain (~160 and ~360 aa for E1 and E2, respectively) and a short C-terminal transmembrane domain (TMD) of ~30 aa. During their synthesis, the E1 and E2 ectodomains are translocated into the ER lumen and their TMDs are inserted into the ER membrane. Both TMDs are composed of two stretches of hydrophobic aa separated by a short polar segment containing fully conserved charged residues. The second hydrophobic stretches function as reinitiation of translocation signals for the downstream protein. Before signal sequence cleavage, both the E1 and E2 TMDs form a hairpin structure. After signal peptidase cleavage, the signal sequence is reoriented toward the cytosol, resulting in a single transmembrane passage (Cocquerel et al. 2002).

The TMDs contribute importantly to the functions of E1 and E2, including membrane anchoring, ER retention, and E1–E2 noncovalent heterodimer formation, which is believed to represent the building block for the viral envelope (reviewed in Voisset and Dubuisson 2004). However, it was shown recently that virion-associated E1 and E2 form large covalent complexes stabilized by disulfide bonds (Vieyres et al. 2010). The presence of disulfide bonds between the envelope glycoproteins suggests that lateral protein–protein interactions might play an active role in the budding of HCV. The ectodomains of E1 and E2 indeed contain numerous highly conserved cysteine residues that may form 4 and 9 disulfide bonds, respectively. In addition, E1 and E2 contain up to 6 and 11 glycosylation sites, respectively. Thus, E1 and E2 maturation and folding is a complex and interdependent process that involves the ER chaperone machinery and disulfide bond formation as well as glycosylation.

Secondary and tertiary structures of the glycoproteins are supposed to be similar among members of the *Flaviviridae* family, suggesting that the HCV envelope glycoproteins belong to the class II fusion proteins (reviewed in Kielian and Rey 2006). Based on disulfide mapping studies and modeling on the known flavivirus glycoprotein E structure, a model of the structural organization of the E2 ectodomain was recently proposed (Krey et al. 2010). This model comprises three domains: Domain 1 (D1) is predicted to contain eight β-strands, is extended at the N terminus by hypervariable region 1 (HVR1), and harbors the majority of known determinants for CD81 interaction. Domain 2 (D2) includes HVR2, and its most conserved part is suggested to act as a fusion loop (aa 119–137). D1 is connected to domain 3 (D3) by a linker region called the intergenotypic variable region (igVR). D3, which includes part of the CD81-binding domain, is connected to the TMD by a flexible stem region. Remarkably, both D2 and D3 appear to be largely intrinsically unstructured, contrasting with the classical view of well-structured viral envelope proteins reported so far. Similar to other intrinsically unstructured proteins, which are expected to adopt different conformations depending on their interaction with specific biological partners, this feature is likely essential for the interaction with E1 as well as the intramolecular interactions required for receptor engagement and membrane fusion. Determination of

the three-dimensional structures of the E1 and E2 ectodomains in association with their biological partners would be key in elucidating the receptor binding and fusion processes mediated by these proteins.

Recent work identified a segment (aa 322–332) in the stem region of E2 (aa 279-331) which is essential for virus entry and harbors a central amphipathic α-helix (see also chapter "Hepatitis C Virus Entry" Zeisel et al., this volume). Due to its location in the stem region, segment 322–332 is likely involved in the reorganization of the glycoprotein complexes taking place during the fusion process (Albecka et al. 2011).

The genes encoding E1 and E2 are particularly variable, and several HVRs have been identified in E2, differing by up to 80 % among HCV genotypes. HVR1 comprises the first 27 aa of E2 and is an immunodominant region that elicits type-specific neutralizing antibodies. Interestingly, despite high variability at the sequence level, HVR1 is a globally basic region with positively charged residues located at specific sequence positions, and the structural properties of this intrinsically unstructured region were found to be quite conserved (Penin et al. 2001). HVR2 (aa 91–97) shows up to 100 % sequence diversity and forms a loop-like structure via flanking cysteine residues within domain 2. The igVR (aa 187–197) varies widely between genotypes but contains a single conserved N-linked sugar moiety and, similar to HVR2, likely forms a disulfide-constrained loop. In contrast to HVR1, HVR2 and igVR are not known targets of the humoral immune response but are essential for structural integrity and function of the HCV glycoprotein heterodimer and may modulate E2 receptor binding (McCaffrey et al. 2011).

3 Nonstructural Proteins

3.1 p7

p7 is a 63-aa integral membrane polypeptide comprising two transmembrane α-helices connected by a positively charged cytosolic loop, while the N and C termini are oriented toward the ER lumen (Carrère-Kremer et al. 2002). It forms hexamers or heptamers with cation channel activity and facilitates virus production, indicating that it belongs to the viroporin family (Clarke et al. 2006; Luik et al. 2009; Chandler et al. 2012 and references therein).

p7 is not required for RNA replication in vitro but is essential for the assembly and release of infectious HCV in vitro as well as productive infection in vivo (reviewed in Steinmann and Pietschmann 2010) (see also chapter "Virion Assembly and Release" by Lindenbach, this volume). However, its precise function is unknown and the role of p7 ion channel activity in viral assembly and release has yet to be elucidated. One possibility is that p7 acts by suppressing acidification of cellular organelles, thereby protecting nascent virions from premature acid-induced conformational changes (Wozniak et al. 2010). Furthermore, there is a growing body of evidence suggesting that p7 is critical for other functions in virus assembly and egress unrelated to its

channel activity (reviewed in Steinmann and Pietschmann 2010). As discussed above and below, p7 likely acts in concert with core, E1 and E2, as well as NS2, especially the first transmembrane segment of NS2, to exert its function(s) (Jirasko et al. 2010; Boson et al. 2011; Ma et al. 2011; Popescu et al. 2011; Stapleford and Lindenbach 2011).

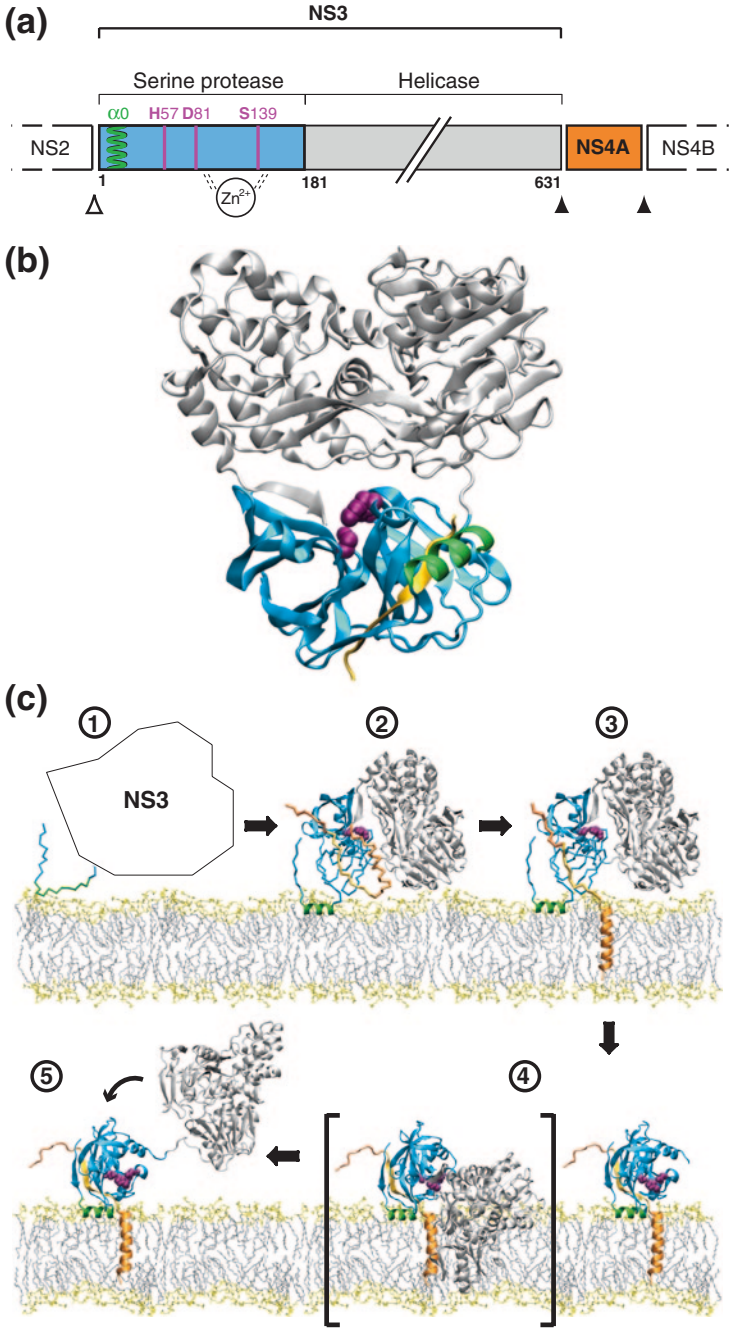
Combined NMR experiments and molecular dynamics (MD) simulations allowed the identification of the secondary structure elements of p7 and the construction of a three-dimensional model of the monomer in a phospholipid bilayer (Montserret et al. 2010). This model revealed an N-terminal α-helix (aa 2–16), a turn involving fully conserved Gly 18, transmembrane α-helix 1 (aa 19–33), a cytosolic loop harboring two fully conserved basic residues at positions 33 and 35, and transmembrane α-helix 2 (aa 40–56), which is slightly bent due to Pro 49, and an unstructured seven-residue C-terminal segment.

The three-dimensional structure of a hexameric p7 channel in a 1,2-diheptanol-sn-glycero-3-phosphocholine (DHPC) short-tail (C7) phospholipid bilayer was solved at 16 Å resolution by single-particle electron microscopy, revealing a highly tilted, flower-shaped protein architecture with six protruding petals oriented toward the ER lumen (Luik et al. 2009). In the modelled structure, the first transmembrane α-helix lines the pore and the N and C termini occupy the "petal tips". More recent modeling of p7 oligomers in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) long-tail (C16:C18) phospholipid bilayer, reflecting the thickness of the ER membrane, revealed a more cylindrical, upright complex (Chandler et al. 2012). Interestingly, comparative evaluation by MD simulations in short- and long-tail phospholipid bilayers revealed the adaptability of the p7 oligomeric structure to membrane thickness. These simulations suggest that p7 forms structurally plastic, minimalist ion channels, compatible with the coexistence of hexameric and heptameric states.

3.2 NS2

Cleavage of the polyprotein precursor at the NS2/NS3 junction is accomplished by a cysteine protease encoded by NS2 and whose function is strongly enhanced by the N-terminal one-third of NS3 (Schregel et al. 2009 and references therein). The NS2 protease itself is dispensable for RNA replication, but cleavage at the NS2/NS3 junction is essential to liberate fully functional NS3 protein and, hence, promote viral RNA replication. The catalytic activity resides in the C-terminal half of NS2 (aa 94–217, $NS2^{pro}$, with His 143, Glu 163, and Cys 184 representing the catalytic triad) while the N-terminal part represents a membrane domain with three putative transmembrane segments (Jirasko et al. 2008, 2010 as well as references therein).

The crystal structure of $NS2^{pro}$ revealed a dimer with two composite active sites (Lorenz et al. 2006) (Fig. 2). Each active site is composed of residues from the two monomers, i.e., His 143 and Glu 163 are contributed by one monomer and Cys 184 by the other. The membrane domain is believed to comprise 3 transmembrane segments (aa 4–23, 27–49, and 72–94) and a small α-helix (aa 61–70)


(a)
NS3
Serine protease
Helicase
α0
H57 D81
S139
NS2
NS4A
NS4B
1
181
631
Zn2+
(b)
(c)
NS3
1
2
3
4
5

◀ **Fig. 4** Structure and membrane association of the HCV NS3-4A complex. **a** Schematic representation of the NS3-4A region of the HCV polyprotein. The NS3 serine protease and NTPase/RNA helicase domains are depicted in *cyan* and *grey*, respectively. Serine protease active site residues His 57, Asp 81, and Ser 139 are highlighted in *purple* and N-terminal amphipathic helix α_0 in *green*. A Zn^{2+} atom coordinated by three cysteine residues and one histidine stabilizes the serine protease structure. NS4A is shown in *orange*. The *white arrowhead* denotes cleavage by the NS2 protease while the *black arrowheads* illustrate *cis* (NS3/NS4A site) and *trans* (NS4A/NS4B site) cleavages by the NS3-4A protease. **b** Structure of the NS3-4A complex, as resolved for a single chain construct comprising NS3 and the central part of NS4A (Yao et al. 1999). The same colors as in panel A are used to highlight the different elements. **c** Dynamic model for the membrane association and structural organization of NS3-4A. (1) Translation of NS3 occurs at the membrane. (2) Amphipathic helix $\alpha_0$folds upon interaction with the membrane interface, followed by folding of the serine protease and helicase domains. (3) Processing at the NS3/NS4A site and posttranslational insertion of the N-terminal segment of NS4A into the membrane. (4) Complete folding of the protease domain and membrane association through helix α_0 and the N-terminal α-helix of NS4A lock the serine protease in a strictly defined position onto the membrane. (5) At the same time, the helicase domain has to move away from the serine protease domain through a rotation of the linker segment connecting the two domains. Adapted from (Brass et al. 2008), where a video illustrating the different steps can be found, and (Morikawa et al. 2011)

whose atomic structures have been solved by NMR, allowing to propose a topology model of full-length, membrane-associated NS2 (Jirasko et al. 2010).

NS2 plays a central organizing role in HCV infectious virus assembly that is independent of its protease activity, but may involve a complex network of interactions with structural and other nonstructural viral proteins (E1 [mediated by E2], E2, p7, NS3, NS5A) (Jirasko et al. 2010; Boson et al. 2011; Stapleford and Lindenbach 2011; Ma et al. 2011; Popescu et al. 2011 and references therein).

3.3 NS3-4A Complex

NS3-4A is a noncovalent complex made of NS3 and the cofactor NS4A. NS3 is a 70 kDa multifunctional protein, with a serine protease located in the N-terminal one-third (aa 1–180) and an NTPase/RNA helicase in the C-terminal two-thirds (aa 181–631). Both enzyme activities have been well characterized, and high-resolution structures have been solved (Yao et al. 1999) (reviewed in De Francesco and Steinkühler 2000; Raney et al. 2010; Morikawa et al. 2011) (Fig. 4).

The NS3-4A protease adopts a chymotrypsin-like fold with two β-barrel subdomains. The structure is stabilized by a Zn^{2+} ion that is coordinated by Cys 97, Cys 99, Cys 145, and His 149. This Zn^{2+} binding site also plays an important role in facilitating processing of the NS2/NS3 site by the NS2 protease (Schregel et al. 2009). The catalytic triad is formed by His 57, Asp 81, and Ser 139 (Fig. 4). The 54-aa NS4A polypeptide functions as a cofactor for the NS3 serine protease. Its central portion (aa 21–32) comprises a β-strand that is incorporated into the N-terminal β-barrel of NS3. The N-terminal hydrophobic portion of NS4A

(aa 1–21) forms a transmembrane α-helix required for the integral membrane association of the NS3-4A complex (Brass et al. 2008) while the C-terminal acidic portion (aa 40–54) comprises a highly negatively charged α-helix that has been shown to interact with other replicase components and to contribute to HCV RNA replication and virus particle assembly (Lindenbach et al. 2007; Phan et al. 2011).

Determinants of NS3-4A protease substrate specificity include an acidic aa at the P6 position, a P1 cysteine (*trans*-cleavage sites) or threonine (*cis*-cleavage site between NS3 and NS4A), and an aa with a small side chain, i.e., alanine or serine, at the P1′ position (consensus cleavage sequence D/E-X-X–X-X-C/T | S/A–X-X–X). However, the recent identification of cellular substrates of the NS3-4A protease has revealed a much more complex scenario. On the one hand, a vast number of cellular proteins display the consensus cleavage sequence and yet only very few are cleaved by NS3-4A. On the other hand, the cellular substrates identified so far have less canonical cleavage sites (reviewed in Morikawa et al. 2011). Therefore, substrate specificity appears to be conferred by additional mechanisms such as the positioning of the NS3-4A protease active site with respect to the membrane (see below).

The NS3 NTPase/RNA helicase is a member of the superfamily 2 DExH/D-box helicases (reviewed in Raney et al. 2010). It couples ATP hydrolysis to the unwinding of double-stranded RNA or of single-stranded RNA regions with extensive secondary structure. The NS3 helicase is essential for HCV RNA replication and also plays a role in viral particle assembly (reviewed in Murray et al. 2008). However, its precise function(s) in the viral life cycle remain(s) elusive. The NS3 helicase unwinds RNA in an "inchworm" or "ratchet-like" fashion (Gu and Rice 2010 as well as references therein). It is unknown why the serine protease and NTPase/RNA helicase domains are physically linked, but evidence for cross-talk between these two essential enzymatic activities is emerging (Beran et al. 2009 and references therein).

Membrane association and structural organization of the NS3-4A complex are ensured in a sequential manner by two determinants: an in-plane amphipathic α-helix at the N terminus of NS3, designated helix α_0 (aa 12–23), and the N-terminal 21 aa of NS4A, which form a transmembrane α-helix (Brass et al. 2008). As shown in Fig. 4, sequential membrane association by these two determinants plays an active role in the processing and structural organization of NS3-4A and positions the serine protease active site in a strictly defined topology with respect to the membrane (Fig. 4). Moreover, one should postulate a second conformation of the NS3-4A complex, with the protease and helicase domains interacting by contacts different from the ones identified in the *cis*-cleavage structure. Indeed, recent studies performed in the NS2B-3 protease of flaviruses and the NS3-4A protease of HCV are in support of a conformational flexibility of the NS3 protease and helicase domains mediated through the interdomain linker segment (Assenberg et al. 2009; Luo et al. 2010; Ding et al. 2011).

Interestingly, NS3-4A is located not only on membranes of the ER and in replication complexes but also, to a minor extent, on mitochondrial or mitochondria-associated membranes, which are thought to be specialized ER sites in close proximity of mitochondria (Wölk et al. 2000; Horner et al. 2011). These

observations may explain how the NS3-4A protease can cleave and thereby inactivate a mitochondrial host protein, the RIG-I adaptor MAVS (also known as Cardif, IPS-1, and VISA) (Meylan et al. 2005) (see also chapter "Innate Immune Responses to Hepatitis C Virus" by Schoggins and Rice, this volume). The NS3-4A protease also cleaves another crucial adaptor protein in innate immune sensing, the TLR3 adaptor TRIF (Li et al. 2005), as well as T cell protein tyrosine phosphatase (Brenndörfer et al. 2009), a modulator of the epidermal growth factor receptor. Hence, the NS3-4A protease plays essential roles not only in the replication but also in the persistence and pathogenesis of HCV (reviewed in Morikawa et al. 2011).

Two first-generation NS3-4A protease inhibitors, telaprevir and boceprevir, have been approved recently for the treatment of chronic hepatitis C (see also chapter "Treatment of Chronic Hepatitis C: Current and Future" by Pawlotsky, this volume). These directly acting antivirals have to be combined with pegylated interferon-α and ribavirin in order to avoid the rapid selection of antiviral-resistant HCV strains (Halfon and Locarnini 2011). Triple therapy comprising pegylated interferon-α, ribavirin, and telaprevir or boceprevir increases sustained virological response rates to ~70 % and allows shortening treatment duration in ~½ of treatment-naïve patients with HCV genotype 1 infection (Jacobson et al. 2011; Poordad et al. 2011).

3.4 NS4B

NS4B is a relatively poorly characterized, hydrophobic 27 kDa protein of 261 aa (reviewed in Gouttenoire et al. 2010a). It is an integral membrane protein comprising an N-terminal portion (aa 1 to ~69), a central part harboring four predicted transmembrane passages (aa ~70 to ~190), and a C-terminal portion (aa ~191 to 261). The N-terminal portion comprises two amphipathic α-helices, AH1 and AH2, extending from aa 3–35 and 42–66, respectively. AH2 has the potential to traverse the membrane bilayer, likely upon oligomerization (Lundin et al. 2003; Gouttenoire et al. 2009a). Site-directed mutagenesis showed that, similar to other HCV nonstructural protein membrane segments (Moradpour et al. 2004a; Penin et al. 2004; Brass et al. 2008), this segment plays an important role in the assembly of a functional replication complex (Gouttenoire et al. 2009a). Interestingly, transmembrane orientation of the N terminus was found to be reduced by coexpression of the other nonstructural proteins, particularly of NS5A, suggesting that the membrane topology of the N-terminal part of NS4B may be dynamic and modulated by protein–protein interactions within the HCV replication complex (Lundin et al. 2006; Gouttenoire et al. 2009a). The C-terminal part comprises a highly conserved amphipathic α-helix (H1, aa 201–213) and a second, membrane-associated 'twisted' amphipathic α-helix (H2, aa 229–253) (Gouttenoire et al. 2009b) as well as two reported palmitoylation sites at the C terminus (Yu et al. 2006). Therefore, membrane association of HCV NS4B is mediated not only by transmembrane domains in its central part but also by determinants for membrane association in the N- and C-terminal portions (AH2, H2, possibly palmitoylation).

NS4B induces the formation of the membranous web, a specific membrane alteration consisting of locally confined membranous vesicles that serves as a scaffold for the HCV replication complex (Egger et al. 2002; Gosert et al. 2003). NS4B interacts with other viral nonstructural proteins and has been reported to bind viral RNA (Einav et al. 2008). In addition, NS4B was reported to harbor NTPase activity (Einav et al. 2004; Thompson et al. 2009) and has been shown to have a role in viral assembly (Jones et al. 2009). Much work remains to be done to further dissect these multiple functions which may be governed by distinct membrane topologies and/or interactions with other viral as well as cellular proteins. Clearly, obtaining a three-dimensional structure of full-length NS4B will be key to gaining further insight into the functions of NS4B.

Similar to the other HCV nonstructural proteins, NS4B has been reported to form oligomers (Yu et al. 2006; Gouttenoire et al. 2010b). Indeed, cross-linking studies provided evidence for formation of at least trimers and suggested that C-terminal palmitoylation plays an important role in this process (Yu et al. 2006). More recently, fluorescence resonance energy transfer and confirmatory coimmunoprecipitation analyses provided evidence for an oligomerization of NS4B in the membrane environment of intact cells (Gouttenoire et al. 2010b). Several conserved determinants were found to be involved in NS4B oligomerization through homotypic and heterotypic interactions. N-terminal amphipathic α-helix AH2 and C-terminal conserved elements were identified as major determinants for NS4B oligomerization (Gouttenoire et al. 2010b; Paul et al. 2011). Mutations that affected the oligomerization of NS4B disrupted membranous web formation and HCV RNA replication, implying that oligomerization of NS4B is required for the creation of a functional replication complex, likely through the induction of membrane curvature and vesicle formation. Indeed, the inducers of replication complexes of other positive-strand RNA viruses have been shown to exert their function as multimeric complexes (reviewed in den Boon et al. 2010).

Taken together, evidence from biochemical, structural and genetic studies as well as electron microscopy indicates that NS4B is a master organizer of HCV replication complex formation. It is no surprise, therefore, that NS4B is also actively being pursued as antiviral target (Rai and Deval 2011).

3.5 NS5A

NS5A is a 447-aa membrane-associated phosphoprotein that plays an important role in modulating HCV RNA replication and particle formation (see also chapter "Virion Assembly and Release" by Lindenbach, this volume). Comparative sequence analyses and limited proteolysis of recombinant NS5A have defined, in addition to its N-terminal membrane anchor, three domains separated by two low complexity sequences (LCS) (Tellinghuisen et al. 2004) (Fig. 5). Domains 1 (D1, aa 36–213; genotype 1b Con1 strain) and 2 (D2, aa 250–342) are primarily involved in RNA replication whereas domain 3 (D3, aa 356–447) is essential for viral assembly (Appel et al. 2008; Tellinghuisen et al. 2008; Kim et al. 2011). In addition, D1 is

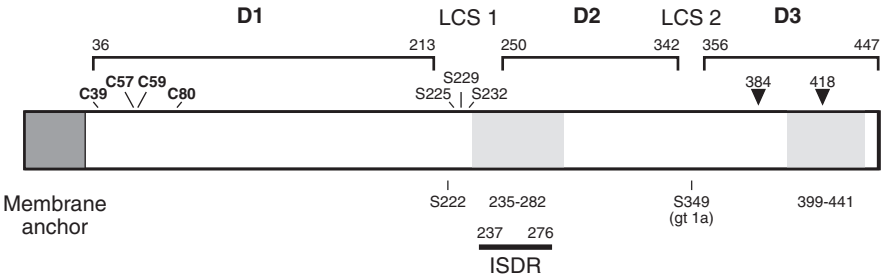


Fig. 5 Schematic representation of HCV NS5A. NS5A is drawn to scale as a *box*. Amino acid positions relate to the HCV Con1 sequence (genotype 1b; GenBank accession number AJ238799; add 1972 amino acids to obtain positions relative to the HCV polyprotein). The domain organization proposed by Tellinghuisen et al. (2004) is shown. Domains 1–3 (D1–D3) are connected by low complexity sequences (*LCS*) 1 and 2. Cysteine residues 39, 57, 59, and 80 coordinate one zinc atom per NS5A protein. The N-terminal amphipathic α-helix, mediating membrane association of NS5A, is highlighted by a *gray box*. Phosphoacceptor sites mapped for genotype 1b (Ser 222; Katze et al. 2000) and genotype 1a HCV isolates (Ser 349; Reed and Rice 1999) are highlighted. Serine residues 225, 229, and 232 that affect hyperphosphorylation of NS5A are also indicated. Two examples each of deletions (Blight et al. 2000; Appel et al. 2005) and *green* fluorescent protein insertions (Moradpour et al. 2004b) found to be tolerated with respect to HCV RNA replication are indicated by *light gray boxes* and *arrowheads*, respectively. ISDR, interferon sensitivity determining region

involved in LD binding (Miyanari et al. 2007) whereas D3 is involved in interaction with the core protein (Masaki et al. 2008). Interestingly, deletions in D2 and D3 are tolerated with respect to RNA replication (Blight et al. 2000; Appel et al. 2005), and viable replicons and viruses harboring GFP insertions in D3 have been established (Moradpour et al. 2004b; Schaller et al. 2007) (Fig. 5).

HCV NS5A can be found in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms. Basal phosphorylation occurs in central and C-terminal parts of NS5A while serine residues 225, 229, and 232 (i.e., Ser 2197, Ser 2201, and Ser 2204 of the genotype 1a strain H polyprotein) in LCS 1 are important for NS5A hyperphosphorylation (Fig. 5). Cell culture-adaptive changes often cluster in the central part of NS5A and target these residues. This observation and the results of studies with kinase inhibitors have led to the assumption that the phosphorylation state of NS5A modulates HCV RNA replication, possibly via regulating interactions with replication—versus assembly-specific host factors (Evans et al. 2004; Neddermann et al. 2004; Appel et al. 2005). The α isoform of casein kinase I (CKIα) (Quintavalle et al. 2007) and casein kinase II (CKII) (Tellinghuisen et al. 2008) can phosphorylate NS5A on serine residues in LCS 1 and on Ser 457 (genotype 2 JFH-1 strain) in D3, respectively. CKII-mediated phosphorylation of Ser 457 (NS5A from the genotype 2 JFH-1 strain is 20 aa longer than that from genotype 1 strains) was found to be essential for assembly of infectious virus particles, at least in the case of the JFH-1 isolate (Tellinghuisen et al. 2008). However, it is likely that additional cellular kinases are involved in generating the different phosphoforms of NS5A (Huang et al. 2007).

NS5A is a monotopic protein anchored to the membrane by an N-terminal amphipathic α-helix embedded in-plane into the cytosolic leaflet of the membrane (Penin et al. 2004 and references therein). This specific feature allows NS5A to associate with a phospholipid monolayer and thus to interact with core on LDs or the LD-ER interface. The crystal structure of D1 immediately following the N-terminal amphipathic α-helix revealed a 'claw-like' dimer with a groove that faces away from the membrane and could accommodate either single- or double-stranded RNA (Tellinghuisen et al. 2005). Each molecule of the dimer coordinates one zinc atom through the four fully conserved cysteine residues, Cys 39, Cys 57, Cys 59, and Cys 80 (Fig. 5). As predicted from the three-dimensional structure, D1 (as well as LCS 1 and part of D2) have been shown to bind RNA as a dimer (Hwang et al. 2010 and references therein). According to one hypothesis, multiple NS5A dimers may form a 'basic railway' on intracellular membranes that would allow tethering as well as sliding of the viral RNA on intracellular membranes and coordination of its different fates during HCV replication (Moradpour et al. 2005). It has been shown that only a small proportion of the HCV nonstructural proteins expressed in cells at a given time are actively engaged in RNA replication (Miyanari et al. 2003; Quinkert et al. 2005). One can easily conceive, therefore, that these proteins may have additional structural functions as arrays or lattices on membranes. The formation of such higher-order structures may explain the extraordinary potency of small molecule inhibitors targeting D1 of NS5A (Gao et al. 2010). Interestingly, a second X-ray structure of NS5A D1 revealed a dimer with a different conformation (Love et al. 2009). It is tempting to speculate that a switch between these alternative conformations may modulate the different roles of NS5A in viral RNA replication and particle assembly.

NS5A D2 (Liang et al. 2007; Hanoulle et al. 2009) and D3 (Verdegem et al. 2011) are natively unfolded monomeric conformers in fast interconversion, with an intrinsic α-helical propensity, suggesting that nascent secondary structures constitute molecular recognition elements and, as such, promote the interaction and stabilization of the conformations with specific viral or host proteins. For example, D2 and D3 were found to interact with and serve as a substrate for cyclophilin A (Hanoulle et al. 2009; Verdegem et al. 2011) (see below).

HCV NS5A has attracted considerable interest because of its potential role in modulating the response to IFN-α therapy (see also chapter "Innate Immune Responses to Hepatitis C Virus" by Schoggins and Rice, this volume). Studies performed in Japan first described a correlation between mutations within a discrete region of NS5A, termed interferon sensitivity determining region (ISDR), and a favorable response to IFN-α therapy (Enomoto et al. 1996). However, this remains a controversial issue. Numerous additional potential functions and interactions have been attributed to NS5A (Macdonald and Harris 2004; de Chassey et al. 2008 and references therein). The remarkably high number of viral and cellular interactants for NS5A is likely due to the natively unfolded nature of D2 and D3. NS5A thus appears to be a hub for protein interactions with high specificities and low affinities. However, only few of these postulated properties of NS5A have been validated in the context of the entire viral life cycle in vitro or of natural HCV infection in vivo.

Interestingly, siRNA screens performed by several independent groups identified phosphatidylinositol 4-kinase IIIα (PI4KIIIα) as an essential host factor involved in HCV RNA replication. Knockdown of PI4KIIIα interferes with membranous web formation and inhibits HCV RNA replication. NS5A interacts with and stimulates PI4KIIIα activity and phosphatidylinositol 4-phosphate (PI4P) production that appears to be essential for HCV replication complex formation (Berger et al. 2011; Lim and Hwang 2011; Reiss et al. 2011 and references therein).

Starting with the observation that cyclosporin A (CsA) inhibits HCV RNA replication in vitro, cyclophilin B was intially identified as target of CsA action (Watashi et al. 2005). More recently, cyclophilin A (CypA) was found to play an essential role in HCV RNA replication and assembly, through interactions with NS5A and eventually also in assembly in an NS2-dependent manner (Ciesek et al. 2009; Kaul et al. 2009; Liu et al. 2009 and references therein). NS5A D2 and D3 were found to interact directly with the active site of CypA, and NMR studies revealed that proline residues in D2 and D3 form a substrate for the peptidyl-prolyl *cis/trans* isomerase activity of CypA (Hanoulle et al. 2009; Verdegem et al. 2011). Moreover, mutations conferring resistance to cyclophilin inhibitors often map to NS5A D2 (Coelmont et al. 2010 and references therein). The exact role of CypA in HCV RNA replication has yet to be defined, although various mechanisms have been proposed, including alterations of NS5A folding that is required to activate the viral replicase (Kaul et al. 2009) and the recruitment of NS5B into the replicase (Liu et al. 2009). Based on the above findings, non-immunosuppressive CsA analogues are currently being developed as antivirals against hepatitis C (Flisiak et al. 2009).

3.6 NS5B

HCV replication proceeds via synthesis of a complementary negative-strand RNA using the genome as a template and the subsequent synthesis of genomic positive-strand RNA from this negative-strand RNA template (see also chapter "Hepatitis C Virus RNA Replication" by Lohmann, this volume). The key enzyme responsible for both of these steps is the NS5B RdRp. This 68 kDa viral enzyme composed of 591 aa has been extensively characterized (Behrens et al. 1996; Lohmann et al. 1997; Lesburg et al. 1999; Simister et al. 2009 and references therein), and NS5B has emerged as a major target for antiviral intervention. The NS5B catalytic domain, formed by the N-terminal 530 aa, contains motifs that are shared by all RdRps, including the hallmark GDD sequence within motif C, and the classical fingers, palm, and thumb subdomain organization of a right hand. A 40-aa linker between the catalytic domain and the C-terminal membrane anchor (aa 570-591, see below) occludes the active site. In vitro, the NS5B catalytic domain is capable of performing copy-back, *de novo* and primer-dependent RNA synthesis, requiring only divalent metals (magnesium or manganese) as cofactors. The catalytic aspartic acids (Asp 220 and Asp 318), located in the palm subdomain, chelate two catalytic metal ions that are responsible

for the polymerization reaction. The single-strand RNA template or primer-template duplex binds in a groove between the fingers and thumb domains that leads directly to the active site. NTPs access this site via a specific tunnel beginning at the backside periphery and extending into the active site located in the palm domain.

A special feature of most HCV RdRp three-dimensional structure models is that extensive interactions between the fingers and thumb subdomains result in a completely encircled active site, with the linker occluding the active site via interaction with the so-called β-flap element of the thumb domain. This likely corresponds to a closed conformation of the enzyme that is adopted for the initiation step of RNA synthesis by a *de novo* mechanism. It was recently reported that the linker is directly involved in the formation of the first dinucleotide primer (Harrus et al. 2010). Transition from this first dinucleotide primer state to the elongation phase of RNA replication requires the removal of the linker and the β-flap element. This is supported by the recently reported co-crystal structure of β-flap-mutated NS5B with primer-template RNA (Mosley et al. 2012). Current structural models do not include an obvious exit path for double-stranded RNA. It is unknown whether the newly synthesized strand emerges base-paired to the template (as double-stranded RNA) or if it is forced to unwind after a few nucleotides in order to leave the active site region.

NS5B is a so-called tail-anchored protein. Posttranslational membrane association is mediated by the C-terminal 21 aa residues, resulting in integral membrane association and cytosolic orientation of the catalytic domain (Schmidt-Mende et al. 2001). The NS5B membrane anchor is dispensable for polymerase activity in vitro but indispensable for RNA replication in cells (Moradpour et al. 2004a). Molecular modeling of membrane-associated NS5B suggests that the RNA-binding groove is stacked onto the membrane interface, thereby preventing access to the RNA template. This inactive form of the RdRp may be activated by a conformational change of the 40-aa linker which connects the catalytic domain and the C-terminal transmembrane segment of NS5B. Such a conformational change would liberate the RNA-binding groove and move the NS5B catalytic domain away from the membrane, while the connecting segment might be involved in binding of viral or cellular factors required for replication complex formation.

4 Conclusions and Perspectives

Much has been learned over the past years about the structure and function of HCV proteins, and most of them are actively being pursued as antiviral targets. However, much work remains to be done with respect to high-resolution structures of full-length membrane proteins, especially core, the envelope glycoproteins, NS2, and NS4B. The study of membrane protein structures still represents a formidable challenge with respect to sample preparation and structural methods. It is likely that novel techniques, such as solid-state NMR, will allow to advance on these important unresolved issues. With respect to the nonstructural proteins, a higher order

structure of the viral replicase will represent an ultimate goal. Such a structure may also reveal insights into the determinants of multifunctionality of the nonstructural proteins, e.g., NS3-4A and NS5A, as well as the different interactions with host factors engaged at a given moment of the HCV life cycle. Ultimately, these efforts should not only yield important new insights into the pathogenesis of hepatitis C, but they should translate into innovative therapeutic and preventive strategies for one of the most common causes of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide.

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Hepatitis C Virus RNA Translation

Michael Niepmann

Abstract After infection of a cell, the positive-strand RNA genome of Hepatitis C Virus (HCV) directly serves as the template for translation in the cytosol. By the use of an internal ribosome entry site (IRES) element in the 5′-untranslated region (5′-UTR) of the viral RNA, the HCV RNA bypasses the need for nuclear processing events like capping and directly recruits the translation apparatus to the viral RNA to start translation of the viral proteins. In this review, I discuss the structure and function of the HCV IRES, focusing on (1) the recruitment of the cellular translation machinery to the IRES, including canonical and noncanonical translation initiation factors, (2) noncanonical RNA-binding proteins that modulate IRES activity, and (3) microRNAs that have an influence on the efficiency of HCV RNA translation.

Abbreviations

HCV	Hepatitis C Virus
IRES	Internal ribosome entry site
UTR	Untranslated region
eIF	Eukaryotic initiation factor
ITAF	IRES trans-acting factor
NSAP1	NS1-associated protein 1
hnRNP	Heterogeneous nuclear ribonucleoprotein
LSm	Like Sm
PTB	Polypyrimidine tract-binding protein
miRNA	MicroRNA
miR-122	MicroRNA-122
Ago	Argonaute

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Contents

1 Introduction

The positive-strand RNA genome of Hepatitis C Virus (HCV) (Poenisch and Bartenschlager 2010) contains a single large open reading frame (ORF) encoding for a polyprotein (see chapter "Hepatitis C Virus Proteins: from Structure to Function" by Moradpour and Penin, this volume). This ORF is flanked by the 5′- and 3′-untranslated regions (UTRs) that contain the signals for replication and translation of the viral RNA (Fig. 1) (Liu et al. 2009b; Niepmann 2009b).

In the 5′-UTR, the stem-loops I and II are involved in replication (Friebe et al. 2001) but also sequences and secondary structures in the core coding region contribute to replication (Vassilaki et al. 2008b). Partially overlapping, the stem-loops II to IV including a few nucleotides of the core coding region constitute the internal ribosome entry site (IRES) that directs the cap-independent initiation of translation of the viral genome (Brown et al. 1992; Tsukiyama-Kohara et al. 1992; Wang et al. 1993). In the 3′-UTR apparently all regions are involved in replication in concert

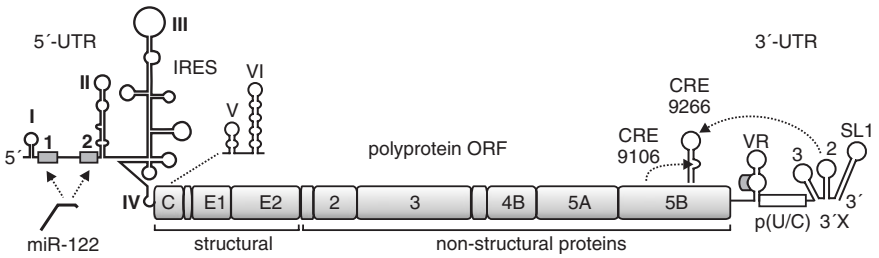

Fig. 1 The HCV genome with the 5′- and 3′-UTRs. The two miR-122 target consensus sequences in the 5′-UTR and one in the variable region (VR) of the 3′-UTR are boxed. Cis-acting replicative elements (CREs) and their interactions as well as the stem-loops in the core-coding region are indicated

with two other cis-acting replicative elements (CREs) within the NS5B coding region [for an overview, see (Liu et al. 2009b)]. Moreover, the 3′-UTR stimulates HCV translation in cis (Song et al. 2006; Bradrick et al. 2006; Bung et al. 2010).

2 HCV IRES Structure and Function

The internal ribosome entry site in the HCV 5′-UTR was functionally identified using dicistronic mRNAs in which the HCV IRES directed translation of the second gene even when the cap-dependent translation of the first gene was inhibited (Tsukiyama-Kohara et al. 1992; Wang et al. 1993). The structure of the IRES (Fig. 2) was then defined by a combination of secondary structure prediction and nuclease mapping as well as genetic and phylogenetic analyses (Brown et al. 1992; Sizova et al. 1998; Honda et al. 1999a; Kolupaeva et al. 2000; Zhao and Wimmer 2001).

The HCV IRES (Fig. 2) is fundamentally different from the IRES elements of the picornaviruses (Niepmann 2009b) in both structure and functionality. The core of the HCV IRES is constituted by the four-way junction at the base of the domain III with the stem-loops IIId, IIIe, IIIf, and IV forming a double pseudoknot structure (Wang et al. 1995). This region can be cleaved by RNase P, suggesting a tRNA-like structure (Nadal et al. 2002).

This central double pseudoknot core domain of the HCV IRES can bind strongly to the platform of the small ribosomal 40S subunit (see Fig. 3a). The bound HCV IRES fits to the surface of the 40S subunit in a stretched conformation that spans from stem-loop II over the core domain to the more apical regions of the stem-loop III (Spahn et al. 2001; Boehringer et al. 2005; Siridechadilok et al. 2005). In the stem-loop III, a pseudoknot-like four-way junction is formed by the IIIabc junction (Kieft et al. 2002). The central IRES core domain with the double pseudoknot positions the initiation codon around position 342 on the 40S ribosomal subunit, with the stem-loop IV unfolding upon binding of the 40S subunit and positioning HCV sequences around the AUG start codon into the mRNA binding cleft (Berry et al. 2011) (red arrow in Fig. 3a). These contacts allow the binding of the HCV IRES to the purified 40S subunit in the absence of any initiation factors or other proteins (Pestova et al. 1998; Kolupaeva et al. 2000; Kieft et al. 2001; Spahn et al. 2001). In addition to the core IRES, also sequences and possibly RNA secondary structures in the core coding region contribute to efficient translation (Vassilaki et al. 2008b) (designated stem-loops V and VI in Fig. 1).

The association of the HCV RNA with the 40S subunit and the association of the two ribosomal subunits largely depend on the magnesium concentration. The concentration of free Mg^{2+} in the cytosol is not easy to determine. The values obtained in different studies with different cells range between 0.2 and 3.8 mM, with a mean over all noninvasive studies of 0.65 mM free Mg^{2+} [reviewed in (Günther 2006)]. At this physiological concentration of 0.65 mM free Mg^{2+} the HCV IRES binds well to the 40S subunit (Kieft et al. 2001), while less than 10 % of the 40S subunits are associated with 60S subunits in the absence of initiation factors (Goss and Harrigan 1986).

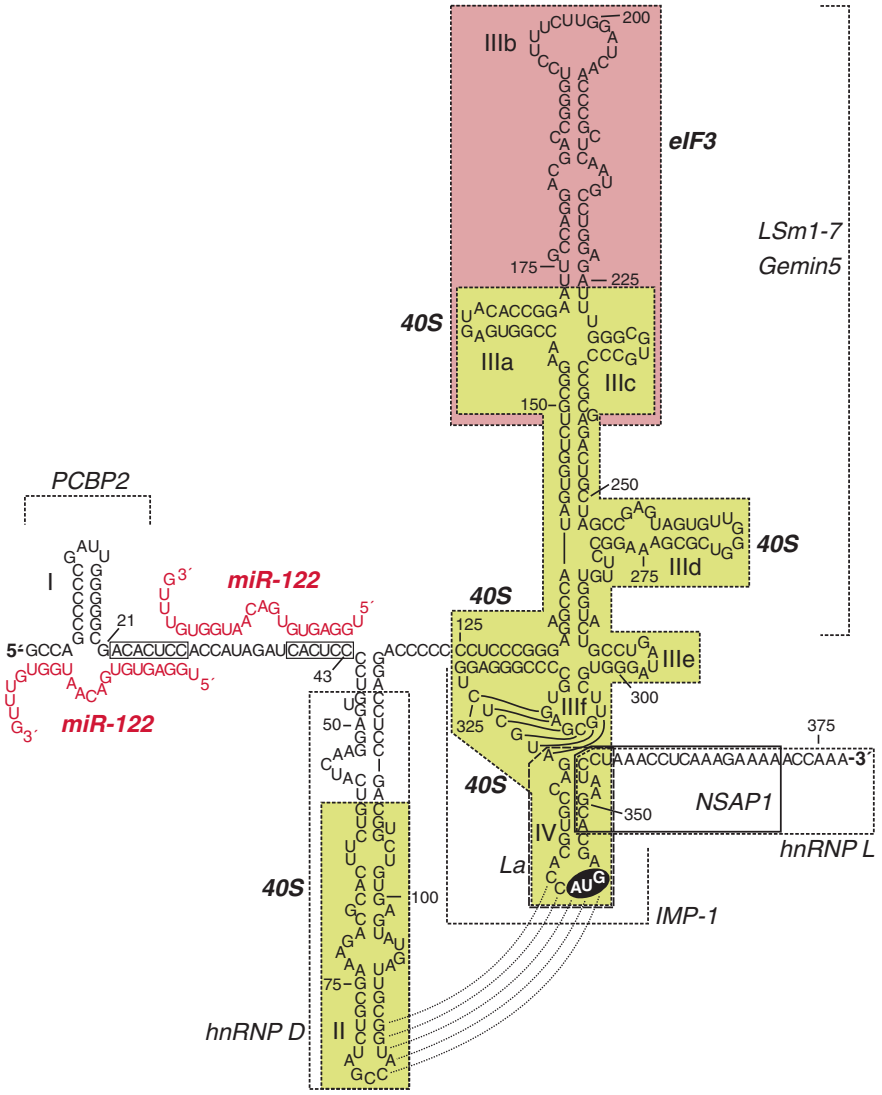

Fig. 2 The HCV 5′-UTR with the IRES. Stem-loops I and II of the 5′-UTR are involved in replication, and stem-loops II – IV plus some core coding nucleotides constitute the IRES that regulates RNA translation. Regions involved in binding to the ribosomal 40S subunit are highlighted in *yellow*, the apical region of stem-loop III that binds eIF3 is highlighted in *red*. Other regions involved in protein binding are indicated; the proteins are discussed in the text. Possible interactions between the apical loops of stem-loop II and IV are indicated. The two miR-122 target consensus sequences in the 5′-UTR are boxed; miR-122 is shown in *red*

To form preinitiation 48S ribosomal complexes that subsequently assemble into translation-competent 80S ribosomes, the HCV IRES requires just three initiation factors, eukaryotic initiation factor (eIF) 3, eIF2, and eIF5. However, HCV

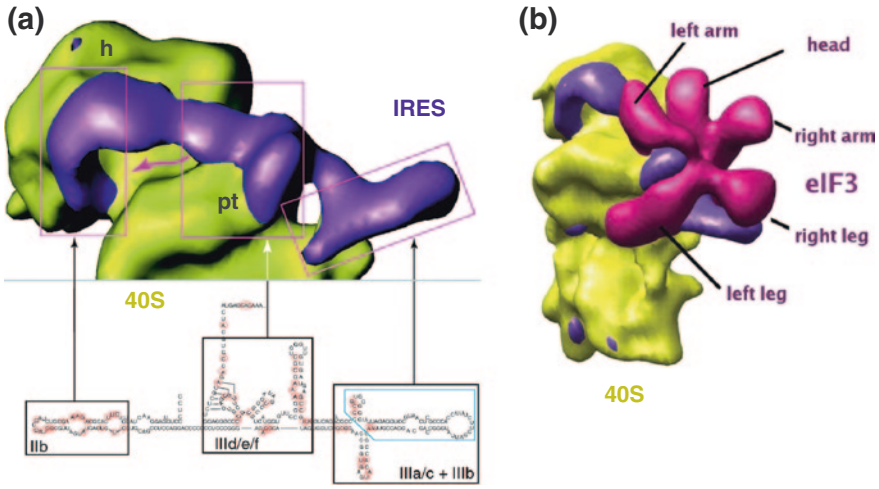

Fig. 3 Binding of the HCV IRES to the ribosomal 40S subunit. The 40S subunit is shown in *yellow*, the HCV IRES in *blue*, and eIF3 in *red*. (**a**) Binding of different IRES regions to the 40S subunit. Stem-loop II binds to the head (h) near the mRNA entry channel on the *left*, the core region of the IRES (IIId/e/f) to the platform (pt), and most of the stem-loop III is sticking out from the 40S subunit. The sequence with the start codon is assumed to bind into the ribosomal entry channel between head and platform (*red arrow*). The interface for binding of the large 60S ribosomal subunit is on the *left* side of the 40S subunit (Boehringer et al. 2005). Modified from Spahn et al. 2001, with permission from the American Association for the Advancement of Science (AAAS). (**b**) Binding of eIF3 (*red*) to the complex of HCV IRES (*blue*) and 40S subunit (*yellow*). The parts of eIF3 are indicated. Modified from Siridechadilok et al. 2005, with permission from AAAS

translation initiation is completely independent of eIF4 group factors (Pestova et al. 1998). eIF3 binds to the apical region of stem-loop III (Sizova et al. 1998; Buratti et al. 1998) and associates with the ribosomal 40S subunit (Jackson et al. 2010) (see Figs. 2 and 3b). eIF2 associates with the initiator tRNA and GTP to form the ternary complex eIF2-GTP-Met-tRNA$_i^{Met}$ which delivers the Met-tRNA$_i^{Met}$ to the 40S subunit. eIF5 then promotes start codon recognition by the ternary complex eIF2-GTP-Met-tRNA$_i^{Met}$ and acts as a GTPase-activator protein for eIF2 (Pestova et al. 1998). Thereby, addition of eIF2-GTP-Met-tRNA$_i^{Met}$ and eIF5 to 40S-IRES-eIF3 complexes is sufficient for the bound 40S subunit to lock onto the HCV initiation codon and to form translation-competent 80S ribosomes upon joining of the large ribosomal 60S subunit (Locker et al. 2007; Terenin et al. 2008; Pestova et al. 2008).

The stem-loop III-eIF3 interaction occurs with lower affinity than the interaction of the IRES with the 40S subunit (Kieft et al. 2001; Ji et al. 2004), but the contacts of eIF3 with the HCV IRES support its binding to the small subunit under cellular conditions where eIF3 is associated with the separate 40S subunit. When the HCV IRES is bound to the 40S surface in the presence of eIF3, the initiation factor largely covers the IRES (Siridechadilok et al. 2005) (Fig. 3b). In contrast to the release of eIF3 upon joining of the ribosomal 60S subunit on normal capped mRNAs, eIF3 remains associated with the ribosomes even after 60S subunit joining (Weinlich et al. 2009).

While stem-loop I of the HCV 5′-UTR is not required for translation (Rijnbrand et al. 1995), stem-loop II makes close contact to the ribosomal surface (Babaylova et al. 2009). The apical half of stem-loop II bends over to contact the 40S subunit near the active site of the ribosome at the 40S–60S interface (see Fig. 3b) and induces conformational changes in the 40S subunit. Thereby, it closes the mRNA binding cleft from the 60S side and restricts the movement of coding RNA within the cleft (Spahn et al. 2001). The apical loop of stem-loop II may interact with the loop of stem-loop IV that contains the AUG start codon (see Fig. 2) and positions the RNA region with the AUG codon in the 40S subunit's decoding groove (Filbin and Kieft 2011), consistent with the inability of the IRES to proceed to 60S subunit joining when the sequence in the apical tip of stem-loop II is modified in a way that it cannot any more interact with the sequence including the AUG in stem-loop IV (Locker et al. 2007; Filbin and Kieft 2011). By modulating 40S conformation, the HCV domain II also promotes the eIF5-induced hydrolysis of the eIF2-bound GTP and eIF2-GDP release and thereby commits the complex to 80S ribosome formation (Locker et al. 2007; Pestova et al. 2008).

The above initiation events at the AUG at position 342 usually result in polyprotein expression. In addition, alternate reading frame (ARF) products that may be expressed by different mechanisms and with different N-terminal sequences (Vassilaki and Mavromara 2009). A Core + 1 product is expressed from a conserved Core + 1 reading frame and can be detected by patient sera (Walewski et al. 2001; Varaklioti et al. 2002). The mechanism of expression was suggested to be a −2 or +1 frameshift when ten consecutive As are present within core codons 9−11(Xu et al. 2001; Boumlic et al. 2011). In the absence of this motif, expression of the core + 1 ORF was proposed to be mediated by a frameshift at Core codon 42 (Boulant et al. 2003) or by internal translation initiation at either codon 26 (Baril and Brakier-Gingras 2005) or at codons 85/87 (Vassilaki and Mavromara 2003; Vassilaki et al. 2008a; Boumlic et al. 2011). However, in vivo-experiments in HuH-7 cells, in SCID mice carrying primary human hepatocytes or in chimpanzees did not reveal evidence for a functional role of a Core + 1 frame product on HCV virus production (McMullan et al. 2007; Vassilaki et al. 2008b). Thus, despite clear evidence for expression of Core + 1 products, their biological roles are not yet clear.

3 Use of Alternative Translation Initiation Factors

eIF2 is a component of the eukaryotic translation initiation apparatus that has no equivalent in bacteria. It routinely delivers the charged initiator tRNA, Met-tRNA$_i^{Met}$ to the ribosomal complexes formed with almost all eukaryotic mRNAs (Jackson et al. 2010). The suppression of the activity of its α-subunit by phosphorylation is one of the major ways to regulate translation in the cell, triggered either by cellular stress conditions or by the innate antiviral defense upon viral infection (see also chapter "Innate Immune Responses to Hepatitis C Virus" by Schoggins and Rice, this volume). However, some viral mRNAs are efficiently translated under these conditions, and this is also the case for HCV RNA, whose translation was reported to be refractory to reduced eIF2-GTP-Met-tRNA$_i^{Met}$ ternary complex availability (Robert et al. 2006).

When the activity of eIF2 is suppressed by the host-cell response to viral infection, HCV obviously has the choice to use alternative factors that can substitute for eIF2 in delivering the charged initiator tRNA to the ribosome. First, the 139 kDa protein eIF5B, which is the eukaryotic homolog of the prokaryotic IF2 (Choi et al. 1998), can substitute for eIF2/eIF5 to promote initiator tRNA-binding to the ribosomal P site (Terenin et al. 2008). This alternative pathway requires only eIF3 and eIF5B as initiation factors and results in translation-competent 80S complexes. A similar pathway has also been reported for the HCV-like IRES of classical swine fever virus (CSFV) (Pestova et al. 2008). Second, a 65 kDa protein now called eIF2D (NM_006893, formerly attributed by mistake to a protein called ligatin), can also substitute for eIF2 in delivering the charged initiator tRNA to the P site of the ribosome in initiation complexes with the HCV IRES in a GTP-independent way (Dmitriev et al. 2010). This eIF2D has a sequence and domain structure that is reminiscent of translation factors since it contains a domain similar to eIF1, and it is also capable of binding elongator tRNAs. Third, the 65 kDa eIF2A protein (NM_032025) (Zoll et al. 2002) mediates translation of HCV RNA under stress conditions (Kim et al. 2011). eIF2A binds to stem-loop IIId of the HCV IRES. Knockdown of eIF2A in the host cells reduced the infectivity of HCV, and the protein was shown to rescue HCV RNA translation under stress conditions. eIF2A binds charged as well as uncharged initiator tRNA with almost similar affinities. The protein was shown to be located in the nucleus of uninfected cells, whereas it relocated to the cytoplasm in stressed or HCV-infected cells (Kim et al. 2011). However, in another study (Dmitriev et al. 2010) the Met-$tRNA_i^{Met}$ binding activity was associated exclusively with the above mentioned eIF2D, but completely separated from native eIF2A upon FPLC-based purification. The reason for this discrepancy is not clear and requires further investigations. In this regard it is worth mentioning that yeast eIF2A interacts with elongation factor 1A to regulate IRES-mediated translation, perhaps making eEF1A an important mediator of translational activation during stress (Reineke et al. 2011). Anyway, these data suggest that HCV has found several ways to escape the suppression of eIF2 activity upon the cellular response to viral infection, and the virus may continue to uncover even more cellular pathways that only become relevant under stress conditions.

4 Contribution of the HCV 3′-UTR to RNA Translation

In analogy to regular mRNAs in which the poly(A)-tail at the 3′-end stimulates translation, HCV translation is stimulated by the viral 3′-UTR in cis, a process that may serve to ensure that only complete, undegraded viral RNA genomes are efficiently translated and replicated. However, initially several conflicting results had been reported about the possible role of the HCV 3′-UTR in RNA translation. The influence of the HCV 3′-UTR on IRES-dependent translation was reported to be positive (e.g., (Ito et al. 1998), not detectable (e.g., (Fang and Moyer 2000) or inhibitory (Murakami et al. 2001) [discussed in (Song et al. 2006)]). Finally, this issue was cleared by using monocistronic HCV reporter RNAs, thereby avoiding the possible collateral cis-activation by additional internal IRES elements present in dicistronic

mRNAs (Jünemann et al. 2007), avoiding cryptic promoters in plasmid DNA and providing authentic HCV RNA 5′- and 3′-ends. The variable region, the poly(U/C)-tract and the most 3′-terminal stem-loop 1 of the highly conserved 3′-X region (see Fig. 1) contribute significantly to translation enhancement, whereas stem-loops 2 and 3 of the 3′-X region are involved only to minor extents (Song et al. 2006; Bradrick et al. 2006; Bung et al. 2010). Thus, also in the HCV 3′-UTR the signals for translation enhancement and the initiation of RNA minus-strand synthesis partially overlap, supporting the idea that these sequences along with viral and cellular factors may be involved in an RNA 3′-5′-end interaction and a switch between translation and RNA replication. Two protein candidates are so far suspected to be involved in 5′- to 3′-end communication, namely the NFAR protein complex (Isken et al. 2003; Isken et al. 2007) and IMP-1 (Weinlich et al. 2009). In addition, the RNA domain 5BSL3.2 (CRE 9266 in Fig. 1) within the NS5B coding sequence interacts with the IRES stem-loop IIId and inhibits IRES-mediated translation (Romero-Lopez and Berzal-Herranz 2012), a process that may also be involved in a switch from translation to replication (see also chapter "Hepatitis C Virus RNA Replication" by Lohmann, this volume).

5 IRES Trans-Acting Factors

Although the HCV IRES can bind to the 40S ribosomal subunit independent of any initiation factor (Pestova et al. 1998; Spahn et al. 2001), it additionally recruits several noncanonical RNA-binding proteins that support optimal IRES activity, like the La protein, NSAP1, hnRNP L and D, IMP-1, Gemin5, LSm1-7, and PCBP2 (see Fig. 4). Such proteins are normally involved in various aspects of cellular RNA metabolism but mostly not involved in the regulation of cap-dependent translation. In most cases, these IRES trans-acting factors (ITAFs) cause translation stimulation on top of the basal IRES activity. The binding of these proteins to different regions of the HCV 5′-UTR and IRES is summarized in Fig. 2, and their properties are depicted in Fig. 4.

An obvious feature of the protein factors modulating HCV IRES activity is that most of them contain multiple RNA-binding domains or motifs. Some ITAFs with multiple RNA-binding domains even oligomerize, such as La protein (Craig et al. 1997) or Poly(rC) binding protein 2, PCBP2 (Bedard et al. 2004). They can therefore likely interact with multiple sites in the RNA and act as RNA chaperones that stabilize a certain IRES tertiary structure. The domain type most frequently found in ITAFs is the RNA recognition motif (RRM) domain, a globular domain which provides a platform for RNA-binding on a plate of β-sheets flanked by α-helices (Kenan et al. 1991; Nagai et al. 1995). Several ITAFs share this domain type, like La protein, NSAP1, hnRNP L, hnRNP D, PTB, and IMP-1. Another important domain type is the KH domain (Makeyev and Liebhaber 2002) that is present in IMP-1 and PCBP2, while some proteins also come with unique RNA-binding domain types, like the La protein with its La motif domain (Martino et al. 2012).

Many of these RNA-binding proteins involved in regulation of HCV RNA translation can also interact with each other. La can interact with LSm1-7 (Maraia and Lamichhane 2011). The SMN complex (that includes Gemin5) can interact with

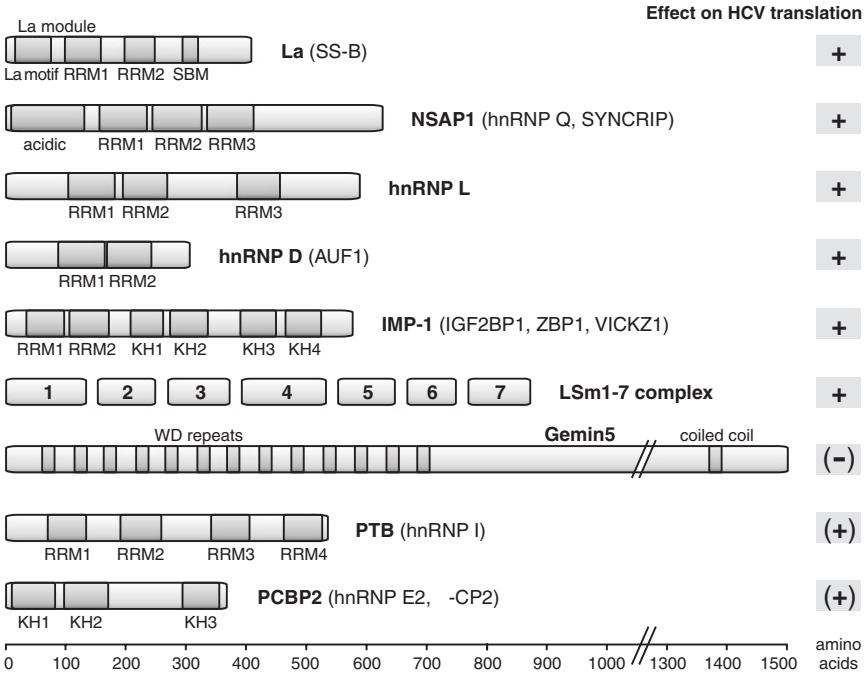

Fig. 4 Noncanonical protein factors that modulate HCV RNA translation. On the *left*, the proteins are shown with their identified RNA-binding domains or motifs. The proteins are: La protein; NSAP1, NS1-associated protein 1; hnRNP L, heterogeneous nuclear ribonucleoprotein L; hnRNP D; IFG2BP1, Insulin-like growth factor 2 binding protein 1; LSm, Like-Sm-proteins 1–7; Gemin5 (involved in the SMN complex); PTB, polypyrimidine tract-binding protein; and PCBP2, Poly(rC)-binding protein 2. The RNA-binding domains depicted in *dark gray* are: the La motif domain; RRM, RNA recognition motif; acidic, acidic domain; SBM, short basic motif; KH, hnRNP K homology domain. On the *right*, the involvement of each protein in modulating the efficiency of translation of the HCV IRES is indicated (+, stimulatory; − inhibitory). Indirect interactions or those requiring further validation are marked with brackets

NSAP1 (Mourelatos et al. 2001). hnRNP D interacts with hnRNP L (Kim et al. 2000; Park et al. 2007), NSAP1 (Moraes et al. 2003) and IMP-1 (Moraes et al. 2003). NSAP1 directly interacts with the purified 40S ribosomal subunit (Park et al. 2011). Moreover, PCBP2 binds to the stem-loop I of the HCV 5′-UTR and to the 3′-UTR, and PCBP2 and PTB appear to interact with La (Fontanes et al. 2009). Besides enhancing circularization and replication, PCBP2 thereby also appears to enhance HCV translation (Wang et al. 2011). These interactions suggest that a network of ITAF proteins may bridge different regions of the HCV 5′-UTR and form a "mediator complex" that facilitates interaction of the HCV IRES with the ribosomal 40S subunit.

For some of the ITAFs, there is sufficient evidence for their involvement in modulation of HCV RNA translation (La, NSAP1, hnRNP L). Few, but convincing reports exist for hnRNP D, IMP-1, LSm1-7, and Gemin5, while for others, there is only weak evidence (PCBP2) or the involvement of the protein in question is controversially discussed (PTB).

5.1 La Protein

La protein is a factor that is involved in processing of eukaryotic precursor tRNAs (Maraia and Lamichhane 2011) and was first characterized as a major nuclear antigenic target (SSB/La) for autoantibodies in patients with Sjögren's syndrome (Chan et al. 1989). The first reports on a role of La protein in binding to a viral RNA described its binding to the genomic RNA of poliovirus (Meerovitch et al. 1993). In the HCV IRES, La protein binds to the region around the initiator AUG comprising the pseudoknot and the stem-loop IV of the HCV IRES and stimulates HCV translation (Ali and Siddiqui 1997; Ali et al. 2000). In the La protein, large regions were identified to be required for binding to the HCV IRES, and the entire La protein was shown to be required for HCV translation stimulation, including amino acid sequences in the N-terminal "La-motif" as well as amino acids near the C-terminus of the protein (Ali et al. 2000; Izumi et al. 2004). A detailed structural and binding study more recently confirmed that indeed most of the La protein is involved in binding to the small stem-loop IV of the HCV IRES. Binding to the stem-loop IV requires the combined action of the La motif domain, RRM 1 and RRM 2, whereas binding of La protein to a simple UUUU homopolymer RNA oligonucleotide (representing its binding site at the 3′-end of its tRNA precursor substrates to be processed) can be accomplished by only the N-terminal "La module" (Martino et al. 2012). In particular, the interaction of La RRM 2 domain with the GCAC motif in the stem-loop IV is essential for the action of La on translation stimulation, and La protein mediates the contact of HCV stem-loop IV with the 40S ribosomal protein S5 (p25) (Pudi et al. 2004). Consistently, a fragment of the La protein comprising amino acids 226–348 can compete La function and reduce the efficiency of 48S complex formation (Costa-Mattioli et al. 2004). Thus, the involvement of different modules of the La protein in binding to the HCV stem-loop IV RNA represents an unprecedented example for binding of an RNA-binding protein to a structured stem-loop RNA motif. However, molecular details of how La protein may actually function in the stimulation of HCV translation are yet to be determined.

5.2 NSAP1

NSAP1 (mice minute virus NS1-associated protein 1) was also characterized as SYNCRIP (synaptotagmin-binding, cytoplasmic RNA-interacting protein) or as hnRNP Q. It has diverse functions in RNA metabolism, including splice regulation (Chen et al. 2008) and translation regulation (Lee et al. 2012). NSAP-1 stimulates HCV RNA translation by binding to the HCV IRES near the Core start codon (Kim et al. 2004). In particular, the downstream portion of stem-loop IV and the A/C-rich sequences immediately downstream (approximately nts 347–374) are bound by NSAP1 (Kim et al. 2004). Moreover, NSAP1 directly interacts with the purified 40S ribosomal subunit. By these interactions, NSAP1 promotes

the correct positioning of the 40S subunit at the HCV initiation codon and facilitates 80S complex formation (Park et al. 2011). Besides its role in HCV translation regulation, NSAP1 may also have a role in HCV replication since NSAP1 knockdown reduces HCV replication (Liu et al. 2009a).

5.3 hnRNP L and hnRNP D

hnRNP L is a protein that acts in the regulation of alternative splicing. hnRNP L binds to intronic or exonic C/A-rich sequences and functions as an activator or repressor of exon usage (Hung et al. 2008). hnRNP L interacts with ACA(C/U)-rich sequences near the 3′ border of the HCV IRES and stimulates HCV translation (Hahm et al. 1998; Hwang et al. 2009). HCV coding sequences between positions 344 and 374 (i.e., a stretch of ~ 30 nts downstream of the initiator AUG at pos. 342) are most important for hnRNP L binding. Thus, these sequences overlap with the binding site characterized for NSAP1, but nothing was reported so far about a possible competition of NSAP1 versus hnRNP L binding to these A/C-rich sequences directly downstream of the stem-loop IV. Moreover, also sequences between 374 and 402 further enhance binding (Hahm et al. 1998), most likely including AC-rich sequences which extend up to position 390 in the core-coding sequence.

hnRNP D (Kajita et al. 1995) (also called ARE/poly(U)-binding/degradation factor 1, AUF1) is a protein involved in destabilization of many mRNAs that have AU-rich elements (AREs) in their 3′-untranslated regions. Association of hnRNP D with these mRNAs recruits other factors to assemble the mRNA degradation machinery and promote rapid mRNA degradation (Gratacos and Brewer 2010). hnRNP D binds to stem-loop II of the HCV IRES and stimulates HCV RNA translation (Paek et al. 2008). Moreover, hnRNP D can interact with hnRNP L (Kim et al. 2000; Park et al. 2007), NSAP1 and IMP-1 (Moraes et al. 2003), indicating that a network of ITAF proteins may bridge different regions of the HCV 5′-UTR, forming a “RNA chaperone” complex. This is reminiscent of the RNA chaperone function of the multi-RRM domain protein PTB that connects different regions of the IRES RNA of foot-and-mouth-disease virus, FMDV, and thereby facilitates initiation factor entry and FMDV RNA translation (Song et al. 2005).

5.4 IMP-1

IMP-1 (insulin-like growth factor 2 mRNA binding protein 1, also called IGF2BP1, ZBP1, or VICKZ1) belongs to a family of RNA-binding proteins implicated in mRNA localization, turnover, and translational control (Yisraeli 2005). IMP-1 was shown to bind to the region comprising nts 315–375 of the 5′-UTR (i.e., stem-loop IV plus ~ 30 coding nts) and to nts 1–151 of the 3′-UTR (i.e., the

variable region and the poly(U/C) tract), but the exact binding sites were not yet characterized in more detail. IMP-1 is associated with the 48S and 80S initiation complexes with any mRNA, either the HCV 5′-UTR or a capped mRNA (Weinlich et al. 2009). Also, eIF3 is present with 48S initiation complexes in any case; however, eIF3 remains associated with 80S ribosomes only in the presence of the HCV RNA, but not with a capped mRNA (Weinlich et al. 2009). Binding of IMP-1 and eIF3 to the HCV IRES can take place on the same RNA molecule, but the two proteins do not interact with each other in the absence of RNA (Weinlich et al. 2009), which is consistent with the finding that IMP-1 interacts with stem-loop IV, while eIF3 interacts with stem-loop III.

IMP-1 enhances HCV IRES-mediated translation initiation (Weinlich et al. 2009). siRNAs targeting IMP-1 mRNA reduced translation of an HCV reporter RNA containing the HCV 5′-UTR independent from the presence or absence of the HCV 3′-UTR, suggesting that the translation stimulation effect of IMP-1 is mediated via its interaction mainly with the HCV 5′-UTR, even if the 3′-UTR was reported to contribute to the stimulation. Thus, like the NFAR proteins which are involved in HCV replication (Isken et al. 2003), IMP-1 can be considered as a candidate protein that is involved in HCV RNA genome circularization. However, the mechanism of action of IMP-1 on HCV translation remains to be determined.

5.5 Gemin5 and LSm1-7

The HCV IRES also interacts with Gemin5 and LSm1-7 proteins. Gemin5 is a peripheral component of the "Survival of Motor Neurons" (SMN) complex that binds to specific sequences in snRNAs and controls the specific loading of Sm proteins during snRNP assembly (Gubitz et al. 2004). The related "Like Sm" (LSm) proteins also form heptameric rings that bind to RNA. The cytoplasmic LSm1-7 complex binds to oligoadenylated 3′-ends of mRNAs and is involved in mRNA decapping and degradation, suggesting a dynamic interaction of the LSm rings with their bound RNAs (Khusial et al. 2005).

Interestingly, the LSm1-7 and Gemin5 proteins function in HCV translation regulation conversely. LSm1-7 binds to the stem-loop III in the HCV IRES and to the poly(U/C)-tract in the 3′-UTR and is required for efficient HCV RNA translation (Scheller et al. 2009). Also, Gemin5 binds to stem-loop III of the IRES. However, in contrast to LSm1-7, Gemin5 downregulates HCV RNA translation (Pacheco et al. 2009). Since the SMN complex (which contains Gemin5) interacts with the Sm proteins (Gubitz et al. 2004) and at least also with the related LSm proteins 10 and 11 (Azzouz et al. 2005), it can only be speculated whether Gemin5 may also be able to control the interaction of the LSm1-–7 complexes with the HCV RNA and thereby somehow exert its negative effect on HCV translation efficiency. LSm1-7 also interacts with La protein in precursor tRNA 3′-end processing (Maraia and Lamichhane 2011), suggesting that such an interaction

may also take place when these factors bind to the HCV IRES, but no such interaction was yet reported. Moreover, SMN interacts with NSAP1 (Mourelatos et al. 2001). Thus, Gemin5 may be involved in a variety of interactions including NSAP1, La protein, and LSm1-7 in controlling HCV translation stimulation in a yet unknown way.

5.6 PTB

Polypyrimidine tract-binding protein (PTB, also called hnRNP I) is a protein involved in splicing regulation and other aspects of cellular RNA metabolism (Xue et al. 2009). Despite a certain tendency to self-interact (Perez et al. 1997; Oh et al. 1998), PTB exists predominantly as a monomer in solution (Song et al. 2005; Monie et al. 2005; Niepmann and Zheng 2006). With its four RRM domains (see Figs. 4b and 5), a single PTB molecule can simultaneously contact different intronic binding sites (Auweter and Allain 2008), a function that is also used when a PTB molecule simultaneously binds different regions of a picornavirus IRES and acts as an RNA chaperone that may support a certain IRES tertiary structure to facilitate initiation factor entry (Song et al. 2005). However, a possible role of PTB in HCV translation is discussed controversially. Some studies had shown a positive influence of PTB on HCV RNA translation (Gosert et al. 2000; Anwar et al. 2000; Fontanes et al. 2009). PTB can also rescue the interference of a small peptide from the N-terminal region of La (amino acids 11–28) with HCV RNA translation (Fontanes et al. 2009), indicating that a network of interactions including La is involved in HCV RNA translation stimulation. In contrast, other studies reported that PTB does not stimulate HCV IRES-driven translation (Tischendorf et al. 2004; Brocard et al. 2007; Nishimura et al. 2008).

6 Micrornas

All of the above-mentioned proteins are expressed rather ubiquitously, making their possible contribution to hepatotropism rather unlikely. In contrast, the microRNA-122 (miR-122) which is expressed preferentially in liver cells and in the human hepatoma cell line HuH-7 (Sempere et al. 2004; Chang et al. 2004; Fu et al. 2005; Landgraf et al. 2007) was found to stimulate HCV propagation (Jopling et al. 2005) and thus may—besides cell surface receptors—contribute to the hepatotropism of HCV (see also chapter "Hepatitis C Virus RNA Replication" by Lohmann, this volume).

microRNAs (miRNAs) regulate eukaryotic gene activity at the posttranscriptional level (Fabian et al. 2010). Processing of miRNA precursors results in ~ 22 bp miRNA duplexes with 3′-overhangs. This miRNA duplex is then unwound, and

the so-called guide strand is incorporated into a microRNA/protein (miRNP) complex, while the opposite (passenger) strand is discarded. The mature miRNA guide strand is fixed with its 5′- and 3′-ends in an Argonaute (Ago) protein (Jinek and Doudna 2009), thereby exposing the so-called seed region near the miRNA's 5′-end (usually miRNA nucleotides 2–8) for base-pairing with a target sequence in the mRNA's 3′-UTR. In addition, also other regions of the miRNA can base-pair with the target (Bartel 2009), but the base pairing of the miRNA with its mRNA target is usually interrupted by some mismatches. Such an interaction of the miRNP effector complex with the target mRNA usually results in translation repression.

In contrast, miR-122 does not repress but stimulates HCV propagation. Between the stem-loops I and II of the highly conserved 5′-UTR of HCV (Fig. 2), there are two sequences complementary to the seed sequence of miR-122, a 7-nucleotide sequence (ACACUCC), and a 6-nucleotide sequence (CACUCC). Moreover, another miR-122 target consensus sequence is present in the otherwise variable region of the HCV 3′-UTR. In HuH-7 cells that are most permissive for HCV replication (see chapter "Cell Culture Systems for Hepatitis C Virus" by Steinmann and Pietschmann, this volume), virus propagation was found to be enhanced by miR-122 interacting with the two target sites in the 5′-UTR, whereas the sequence in the 3′-UTR was dispensable (Jopling et al. 2005). This effect of the liver-specific miR-122 might contribute to the tissue tropism of HCV and may also be of therapeutic interest since HCV levels in infected chimpanzees were shown to be reduced during anti-miR-122 treatment (Lanford et al. 2010) (see also chapter "Hepatitis C Virus-Specific Directly Acting Antiviral Drugs" by Delang et al., this volume).

Conversely, another microRNA may repress extrahepatic HCV replication. In stem-loop II, there is a binding site for miR-199a*. Since stem-loop II is involved in both translation and replication, it is not surprising that the invasion of the stem-loop II secondary structure by miR-199a* reduces HCV replication efficiency (Murakami et al. 2009). Since miR-199a* is expressed at moderate levels in several human tissues (Lagos-Quintana et al. 2002; Liang et al. 2007; Landgraf et al. 2007), whereas its expression in human liver is rather low (Liang et al. 2007; Murakami et al. 2009), it had been speculated that a possible suppression of HCV replication by miR-199a* in extrahepatic tissues may indirectly contribute to HCV liver tropism (Pietschmann 2009).

6.1 microRNA-122

miR-122 appears to promote HCV RNA genome accumulation by more than one mode of action. Based on the original finding that miR-122 stimulates HCV RNA accumulation in cells, it was first proposed that HCV RNA replication, but not translation, is the primary mode-of-action of miR-122 (Jopling et al. 2005). However, in the dicistronic HCV replicon system employed by the authors, an additional internal picornavirus IRES element served to drive expression of the

HCV non-structural proteins required for replication. Such an internal picornavirus IRES may transfer captured initiation factors (and possibly ribosomes) to the 5′-terminal translation initiation site (Jünemann et al. 2007). This effect may then camouflage possible negative effects of mutations under investigation in the 5′-terminal HCV IRES. In fact, by using a monocistronic reporter system, it was shown that miR-122 also stimulates HCV RNA translation, while not excluding other, additional modes of miR-122 action (Henke et al. 2008; Niepmann 2009a). The effect of miR-122 on HCV translation was confirmed in subsequent studies (Jangra et al. 2010; Roberts et al. 2011; Wilson et al. 2011). The overall translation efficiency directed by the HCV 5′-UTR is highest during the G_0 and G_1 phases of the cell cycle, and the superimposed stimulation by miR-122 is high in G_0, G_1 and G_2/M, making the overall translation efficiency highest in the normal state of the hepatocytes (Fehr et al. 2012). Moreover, miR-122 could bind to additional target sites in the HCV genome, including one in the NS5B coding region. However, addressing the NS5B miR-122 target consensus site and the one in the 3′-UTR did not stimulate but rather slightly impair HCV RNA translation and replication (Nasheri et al. 2011).

A sequence that partially overlaps with the miR-122 target sites in the HCV 5′-UTR can hybridize to a complementary sequence in the Core protein coding region (nts 24 - 38) and thereby inhibit HCV RNA translation (Honda et al. 1999b; Wang et al. 2000; Kim et al. 2003). In the rabbit reticulocyte lysate in vitro-translation system, stimulation of HCV IRES-dependent translation by miR-122 operates simply by displacement of this inhibitory long-range RNA–RNA interaction; thereby, single-stranded miR-122 guide strand or shorter RNA oligonucleotides indirectly stimulate HCV translation (Goergen and Niepmann 2012). Indeed, miR-122 can induce a switch in the conformation of the HCV 5′-UTR from a “closed” to an “open” conformation (Diaz-Toledano et al. 2009). In contrast, in cells this interaction plays a negligible role since only duplex miR-122 precursors of the correct length are effective in cells (Goergen and Niepmann 2012). Thus, other mechanisms must account for the effect of miR-122. Since Ago proteins fit the single-stranded guide strand between their PAZ and MID/PIWI domains and thereby pose steric constraints on the overall length of the guide strand (Jinek and Doudna 2009), it appeared most likely that the effector complex involved in the stimulation of HCV propagation contains an Ago protein. An RNAi-based screen for factors involved in the complete HCV life cycle revealed genes involved in microRNA biogenesis and effector function (Dicer1, Drosha, DGCR8, Ago1-4) to be required for efficient overall HCV replication (Randall et al. 2007). Consistently, Ago knockdown decreased HCV translation efficiency in cells containing miR-122 (Roberts et al. 2011; Wilson et al. 2011), and Ago2 colocalizes with miRNA-122 and replicating HCV RNA in perinuclear weblike structures in HuH-7 cells (Berezhna et al. 2011), supporting the idea that Ago protein may be involved in mediating the effect of miR-122 on the HCV RNA.

In addition to translation stimulation, miR-122 was found to also have other functions in HCV RNA metabolism. Mutations in the miR-122 target sites in the 5′-UTR were shown to have a much more pronounced effect on overall HCV virus

yield compared with mutations in the IRES stem-loop IIId, which have a quantitatively similar effect on mere translation. These findings indicated that another, additional mechanism contributes to miR-122 action on HCV RNA accumulation (Jangra et al. 2010). The elongation phase of HCV RNA synthesis was tested for effects of miR-122 but found to be not stimulated by miR-122 (Villanueva et al. 2010). However, miR-122 protects HCV RNA against nucleolytic degradation (Shimakami et al. 2012a). Actually, the binding of miR-122 with their seed sequences and also internal miRNA nucleotides 3' of its seed sequence to the two target sites in the HCV 5′-UTR results in a largely double-stranded RNA complex at 5′-end of the HCV RNA (Machlin et al. 2011; Shimakami et al. 2012b) (compare Fig. 2). This RNA–protein complex is required to protect the HCV RNA from degradation in an Ago2-dependent manner and facilitates the production of infectious virus (Shimakami et al. 2012a; Shimakami et al. 2012b).

The biogenesis of the miR-122-Ago protein complexes involved in HCV RNA propagation may resemble the canonical way of effector complex biogenesis. Knockdown of both Dicer and TRBP reduce HCV RNA accumulation in HuH-7 cells which contain endogenous miR-122. However, when miR-122 duplex is provided ectopically, the need for processing of endogenous pre-miRNA by Dicer is bypassed, and the Dicer knockdown has no effect on HCV RNA accumulation. In contrast, depletion of TRBP reduces miR-122-dependent HCV RNA accumulation even when mature duplex miR-122 precursors are supplied ectopically, whereas single-stranded miR-122 guide strand had no effect (Zhang et al. 2012). Thus, we can assume that RISC-loading complexes that include TRBP and Ago acquire the duplex miR-122 precursor, unwind it, and load the mature miR-122 guide strand onto the HCV RNA where it functions in stabilization of the HCV RNA and translation stimulation (Fig. 5). However, the molecular details of the functional interaction of miR-122 with the HCV RNA remain to be elucidated.

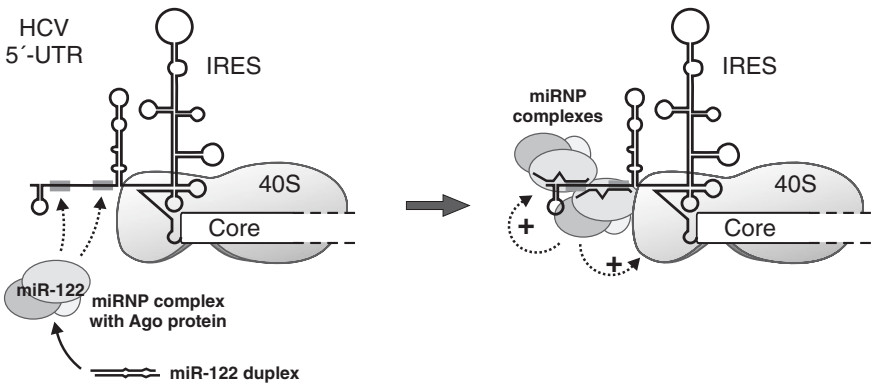

Fig. 5 Hypothetical mechanism of stimulation of HCV RNA replication by miR-122. miR-122 is most likely unwound from its duplex precursor and loaded onto the two miR-122 target sites (*gray boxes*) in the HCV 5′-UTR by a complex containing an Ago2 protein. The microRNA-protein complex on the HCV RNA stabilizes the RNA and stimulates translation

7 Concluding Remarks

Many cellular factors bind to the HCV 5′-UTR including the IRES. Considering the structure of the IRES-40S-eIF3 complex (Fig. 3b), it is difficult to envision how all these factors might bind to the IRES. However, on the one hand, in many cases it is not yet clear at which time point during the viral replication cycle they actually bind. On the other hand, La protein, IMP-1, hnRNP L, and NSAP1 all bind to slightly different sequences of the core and stem-loop IV region. The structure of stem-loop IV RNA is not resolved (Fig. 3), but it is assumed that stem-loop IV is unfolded, and the RNA region that contains the initiator AUG is threaded into the entry channel of the 40S subunit (indicated by the red arrow in Fig. 3a) (Spahn et al. 2001; Filbin and Kieft 2011; Berry et al. 2011). Moreover, hnRNP D binds to the stem-loop II which actually is close to the entry channel, and a possible interaction of hnRNP D with hnRNP L and many other protein–protein interactions (see above) may act in concert to place the HCV RNA region containing the start codon more efficiently into the ribosomal entry channel. Also, at the solvent side of the 40S subunit (the right side of the 40S in Fig. 3), a tip of the stem-loop III of the HCV IRES is visible in the 40S-IRES-eIF3 complex, leaving a possibility that also the LSm ring binds to the tip of the IRES stem-loop III in that complex. Finally, the RNA region including stem-loop I and the sequence between stem-loops I and II is not resolved (Fig. 3), but it can be assumed to be close to the left arm and left leg of eIF3. This could be the place where miR-122-protein complexes act on the ribosome to stimulate RNA translation. Thus, many interactions still have to be elucidated to obtain more detailed information on the actual function of this complex apparatus in translation initiation via the HCV IRES and eventually also in regulating viral RNA replication and assembly.

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Hepatitis C Virus RNA Replication

Volker Lohmann

Abstract Genome replication is a crucial step in the life cycle of any virus. HCV is a positive strand RNA virus and requires a set of nonstructural proteins (NS3, 4A, 4B, 5A, and 5B) as well as cis-acting replication elements at the genome termini for amplification of the viral RNA. All nonstructural proteins are tightly associated with membranes derived from the endoplasmic reticulum and induce vesicular membrane alterations designated the membranous web, harboring the viral replication sites. The viral RNA-dependent RNA polymerase NS5B is the key enzyme of RNA synthesis. Structural, biochemical, and reverse genetic studies have revealed important insights into the mode of action of NS5B and the mechanism governing RNA replication. Although a comprehensive understanding of the regulation of RNA synthesis is still missing, a number of important viral and host determinants have been defined. This chapter summarizes our current knowledge on the role of viral and host cell proteins as well as cis-acting replication elements involved in the biogenesis of the membranous web and in viral RNA synthesis.

Abbreviations

HCV	Hepatitis C virus
IRES	Internal ribosome entry site
nts	Nucleotides
NTR	Nontranslated region
CRC	Crude replication complex
CRE	Cis-acting replication element
MW	Membranous web
DMV	Double membrane vesicle
MMV	Multi membrane vesicle
RdRp	RNA-dependent RNA polymerase
Ago2	Argonaute protein 2

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Contents

1 Introduction

Hepatitis C virus (HCV) is an enveloped positive-strand RNA virus belonging to the genus *Hepacivirus* in the family *Flaviviridae* (van Regenmortel et al. 2000). The genome of HCV encompasses a single ~9,600 nts long RNA molecule containing one large open reading frame (ORF) that is flanked by nontranslated regions (NTRs), important for viral RNA translation, and replication. The 5′NTR contains an internal ribosome entry site (IRES), enabling viral RNA translation in the absence of a cap structure. HCV proteins generated from the polyprotein precursor are cleaved by cellular and viral proteases into at least 10 different products (for further details see chapter "Hepatitis C Virus Proteins: From Structure to Function", this volume): core, envelope glycoproteins E1 and E2, p7, and the nonstructural proteins (NS) NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Core to NS2 is primarily involved in the formation of infectious virus (see chapter "Virion Assembly and Release", this volume), whereas the nonstructural proteins NS3 to NS5B are primarily involved in viral RNA replication, forming the viral replication complex (Bartenschlager et al. 2010), which will be the focus of this chapter. NS3 is a multifunctional protein, consisting of an aminoterminal protease domain required for processing of the NS3 to NS5B region (Bartenschlager et al. 1993) and a carboxyterminal helicase/nucleoside triphosphatase domain (Suzich et al. 1993; Kim et al. 1995). NS4A is a cofactor that activates the NS3 protease function by forming a heterodimer (Bartenschlager et al. 1995). The hydrophobic protein NS4B induces vesicular membrane alterations involved in RNA replication (reviewed in Gouttenoire et al. 2010a). NS5A is a phosphoprotein capable of RNA binding (Huang et al. 2005; Foster et al. 2010), which seems to play an important role in regulating viral replication and assembly (Appel et al. 2008; Tellinghuisen et al. 2008; Masaki et al. 2008). It exists in two phosphorylation variants (Tanji et al. 1995; Kaneko et al. 1994); a basally (hypo-)phosphorylated form (p56), which is supposed to be important for RNA replication (Appel et al. 2005b; Blight et al. 2000) and a hyperphosphorylated variant (p58), probably involved in assembly.

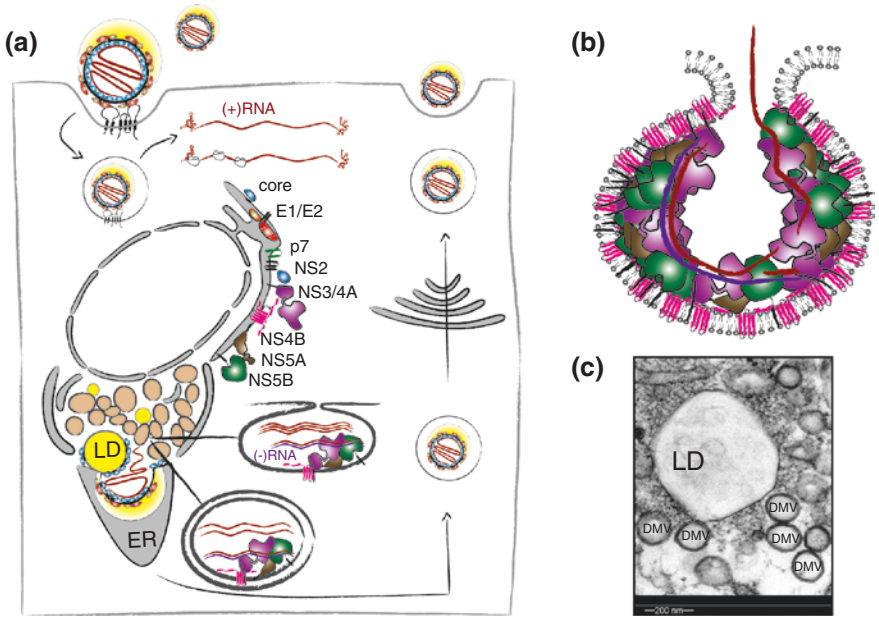

Fig. 1 **a** Schematic representation of the HCV replication cycle. Different potential substructures harboring the replication sites based on biochemical evidence as shown in (**b**) and DMVs recently detected by EM (**c**) are indicated. **b** Model of the HCV replication complex based on biochemical evidence (Quinkert et al. 2005). Multiple copies of the nonstructural proteins serve as structural components of a vesicular structure, containing probably only one replication intermediate and several progeny positive strands. Only a minor subfraction of the nonstructural proteins is supposed to have a function in RNA synthesis. A pore should allow the access of nucleotides and the exit of RNA. **c** Electron micrograph of DMVs in HCV-infected cells 16 h after infection (provided by Inès Romero-Brey unpublished). *LD* lipid droplet, *DMV* double membrane vesicle

NS5B is the RNA-dependent RNA polymerase of HCV, the key enzyme of viral RNA synthesis (Behrens et al. 1996; Lohmann et al. 1997).

After RNA translation and polyprotein processing NS3 to NS5B induce distinct membrane alterations, harboring the sites of viral RNA replication (Gosert et al. 2003; Fig. 1a), which is a typical feature of all positive-strand RNA viruses [reviewed in (Miller and Krijnse-Locker 2008)]. The minimal genetic unit necessary and sufficient for RNA replication has been defined by subgenomic replicons and encompasses the NTRs as well as the NS3 to 5B coding region (Lohmann et al. 1999a). The first step of RNA synthesis generates a negative-strand genome, which serves as template for progeny positive-strand RNA that is produced in 5- to 10-fold excess. It is generally assumed that RNA synthesis of complementary strands initiates at the very 3′terminus of the template strand by de novo initiation of RNA synthesis and that RNA replication involves a double-stranded RNA intermediate, although clear experimental proof for both assumptions is missing. The newly synthesized positive-strand RNA is either re-entering a new translation/replication cycle or is packaged into virions. This chapter summarizes

our current knowledge on HCV-induced membrane alterations, the role of the nonstructural proteins and cis-acting elements in distinct steps of RNA synthesis and provides a selective review of some important host factors involved in these processes.

2 Ultrastructure of the HCV Replication Sites

2.1 Model of the HCV Replication Complex

The establishment of robust cell culture models for HCV (see chapter "Cell Culture Systems for Hepatitis C Virus", this volume) provided the first opportunity to analyze structure and functions of the viral replication sites in detail. Early pioneering EM-studies of liver tissue from infected patients and chimpanzees indicated that HCV, like other positive-strand RNA viruses, induced membrane alterations in infected hepatocytes (Jackson et al. 1979; Shimizu et al. 1990; Shimizu 1992). Later it was shown that the expression of viral nonstructural proteins, particularly NS4B, indeed resulted in the induction of vesicle accumulations, which were designated the membranous web (Egger et al. 2002). These data were confirmed using cell lines harboring persistent subgenomic replicons (Gosert et al. 2003). Immunofluorescence analysis of the localization of viral nonstructural proteins revealed an ER-like distribution with distinct dot-like structures in these replicon cells also co-localizing with newly synthesized viral RNA (Gosert et al. 2003). At the ultrastructural level, these dot-like structures correspond to accumulations of vesicles, which stained positive for viral nonstructural proteins (Gosert et al. 2003). It seems likely that individual vesicles within the membranous web represent the sites of viral RNA replication. Based on analogy with related viruses, it is furthermore assumed that the vesicles are invaginations from the ER membrane, which are connected with the cytoplasm by a small pore allowing the exchange of small membrane-impermeable molecules like nucleotides for RNA synthesis [Fig. 1b; (Welsch et al. 2009)].

This model was further supported by biochemical studies of membrane preparations from replicon cells, so-called crude replication complexes (CRCs) and by selective permeabilization of replicon cells (Ali et al. 2002; Hardy et al. 2003b; Aizaki et al. 2004; El Hage and Luo 2003; Lai et al. 2003; Shi et al. 2003; Miyanari et al. 2003). CRCs and permeabilized replicon cells are capable of RNA synthesis in vitro and this process was shown to be resistant to treatment with proteases and nucleases (Quinkert et al. 2005; Miyanari et al. 2003; Targett-Adams et al. 2008), as well as to Triton X-100 treatment at 4°C (Aizaki et al. 2004; Shi et al. 2003). These results suggested that viral replication complexes were protected by detergent resistant membranes, most likely resembling those vesicular structures indicated by the EM analysis. To allow access of nucleotides to the sites of RNA synthesis, these vesicles should contain an opening allowing access for nucleotides, but small enough to protect the replication sites from nucleases and proteases

(Fig. 1b; Quinkert et al. 2005; Miyanari et al. 2003). Protease and nuclease digests of CRCs (Quinkert et al. 2005) and in permeabilized replicon cells (Miyanari et al. 2003) furthermore demonstrated that all of the negative-strand RNA, ~50 % of the positive-strand RNA, but less than 5 % of the nonstructural proteins were protected, indicating that the majority of NS-protein copies seem not to be involved in the formation of replication sites. Based on these data, it was assumed that an active replication site contained only one copy of negative-strand RNA, which might be part of a double-stranded replication intermediate (Targett-Adams et al. 2008), several copies of positive-strand RNA and 500–2,000 copies of nonstructural proteins [Fig. 1b (Quinkert et al. 2005)].

2.2 DMVs and MMVs

This simplistic model of the HCV replication complex has been challenged in several ways by more recent studies. First, it was shown that the majority of membrane alterations found in HCV-infected cells were not single membrane vesicles, but more complex structures, shelled by two or more membranes and termed double-membrane vesicles (DMVs; Fig. 1c) and multi membrane vesicles (MMVs) (Ferraris et al. 2010; Reiss et al. 2011), similar to membrane alterations identified for coronaviruses (Knoops et al. 2008; Gosert et al. 2002) and picornaviruses (Belov et al. 2012). HCV double-stranded RNA and nonstructural proteins have been found inside DMVs and DMV abundance clearly correlates with viral RNA replication (Ferraris et al. 2010; Romero-Brey et al. 2012), arguing for a functional role of these structures. However, connections of the vesicles to the cytoplasm were rarely observed; therefore, it is not clear how nucleotides get into and newly synthesized RNA out of the DMVs. The simple "ER-invagination model" (Fig. 1b) that was convincingly shown for the related Dengue virus (Welsch et al. 2009) was furthermore challenged by three-dimensional reconstructions, demonstrating that DMVs originate from protrusions rather than invaginations of the ER, with the outer membrane connected by a neck to the ER membrane (Romero-Brey et al. 2012). How this topology of DMVs can be linked to previous models of HCV and flavivirus replication sites (Welsch et al. 2009) is an open question. In case of picornaviruses, RNA replication takes place preferentially at complex single membrane structures originating from the cis-Golgi, which are later transformed into DMVs by membrane wrapping processes (Belov et al. 2012) with yet to be defined function. In case of SARS coronavirus, it has been suggested that active RNA replication occurs in circular single membrane structures known as convoluted membranes (Knoops et al. 2008). DMVs probably originate from these replication sites and represent a final storage compartment to hide replication intermediates from recognition by the innate immune response (Knoops et al. 2008). Similar models might be true for HCV, since DMVs are the dominant species of membrane alterations only at time points later than 16 h after transfection or infection (Paul et al. 2011; Romero-Brey et al. 2012).

2.3 Biogenesis of the Membranous Web

2.3.1 Viral Determinants

The term membranous web was originally established to designate distinct tightly packed vesicle accumulations induced by expression of NS4B (Egger et al. 2002). However, meanwhile, this term is used to generally subsume distinct membrane alterations induced by the HCV nonstructural proteins, containing the sites of viral RNA synthesis (Gosert et al. 2003). The membranous web most likely originates from the ER, as indicated by ultrastructural studies, biochemical evidence and cellular marker proteins, partially co-localizing with the viral replication sites (Gosert et al. 2003; Egger et al. 2002; Miyanari et al. 2003; El Hage and Luo 2003; Romero-Brey et al. 2012). In addition, the early endosomal marker Rab5 has also been found to colocalize with viral NS proteins, suggesting that the formation of HCV replication sites engages several organelles (Stone et al. 2007). Interestingly, the morphology of the membranous web is not depending on RNA replication, but is solely driven by the nonstructural proteins NS3 to NS5B, presumably in concert with cellular factors, since no obvious differences have been found between ectopic protein expression models, cells harboring replicons or infected cells (Romero-Brey et al. 2012; Egger et al. 2002; Gosert et al. 2003; Reiss et al. 2011; Ferraris et al. 2010; Paul et al. 2011).

NS4B has been identified as the main driver of the biogenesis of the membranous web, because sole expression of NS4B induced structures most closely resembling expression of NS3 to 5B (Egger et al. 2002). NS4B is predicted to contain four central transmembrane segments flanked by N- and C-terminal regions attached to the membrane by amphipathic α-helices (Lundin et al. 2003; Gouttenoire et al. 2009b; Gouttenoire et al. 2009a) (see also chapter "Hepatitis C Virus Proteins: From Structure to Function", this volume). Recent studies have shown that NS4B can oligomerize, thereby probably forming the scaffold of membranous vesicles (Yu et al. 2006; Gouttenoire et al. 2010b; Paul et al. 2011). Oligomerization is mediated by homotypic and especially heterotypic interactions involving the N-terminal amphipathic helix 2 and the C-terminus of the protein (Paul et al. 2011; Gouttenoire et al. 2010b). Mutations residing in the NS4B C-terminal region that are impaired in NS4B self-interaction and expressed in the context of the NS3 to NS5B polyprotein indeed generate aberrant DMV structures, supporting the notion that DMVs play an essential role in RNA replication (Paul et al. 2011; Aligo et al. 2009). However, NS4B is not the only determinant of membranous web morphogenesis and more recent data show that also the sole expression of NS3/4A, NS5A, and even NS5B gives rise to distinct vesicular membrane rearrangements (Romero-Brey et al. 2012), suggesting that the role of other nonstructural proteins in this process has been underrated. Interestingly, NS3/4A, NS4B, and NS5B induce single membrane vesicles, which are different from the DMVs and MMVs observed upon expression of the entire replicase module NS3–5B (Romero-Brey et al. 2012). In contrast, only NS5A induced vesicles containing several lipid bilayers and occasionally vesicles containing a pair of membranes morphologically identical to DMVs (Romero-Brey et al. 2012). Although we currently do not understand the role of these DMVs in HCV replication, these novel

data clearly indicate that morphogenesis of the membranous web is complex and engages a concerted action of several nonstructural proteins. NS4B might therefore serve as the major scaffold in this process, modulated mainly by NS5A with the help of NS3/4A and NS5B. A critical role of NS4B-NS5A interactions in web formation is also indicated by genetic studies (Paul et al. 2011) and by the physical interaction between NS3/4A and the C-terminal region of NS4B (Aligo et al. 2009).

2.3.2 Host Factors

In addition to viral proteins also several host factors have been shown to contribute to membranous web formation. Recently, the lipid kinase phosphatidylinositol 4-kinase III alpha (PI4KIIIα, PIK4CA, PI4KA) has been identified by several siRNA screens as a cellular protein essential for HCV RNA replication (Vaillancourt et al. 2009; Borawski et al. 2009; Li et al. 2009; Tai et al. 2009; Berger et al. 2009; Reiss et al. 2011). NS5A and NS5B interact with PI4KIIIα and activate its lipid kinase activity, giving rise to elevated intracellular phosphatidylinositol 4-phosphate (PI4P) levels (Reiss et al. 2011; Berger et al. 2011). Silencing of PI4KIIIα results in reduced DMV size and absence of MMV formation, suggesting that this enzyme is critically involved in web morphology (Reiss et al. 2011), probably mediated by PI4P. More recent data, furthermore, suggest that PI4KIIIα modulates NS5A phosphorylation, by promoting p56 synthesis, which might regulate the structure of the HCV replication sites as well (Reiss and Lohmann unpublished data).

The HCV replication cycle is, furthermore, tightly linked to host cell lipids in various other ways, which are mostly not well understood (reviewed in Alvisi et al. 2011). HCV alters expression of genes involved in cellular lipid metabolism, resulting in accumulation of intracellular lipids (Diamond et al. 2010; Blackham et al. 2010; Su et al. 2002), which is critical for viral RNA replication (Kapadia and Chisari 2005). On the one hand, increased lipid levels might be required to generate the membrane proliferations necessary to form the HCV replication sites, on the other hand, they might be necessary for protein modifications. FBL2, for example, is a geranylgeranylated protein interacting with NS5A and geranylgeranylation was shown to be critical for HCV RNA replication (Wang et al. 2005). NS4B is supposed to be palmitoylated at two cysteine residues at the C-terminus and this modification seems to facilitate oligomerization (Yu et al. 2006).

Lipid droplets (LDs) are also often found in ultrastructural studies of the membranous web (LDs, Fig. 1a, c). LDs are cellular storage organelles for neutral lipids surrounded by a phospholipid monolayer (Martin and Parton 2006). HCV core (Barba et al. 1997; Moradpour et al. 1996; McLauchlan et al. 2002) and NS5A (Shi et al. 2002; Brass et al. 2002) are associated to LDs, because membrane attachment of both proteins is mediated by an amphipathic helix, capable of association with membrane mono- and bilayers. LDs are currently believed to play a central role in the coordination of viral RNA synthesis and virion morphogenesis by physically associating replication and assembly sites (Miyanari et al. 2007; reviewed in Bartenschlager et al. 2011; see also chapter "Virion Assembly and Release", this volume). An interaction of core and NS5A domain III, which is probably regulated

by phosphorylation, has been shown to be critical for assembly of infectious virus, by recruiting both proteins to the same LDs (Appel et al. 2008; Tellinghuisen et al. 2008; Masaki et al. 2008). Although core seems to be the main driver in recruiting the viral replication sites to LDs (Miyanari et al. 2007), viral double-stranded RNA has been found surrounding LDs also in the absence of core, suggesting an additional role of these organelles in RNA replication (Targett-Adams et al. 2008).

Autophagy has also been suggested to contribute to the biogenesis of the membranous web and a very recent study even proposed that HCV RNA replication mainly takes place on autophagosomal membranes (Sir et al. 2012). Indeed, DMVs induced by HCV share morphological similarities with autophagosomes and colocalize with autophagosomal markers in some studies (Ferraris et al. 2010; Guevin et al. 2010). However, the functional role of autophagy in the HCV life cycle is still controversially discussed (reviewed in Dreux and Chisari 2011). Silencing of autophagy components indeed impaired HCV replication, but only very early in infection and not in persistent replication (Dreux et al. 2009; Guevin et al. 2010), suggesting an important role in translation of the viral RNA (Dreux et al. 2009), rather than in membranous web biogenesis. Autophagy has, furthermore, been discussed to be involved in subversion of innate immune responses against HCV (Ke and Chen 2011) and in production of infectious virus (Tanida et al. 2009).

In summary, the biogenesis of the membranous web is a complex process involving not only NS4B but all HCV nonstructural proteins and several host factors. In the light of the complexity of these membrane alterations, including DMVs, MMVs, LDs, and autophagosomes, we are far from understanding their precise functions in viral RNA replication.

3 RNA Synthesis

3.1 Limitations of Current Model Systems

Most of our knowledge on the distinct contribution of viral proteins to viral RNA synthesis is based on biochemical studies and structural analyses of individually expressed and purified proteins, particularly NS5B. This has been complemented by reverse genetics using replicons or infectious virus, particularly to dissect the function of cis-acting elements (see also chapter "Hepatitis C Virus RNA Translation", this volume). However, requirements of RNA replication in cell culture are complex, involving polyprotein processing, membranous web induction, interaction of cis-acting RNA elements (CREs) with proteins, RNA synthesis, and so on. This entire process seems to involve several cis-functions mediated by proteins synthesized on their original template, indicated by the limited possibility to rescue lethal mutants by transcomplementation, which is currently only possible for distinct mutations in NS5A and NS4B (Appel et al. 2005a; Jones et al. 2009; Fridell et al. 2011). Therefore, most mutations interfering with any step in the process of RNA synthesis will finally result in an abrogation of RNA synthesis, without providing further

mechanistic insight. Some limited information into the complex interactions between nonstructural proteins has been gained by studies using intergenotypic chimeras (e.g. Binder et al. 2007) and by the selection of pseudoreversions, rescuing replication deficient mutants and thereby providing genetic evidence for functional interactions (e.g. Lindenbach et al. 2007; Paredes and Blight 2008). Still, there are some major discrepancies between biochemistry and cell culture such as the promiscuity of viral proteins regarding template choice in vitro compared to the strict requirement for almost invariant cis-acting elements to allow RNA replication in cell culture. Biochemical studies on CRCs purified from replicon cells failed to close this gap. CRCs predominantly synthesize a single, full-length RNA product of predominantly positive polarity (Hardy et al. 2003a) and probably also some negative-strand RNA (Ali et al. 2002). RNA synthesis in this system requires at least helicase and polymerase activity as shown by specific inhibitors (Hardy et al. 2003a), involves de novo initiation of RNA synthesis (Hardy et al. 2003a) and gives rise to single- and double-stranded RNA products (Lai et al. 2003). However, inhibition of NS5B is mainly achieved by chain terminating nucleotides in this system (Migliaccio et al. 2003; Lai et al. 2003), whereas several classes of allosteric nonnucleosidic inhibitors of NS5B and heparin failed to inhibit RNA synthesis (Ma et al. 2005), suggesting that CRCs contain stable complexes of the replicase bound to its RNA template. Therefore, it has not been possible to feed exogenous templates into CRCs, which strongly limits their use in mechanistic studies. Furthermore, this system is not accessible to reverse genetics, since active replication in cell culture is a prerequisite of CRC production, thereby precluding the study of mutations affecting defined steps of RNA replication. We are therefore currently lacking adequate in vitro models to address more specific interactions between HCV nonstructural proteins and their specific template, particularly regarding the complex initiation of positive- and negative-strand RNA synthesis.

3.2 Cis-Acting RNA Elements

CREs are mainly, but not exclusively, found in NTRs at the termini of the viral positive- and negative-strand RNA (Fig. 2). The RNA secondary structures and the functional roles of most cis-acting elements in the HCV genome have been mapped extensively in vitro and in cell culture, but the distinct mechanistic functions of individual stem loops are not known due to the lack of appropriate in vitro models.

3.2.1 The 3′end of the Positive-Strand RNA

The 3′NTR is essential for viral RNA replication (Friebe and Bartenschlager 2002), presumably for the initiation and regulation of negative-strand synthesis (Binder et al. 2007). It is composed of a variable region, a polyU/UC tract of variable length and a highly conserved 98-bases element designated X-tail or 3′X, encompassing the 3′end of the viral genome (Fig. 2b; Tanaka et al. 1995; Kolykhalov et al. 1996).

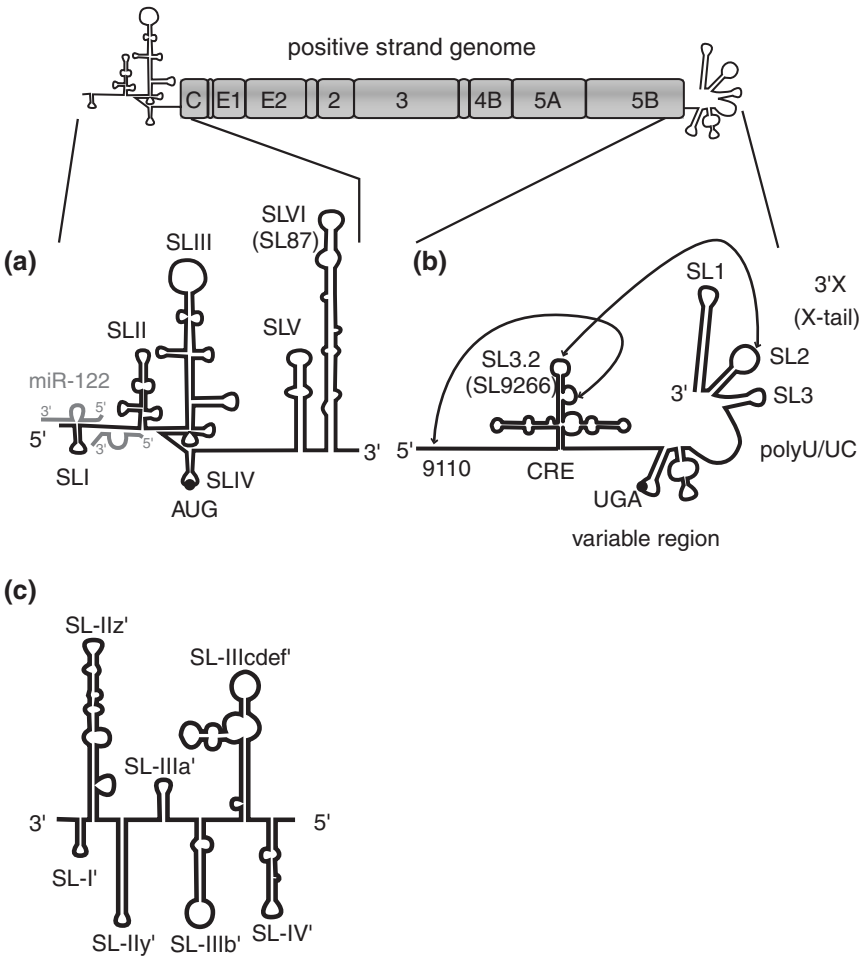

Fig. 2 Schematic representation of cis-acting replication elements. **a** 5′ end of the viral positive strand (Honda et al. 1996). Two copies of miR-122 binding to the 5′NTR are shown in *grey*. **b** 3′end of the viral positive strand (Blight and Rice 1997). Long range interactions of SL3.2 with sequences around 9,110 (Tuplin et al. 2012) and with the loop region of SL2 (Friebe et al. 2005) are indicated by arrows. **c** 3′ end of the viral negative strand (Smith et al. 2002; McMullan et al. 2007). Alternative nomenclatures of some structures are given in *brackets*

The variable region is predicted to form two stem-loop structures, which partly overlap with the very 3′-terminal region of the NS5B coding sequence. Deletion of the variable region results in replicons with significantly reduced replication efficiency, suggesting that this part of the 3′NTR is not essential, but contributes to efficient RNA replication (Friebe and Bartenschlager 2002; Yi and Lemon 2003a). The length of the polyU/UC tract varies between 30 and 90 nts among HCV isolates (Kolykhalov et al. 1996). It is composed of homopolymeric uridine stretches

interspersed by single cytosines. Uridine cannot be replaced by other homopolymeric nucleotides; however, a minimal length of 26–33 consecutive uridines is essential and sufficient for efficient RNA replication in cell culture (Friebe and Bartenschlager 2002; You and Rice 2008). Interruption of this minimal U homopolymer by C residues is deleterious for replication, but the position of homopolymeric U within the polyU/UC tract is flexible (You and Rice 2008; Yi and Lemon 2003a). This polyU stretch might provide a binding platform for viral and cellular proteins (Friebe and Bartenschlager 2002; You and Rice 2008), since NS3 helicase, NS5A, and NS5B have been shown to preferentially bind to polyU (Gwack et al. 1996; Huang et al. 2005; Lohmann et al. 1997). However, the distinct functional role of the polyU/UC region in viral RNA synthesis has not been clarified yet.

The 98-nt X-tail is almost invariant among HCV isolates and is supposed to contain the main regulatory elements required for negative-strand synthesis (Kolykhalov et al. 1996; Tanaka et al. 1995). It comprises three experimentally validated stem-loop structures (Blight and Rice 1997), which are all essential for viral replication (Friebe and Bartenschlager 2002; Yi and Lemon 2003b; Yi and Lemon 2003a) and barely tolerate mutations (Yi and Lemon 2003b; Yi and Lemon 2003a), indicating that not only the structures, but also the sequences are critical for RNA replication. The very 3′end of the HCV genome is a uridine residue in all HCV isolates analyzed so far and base paired in the very stable 3′-terminal SL1 (Fig. 2b). The terminal U can be replaced by C, in line with the requirements of the RdRp to initiate RNA synthesis with a purine base (G or A) (Cai et al. 2004). However, mutant replicons recovered from cell culture revealed reversions to U or even contained additions of U residues in all cases, suggesting a strong selective pressure for a terminal U (Cai et al. 2004; Yi and Lemon 2003b).

Another CRE, designated SL3.2 or SL9266, has been identified within the NS5B coding region. Stem-loop 3.2 is part of a larger predicted cruciform like secondary structure (Fig. 2b; You et al. 2004) and engaged in a kissing-loop interaction with SL2 in the X-tail. This interaction encompasses 7–8 complementary nts in the loop regions and is essential for RNA replication (Friebe et al. 2005). The position of SL3.2 can be moved into the 3′NTR and complementarity between the loop sequences of SL3.2 and SL2 was shown to be more important than the precise sequence (Friebe et al. 2005), arguing for a functional role of a pseudoknot structure at the 3′end of the genome. More recent studies suggest that the bulge region of SL3.2 can form an independent alternative pseudoknot structure with upstream sequences around nucleotide 9,110 (Diviney et al. 2008; Tuplin et al. 2012). Since both interactions of SL3.2 seem mutually exclusive, they might support a functional switch in the HCV replication cycle, e.g. from translation to replication (Tuplin et al. 2012).

3.2.2 The 5′NTR and the 3′end of the Negative-Strand RNA

The 5′NTR encompasses 341–342 nts and has a dual function in the HCV replication cycle; first in the positive strand by functioning as an IRES driving RNA translation, and thus polyprotein synthesis (see chapter “Hepatitis C Virus RNA Translation”, this

volume) and second in the negative strand, providing CREs proposed to direct progeny positive-strand synthesis. Interestingly, the 5′NTR and the complementary 3′end of the negative-strand RNA have been shown to adopt very different secondary structures, in line with their different functions in RNA translation and replication, respectively (Fig. 2a and c, respectively) (Honda et al. 1996 compared to Smith et al. 2002; Schuster et al. 2002). Genetic analyses mapped the minimal region required for RNA synthesis to the 3′-terminal 125 nts, comprising SL-I' and SL-IIz' (Fig. 2c; Friebe and Bartenschlager 2009; Friebe et al. 2001). SL-IIy' is important for efficient RNA replication, whereas the remaining stem loops only seem to have auxiliary functions (Friebe and Bartenschlager 2009). The distinct mechanistic roles of the stem-loop structures at 3′end of the negative strand are still ill defined. In case of SL-IIz', the structure of the stem rather than the discrete sequence seems to be the major determinant. Still it is interesting to note that two miR-122 seed sequences are located in the complementary region of SL-IIz' in the 5′NTR (Fig. 2a; Jopling et al. 2005), suggesting a role of miR-122 in the proper formation of RNA secondary structures. In case of SL-I' only the stem structure is essential for replication of genotype 1b, whereas genotype 2a is also sensitive to sequence alterations not affecting the stem structure (Friebe and Bartenschlager 2009; Luo et al. 2003). The 3′end of the HCV negative-strand genome encompasses a short single stranded region adjacent to SL-I' terminating mostly with C and sometimes with U in clinical isolates, again reflecting the need of the RdRp to initiate RNA synthesis with a purine base (Cai et al. 2004). However, after multiple rounds of replication in cell culture a terminal C was found to be replaced by U (Cai et al. 2004), suggesting that A is the preferred initiating nucleotide for positive- and negative-strand synthesis in cell culture.

Additional conserved stem-loop structures have been found in the core coding sequence. These sequences are not contained in subgenomic replicons, and therefore seem not to be essential for RNA synthesis (Lohmann et al. 1999a). However, disruption of SLVI/SL87 in full-length viral genomes caused severe replication defects in vivo and in cell culture, suggesting that this stem loop has important functions in the regulation of viral RNA synthesis (Fig. 2a; Vassilaki et al. 2008; McMullan et al. 2007).

3.3 The Viral RNA Polymerase NS5B

The viral RdRp NS5B is the driving force of RNA synthesis. The active enzyme can be expressed heterologously with recombinant baculovirus (Behrens et al. 1996) or in *E. coli* (Al et al. 1998) and a huge number of studies have shed light on the structure and biochemical properties of NS5B in vitro. The enzyme consists of a catalytic domain, followed by a linker sequence and a C-terminal membrane insertion sequence, which is essential for RNA replication in cell culture (Fig. 3a; Ivashkina et al. 2002; Moradpour et al. 2004), but seems not to significantly contribute to RNA synthesis in vitro (Lohmann et al. 1997; Yamashita et al. 1998). Removal of the membrane insertion sequence, comprising the C-terminal 21 amino acid residues, increases solubility

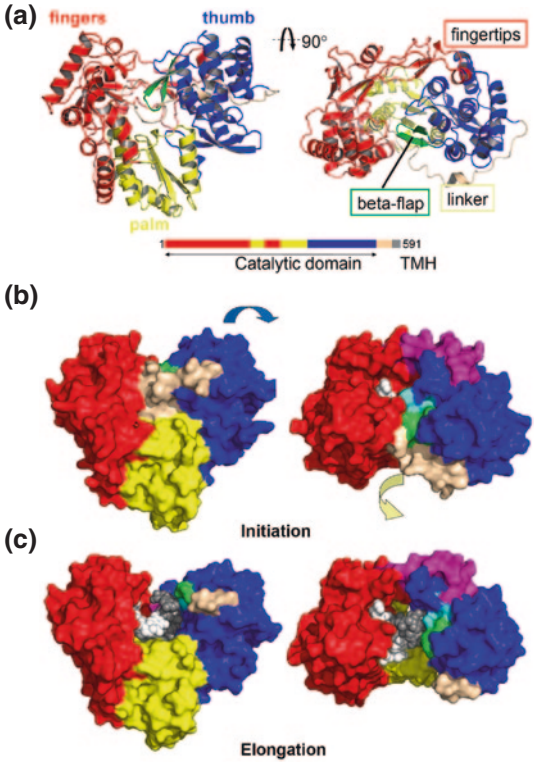

Fig. 3 Structure of the HCV polymerase in *front* views (*left panels*) and *top* views (*right panels*) (Bressanelli et al. 1999). **a** *Ribbon model* of HCV NS5B indicating the fingers (*red*), thumb (*blue*), palm (*yellow*) and linker (*wheat*) subdomains. Note the contact of fingertips and thumb, resulting in a closed structure. The beta flap in the thumb domain is indicated by *green color*. **b** Space filling model of the same structures as in (**a**) with template RNA (*light gray*) modeled. This structure represents the closed conformation, capable of binding the single-stranded template RNA and the two initiating nucleotides. Structural movements of the thumb and linker domains required for the transition to elongation are indicated by a *blue* and *wheat colored arrow*, respectively. **c** Crystal structure of the elongation mode of NS5B in a complex with a double-stranded replication intermediate consisting of the template RNA (*light gray*) and newly synthesized RNA (*dark gray*)

and facilitates purification of NS5B; therefore, most biochemical and all structural studies so far used the so-called NS5BΔC21 enzyme (Ferrari et al. 1999) or even C-terminal deletions encompassing up to 60 amino acid residues (Leveque et al. 2003).

Structural analysis of the catalytic domain revealed a so-called right hand shape, common to many single-subunit polymerases, with fingers, thumb, and palm subdomains [Fig. 3a (Ago et al. 1999; Lesburg et al. 1999; Bressanelli et al. 1999)]. All regular structures published until very recently reveal a closed conformation, encircled on one side by the "fingertips", which is a hallmark of viral RdRps, and on the other side by the linker and the so-called beta flap (or β-hairpin). The latter is specific to *Flaviviridae* RdRps [reviewed in (Lescar and Canard 2009)], but the linker or a variation thereof is common to de novo

initiating RdRps (Butcher et al. 2001). In HCV NS5B, its functional role has not been fully clarified yet. Deletion of the entire linker (so-called ΔC47 or ΔC60 constructs) strongly enhances polymerase activity in vitro and stimulates de novo initiation, suggesting a negative regulatory function (Leveque et al. 2003), whereas a recent study suggests that the linker plays an active role in the very first step of de novo initiation (Harrus et al. 2010). Anyhow, the closed conformation is supposed to represent the initiation state of the polymerase, since the catalytic core only provides space for a single-stranded RNA template and nucleotides for de novo initiation of RNA synthesis, but not for a double-stranded RNA (Fig. 3b; Simister et al. 2009). The closed conformation, therefore, seems to be actively inhibit primer-dependent RNA synthesis by forming a "locked" structure preventing access of primer-template complexes (Chinnaswamy et al. 2008; Ranjith-Kumar et al. 2003).

3.3.1 Template Requirements and Initiation Modes

Purified NS5B can initiate RNA synthesis by a primer-dependent mechanism or de novo (Behrens et al. 1996; Lohmann et al. 1997; Luo et al. 2000; Zhong et al. 2000; Sun et al. 2000). De novo initiation at the 3′end of the viral positive- and negative-strand RNA is likely to be the physiological mode of initiation of RNA synthesis in infected cells, although this has not been experimentally validated yet. De novo initiation in vitro requires a terminal purine, but is most efficient with a G (Zhong et al. 2000). It can take place even on homopolymeric polypyrimidine templates at high nucleotide concentrations (>50 μM; Luo et al. 2000), suggesting that no specific cis-acting elements are required. However, a stable secondary structure and a single-stranded sequence of at least three nucleotides has been shown to be optimal for de novo initiation on nonhomopolymeric templates (Kao et al. 2000), although a secondary structure is not absolutely required (Shim et al. 2002). The 3′end of the HCV negative-strand genome consists of a stem loop with a single-stranded overhang, thereby corroborating the ideal structure for de novo initiation (Fig. 2b). Interestingly, the 3′end of the HCV positive-strand genome is buried within a stable stem structure, which cannot bind to the closed initiation conformation of NS5B (Fig. 2a). In contrast, the positive-strand genome of the related pestiviruses terminates with 3–5 C residues adjacent to a stem loop, representing in theory an ideal template for de novo initiation by the polymerase (Yu et al. 1999). Indeed, it has been shown that the 3′end of the HCV negative strand is an excellent template for de novo initiation, whereas the 3′end of the positive-strand hardly gives rise to terminal de novo initiation (Reigadas et al. 2001; Binder et al. 2007), suggesting that auxiliary factors might be required to initiate negative-strand synthesis by NS5B, thereby probably allowing a tight regulation of this process.

However, de novo initiation by NS5B in vitro is not limited to the 3′end of the template, but can also take place internally (Binder et al. 2007; Shim et al. 2002) and on circular templates (Ranjith-Kumar and Kao 2006). This suggests that NS5B

in solution is in an equilibrium between the closed conformation observed with all crystal structures and an open conformation capable of binding to internal or circular initiation sites (Ranjith-Kumar and Kao 2006) and to primer templates (Lohmann et al. 1997; Behrens et al. 1996). Mn^{2+} ions seem to stabilize the closed conformation, thereby favoring terminal de novo initiation (Ranjith-Kumar et al. 2002).

3.3.2 Steps of RNA Synthesis

RNA synthesis by NS5B in vitro can be separated into distinct steps, namely RNA binding, initiation, elongation, and termination. NS5B binds to a plethora of heteropolymeric RNA templates with no clear specificity for virus-derived sequences, while the binding to homopolymers follows a distinct pattern (polyU > polyG > polyA > polyC) (Lohmann et al. 1997). RNA binding seems to be a very slow and inefficient process and accounts primarily for the low apparent specific activity of the enzyme in vitro (Liu et al. 2006). The enzymatic core of NS5B protects 8–10 nts from RNase digest (Kim et al. 2000) and binds to single-stranded RNAs of >7 nts with high affinity (Kim et al. 2005).

De novo initiation of RNA synthesis involves several steps, which have been characterized by biochemical studies, supported by structural evidence (Fig. 3b; Harrus et al. 2010). After binding of a single-stranded template and the first two nts matching to the 3′end, a dinucleotide primer is synthesized, requiring high concentrations of the priming nts (Ferrari et al. 2008). These dinucleotide primers are produced in great abundance and accumulate in vitro, suggesting that they dissociate rapidly from the NS5B-template complex, whereas progression to processive elongation seems to be inefficient and rate limiting (Harrus et al. 2010; Shim et al. 2002). This initial primer synthesis seems to be facilitated by a very closed conformation, since a very high de novo initiation efficiency observed for NS5B from isolate JFH-1 correlated with a particularly closed structure (Simister et al. 2009). It, furthermore, requires a "platform" to support the first nucleotide, which has to move out of the active center upon addition of the third base. This platform has not been clearly identified in the structure of the polymerase, but it might be provided by the C-terminal linker sequence (Harrus et al. 2010) or by the beta flap in coordination with a GTP bound close to the active site, as suggested for pestiviruses (Lescar and Canard 2009; Choi et al. 2004; D'Abramo et al. 2006). Such a role of coordinating GTP in stabilizing the initiation complex might also explain the strong stimulating effect of high GTP concentration on de novo initiation of NS5B in vitro (Lohmann et al. 1999b; Harrus et al. 2010; Ranjith-Kumar et al. 2003).

The switch from primer synthesis to processive elongation requires high concentrations of the third base to be incorporated (Ferrari et al. 2008), and is furthermore facilitated by high GTP concentrations (Harrus et al. 2010). This switch requires a major conformational change in the polymerase structure, resulting in a move of the "priming platform" and an opening of the entire enzymatic core. The C-terminal linker is removed to accommodate a double-stranded RNA, allowing egress of the template-primer duplex, and the fingertips shift and adjust their contacts with the

thumb (Fig. 3c). The existence of such an "open" conformation is supported by data from a genotype 2a NS5B (Biswal et al. 2005) and by a recent structure of NS5B in complex with a primer template, which was obtained after deletion of the beta flap (Mosley et al. 2012). In this structure, the C-terminal linker is disordered and no longer occludes the exit from the catalytic site and the thumb domain is moved relative to palm and fingers by 20°, generating a large cavity capable of binding double-stranded RNA (Mosley et al. 2012). Position 405 in the thumb domain seems to be a central switch in the transition from initiation to elongation, stabilizing a closed conformation for dinucleotide synthesis, on the one hand, and facilitating the transition to the open conformation, on the other hand, (Scrima et al. 2012) and this position is also critical for efficient RNA replication in cell culture (Schmitt et al. 2011). It should be noted that the C-terminal linker, supposed to take part in the major conformational switch to elongation, is directly connected to the transmembrane segment of NS5B (Ivashkina et al. 2002) and thereby will probably cause a repositioning of the entire enzyme relative to the membrane.

Once RNA synthesis is initiated, NS5B elongates the nascent RNA by about 100–400 nts per minute and is capable to processively copy an entire RNA genome in vitro (Oh et al. 1999; Lohmann et al. 1998; Simister et al. 2009), suggesting that no helicase is required to resolve secondary structures. In this stage, the polymerase is tightly bound to its template and elongation complexes can even be stalled and purified (Jin et al. 2012). Elongation requires only low nucleotide concentrations compared to the initiation reaction (Jin et al. 2012). Little is known about termination of RNA synthesis; however, the polymerase might just fall off after approaching the end of the template.

RNA synthesis by NS5B is error prone and provides the molecular basis of the high genetic variability of HCV isolates. A recent study revealed a high error rate of ca. 10^{-3} per site with a strong bias toward G:U/U:G mismatches for NS5B in vitro, which was corroborated by an observed 75-fold difference in transitions over transversions in vivo (Powdrill et al. 2011).

Although the polymerase is capable of de novo initiation and copying an entire genome in vitro without the help of other factors, there are still some open questions and discrepancies between the properties of NS5B in vitro and replication in cell culture. The overall slow and inefficient initiation reaction, requiring very high nucleotide concentrations in vitro, suggests that this process might be facilitated by auxiliary factors in vivo (Harrus et al. 2010). It is, furthermore, puzzling that the 3′end of the positive-strand RNA is not a bona fide template for de novo initiation, suggesting that initiation of negative-strand synthesis might be a tightly regulated process, requiring additional co-factors. The absence of a clear specificity for viral templates in vitro is in striking contrast to the importance of the CREs for replication in cell culture. Finally, NS5B shows a strong preference for G as initiating nucleotide in vitro, but constitutively initiates negative-strand synthesis with A in vivo and even tends to convert initiation of positive-strand synthesis from G to A in cell culture (Cai et al. 2004). Taken together, analysis of purified NS5B in vitro as well as structural studies provided a number of important insights into the mechanisms of HCV RNA synthesis, but did not reveal the complex regulation of this process in vivo.

3.4 Contribution of Other Viral Proteins to RNA Synthesis

Although it is clear from reverse genetics studies that NS3/4A and NS5A have essential functions in RNA replication, their distinct contribution to RNA synthesis is still unresolved. A study using intergenotypic replicon chimeras found that NS3 helicase, NS5A, and NS5B are required to recognize genotype-specific signals for positive-strand synthesis, suggesting that a complex of these proteins is engaged in initiation of RNA synthesis (Binder et al. 2007). However, deeper mechanistic insights are currently limited due to the lack of appropriate model systems. Since the individual functions of each nonstructural protein have been described in detail in the chapter "Hepatitis C Virus Proteins: From Structure to Function", this volume, the present chapter will focus on the knowledge about their interactions.

The role of the NS3 NTPase/helicase (NS3h) in RNA synthesis is particularly enigmatic. The helicase activity might be involved in initiation of RNA synthesis, e.g. by resolving strong stem-loop structures at the 3′end, thereby generating a template accessible to de novo initiation by NS5B. In addition, NS3h could support NS5B in the elongation phase by unwinding double-stranded replication intermediates. Finally, a ssRNA translocase activity might help to strip proteins off the RNA or deliver RNA for packaging into virions (Gu and Rice 2010). However, none of these functions could yet be validated in cell-based assays. Still, a number of cross-talks between NS3h and other nonstructural proteins have been demonstrated in vitro, which might point to important regulatory functions in vivo. On the one hand, the helicase activity of NS3 is regulated by the NS3 protease domain and by NS5B (Jennings et al. 2008; Zhang et al. 2005), on the other hand, NS3h stimulates the RdRp in vitro (Piccininni et al. 2002), suggesting that NS3 and NS5B indeed might function together.

An important regulatory role for the replicase has recently been assigned to NS4A, the cofactor of NS3 protease. Genetic evidence points to the C-terminal region functioning as an electrostatic switch, regulating NS3 protease function and NS5A phosphorylation (Lindenbach et al. 2007).

NS4B, besides its previously discussed role in organizing the membranous web, might also have more distinct roles in RNA synthesis. NS4B has been shown to inhibit NS5B in vitro (Piccininni et al. 2002). NS4B is furthermore capable of binding RNA (Einav et al. 2008; Einav et al. 2004) and has an NTPase activity; however, the role of the latter two functions has not been clarified yet. In addition, genetic evidence for an interaction of NS4B with NS3 has been reported (Paredes and Blight 2008), which might regulate RNA replication beyond the morphogenesis of the replication sites.

Essential functions of NS5A in viral RNA synthesis, particularly of the hypophosphorylated variant, are clearly suggested from genetic studies. Many cell culture adaptive mutations increasing RNA replication efficiency of genotype 1 isolates and reducing the level of hyperphosphorylated NS5A are found in NS5A (for further details see chapters "Cell Culture Systems for Hepatitis C Virus" and "Hepatitis C Virus Proteins: From Structure to Function", this volume). The analysis of the mechanistic role of NS5A is particularly hampered by the existence of these different

phospho-isoforms. The determinants involved in regulation of phosphorylation are ill defined as is the case for most of the phosphorylation sites and their role in viral RNA synthesis. However, the synthesis of p58 requires at least an NS3-5A polyprotein (Koch and Bartenschlager 1999); therefore, different purified phospho-isoforms have not been accessible for in vitro assays yet and it can be assumed that heterologously expressed NS5A might not be properly phosphorylated. Still, in vitro studies suggest that low doses of NS5A stimulate NS5B, whereas high doses are inhibitory to the polymerase (Shirota et al. 2002; Quezada and Kane 2009). An inhibitory function of NS5A was also found in a cell-based assay (Ranjith-Kumar et al. 2011), suggesting a regulatory role of NS5A for RNA synthesis. The most distinct biochemical property of NS5A that could be envisaged in RNA synthesis is its RNA-binding activity, which resides in domain 1 (Huang et al. 2005) and is modulated by domains 2 and 3 (Foster et al. 2010). One of the available crystal structures of NS5A domain 1, indeed, suggests that NS5A dimers form a basic cleft capable of accommodating RNA (Tellinghuisen et al. 2005), which might play a role in RNA transport, e.g. from replication to assembly sites (see also chapters "Hepatitis C Virus Proteins: From Structure to Function" and "Virion Assembly and Release", this volume). In addition, NS5A interacts with and recruits a plethora of host cell proteins, which might be directly or indirectly involved in RNA synthesis (see below).

3.5 Host Factors Involved in RNA synthesis

Several high content screening approaches, including recent siRNA screenings brought up a huge number of cellular proteins which are supposed to be involved in HCV RNA replication (e.g. Li et al. 2009; Tai et al. 2009). Host factors involved in the morphogenesis of the membranous web, like PI4KIIIα, have been described above; therefore, this part will focus on some of those which might be directly involved in RNA synthesis.

The human VAMP-associated protein A (hVAP-A) and its isoform hVAP-B were identified in yeast two-hybrid screens using NS5A as a bait and were shown to interact with NS5A and NS5B (Gao et al. 2004; Hamamoto et al. 2005). Because of their role in cellular vesicle transport hVAPs are discussed to be involved in the formation of viral membrane rearrangements (reviewed in Moriishi and Matsuura 2007). Interestingly, hVAP-A was found to bind only to hypophosphorylated, but not to hyperphosphorylated NS5A (Evans et al. 2004) and is still the only known host protein differentially interacting with the phospho-isoforms of NS5A. Therefore, hVAP-A was suggested to have a more direct role in RNA synthesis, e.g. regulation of viral replicase activity in a NS5A phosphorylation-dependent manner (Evans et al. 2004).

Cyclophilins are peptidyl-prolyl cis/trans isomerases and their essential role in HCV replication was identified by the inhibition of HCV replication upon cyclosporin A treatment of replicons (Watashi et al. 2003). Initially, cyclophilin B was found to interact with NS5B, regulating template binding of the polymerase (Watashi et al. 2005). More recent results rather suggest that cyclophilin A (CyPA)

is critical for viral RNA replication (Kaul et al. 2009; Liu et al. 2009) and resistance to cyclophilin inhibitors point to NS5A as the main target site of CyPA (Yang et al. 2010; Kaul et al. 2009). CyPA has, furthermore, been shown to directly convert proline residues in NS5A domains 2 and 3 (Coelmont et al. 2010; Verdegem et al. 2011), probably inducing conformational changes critically involved in the function of NS5A. Dependence on CypA can be overcome in part by reducing the cleavage kinetics at the NS5A/NS5B junction, arguing that a kinetically controlled folding step of NS5A plays an important role for viral replicase activity (Kaul et al. 2009).

The liver specific microRNA miR-122 is one of the most remarkable host factors of HCV, regulating RNA abundance in cell culture (Jopling et al. 2005) and in vivo (Lanford et al. 2010). MiR-122 is a critical factor restricting viral replication in cultured cells (Narbus et al. 2011) and might substantially contribute to the liver tropism of HCV. MiR-122 binds to two seed sequences in the 5′NTR, forming an unconventional micro-RNA/target complex, encompassing the 5′end of the viral genome, thereby probably preventing degradation by RNases and/or induction of innate immune responses (Fig. 2a; Machlin et al. 2011). Indeed, miR-122 has been found to stabilize the viral genome in an Ago2-dependent manner (Shimakami et al. 2012). In contrast to conventional microRNA functions on mRNA, miR-122 also stimulates translation of the viral RNA (see chapter "Hepatitis C Virus RNA Translation", this volume).

Cellular RNA-binding proteins are also supposed to serve important functions at different steps of HCV replication. However, 26 cellular proteins specifically binding to the IRES (Lu et al. 2004) and more than 70 interacting with the 3′NTR (Harris et al. 2006) have been identified in proteomic studies; therefore a detailed description of all of them is beyond the scope of this chapter, but several of them are discussed in the chapter "Hepatitis C Virus RNA Translation", this volume, with respect to their role in translation. Little is known about the role of cellular RNA-binding proteins in RNA replication, however, the NF/NFAR proteins have been shown to mediate interactions between the 5′ and 3′NTR of the viral positive-strand RNA and might be involved in the regulation of translation and replication of HCV and the related pestiviruses (Isken et al. 2007; Isken et al. 2003).

4 Intracellular Dynamics of RNA Synthesis

Little is known about the dynamics of HCV RNA replication in vivo and the HCV RNA copy number in infected hepatocytes. This is particularly due to the difficulties in detecting viral antigens and RNA in the liver, arguing for overall relatively low viral RNA and protein levels. However, mathematic modeling of viral decline after therapy revealed that about 10^{12} virions are produced per day in infected individuals (Neumann et al. 1998), suggesting also a relatively high dynamics of RNA replication in the infected liver. Previous studies in chimpanzees determined $10^3 - 3 \times 10^5$ genomes per μg of total liver RNA (equivalent

to ~10^4–10^5 cells), depending on the sample. Therefore, it was assumed that 0.1–30 % of hepatocytes are infected, assuming that an infected cell should contain at least 10 positive-strand RNA genomes to maintain persistent replication (Bigger et al. 2004). Recent studies using highly sensitive two-photon microscopy found that up to 20 % of human hepatocytes are infected, most of the antigen positive cells containing clearly detectable amounts of double-stranded RNA, arguing for much higher HCV RNA copy numbers than 10 per infected cell (Liang et al. 2009).

In cell culture, the dynamics of RNA synthesis is highly variable in transient replication models, depending on the viral isolate, the permissiveness of the host cells, host cell growth, and so on. (see chapter "Cell Culture Systems for Hepatitis C Virus", this volume). Detailed replication kinetics are only available for the most efficient system, replication of the genotype 2a isolate JFH-1 in Huh-7 cells, either upon transfection with subgenomic replicons (Binder et al. 2007) or after virus infection (Keum et al. 2012), revealing very similar results. First viral negative-strand RNAs are detectable 4 or 6 h after transfection or infection, respectively. After this time point, negative-strand RNA levels increase exponentially, reaching a plateau at 24–48 h and slightly declining later on. Most (>90 %) of the incoming positive-strand RNA is degraded within the first hours in both model systems reaching a minimum at the onset of negative-strand synthesis (Binder et al. 2007; Keum et al. 2012). At this time point, positive- to negative-strand ratios are ~1:1, then exponential positive-strand synthesis starts and parallels negative-strand synthesis, again reaching a plateau 24–48 h after transfection/infection, with a positive- or negative-strand ratio of ~10:1. This ratio stays constant in case of the replicon (Binder et al. 2007), but interestingly increases up to 100:1 in case of the infection model (Keum et al. 2012). These data overall suggest that only a minority of incoming positive strands are entering into a productive replication cycle, starting with a 1:1 conversion into probably double-stranded replication intermediates (Targett-Adams et al. 2008). The initial lag-phase of 4–6 h might represent the time required for polyprotein translation, generation of the membranous replication compartment and RNA synthesis (100–400 nts/min. in vitro) (Lohmann et al. 1998; Simister et al. 2009). Asymmetric RNA synthesis is established within a few hours later, rapidly reaching the 10:1 excess of positive-strand RNA typical for all positive-strand RNA viruses. The plateau of RNA synthesis reached within 24–48 h for JFH1 probably reflects restrictions by the host cell, e.g. due to limiting host factors involved in RNA synthesis (Lohmann et al. 2003). Less efficient genotype 1 replicons exhibit a much slower replication kinetics with no clear exponential phase and reach steady-state replication levels at much later time points (Binder et al. 2007; Krieger et al. 2001). The strong increase of positive-to-negtive-strand ratios observed late in infection, but not for replicons, is counterintuitive, since positive-strand genomes should rather be depleted due to the secretion of virions (Keum et al. 2012). However, negative-strand synthesis, in contrast to positive-strand synthesis, probably depends on the continuous generation of new replication sites by positive-strands entering into new translation/RNA replication cycles, which might be limited during the full viral life cycle because of the competition with virion production.

In steady-state cultures of HCV replicon cells, viral RNA and protein amounts have been thoroughly quantified and seem quite similar for different cell clones and viral isolates, arguing for a balance between cell growth and viral replication which is dictated by the selective pressure (see chapter "Cell Culture Systems for Hepatitis C Virus", this volume). Viral negative strands are the most limiting component in replicon cells with less than 100 copies per cell on average, which also represents by definition the maximal number of active replication sites per cell (Quinkert et al. 2005). In contrast, more than 1,000,000 copies of nonstructural proteins are found, indicating that not all vesicular structures seen in EM or antigen positive dots in IF studies can represent active replication complexes (Quinkert et al. 2005). Based on their resistance to proteases, only a subfraction of less than 5 % of these protein copies seem to be engaged in the formation of viral replication sites (Miyanari et al. 2003; Quinkert et al. 2005). Interestingly, roughly 1,000–5,000 positive-strand RNA molecules per cell were reported for transient and steady-state cell cultures and this number might represent a limit of Huh-7-based cell cultures (Quinkert et al. 2005; Keum et al. 2012; Blight et al. 2002).

Mathematic modeling revealed that the sequestration of viral RNA into membranous replication compartments might be an important factor in restraining viral RNA synthesis and defining these steady-state levels (Dahari et al. 2007). Since the half lives of viral NS-proteins and viral positive-strand RNA in replicon cells have been shown to be 11–16 h (Pietschmann et al. 2001; Pause et al. 2003), it can be estimated that only about 1,000 positive-strand RNA molecules are synthesized per day per cell by ca. 100 replicase complexes (Quinkert et al. 2005). In consequence, each newly synthesized positive strand has to be excessively translated to yield the ascertained surplus of proteins, whereas RNA synthesis is a rather rare event, which most likely is achieved only by a few nonstructural protein copies (Fig. 1b). However, it is currently unclear, which mechanisms render a few replicase copies active and the majority inactive.

5 Conclusions and Future Perspectives

Based on our still patchy understanding described above, it is tempting to summarize our current knowledge on viral RNA replication in a tentative succession of events. After release of the viral genome into the cytoplasm of the host cell, the positive-strand RNA is translated giving rise to numerous polyprotein copies at the ER-membrane, which are co- and post-translationally processed into structural and nonstructural proteins. A fraction of NS3/4A, NS4B, NS5A, and NS5B drives the formation of membrane alterations, mainly DMVs, supported by several host factors, e.g. PI4KIIIα. It seems likely that active replication sites are connected to the cytoplasm to allow access of nucleotides and release of newly synthesized RNA (Fig. 1a, b). Negative-strand synthesis is most likely initiated within such vesicular structures, resulting in a probably (partially?) double-stranded replication intermediate. Since most nonstructural proteins cannot be complemented in trans, it can

be assumed that RNA synthesis is initiated from a protein complex translated from the same RNA molecule. This protein complex might assemble at the polyU tract of the genome and probably contains the entire set of NS3 to 5B and some host factors. Initiation of negative-strand synthesis involves cis-acting elements within NS5B and the 3′NTR and might be the most tightly regulated step in the entire process of RNA replication. Since the 3′end of the positive strand is buried in a strong stem, it is not a template for de novo initiation of RNA synthesis by the polymerase; therefore, it seems likely that additional factors like the viral helicase might be essential at this step. NS5B initiates RNA replication by production of a dinucleotide primer, then undergoes a huge conformational change and processively copies the entire genome. Currently it is not clear, whether primer synthesis really takes place at the 3′end or at a different site of the genome, as in case of poliovirus (Paul et al. 2000), with a subsequent transfer of the polymerase/primer complex to the 3′end. Due to the low and limiting number of negative-strand RNA, it is furthermore tempting to speculate that negative-strand synthesis can be initiated only once from a positive strand by a cis-acting protein complex translated on the same RNA. In this scenario, initiation of negative-strand RNA would essentially require a preceding translation of the positive-strand RNA, resulting in the formation of a new replication vesicle and each replication site would indeed contain only one negative-strand RNA/replication intermediate (Fig. 1b). At least in vitro, synthesis of progeny positive strands can directly be initiated by the polymerase due to a favorable 3′ terminal structure of the negative strand, thereby probably allowing multiple initiation cycles, resulting in an overall surplus of positive-strand RNA. The progeny positive-strand RNA is released into the cytoplasm by a yet to be defined mechanism, which could involve NS5A and the translocase function of the NS3 helicase. Mechanisms terminating the lifespan of an active replication site are not known so far. However, the connection to the cytoplasm might simply be constricted after a certain time, sequestering the replication intermediates inside DMVs to prevent recognition by innate immunity, as suggested for arteriviruses (Knoops et al. 2011).

Many of the mechanisms in this model are hypothetical and require experimental validation. Particularly, enigmatic are the transition from translation to replication, the role of the NS3 helicase, the function of NS5A and the distinct roles of its phospho-isoforms, the interplay of CREs with viral and host proteins, the initiation and regulation of RNA synthesis, and the morphology of active replication sites, just to name a few. The complex linkage between translation/replication and cis-/trans-acting functions in the replication cycle of positive-strand RNA viruses severely impedes the analysis of individual steps of RNA synthesis in cell culture models. Therefore, further mechanistic insights will require more sophisticated in vitro models allowing further dissecting the complexities governing HCV RNA synthesis.

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Virion Assembly and Release

Brett D. Lindenbach

Abstract Hepatitis C Virus (HCV) particles exhibit several unusual properties that are not found in other enveloped RNA viruses, most notably their low buoyant density and interaction with serum lipoproteins. With the advent of systems to grow HCV in cell culture, the molecular basis of HCV particle assembly and release can now be addressed. The process of virus assembly involves protein–protein interactions between viral structural and nonstructural proteins and the coordinated action of host factors. This chapter reviews our current understanding of these interactions and factors.

Contents

1 The Curious Nature of HCV Virus Particles

To understand the process of how hepatitis C virus (HCV) particles are assembled, it is important to first review the structure and physical properties of infectious virus particles. Serum-derived HCV particles are often complexed with antibodies and other serum components, making their characterization difficult; nevertheless, much has been learned through the study of chimpanzee serum samples with high specific

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infectivity. The advent of HCV cell culture (HCVcc) systems (reviewed in the chapter "Cell Culture Systems for Hepatitis C Virus" by Steinmann and Pietschmann, this volume) now allows functional virus particles to be grown, purified, and characterized in much greater detail. However, it must be noted that the physical properties of particles produced in cultured hepatoma cell lines differ from those produced in vivo or in primary human hepatocytes (Lindenbach et al. 2006; Podevin et al. 2010). Thus, our understanding of HCV particles, and therefore virus assembly, remains incomplete.

HCV particles are enveloped and contain the viral core protein, which likely combines with the viral genome to form a nucleocapsid, and two surface glycoproteins, E1 and E2 (reviewed in the chapter "Hepatitis C Virus Proteins: From Structure to Function" by Moradpour and Peinin, this volume). A hypothetical model of an HCV particle is shown in Fig. 1a. Infectious, serum-derived particles have diameters between 30 and 80 nm (Bradley et al. 1985; He et al. 1987; Yuasa et al. 1991), while highly purified HCVcc particles have diameters between 60 and 75 nm (Gastaminza et al. 2010; Merz et al. 2011). By electron microscopy (EM), HCVcc particles are pleomorphic, contain electron-dense cores, and lack discernible surface features (Wakita et al. 2005; Gastaminza et al. 2010; Merz et al. 2011).

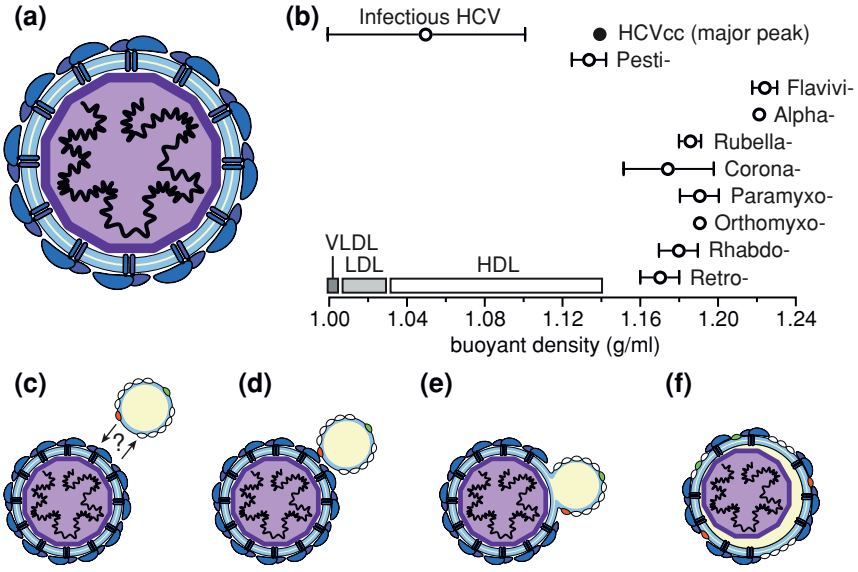

Fig. 1 HCV particles interact with low-density lipoproteins. **a** A model of an HCV particle, based on the structure of flaviviruses. The surface of the enveloped virus particle is decorated with the viral E1-E2 glycoproteins. Within the virus particle is a nucleocapsid formed by core protein and the viral RNA genome. **b** A comparison of enveloped RNA virus buoyant densities. Range bars indicate the buoyant density of viruses within each taxonomic group (data obtained from the International Committee on Taxonomy of Viruses website). The buoyant density of serum lipoproteins are indicated at the bottom for comparison. **c–f**. Illustrations show putative interactions between HCV particles and VLDL particles. See text for further description

The entry of HCV particles is dependent on the low pH of endosomal compartments (Tscherne et al. 2006), suggesting that the viral glycoproteins undergo acid-dependent conformational change, perhaps similar to the type II fusion mechanism of the flavivirus E protein (Bressanelli et al. 2004; Modis et al. 2004) (for further details see chapter "Hepatitis C Virus Entry" by Zeisel et al, this volume). However, HCVcc particles are remarkably resistant to low pH, indicating that virus particles may need to undergo a priming event before they become pH-responsive.

A key feature of infectious HCV particles is that they exhibit unusually low buoyant densities compared to other enveloped RNA viruses, while HCV particles with higher buoyant densities are less infectious (Fig. 1b). Highly infectious virus particles present in chimpanzee serum were found to have densities between 1.03 and 1.10 g/ml (Bradley et al. 1991; Hijikata et al. 1993b). Similarly, HCVcc particles with high specific infectivity have a peak buoyant density of approximately 1.10 g/ml (Cai et al. 2005; Lindenbach et al. 2005), although most cell culture-produced particles have low specific infectivity and buoyant densities near 1.15 g/ml (Cai et al. 2005; Lindenbach et al. 2005; Wakita et al. 2005; Zhong et al. 2005; Yi et al. 2006).

The low buoyant density of infectious HCV particles is thought to be due to their interaction with serum lipoproteins (Thomssen et al. 1992; Prince et al. 1996; André et al. 2002; Nielsen et al. 2006). Consistent with this, Apolipoprotein (Apo) AI, ApoB, ApoC1, and ApoE associate with serum-derived HCV particles (Thomssen et al. 1992; Kono et al. 2003; Nielsen et al. 2006). ApoE and ApoC1 have also been found in association with HCVcc (126, 568, 571), although reports of ApoB association have been variable (Chang et al. 2007; Meunier et al. 2008; Merz et al. 2011). Furthermore, lipid profiling revealed that highly purified HCVcc particles contain lipid and cholesterol contents similar to low-density lipoproteins (LDL) and very-low density lipoproteins (VLDL) (Merz et al. 2011).

The interaction of HCV particles with serum lipoproteins has led to the hypothesis that the virus exists as a hybrid "lipoviral" particle (LVP), which may protect virus particles from neutralizing antibodies (André et al. 2002). However, the precise nature of virus particle-lipoprotein association remains unclear and requires deeper understanding of three unanswered questions. First, what is the molecular basis for interaction of HCV particles with serum lipoproteins (Fig. 1c)? Second, what are the stoichiometric ratios of viral structural proteins and lipoprotein components within an infectious virus particle? Third, do virus particles and serum lipoproteins transiently or stably interact as separate particles, perhaps through specific protein–protein interactions (Fig. 1d), or are they in fact hybrid particles that share a single envelope (Fig. 1e and f)? While LVPs are frequently depicted in reviews and primary research articles as in Fig. 1f, it is unclear how neutral lipids and cholesterol esters would be enveloped by a charged phospholipid bilayer, suggesting that structures topologically similar to Fig. 1d or e are more likely.

One experiment that supports the two-particle model (Fig. 1d) is that HCVcc particles chemically stripped of cholesterol lose their infectivity, which can be restored by adding back exogenous cholesterol (Aizaki et al. 2008). Second, the buoyant density of HCV particles in serum rapidly shifts in relation to dietary triglycerides, suggesting that the interaction of HCV particles with serum

lipoproteins is transient and exchangeable (Felmlee et al. 2010). On the other hand, it is not clear from EM images whether purified HCVcc particles are decorated with VLDL/LDL particles (Gastaminza et al. 2010; Merz et al. 2011). Also, as described below, the production of HCVcc particles is dependent on many—but not all—components of the VLDL assembly pathway, suggesting that the interaction with lipoproteins begin at an early stage of virion assembly. Thus, while our knowledge of virus structure and virus assembly is still incomplete, advances in either area should inform our understanding of both areas.

2 Key Viral and Cellular Players in Assembly

2.1 Viral Structural Proteins

As mentioned, the HCV core, E1, and E2 proteins are structural components of virus particles. Interestingly, these proteins are targeted to distinct places within the cell, which suggests that virus particle assembly is regulated, at least in part, by the coordinated localization of viral structural proteins (see also chapter "Hepatitis C Virus Proteins: From Structure to Function" by Moradpour and Penin, this volume).

2.1.1 Core Protein

HCV core protein is generated from the viral polyprotein through C-terminal signal peptidase cleavage and subsequent C-terminal trimming by signal peptide peptidase (Santolini et al. 1994; Yasui et al. 1998; McLauchlan et al. 2002; Okamoto et al. 2004). Mature core protein consists of an N-terminal RNA-binding region (Domain I, ~120 amino acids) and a C-terminal membrane-binding region (Domain II, ~50 amino acids). Core protein forms homodimers (Boulant et al. 2005), which may be stabilized by an intermolecular disulfide bond (Kushima et al. 2010), as well as higher order multimers (Matsumoto et al. 1996; Kunkel et al. 2001; Klein et al. 2004). In vitro, core protein can interact with structured RNAs to form nucleocapsid-like structures (Kunkel et al. 2001; Klein et al. 2004), although preformed capsids have not been definitively identified in virus-producing cells. One possibility is that sequestration of core protein from viral RNA serves to prevent premature nucleocapsid formation, and that encapsidation occurs concurrent with budding.

Core protein interacts with cellular membranes through two amphipathic helices located within Domain II, as well as palmitoylation of a conserved cysteine residue (Boulant et al. 2005; Boulant et al. 2006; Majeau et al. 2009). This mode of peripheral membrane interaction allows mature core protein to migrate to the surface of lipid droplets (LDs) (Moradpour et al. 1996; Barba et al. 1997; McLauchlan et al. 2002; Boulant et al. 2006). LDs are cellular lipid storage organelles that contain a hydrophobic core of neutral lipids and cholesterol esters surrounded by a phospholipid monolayer that is derived from the outer leaflet of the

endoplasmic reticulum (ER). Targeting of core protein to LDs requires the MAPK-regulated cytosolic phospholipase A2, PLA2G4A (Menzel et al. 2012), and may be enhanced by the cellular enzyme diacylglycerol acetyltransferase 1, DGAT1 (Herker et al. 2010). Thus, core trafficking is functionally tied to specific lipid metabolism events. Furthermore, mutations in core protein that disrupt LD trafficking also abrogate virus production, indicating that LD localization is necessary for virus assembly (Boulant et al. 2007; Miyanari et al. 2007; Shavinskaya et al. 2007). Thus, targeting to LDs may serve to sequester core prior to virus assembly. Retrieval of core from LDs appears to involve recruitment of clathrin adapter protein complex 2 via a specific YXXø motif in core protein (Neveu et al. 2012), as well as specific interactions between viral NS proteins (described below).

To examine core trafficking during virus assembly, Counihan and colleagues developed methods to fluorescently label and image functional, tetracysteine-tagged core protein in live, virus producing cells (Counihan et al. 2011). These data, together with a subsequent paper by Coller and colleagues, show that core is recruited from LDs into virus particles that co-traffic within the secretory pathway in association with ApoE, and that the recruitment of core into this pathway is dependent on interactions between NS2 and NS3-4A (Counihan et al. 2011; Coller et al. 2012).

2.1.2 Envelope Glycoproteins

The HCV E1 and E2 glycoproteins are the major viral structural proteins expressed on the surface of virus particles. During their synthesis, E1 and E2 are translocated into the endoplasmic reticulum (ER), where they interact to form noncovalent heterodimers (Dubuisson et al. 1994; Duvet et al. 1998; Rouillé et al. 2006). E1-E2 dimerization is mediated via their C-terminal transmembrane (TM) domains (Op De Beeck et al. 2000; Patel et al. 2001; Ciczora et al. 2005; Ciczora et al. 2007) and regions within their ectodomains (Yi et al. 1997; Drummer and Poumbourios 2004; Albecka et al. 2011). Native heterodimer formation is a slow process, with the folding of each glycoprotein dependent on the other (Michalak et al. 1997; Patel et al. 2001; Brazzoli et al. 2005) as well as cellular chaperones (Dubuisson and Rice 1996). It has been predicted that E1-E2 heterodimer functions as a class II fusion protein complex (Yagnik et al. 2000); however, formal proof of this will require high-resolution structural information of the glycoprotein complex.

The ectodomain of E2 can independently fold into a near-native form that binds cellular receptors and is recognized by conformation-specific antibodies. Characterization of the recombinant E2 ectodomain revealed that it contains three β-sheet-rich domains separated by regions of random coil and β-turns (Whidby et al. 2009; Krey et al. 2010). Furthermore, the overall structure of E2 is the same at both acidic and neutral pH (Whidby et al. 2009; Krey et al. 2010), suggesting that E2 does not undergo pH-dependent conformational changes on its own. Because E1 does not fold properly in the absence of E2, little is known about the structure of the E1 ectodomain.

In HCVcc-producing Huh-7 cells (see chapter "Cell Culture Systems for Hepatitis C Virus" by Steinmann and Pietschmann, this volume) and other cell lines, E1-E2 heterodimers are retained within the ER; the major determinants of ER-retention reside within the E1-E2 TM domains (Dubuisson et al. 1994; Cocquerel et al. 1998; Duvet et al. 1998; Cocquerel et al. 1999; Cocquerel et al. 2002; Ciczora et al. 2005; Rouillé et al. 2006). However, when expressed in polarized Caco-2 or HepG2 cells, a fraction of E1-E2 heterodimers is secreted in association with chylomicron-like and VLDL-like lipoproteins, respectively (Icard et al. 2009). These data suggest that the HCV glycoproteins contain undetermined signatures for lipoprotein association.

The development of HCVcc systems has allowed the functional, virion-associated forms of E1-E2 to be partially characterized. HCVcc-associated E1-E2 contain both high mannose and complex N-linked glycans, indicating that virus particles transit through the Golgi (Vieyres et al. 2010). Although intracellular E1-E2 forms non-covalent heterodimers, virion-associated E1-E2 are found in large, natively folded, disulfide-linked complexes (Vieyres et al. 2010). These covalent linkages may contribute to the acid-resistance of HCV particles (Tscherne et al. 2006) and suggest that disulfide rearrangement may be necessary to prime HCV particles for low pH-mediated fusion.

2.2 Viral Nonstructural Proteins

2.2.1 The p7 Ion Channel Protein

The small integral membrane p7 protein is considered to be a nonstructural (NS) protein, although definitive evidence is lacking whether or not p7 is virus-associated. This protein is required for the production of infectious virus particles and appears to play at least two essential roles in assembly and maturation (Sakai et al. 2003; Jones et al. 2007; Steinmann et al. 2007; Wozniak et al. 2010).

First, p7 is required for an early stage of virus assembly through interaction with NS2 (Jirasko et al. 2010; Boson et al. 2011; Ma et al. 2011; Popescu et al. 2011; Stapleford and Lindenbach 2011; Tedbury et al. 2011). The second major role of p7 involves its ability to oligomerize to form hexameric and heptameric cation-specific ion channels (Griffin et al. 2003; Pavlovic et al. 2003; Premkumar et al. 2004; Clarke et al. 2006; Luik et al. 2009; Montserret et al. 2010). Remarkably, p7 equilibrates pH gradients within the secretory and endolysosomal compartments of virus-producing cells (Wozniak et al. 2010). A p7 mutant lacking this activity was unable to produce infectious virus particles, but could be complemented by expressing the influenza M2 viroporin or by inhibiting the vacuolar-type H^+-ATPase with Bafilomycin A1 (Wozniak et al. 2010). These data strongly suggest that the ion channel activity of p7 acts as a viroporin to protect virus particles from premature exposure to low pH during virus maturation and egress.

2.2.2 NS2

NS2 is a polytopic membrane protein that contains three N-terminal TM domains and a C-terminal cysteine protease domain (Grakoui et al. 1993; Hijikata et al. 1993a; Lorenz et al. 2006; Jirasko et al. 2008; Schregel et al. 2009; Jirasko et al. 2010). Folding of the cysteine protease domain requires homodimerization to form a single enzyme with two composite active sites at the dimer interface (Lorenz et al. 2006). The only known substrate of this protease is the NS2/3 junction. While NS2 is dispensable for RNA replication of subgenomic replicons, NS2 protease activity is required for polyprotein processing and RNA replication of full-length HCV genomes (Kolykhalov et al. 2000; Welbourn et al. 2005). By using bicistronic constructs to overcome the requirement of NS2–3 cleavage for genome replication, it was shown that the NS2 protease domain, but not NS2 protease activity, is required for virus assembly (Jones et al. 2007; Jirasko et al. 2008).

NS2 plays an essential role at an early stage of virus assembly, bringing together E1-E2 glycoprotein complex, p7, and the NS3–4A enzyme complex (Phan et al. 2009; Jirasko et al. 2010; Boson et al. 2011; Ma et al. 2011; Popescu et al. 2011; Stapleford and Lindenbach 2011). Interestingly, many of these protein–protein interactions map to the N-terminal TM domains of NS2, suggesting that NS2 serves to bring together proteins within the plane of the ER membrane. By combining genetic analysis of NS2 with live cell imaging of functional core protein, Counihan and colleagues showed that the interaction between NS2 and NS3–4A is required to recruit core protein from LDs into sites of virus assembly (Counihan et al. 2011). Similarly, immunofluorescent studies on fixed samples showed that the interaction between NS2 and p7 is required to localize NS2 and core-containing LDs to putative sites of virus assembly (Jirasko et al. 2010; Boson et al. 2011; Popescu et al. 2011; Tedbury et al. 2011). Together, these data indicate that a p7-NS2 complex mediates the early stages of virus assembly through protein–protein interactions. Given that p7 and NS2 form hexamers and dimers, respectively, one intriguing possibility is that a higher order structure of p7-NS2 oligomers may template similar patterns of protein–protein interaction within the viral structural proteins during virion morphogenesis.

2.2.3 NS3–4A

The NS3-4A enzyme complex is essential for HCV polyprotein processing and genome replication. As reviewed in the chapter "Hepatitis C Virus Proteins: From Structure to Function" by Moradpour and Penin, this volume, NS3 contains an N-terminal serine protease domain and a C-terminal NTPase/RNA helicase domain (Morikawa et al. 2011). The small NS4A protein functions as a cofactor for both NS3 serine protease and RNA helicase activities (Failla et al. 1994; Bartenschlager et al. 1995; Lin et al. 1995; Kuang et al. 2004; Beran et al. 2009). NS4A contains an N-terminal TM domain, a central peptide that intercalates into the NS3 serine protease domain, and a C-terminal acidic peptide. Mutagenesis of

the C-terminal acidic domain showed that this region has dual roles in RNA replication and virus assembly, perhaps through its ability to modulate NS3 RNA helicase activity (Beran et al. 2009; Phan et al. 2011).

NS3–4A has been implicated in virus assembly through genetic and biochemical studies. Specifically, mutations in NS3 that enhance RNA replication cause defects in virus assembly (Pietschmann et al. 2009), suggesting that NS3 may contribute to the switch between RNA replication and virus assembly. Furthermore, mutations in the NS3 helicase domain were shown to suppress defects in virus assembly caused by mutations and/or genetic incompatibilities in NS2, NS3, or NS4A (Ma et al. 2008; Phan et al. 2009; Jirasko et al. 2010). As indicated above, the interaction between NS2 and NS3–4A is involved in recruiting LD-associated core protein into virus assembly (Counihan et al. 2011). Given that core protein is an RNA binding protein and that RNA helicases are processive RNA motors, one attractive hypothesis is that the NS3–4A RNA helicase packages HCV RNA during virus assembly. Consistent with this, genetic and biochemical evidence indicate that interaction between the NS3 helicase domain and core protein is essential for virus assembly (Jones et al. 2011; Mousseau et al. 2011). Nevertheless, definitive evidence is needed to show whether NS3–4A helicase activity is directly involved in nucleocapsid formation.

2.2.4 NS5A

NS5A is an RNA-binding phosphoprotein that plays multiple roles in the virus life cycle. It contains three domains: domain I, which folds into an unusual structure and mediates homodimerization; domain II, which is conserved but likely natively unfolded; domain III, which is less conserved and is tolerant of large insertions and deletions; and two low complexity regions that separate these domains.

NS5A plays essential roles in virus particle assembly, largely through determinants in domain III (Appel et al. 2008; Tellinghuisen et al. 2008; Kim et al. 2011). Specifically, virus assembly requires phosphorylation of a serine residue within domain III by casein kinase IIα (Tellinghuisen et al. 2008). Furthermore, genetic and biochemical data indicate that domain III encodes determinants for transient or weak association with the p7-NS2 complex (Jirasko et al. 2010; Ma et al. 2011; Popescu et al. 2011; Scheel et al. 2012). Similar to NS3, mutations in NS5A that enhance RNA replication cause decreases in virus assembly, suggesting that NS5A may contribute to the switch between replication and assembly (Pietschmann et al. 2009).

Virus particle assembly requires the recruitment of NS5A to LDs, where it interacts with core protein (Miyanari et al. 2007; Appel et al. 2008; Masaki et al. 2008). In addition to interacting with other viral proteins, NS5A interacts with ApoE, an apolipoprotein (Apo) that is required for virus assembly (Evans et al. 2004; Benga et al. 2010; Cun et al. 2010), and Annexin A2, a cellular membrane sorting protein that enhances virus assembly (Backes et al. 2010).

2.2.5 NS4B and NS5B

NS4B is a polytopic membrane protein that plays an essential role in the formation of HCV RNA replication complexes (Gouttenoire et al. 2010). In addition, genetic analysis of NS4B revealed a point mutation in the C-terminal region that increased HCVcc titers but had minimal effects on RNA replication (Jones et al. 2009). It is not yet clear whether NS4B plays a direct or indirect role in virus assembly.

NS5B is the RNA-dependent RNA polymerase that replicates the viral genome (chapter "Hepatitis C Virus RNA Replication" by Lohmann, this volume). It too has been implicated in virus assembly through genetic approaches. Specifically, a mutation at a surface loop residue in the "fingers" subdomain of NS5B was able to suppress virus assembly defects caused by genetic incompatibility of a heterologous p7 within a chimeric genome (Gouklani et al. 2012). Again, it remains to be determined whether this represents a direct or indirect role for NS5B in virus assembly.

2.3 Cellular Factors

2.3.1 VLDL Assembly

HCV particle assembly appears to share numerous features with the pathway of VLDL/LDL assembly. It is therefore worthwhile to briefly review the mechanisms of this pathway.

LDL particles are synthesized in hepatocytes as VLDL particles, which traffic lipids and cholesterol as they circulate through the blood. The surface of VLDL and LDL particles are coated with a single copy of ApoB, a large (≈4500 amino acids), essential, amphipathic glycoprotein; VLDL and LDL particles also transiently associate with the exchangeable apolipoproteins ApoA5, ApoC1, ApoC2, ApoC3, ApoE.

Biosynthesis of VLDL appears to occur in two steps. In the first step, newly synthesized ApoB acquires and encases lipids as it is translocated into the ER to form a VLDL precursor. Proper folding of ApoB requires the co-translational transport of lipids into the ER by microsomal triglyceride transfer protein (MTP) (Sakata et al. 1993; Yao et al. 1997). In the second step, the VLDL precursor undergoes further lipidation steps to form mature VLDL particles, which are secreted through the Golgi in a COPII-dependent manner (Gusarova et al. 2003; Siddiqi 2008). The mechanisms of secondary lipidation are still under investigation. Some models posit that VLDL precursors acquire lipids via fusion with (or catabolism of) ER-resident, ApoE- and ApoC-containing LDs, which may be produced by MTP and/or CideB (Rustaeus et al. 1998; Wang et al. 2007; Ye et al. 2009). However, direct evidence and mechanistic details of this fusion seems to be lacking. Alternatively, other evidence indicates that secondary lipidation occurs in a post-ER compartment, most likely the Golgi (Stillemark et al. 2000; Gusarova et al. 2003; Gusarova et al. 2007; Blasiole et al. 2008). Part of this debate stems from the fact that VLDL assembly has

been studied in different animal and cell culture systems, which may emphasize one part of a pathway over another. In this regard, it is notable that Huh-7 cells, which robustly support HCV RNA replication, do not produce authentic VLDL (Yamamoto et al. 1987; Meex et al. 2011); rather, they produce VLDL-like particles that are underlipidated, which may explain why HCVcc particles produced in Huh-7 cells have higher buoyant density and lower specific infectivity than HCV produced in bona fide hepatocytes (Lindenbach et al. 2006; Podevin et al. 2010).

Despite the above caveats of studying VLDL production in Huh-7 cells, several lines of evidence show that the assembly of HCVcc particles and VLDL are closely linked. First, small molecule inhibitors of MTP block virus particle production (Huang et al. 2007; Gastaminza et al. 2008; Nahmias et al. 2008; Jiang and Luo 2009). While some groups have reported that ApoB expression is required for HCV assembly (Huang et al. 2007; Gastaminza et al. 2008; Nahmias et al. 2008), Jiang and Luo found that HCV assembly was not dependent on ApoB expression, but was highly dependent on the small exchangeable protein ApoE (Jiang and Luo 2009). Similarly, Coller and colleagues showed that nascent virus particles traffic through the secretory pathway in association with ApoE, but not with ApoB (Coller et al. 2012). These are intriguing findings, since ApoB expression is essential for VLDL assembly, suggesting that HCV assembly may actually depend on ApoE-containing ER-luminal LDs rather than VLDL particle formation. Consistent with the essential role for ApoE in HCV production, mouse hepatoma cells were shown to produce infectious HCVcc particles in an ApoE-dependent manner (Long et al. 2011). In addition, intracellular HCVcc particles were shown to be immunoprecipitated and neutralized by antibodies against another small exchangeable apolipoprotein, ApoC1, indicating that they also associate at an early stage of virus production (Meunier et al. 2008).

2.3.2 ESCRT Pathway and Endosomal Release

The endosomal-sorting complex required for transport (ESCRT) pathway is a cellular machinery for the outward budding and fission of vesicles away from the cytoplasm, and is centrally involved in the formation of multivesicular bodies (Henne et al. 2011). In addition, many enveloped viruses utilize this pathway for budding into extracellular compartments (Welsch et al. 2007). Three groups found that the secretion of infectious HCVcc particles is dependent on components of the ESCRT pathway, although intracellular infectious virus particles were assembled when late steps of the ESCRT pathway were inhibited (Corless et al. 2010; Ariumi et al. 2011; Tamai et al. 2012). One interpretation of these data is that the ESCRT-III pathway is involved in resolving the terminal membrane fission event and that incompletely budded virus particles can be released during experimental preparation of cell extracts. An alternative interpretation is that the ESCRT pathway is required for a post-assembly step of virus release, perhaps through trafficking of virus particles into a late endosomal/multivesicular body compartment. In this regard, Tamai and coworkers propose that Hrs contributes to the release of virus particles through fusion of multivesicular bodies with the plasma membrane, similar to the release

of exosomes (Tamai et al. 2012). Consistent with an endosomal trafficking of virus particles, Lai and colleagues found evidence that HCVcc particles traffic through early and late endosomal compartments (Lai et al. 2010), although the timing of these experiments made it difficult to know whether the immunolabeled particles were on their way in or out of cells. More compelling evidence was provided by Coller and colleagues, who showed core protein trafficking with markers of recycling endosomal compartments, Rab11, transferrin, and dextran in live, virus-producing cells (Coller et al. 2012). These results are consistent with the flow of virus particles through the secretory pathway into a sorting endosomal compartment.

2.3.3 Other Host Factors

As mentioned above, trafficking of core protein to LDs requires PLA2G4A and is enhanced by DGAT-1 (Herker et al. 2010; Menzel et al. 2012). Interestingly, the block in virus assembly caused by a pharmacologic inhibitor of PLA2G4A was overcome by exogenous addition of arachidonic acid, the product of this enzyme. These data implicate specific lipid products in the pathway of virus assembly.

Another host factor involved in core protein trafficking is AP2M1, the μ1 subunit of clathrin adapter protein complex 2 (Neveu et al. 2012). This was a surprising result, as AP2M1 is involved in recruiting cargo into clathrin-mediated endocytosis at the plasma membrane. Further work will be needed to clarify the role of this pathway in virus assembly.

Backes and colleagues identified Annexin A2, a phospholipid-binding protein that anchors membranes to the actin cytoskeleton, within partially purified HCV RNA replication complexes (Backes et al. 2010). Surprisingly, knockdown of this gene had no effect on viral genome replication, but significantly inhibited the assembly of infectious virus particles within cells. Furthermore, Annexin A2 was found to bind to NS5A domain III that has been implicated in virus assembly (Backes et al. 2010).

The stress granule proteins G3BP1, TIA-1, and TIAR were recently implicated in virus particle production (Garaigorta et al. 2012). Knockdown of these genes moderately inhibited viral RNA replication but had stronger effects on virus assembly and release. Their mechanism of action is unknown, but may be independent of their roles in stress granule formation (Garaigorta et al. 2012).

3 Mechanism of Virus Particle Assembly

The details of HCV particle assembly are not yet fully clear, but must involve the coordinated action of the ER-resident E1-E2 glycoprotein complex, recruitment of LD-associated core protein to package viral RNA, and several viral and host factors described above. HCV particles form through budding into the ER, similar to other members of the *Flaviviridae*. Consistent with this, Gastaminza and colleagues showed that infectious HCVcc particles accumulate within cells when

treated with Brefeldin A, a potent inhibitor of ER-Golgi transport (Gastaminza et al. 2008). As described above, the interaction between LD-associated core and NS5A proteins is important at an early step of this process, perhaps by shifting RNA out of replication and into virus assembly (Miyanari et al. 2007; Appel et al. 2008; Masaki et al. 2008). Similarly, NS2 brings together the viral E1-E2 glycoprotein complex, p7, and the NS3-4A enzyme complex (Phan et al. 2009; Jirasko et al. 2010; Boson et al. 2011; Ma et al. 2011; Popescu et al. 2011; Stapleford and Lindenbach 2011), and the interaction between p7-NS2 and NS3–4A is necessary to recruit core protein from LDs into sites of virus assembly (Boson et al. 2011; Counihan et al. 2011). Given that preformed capsids have not been identified, nucleocapsid formation likely takes place in concert with budding. Furthermore, virus particle assembly may be intimately coordinated with RNA replication, as seen for other members of this virus family (Khromykh et al. 2001; Welsch et al. 2009). A model that brings together these considerations is presented in Fig. 2.

4 Maturation and Release of Virus Particles

As indicated above, E1-E2 present on extracellular virus particles contain some complex modifications (Vieyres et al. 2010), indicating that virus particles pass through the Golgi. Consistent with this, Coller and colleagues found that core protein trafficked to Golgi in a virus assembly-dependent manner prior to being released at the plasma membrane in a VAMP1-dependent manner (Coller et al. 2012). During egress, HCV particles depend on p7 to neutralize acidic compartments within the secretory pathway (Wozniak et al. 2010).

Gastaminza and colleagues showed that nascent, intracellular HCVcc particles have a higher buoyant density than extracellular particles and acquire their low

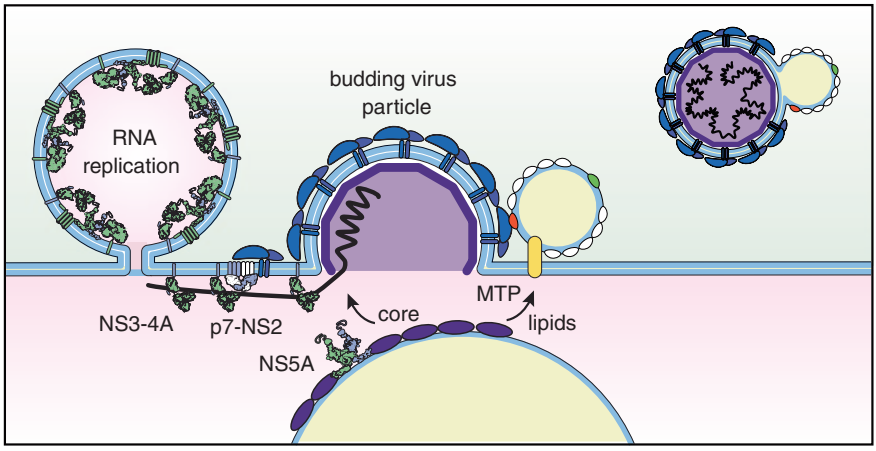

Fig. 2 Model of HCV particle assembly. See text for further description

buoyant density in a post-ER compartment (Gastaminza et al. 2006; Gastaminza et al. 2008). These data imply that virus particles undergo post-synthetic lipidation, similar to the VLDL assembly pathway.

In addition to producing extracellular virus particles, HCV has been reported to directly infect neighboring cells without releasing detectable virus particles or requiring the canonical HCV entry factors (Timpe et al. 2008; Witteveldt et al. 2009). The structural protein requirements and assembly and release pathways for cell–cell HCV transmission are not currently understood.

5 Conclusions

Much has been learned about the assembly and release of HCV particles in the past 7 years of research with HCVcc systems. Essential protein–protein interactions have been defined, and numerous host factors have been implicated. However, the structure of HCV particles and their interaction with lipoproteins remain to be addressed, and key questions need to be examined by using bona fide hepatocytes that make authentic lipoproteins. There is yet much work to be done!

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Innate Immune Responses to Hepatitis C Virus

John W. Schoggins and Charles M. Rice

Abstract The innate immune response provides the first line of defense against invading viral pathogens. Incoming viruses are sensed by dedicated host factors that, when triggered, initiate multiple signal transduction pathways. Activation of these pathways leads to the induction of highly orchestrated transcriptional programs designed to limit virus replication and spread. In recent years, our understanding of innate immune responses targeting hepatitis C virus (HCV) has increased substantially, largely due to the development of new systems and methodologies to study HCV–host interactions in vitro and in vivo. However, significant gaps still remain. Here, we aim to provide a comprehensive view of the innate immune response to HCV, focusing primarily on knowledge gained from cell culture models of HCV infection, as well as data from human patients infected with HCV. While some paradigms of the host response to HCV revealed in cell culture translate to human infection in vivo, others are less clear. Further insight into the similarities and differences in these systems will not only reveal directions for future studies on HCV immunity, but may also guide the development of novel strategies to control HCV and other viral infections.

Contents

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1 Introduction

Hepatitis C continues to exert a heavy disease burden across the globe, with 3–4 % of the population chronically infected with the hepatitis C virus (HCV) (Lavanchy 2009). Until recently, standard of care for chronic HCV infection included a regimen of interferon (IFN) alpha in combination with ribavirin (see also Treatment of Chronic Hepatitis C: Current and Future by Pawlotsky). For genotype 1 viruses, the most resistant to treatment, this regimen affords an average of 40–50 % sustained virological response (SVR) (McHutchison et al. 2009), which is defined as undetectable viral load six months after termination of treatment. The recent introduction into the clinic of direct-acting antivirals targeting the viral NS3 protease is yielding promising results by increasing SVR rates in both treatment-naïve patients and nonresponders (Lamarre et al. 2003; Welsch et al. 2012).

Despite increasingly effective treatment regimens, significant gaps in our understanding of the basic biology of HCV infection and immune activation remain. These deficiencies are highlighted clinically by the fact that only 30 % of acutely infected individuals are able to clear the virus (Lavanchy 2009). The remaining 70 % of infected individuals progress to chronicity, largely due to inadequate control by the host immune response. Recent advances in our understanding of the innate immune responses targeting HCV highlight a complex interplay between host-directed mechanisms of controlling virus replication and virus-directed mechanisms of counteracting the host response. Indeed, treatment with IFN is aimed at tipping the balance of the immune response in favor of the host, and success depends on numerous factors, including HCV genotype and host genetics. A deeper understanding of the interplay between virus and host innate immune response has required the development of new viral tools and cellular systems to dissect mechanisms of immune-mediated control. These include recombinant HCV generated from infectious molecular clones and a small variety of immortalized and primary human hepatocyte cell culture systems. The in vitro models systems have, in turn, complemented clinical and translational studies to gain insight into innate mechanisms controlling HCV infection in primate models and infected patient populations.

2 Innate Immune Response to Viral Infection

To defend against invading viral pathogens, the host cell relies on both membrane bound and cytoplasmic receptors to sense viral nucleic acid and/or viral proteins (Fig. 1). The major players in viral sensing include members of the toll-like receptor family (TLRs), the RIG-I-like receptors (RLRs), and the viral DNA sensors (Kawasaki et al. 2011). Across the viral phylogeny, all of these viral sensing families have been implicated in host recognition of numerous viruses. Downstream of the viral sensors are adaptor proteins that promote the antiviral signaling program. The TLRs rely on MyD88 and TRIF as adaptors (O'Neill and Bowie 2007). RLRs use the mitochondria-localized MAVS (also known as IPS-1, VISA, Cardif) (Kawai et al. 2005; Meylan et al. 2005; Seth et al. 2005; Xu et al. 2005),

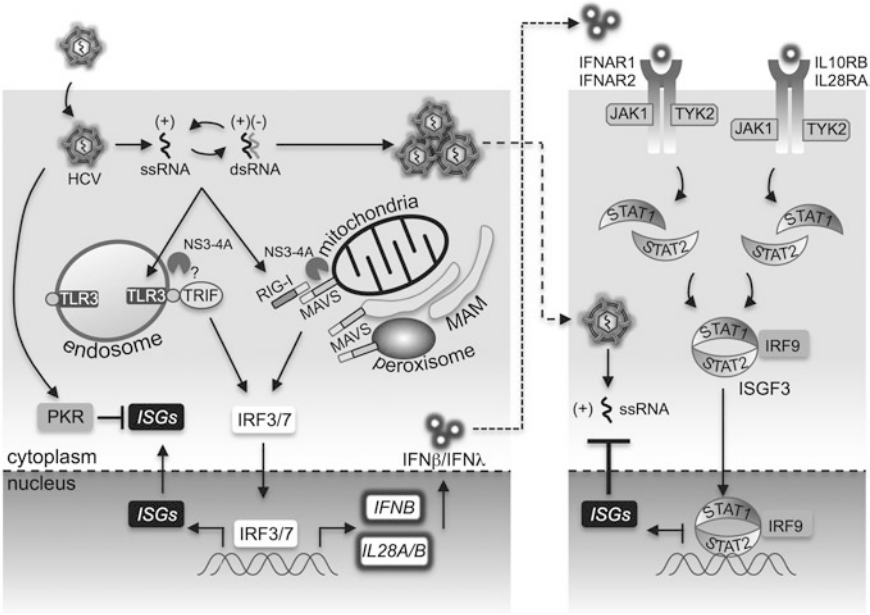

Fig. 1 Overview of the innate immune response to HCV. Incoming or replicating viral genomes are detected by pattern recognition receptors such as RIG-I. Activation signals are transmitted through the RIG-I adaptor MAVS, which triggers activation of IRF3 and or IRF7. Type I/III IFNs and a subset of ISGs are transcribed. IFNs are secreted from the infected cell and act on neighboring cells through the JAK/STAT pathway, leading to widespread ISG induction. Two viral mechanisms of immune evasion are depicted: a) cleavage of MAVS and TRIF adaptors by HCV NS3/4A and b) HCV-mediated activation of PKR, which suppresses translation of ISG mRNAs and renders the cells less responsive to IFN

and several viral DNA sensors signal through STING (Ishikawa and Barber 2008; Ishikawa et al. 2009; Unterholzner et al. 2010). For most sensors, adaptor engagement leads to activation of kinases that phosphorylate signaling molecules to promote downstream transcription. For example, viral RNA sensing by RLRs leads to MAVS-mediated activation of the TBK1 kinase, which phosphorylates interferon regulatory factor 3 (IRF3) and IRF7 and triggers their translocation to the nucleus (Fitzgerald et al. 2003; Sharma et al. 2003). Similar signaling also occurs by viral RNA engagement of the TLR3-TRIF pathway.

The downstream effect of these signaling pathways is transcriptional induction of antiviral type I and III interferons (IFNs). The human genome encodes 14 unique type I IFN genes, 1 for IFNβ and 13 for the various IFNα subtypes (Pestka 2007). For type III IFNs, or IFNλ, humans have 3 genes, *IL29*, *IL28A*, and *IL28B*, which encode IFNλ1, IFNλ2, and IFNλ3 proteins, respectively. In addition to IFNs, other proinflammatory cytokines can be triggered, depending on the incoming virus and activation pathway. Once IFNs are produced, they are secreted from the cell and act in an autocrine or paracrine fashion to promote antiviral signaling. Type I IFNs bind a heterodimeric receptor consisting of IFNAR1 and IFNAR2 (Uze et al. 2007), while type III IFNs bind a heterodimeric receptor consisting of IL10-R2

(also known as IL10RB) and IL28RA (Kotenko et al. 2003; Sheppard et al. 2003). Binding of IFN to the receptor ectodomain results in recruitment of JAK1 and TYK2 kinases to the cytoplasmic tails. Kinase activation leads to phosphorylation and subsequent dimerization of STAT1 and STAT2. STAT1/STAT2 heterodimers associate with IRF9 to form the transcriptionally active ISGF3 complex, resulting in the transcription of numerous interferon-stimulated genes (ISGs) (Stark and Darnell 2012). Collectively, these genes facilitate both clearance of virus from infected cells and protection of neighboring, uninfected cells from incoming viral progeny. They also act to recruit effector immune cells to the site of infection and promote the adaptive immune response. Thus, once IFN pathways are activated, the downstream response has enormous breadth, as noted by the existence of numerous type I/III IFN genes and many hundreds of ISGs. In contrast, relatively fewer upstream sensors and adaptors have been discovered, suggesting an inherent and requisite pleiotropy in their function. Indeed, host defenses against dozens of well-studied viruses have been shown to funnel through the relatively small number of RLRs, TLRs, and DNA sensors (Keating et al. 2011; Kumar et al. 2009; Loo and Gale 2011).

3 Cellular Mechanisms to Sense HCV Infection

Accumulating evidence suggests that RLRs, particularly RIG-I, dominate the cellular response to HCV infection. The RLRs consist of RIG-I (encoded by *DDX58)*, MDA5 (encoded by *IFIH1*), and LGP2 (encoded by *DHX58*). RIG-I and MDA5 recognize a variety of viral RNA pathogen-associated molecular patterns (PAMPs) (Loo and Gale 2011), while LGP2 has been implicated in regulating the function of RIG-I and MDA5 (Rothenfusser et al. 2005; Satoh et al. 2010; Yoneyama et al. 2005). Engagement of RIG-I and MDA5 by RNA occurs through the repressor domain, leading to activation of two amino terminal caspase recruitment (CARD) domains. The CARD domains, which are absent in LGP2, trigger downstream signaling by activating MAVS. Less than 10 years have passed since the discovery of RIG-I, MDA5, and other downstream components of antiviral immunity. Since then, a wealth of biochemical and genetic data has yielded new insight into the mechanisms of innate immune activation mediated by viral nucleic acid sensors. Examples include: the recent crystal structure of RIG-I in complex with double stranded RNA (Jiang et al. 2011; Kowalinski et al. 2011; Luo et al. 2011), biochemical insight into the composition and signaling potential of PAMPS that bind RIG-I (Binder et al. 2011; Hornung et al. 2006; Pichlmair et al. 2006), the finding that MAVS-mediated antiviral signaling is linked to mitochondrial membrane potential (Koshiba et al. 2011), and studies on the ability of MAVS to form prion-like aggregates (Hou et al. 2011). Additional data indicate that antiviral signaling through MAVS is not exclusive to mitochondria, but also emanates from MAVS localized on peroxisomes and mitochondrial-associated endoplasmic reticulum membranes (Dixit et al. 2010; Horner et al. 2011). Here, we focus specifically on the role of RLR signaling pathways in HCV infection, both in cell culture models and in vivo (Fig. 2).

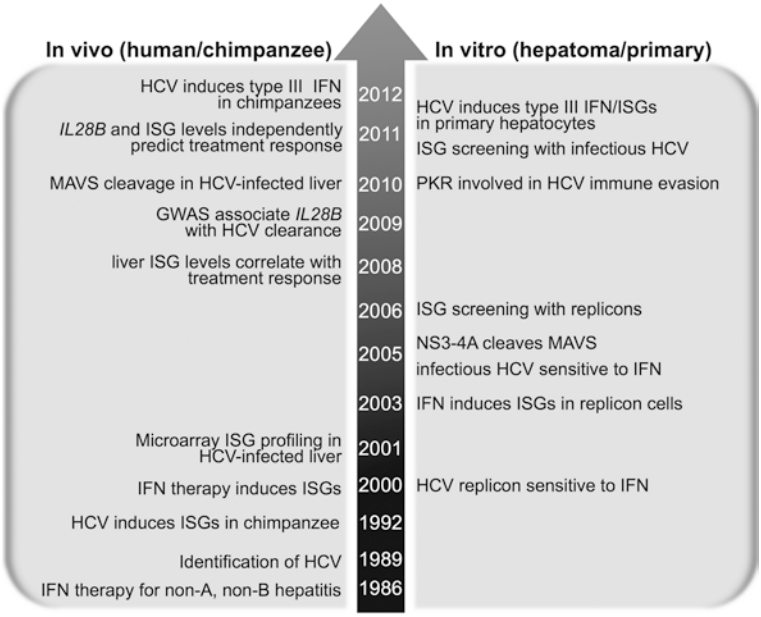

Fig. 2 Timeline of major discoveries in innate immunity to HCV

Nearly simultaneously with the discovery of RIG-I as a trigger for IFN production in response to the viral dsRNA mimic polyI:C (Yoneyama et al. 2004), RIG-I was also implicated as a factor controlling cell permissiveness to HCV replication. In a complementation screen, a cDNA encoding RIG-I restored antiviral signaling in Huh7.5 cells (Sumpter et al. 2005), an immortalized human hepatoma line that was derived from parental Huh7 cells based on its ability to support high levels of HCV replication (Blight et al. 2002). Genetic analysis correlated the Huh7.5 defect in antiviral signaling to a loss-of-function missense mutation in the RIG-I coding sequence (Sumpter et al. 2005). Additional genetic evidence for the role of RIG-I in HCV detection was obtained by showing that RIG-I-deficient mice are impaired in their ability to trigger IFN pathways in response to stimulation with HCV PAMPs (Saito et al. 2008). Similarly, knockdown of RIG-I in IFN-competent human hepatoma lines results in impaired IFNβ induction in response to HCV infection (Eksioglu et al. 2011).

Interestingly, some Huh7 derivatives are highly permissive to HCV replication but do not have defects in RIG-I pathways. When a panel of Huh7 lines with altered IRF3 responsiveness were infected, there were no major changes in HCV replication (Binder et al. 2007). Similarly, reconstitution of wild-type RIG-I signaling in the highly permissive Huh7.5 line had little effect on IRF3 activation and HCV replication. In contrast, constitutively active RIG-I stimulated IRF3 pathways and inhibited HCV replication. These data suggest that RIG-I is not the only factor controlling cell line permissiveness to HCV replication (Binder et al.

2007; Feigelstock et al. 2010). Recently, Huh7.5 cells were shown to have upregulated Hedgehog signaling that correlates with increased virus replication (Choi et al. 2011), but it is unclear whether crosstalk exists between Hedgehog and RIG-I pathways. A role for RIG-I in detecting HCV in primary human hepatocytes has also recently been proposed. In primary cell cultures derived from peritumoral liver tissue, knockdown of RIG-I resulted in a decrease in HCV-induced IFNβ production with a concomitant increase in HCV RNA levels (Yang et al. 2011). As an in vivo correlate, gene expression studies in liver biopsies from HCV-infected patients showed altered RIG-I mRNA levels when compared to control samples (Vilasco et al. 2006).

In contrast to RIG-I, little is known about a potential role for MDA5 in the host response to HCV. Other members within the same viral family, the *Flaviviridae*, including dengue virus, West Nile virus, and Japanese encephalitis virus, have been shown to activate host responses through combined RIG-I and MDA5 signaling (Loo and Gale 2011). Ectopic expression of MDA5 in Huh7 cells confers antiviral activity against cell culture-derived HCV (Schoggins et al. 2011). While the nature of this MDA5-mediated state has not been uncovered, it may be linked to direct activation of IRF3, similar to what has been reported with constitutively activated RIG-I (Binder et al. 2007). This hypothesis is substantiated further by the recent development of MDA5 transgenic mice, which express multiple copies of the murine *Ifih1* gene. These mice exhibit a chronic type I IFN signature and are resistant to lethal challenge with vesicular stomatitis virus (Crampton et al. 2012). Moreover, in human fetal liver cultures, which do not have the same immunodeficiencies as immortalized hepatoma lines, HCV replication was enhanced in cells expressing paramyxovirus V proteins (Andrus et al. 2011). The V proteins are thought to interfere with IFN pathways at the level of viral sensing through MDA5 and IFN signaling (Ramachandran and Horvath 2009). Thus the reproducible effect of V proteins on HCV replication across multiple donor tissues highlights a potential role for MDA5 in innate responses to HCV. The ability of MDA5 to sense infectious HCV has not been shown. Given that MDA5-deficient mouse embryonic fibroblasts are still competent to trigger the IFNβ promoter in response to stimulation with HCV RNA (Saito et al. 2008), it may be unlikely that MDA5 serves as an HCV sensor. However, since MDA5 is itself induced by interferon, it may be produced upon HCV infection and contribute to a second-wave antiviral response, similar to a mechanism proposed during West Nile virus infection (Fredericksen et al. 2008).

The role of TLR3 in innate immune responses to HCV has been studied more than MDA5, but not to the same extent as RIG-I. Ectopic expression of TLR3 in TLR3-deficient Huh7 cells renders the cells competent to establish antiviral signaling upon HCV infection (Eksioglu et al. 2011; Li et al. 2012; Wang et al. 2009). Conversely, knockdown of TLR3 in TLR3-replete LH86 hepatoma cells results in impaired IFNβ production upon HCV infection; however, the impact of TLR3 knockdown on HCV replication in these cells was not reported (Eksioglu et al. 2011). While TLR3 appears to be sufficient to trigger antiviral signaling in response to HCV infection, clear genetic evidence demonstrating a

RIG-I-independent requirement for TLR3 in HCV sensing is lacking. An alternative mechanism may involve crosstalk between TLR3 and RIG-I pathways. In a parainfluenza virus 5 infection model, overexpression of TLR3 led to increased RIG-I levels and enhanced cytokine production (Manuse and Parks 2010). A similar TLR3-mediated RIG-I induction may contribute to the results obtained in HCV studies. The contribution of TLR3 to HCV-mediated immune activation will likely need to be resolved by suppressing expression of TLR3 and RIG-I alone and in combination in a cell line that naturally expresses both proteins and is permissive to HCV infection. Alternatively, future progress in the development of mouse models of HCV infection may permit a more careful and robust analysis in mice lacking these and other innate immune activation pathways.

4 HCV Countermeasures to Block Innate Immune Activation

Like many viruses, HCV has evolved mechanisms to counteract the host immune response. Indeed, validation of RIG-I as a primary HCV sensor has relied primarily on in-depth analysis of RIG-I signaling inactivation via cleavage of MAVS by the HCV NS3-4A protease. NS3-4A has serine protease activity that contributes to HCV polyprotein processing and is required for replication (Bartenschlager and Lohmann 2000). The protease activity of NS3-4A also releases MAVS from membranes to which it is anchored, thereby preventing signaling mediated through RIG-I and IRF3 (Foy et al. 2003; Li et al. 2005b; Meylan et al. 2005). Genetic analysis has shown that the NS3-4A-mediated MAVS cleavage event requires hydrophobic acids in the N-terminal NS3 amphipathic helix $\alpha(0)$ (Horner et al. 2012).

While mitochondrial membranes are reported as the main sites for MAVS localization (Seth et al. 2005), recent evidence points to other cellular membranes bearing MAVS. These include peroxisomes and the mitochondrial-associated membranes between mitochondria and peroxisomes, the latter of which are targets of NS3-4A-mediated MAVS cleavage (Dixit et al. 2010; Horner et al. 2011). The formation of higher order MAVS structures, including oligomers and prion-like fibrils, has also been implicated as a requirement for antiviral signaling (Baril et al. 2009; Onoguchi et al. 2010; Tang and Wang 2009). In one study, overexpression of NS3-4A was capable of inhibiting MAVS self-association (Tang and Wang 2009). However, the role of NS3-4A-mediated disruption of MAVS oligomerization or aggregation in the context of HCV infection has not been addressed in detail.

Of clinical relevance, several protease inhibitors targeting NS3-4A have been shown to restore antiviral signaling in dsRNA-stimulated or HCV-infected cells by preventing MAVS cleavage (Johnson et al. 2007; Jouan et al. 2010; Liang et al. 2008). Thus, the benefit of protease inhibitors may be two-fold: direct suppression of the HCV life cycle by disruption of HCV polyprotein processing and enhancement of innate signaling by preventing MAVS cleavage. However, the concentration of NS3-4A inhibitor required to see effects on innate signaling surpasses the

therapeutic doses (Liang et al. 2008), raising speculation about the relevance of this proposed mechanism in HCV-infected patients undergoing treatment with direct-acting antivirals.

Direct in vivo evidence for NS3-4A-mediated MAVS cleavage has also been obtained by Western blotting of liver samples from patients chronically infected with HCV (Bellecave et al. 2010). Interestingly, cleavage of MAVS by HCV and other hepaciviruses has been implicated in shaping the evolution of MAVS. Unlike human MAVS, several primate species express MAVS that is resistant to NS3-4A-mediated cleavage. The resistant variant often relies on a single point mutation in amino acid 506, suggesting that convergent evolution at this site may have allowed multiple primate species to escape antagonism by paleo-hepaciviruses, which have yet to be identified (Patel et al. 2012).

Data on the ability of NS3-4A to cleave the TLR3 adaptor TRIF are currently conflicting. NS3-4A cleavage of TRIF was reported in both cell-free and cell culture systems (Li et al. 2005a). In these experiments, TRIF cleavage resulted in impaired signaling upon cellular stimulation with dsRNA. Additional studies were unable to corroborate these findings, but did confirm NS3-4A-mediated cleavage of MAVS (Dansako et al. 2007, 2009). Other HCV proteins, including core, E2, NS4B, and NS5A, have been implicated in immune evasion processes, the details of which have been the subject of recent reviews (Horner and Gale 2009; Thimme et al. 2012). Collectively, these HCV proteins have been suggested to interfere with IFN signaling or downstream IFN effector functions. HCV has also been suggested to induce autophagy as a mechanism to suppress IFN responses (Ke and Chen 2011). This study mostly relied on induction of IFNβ promoter reporter plasmids in HCV-infected cells lacking various autophagy-related genes. Confirmation of each of these findings in immune-competent cell culture models of HCV infection would be useful in determining whether these mechanisms are bona fide immune evasion strategies.

A recent study showed that infectious HCV was able to antagonize downstream IFN pathways by promoting the phosphorylation of the RNA-dependent protein kinase PKR (Garaigorta and Chisari 2009), a constitutively expressed and interferon-induced effector with broad antiviral activity. In HCV-infected Huh7 cells, phosphorylation of PKR results in phosphorylation and subsequent inactivation of the eukaryotic translation initiation factor eIF2α. The result is a decrease in translation of antiviral ISG mRNAs with concomitant increase in HCV resistance to the effects of IFN post-infection. From the perspective of the virus, this downstream IFN evasion mechanism may serve as a back up system to counteract leaky IFN induction due to inefficient upstream antagonism of MAVS cleavage by NS3-4A. Alternatively, HCV may allow a low level of IFN signaling breakthrough to limit rampant virus spread and progression to a fulminant state. Under these conditions, downstream ISG evasion through this PKR-dependent pathway would be sufficient for the virus to maintain a minimally replicative state without excessive cellular damage.

In a separate study using the HCV-permissive cell line Huh7.25.CD81, PKR was implicated as a sensor that detects HCV to induce IRF3-specific genes independently of RIG-I (Arnaud et al. 2011). Among these genes, ISG15 was shown

to negatively impact IFN induction by interfering with RIG-I ubiquitination. The authors suggest that HCV induces ISG15 as an early IFN evasion mechanism, which would support other claims that ISG15 promotes HCV replication (Broering et al. 2010; Chen et al. 2010).

5 Host Responses to HCV Infection

The cellular response to HCV infection is largely dominated by induction of antiviral IFNs, ISGs, and proinflammatory cytokines and chemokines. Much of our current understanding of these responses has come from in vivo studies in chimpanzees and humans. Only recently has progress been made using in vitro systems to corroborate and mechanistically dissect in vivo observations (Fig. 2).

One of the first indications that HCV induces a host immune response was the discovery that chimpanzees acutely infected with HCV had elevated mRNA levels of HLA-C and IFI6 (Kato et al. 1992), two of the first interferon-induced genes to be discovered. Since this observation 20 years ago, our understanding of host responses in HCV-infected humans and chimps has increased substantially with the introduction of genome-wide profiling technologies such as microarrays. Broad ISG induction has been observed in HCV-infected humans and chimps and in humans co-infected with HCV and HIV (Bigger et al. 2001; Chen et al. 2005; Lempicki et al. 2006; Smith et al. 2003; Su et al. 2002). Similarly, activation of innate pathways has been shown in the immunodeficient Alb-uPA human liver chimeric mouse model of HCV infection (Walters et al. 2006). Given the reproducibility of HCV-mediated ISG induction in several models, there is surprisingly little evidence to support a link to type I IFN gene expression. Highly sensitive RT-PCR assays failed to detect differences in intrahepatic type I IFN expression between patients chronically infected with HCV and those exhibiting other nonviral liver pathology (Abbate et al. 2003; Mihm et al. 2004). In chimpanzees, intrahepatic type I IFN gene expression can be detected in the acute phase, but it generally decreases after several weeks of infection (Shin et al. 2006). Interestingly, one study found small but statistically significant increases in type III IFN (IL28A/B) gene expression when comparing liver tissue from chronically infected HCV patients to tissue from uninfected controls with nonviral liver disease (Mihm et al. 2004). In chimpanzees, acute HCV infection results in a robust induction of intrahepatic IL28A/B mRNA (Thomas et al. 2012). Similar findings in humans are experimentally difficult to obtain, given that acute infections are often asymptomatic and liver biopsies are not clinically indicated. Nonetheless, in vivo gene expression data from HCV-infected humans and chimpanzees may point to a stronger correlation between ISG levels and type III IFNs rather than type I IFNs (Thomas et al. 2012).

Recently, host responses to HCV infection have been examined in primary human hepatocyte cultures and largely validate in vivo findings. HCV infection of primary hepatocytes from adult liver tissues was shown to induce high levels of

ISG expression, but only low levels of IFNβ were detected (Yang et al. 2011). In a separate study, primary human hepatocyte cultures infected with HCV exhibited marked induction of IL28A/B mRNA, but only modest IFNα/β mRNA (Thomas et al. 2012). Nearly identical findings were observed when primary human fetal liver cultures were infected with HCV (Marukian et al. 2011). These latter two studies also reported a global IFN signature comprised of widespread ISG induction following HCV infection. The correlation between innate responses to HCV in primary hepatocyte cultures and in vivo provide compelling data to suspect a prominent role for type III IFNs in the host response to HCV infection. Because primary human hepatocyte cultures and liver biopsies contain a subset of nonhepatic cells, profiling gene expression on the single cell level will be critical in deciphering the relative contribution of hepatocytes to the induction of type III IFNs and ISGs.

5.1 Host Responses to Therapeutic IFNα Treatment

Type I IFN, specifically IFNα, has been the principal component of HCV treatment for more than 20 years and its antiviral effects on HCV are well documented in vitro and in vivo. Replicating HCV genomes launched from RNA or DNA are known to be highly sensitive to IFN-mediated inhibition in cell culture (Blight et al. 2000; Chung et al. 2001; Frese et al. 2001; Guo et al. 2001). This inhibitory effect correlates with ISG induction (Lanford et al. 2003; Zhu et al. 2003), and specifically inhibition of HCV genome translation (Wang et al. 2003). Similar effects of type III IFNs have been described in replicon-bearing cell lines (Marcello et al. 2006; Zhu et al. 2005). Cell culture-derived infectious HCV is also inhibited by pretreatment of Huh7 cells with type I and III IFNs, but their kinetics of inhibition are distinct (Cai et al. 2005; Lindenbach et al. 2005; Marcello et al. 2006).

Early studies on therapeutic IFN responses in patients chronically infected with HCV confirmed ISG induction by showing on-treatment increases of MX1 mRNA in PBMC (Meier et al. 2000) and 2'-5' oligoadenylate synthetase activity in serum (Murashima et al. 2000). Particularly compelling are reports that have correlated ISG induction patterns with treatment outcome. Transcriptional profiling of IFN-treated PBMC from treatment-naïve patients chronically infected with HCV showed a global ISG induction that was significantly greater in future responders compared to nonresponders (He et al. 2006). Similar results were found using PBMC from HCV-infected patients undergoing IFN therapy (Taylor et al. 2007). Importantly, the correlation between treatment response and ISG levels has also been corroborated in a study using paired pre/post-treatment liver biopsies (Sarasin-Filipowicz et al. 2008). Prior to treatment, intrahepatic ISG mRNA levels are low in patients who will eventually respond to treatment, but increase dramatically after the first IFN injection. Conversely, baseline ISG mRNA levels are elevated in future nonresponders and fail to increase after therapy begins. Similar

to human nonresponders, chimpanzees chronically infected with HCV also have a poor response to exogenous IFN treatment (Lanford et al. 2007). These studies suggests that a low level of innate immune activation in chronically infected patients is counterproductive to successful treatment outcome. Mechanistically, this may be due to establishment of a cellular state that is refractory to additional IFN stimulation (Sarasin-Filipowicz et al. 2009) or induction of IFN-induced negative regulators that thwart treatment responses (Huang et al. 2007; Randall et al. 2006).

5.2 *Genetic Variation at IL28B: Impact on Host and Treatment Responses*

The impetus for the aforementioned studies on type III IFN induction in primary human hepatocytes (Marukian et al. 2011; Thomas et al. 2012) was largely born out a series of genetic studies published in 2009 that implicated the human *IL28B* gene in the immune response to HCV. Genome-wide association studies showed that genetic variation near the *IL28B* locus strongly correlated with both spontaneous and treatment-induced HCV clearance (Ge et al. 2009; Rauch et al. 2010; Suppiah et al. 2009; Tanaka et al. 2009; Thomas et al. 2009). The two main single nucleotide polymorphisms (SNPs) identified in these studies were rs8099917 and rs12979860, and both are upstream of the IL28B coding region. *IL28B* genetic variation turned out to be a stronger predictor of response than other clinical factors such as age, baseline viral load, fibrosis stage, or ethnicity (Ge et al. 2009; Suppiah et al. 2009). *IL28B* SNP correlations to treatment outcomes in liver transplantation settings have also yielded highly interesting findings. The collective result across several studies indicates that *IL28B* genetic variation is associated with post-transplant treatment success when favorable alleles are homozygous in either the donor liver, the recipient patient, or both (Charlton et al. 2011; Coto-Llerena et al. 2011; Eurich et al. 2011; Fukuhara et al. 2010; Motomura et al. 2011). These results indicate that the impact of the *IL28B* SNP to treatment-induced clearance is not necessarily liver specific and involves extra-hepatic contributions. While a mechanism to explain the impact of *IL28B* genetic variation on HCV clearance is still under active pursuit, several correlative findings are providing novel insight.

Gene expression studies show substantial variation with respect to the impact of *IL28B* polymorphisms on *IL28B* mRNA levels. Peripheral blood mononuclear cells (PBMC) from treatment-naïve HCV-infected patients homozygous for the favorable rs8099917 SNP had a modest but statistically significant increase in *IL28B* mRNA levels when compared to PBMC from patients heterozygous or homozygous for the unfavorable allele (Tanaka et al. 2009). Similarly, in a cohort of 49 healthy individuals, the nonresponder genotype at the rs8099917 SNP was associated with a modest but statistically significant decrease in *IL28B*

mRNA in whole blood when compared to responder genotype (Suppiah et al. 2009). *IL28B* mRNA expression in liver tissue is variable depending on the study. Homozygosity for the unfavorable genotype at the rs12979860 SNP correlated with lower intrahepatic *IL28B* mRNA levels (Dill et al. 2011). In contrast, other studies show that intrahepatic levels of *IL28B* mRNA were not statistically different with respect to *IL28B* alleles (Honda et al. 2010; Urban et al. 2010). However, in a liver transplantation study, homozygosity for the favorable rs8099917 allele in explanted livers was associated with higher expression of *IL28B* mRNA (Fukuhara et al. 2010). Summarily, these studies indicate a potential link between *IL28B* polymorphisms and *IL28B* expression levels, but the variability across data sets suggests more cohorts will need to be studied to solidify this conclusion. Moreover, the cohorts studied to date were all chronically infected. Studies in acutely infected patients may provide more definitive insight with respect to the impact of *IL28B* SNPs on *IL28B* mRNA levels, particularly since primary human hepatocyte cultures and liver samples from acutely infected chimpanzees exhibit markedly high *IL28B* gene induction post-infection (Marukian et al. 2011; Thomas et al. 2012). An additional factor to consider is IL28B (IFNλ3) protein potency. Gene expression levels that differ by less than two-fold may rarely reach statistical significance in human trials, but small changes may have dramatic effects in vivo due to the highly pleiotropic nature of IFN signaling.

Given the known role of type III IFNs in antiviral immunity, it is reasonable to predict that ISG levels in HCV-infected patients may correlate with *IL28B* genetic variation. Indeed, when intrahepatic ISG expression was stratified by the genotypes at both rs8099917 (91 patients) and rs12979860 (25 patients) SNPs, a correlation between high ISG levels and unfavorable IL28B SNP genotypes was observed (Honda et al. 2010; Urban et al. 2010). A cohort of 133 HCV-infected patients showed similar effects of the rs12979860 SNP on selected ISGs, but levels of other ISGs were not significant (Abe et al. 2011). One limitation to these studies was that the data sets were not stratified by treatment outcome. When 109 chronically infected patients were assessed for intrahepatic ISG levels as they relate to both *IL28B* genotype and treatment outcome, nonresponders had elevated ISG levels prior to treatment, regardless of whether they carried the favorable or unfavorable *IL28B* genotypes (Dill et al. 2011). Multivariate statistical analyses in this study indicated that ISG levels and *IL28B* genotype were independent predictors of response, a conclusion corroborated in two other studies (Asahina et al. 2012; Naggie et al. 2012). Moreover, a 4-ISG classifier consisting of *IFI27*, *ISG15*, *RSAD2*, and *HTATIP2* provided a more accurate prediction of response than *IL28B* genotype (Dill et al. 2011), as did MX1 liver immunostaining (McGilvray et al. 2012). Together, the results suggest that while there may be no direct link between *IL28B* genetic variation and ISG expression levels in chronically infected patients, obtaining both sets of clinical parameters could be informative for determining patient treatment options.

Aside from *IL28B* mRNA levels and overall ISG expression, the impact of a putative *IL28B* coding mutation has also been examined. The rs12979860 *IL28B* SNP was originally found to be in tight linkage disequilibrium with a SNP in the

coding sequence that confers a lysine to arginine substitution at amino acid 70 (Ge et al. 2009). Although altered cytokine function due to a missense mutation is an attractive hypothesis to explain *IL28B* SNP effects, in vitro studies did not find any functional differences between wild type and mutant IL28B (IFNλ3) proteins with respect to IFN signaling or ISG induction (Urban et al. 2010).

To date, the most significant clinical parameter affected by *IL28B* genetic variation is viral kinetics. Homozygosity for the favorable *IL28B* alleles confers a more rapid treatment-induced decline in first phase viral kinetics that continues into the second phase (Lindh et al. 2011; Naggie et al. 2012; Scott et al. 2011; Thompson et al. 2010). How this reproducible effect of *IL28B* polymorphisms on viral kinetics relates to the host immune response is not clear. Impaired NK cell immunity has been implicated (Naggie et al. 2012), but additional studies are needed to solidify our understanding of the mechanisms by which *IL28B* genetic variation impacts HCV clearance.

While this chapter was in press, a genetic study was published showing that SNPs at *IL28B* were associated with a new gene interferon, *IFNL4*, that encodes *IFNλ4* protein (Prokunina-Olsson et al 2013) . A dinucleotide variant, denoted ss469415590, at this region was strongly associated with HCV clearance, similar to SNPs at *IL28B*. Interestingly, the allelic variant that generates *IFNL4* protein correlated with unfavorable *IL28B* SNPs, while the variant that introduced a premature stop codon in *IFNL4* was more closely correlated with favorable *IL28B* SNPs. In functional assays, *IFNL4* had antiviral activity against HCV replicons and was capable of stimulating ISG expression. These new findings suggest that HCV clearance may be negatively impacted by the presence of *IFNL4*. Additional studies will be needed to understand the cellular mechanisms underlying these observations.

5.3 Interferon-Stimulated Genes Targeting HCV

The mechanisms of IFN action during therapeutic HCV treatment are not fully understood, but antiviral ISGs are considered the primary effectors of the response. In recent years, progress has been made toward identifying and characterizing which among the many hundreds of ISGs contribute to suppression of HCV infection. In two small-scale screens, 18 or 29 ISGs were tested by overexpression for activity against genotype 1b HCV subgenomic replicons. Several ISGs, including, *GBP1*, *IFI6*, *IFI27*, *IRF1*, *IRF9*, *ISG20*, *MX1*, *OASL*, *PKR*, and *RSAD2* (also known as viperin), significantly reduced replicon activity(Itsui et al. 2006; Jiang et al. 2008). The antiviral effects of viperin and *IRF1* were independently confirmed in the replicon system (Helbig et al. 2005; Kanazawa et al. 2004), and viperin was implicated in blocking infectious HCV replication through putative interactions with viral NS5A protein (Helbig et al. 2011; Wang et al. 2012). Characterization of *GBP1* showed that its overexpression in cells already infected with HCV results in decreased intracellular HCV copy number and reduced release of HCV core into cell supernatants. GBP1 interacted with HCV

NS5B when both proteins were overexpressed in HEK293 or used as bait and target in a yeast two-hybrid assay (Itsui et al. 2009). Similar to *GBP1*, overexpression of *IFIT1* or *IFITM1* in cells already infected with HCV resulted in decreased HCV replication; however, expression of these genes prior to infection had no effect on viral replication (Raychoudhuri et al. 2011).

In a larger screening effort, over 380 ISGs were tested for their ability to inhibit infectious genotype 2a HCV and other RNA viruses when expressed prior to infection (Schoggins et al. 2011). Statistically significant reductions in virus replication were demonstrated for at least 25 genes, many of which are uncharacterized. With respect to HCV, several genes showed a range of inhibitory effects. Modest effectors included *DDIT4*, *NT5C3*, *IFI44L*, *MAP3K14*, and *OASL*, while the more potent effectors were *RIG-I* (also known as *DDX58*), *MDA5* (also known as *IFIH1*), *IRF1*, *IRF2*, *IRF7*, all of which are components of antiviral signaling. Very few genes that were previously identified to target subgenomic replicons had significant inhibitory effects when screened against infectious HCV (Itsui et al. 2006; Jiang et al. 2008; Schoggins et al. 2011). This discrepancy may be due to differences in HCV genotype or other experimental parameters. More likely, however, the lack of correlation suggests that replicons are generally more sensitive to ISGs than infectious virus, a hypothesis supported by the observation that replicon-bearing cell lines are more responsive to IFN than HCV-infected cells (Garaigorta and Chisari 2009). Interestingly, the subset of anti-HCV effectors identified in this large screening effort did phenocopy when tested against genotype 2a replicons, and their mechanisms of action largely converged on inhibition of HCV primary translation (Schoggins et al. 2011). The potential of this ISG subset to inhibit HCV in primary human fetal liver cultures has also been confirmed (Schoggins and Rice, unpublished data), suggesting that these ISGs may play a role in anti-HCV responses in vivo.

Since this large screening effort was based on overexpression, the results indicate that the identified ISGs are sufficient to inhibit HCV, but additional studies are needed to determine whether these effectors are necessary for cell intrinsic or IFN-induced control mechanisms. One strategy is to carry out a knockdown "rescue" screen in the context of IFN treatment to identify genes responsible for the antiviral effect. Recently, a genome-wide siRNA screen of this type was performed on IFN-treated HCV genotype 1b replicon-bearing cell lines (Zhao et al. 2012). Surprisingly, few ISGs had strong effects in this assay. Instead, the predominant group of knockdown hits were enriched in genes belonging to mRNA processing and translation initiation. In a smaller and more targeted knockdown screen, 60 ISGs were selected as potential IFN-induced effectors against HCV replicons (Metz et al. 2012). A rescue screen in which these 60 ISGs were knocked down prior to IFN treatment revealed 7 genes that contributed to IFN antiviral action against HCV, including *IFIT3*, *IFITM1*, *IFITM3*, *PLSCR1*, *TRIM14*, *RNASEL*, and *NOS2*. Interestingly, no individual gene knockdown could not entirely rescue the effects of IFN. This finding is consistent with the widely accepted hypothesis that the IFN system works in a combinatorial fashion, with multiple ISGs contributing to the antiviral response but no single ISG serving as a "magic bullet". Indeed,

when selected anti-HCV ISGs are overexpressed or knocked down in combination, their combined effects are typically greater than the effect of either gene alone (Metz et al. 2012; Schoggins et al. 2011). Summarily, these recent screening efforts are just beginning to scratch the surface with respect to our understanding of how the complex and highly pleiotropic IFN system exerts its antiviral effects against HCV. Screens in hepatoma cell lines have been useful for the first stages of anti-HCV ISG identification. However, additional studies in primary human hepatocytes, as well as correlation to data sets from HCV-infected patients will be critical in deciphering mechanisms of individual ISGs. Insight into ISG mechanisms of action may provide a foundation for the development of novel therapies that coincide with the goal of IFN-free treatment regimens.

6 Conclusion

While the field of HCV innate immunity is relatively young, significant advances have been made in recent years. Many of these advances have uncovered important paradigms that have impacted the larger virology and immunology communities. With the advent of cell culture models to study all aspects of the HCV life cycle, host immune responses and viral evasion mechanisms can also be addressed. Recent studies in primary hepatocyte culture systems are beginning to corroborate many of the findings observed in both human and chimpanzee models of infection. Moreover, powerful profiling technologies such as microarray, genome-wide association studies, and expressional or knockdown screens have provided a wealth of new data to confirm existing hypotheses and generate new ones regarding the intricate methods the host uses to control HCV infection and the countermeasures HCV employs to evade the host response. As advances in cellular models of HCV infection converge with clinical/translational data from in vivo studies, our understanding of the complexities of the innate immune responses targeting HCV will hopefully contribute to better treatment regimens, and ultimately, widespread curing of chronically infected individuals.

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Adaptive Immune Responses in Hepatitis C Virus Infection

Christoph Neumann-Haefelin and Robert Thimme

Abstract The adaptive immune response plays a central role in the outcome of hepatitis C virus (HCV) infection. Indeed, spontaneous viral clearance is associated with an early neutralizing antibody response as well as vigorous and sustained HCV-specific CD4+ and CD8+ T cell responses. In persistent HCV infection, however, all three components of the antiviral adaptive immune response fail due to different viral evasion strategies. In this chapter, we will describe the components of a successful immune response against HCV and summarize the mechanisms of immune failure. We will also highlight characteristics of protective CD8+ T cell responses which is the key factor to the design of an efficacious vaccine.

Contents

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1 Successful Adaptive Immune Response Against Hepatitis C Virus

The adaptive immune response against HCV includes all components of the adaptive immune system, specifically antibodies, CD4+ T cells, and CD8+ T cells (Fig. 1). All three components have been shown to be associated with viral clearance. Thus, it is most likely that a well-coordinated interaction of the different immune cells is essential for a successful immune response against HCV, however, little is known about the precise interaction of this cross-talk.

1.1 Neutralizing Antibodies

Immunocompetent patients develop HCV-specific antibodies at an early phase of infection, mostly prior to or around the peak of liver enzymes. Most of these antibodies, however, have no antiviral activity. Indeed, only a small subset of HCV-specific antibodies is able to prevent virus binding, entry or post-entry steps of the viral lifecycle (see also chapter "Hepatitis C Virus Entry" by Zeisel et al. this volume). These antibodies may prevent viral infection and spread and are therefore termed neutralizing antibodies.

Neutralizing antibodies target linear as well as conformational discontinuous epitopes mainly located within the envelope glycoproteins E1 and E2. A hotspot of neutralizing epitopes is located in and close to the hypervariable region 1 (HVR1)

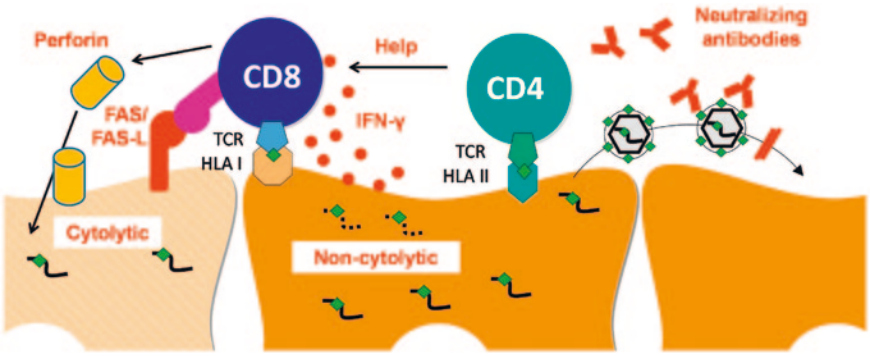

Fig. 1 Successful adaptive immune response against HCV. Neutralizing antibodies, HCV-specific CD4+, and CD8+ T cells are involved in the adaptive immune response against HCV. Neutralizing antibodies prevent HCV transmission by blocking viral binding, entry, or post-entry steps. CD4+ T cells recognize viral antigens that are presented by HLA class II alleles via their T cell receptor (TCR). They provide important help to CD8+ T cells. HCV-specific CD8+ T cells recognize viral antigens that are presented on HLA class I molecules. They act non-cytolytically, e.g., by secreting antiviral cytokines such as IFN-γ, as well as cytolytically, e.g., through perforin secretion and engaging the FAS/FAS-L pathway

of E2, and the high viral quasispecies variability in this region, which is a consequence of replication by the error-prone RNA-dependent RNA polymerase, has been attributed to viral evasion from the neutralizing antibody response (see below). The viral regions targeted by neutralizing epitopes have important functions in virus binding and entry, such as binding to the host cell receptors CD81 and SRB1, but may also be involved in important post-attachment steps (Sabo et al. 2011).

While strong data indicates the neutralizing activity of these antibodies in vitro, their efficiency in vivo is less understood. First evidence for a protective role of neutralizing antibodies came from the chimpanzee animal model of HCV infection, where transfer of neutralizing antibodies or vaccination with envelope proteins led to partial protection against a homologous viral challenge (Farci et al. 1994). A similar protective role of neutralizing antibodies could not be confirmed in HCV-infected patients for a long time, since many studies failed to demonstrate neutralizing antibodies in patients with acute-resolving infection, while neutralizing antibodies were detectable in patients after persistent infection had been established (Bartosch et al. 2003; Logvinoff et al. 2004; Netski et al. 2005; Kaplan et al. 2007). However, clear evidence for an important role of neutralizing antibodies in viral clearance came from a study that used homologous viral assays to study the neutralizing antibody response in patients that were infected with a known viral inoculum in a single-source outbreak of HCV infection (HCV genotype 1b strain AD78, transmitted through a contaminated anti-D immunoglobulin preparation) (Pestka et al. 2007). In this study, acute-resolving HCV infection was associated with an early development of neutralizing antibodies, while persistent infection was associated with a delayed induction of neutralizing antibodies (Pestka et al. 2007). It is important to note, however, that viral clearance can also occur in the absence of neutralizing antibodies, and even in agammaglobulinaemic patients (Adams et al. 1997), indicating that neutralizing antibodies do not necessarily have an essential role in HCV clearance.

1.2 CD4+ T Cells

HCV-specific CD4+ T cells that recognize viral antigens in the context of HLA class II molecules play an important role in viral control. This is supported by at least three different observations. First, viral clearance has been linked to strong, broadly directed, and sustained HCV-specific CD4+ T cell responses (Diepolder et al. 1995; Missale et al. 1996; Diepolder et al. 1997; Day et al. 2002; Schulze zur Wiesch et al. 2005). Second, certain HLA class II alleles have been associated with viral clearance. These HLA class II alleles include DRB1*1101 and DQB1*0301; both have been described to be protective in multiple studies in diverse populations (Schmidt et al. 2011). Studies in well-defined single-source infection cohorts identified additional protective HLA class II alleles such as DRB1*01, DRB1*04, and DRB1*15 (Schmidt et al. 2011). In contrast to CD8+ T cell epitopes, CD4+ T cell epitopes are highly promiscious, indicating that they can be recognized in the context of multiple HLA class II alleles. Thus, it is difficult to clarify the impact of defined CD4+ T cell

epitopes in the protective role of certain HLA class II alleles. It is important to point out, however, that the majority of HCV-specific CD4+ T cell responses identified in comprehensive analyses were restricted by HLA class II alleles that have been associated with viral control (Day et al. 2002; Schulze zur Wiesch et al. 2005). Third, CD4+ depletion studies in the chimpanzee model have revealed strong evidence for the important role of HCV-specific CD4+ T cells in viral control. Indeed, after the antibody-mediated depletion of CD4+ T cells, chimpanzees temporarily controlled viremia, indicating that this initial control was mediated by CD8+T cells. However, viral titers increased again and this was associated with the evolution of viral escape mutations in CD8+ T cell epitopes (Grakoui et al. 2003). These data support the concept that HCV-specific CD8+ T cells are the main antiviral effector cells, while HCV-specific CD4+ T cells have important helper functions and help to prevent viral escape from the CD8+ T cell response. This concept is also in agreement with the earlier observation that the loss of initially strong HCV-specific CD4+ T cell responses can be associated with viral recurrence even months after apparent viral control (Gerlach et al. 1999). Interestingly, a recent study convincingly demonstrated that broadly directed HCV-specific CD4+ T cell responses are primed irrespective of the outcome of infection. However, these responses rapidly disappear in patients with persistent infection (Schulze Zur Wiesch et al. 2012). These combined data suggest that the loss of CD4+ T cell help is an important mechanism in CD8+ T cell failure and an important determinant of viral persistence. Indeed, in patients with chronic HCV infection, virus-specific CD4+ T cell responses are rarely detectable (Day et al. 2003; Ulsenheimer et al. 2006; Lucas et al. 2007).

1.3 CD8+ T Cells

Virus-specific CD8+ T cells are key players in the antiviral immune response and have been shown to be the main adaptive effector cells involved in HCV clearance. Indeed, in patients with acute HCV infection, viral load remains at high titers during the first weeks of infection. After 6–8 weeks, multi-specific, virus-specific CD8+ T cells appear in the peripheral blood and this is temporally linked to the rise of liver enzymes, clinical symptoms, and a sharp decline in viral load (Gruener et al. 2000; Lechner et al. 2000; Thimme et al. 2001; Cox et al. 2005a, b). A very similar course has been observed in infected chimpanzees, where the emergence of intrahepatic HCV-specific CD8+ T cells 6–8 weeks after infection correlates with viral control (Cooper et al. 1999; Thimme et al. 2002). Importantly, in this model it has been recently shown that the so far unexplained delay in the HCV-specific CD8+ T cell response is indeed due to a delayed priming of HCV-specific CD8+ T cells, and not due to a delayed homing of the HCV-specific CD8+ T cells to the liver (Shin et al. 2011).

In addition to the temporal link between viral control and the HCV-specific CD8+ T cell response, there is also direct experimental evidence showing that CD8+ T cells are the main effector cells in HCV infection: after antibody-mediated

depletion of CD8+ T cells in chimpanzees, viral load remained at high levels for a prolonged time period. A decline and finally viral clearance was only observed after CD8+ T cells recovered and HCV-specific CD8+ T cells emerged in these animals (Shoukry et al. 2003).

Further supporting the important role of CD8+ T cells in viral clearance, certain HLA class I alleles such as B27 or B57 are associated with spontaneous resolution of acute HCV infection, and this protective effect could be linked to dominant CD8+ T cell epitopes restricted by these HLA class I alleles (Schmidt et al. 2011).

HCV-specific CD8+ T cells mediate their antiviral effects through two different effector mechanisms. First, they can act cytotoxically, e.g., they kill infected target cells (mainly hepatocytes) that present viral antigens by HLA class I molecules on their cell surface. Cytotoxicity can be mediated either by cell-bound receptors such as FAS and its ligand FAS-L or by paracrine secretory factors such as perforin. Second, HCV-specific CD8+ T cells contribute to viral control by non-cytolytic mechanisms, e.g., secretion of antiviral cytokines such as Interferon-gamma (IFN-γ) and Tumor Necrosis Factor-alpha (TNF-α). Importantly, experiments performed in an HLA-A2 expressing HCV replicon cell model demonstrated that HCV-specific CD8+ T cells perform their antiviral effects primarily through non-cytolytic mechanisms, especially IFN-γ secretion, while cytolytic effector functions were only observed at high effector-to target ratios (Jo et al. 2009). HCV replicon cells are also sensitive to cytolysis by perforin; however, most HCV-infected patients display virus-specific CD8+ T cells with low perforin secretion, indicating that this effector pathway may play a minor role in natural HCV infection (Jo et al. 2012).

Of note, a newly identified subset of HCV-specific CD8+ T cells is characterized by the production of IL-17 as well as a high expression of CD161 and the liver homing chemokine receptor CXCR6 (Northfield et al. 2008; Billerbeck et al. 2010). These cells may have a protective effect in chronic HCV infection, since they have been found in higher frequencies in patients with mild stages of liver inflammation or fibrosis. Interestingly, HCV-specific IL-17 producing CD8+ T cells do not overlap with HCV-specific IFN-γ producing CD8+ T cells in their epitope repertoire (Grafmueller et al. 2012).

2 Failure of the HCV-Specific Adaptive Immune Response

In patients with acute persistent infection, HCV-specific CD8+ T cell responses are typically weak, target only a small number of viral epitopes, and are often lost during establishment of chronic infection (Gruener et al. 2000; Lechner et al. 2000; Thimme et al. 2001; Cox et al. 2005a, b). Several mechanisms have been suggested to contribute to the failure of the HCV-specific adaptive immune response (Fig. 2). We will first summarize evasion strategies from neutralizing antibodies, and will then discuss some major mechanisms of T cell failure, including viral escape mutations, T cell dysfunction, and suppression by regulatory T cells.

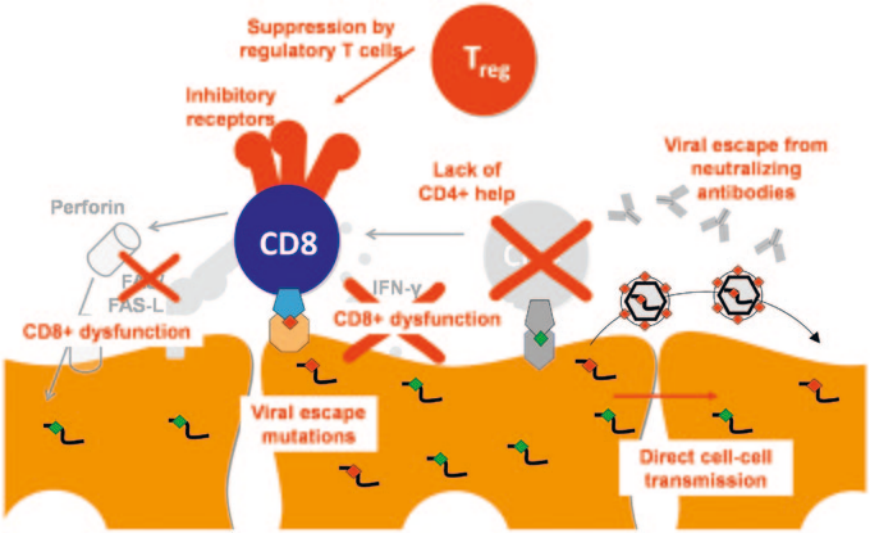

Fig. 2 Failure of the HCV-specific adaptive immune response. The mechanisms that contribute to the failure of the adaptive immune response are displayed in *red*. Evasion from neutralizing antibodies is mediated by viral escape mutations as well as direct cell–cell transmission, avoiding contact with neutralizing antibodies. CD8+ T cell dysfunction is characterized by impaired production of antiviral cytokines as well as impaired cytotoxicity. Expression of inhibitory receptors, suppression by regulatory T cells, as well as lack of CD4+ help may contribute to CD8+ T cell dysfunction. Viral escape mutations in CD8+ T cell epitopes further contribute to CD8+ T cell failure

2.1 Evasion from Neutralizing Antibodies

HCV is an RNA virus with a high replication rate and its RNA-dependent RNA polymerase NS5B lacks a proof-reading mechanism (see also chapters "Hepatitis C Virus RNA Replication" by Lohmann and "The Origin of Hepatitis C Virus" by Simmonds, this volume). As a consequence, multiple slightly different viral variants (quasispecies) circulate in a single patient that can escape adaptive immune responses.

HCV-specific neutralizing antibodies occur in the majority of patients with chronic HCV infection, however, multiple mechanisms contribute to viral evasion from the humoral immune response (Di Lorenzo et al. 2011). Indeed, evolution of viral quasispecies that display mutations within targeted epitopes and thus confer viral escape from neutralizing epitopes has been demonstrated by multiple studies (Farci et al. 1996; von Hahn et al. 2007; Dowd et al. 2009). Interestingly, some of these escape mutations act through an altered use of HCV entry factors (Fofana et al. 2012). In addition to viral escape, interactions of HCV glycoproteins with lipoproteins (especially HDL) and scavenger receptor B1 (SR-B1), as well as specific glycans on E2, protect HCV particles from neutralizing antibodies.

Another important evasion mechanism from neutralizing antibodies is direct cell–cell transmission that may strongly contribute to viral spread in the infected liver (Timpe et al. 2008; Brimacombe et al. 2011) (see also chapter "Hepatitis C Virus

Entry" by Zeisel et al. this volume). Indeed, the important roles of both, viral escape as well as cell–cell transmission, in evasion from the neutralizing antibody response has been supported in a recent study performed in patients who underwent liver transplantation (Fafi-Kremer et al. 2010). Indeed, re-infection of the liver graft (which is nearly universally observed after transplantation) included only few viral quasispecies that were present in the explanted liver. The quasispecies that established re-infection were indeed resistant to homologous neutralizing antibodies, indicating viral escape, while the viral quasispecies that were lost after transplantation were sensible to neutralization by homologous antibodies. This indicates that these viral quasispecies were transmitted by cell–cell transmission prior to transplantation, a transmission mode that is no longer applicable in the transplant setting (Fafi-Kremer et al. 2010).

It is also important to note that most neutralizing antibodies show little cross-neutralization of heterologous viral strains; thus, identification of neutralizing antibodies with broad cross-neutralizing activity is an important prerequisite for the use of neutralizing antibodies in prophylactic or therapeutic vaccination strategies.

2.2 Viral Escape from T Cell Responses

Some of the variants also escape the virus-specific CD8+ T cell response and are thus positively selected in the host (Bowen and Walker 2005). Indeed, three different patterns of viral escape can occur. First, mutations at the HLA class I binding anchors of CD8+ T cell epitopes, usually located at amino acid position 2 or 3 and the C-terminus, interfere with epitope binding to the HLA class I molecule, preventing antigen presentation. Second, mutations within the T cell receptor contact residues of the epitope, usually located in the central part of CD8+ T cell epitopes, impair recognition by the epitope-specific CD8+ T cells (Soderholm et al. 2006). Third, mutations in the flanking regions of an epitope can disrupt antigen processing by the proteasome and other peptidases, resulting in a lack of antigen presentation (Seifert et al. 2004; Timm et al. 2004; Kimura et al. 2005).

Viral escape has been described in chimpanzees and patients nearly two decades ago (Weiner et al. 1995; Chang et al. 1997). In chimpanzees, the emergence of viral escape mutations in CD8+ T cell epitopes correlated with viral persistence (Erickson et al. 2001). However, the fact that these escape mutations were first detectable after development of chronic infection in these chimpanzees, has led to the hypothesis that the occurrence of viral escape mutations is rather the consequence than the cause of viral persistence. More recent studies in humans observed viral escape mutations in patients with acute persistent infection, but not in patients with acute-resolving infections (Timm et al. 2004; Cox et al. 2005a, b; Tester et al. 2005). Selection of viral escape mutations occurred early in acute infection and remained fixed thereafter, indicating that viral escape may indeed be a causative mechanism of CD8+ T cell failure and viral persistence (Cox et al. 2005a, b).

Some immunodominant HCV-specific CD8+ T cell responses reproducibly lead to viral escape in patients with persistent infection who express the restricting HLA

class I allele, resulting in HLA class I associated viral sequence polymorphisms that are also referred to as HLA footprints in the viral genome (Timm et al. 2007, Rauch et al. 2009; Lange et al. 2010; Ruhl et al. 2011). Interestingly, most of these HLA footprints have been observed for HLA-B alleles, while less HLA footprints were identified for HLA-A alleles. It has been suggested that HLA-B alleles may be dominant in driving viral escape in human viral infections (Kiepiela et al. 2004). However, the mechanisms of this dominant effect remain elusive. Many viral escape mutations have a negative impact on viral replication capacity or other steps of the viral life cycle and thus come at a 'fitness cost' for the virus. In consequence, these viral escape mutations revert to wild-type after transmission of the virus to an individual that does not carry the restricting HLA class I allele and thus does not target the epitope region (Timm et al. 2004; Ray et al. 2005). In contrast, some viral escape mutations do not have a significant viral fitness cost, and are thus maintained even after transmission to patients that are negative for the restricting HLA class I allele. This leads to an accumulation of these variants in the population and finally the extinction of the respective epitope from the virus-specific epitope repertoire as observed for some HIV-specific CD8+ T cell epitopes (Neumann-Haefelin et al. 2008a; Kawashima et al. 2009).

Next to its relevance for the phylogenetic viral evolution, viral fitness costs of viral escape mutations may also have an important influence in the outcome of infection. Indeed, CD8+ T cell responses that require viral escape mutations associated with high viral fitness costs may lead to superior viral control and thus higher rates of viral clearance, similar to what has been observed in HIV infection (McMichael 2007; Goulder and Watkins 2008). Importantly, in this context, a recent study performing next-generation sequencing of longitudinal viral samples from patients with acute HCV infection identified two distinct bottlenecks in viral evolution. The first bottleneck occurred directly after transmission, with only one to two viral variants successfully establishing infection. The second viral bottleneck occurred approximately 100 days after infection and was characterized by a decline in viral diversity. It resulted in viral clearance in some individuals. However, in others, the emergence of a new viral population with a small number of mutations, most likely due to viral escape from CD8+ T cell epitopes contributed to viral persistence (Bull et al. 2011). These observations provide further evidence that successful evolution of viral escape mutations in this critical phase of infection may be indeed a prerequisite of chronic infection. A lack of CD4+ help may contribute to the evolution of viral escape mutations (Grakoui et al. 2003); in addition, limited T cell receptor diversity may also facilitate viral escape (Meyer-Olson, et al. 2004).

Importantly, viral escape mutations are present in only 50–70 % of targeted CD8+ T cell epitopes in chronic HCV infection (Cox et al. 2005a, b; Neumann-Haefelin et al. 2008a, b). Thus, viral escape is not universal and it is clearly not the only mechanism of CD8+ T cell failure in HCV infection. Indeed, it has been observed in patients with acute persistent HCV infection that viral escape does not occur in the context of CD8+ T cell responses that are functionally impaired (Urbani et al. 2005). Recent studies performed in patients with chronic HCV infection support the hypothesis that viral escape and T cell dysfunction are two complementary mechanisms of CD8+ T cell failure in persistent infection and that each of these

two mechanisms may account for CD8+ T cell failure in approximately 50 % of epitopes (Rutebemberwa et al. 2008; Bengsch et al. 2010; Kasprowicz et al. 2010).

In contrast to CD8+ T cells, viral escape is likely not a major determinant of HCV-specific CD4+ T cell failure (Fleming et al. 2010; Fuller et al. 2010). This is in agreement with the observation that HCV-specific CD4+ T cell responses are very weak and dysfunctional in chronic infection, and also in agreement with the concept that HCV-specific CD4+ T cells primarily have a helper function rather than strong direct antiviral activity.

2.3 T Cell Dysfunction

The dysfunction of HCV-specific T cells is a hallmark of chronic infection. Indeed, HCV-specific CD8+ T cells have been shown to be impaired in cytotoxicity, production of antiviral cytokines, and antigen-triggered proliferation (Wedemeyer et al. 2002; Spangenberg et al. 2005). These dysfunctional HCV-specific CD8+ T cells are characterized by a high expression of the cell surface receptor programmed cell death 1 (PD-1) (Golden-Mason et al. 2007; Penna et al. 2007; Radziewicz et al. 2007; Radziewicz et al. 2008). PD-1 was initially identified in the persistent LCMV mouse model as a marker for functionally exhausted CD8+ T cells (Barber et al. 2006), but has now been implied in CD8+ T cell exhaustion in several human viral infections, including HIV (Day et al. 2006), HBV (Isogawa et al. 2005), and HCV infection. PD-1 ligation by its two receptors PD-L1 and PD-L2 represses the cell survival factor Bcl-xL as well as Tbet, GATA-3, and Eomes, that act as transcription factors for effector cell genes (Keir et al. 2008).

Importantly, PD-1 expression correlates with HCV viral loads (Penna et al. 2007) and is even higher on intrahepatic HCV-specific CD8+ T cells compared to peripheral HCV-specific CD8+ T cells (Golden-Mason et al. 2007; Radziewicz et al. 2007; Nakamoto et al. 2008; Radziewicz et al. 2008). In consequence, intrahepatic HCV-specific CD8+ T cells are prone to apoptosis (Radziewicz et al. 2008).

Function of HCV-specific CD8+ T cells can be improved by the blockade of the PD-1 pathway (Golden-Mason et al. 2007; Penna et al. 2007; Radziewicz et al. 2007), however, a complete functional restoration cannot be achieved by PD-1 blockade alone. Indeed, next to PD-1, a panel of additional inhibitory receptors have been identified in the LCMV mouse model (Blackburn et al. 2009), and subsequent studies in the HCV field identified the inhibitory receptors CTL-A4 (Nakamoto et al. 2008; Nakamoto et al. 2009), Tim-3 (Golden-Mason et al. 2009; McMahan et al. 2010), 2B4 (Schlaphoff et al. 2011), CD160, and KLRG1 (Bengsch et al. 2010) to be upregulated on exhausted HCV-specific CD8+ T cells. Blockade of several of these inhibitory pathways resulted in a more effective functional restoration compared to blockade of PD-1 alone (Nakamoto et al. 2009; McMahan et al. 2010). These data provide a rationale for the development of new immunotherapeutic approaches in chronic HCV infection.

Interestingly, the coexpression of inhibitory receptors such as PD-1, 2B4, CD160, and KLRG1 is observed in about half of the HCV-specific CD8+ T cell responses in chronic infection. This exhaustive phenotype is associated with low and intermediate levels of CD127 expression and absence of sequence variations within the corresponding epitopes, indicating ongoing antigen triggering. In contrast, a low expression of inhibitory receptors by the remaining HCV-specific CD8+ T cells occurrs in concert with a high CD127 expression and presence of viral sequence variations within the corresponding epitopes (Bengsch et al. 2007; Golden-Mason et al. 2007; Radziewicz et al. 2007; Bengsch et al. 2010). In sum, these results suggest that T cell exhaustion contributes to the failure of about half of HCV-specific CD8+ T cell responses and that it is determined by a complex interplay of immunological (e.g. T cell differentiation) and virological (e.g. ongoing antigen triggering) factors.

The mechanisms that contribute to the expression of inhibitory receptors and T cell dysfunction are currently not well understood. Factors that have been implied in this process are continuous antigen triggering, a lack of CD4+ T cell help, as well as the action of regulatory T cells.

HCV-specific CD4+ T cells are detectable in very low frequencies in chronic infection. Since only few HLA class II tetramers are available for the analysis of HCV-specific CD4+ cells, and since HLA class II tetramers techniques are more demanding in comparison to HLA class I tetramers used for CD8+ T cell analysis, only few studies have addressed the HCV-specific CD4+ T cell phenotype and function so far. These studies indicate that HCV-specific CD4+ T cells present in chronic HCV infection may display an exhausted phenotype and may be characterized by a high expression of inhibitory receptors, similar to the observed phenotype of HCV-specific CD8+ T cells (Golden-Mason et al. 2009; Raziorrouh et al. 2011).

2.4 Regulatory T Cells

Different types of regulatory T cells (T_{regs}) are involved in HCV immunology, and T_{regs} have been implicated in both, inhibition of HCV-specific T cells in the acute phase of infection, contributing to T cell failure and chronic evolution of infection, as well as protection from immunopathology during chronic infection (Alatrakchi and Koziel 2009). One well-defined T_{reg} subset consists of CD4+ T cells that are positive for CD25 (that is also expressed by activated T cells) and the transcription factor Foxp3. In patients with chronic HCV infection, CD4+ CD25+ (Foxp3+) T_{regs} have a higher frequency compared to individuals with resolved infection or healthy controls (Sugimoto et al. 2003; Cabrera et al. 2004; Boettler et al. 2005). These T_{regs} suppress the proliferation and cytokine secretion of HCV-specific CD8+ effector T cells in a cell–cell contact-dependent manner in vitro. In some settings, the inhibitory action may also depend on cytokines such as IL10 or TGFβ (Rushbrook et al. 2005; Bolacchi et al. 2006).

There is evidence for an antigen-specific induction of CD4+ CD25+ T_{regs}. Indeed, CD4+ CD25+ Tregs could be induced by HCV-derived peptides; the number of inducing peptides was rather low but overlapped partially with previously described effector epitopes (Li et al. 2009). In accordance with this finding,

HCV-specific T_{regs} could also be stained using HLA class II peptide tetramers (Ebinuma et al. 2008). Induction of T_{regs} may be favored by increased levels of TGFβ, possibly produced by infected hepatocytes (Hall et al. 2010). Interestingly, there may be a differential role of different HCV proteins in the induction of T_{regs}. For example, immunization of mice with the core protein resulted in the induction of T_{regs}, while immunization with NS3 protein led to the induction of effector T cells (Krishnadas et al. 2010). This finding correlated with the selective generation of core-specific T_{reg} clones from chronically infected patients, but not from individuals with resolved HCV infection (Langhans et al. 2010). Of note, a recent report suggests that T_{regs} may be induced by certain viral epitope variants, mechanistically linking two different mechanisms of T cell failure, viral escape and enhanced T_{reg} induction (Cusick et al. 2011). In contrast to the antigen-specific induction of T_{regs}, the inhibitory action of T_{regs} may be less antigen-specific. Indeed, CD4+ CD25+ T_{regs} isolated from HCV-infected patients have been shown to suppress EBV- and CMV-specific CD8+ T cells to a similar level as compared to HCV-specific CD8+ T cells (Boettler et al. 2005; Rushbrook et al. 2005), although preferential suppression of HCV-specific CD8+ T cells was observed in another study (Ebinuma et al. 2008). *In vivo*, specific inhibition may be largely mediated by the strong attraction of T_{regs} to the liver of HCV-infected patients (Ward et al. 2007), a process that may be induced by HCV through an elevated expression of chemokines such as CCL17 and CCL22 (Riezu-Boj et al. 2010).

Several studies support the concept that CD4+ CD25+ T_{regs} limit immunopathology in the chronic phase of infection. For example, the frequency, production of inhibitory cytokines, and inhibitory potency of CD4+ CD25+ T_{regs} was shown to be enhanced in chronically infected patients with normal aminotransferase levels as compared to patients with elevated levels (Bolacchi et al. 2006; Itose et al. 2009). In addition, the frequency and function of CD4+ CD25+ T_{regs} has been demonstrated to correlate inversely with liver inflammation (Bolacchi et al. 2006) as well as fibrosis (Claassen et al. 2010; Sturm et al. 2010). Interestingly, CD4+ CD25+ Tregs were concentrated in necro-inflammatory areas in close contact to CD8+ T cells, supporting the cell-cell contact dependent mode of inhibition (Sturm et al. 2010). In the chimpanzee model, the frequency and function of CD4+ CD25+ T_{regs} was not only elevated in animals with chronic infection, but also in animals that had recovered from infection (Manigold et al. 2006). Although this finding differs from findings in humans, it may indicate that T_{regs} also control HCV-specific (memory) T cells and thus prevent immunopathology after resolution of infection.

The role of regulatory T cells during acute infection is less understood. Indeed, there are only few studies that performed an in-depth analysis of T_{regs} in acute infection. Strikingly, these studies did not observe a difference in T_{reg} frequencies between individuals that cleared infection or developed chronic infection (Smyk-Pearson et al. 2008; Heeg et al. 2009). One study observed that resolution of infection was associated with a relative loss of in vitro suppression by T_{regs} at a late time point (approx. 6 months after presentation) (Smyk-Pearson et al. 2008). It is unclear, however, if this change in T_{reg} function has an impact on infection outcome, or if it is rather an effect of differential antigenic stimulation in those individuals that clear the infection in comparison to those that experience chronic antigenic stimulation.

In addition to CD4+ CD25+ T_{regs}, CD8+ T_{regs} have also been identified in HCV infection (Billerbeck and Thimme 2008). HCV peptide-specific CD8+ T_{regs} that produce high amounts of IL10 and/or TGFβ and suppress HCV-specific CD8 effector T cells in vitro have been observed in chronically HCV-infected patients (Accapezzato et al. 2004; Alatrakchi et al. 2007). Similar to CD4+ CD25+ T_{regs}, IL10 producing CD8+ T_{regs} might also be involved in prevention of liver damage in chronic HCV infection (Abel et al. 2006). Interestingly, Foxp3+ CD8+ T_{regs} could easily be induced from the peripheral blood of chronically HCV-infected patients, indicating that further subsets of T_{regs} may play a role in HCV immunology (Billerbeck and Thimme 2007).

3 Protective CD8+ T Cell Responses

For vaccine design, it is essential to identify protective CD8+ T cell epitopes as attractive vaccine targets and to understand the mechanisms of protective CD8+ T cell responses in more detail. In the last few years, our knowledge about CD8+ T cell responses that are restricted by protective HLA class I alleles has substantially expanded, and we will therefore address this issue in more detail. Indeed, the HLA class I alleles most consistently associated with viral clearance are HLA-B27 and HLA-B57 (Schmidt et al. 2011). HLA-B27 has been demonstrated to have a strong protective effect in a cohort of Irish women who were infected with the same HCV genotype 1b inoculum through a contaminated anti-D immunoglobulin preparation (McKiernan et al. 2004). Protection by HLA-B27 was also confirmed in a large study analyzing the effect of HLA alleles on the need for HCV-related liver transplantation in the United States Organ Procurement and Transplantation Network database (Hraber et al. 2007). HLA-B57 has been identified to be protective in several US cohorts that were diverse regarding host ethnicity and viral genotype (Thio et al. 2002; Kuniholm et al. 2010; Kim et al. 2011). In addition, protective effects have been described for a number of additional HLA class I alleles, including HLA-A3 and -A11, although these associations have not been confirmed in other cohorts (Thio et al. 2002; McKiernan et al. 2004).

The identification of immunodominant CD8+ T cell epitopes that are restricted by these HLA class I alleles has allowed novel insights into the mechanisms of protective CD8+ T cell responses. For example, an immunodominant HLA-B27 restricted CD8+ T cell epitope located in NS5B is targeted in nearly all HLA-B27+ patients with an HCV genotype 1 infection (Neumann-Haefelin et al. 2006). The few HLA-B27+ patients who progress to chronic HCV genotype 1 infection, however, develop viral escape mutations within this epitope. Viral escape in this protective HLA-B27 restricted epitope cannot occur easily. Indeed, escape mutations at the main HLA-B27 binding anchors of the epitope are not tolerated by the virus since mutations at these positions would abrogate viral replication (viral fitness cost). In contrast, escape mutations at other positions of the epitope (e.g., positions that serve as T cell receptor recognition sites) can occur. However, due to a broad T cell cross-recognition of

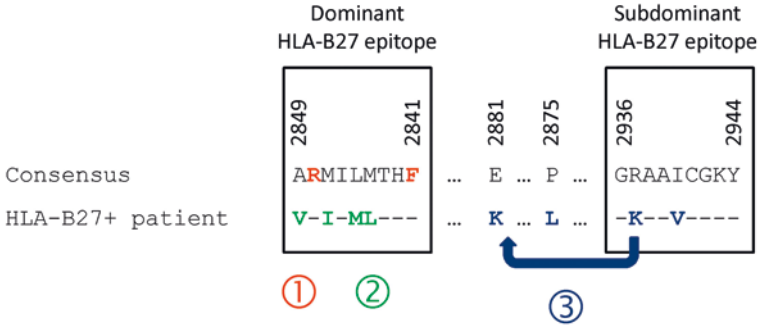

Fig. 3 Complex viral escape from HLA-B27 restricted CD8+ T cell response against the dominant NS5B 2841-2849 epitope and the subdominant NS5B 2936-2944 epitope. Viral escape in this HLA-B27+ patient requires the evolution of 8 mutations: In the dominant epitope, mutations at the HLA-B27 binding anchors (*red*) are not tolerated due to viral fitness costs (1) Mutations at the T cell receptor contact residues are possible, however, four mutations are required for complete escape due to broad cross-recognition of variants (2) In the subdominant epitope, certain mutations require the co-evolution of additional compensatory mutations at positions 2881 and 2875 approximately 60 amino acids upstream of the epitope

viral variants, several mutations within the epitope are required to fully escape from the epitope-specific CD8+ T cell responses (Dazert et al. 2009). Interestingly, viral escape from another, subdominant HLA-B27 epitope in NS5B also required the evolution of several mutations at least in some patients: A mutation of the HLA-B27 binding anchor of this epitope was only tolerated by the virus in combination with two compensatory mutations outside of the epitope (Neumann-Haefelin et al. 2011). In sum, our studies imply that HLA-B27 is associated with viral clearance since viral escape from the HLA-B27 restricted CD8+ T cell response requires a complicated viral evolution that cannot evolve rapidly, giving the CD8+ T cell response enough time to clear the virus before viral escape can occur (Fig. 3).

This concept is supported by recent next-generation sequencing studies that demonstrate that viral evolution passes through a 'bottleneck' due to CD8+ T cell pressure approximately 100 days after infection (Bull et al. 2011). In this scenario, the need for multiple HLA-B27-driven escape mutations may be the cork in the bottleneck of viral evolution and ultimately lead to viral clearance.

Importantly, this need of complicated viral evolution for viral escape is not unique to HLA-B27, but has now also been confirmed for other protective HLA class I alleles. For HLA-B57, two immunodominant CD8+ T cell epitopes have been described that are located in E2 and NS5B, respectively (Kim et al. 2011). Viral escape from the NS5B epitope requires a compensatory mutation three amino acids upstream of the epitope in many patients (Oniangue-Ndza et al. 2011). Similarly, two immunodominant HLA-A3 CD8+ T cell epitopes have been identified in NS3 and NS5B, respectively. Escape from the HLA-A3 epitope in NS3 involves two mutations at the C-terminal amino acids of the epitope. One of these mutations mediated viral escape, while the second mutation compensates for fitness

cost associated with the latter mutation (Fitzmaurice et al. 2011). In sum, these findings suggest that protective CD8+ T cell epitopes have two important characteristics: First, they are targeted by strong, immunodominant CD8+ T cell responses that exert a high selective pressure. Second, viral escape in these epitopes is a complex situation, further narrowing the bottleneck viral evolution needs to pass through in the most critical phase of infection and thus allows a high rate of viral clearance.

4 Implications for Vaccine Design

The more detailed characterization of the HCV-specific immune response and the better understanding of its failure in persistent infection that have evolved in the past few years have important implications for vaccine design. Indeed, from our current concept of HCV-specific adaptive immunity it may be necessary to include several components of the adaptive immune response in a successful vaccination strategy. For example, vaccine-induced CD8+ T cell responses may fail if sufficient CD4+ T cell help is missing. In addition, previous T cell vaccination trials usually targeted multiple, mostly HLA-A2 restricted CD8+ T cell epitopes. In contrast to this concept, however, recent findings rather support the induction of single immunodominant CD8+ T cell responses that target conserved viral regions and display broad cross-recognition of possible viral variants, thus preventing viral escape. These CD8+ T cell responses will likely be achieved more successfully on an HLA background other than HLA-A2. A promising approach may be the induction of HCV-specific CD4+ and CD8+ T cell responses through a recombinant adenoviral vector expressing the nonstructural HCV proteins. In healthy volunteers, this approach led to the induction of multi-specific, polyfunctional long-lived central, and effector memory cells (Barnes et al. 2012).

Regarding therapeutic immunostrategies, restoration of CD8+ T cell function may be a promising concept. Restoration of CD8+ T cell function will likely require more than just the blockade of one inhibitory pathway such as PD-1. More likely, several inhibitory pathways have to be targeted simultaneously. This procedure may restore both, CD8+ and CD4+ function and may thus allow viral clearance. Interestingly, a case of spontaneous resolution of chronic HCV infection was recently described to be associated with the emergence of neutralizing antibodies as well as restoration of T cell function (Raghuraman et al. 2012), indicating that immunomodulatory strategies may be indeed a realistic approach.

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Liver Injury and Disease Pathogenesis in Chronic Hepatitis C

Daisuke Yamane, David R. McGivern, Takahiro Masaki and Stanley M. Lemon

Abstract Chronic hepatitis C virus (HCV) infection is a leading cause of liver-specific morbidity and mortality in humans, including progressive liver fibrosis, cirrhosis, and hepatocellular carcinoma. It has also been associated with altered function in other organs, including those of the endocrine, hematopoietic, and nervous systems. Disease results from both direct regulation of cellular metabolism and signaling pathways by viral proteins as well as indirect consequences of the host response to HCV infection, including inflammatory responses stemming from immune recognition of the virus. Recent in vitro studies have begun to reveal molecular mechanisms responsible for virus-induced changes in cell metabolism and cellular kinase cascades that culminate in pathologic consequences in the liver, such as steatosis, insulin resistance, and carcinogenesis. Here we discuss how these findings may be relevant to disease pathogenesis in patients, and suggest future directions in the field.

Contents

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1 Chronic HCV Infection and Liver Disease

Persistent HCV infection is typically associated with chronic inflammatory changes within the liver. These reflect an unresolved wound healing response that includes abnormal production of extracellular matrix proteins and progressive fibrosis, the pathogenesis of which is only partially understood. As the disease advances, over a period of years to decades, the liver architecture is disrupted and liver function is inexorably diminished. Fibrotic changes are generally confined to the portal tracts early in the disease process, but with progression there is extension to the centrilobular area with "bridging" between adjacent portal tracts. In a significant proportion of infected patients, the process culminates in frank cirrhosis, in which the basic architecture of the liver is disrupted by fibrotic tissue interspersed with nodules of regenerating hepatocytes. Cirrhosis often develops silently, becoming symptomatic only at a late stage in its development, and it is inherently procarcinogenic. Individuals with hepatitis C-associated cirrhosis are thus at particularly high risk for developing hepatocellular carcinoma (HCC).

To a large extent, these pathologic consequences of HCV infection are likely to result from an active, but ultimately ineffective immune response to the virus that causes liver injury, but fails to eliminate the infection (see chapter "Adaptive Immune Responses in Hepatitis C Virus Infection" by Neumann-Haefelin and Thimme, this volume). This is consistent with the fact that very sensitive multiphoton microscopy methods detect viral antigen in only a small minority (~5–20 %) of hepatocytes (Liang et al. 2009). However, there is continuing controversy about the relative importance of such indirect, immune-mediated mechanisms versus more direct effects stemming from the expression of viral proteins in the development of fibrosis as well as liver cancer. Oxidative stress is an important aspect of HCV pathogenesis, as described in greater detail below, and may result directly from the expression of viral proteins as well as from inflammation related to immune recognition of the virus. Similarly, alterations in intrahepatic lipid metabolism, steatosis, and insulin resistance have been attributed to both virus-specific and nonspecific causes. A paucity of animal models of HCV-related disease continues to fuel this uncertainty, which is unlikely to be resolved until better animal models become available (the current status of animal models is described in the chapter "Cell Culture Systems for Hepatitis C Virus" by Steinmann and Pietschmann, this volume).

2 Fibrosis and Chronic HCV Infection

Chronic inflammation drives fibrogenesis, a process in which there is increased deposition of extracellular matrix proteins leading to fibrotic scarring and ultimately cirrhosis. The perisinusoidal hepatic stellate cell (HSC) plays a central role in this. Fundamental to wound healing within the liver, quiescent HSC that are resident within the liver undergo transformation to become proliferative, contractile myofibroblasts (MFs) [reviewed in Hernandez-Gea and Friedman (2011)]. MFs are

the dominant source of extracellular matrix within the liver, and while they may arise from other cell types, they are derived primarily from HSCs. The activation of HSCs and their transformation to MFs is intimately linked to the immune response to the virus, and both positively and negatively regulated via multiple growth factors, cytokines, and chemokines, including in particular transforming growth factor β (TGF-β) and platelet-derived growth factor (PDGF), tumor necrosis factor alpha (TNF-α), and monocyte chemotactic protein-1 (MCP-1 or CCL2), all of which are thought to act positively to enhance fibrogenesis (Hernandez-Gea and Friedman 2011). Interferon-γ, on the other hand, produced both by NK cells and virus-specific T cells, may negatively regulate fibrosis by inducing phosphorylation of STAT1. Oxidative stress is potent stimulus to HSC activation and is generated in large part by the inflammatory immune response (Brenner 2009). During chronic infection, intrahepatic α/β or γ/δ T, NK, and NKT lymphocytes sustain inflammation and may thus contribute to the continued progression of fibrosis.

However, the expression of specific viral proteins may play a role in HSC activation and initiation of fibrosis, acting directly on quiescent HSC to induce their transformation to MFs. CD81 expressed by HSCs may be bound by the E2 envelope glycoprotein, inducing matrix metalloproteinase 2 that degrades the normal extracellular matrix (Mazzocca et al. 2005). There is also indirect evidence that both structural and nonstructural proteins of HCV may stimulate HSC proliferation and/or increased production of chemokines and cellular adhesion molecules (Bataller et al. 2004). In vitro studies also suggest that the expression of TGF-β and other pro-fibrotic signals may be induced in hepatocytes by HCV infection (Schulze-Krebs et al. 2005). A key question that remains unanswered is whether hepatocytes are the only cell type infected by HCV in the liver, or whether HCV has the potential to infect HSCs or immune cells infiltrating the liver.

The transcription factor NF-κB plays a central role in regulating both the inflammatory response to HCV within the liver, as well as linking inflammation to fibrosis and carcinogenesis (Luedde and Schwabe 2011; Sun and Karin 2008). NF-κB may be activated when HCV infection is sensed by pathogen recognition receptors of the innate immune system, including retinoic acid-inducible gene I (RIG-I) and Toll-like receptor 3 (TLR3), both of which trigger signaling pathways that lead to activation of NF-κB as well as interferon regulatory factor 3 (IRF-3) (Saito et al. 2008; Wang et al. 2009c) (see chapter "Innate immune responses to Hepatitis C Virus" by Schoggins and Rice, this volume). Other studies implicate HCV-induced endoplasmic reticulum stress (Waris et al. 2002), or suggest that the expression of HCV-encoded proteins may directly activate NF-κB signaling (Dolganiuc et al. 2004; Sato et al. 2006; Waris et al. 2003). Similarly, both core and NS3 have been reported to stimulate IL-1 receptor-associated kinase (IRAK) activity in different types of cells, while phosphorylation of p38 and activation of extracellular regulated kinase (ERK) and c-jun N-terminal kinase (JNK) may result from TLR2-mediated sensing of infection (Dolganiuc et al. 2004). All of these responses are likely to promote a fibrogenic response in the continuing presence of infection. However, many of these studies need to be interpreted with caution as their conclusions are based on in vitro systems in which viral protein expression may be many fold higher than it is in the liver in chronic hepatitis C (Liang et al. 2009).

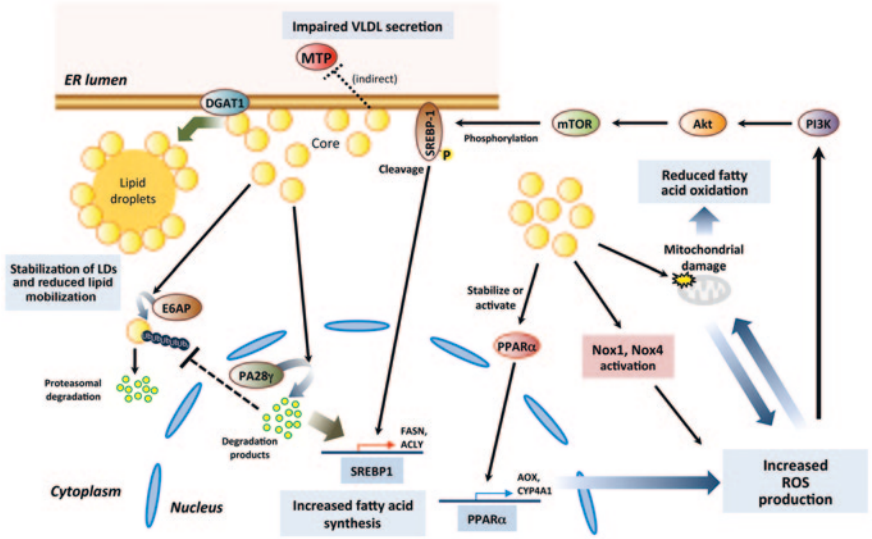

Fig. 1 Potential mechanisms underlying steatosis induced by the HCV core protein. Steatosis results from enhanced lipogenesis, increased stability of lipid droplets (LDs), reduced lipoprotein secretion, and altered mitochondrial function. Core protein localizes to lipid droplets in a DGAT1-dependent manner, slows turnover of LDs, and causes their accumulation in the cytoplasm. Increased transactivation of SREBP-1 by core protein occurs through PA28γ-dependent fashion in nucleus and ROS-activated phosphorylation-dependent activation of SREBP-1 through the PI3K-Akt-mTOR pathway. PA28γ also suppresses the ubiquitin (Ub)-dependent proteasomal degradation of core protein by E6AP, maintaining a high abundance of core protein in the cytoplasm. Oxidative stress induced by core occurs through (1) mitochondrial damage resulting from its interactions with the mitochondrial outer membrane, (2) activation of NADPH oxidase in TGF-β1-dependent or independent manner, or (3) persistent activation of PPARα, which enhances the expression of lipid oxidation enzymes. Reduced fatty acid oxidation resulting from mitochondrial injury also contributes to increased fatty acid accumulation

3 Steatosis in Chronic Hepatitis C

Hepatic steatosis (fatty liver) is frequently associated with HCV infection. This is characterized by the cytoplasmic accumulation of lipid droplets (LDs), mainly composed of triglyceride and cholesteryl ester, and results from multiple inter-related effects of the virus on cellular metabolism as depicted in Fig. 1. Steatosis is more frequently observed in patients with chronic hepatitis C than hepatitis B (Altlparmak et al. 2005; Machado et al. 2011; Persico et al. 2009), and a number of studies suggest that it correlates with both the progression of hepatic fibrosis and development of HCC (Adinolfi et al. 2001; Leandro et al. 2006; Ohata et al. 2003; Pekow et al. 2007). The composition of triglycerides in the liver is uniquely and significantly enriched in carbon monounsaturated (C18:1) fatty acids in chronic hepatitis C (Moriya et al. 2001b). This is distinct from what occurs in obese patients and

suggests that it may be induced via a virus-specific mechanism. A similar conclusion is suggested by a uniquely high prevalence of steatosis in patients infected with genotype 3 HCV, in whom the degree of steatosis correlates well with viral load and sustained antiviral responses to therapy frequently coincide with resolution of the fatty liver (Adinolfi et al. 2001; Poynard et al. 2003; Rubbia-Brandt et al. 2001). A direct viral cause of steatosis is also supported by cell culture studies showing increased LD synthesis following HCV infection (Barba et al. 1997; Hope and McLauchlan 2000), and studies in chimpanzees showing virus induction of lipogenesis (Su et al. 2002). Rates of sustained virological response (SVR) to interferon-based therapy are lower in patients with severe steatosis (Patton et al. 2004; Westin et al. 2007), making it particularly important to understand the underlying pathogenetic mechanisms.

Both in patients as well as chimpanzees with HCV infection, there is increased hepatic expression of sterol regulatory element-binding protein-1 (SREBP-1) and enhanced transcription of SREBP-1-related genes involved in lipogenesis (Fujino et al. 2010; Su et al. 2002). It is likely that HCV proteins directly alter lipid metabolism in infected cells, as the intrahepatic expression of full-length genotype 1b HCV polyprotein induces steatosis in transgenic mice (Alonzi et al. 2004; Lerat et al. 2002). In at least one of these transgenic lineages, FL-N/35 mice (Lerat et al. 2002), the level of HCV protein expression was comparable to that in human subjects, in whom HCV proteins are typically very difficult to detect by immunoblotting or histochemical approaches (Liang et al. 2009). The development of steatosis and an increased frequency of HCC in FL-N/35 mice, in the absence of an immune response or inflammation, provides compelling evidence that HCV proteins expressed in the liver may play key roles in disease pathogenesis. Not all strains of mice appear to be equally susceptible to these effects of HCV protein expression, however, implying a potentially important role for host genetics.

HCV proteins could promote steatosis by increasing lipogenesis and fatty acid uptake, or decreasing fatty acid oxidation and lipoprotein secretion (Fig. 1). FL-N/35 transgenic mice have lower plasma triglyceride levels than control mice due to impaired microsomal triglyceride transfer protein (MTP) activity, and they demonstrate enhanced cleavage of SREBP-1c mediated by site-1 protease leading to increased expression of SREBP-1 regulated genes, such as ATP citrate-lyase, fatty acid synthase, and hepatic stearoyl-CoA desaturase 1 (Lerat et al. 2009). HCV infection also activates SREBPs in cell culture by stimulating its transcription and phosphorylation in addition to proteolytic cleavage (Waris et al. 2007).

Among HCV proteins, the core protein stands out as potentially important in the pathogenesis of hepatic steatosis and HCC in transgenic mice. A number of studies have confirmed an association between core expression and steatosis in transgenic mice (Lerat et al. 2002; Moriishi et al. 2007; Moriya et al. 1998; Naas et al. 2005; Tanaka et al. 2008), while others have found no such phenotype in transgenic mice expressing HCV structural proteins including core (Honda et al. 1999; Kawamura et al. 2006; Kawamura et al. 1997). This may reflect the influence of different genetic backgrounds in these transgenic mice, the level of transgene expression, or possibly the choice of promoters used to drive expression of transgenes. Importantly, however, the ectopic expression of core by itself

in cultured cells can recapitulate steatogenic effects observed in transgenic mice models, including MTP inhibition (Perlemuter et al. 2002; Yamaguchi et al. 2005) and activation of SREBP-1 (Moriishi et al. 2007). These observations underscore the importance of core as a principal regulator of HCV-associated steatosis. Consistent with this, Waris et al. (2007) demonstrated greater stimulation of SREBP-1 and SREBP-2 activity with genotype 3 core protein than the genotype 1 protein (Fig. 1), which correlates with the strong association of genotype 3 with steatosis in patients mentioned above.

Moriishi et al. (2003, 2007, 2010) have proposed that cellular pathways involved in degradation of the core protein might be important with respect to lipogenesis (Fig. 1). Core is normally degraded through interactions with E6-associated protein (E6AP), a HECT domain E3 ubiquitin ligase, or PA28γ, a proteasome activator, in the cytoplasm and nucleus, respectively (Moriishi et al. 2003; Shirakura et al. 2007; Suzuki et al. 2009). Significantly, transgenic mice expressing the core protein, but defective for PA28γ expression, do not exhibit increased SREBP-1 transactivation and are protected from hepatic steatosis (Moriishi et al. 2007). Core protein is normally found primarily within the cytoplasm, but it is most abundant in the nuclei of hepatocytes in the PA28$\gamma^{-/-}$ knockout mice. Consistent with this, core is found in the nucleus in cell cultures treated with a proteasome inhibitor, MG-132. This suggests that core is normally transported to the nucleus where it undergoes rapid PA28γ-dependent degradation (Moriishi et al. 2003). The predominant nuclear localization of core in PA28$\gamma^{-/-}$ liver may reflect a requirement for PA28γ to activate the trypsin-like peptidase of the 20S proteasome (Li and Rechsteiner 2001). These results suggest that core may need to be processed in a PA28γ-dependent manner to stimulate lipogenesis. However, PA28γ appears to have a contrary, protective role in E6AP-mediated degradation of core, as knockdown of PA28γ lessens the abundance of core within the cytoplasm (Moriishi et al. 2010). Thus, it is possible that the absence of cytoplasmic core in the liver of PA28$\gamma^{-/-}$ mice may result from accelerated ubiquitin-mediated degradation of cytoplasmic core protein (Fig. 1).

Other work suggests that the steatogenic effect of core requires diacylglycerol acyltransferase 1 (DGAT1). Core is localized on the surface of cytoplasmic lipid droplets (Barba et al. 1997; Hope and McLauchlan 2000), which have proven to be important in the assembly of infectious virus (Masaki et al. 2008; Miyanari et al. 2007) (see chapter "Virion Assembly and Release" by Lindenbach, this volume). Core protein transport to the lipid droplet and production of infectious virus is dependent upon the activity of DGAT1, but not DGAT2 (Herker et al. 2010). Consistent with this, in murine embryo fibroblasts lacking DGAT1, core protein does not associate with lipid droplets (Harris et al. 2011). Interestingly, the translocation of core to lipid droplets results in an increase in their stability, as indicated by delayed turnover of triglyceride and cholesteryl ester in the presence of core (Harris et al. 2011). The stabilizing effect of core on lipid droplets might contribute to enhanced cellular abundance of lipids (Fig. 1), as core transgenic mice lacking DGAT1 do not develop steatosis. While DGAT1 mediates core-induced steatosis, the over-expression of DGAT2, but not DGAT1, independently induces hepatic steatosis in mice (Monetti et al. 2007). There is no known

cross-talk between PA28γ and DGAT1, but PA28γ-mediated inhibition of E6AP-dependent degradation of core could facilitate core interactions with DGAT1 in the cytoplasm, thereby allowing core to translocate to lipid droplets (Fig. 1).

However, the interpretation of these data demands caution, as these studies rely heavily on protein over-expression. The abundance of core may thus be higher in these studies than it is normally in vivo, and both its localization and function could be affected by the absence of other viral proteins, such as NS3 or NS5A, with which it normally interacts (Masaki et al. 2008; Miyanari et al. 2007; Mousseau et al. 2011; Shi et al. 2002). Also, the potential involvement of other viral proteins should not be disregarded, as NS2 and NS4B are also capable of inducing accumulation of lipid droplets in cell culture (Oem et al. 2008; Waris et al. 2007). Steatosis is likely to result from effects mediated by multiple HCV proteins.

4 Insulin Resistance and HCV Infection

Insulin resistance and type 2 diabetes mellitus are associated with chronic hepatitis C to a greater extent than other chronic liver diseases, including chronic hepatitis B (Hui et al. 2003; Mason et al. 1999). The severity of insulin resistance correlates with poor response to interferon-based therapies and an increased risk of fibrosis (D'Souza et al. 2005; Muzzi et al. 2005; Ratziu et al. 2003) as well as HCC (Veldt et al. 2008; Wang et al. 2009b). Insulin resistance also has been implicated causally in steatosis and fibrosis (Fartoux et al. 2005). Importantly, insulin resistance and type 2 diabetes mellitus appear to occur as a consequence of the infection, because they may resolve with successful antiviral therapy (Arase et al. 2009; Kawaguchi et al. 2007; Simo et al. 2006).

Potential points of interaction of the virus with insulin signaling pathways have been sought in clinical studies, transgenic mice, and cell culture (Fig. 2). These studies have focused mainly on the insulin receptor, insulin receptor substrate 1 (IRS-1), and downstream phosphoinositol-3 kinase (PI3K)-Akt signaling. In patients with chronic hepatitis C, insulin receptor and IRS-1 expression were increased several fold compared with control subjects, but the receptor-IRS-1 interaction, IRS-1 phosphorylation, downstream PI3K activity, and phosphorylated Akt levels were significantly decreased (Aytug et al. 2003). Core transgenic mice also exhibit insulin resistance with significantly increased basal serum insulin levels and a several-fold increase in islet area (Shintani et al. 2004). In these animals, a loss of IRS-1 phosphorylation was restored by administrating anti-TNFα antibody. TNFα initiates signaling cascades that lead to insulin resistance within the liver by activating JNK kinases (Xu et al. 2008) and S6K1 through IKKβ (Zhang et al. 2008), and induces serine phosphorylation of IRS-1 that interferes with insulin-induced tyrosine phosphorylation (Kanety et al. 1995) (Fig. 2). Importantly, core protein expression triggers TNFα promoter activity in cell culture in a PA28γ-dependent manner in association with impaired insulin signaling

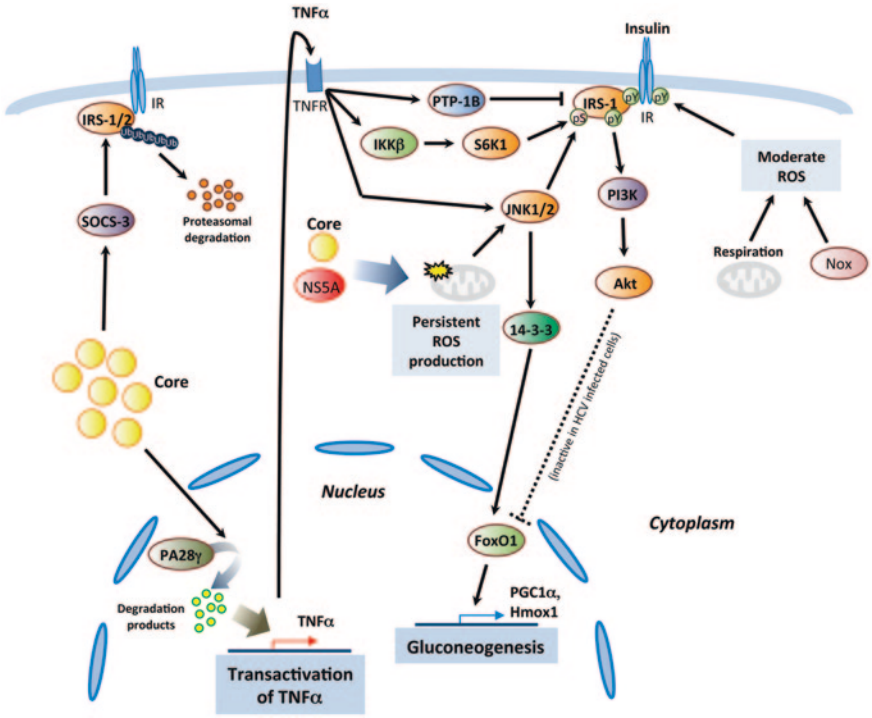

Fig. 2 Potential HCV interactions with insulin signaling. Insulin signaling normally occurs through the insulin receptor (IR)-IRS-PI3K-Akt pathway which leads to inhibition of FoxO1-mediated transcription by facilitating cytosolic translocation of the transcription factor from the nucleus following its phosphorylation at Ser319. Core protein may interfere with IRS-1/2 signaling by (1) enhancing SOCS-3 mediated proteasomal degradation, (2) upregulation of TNFα synthesis in PA28γ-dependent fashion, or (3) inducing persistent production of ROS due to mitochondrial injury, resulting in activation of JNK. JNK in turn phosphorylates 14-3-3, a cytoplasmic binding partner of FoxO1, facilitating its movement to the nucleus and subsequent activation of FoXO1-mediated transcription leading to gluconeogenesis. Thus, in vitro studies suggest that FoxO1 transcription is regulated primarily by an Akt-independent pathway in the context of HCV infection. TNFα negatively regulates tyrosine phosphorylation of IRS-1 by increasing PTP-1B expression and JNK1/2 and IKKβ-mediated S6K1 activity, which phosphorylates IRS-1 (pS) and thus interferes with tyrosine phosphorylation (pY)-mediated signal transduction. Tyrosine phosphorylation of IR is normally maintained by modest levels of endogenous ROS produced by mitochondria or NADPH oxidases (Nox), which oxidizes the IR beta-chain of IR and induces its autophosphorylation. Dysfunction of mitochondria associated with HCV infection may disable these normal regulatory functions

(Miyamoto et al. 2007). This finding may be of clinical relevance as TNFα levels are frequently elevated in patients with chronic hepatitis C (Polyak et al. 2001).

Oxidative stress (see Sect. 5) correlates strongly with the homeostasis model assessment-insulin resistance (HOMA-IR) score (Mitsuyoshi et al. 2008), and it is possible that insulin resistance might result from altered redox state regulation of the phosphorylation of kinases involved in insulin signaling (Bonnard et al. 2008; Yu et al. 2006). Numerous studies suggest that reactive oxygen species (ROS) generated

from mitochondria have an important influence on insulin signaling (Fig. 2). Insulin activates downstream signaling cascades by inducing tyrosine phosphorylation of its receptor, a process which requires modest elevation of ROS. H_2O_2 produced by mitochondrial respiration or NADPH oxidases are considered the major sources of endogenous ROS required for this (Storozhevykh et al. 2007). However, chronic exposure to high levels of ROS can result in insulin resistance (Anderson et al. 2009; Houstiset al. 2006), probably by inducing sustained autophosphorylation of the insulin receptor and activation of stress-response kinases, such as JNK, protein kinase C (PKC), and IKK, which can inactivate IRS-1 and -2 through serine phosphorylation (Lowell and Shulman 2005). Overproduction of ROS, leading to insulin resistance, can be triggered by mitochondrial damage due directly to expression of HCV proteins as well as mitochondrial hyperpolarization caused by chronic exposure to high glucose (Yu et al. 2006). Conversely, insulin resistance can trigger mitochondrial dysfunction by hyperactivating the transcription factor FoxO1, that in turn transactivates expression of Hmox1, thereby disrupting the respiratory chain and ATP production in mitochondria (Cheng et al. 2009). These studies suggest a very complex cause and effect relationship between insulin resistance and mitochondrial dysfunction centering on FoxO1.

FoxO1 regulates the expression of multiple genes involved in gluconeogenesis and lipid metabolism. It is regulated by IRS-1/2 signaling through Akt, which phosphorylates FoxO1, leading to its export from the nucleus (Dong et al. 2008). NS5A and, to a lesser extent, NS4A have been suggested to induce sustained activation of FoxO1-mediated transcription by inducing increases in its nuclear abundance (Deng et al. 2011) (Fig. 2). While Akt is activated in HCV-infected cells (and might be expected to suppress FoxO1 transcription), NS5A expression induces a state of oxidative stress that activates the stress-related kinase JNK. JNK in turn acts to phosphorylate 14-3-3, a cytoplasmic FoxO1 binding partner. This results in the dissociation of FoxO1 from 14-3-3, and the movement of FoxO1 to the nucleus where it can mediate transcription (Fig. 2). Thus, increases in gluconeogenesis that have been observed in cells over-expressing NS5A (or infected with HCV) can be blocked both by JNK inhibitors and ROS scavengers (Deng et al. 2011). Core protein, another potent ROS inducer, also activates JNK and induces sustained activation of FoxO1 transcription (Banerjee et al. 2010; Banerjee et al. 2008; Okuda et al. 2002). However, it is not clear whether either NS5A or core is expressed in sufficient abundance within hepatocytes in vivo to directly mediate such changes in FoxO1 transcription. Nonetheless, these observations explain how increased ROS production can be a primary cause of insulin resistance. HCV infection induces ROS production in cultured cells by causing mitochondrial injury (Deng et al. 2008), and, as discussed below, oxidative stress and increased ROS abundance are typically present within the chronically infected liver.

Degradation of IRS-1 leading to depletion of glucose transporter type 4 (GLUT4) expression may exacerbate increased gluconeogenesis (Bose et al. 2012). Other mechanisms may also be involved in HCV-associated insulin resistance, including possibly suppressor of cytokine signaling 3 (SOCS-3)-mediated ubiquitination and degradation of IRS-1 and -2 (Kawaguchi et al. 2004), or

dephosphorylation of Akt by protein phosphatase 2A (PP2A), which is overexpressed in the liver of HCV transgenic mice as well as HCV-infected patients (Bernsmeier et al. 2008) (Fig. 2). In addition, a recent study has demonstrated cross-talk between insulin signaling and interferon pathways. IRS-2 depletion increases tyrosine phosphatase PTP-1B activity, thereby negatively regulating JAK-STAT1 signaling due to loss of STAT1 phosphorylation (Garcia-Ruiz et al. 2012). This might be relevant to the increased rates of failure of interferon therapy in patients with insulin resistance, as IRS-1/2 abundance is frequently reduced.

5 HCV-Induced Oxidative Stress

Oxidative stress is more frequently associated with HCV than HBV infection (Fujita et al. 2008), and is accompanied by glutathione depletion (Barbaro et al. 1999b), increased oxidative DNA damage (Cardin et al. 2001; Fujita et al. 2007) and lipid peroxidation (Farinati et al. 1995), both in the liver and blood cells. Increased oxidative stress is thought to occur as a consequence of chronic inflammation, alteration of cellular oxidative enzymes, depletion of antioxidants and/or viral protein expression. Especially core and some nonstructural proteins are potent inducers of ROS in vitro (Garcia-Mediavilla et al. 2005; Gong et al. 2001; Korenaga et al. 2005; Li et al. 2002; Okuda et al. 2002; Thoren et al. 2004) and in transgenic mice (Machida et al. 2006; Moriya et al. 2001a; Tanaka et al. 2008; Wang et al. 2009a).

A fraction of the core protein localizes to the mitochondrial outer membrane, where it interacts with prohibition (Tsutsumi et al. 2009) and suppresses function of electron transport complex I, leading to increased Ca^{2+} influx and subsequent ROS production (Korenaga et al. 2005; Li et al. 2007). Breakdown of the mitochondrial outer membrane caused by core expression may be dependent upon activation of PPARα, followed by transactivation of genes involved in lipid oxidation, such as acyl-CoA oxidase and CYP4A1, as PPARα$^{-/-}$ core transgenic mice do not exhibit such abnormalities (Tanaka et al. 2008). Persistent activation of PPARα induced by core protein culminates in inhibition of the β-oxidation of lipids, and may contribute to steatosis in core transgenic mice.

In addition to mitochondrial ROS production, cellular oxidative enzymes, NADPH oxidases (Nox), are induced during HCV infection and may act as important sources of ROS, generating superoxides by catalyzing oxidation of NADPH (de Mochel et al. 2010). Nox4 contributes to TGF-β1-dependent ROS production in HCV-infected cell cultures (Boudreau et al. 2009). The transactivation of TGF-β1 by core protein may involve NFκB and/or the MAPK pathway (Lin et al. 2010; Taniguchi et al. 2004), although there are conflicting opinions as to whether TGF-β1 activation is a cause or effect of ROS production mediated by Nox4. This is potentially relevant to fibrosis, as TGF-β promotes the transformation of HSC to MF. Another study has shown that while Nox1 and Nox4 are markedly induced in cell culture as well as liver tissue from chronic hepatitis C patients, only Nox4 has the ability to translocate to the nucleus where it causes accumulation of nitrotyrosine, a marker for peroxynitrite production

(de Mochel et al. 2010). Immune cells involved in the inflammatory response to HCV infection also contribute to the development of oxidative stress.

A direct link between oxidative stress and steatosis has been suggested by Waris et al. (2007), who demonstrated that lipogenesis could be initiated by oxidative stress via the induction of PI3K-Akt signaling leading to activation of SREBP-1 and SREBP-2. Antioxidants, PI3K inhibitors, and calcium signaling inhibitors all suppressed HCV-induced transactivation and phosphorylation of SREBPs, consistent with a role for PI3K-Akt signaling and downstream involvement of mammalian target of rapamycin (mTORC1) (Laplante and Sabatini 2009; Wang and Sul 1998). This pathway is likely to contribute to insulin resistance associated with HCV infection as discussed above. Importantly, fatty liver is more prone to oxidation than normal tissue because of the enhanced accumulation of polyunsaturated fatty acids that are susceptible to peroxidation. Lipid peroxidation can rapidly spread via a chain reaction once initiated through interaction with ROS (Cheeseman and Slater 1993). Steatogenic effects initiated by oxidation may be amplified through such a mechanism. Lipid peroxidation is commonly detected in liver from patients with chronic hepatitis C (Barbaro et al. 1999a; Farinati et al. 1995), but significantly higher levels of protein adducts with lipid peroxidation products, malondialdehyde, 4-hydroxy-2-nonenal, and 4-hydroxy-2-hexanal, are found in patients with steatosis (Kitase et al. 2005). Multiple manifestations of injury associated with chronic hepatitis C might thus stem from persistent unresolved oxidative stress.

6 Hepatitis C-Associated Cancer

6.1 Hepatocellular Carcinoma

In the United States, HCC rates have risen faster than any other cancer over the past few decades, primarily as a result of chronic HCV infection (El-Serag and Rudolph 2007). While a lack of long-term studies makes it difficult to estimate the overall risk of HCC development in HCV-infected persons, in those patients who have progressed to cirrhosis, the annual risk for cancer development is 1–4 %. The mechanisms by which HCV promotes liver cancer are not well understood and, as with fibrosis and disturbances in lipid metabolism discussed above, the lack of a small animal model of chronic HCV infection has hampered progress in this direction.

The development of HCC in the setting of chronic HCV infection shares features in common with liver cancer arising due to other causes, such as alcohol, HBV infection, and nonalcoholic fatty liver disease (NAFLD). It is a multistep process, and as with other disease manifestations of HCV infection discussed above, the extent to which direct virus-specific mechanisms versus indirect effects of inflammation and accelerated hepatocellular turnover remain uncertain (Lemon and McGivern 2012). In patients with chronic hepatitis C, cancer typically (but not always) arises in the setting of advanced fibrosis and cirrhosis (Lok et al. 2011). As discussed above, the transcription factor NF-κB is a central regulator of the inflammatory responses

that lead to hepatic fibrogenesis and it is frequently activated in HCV-infected liver (Tai et al. 2000). NF-κB functions in multiple cell types within the liver, and there is abundant evidence that its activation promotes the activation, survival, and pro-fibrogenic inflammatory responses of HSCs and hepatic MFs (Luedde and Schwabe 2011).

HCCs are highly heterogeneous between patients and frequently exhibit aneuploidy, suggesting a role for mechanisms that promote genomic instability (Thorgeirsson and Grisham 2002). ROS present within the infected liver (see above) are likely to contribute to chromosomal damage, and thus may promote the development of cancer. Alcohol ingestion adds to the level of oxidative stress in the liver, potentially explaining synergy between alcohol and HCV infection in hepatic carcinogenesis. As discussed above, steatosis also correlates with an increased risk of cancer in the HCV-infected patient, consistent with an important role for oxidative stress. Thus, the development of HCC is very likely to represent a downstream result of the pathogenetic processes described in the preceding chapters that lead to abnormalities in lipid metabolism, steatosis, inflammation, and fibrosis.

Apoptosis of infected hepatocytes is likely to result from immune responses to the virus (Guidotti and Chisari 2006), and this could be an important factor in the development of cancer. The ability of the liver to regenerate following injury is unusual among adult organs, and HCC of all causes typically arises in an environment of chronic disease in which there are repeated cycles of hepatocyte death and regeneration. Recent studies suggest that chronic hepatocellular injury and compensatory proliferation may itself drive liver carcinogenesis. Knockdown of the pro-apoptotic p53-upregulated modulator of apoptosis (PUMA) reduces the incidence of HCC in mice challenged with the carcinogen diethylnitrosamine (Qiu et al. 2011). This finding might seem paradoxical at first glance, as apoptosis is often considered to be anticarcinogenic and cancer cells are often resistant to apoptosis. However, in the context of chronic liver disease and the early stages of tumorigenesis, apoptotic cell death and stimulation of regenerative cell growth may allow amplification of previously quiescent cells in which chromosomal damage has occurred. NF-κB activation may contribute to the proliferation and survival of such cells (Luedde and Schwabe 2011). Continuous cycling of these events in an environment of oxidative stress over a period of many years may permit accumulation of mutations in some cells, contributing eventually to their escape from normal growth controls and leading to a transformed phenotype. In agreement with this model, cancer has been observed in mice with constitutive genetic stimulation of hepatocyte turnover (Yamaji et al. 2010). In this model, HCV infection (or any other cause of chronic liver injury) is only indirectly associated with carcinogenesis, since the HCC would originate from an uninfected hepatocyte or hepatocyte progenitor.

However, there is evidence for more direct involvement of the virus in hepatocellular carcinogenesis. HCC occurs at high frequency in several HCV transgenic mouse lineages in which the HCV proteins are expressed specifically in the liver, either individually or together as part of the HCV polyprotein. The mouse genetic background seems to be important for the cancer phenotype, and C57BL/6 mice are particularly prone to development of HCC following expression of HCV proteins (Klopstock et al. 2009). This is interesting with respect to the relationship

between steatosis and HCC discussed above, as nontransgenic C57BL/6 mice are particularly prone to develop steatosis as they age (Lerat et al. 2002). Transgenic mouse studies implicate both structural and nonstructural proteins of HCV in carcinogenesis. Mice expressing a high abundance of a genotype 1b core protein developed hepatic adenomas at 12 months of age, and poorly differentiated HCC after 16 months (Moriya et al. 1998). This phenotype was not observed in nontransgenic littermates and was more frequent in males (26–31 %) than females (0–14 %). Steatosis was observed from the age of 3 months in the transgenic mice. In addition, the FL-N/35 mice discussed above, in which there is constitutive, low level, liver-specific expression of the entire polyprotein of a genotype 1b HCV also demonstrate age- and sex-dependent steatosis and development of hepatocellular adenoma and carcinoma (Lerat et al. 2002). Cancer was observed at higher rates in FL-N/35 mice than in other transgenic lineages that expressed only the structural proteins, suggesting a contribution of the nonstructural proteins to carcinogenesis. Consistent with this, a specific role for NS5A is suggested by an increased frequency of HCC in NS5A transgenic mice (Wang et al. 2009a). Importantly, HCV transgenic mice are more susceptible than their nontransgenic littermates to cancer development following a variety of secondary insults, including iron overload (Furutani et al. 2006), diethylnitrosamine exposure (Kamegaya et al. 2005), infection with Helicobacter hepaticus (Fox et al. 2010), aflatoxin (Jeannot et al. 2012), or alcohol (Machida et al. 2009).

A common feature of these transgenic models is that cancer develops in the absence of inflammation or immune recognition of the transgene. This speaks to the importance of viral proteins in HCV-related carcinogenesis, and could result from the presence of oxidative stress in the transgenic liver. A related possibility is that HCV protein expression may render the hepatocyte more susceptible to chromosomal damage and genomic instability through modulation of host cell cycle checkpoints. Such a hypothesis is supported by in vitro studies of HCV, which have revealed multiple interactions of HCV proteins with host proteins that act as tumor suppressors or otherwise control cellular proliferation (McGivern and Lemon 2009). These interactions may specifically impair the ability of infected cells to respond to DNA damage caused by the oxidative stress induced by HCV replication (Korenaga et al. 2005; Okuda et al. 2002), thereby allowing a genetically damaged cell to continue to proliferate.

Mutation or loss of p53, a master regulator of the cell that plays a critical role in the response to chromosomal DNA damage, is a common feature of HCC (Hussain et al. 2007). Various studies suggest that the HCV core protein (Kao et al. 2004; Kwun et al. 2001), NS3 (Ishido and Hotta 1998; Tanaka et al. 2006), and NS5A protein (Majumder et al. 2001; Qadri et al. 2002) interact with p53 in vitro, and may modulate p53 function (Kwun et al. 2001). This could leave the cell more susceptible to DNA damage as a result of oxidative stress or environmental insults. However, although intriguing, the results of these studies should be interpreted with caution since they have generally used protein overexpression systems or cell lines that may not be physiologically relevant.

Another cellular protein commonly mutated in HCC is the retinoblastoma tumor suppressor protein (Rb). Rb abundance is negatively regulated in cultured human

hepatoma cells supporting replication of subgenomic and full-length genotype 1b HCV replicons (Munakata et al. 2005), and also in cells infected with laboratory strains of HCV (McGivern et al. 2009; Munakata et al. 2007). The HCV polymerase, NS5B, associates with Rb through a motif with homology to the LXCXE motif found in cellular and viral Rb-binding proteins (Munakata et al. 2005). Further mechanistic studies demonstrated that the association of Rb with NS5B results in the recruitment of the ubiquitin ligase E6AP, targeting Rb for ubiquitin-dependent proteasomal degradation (Munakata et al. 2007). This targeting of Rb for degradation may be important for HCC development, since loss of Rb is a key event for promoting tumorigenesis (Mayhew et al. 2007; McClendon et al. 2011). Rb is important in regulation of the cell cycle, and required for certain checkpoints, thus its loss as a result of NS5B expression may render the infected hepatocyte more vulnerable to DNA damage. Such a model of HCV-related carcinogenesis does not require continued expression of NS5B for maintenance of the malignant phenotype (McGivern and Lemon 2009).

Other studies suggest that HCV may interfere with cellular pathways controlling differentiation. In the case of the Wingless/Int (Wnt) signaling pathway, NS5A protein causes stabilization of β-catenin either by direct interaction (Milward et al. 2010; Park et al. 2009) or by interaction with the p85 subunit of PI3K to modulate downstream signaling pathways, ultimately leading to β-catenin stabilization (Park et al. 2009; Street et al. 2005). In addition, the production of Hedgehog (Hh) ligands is increased in patients with chronic hepatitis C as well as in cultured hepatoma cells infected with HCV. Aberrant production of Hh ligands may impact Hh-responsive cell populations in the liver that expand during fibrosis and cirrhosis, including MFs and epithelial progenitors (Pereira Tde et al. 2010).

Finally, there is recent evidence that HCV infection can directly promote apoptosis. Infection of Huh-7 hepatoma cells with laboratory strains of HCV such as JFH1 or its derivatives results in a fraction of the cells undergoing apoptosis (Deng et al. 2008; Kannan et al. 2011; Walters et al. 2009), while HCV infection sensitizes Huh-7 cells to TRAIL-dependent apoptosis (Lan et al. 2008). HCV infection of chimeric SCID/Alb-uPA mice bearing human liver tissue results in disturbed lipid metabolism, endoplasmic reticulum and oxidative stress, and increased hepatocellular apoptosis (Joyce et al. 2009), all of which could contribute to the development of cancer.

Whether different genotypes or subtypes of HCV vary in their ability to cause cancer is a difficult question to answer since studies must take into account a number of potentially confounding factors such as duration of infection, age, and sex of the patient. More cancers tend to be associated with genotype 1 strains of HCV but this association may be related to the worldwide prevalence of this genotype. Some studies have found no association between HCV genotype and cancer risk (Ryu et al. 2009; Seto et al. 2010), while others suggest that infection with genotype 1b may confer a greater risk of HCC (Bruno et al. 2007; Raimondi et al. 2009). Specific aminoacid polymorphisms in core in genotype 1b HCV (especially Gln70 and Leu91) have been associated with a higher risk for cancer (Akuta

et al. 2007; Hu et al. 2009; Kobayashi et al. 2010; Nakamoto et al. 2010). The mechanism(s) driving the differences in HCC risk are not clear, but an interesting possibility is that these polymorphisms act to regulate expression of an N-terminally truncated core protein referred to as p8 minicore (Eng et al. 2009). The biological properties of this protein are not well characterized, and it could function to promote carcinogenesis (Ahmad et al. 2011).

Not all patients who develop cirrhosis will go on to develop cancer, and it is very likely that this is controlled at least in part by host genetic variation. Genome-wide association studies have identified polymorphisms that associate with enhanced susceptibility to HCC in patients with chronic HCV (Kumar et al. 2011; Miki et al. 2011), but the underlying mechanisms remain to be elucidated. The identification of host genetic factors may permit more effective surveillance of chronic HCV patients at risk for HCC development. This would be an important advance, since early diagnosis and intervention have a profound impact on patient survival.

6.2 Lymphoproliferative Disorders

Findings from a variety of epidemiologic studies suggest that patients who are seropositive for anti-HCV are at significantly increased risk of developing non-Hodgkin lymphoma (NHL), particularly B cell NHL, compared to controls (Dal Maso and Franceschi 2006; Giordano et al. 2007; Gisbert et al. 2003). This risk is higher in HCV-infected patients with mixed cryoglobulinemia (MC) (see below) and vasculitis (Monti et al. 2005; Saadoun et al. 2005). Recent studies suggest that effective antiviral therapy might be beneficial in the management of HCV-positive low-grade NHL (Gisbert et al. 2005; Hermine et al. 2002), which would be consistent with a direct causative role for the virus, but the pathogenesis of HCV-associated lymphoma remains unclear. One possibility is that persistent antigenic stimulation associated with an infection that persists for decades may lead to antigen-dependent, benign clonal B cell lymphoproliferation, and eventually malignant transformation (Gisbert et al. 2005; Sansonno et al. 1998). The B lymphocyte stimulator (BLyS) receptor-ligand system may be involved in this process. BLyS is a critical survival factor for B cells, promoting their activation and maturation, and its abnormal production allows the survival of autoreactive B cells, thus triggering B cell lymphoproliferative disorders (De Vita et al. 2008). Perhaps relevant to its development, BLyS ligand-receptor activity is increased in HCV-induced clonal B cell proliferation, including NHL and MC (Landau et al. 2009). A second possibility is that HCV may be capable of directly infecting B cells, where it has been proposed to induce a mutator phenotype (Machida et al. 2004). Interestingly, peripheral blood mononuclear cells from patients with chronic HCV infection often show rearrangement of the bcl-2 gene with t(14;18) translocation, a common chromosomal translocation in B cell lymphomas (Kitay-Cohen et al. 2000).

7 Extrahepatic Manifestations of HCV Infection

Although HCV is generally considered to be an hepatotropic virus, various clinical manifestations of persistent infection involve other organ systems, including the endocrine system, lymphatic system, central and peripheral nerve systems, eyes, kidneys, heart, blood vessels, skin, and joints (Sène et al. 2004). In many cases, these may have a considerable influence on the patient's quality of life, approach to treatment, and, in some, risk of death. Mixed cryoglobulinemia (MC), lymphoproliferative disorders (see above), glomerulonephritis (GN), and sicca complex (Sjögren syndrome) are the most representative and substantial extrahepatic manifestations.

7.1 Mixed Cryoglobulinemia and Glomerulonephritis

Cryoglobulins are immunoglobulins that become insoluble below 37 °C and form high-molecular weight aggregates (Dammacco and Sansonno 1997). MC is a well-documented manifestation of HCV infection and may be present in up to 60 % of HCV-infected patients, but clinically evident manifestations develop in only 5–20 % of these patients (Dammacco et al. 1993; Lunel et al. 1994; Pawlotsky et al. 1994). MC associated with HCV infection may present with various manifestations including vasculitis, purpura, arthritis, sicca syndrome, neuropathy, and GN (Ferri et al. 2004). Therefore, patients with MC symptoms should be screened for HCV infection.

HCV-associated vasculitis is immune-complex in origin, and characterized by the intravascular deposition of circulating immunoglobulin (IgG and IgM) complexes containing HCV particles (Agnello and Abel 1997; Agnello et al. 1992; Sansonno et al. 1995; Sansonno et al. 2003). Chronic stimulation of B lymphocytes resulting from persistent HCV infection, or possibly direct infection of B cells as discussed above, may be responsible for oligo/monoclonal expansions of cells producing antibodies, including rheumatoid factor (IgM anti-IgG), that are present in the cryoglobulins (Dammacco et al. 2000; Pal et al. 2006). Antiviral suppression of viral replication results in a significant improvement in cryoglobulinemia and associated vasculitis, supporting a causal relationship between infection and MC (Ferri et al. 1993; Misiani et al. 1994; Saadoun et al. 2006). For patients with severe vasculitis, including GN with renal insufficiency, the anti-CD20 antibody rituximab has been used before initiation of antiviral therapy (Saadoun et al. 2008), and has been reported to reduce signs of vasculitis (Cacoub et al. 2008).

The association between HCV infection and renal insufficiency is also well established (Dalrymple et al. 2007). Immune complexes containing viral RNA have been detected along capillary walls and in subepithelial regions of glomeruli (Okada et al. 1996; Stokes et al. 1997). These immune complexes may activate mesangial TLR3, leading to the amplification of inflammatory cytokine production and contributing to the establishment of glomerulopathy by facilitating intrarenal migration and activation of inflammatory cells through the induction of intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1),

and macrophage colony-stimulating factor (M-CSF) (Merkle et al. 2012; Wörnle et al. 2006). Interferon-based antiviral therapies are effective to some extent in patients with HCV-related GN (Fabrizi et al. 2007), and it is likely that these patients will benefit from direct-acting antivirals as well.

Sicca complex, manifested by dryness of mucous membranes, is also observed in patients with MC vasculitis (Ferri et al. 2004). An association has also been suggested between HCV infection and Sjögren syndrome, an autoimmune disease that involves destruction of the salivary and lachrymal glands (Haddad et al. 1992), but this remains under debate. Peripheral neuropathy and rheumatologic disorders such as polyarthralgia and arthritis have also been reported in patients with HCV infection (Sène et al. 2004). However, these manifestations could be part of the MC syndrome (Ferri et al. 2004).

7.2 Central Nervous System Manifestations

Mild neuropsychiatric disturbances and loss of cognitive function also occur in some patients with chronic hepatitis C in the absence of hepatic encephalopathy due to metabolic causes or frank depression (Forton et al. 2006). While it is difficult to assess the significance of these findings in patients with underlying liver disease in whom a history of substance abuse is common, they may be accompanied by abnormalities in magnetic resonance scans of the brain. Positron emission tomography (PET) scans also suggest the presence of microglial inflammation in some of these patients (Grover et al. 2012). The possibility that virus may directly infect tissues of the central system was initially suggested by differences in quasispecies distribution among viruses sampled from the liver, brain, and serum of patients (Forton et al. 2004). More recent evidence supports the possibility that HCV may infect endothelial cells that constitute the blood–brain barrier, potentially enhancing their permeability and altering their barrier function (Fletcher et al. 2012). Related CNS-derived cell lines express the receptors required for HCV entry and are permissive for HCV replication (Fletcher et al. 2012; Fletcher et al. 2010). This is an emerging area of investigation that may prove important if CNS infection provides a sanctuary for the virus against antiviral therapies.

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Hepatitis C Virus-Specific Directly Acting Antiviral Drugs

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Abstract The major targets for direct-acting antivirals (DAAs) are the NS3/4A protease, the NS5A protein, and the NS5B polymerase. The latter enzyme offers several target sites: the catalytic domain for nucleoside/nucleotide analogs and different allosteric sites for non-nucleoside inhibitors. Two protease inhibitors have already been approved and more than 40 new NS3/4A, NS5A, or NS5B inhibitors are in development pipeline. Not only these agents can achieve very high cure rates when combined with PEG-IFN and RBV, but have also started to provide promising results when combined in IFN-free, all-oral combinations. In addition to the more canonical drug targets, new alternative viral targets for small molecule drug development are emerging, such as p7 or NS4B. Current research is focusing on defining the most efficacious DAA combination regimens, i.e., those which provide the highest rates of viral eradication, broadest spectrum of action, minimal or no clinical resistance, shortest treatment duration, and good tolerability.

Contents

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1 Introduction

With an estimated prevalence of HCV infection of 2 % worldwide (Shepard et al. 2005), chronic infection with hepatitis C virus (HCV) represents a major public health threat and a significant challenge for the physician and the patient. Until recently, the therapeutic regimens used to treat chronic infection have been based on the use of different forms of ***pegylated interferon-α*** (PEG-IFN) combined with the broad-spectrum antiviral ***ribavirin*** (RBV). These regimens not only are poorly tolerated and contraindicated in a high number of patients, but their effectiveness in eradicating the infection is limited to a fraction of the individuals who are eligible for therapy (see chapter "Treatment of Chronic Hepatitis C: Current and Future" by Pawlotsky, this volume, for further details). Therefore, there is an obvious and urgent need to develop more effective and tolerated treatments.

In order to address this need, new direct-acting antiviral agents (DAAs) are being developed that target specific HCV enzymes and proteins. With the recent approval of the first two NS3/4A oral protease inhibitors [***boceprevir*** (Chang et al. 2012) and ***telaprevir*** (Forestier and Zeuzem 2012)], for the treatment of HCV genotype 1 infections in combination with a PEG-IFN/RBV backbone, we have witnessed a tremendous advance in the pharmacotherapy of chronic hepatitis C. With this new triple therapy regimen, the patient cure rates for HCV genotype 1 infections have increased from around 20–30 % to around 70–80 %, while significantly reducing treatment duration (Lee et al. 2012). However, these DAA-containing regimens still exhibit severe limitations: (1) they increase the spectrum of serious side effects associated with anti-HCV therapy; (2) they are associated with a huge pill-burden and complex dosing schedule; (3) they are limited to genotype 1 infections; (4) selection of DAA-resistant viral variants does occur in patients who respond poorly to the PEG-IFN/RBV component of the triple therapy (for further clinical details see chapter "Treatment of Chronic Hepatitis C: Current and Future" by Pawlotsky, this volume).

In order to overcome these limitations, a number of other DAAs are in development with the objective to develop therapeutic regimens that are more efficacious and convenient, better tolerated, active on all viral genotypes, and with a negligible likelihood to develop viral resistance. These are primarily targeted at the NS3/4A protease, NS5A protein, or NS5B RNA-dependent RNA polymerase. Additionally, other, less-studied viral proteins, such as the ion channel formed by p7 or the replication complex protein NS4B have been recently demonstrated to be "druggable", providing potential new targets for pharmacological intervention (further details about the molecular properties of HCV proteins are given in chapter "Hepatitis C Virus Proteins From Structure to Function"by Moradpour and Penin, this volume). The ultimate goal of the drug discovery effort in HCV research will be that of developing a highly efficacious, IFN-free all-oral therapy and—to this aim—several clinical trials, combining only oral antivirals, have started to show very promising results.

In this chapter, we summarize the progress toward the development of novel HCV-targeted antiviral agents targeting the NS3/4A serine protease, the NS5A protein, the NS5B RNA-dependent RNA polymerase, the p7 ion channel, or the NS4B protein, with particular emphasis on those compound classes or combinations that have shown the most encouraging antiviral activity in the clinic.

2 Inhibitors of the NS3/4A Serine Protease

NS3 is a multifunctional protein that contains a serine protease domain in its ~180 N-terminal amino acids. The remainder of the protein encompasses an RNA helicase. The NS3 serine protease domain is, in turn, one component of a heterodimeric serine protease that requires the noncovalently associated viral protein NS4A for optimal catalytic activity (Failla et al. 1994). Accordingly, this viral enzyme is currently referred to as the NS3/4A protease (detailed in the chapter "Hepatitis C Virus Proteins: From Structure to Function" by Moradpour and Penin, this volume). The activity of the NS3/4A protease is responsible for the proteolytic cleavage of the viral polyprotein at the four junctions NS3-NS4A, NS5A-NS5B, NS4A-NS4B, and NS4B-NS5A (Tomei et al. 1993). The protease cofactor, NS4A, is a relatively small protein, consisting of only 54 residues. The first ~20 residues of NS4A are hydrophobic and form a transmembrane α-helix that is thought to anchor the NS3/4A protease/helicase complex to the outer membrane of the endoplasmic reticulum. A short, central domain of NS4A, amino acids 21–34, are directly implicated in the interaction with the NS3 protease domain and critically required for full enzymatic activity (Failla et al. 1995; Lin et al. 1995). Similar to other trypsin-like serine proteases, NS3/4A is made of two domains both composed of a ß-barrel and two short α-helices with the catalytic triad located in a crevice formed at the interface between the two domains (Kim et al. 1996; Love et al. 1996; Yan et al. 1998) (Fig. 1a). The central region of NS4A is an integral part of the protease domain. It forms one strand of an eight-stranded ß-barrel and increases the enzymatic activity of the protease by stabilizing the N-terminal domain of NS3 and by contributing to the formation of the substrate recognition site (De Francesco and Steinkuhler 2000).

The NS3/NS4A-dependent cleavage sites of the HCV polyprotein have the consensus sequence Asp/Glu-$(Xaa)_4$-Cys/Thr↓Ser/Ala-$(Xaa)_2$-Leu/Trp/Tyr, with the most efficient cleavage occurring after cysteine. The primary specificity of a protease is defined by the side chain of the amino acid that precedes the scissile bond (i.e., the P_1 position; we follow the nomenclature of Schechter and Berger (Schechter and Berger 1967) in designating the cleavage sites as P_3-P_2-P_1 … P_1'-P_2'-P_3', with the cleaved peptide bond between P_1 and P_1' and the C-terminus of the substrate on the prime site). The specificity for the P_1 amino acid is imposed by the features of the complementary S_1 pocket on the enzyme. The S_1 pocket of the NS3/4A protease is shallow and closed at its bottom by a bulky phenylalanine residue, explaining the preference of the NS3 protease for Cys in the substrate P_1 position. In general, the substrate binding site of the NS3/4A serine protease appears to be relatively flat and featureless. Because of the absence of well-defined cavities in the substrate recognition site, the substrate sequence is specifically recognized owing to a series of weak molecular interactions distributed along a rather large surface. This mechanism of substrate recognition, more reminiscent of a protein-protein interaction compared to that typical for enzyme small molecule-substrate docking, has represented a very difficult starting point for the development of small molecule inhibitors of the enzyme. In spite of the formidable challenges encountered in the discovery of inhibitors of this target, a number of highly potent and efficacious active site inhibitors have now been developed.

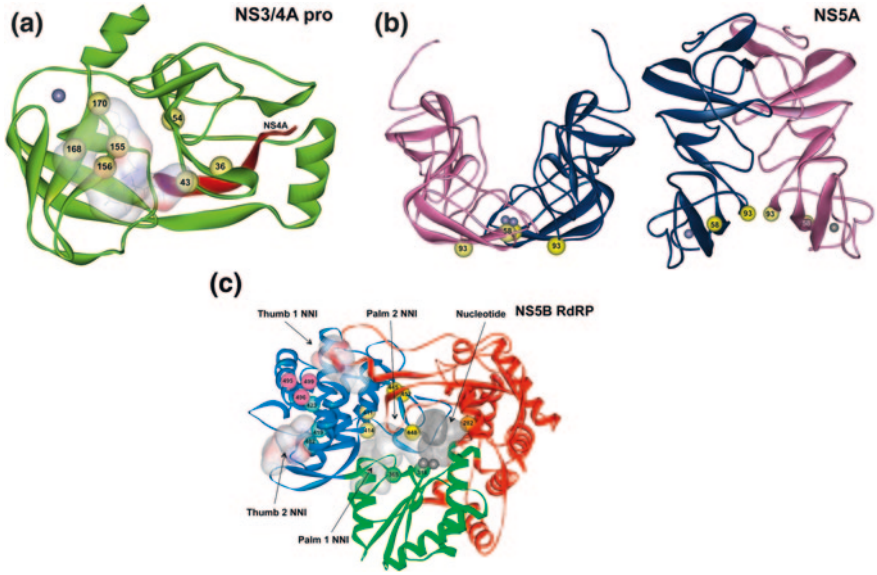

Fig. 1 Three-dimensional structures of the main targets for the development of HCV-specific direct-acting antivirals. **a** NS3/4A protease domain [PDB entry: 3SU3]. NS3 is depicted in *green* color. The NS4A cofactor is shown in *red*. The structural Zn^{++} ion is modeled in *gray*. Vaniprevir, a P_4-P_2 macrocyclic noncovalent NS4/4A protease inhibitor, is bound in the enzyme active site. The amino acids corresponding to the main resistance mutations are highlighted and numbered. **b** Two alternative dimeric conformations of NS5A protein Domain I [PDB entry: 1ZH1 (*left*) and 3FQM (*right*)]. The structural Zn^{++} ion is modeled in *gray*. Amino acids corresponding to main resistance mutations are highlighted and numbered. Substitutions of Y93 represent primary mutations, whereas mutations of position P58 act as a secondary mutation (Fridell et al. 2010). Other positions that are mutated in NS5A-resistant HCV variants (i.e., M28, Q30, L31, and P32) are not visible in the Domain I crystallographic structures. **c** Structure of the HCV RNA-dependent RNA polymerase and binding sites for nucleoside/nucleotide and non-nucleoside polymerase inhibitors. The palm, fingers, and thumb domains are colored *green*, *red,* and *red blue*, respectively. Mn^{++} ions in the active site are modeled in *gray*. Amino acids corresponding to resistance mutations in different regions have a different color: *pink* for P495, P496, and V499 (Thumb I site), *light blue* for L419, M423, and I482 (Thumb II site), *yellow* for N411, M414, Y448, and Y452 (Palm I site/beta-hairpin loop), and *green* for C316 and S365 (Palm II site)

From a chemical point of view, NS3/4A protease inhibitors can be divided into three main categories (Fig. 2): (1) linear peptidomimetics with an alpha-ketoamide group that bind the enzyme active site covalently but reversibly, (2) linear noncovalent peptidomimetic inhibitors, and (3) macrocyclic noncovalent peptidomimetic inhibitors. Macrocyclic inhibitors can be further classified as P_3-P_1 macrocycles or P_4-P_2 macrocycles. The clinical proof of concept for NS3/4A protease inhibitors was first achieved with ***ciluprevir* (*BILN 2061*)**, a P_3-P_1 macrocyclic inhibitor that showed substantial antiviral activity in patients with HCV genotype 1 (Lamarre et al. 2003). However, ciluprevir was not further developed due to serious cardiotoxicity observed in a preclinical model (Goudreau and Llinas-Brunet 2005). The first NS3/4A inhibitors that received approval for use in chronic hepatitis C, telaprevir and boceprevir, both belong to the linear covalent inhibitor class. In the scientific community, telaprevir and boceprevir are

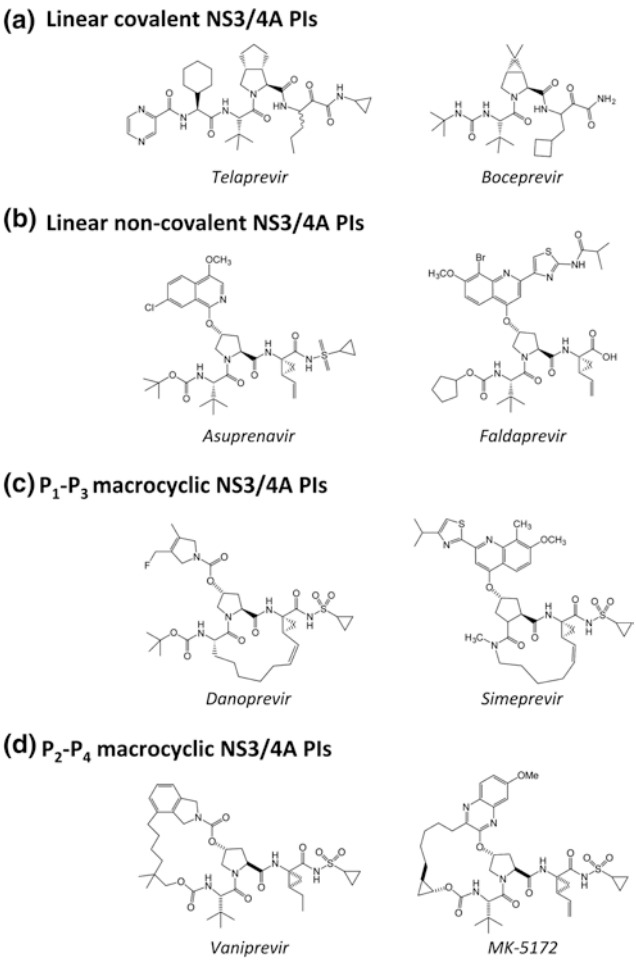

Fig. 2 Chemical structures of selected NS3/4A serine protease inhibitors

also often referred to as "first-wave" NS3/4A protease inhibitors in order to distinguish them from the second-wave and/or second-generation of agents being developed.

2.1 First-Generation NS3/4A HCV Protease Inhibitors

First-generation NS3/4A protease inhibitors are defined as agents that (1) display potent antiviral activity on HCV genotype 1, but (2) oppose a low genetic barrier to selection of resistant viral variants, and (3) have a restricted spectrum of action on other viral genotypes, especially genotype 3. First-generation NS3/4A inhibitors can be in turn classified as belonging to the "first wave" (linear covalent inhibitors) or to the "second wave" (linear noncovalent or macrocyclic inhibitors).

2.1.1 First-Wave NS3/4A HCV Protease Inhibitors: Boceprevir and Telaprevir

Several groups have derived covalent serine protease inhibitors from known substrates by replacing the scissile amide bond with an electrophilic moiety. As a consequence of the catalytic action of the enzyme, such a molecular "warhead" is able to form a covalent adduct with the catalytic serine residue (Edwards and Bernstein 1994). Compounds with this mechanism of action are also referred to as "serine-trap inhibitors". Several biopharmaceutical groups have reported a series of substrate-based, electrophile-based peptidic or peptidomimetic inhibitors of the NS3/4A protease, with electrophilic groups that included aldehydes, boronates, lactams, α-keto amides, and α-keto acids [reviewed in (Chen and Tan 2005)]. Here, we will focus on α-keto amides, since this is the warhead of telaprevir and boceprevir.

One very important mechanistic feature of α-keto amide protease inhibitors is that, in contrast to some other classes of covalent serine traps, the covalent bond formed with the enzyme catalytic serine is entirely reversible. Briefly, this compound class binds to the enzyme active site via a 2-step mechanism, wherein the initial transient formation of a noncovalent enzyme-inhibitor complex is followed by a slow rearrangement to a covalently bound hemiketal complex. The covalent complex undergoes then a very slow dissociation step. Unlike noncovalent inhibitors, covalently bound inhibitor-NS3/4A complexes are thus very long-lived, with half-lives ranging from several minutes to hours. This particular property might at least in part explain the striking antiviral efficacy associated with some of the compound in this class in spite of relatively weak binding affinities.

The evolution of α-ketoamide-based inhibitors of the NS3/4A protease, described in detail in a number of reviews (Chen and Tan 2005; Lin et al. 2006), has culminated in the development of ***boceprevir*** (Chang et al. 2012) and ***telaprevir*** (Forestier and Zeuzem 2012) (Fig. 2a). In May 2011, the NS3/4A protease inhibitors telaprevir and boceprevir gained approval by the US Food and Drug Administration for the treatment of patients chronically infected with HCV genotype 1, in combination with PEG-IFN and RBV. Both agents greatly improve rates of sustained viral response (SVR) among treatment-naïve as well as treatment-experienced patients. While addition of either one of these new agents to the standard PEG-IFN/RBV therapy has pushed SVR rates to unprecedented levels, these drugs still have a number of liabilities, including serious side effects (rash for telaprevir, dysgeusia for boceprevir, and anemia for telaprevir and boceprevir), suboptimal pharmacokinetic properties (dosing every 8 h is required), and low barrier to resistance (Aghemo et al. 2012) (described in more detail in the chapter "Treatment of Chronic Hepatitis C: Current and Future" by Pawlotsky, this volume). Mutations associated with in vitro as well as clinical resistance to telaprevir or boceprevir were identified at several positions close to the NS3 protease active site (Fig. 1a), including V36A/M/L, T54A/S, R155K/M/S/T, A156S (all four conferring low- to medium-level resistance), and A156T/Y (conferring high-level resistance) (Kieffer et al. 2007; Kwo et al. 2010; Sarrazin et al. 2007; Susser et al. 2009). Additionally, these agents have a very limited spectrum of action with respect to the different viral genotypes. For example, in clinical trials, telaprevir displayed activity against HCV genotype 2, but not genotype 3 (Foster et al. 2011a). Moreover, a small,

randomized trial enrolling genotype 4 patients showed that addition of telaprevir to PEG-IFN/RBV only had a modest effect on SVR rates (Benhamou et al. 2009).

2.1.2 Second-Wave NS3/4A Protease Inhibitors

A number of so-called second-wave NS3/4A protease inhibitors are currently being studied in Phase II or III clinical trials. They include: linear noncovalent inhibitors ***faldaprevir/BI 201335*** (White et al. 2010), ***asunaprevir/BMS-650032*** (McPhee et al. 2012b), ***sovaprevir/ACH-1625*** (Agarwal et al. 2012), and ***GS-9451*** (Sheng et al. 2012a); P_3-P_1 macrocyclic inhibitors ***simeprevir/TMC435*** (Lin et al. 2009), ***danoprevir/RG7227/ITMN-191*** (Seiwert et al. 2008), ***ABT-450*** (Wagaw et al. 2009), and ***GS-9256*** (Sheng et al. 2012b); and P_4-P_2 macrocyclic inhibitor ***vaniprevir/MK-7009*** (Liverton et al. 2010) (Fig. 2b–d). These agents are characterized by very potent activity on HCV genotype 1 [typically, low-nM EC_{50} in the replicon system (chapter "Cell Culture Systems for Hepatitis C Virus" by Steinmann and Pietschmann, this volume)] as well as antiviral efficacy on HCV genotype 1 patients similar to that of boceprevir or telaprevir (Lee et al. 2012; Sarrazin et al. 2012; Schaefer and Chung 2012). They differ from their first-wave counterpart in that they do not have the chemical reactivity required to make a covalent bond to their target and are therefore expected to display fewer- and less severe side effects. In addition, these agents have substantially improved pharmacokinetic profiles, which allows for less frequent dosing, typically once a day [*qd*: simeprevir (Reesink et al. 2010), faldaprevir (Manns et al. 2011), sovaprevir (Agarwal et al. 2012), ABT-450 (Poordad et al. 2012a), and GS-9451 (Dvory-Sobol et al. 2012)] or twice a day [*bid:* vaniprevir, danoprevir (Forestier et al. 2011), asuprenavir (Pasquinelli et al. 2012), and GS-9256 (Zeuzem et al. 2012a)]. It should be pointed out that low-dose ritonavir boosting is used with danoprevir (Reddy et al. 2012) and ABT-450 (Lawitz et al. 2010a) in order to decrease dosing frequency and to reduce drug-associated side effects.

Although second wave-first generation NS3/4A HCV protease inhibitors tend to have a significantly broader spectrum of action on the different HCV genotypes compared to the inhibitor of the first wave, these agents cannot be considered pan-genotypic antivirals since they do not exhibit the same potency across all viral genotypes. For example, simeprevir and faldaprevir have been reported to have a rather broad spectrum of action in biochemical assays, with variable in vitro activity against NS3/4A protease of HCV genotypes 1–6 (Reesink et al. 2010; White et al. 2010). For both compounds, the lowest inhibitory potency was observed against genotype 3. Simeprevir has also been investigated in patients infected with genotypes 2–6 viruses. In line with the in vitro data, medium to high antiviral activities (over -2.0 $\log_{10}$ IU/ml in all instances) were observed against genotypes 2, 4, 5, and 6, whereas the drug had no effect in patients infected with HCV genotype 3 (Moreno et al. 2012).

Along with the restricted genotype coverage, the genetic barrier posed to resistance by all first-generation NS3/4 protease inhibitors is low and extensive cross-resistance is observed between the different compounds or compound classes (Sarrazin and Zeuzem 2010). First-wave and second-wave NS3/4A protease inhibitors show slightly different resistance mutation patterns. In particular, mutations of Val 36 (V36A/M) or Thr 54

(T54S/A) have been exclusively observed in association with covalent, linear inhibitors of the first wave. Mutations at position Arg 155 (R155K/T/Q) have been shown to confer broad cross-resistance to all first-generation inhibitors. Conversely, mutations of Asp 168 (D168/E/G/H/T/Y) are specifically found to confer mutation to noncovalent peptidomimetic inhibitors, regardless whether of linear or macrocyclic structure (Dvory-Sobol et al. 2012; Lagace et al. 2012; Lenz et al. 2010; Lin et al. 2004; McPhee et al. 2012a; Sarrazin and Zeuzem 2010). This is believed to occur because of the interaction between the very large P2 substituent, common to all these inhibitors, with D168 in the enzyme active site (Romano et al. 2012). Notably, D168 is one of the few active site residues not entirely conserved among HCV genotypes, being replaced by glutamine in isolates belonging to genotype 3. This could partly explain why HCV genotype 3 is "naturally resistant" to virtually all first-generation, second-wave protease inhibitors (Trozzi et al. 2003). Agents belonging to this second-wave of first-generation NS3/4A protease inhibitors have showed high rates of SVR in HCV genotype 1 patients, when used in combination with PEG-IFN and RBV, of the same order of magnitude or higher than those reported for boceprevir or telaprevir triple-combination regimens (Lee et al. 2012; Schaefer and Chung 2012). Simeprevir and faldaprevir are currently in Phase III clinical evaluation for their use in PI/PEG-IFN/RBV combination to treat HCV genotype 1 chronic infection.

2.2 Second-Generation NS3/4A Protease Inhibitors

Second-generation NS3/4A protease inhibitors are defined as agents that (1) pose a high barrier to the development of viral resistance, (2) retain activity against the viral variants that are resistant to first-generation compounds, and (3) are active across the different genotypes, including HCV genotype 3.

MK-5172 (Fig. 2d) is a second-generation NS3/4A protease inhibitor with pan-genotype antiviral activity and improved resistance profile (Summa et al. 2012). Importantly, in biochemical assays, this agent maintained potent antiviral activity against the predominant amino acid substitutions known to confer resistance to first-generation protease inhibitors. Thus, MK-5172 remained highly active against HCV variants harboring mutations of Arg 155 or Asp 168. A recent crystallographic study analyzing the molecular basis of drug resistance against NS3/4A protease inhibitors revealed that telaprevir, danoprevir, and vaniprevir interact directly with sites that confer resistance upon mutation, whereas MK-5172 interacts in a unique conformation with the catalytic triad (Romano et al. 2012). This novel mode of MK-5172 binding explains its retained potency against the two multidrug-resistant variants, R155K and D168A. Results from early clinical trials are essentially in line with its in vitro properties. Thus, no viral breakthrough has been observed in HCV genotype 1-infected patients who received this drug alone for 7 days and antiviral activity persisted for several days beyond the treatment period (Brainard et al. 2010), suggesting a higher barrier to resistance compared to first-generation inhibitors. In the same trial, patients infected with HCV genotype 3 showed a robust antiviral response at the higher drug doses. Longer drug administration periods will be required to draw final conclusions regarding the in vivo resistance profile and genotype 3 efficacy of MK-5172.

ACH-2684, a P_3-P_1 macrocyclic inhibitor, is another second-generation HCV protease inhibitor reported to be in Phase I clinical trial. ACH-2684 showed potent biochemical activity against genotype 1–6 viruses and against known resistant variants (Huang et al. 2010). Additional examples of second-generation HCV protease inhibitors have started to fill the preclinical research space [see for example (Kazmierski et al. 2012)].

3 NS5A Inhibitors

HCV NS5A is a multifunctional protein that is essential for HCV RNA replication complex and required for virion assembly (see chapters "Hepatitis C Virus RNA Replication" by Lohmann and "Virion Assembly and Release" by Lindenbach, this volume). It is a phosphoprotein expressed in basally and hyperphosphorylated forms (p. 56 and p. 58, respectively) (Huang et al. 2007). The NS5A protein structure consists of three domains: Domain I (aa 1–213), Domain II (aa 250–342), and Domain III (aa 356–447). While Domains II and III have not been structurally characterized, the crystal structure of Domain I has been crystallized in alternative dimer forms (Love et al. 2009; Tellinghuisen et al. 2005), both containing zinc- and RNA-binding motifs (Fig. 1b). NS5A has been shown to interact with a variety of host proteins (Macdonald and Harris 2004), including a critical interaction with phosphatidylinositol 4-kinase IIIα (PI4KIIIα), a cellular lipid kinase that is absolutely required for HCV replication (Reiss et al. 2011). Not being associated to any measurable enzymatic activity, NS5A has been considered "not druggable" for a very long time. In recent years, however, compounds acting on NS5A have emerged as efficacious inhibitors of HCV replication, with specific examples displaying in vitro anti-HCV activity in the low pM range. The first NS5A inhibitors were discovered by replicon-based high-throughput screening (Conte et al. 2009; Lemm et al. 2010). The initial lead compounds (Fig. 3a) had moderate potency and narrow anti-HCV activity, mainly on genotype 1b. Subsequent medicinal chemistry efforts (Lemm

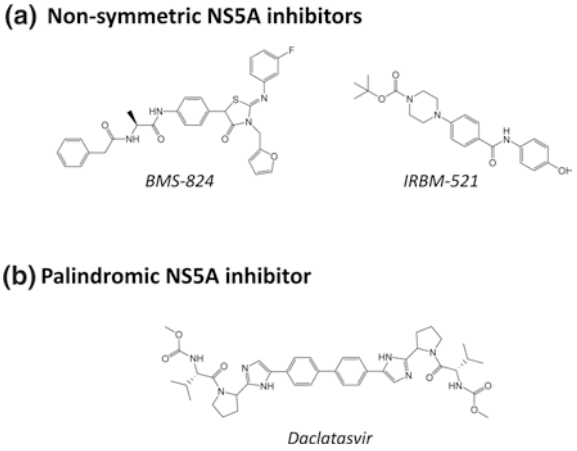

Fig. 3 Chemical structures of selected NS5A protein inhibitors

et al. 2011) resulted in the discovery of extremely potent compounds characterized by a very peculiar, highly symmetrical dimeric structure (Fig. 3b). The most studied of this "palindromic" NS5A inhibitor class is ***daclatasvir/BMS-790052*** (Gao et al. 2010), a highly optimized NS5A inhibitor, which exhibited 50 % effective concentration (EC_{50}) values of 50 and 9 pM against replicon of genotype 1a and 1b, respectively. Moreover, daclatasvir exhibited low picomolar activity against replicons harboring NS5A from a broad range of HCV genotypes (McPhee et al. 2012b), thus indicating its pan-genotypic potential.

It should be pointed out that the precise mechanism of action of the so-called "NS5A-inhibitors" is not completely understood. They were initially claimed to be NS5A inhibitors mainly based on the selection of specific resistant mutations that mapped in NS5A Domain I (Conte et al. 2009; Lemm et al. 2010). In particular, changes corresponding to variants of NS5A Tyr93 (Y93H/C/N) were found by different groups to be most common mutations conferring broad resistance to this class of antivirals. Interestingly, Tyr93 is found near the protein dimer interface (Fig. 1b), leading to speculate that NS5A inhibitors might act by modulating NS5A monomer/dimer equilibrium (Conte et al. 2009). However, the palindromic topology of daclatasvir and related compounds rather suggests a binding interaction with NS5A in which the inhibitor interacts across the dimer interface, making simultaneous contacts to both protein monomers (Belda and Targett-Adams 2012). This could at least partly explain the extraordinary potency observed for the palindromic inhibitor series. Among the reported properties of NS5A inhibitors are the ability to inhibit NS5A hyperphosphorylation (Lemm et al. 2010) and to cause the redistribution of NS5A from the HCV membranous web to different subcellular localizations, thus impeding the formation of new replication complexes (Qiu et al. 2011; Targett-Adams et al. 2011) (see also chapter "Hepatitis C Virus RNA Replication" by Lohmann, this volume). NS5A inhibitors were also shown to interfere with the accumulation of phosphatidylinositol 4-phosphate (PI4P) in the membranous HCV replication compartment, the so-called membranous web (Berger et al. 2012; Reghellin et al. 2012). Membranous web PI4P is produced, in HCV-infected or replicon cells, by PI4KIIIα, a phosphatidylinositol-specific kinase that is specifically recruited and activated by the interaction with NS5A (Reiss et al. 2011). These new data indirectly suggest that interaction of these antiviral agents with NS5A might also interfere with the recruitment and/or activation of PI4KIIIα by HCV NS5A.

Clinically, inhibition of NS5A has been associated with steep reductions in HCV RNA in monotherapy (Gao et al. 2010) and enhanced SVR rates when combined with PEG-IFN and RBV (Pol et al. 2012). When patients with chronic HCV genotype 1 infection were administered single doses of 1, 10, and 100 mg of daclatasvir, viral load dropped by up to 3.6 $\log_{10}$ IU/ml. Strikingly, the drop in viremia observed at the highest dose was sustained for 144 h post-dose in two patients infected with genotype 1b virus (Gao et al. 2010). In spite of the striking antiviral activity, the genetic barrier to resistance for this drug is low, and resistant variants are selected readily in vitro as well as in patients, with the more relevant substitutions found at NS5A residues 28, 30, 31, and 93 for genotype 1a and residues 31 and 93 for genotype 1b (Fridell et al. 2011). A recent Phase II trial highlighted the efficacy of daclatasvir in combination with PEG-IFN/RBV. All subjects received

48 weeks of triple therapy. SVR rates for 10 and 60 mg doses were 83 %, whereas the standard therapy only achieved an SVR of 25 % (Pol et al. 2012) (for further details see chapter "Treatment of Chronic Hepatitis C: Current and Future" by Pawlotsky, this volume). Daclatasvir is currently being evaluated in IFN-free regimens in combination with sofosbuvir (nucleotide polymerase inhibitor), asunaprevir (protease inhibitor), and/or BMS791325 (non-nucleoside polymerase inhibitor) (Everson et al. 2012; Sulkowski et al. 2012a).

GS-5885 (Lawitz et al. 2012) is a more recently reported NS5A inhibitor with EC_{50} values of 34 pM against genotype 1a and 4 pM against genotype 1b replicons. Antiviral activity of once-daily dosing (1 to 90 mg) of GS-5885 for 3 days in patients with chronic genotype 1 HCV infection resulted in median maximal reductions in viral RNA ranging from 2.3 to 3.3 $\log_{10}$ IU/ml. HCV RNA sequencing following GS-5885 dosing revealed a pattern of resistance mutations virtually identical to what observed with daclatasvir. Like daclatasvir, GS-5885 is also evaluated in IFN-free regimens in combination with sofosbuvir (nucleotide polymerase inhibitor) and ribavirin (Gane et al. 2012b).

Other NS5A inhibitors in clinical development include: ***ABT-267, GSK2336805, PPI-561*** (Phase II) and ***ACH-2928, ACH-3102, PPI-668, IDX719,*** and ***MK-8742*** (Phase I) (Belda and Targett-Adams 2012). It is worth pointing out that ACH-3102 and MK-8742 are two early-stage NS5A inhibitors that (1) show a relatively higher barrier to resistance and (2) retain substantial levels of potency against resistance mutations that affect early NS5A inhibitors, such as daclatasvir and GS-5885 (Liu et al. 2012; Yang et al. 2012). In analogy with the evolution of NS3/4A protease inhibitors, these novel agents can be viewed as "second-generation" NS5A inhibitors (Belda and Targett-Adams 2012).

4 Inhibitors of the NS5B Polymerase

The HCV NS5B RNA-dependent RNA polymerase (RdRp) catalyzes the synthesis of a complementary minus-strand RNA, using the (incoming) RNA genome as a template, and subsequently the synthesis of new progeny genomic plus-strand RNA from the minus-strand RNA template (detailed in chapter "Hepatitis C Virus RNA Replication" by Lohmann, this volume). Similar to other nucleic acid polymerases, NS5B has the typical right-hand polymerase structure, consisting of a thumb domain and a fingers domain, both encircling the enzyme active site located within the palm domain (Bressanelli et al. 1999; Lesburg et al. 1999) (Fig. 1c). The evolutionarily very well-conserved active site includes a GDD motif, implicated in coordinating two Mg^{++} ions required to bind the nucleotide substrate(s) and to assist the enzymatic reaction (Fig. 1c). Inhibitors of the NS5B RdRp are classified into nucleoside (NI), nucleotide, and non-nucleoside (NNI) inhibitors (Membreno and Lawitz 2011) (Fig. 4).

The nucleoside/nucleotide inhibitors are mimics of the natural polymerase substrates and bind to the NS5B active site, causing chain termination. Importantly, because of the active site conservation, they have similar efficacy across all HCV genotypes and

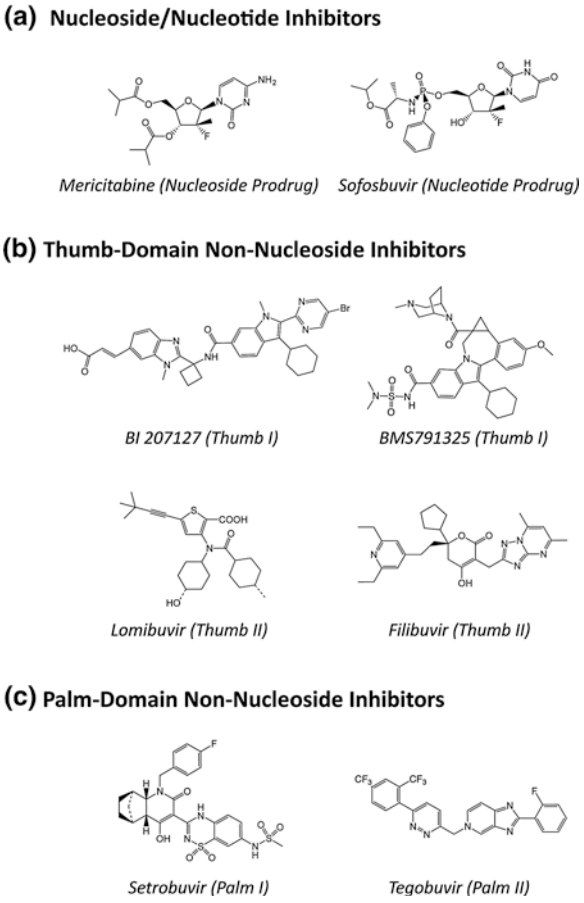

Fig. 4 Chemical structures of selected inhibitors of the NS5B RNA-dependent RNA polymerase

isolates (Ludmerer et al. 2005). For the same reason, nucleoside/nucleotide inhibitors are associated with a high barrier to development of drug resistance (McCown et al. 2008). NNIs bind to one of at least four, less conserved, allosteric sites, resulting in the inhibition of enzyme conformational changes that are necessary for the enzyme catalytic function (Tomei et al. 2003; Tomei et al. 2004). In contrast to NIs, these molecules have shown a restricted spectrum of activity against the various HCV genotypes (Ludmerer et al. 2005) and present a very low barrier to emergence of resistance (McCown et al. 2008). So far, NNIs have targeted four main allosteric sites in the NS5B polymerase: thumb domains I and II and palm domains I and II (depicted in Fig. 1c).

4.1 Nucleoside Inhibitors

HCV-specific nucleoside inhibitors (NI) are ribonucleoside analogs that need to be converted to their corresponding 5′-triphosphate by cellular kinases in the

cytoplasm of infected cells, thereby mimicking natural polymerase substrates (Carroll and Olsen 2006). NIs with anti-HCV activity possess a free 3′-hydroxyl function and may therefore not be considered as obligate chain terminators. Virtually, all nucleoside/-tide inhibitors in development contain modifications at the sugar 2′-position. It is thus conceivable that these compounds may act as virtual chain terminators because of steric hindrance exerted by 2′-*C*-methyl or 2′-fluoro groups. The primary mutation identified to be associated with decreased susceptibility to a broad range of 2′-modified nucleoside/-tide analogs is NS5B S282T (Le Pogam et al. 2006a; Migliaccio et al. 2003; Pawlotsky et al. 2012) (Fig. 1c). This mutation dramatically reduces HCV replication capacity, explaining at least in part the high barrier to resistance posed by these agents (Pawlotsky et al. 2012).

Valopicitabine **(*NM283*)** is an oral prodrug (3′-*O*-valine ester) of 2′-*C*-methylcytidine (***NM107***) (Pierra et al. 2006) and the first nucleoside analog inhibitor of HCV that entered clinical trials. In Phase II clinical trials in HCV-infected patients, valopicitabine was combined for 48 weeks of treatment with PEG-IFN-α. The decline in viral load was not significantly different when compared to the standard of care group, i.e., PEG-IFN and RBV (Afdhal et al. 2007). Whereas HCV replicons resistant to 2′-*C*-methylcytidine carry the S282T mutation, no selection of resistant viruses was observed in patients treated with this nucleoside analog (Lawitz et al. 2006). This is consistent with the low antiviral effect exerted by this drug in the clinical trials. Based on the low-benefit profile observed in clinical trials, the development of valopicitabine was stopped.

Following 2′-*C*-methylcytidine, various other 2′-modified nucleoside analogs targeting the HCV NS5B polymerase have been reported to inhibit HCV replication in vitro: these include 2′-*O*-methylcytidine (Carroll et al. 2003), 2′-*C*-methyladenosine (Carroll et al. 2003), 2′-*C*-methylguanosine (Migliaccio et al. 2003), 7-deaza-2′-*C*-methyladenosine (Olsen et al. 2004), 2′-deoxy-2′-fluoro-2′-*C*-methylcytidine (PSI-6130) (Stuyver et al. 2006), and 2′-deoxy-2′-spirocyclopropylcytidine (TMC647078) (Berke et al. 2011). Except for PSI-6130, the parent drug of RG7128, none of these nucleoside analogs are further developed.

Mericitabine/RG7128 (Fig. 4a) is a di-isobutyl ester prodrug of PSI-6130, a cytidine nucleoside analog (β-*D*-2′-deoxy-2′-fluoro-2′-*C*-methylcytidine) (Stuyver et al. 2006). PSI-6130 is metabolized intracellularly to the 5′-triphosphate of β-D-2′-deoxy-2′-fluoro-2′-*C*-methylcytidine and also, following deamination, to the triphosphate of β-D-2′-deoxy-2′-fluoro-2′-*C*-methyluridine (PSI-6206) (Ma et al. 2007). Both triphosphates are incorporated as nonobligate chain terminators into RNA synthesized by purified NS5B (Murakami et al. 2007). Replicons resistant to PSI-6130 carry the S282T mutation, as is the case for valopicitabine-resistant replicons. Interestingly, this mutation results only in a low level (three to fourfold) of in vitro resistance and emergence of resistance is slower than for other classes of DAAs (Pawlotsky et al. 2012). Mericitabine has been shown to be active against genotype 1 and 4 HCV and is currently in Phase II clinical development in triple combination with PEG-IFN and RBV (Le Pogam et al. 2010). Mericitabine is also being evaluated in early-stage clinical trials as a component of IFN-free, all-oral regimen in combination with the NS3/4A protease inhibitor danoprevir

(Gane et al. 2010; Pawlotsky et al. 2012). In line with the high barrier to resistance observed in vitro, no evidence of genotypic resistance to mericitabine has been detected by population or clonal sequence analysis in any baseline or on-treatment samples collected from >600 patients enrolled in Phase I/II trials of mericitabine administered as monotherapy, in combination with PEG-RBV, or in combination with danoprevir (Pawlotsky et al. 2012) (see also chapter "Treatment of Chronic Hepatitis C: Current and Future" by Pawlotsky, this volume).

4.2 Nucleotide Inhibitors

Nucleotide polymerase inhibitors are liver-targeted prodrugs designed to enhance formation of its active triphosphate in the liver, while minimizing systemic exposure of the nucleotide drug and its nucleoside metabolite. Such prodrugs of nucleotide analogs (i.e., nucleoside 5′-monophosphates) are preferentially cleaved by hepatic enzymes, thereby efficiently releasing their nucleoside monophosphate in liver cells. The rate-limiting step for metabolic activation of nucleoside analogs, the initial phosphorylation to a nucleoside monophosphate, is bypassed, resulting in higher levels of nucleoside triphosphates in the cell.

Sofosbuvir/GS-7977 (Fig. 4a) is currently the most advanced NS5B polymerase inhibitor in clinical development (Phase III). It is a chirally pure isomer of PSI-7851, a phosphoramidate prodrug of a uridine nucleotide analog (beta-D-2′-deoxy-2′-fluoro-2′-C-methyluridine monophosphate) (Murakami et al. 2008). It was previously shown that PSI-7851 exists as a 1:1 mixture of two diastereoisomers, PSI-7976 and PSI-7977, at the phosphorus center of the phosphoramidate moiety. PSI-7977, the Sp isomer, was found to inhibit HCV replication more effectively than PSI-7976 (Rp isomer) in replicon-based assays. The S282T mutation was the common resistance mutation emerging during resistance selection in vitro (Lam et al. 2012). While this mutation conferred resistance to sofosbuvir in genotype 1 replicons, it only caused a very minor shift in potency in genotype 2a, thus suggesting that additional mutations in genotype 2a NS5B are required for the resistant phenotype (Lam et al. 2012). Phase II clinical studies revealed that addition of sofosbuvir to PEG-IFN and RBV for 12 weeks resulted in SVR rates in excess of 90 % for naïve genotype 1 HCV infected patients (Kowdley et al. 2012b). Furthermore, IFN-free, all-oral sofosbuvir/RBV demonstrated consistent antiviral suppression across HCV genotypes (Gane et al. 2012b). Viral resistance was hardly observed in any clinical studies using sofosbuvir, confirming the high genetic barrier to resistance. Sofosbuvir is currently studied in IFN-free combinations with a number of other DAAs, including NS3/4A protease inhibitors (GS-938, simeprevir) and NS5A inhibitor (daclatasvir, GS-5885). Striking preliminary data have been reported from an ongoing Phase II study examining a 12 weeks course of therapy with sofosbuvir in combination with GS-5885 and ribavirin in patients with genotype 1 chronic HCV infection. In this trial, among treatment-naïve patients receiving this combination, 100 % (n = 25/25) remained HCV

RNA undetectable 4 weeks after completing therapy (SVR4) (Gane et al. 2012a). The very high efficacy of the combination between sofosbuvir and an NS5A inhibitor was also demonstrated in a trial where 12 weeks viral elimination rates (SVR12) of greater than 93 % were observed in previously untreated patients infected with HCV genotype 1, 2, or 3 (Sulkowski et al. 2012b). Importantly, in this trial, the inclusion of RBV did not influence the virological outcome.

GS-938 (formerly PSI-938) is a prodrug of 2′-F-2′-C-methylguanosine monophosphate and was created to be an optimal partner DAA for pyrimidine nucleotide GS-7977 (Lam et al. 2011b). The nucleotides employ different prodrug cleavage pathways, largely independent phosphorylation pathways, competition with separate endogenous nucleotide pools (purine/pyrimidine), and complementary resistance profiles. No resistant genotype 1a and 1b replicons could be selected. In contrast, various mutations in NS5B were identified in resistant genotype 2a replicons. Phenotypic characterization showed that single mutations were not able to confer resistance to GS-938, but that a combination of three amino acid changes, S15G/C223H/V321I, was required (Lam et al. 2011a). Interestingly, no cross-resistance was observed with 2′-modified nucleoside/-tide analogs such as PSI-6130, GS-7977, BMS-986094, and IDX-184. Conversely, GS-938 remained fully active against both the S96T and S282T replicons (Lam et al. 2011b). The potential of the combination of GS-938 and GS-7977 was studied in the NUCLEAR clinical trial (Lawitz et al. 2011). The 14 days proof-of-concept study demonstrated excellent safety/tolerability, lack of antagonism (pharmacokinetic or antiviral), and no virologic breakthrough. However, in the subsequent Phase IIb Quantum study all treatment arms containing GS-938 were discontinued because abnormal liver function tests were observed in some patients receiving GS-938 (Press release Pharmasset, 16 December 2011).

A novel, double prodrug approach was used to synthesize nucleotide analog ***BMS-986094*** (formerly INX-08189). A phosphoramidate ProTide motif and a 6-O-methoxy base moiety were combined to generate a lipophilic prodrug of the monophosphate of 2′-C-methyl guanosine (McGuigan et al. 2010). BMS-986094 is highly active in HCV replicon assays with an EC_{50} of 1–10 nM (genotypes 1a, 1b, 2a) (Vernachio et al. 2011), and replicons resistant to BMS-986094 carried the S282T mutation in NS5B, resulting in an approximately 10-fold reduction in sensitivity to the drug. Following 7 days of monotherapy, a median reduction of -4.25 $\log_{10}$ in HCV RNA levels was achieved (Rodriguez-Torres et al. 2011). Clinical development of BMS-986094 was stopped, however, due to adverse effects involving heart and kidney toxicity.

IDX184 is a different nucleotide prodrug also designed to deliver high concentrations of 2′-C-methyl guanosine 5′-monophosphate (MP) in hepatocytes (Zhou et al. 2011). In spite of the relatively encouraging clinical results, development of this drug was significantly slowed down in light of the severe toxicity associated with BMS-986094, with which IDX184 shares the parent nucleotide (2′-C-methyl guanosine MP). Other nucleotide polymerase inhibitors are currently studied in preclinical, Phase I and II clinical trials including ***PSI-353661*** (prodrugs of 2′-C-methylguanosine monophosphate), ***GS-6620*** (nucleotide prodrug of C-nucleoside analog), and ***ALS-2200*** (structure unknown).

4.3 Non-nucleoside Inhibitors

Screening of inhibitor libraries has resulted in the discovery of several classes of allosteric non-nucleoside inhibitors (NNIs). The hallmark of all allosteric HCV NNIs described so far is that, in contrast to active site nucleoside inhibitors, they are noncompetitive with NTP substrates and inhibit the polymerase at a stage preceding the elongation reaction (Gu et al. 2003; Tomei et al. 2003; Tomei et al. 2004). To date, at least four distinct allosteric binding sites are known to be targeted by NNIs; two of them, dubbed "thumb I" and "thumb II", are located on the polymerase thumb domain, whereas the other two sites, "palm I" and "palm II", are close to the active site cavity and involve primarily amino acids from the palm domain (Fig. 1c).

4.3.1 Thumb I Inhibitors

The thumb I NNI binding site is located in the thumb domain 30 Å away from the active site (Fig. 1c). Several structurally related inhibitors have been shown to bind to this site (Beaulieu 2006). This class of inhibitors interrupts the intramolecular contacts between the thumb and the finger-tip loop, thereby preventing the formation of a productive enzyme complex for RNA synthesis (Di Marco et al. 2005; Kukolj et al. 2005). For this reason, these agents are also known as "finger-loop" inhibitors and are characterized by having a common benzimidazole or indole chemical core (Fig. 4b). The in vitro antiviral efficacy of finger-loop inhibitors was originally demonstrated in HCV subgenomic replicons (Kukolj et al. 2005; Tomei et al. 2003), where drug resistant variants carrying mutations at positions P495, P496, and T389 could be readily selected (Delang et al. 2012; Kukolj et al. 2005; Tomei et al. 2003) (Fig. 1c). Optimization of cellular permeability by conversion of the benzimidazole scaffold to the more lipophilic indole scaffold provided further enhancement of the antiviral activity, leading eventually to anti-HCV clinical activity (Beaulieu et al. 2012; Brainard et al. 2009; Devogelaere et al. 2012; Larrey et al. 2009; Narjes et al. 2011). Notably, agents belonging to this class of NNIs display reduced activity against genotype 1a HCV compared to genotype 1b (Brainard et al. 2009; Erhardt et al. 2009; Larrey et al. 2009). Several such indole-based inhibitors (***BI 207127, TMC647055, and BMS791325***) are currently being investigated in clinical trials (Beaulieu et al. 2012; Devogelaere et al. 2012; Larrey et al. 2012; Larrey et al. 2009). The most advanced thumb site I-targeting NNI is ***BI 207127*** (Fig. 4b). It is currently in Phase II clinical development. In line with the in vitro findings, BI 207127-resistant mutants that encode P495 substitutions were observed in 11 % of patients who received a 5 days monotherapy (Larrey et al. 2009). BI 207127 in combination with PEG-IFN and RBV demonstrated strong antiviral activity with a favorable safety and tolerability profile (Larrey et al. 2012). In a Phase IIb IFN-free clinical trial (Zeuzem et al. 2012b), BI 207127 was studied in combination with the NS3/4Aprotease inhibitor BI 201335

together with RBV for 28 weeks. This IFN-free combination, which included 362 treatment-naïve patients with genotype-1 HCV infection, resulted in virus elimination with 70 % of patients overall, compared with 85 % seen in the GT-1b patient subgroup. More strikingly, a 12 weeks IFN-free and RBV-free triple therapy with daclatasvir, asunaprevir, and BMS-791325, another thumb I NNI, achieved 94 % (n = 15/16) 4 weeks post-treatment response (SVR4) in treatment-naïve patients infected with HCV genotype 1 viruses (Everson et al. 2012). Importantly, although preliminary, these results demonstrate the potential for thumb I NNIs to be successfully utilized in an IFN-free all-oral regimen, at least for selected patients.

4.3.2 Thumb II Inhibitors

Thiophene carboxylic acid derivatives were reported to inhibit NS5B RdRp activity and HCV RNA replication in the replicon cell culture system. Crystallographic studies revealed the existence of a hydrophobic cavity located at the base of the thumb domain of NS5B (Wang et al. 2003). Like thumb I NNIs, thiophene carboxylic acid derivatives are noncompetitive with nucleotide incorporation and inhibit an initiation step of RNA synthesis by interfering with conformational changes that are likely required for processive elongation of the replicating strand. Thiophene-based inhibitors were found to select for M423, L419, and I482 resistant mutants in replicon cell culture experiments (Le Pogam et al. 2006b). ***Lomibuvir/VX-222*** (Fig. 4b) exhibits low micromolar antiviral activity against HCV 1a and 1b isolates in replicon assays. Upon 3 days of monotherapy, lomibuvir achieved a mean reduction in HCV RNA ranging from 3.1 to 3.4 $\log_{10}$ with similar activity in genotype 1a- and 1b-infected patients (Rodriguez-Torres et al. 2010). Lomibuvir is also studied in combination with protease inhibitor telaprevir. In particular, combining lomibuvir and telaprevir with PEG-IFN + RBV resulted in overall cure rates of up to 90 % (Nelson et al. 2011) with no on-treatment viral breakthroughs observed. A back-up drug for lomibuvir, ***VX-759***, resulted in a significant reduction in viral load in treatment-naïve genotype 1 infected patients during a proof-of-concept study (Cooper et al. 2009). On-treatment rebound of viremia suggested the emergence of resistant strains. Genotypic analysis of clinical isolates confirmed mutations at positions L419, M423, and I482. VX-759 is currently being evaluated in Phase II clinical trials.

In addition to thiophene-derived carboxylic acids, other classes of molecules have been identified to target this allosteric site. ***Filibuvir/PF-868554*** (Fig. 4b), a ***dihydropyranone*** derivative, is a potent and selective HCV inhibitor in vitro (Li et al. 2009). It exerts strong in vitro antiviral activity against the HCV 1b replicon (EC_{50} of 0.075 μM), and reduced activity against the 1a replicon (EC_{50} of 0.39 μM). The predominant in vitro resistance mutation is M423T (Shi et al. 2009). Other amino acid mutations, M426T and I482T, were detected at a much lower frequency. Interestingly, the resistance mutations at residues L419, M423, and I482 had different effects on the level of inhibition of RNA synthesis by filibuvir or by lomibuvir (Yi et al. 2012). It was therefore hypothesized that the

binding sites of filibuvir and lomibuvir are partially overlapping, but not identical. In treatment-naïve genotype 1 patients, addition of filibuvir to PEG-IFN and RBV was associated with a maximal mean reduction in HCV RNA of 4.7 $\log_{10}$ IU/mL at day 28 (Jacobson et al. 2009). However, a high relapse rate was observed, probably due to the short treatment duration. Sequence analysis of the NS5B coding region identified residue M423 as the predominant site of mutation (Wagner et al. 2011). Currently, filibuvir is in Phase II of clinical development.

Another class of non-nucleoside polymerase inhibitors that interacts with thumb domain II is the class of the ***pyranoindole*** derivatives (i.e., ***HCV-371*** and a follow-up compound ***HCV-086***). It entered clinical development, but failed to demonstrate significant efficacy and development was therefore discontinued (Howe et al. 2004; Howe et al. 2006). Recently, substituted ***N-phenylbenzenesulphonamides*** were also shown to inhibit HCV genotype 1a and 1b replication in vitro (May et al. 2012).

4.3.3 Palm I Inhibitors

Palm domain I is located at the junction of the palm and the thumb domain of NS5B and is in relatively close proximity to the catalytic site. The first class of palm domain I inhibitors was originally discovered by GlaxoSmithKline and is characterized by a ***benzothiadiazine*** scaffold. Akin to the thumb domain targeting compounds, benzothiadiazine-based compounds inhibit RNA synthesis before formation of an elongation complex. In vitro benzothiadiazines select for M414-resistant mutants (Nguyen et al. 2003).

The benzothiadiazine inhibitor ***setrobuvir/RG7790*** (formerly ANA598; Fig. 4c) exhibits low nanomolar potency against genotype 1 HCV replicons (Thompson et al. 2009). In Phase I studies, RG7790, dosed for 3 days as monotherapy, resulted in a median viral load decline of 2.4 $\log_{10}$ in treatment-naïve genotype 1 infected patients (Lawitz et al. 2009). No patient showed evidence of viral rebound while on setrobuvir. A Phase II clinical trial in which treatment-naïve genotype-1 HCV infected patients were treated with a combination of PEG-IFN, RBV, and RG7790 revealed that addition of RG7790 resulted in a more rapid viral clearance than PEG-IFN and RBV alone (Lawitz et al. 2010b). A low viral breakthrough rate of <2 % was observed.

Benzothiadiazine inhibitors ***ABT-333*** and ***ABT-072*** have been studied in vitro and in Phase I clinical trials. The most frequently observed in vitro resistance mutations selected by ABT-333 and ABT-072 were C316Y, M414T, Y448H/C, or S556G. Combinations of ABT-333 or ABT-072 with PEG-IFN and RBV both resulted in significantly greater antiviral activity in treatment-naïve patients than PEG-IFN and RBV alone (SVR of 63, 52, and 9 %, respectively) (Poordad et al. 2012b). Based on these results, ABT-333 was selected for further development in an IFN-free regimen. In the so-called Aviator Phase IIb trial, 12 weeks of treatment with the IFN-free multidrug combination of ABT-333, ritonavir-boosted protease inhibitor ABT-450, ABT-267 (NS5A inhibitor), and ribavirin resulted

in a striking 97.5 % (n = 77/79) or 93.3 % (n = 42/45) SVR12 in genotype 1 treatment-naïve patients or previous null responders, respectively (Kowdley et al. 2012b).

Acylpyrrolidines are yet another class of palm site I binding compounds (Slater et al. 2007). The activity of acylpyrrolidines is affected by the M414T mutation, suggesting that these compounds bind within palm domain I (Pauwels et al. 2007). ***GSK625433*** was advanced into Phase I clinical trials, but this study was halted because of adverse effects noted in long-term mouse carcinogenicity studies (Gray et al. 2007).

4.3.4 Palm II Inhibitors

The palm II NNI binding site partially overlaps with the palm I site and is located in proximity to the active site and the junction between the palm and thumb domain. A class of ***benzofurans*** were identified as potent inhibitor of in vitro HCV replication (Gopalsamy et al. 2006). These molecules select for resistant mutants at residues L314, C316, I363, S365, and M414 (Howe et al. 2008) (Fig. 1c). ***HCV-796*** showed significant activity in early-stage clinical trials (Kneteman et al. 2009). The development of HCV-796 was halted, however, because elevated liver enzyme levels were detected in some patients treated with this agent.

A class of ***imidazopyridines*** exerts potent in vitro antiviral activity against HCV. Drug resistant variants carry mutations in palm domain II (C316Y) as well as in the β-hairpin loop (C445F, Y448H, Y452H) (Fig. 1c). This β-hairpin loop is located in close proximity to the catalytic active site and is believed to be involved in primer-independent initiation of RNA replication (Hong et al. 2001). The fact that the imidazopyridines, in contrast to other NNIs, do not inhibit the enzymatic activity of the purified RdRp, suggested that this class of molecules targets the enzyme via a unique mechanism. Recent data indeed revealed that upon intracellular activation by CYP1A, the resulting metabolite of tegobuvir, after forming a conjugate with glutathione, directly and specifically interacts with NS5B (Hebner et al. 2012). Within this class of imidazopyridines, ***tegobuvir/GS-9190*** (Fig. 4c) has demonstrated antiviral activity in HCV-infected patients. In Phase II studies tegobuvir is evaluated in combination with GS-9256, a protease inhibitor, when used as: (1) a dual antiviral therapy; (2) a three-drug, all-oral regimen with RBV; or (3) a four-drug regimen with RBV and PEG-IFN. Median maximal reductions in HCV RNA were 4.1 $\log_{10}$ IU/mL for the dual therapy, 5.1 $\log_{10}$ IU/mL for the triple therapy, and 5.7 $\log_{10}$ IU/mL for the quadruple therapy during 28 days of treatment (Zeuzem et al. 2012b). No viral breakthroughs were observed in patients treated with a four-drug regimen, whereas it was observed in the dual and triple therapy arms. The highest rate of resistance mutations was detected in the dual therapy arm. Results from this study demonstrated that addition of RBV improved the antiviral activity, delayed resistance emergence, and resulted in a greater proportion of patients achieving an RVR. In another clinical study, treatment with an all-oral, IFN-free quad regimen containing tegobuvir, protease inhibitor GS-9451, NS5A

inhibitor GS-5885 and RBV resulted in high SVR rates (Sulkowski et al. 2012b) with relapse and viral breakthrough being limited to GT1a infected patients.

5 Emerging Targets for DAA Development

5.1 The p7 ion Channel

The HCV p7 protein is a "viroporin" (virus encoded ion channel) that is critical for HCV virus infection (see chapters "Hepatitis C Virus Proteins From Structure to Function" by Moradpour and Penin and "Virion Assembly and Release" by Lindenbach, this volume). It is a small transmembrane protein that forms hexameric channels (Luik et al. 2009). The N and C termini are oriented toward the ER lumen, whereas the connecting loop faces the cytoplasm (Carrere-Kremer et al. 2002). P7 is critical for the release of infectious virions in vitro and in vivo (Sakai et al. 2003; Steinmann et al. 2007a). When its ion channel activity is blocked, virus production is significantly reduced (Steinmann et al. 2007b). A number of HCV p7 inhibitors have been identified.

Viroporin inhibitors were first approved 40 years ago for influenza A antiviral therapy. ***Adamantanes*** (***amantadine*** and ***rimantadine***) were shown to block influenza A infection by inhibition of the M2 ion channel activity. Interestingly, adamantanes are also able to inhibit H^+ transport through p7 ion channels (Griffin et al. 2003). Binding of amantadine to the p7 ion channel prevents opening of the channel that is mediated by low pH. The antiviral effect of amantadine in a HCV-infected cell culture system was shown to be genotype-dependent (Griffin et al. 2008; Steinmann et al. 2007b). In contrast, rimantadine could inhibit virus infectivity of different gentoypes. Adamantane resistance is conferred by a L20F mutation in p7 (Foster et al. 2011b). Interestingly, this mutation is also observed in HCV infected patients unresponsive to PEG-IFN, RBV, and amantadine treatment. In 1997, it was reported that amantadine treatment could induce a sustained biochemical and virological response in HCV infected patients who previously failed to respond to IFN-α monotherapy (Smith 1997). Moreover, data from meta-analysis support a specific antiviral effect in vivo (Deltenre et al. 2004). However, the possible benefit of amantadine monotherapy, or in combination with PEG-IFN/RBV remains controversial since these findings could not be confirmed in some other studies (Pessoa et al. 2012; von Wagner et al. 2008). The lack of efficacy for adamantanes in vivo can possibly be explained by the proposed minimal genetic barrier to resistance, since adamantane resistance is conferred by a single mutation that has little consequence for viral replication fitness.

Inhibition of the p7 ion channel activity was also demonstrated with various ***long-alkylated iminosugar*** derivatives (Steinmann et al. 2007b), molecules that are also known to inhibit ER α-glucosidase I and II. Long-alkylated iminosugars prevent p7 channel oligomerization by intercalating between p7 protomers. Resistance to alkylated iminosugars is mediated by a F25A mutation in p7 (Foster

et al. 2011b). In a Phase II clinical trial, iminosugar ***UT-231b*** did not demonstrate efficacy in HCV infected patients who previously failed standard therapy (United Therapeutics company website, clinical trial identifier NCT00069511).

Acylguanidine BIT225 (N-[5-(1methyl-1H-pyrazol-4-yl)-napthalene-2-carbonyl]-guanidine) was identified in a rational drug design program based on amiloride derivatives. It inhibits p7 ion channel activity in planar lipid bilayers and is active in vitro against bovine viral diarrhea (BVDV), an HCV-related virus (chapter "The Origin of Hepatitis C Virus" by Simmonds, this volume) that also encodes a p7 ion channel (Luscombe et al. 2010). BIT225 was shown to result in synergistic antiviral activity in combination with PEG-IFN + RBV and with nucleoside polymerase inhibitors in BVDV assays (Luscombe et al. 2010). In a Phase IIa clinical trial (Tanwandee et al. 2011), chronic HCV patients were administered BIT225 for 28 days in combination PEG-IFN plus RBV. Patients then continued on with SOC with PEG-IFN/RBV for a further 44 weeks. 87 % of patients receiving BIT225 achieved a complete early virological response [cEVR, defined as viral load below the level of detection (<50 IU/ml) at 12 weeks], versus 63 % for PEG-IFN and RBV alone. BIT225 also targets HIV-1 Vpu and could thus have potential in the treatment of HCV and HIV-1 coinfected patients (Khoury et al. 2010).

5.2 The NS4B Protein

HCV replication appears to be associated with intracellular membrane structures, the so-called membranous web (chapter "Hepatitis C Virus RNA Replication" by Lohmann, this volume). This structure is believed to be induced by the NS4B protein. NS4B is a highly hydrophobic protein consisting of an N-terminal part, a central part harboring four transmembrane segments and a C-terminal part (Gouttenoire et al. 2010). It is an integral membrane protein with the N- and C-terminal parts located on the cytosolic side of the ER membrane (chapter "Hepatitis C Virus Proteins From Structure to Function" by Moradpour and Penin, this volume). NS4B is also required to assemble the other viral nonstructural proteins within the apparent sites of RNA replication. NS4B and HCV RNA have been shown to colocalize to the membranous web, suggesting that NS4B is in intimate contact with viral RNA in the context of authentic viral RNA replication (El Hage and Luo 2003). NS4B binding to HCV RNA has a preference for the 3′-terminus of the HCV negative RNA strand (Einav et al. 2008). This region harbors a highly conserved secondary structure that is possibly recognized by HCV NS4B. Two classes of NS4B inhibitors have been recently identified: one class that inhibits the binding of NS4B to HCV RNA and a second class that disrupts interactions of NS4B with membranes [reviewed in (Rai and Deval 2011)].

Clemizole hydrochloride, a first-generation antihistamine, is the most advanced HCV NS4B inhibitor. Its ability to inhibit the binding of NS4B to HCV RNA was discovered during a screening with a microfluidic RNA-binding assay. It inhibits RNA binding with an IC_{50} of 24 nM and has a modest inhibitory effect on HCV RNA replication (EC_{50} for viral replication is ~8 μM for genotype 2a). In contrast,

clemizole might not be active against HCV genotype 1 (EC_{50} > 20 μM) (Einav et al. 2010). Interestingly, the in vitro antiviral activity of clemizole was shown to be synergistic with HCV protease inhibitors, whereas combinations with IFN, ribavirin, or polymerase inhibitors were found to be additive (Einav et al. 2010). It is speculated that the observed synergy could be due to an interaction between HCV NS4B and NS3, possibly involving conformational changes. Clemizole resistant variants carry mutations at position W55 and R214 in the NS4B protein and mutations in the 3′-terminus of the negative strand HCV RNA (Einav et al. 2008). The combination of clemizole with a protease inhibitor decreased the emergence of resistance against the latter. A Phase I proof-of-concept study, evaluating the safety and efficacy of clemizole as a single agent therapy in treatment-naïve HCV infected patients, is ongoing. Preliminary data reveal that clemizole, while mostly not active in monotherapy, results, when combined with PEG-IFN and RBV, in a more efficacious reduction in viral load than PEG-IFN and RBV alone (Choong et al. 2010). Clemizole-related molecules in which the benzimidazole core is replaced by an indazole core have been reported to be more potent in vitro than clemizole (Einav et al. 2010).

During a large-scale screen for small molecules that bind to recombinant NS4B, different compound classes were identified that were able to inhibit HCV replication. One of these classes, the ***pyrazolopyrimidine*** family, was further studied. ***Anguizole***, 7-[chloro(difluoro)methyl]-5-furan-2-yl-N-(thiophen-2-ylmethyl)pyrazolo[1,5a] pyrimidine-2-carboxamide, is active against genotype 1a and 1b, but is not active against genotype 2 (Bryson et al. 2010). The most commonly selected resistance mutation in vitro is H94R in NS4B, which results in a 37-fold resistance when reintroduced into a wild-type backbone. Anguizole treatment results in an altered subcellular localization pattern of NS4B. It specifically interacts with the N-terminal amphipathic helix AH2 of NS4B, and thereby inhibits the ability of AH2 to aggregate lipid vesicles. Another molecule that abrogates the ability of AH2 to aggregate lipid vesicles is ***C4*** (5-(N-methyl-N-isobutyl)amiloride) (Cho et al. 2010). In contrast to anguizole, molecule C4 is active against HCV genotype 2a in vitro. Furthermore, as determined by atomic force microscopy, C4 inhibits AH2 oligomerization which is not observed for anguizole. Both AH2 inhibitors were not able to inhibit NS4B binding to HCV RNA.

6 Concluding Remarks

In the last few years, vast progress has been made in our understanding of the HCV replication cycle and in the development of small molecule-based therapeutics for the treatment of chronic hepatitis C. The ultimate goal is to develop a well-tolerated therapeutic regimen, with minimal contraindications, efficacious against all HCV genotypes and eradication of the virus with no or minimal risk of developing drug resistance. Ideally, such therapy would no longer require the use of PEG-IFN or RBV, which are associated with inconvenient dosing and

numerous side effects. Results from recent clinical trials have firmly established the concept that a permanent cure can be achieved with IFN-free combinations of DAAs (Assis and Lim 2012; Ferenci 2012). While many novel DAAs are still being evaluated in combination with the PEG-IFN/RBV backbone, the race for IFN-free HCV therapies has started and several DAA-based, IFN-free drug combinations are being evaluated in Phase I and II clinical trials (Assis and Lim 2012; Chatel-Chaix et al. 2012). Drugs to be used in an IFN-free combination must have nonoverlapping resistance profiles. This is achievable because the DAAs under development target different viral proteins or even different drug-binding sites within the same protein. More than 40 new NS3/4A, NS5A, or NS5B inhibitors are in the development pipeline. Compelling preclinical as well as clinical evidence indicates that the current generation of nucleoside/nucleotide inhibitors of the NS5B polymerase has a broad spectrum of action on the different viral genotypes and that this is accompanied by a very high barrier to development of resistance. In light of these properties, nucleoside/nucleotide polymerase inhibitors have the potential to become the backbone of future DAA combination therapies. IFN-free combination of a nucleotide polymerase inhibitor with NS5A inhibitors have started to show viral eradication rates approaching 100 % in patients infected with different viral genotypes (Gane et al. 2012a; Sulkowski et al. 2012b). Although nucleotide polymerase inhibitors show the promise to become the cornerstone of future HCV therapy, we should also take note that—like is the case for IFN and RBV—inclusion of a nucleotide in a DAA combination regimen is not an absolute requirement. Recent clinical data have indicated that it is possible to achieve SVR in >90 % of patients by administering DAA cocktails that include an NS3/4A protease inhibitor, an NS5A inhibitor, and an NS5B NNI (Everson et al. 2012; Kowdley et al. 2012a). Second-generation NS3/4A protease inhibitors and second-generation NS5A inhibitors, with broader spectrum of action and high barrier to resistance, have now entered the development pipeline, thus expanding the number of possible ingredients for an effective combination regimen. Furthermore, the already-rich repertoire of HCV-specific DAAs is further widening, with the p7 ion channel and the NS4B protein emerging as potential drug targets. What DAA combination will turn out to be more successful and most convenient for which patients is the question that needs to be addressed in current and future research.

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Treatment of Chronic Hepatitis C: Current and Future

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Abstract Resolution of the three-dimensional structures of several Hepatitis C virus (HCV) proteins, together with the development of replicative cell culture systems, has led to the identification of a number of potential targets for direct-acting antiviral agents (DAA). Numerous families of drugs that potently inhibit the HCV life cycle in vitro have been identified, and some of these molecules have reached early to late clinical development. Two NS3-4A protease inhibitors, telaprevir and boceprevir, were approved in Europe and the United States in 2011 in combination with pegylated interferon (IFN)-α and ribavirin for the treatment of chronic hepatitis C related to HCV genotype 1. A number of other DAAs are at the clinical developmental stage in combination with pegylated IFN-α and ribavirin or with other DAAs in IFN-free regimens, with or without ribavirin. They include second-wave, first-generation, and second-generation NS3-4A protease inhibitors, nucleoside/nucleotide analogue inhibitors, and non-nucleoside inhibitors of HCV RNA-dependent RNA polymerase, inhibitors of nonstructural protein 5A and host-targeted agents, such as cyclophilin A inhibitors and microRNA-122 antagonists. The proof of concept that IFN-free regimens can lead to HCV eradication has recently been brought. This chapter provides an overview of the current treatment of HCV infection and discusses the future of HCV therapy with new anti-HCV drugs.

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Contents

1 Introduction

Treatment of chronic hepatitis C has been based for more than 10 years on the combination of pegylated interferon (IFN)-α and ribavirin, administered for 24 or 48 weeks. The endpoint of hepatitis C virus (HCV) infection therapy is the sustained virological response (SVR), characterized by undetectable HCV RNA (<10–15 international units [IU]/mL) 24 weeks after the end of treatment, which corresponds to viral eradication in more than 99 % of cases (Swain et al. 2010). This therapy yielded viral eradication in approximately 80 and 40–50 % of patients infected with HCV genotypes 2–3 and 1, respectively (European Association for the Study of the Liver 2011; Ghany et al. 2011; Ghany et al. 2009). Thanks to the development of new model systems (see chapters "Cell Culture Systems for Hepatitis C Virus" and "Animal Models for Hepatitis C", this volume), the multiple steps of the HCV lifecycle have now been unravelled, allowing for the identification of new anti-HCV drugs that block them. Two NS3-4A protease inhibitors, telaprevir (Vertex/Janssen) and boceprevir (Merck), have been approved in combination with pegylated IFN-α and ribavirin for the treatment of chronic HCV genotype 1 infection. A number of other antiviral drugs, including direct-acting antiviral (DAA) agents and host-targeted agents (HTAs) have reached preclinical or early to late clinical development. This chapter provides an overview of the current treatment of HCV infection and discusses the future of HCV therapy with new anti-HCV drugs.

2 Current Treatment of Chronic Hepatitis C

2.1 HCV Genotype Non-1

In patients infected with HCV genotypes 2, 3, 4, 5, and 6 (for genotypes, see chapter "The Origin of Hepatitis C Virus", this volume), the standard treatment of chronic hepatitis C still remains the combination of pegylated IFN-α and ribavirin (European Association for the Study of the Liver 2011). Pegylated IFN-α2a and pegylated

IFN-α2b must be administered at the doses of 180 μg/week or 1.5 μg/kg/week, respectively. For patients infected with genotype 2 or 3, the dose of ribavirin is 0.8 g/day and treatment duration 24 weeks; however, patients with genotypes 2 and 3 with baseline factors suggesting low responsiveness should receive weight-based ribavirin. In patients infected with genotypes 4, 5, and 6, the dose of ribavirin is based on body weight and treatment should last up to 72 weeks, depending on the on-treatment virological response (European Association for the Study of the Liver 2011).

HCV RNA levels should be measured at baseline, weeks 4, 12, and 24, at the end of treatment, and 24 weeks after treatment withdrawal (European Association for the Study of the Liver 2011; Chevaliez et al. 2012). A rapid virological response (RVR) is defined as undetectable HCV RNA at week 4; an early virological response (EVR) as HCV RNA detectable at week 4, but undetectable at week 12; and a slow or delayed virological response (DVR) as HCV RNA detectable at week 12 but undetectable at week 24. Patients who have an RVR probably need no more than 24 weeks of therapy, except for those infected by an HCV genotype other than 2 or 3 and who have a high baseline HCV RNA level (>400,000–800,000 IU/mL). In patients infected with genotype 2 or 3 who have a low baseline HCV RNA level and who experience an RVR, 16 weeks of therapy could be sufficient. Patients who achieve an EVR require 48 weeks of therapy, whereas patients with a DVR appear to benefit from 72 weeks of treatment. Patients with less than a 2 Log_{10} decline in HCV RNA at week 12 are unlikely to experience sustained viral eradication and should be taken off therapy (European Association for the Study of the Liver 2011; Chevaliez et al. 2012).

2.2 HCV Genotype 1

The new standard of care for patients infected with HCV genotype 1 is the combination of pegylated IFN-α, ribavirin, and an NS3-4A protease inhibitor, either telaprevir or boceprevir (Ghany et al. 2011).

2.2.1 Telaprevir-Based Therapy

Phase III Trials in Treatment-Naïve Patients

The Phase III ADVANCE trial included 1,088 treatment-naïve patients infected with HCV genotype 1 (Table 1) (Jacobson et al. 2011). Telaprevir [750 mg three times per day (tid)] was administered for 12 or 8 weeks in combination with pegylated IFN-α2a (180 μg/week) and ribavirin (1,000 or 1,200 mg/day according to body weight). The administration of pegylated IFN-α2a and ribavirin was continued until week 24 in patients who achieved an extended rapid virologic response (eRVR), defined as an undetectable HCV RNA (<10 IU/mL) at week 4 of therapy that was still undetectable at week 12. Patients without an eRVR received pegylated IFN-α2a and ribavirin until week 48. Patients in the control arm received pegylated IFN-α2a

Table 1 Summary of virological results in the ADVANCE trial

	T12PR N = 363 (%)	T8PR N = 364 (%)	PR48 N = 361 (%)
SVR (overall)	75	69	44
Rapid virological response			
RVR	68	66	9
eRVR	58	57	8
SVR according to eRVR			
eRVR+	89	83	97
eRVR−	54	50	39
Relapse			
Overall	9	9	28
Completed regimen	6	7	27
Virologic failure	8	13	NA

SVR sustained virological response; *RVR* rapid virological response, i.e., undetectable HCV RNA at week 4; *eRVR*: extended rapid virological response, i.e., undetectable HCV RNA at week 4 that remains undetectable at week 12. Virological failure: patients who meet the stopping rule (HCV RNA >1,000 IU/mL at week 4, HCV RNA decline <2 Log_{10} at week 12, HCV RNA detectable at week 24–40), patients with HCV RNA >1,000 IU/mL at week 12 even if HCV RNA decline ≥2 Log_{10}, and patients with detectable HCV RNA at the end of treatment. *T12PR*: 12 weeks of telaprevir, pegylated IFN-α and ribavirin, followed by response-guided pegylated IFN-α and ribavirin (until week 24 or 48); *T8PR* 8 weeks of telaprevir, pegylated IFN-α and ribavirin, followed by response-guided pegylated IFN-α and ribavirin (until week 24 or 48); *PR48* (*control arm*) pegylated IFN-α and ribavirin 48 weeks. *NA* not applicable

and ribavirin for 48 weeks. The stopping rule for telaprevir was an HCV RNA level >1,000 IU/mL at week 4; the stopping rule for all study drugs was an HCV RNA level decline <2 Log_{10} IU/mL at week 12, or detectable HCV RNA at weeks 24–40. SVR rates in the ADVANCE trial were significantly higher with than without telaprevir: 75 and 69 % in the 12 and 8 week telaprevir arms versus 44 % in the control arm, respectively ($p < 0.0001$ for both comparisons with the control arm) (Table 1) (Jacobson et al. 2011). In the 12-week telaprevir arm, 58 % of the patients achieved an eRVR and were treated for 24 weeks; they achieved SVR in 89 % of cases, whereas only 54 % of patients without an eRVR, who were treated for 48 weeks, cleared HCV. In addition, relapses were less frequent in the telaprevir arms than in the control arm (9 % in both telaprevir arms versus 28 %, respectively). Virological failure was observed in 13 % of cases in the 8-week telaprevir arm and 8 % in the 12-week telaprevir arm (Jacobson et al. 2011). The difference was explained by a higher breakthrough rate after telaprevir discontinuation in the former group, suggesting that longer telaprevir administration prevents subsequent failures.

Another Phase III trial with telaprevir in treatment-naïve patients, ILLUMINATE, was aimed to assess whether 24 weeks of therapy were sufficient in patients with an eRVR (Sherman et al. 2011). Telaprevir was administered for 12 weeks in combination with pegylated IFN-α2a and ribavirin at the same doses as in ADVANCE. Pegylated IFN-α2a and ribavirin were continued after telaprevir discontinuation. Approximately 60 % of the patients achieved an eRVR; they were then randomized at week 20 into either 24 or 48 weeks of total treatment duration. The global SVR rate of the trial was 72 %, including patients with and without an eRVR (Sherman

et al. 2011). Patients with an eEVR achieved SVR in 92.0 and 87.5 % in the 24- and 48-week treatment arms, respectively, confirming that patients who achieve an eRVR should not receive more than 24 weeks of therapy. Sixty-four percent of the patients who did not achieve an eRVR and were assigned to receive 48 weeks of treatment achieved an SVR (Sherman et al. 2011).

Phase III Trials in Treatment-Experienced Patients

Non-responders to a first course of pegylated IFN-α and ribavirin can be categorized as null-responders, who achieve a less than 2 Log_{10} HCV RNA level decline during the first 12 weeks of therapy, and partial responders, who achieve a 2 Log_{10} or more HCV RNA level decline at week 12, but keep detectable HCV RNA throughout treatment. Responder-relapsers achieve undetectable HCV RNA on pegylated IFN-α and ribavirin treatment, but they relapse after its cessation (European Association for the Study of the Liver 2011).

The Phase III, randomized, double-blind, placebo-controlled REALIZE trial was conducted in 662 treatment-experienced patients infected with HCV genotype 1, including responder-relapsers, partial responders, and null responders (Table 2) (Zeuzem et al. 2011). They received the triple combination of telaprevir (750 mg tid), pegylated IFN-α2a (180 μg/week), and ribavirin (1,000–1,200 mg/day according to body weight). The patients were randomized to start the three drugs simultaneously, or after a 4 week lead-in period with pegylated IFN-α and ribavirin only. In both arms, telaprevir was administered for 12 weeks and pegylated IFN-α and ribavirin were continued until week 48. Patients in the control arm received pegylated IFN-α and ribavirin for 48 weeks. Telaprevir was discontinued if the HCV RNA level was >100 IU/mL at week 4 of administration. The SVR rates were not different in the two telaprevir arms: 64 % without lead-in versus 66 % with a lead-in. They were significantly higher than in the control arm (17 %, $p < 0.001$ for both comparisons) (Zeuzem et al. 2011). As shown in Table 2, SVR rates were not different with or without a lead-in period, whatever the response to the first course of therapy. When SVR rates from both telaprevir arms were pooled, they were always significantly higher than those in the control arm: 31 versus 5 % in prior null responders, 57 versus 15 % in prior partial responders, and 86 versus 24 % in prior responder-relapsers, respectively (Zeuzem et al. 2011).

Safety with Telaprevir

Telaprevir use was associated with two key adverse reactions: skin disorders, including rash and pruritus, and anemia. These adverse events were frequent, sometimes severe, and in some cases treatment-limiting. In the ADVANCE trial, rash was more frequently observed in the 12-week telaprevir arm than in the control arm (56 versus 37 %, respectively) (Jacobson et al. 2011). This was also the case in the REALIZE trial (37 and 36 % in the telaprevir arms versus, 19 % in the control arm) (Zeuzem et al. 2011). Approximately 90 % of all rashes were

Table 2 Summary of virological results in the REALIZE trial

	T12PR48 N = 266 (%)	Lead-in T12PR48 N = 264 (%)	PR48 N = 132 (%)
Previous response-relapse			
SVR			
All patients	83	88	24
Undetectable HCV RNA at week 8	90	92	100
Relapse at week 72	7	7	65
Virologic failure	1	1	26
Previous partial response			
SVR			
All patients	59	54	15
Undetectable HCV RNA at week 8	68	58	NA
Relapse at week 72	21	25	0
Virologic failure	18	19	70
Previous null response			
SVR			
All patients	29	33	5
Undetectable HCV RNA at week 8	59	68	100
Relapse at week 72	27	25	60
Virologic failure	57	47	84

SVR sustained virological response. *Virological failure* patients who meet the stopping rule (HCV RNA >1,000 IU/mL at week 4, HCV RNA decline <2 Log_{10} at week 12, HCV RNA detectable at week 24–40), patients with HCV RNA >1,000 IU/mL at week 12 even if HCV RNA decline ≥2 Log_{10}, and patients with detectable HCV RNA at the end of treatment. *T12PR48* 12 weeks of telaprevir, pegylated IFN-α and ribavirin, followed by 36 weeks of pegylated IFN-α and ribavirin; Lead-in *T12PR48* 4 weeks of lead-in with pegylated IFN-α and ribavirin only, followed by 12 weeks of telaprevir, pegylated IFN-α and ribavirin, followed by 32 weeks of pegylated IFN-α and ribavirin; *PR48* (*control arm*) pegylated IFN-α and ribavirin 48 weeks

mild or moderate (grades 1 and 2), whereas 6 % of patients experienced severe (grade 3) rash, leading to telaprevir discontinuation. Among the more than 3,000 patients treated with telaprevir worldwide, 3 cases suggestive of Stevens–Johnson syndrome and 11 cases suggestive of drug reaction with eosinophilia with systemic symptoms (DRESS syndrome) have been reported, none of which were lethal (Picard and Cacoub 2012). The majority of rashes occurred during the first 4 weeks, with a median time of onset at 22 days. More recently, two fatal cases were reported (one toxic epidermal necrolysis and one DRESS syndrome) in patients who did not stop telaprevir after the onset of the dermatological symptoms.

Hemolytic anemia induced by ribavirin is aggravated by the addition of telaprevir, as a result of bone marrow suppression. Patients treated with the triple combination of pegylated IFN-α, ribavirin, and telaprevir more often had severe anemia, defined by hemoglobin ≤10.0 g/dL, than those receiving pegylated IFN-α and ribavirin alone (36 versus 14 %, respectively). In both the ADVANCE and the REALIZE trials, nausea and diarrhea were also more frequent in the telaprevir arms than in the control arm (difference ≥10 %) (Jacobson et al. 2011; Zeuzem et al. 2011).

Practical Use of Telaprevir

The triple combination of pegylated IFN-α, ribavirin, and telaprevir is indicated in patients infected with HCV genotype 1, who qualify for therapy. Telaprevir is administered for 12 weeks in combination with pegylated IFN-α2a or -α2b and ribavirin, followed by pegylated IFN-α and ribavirin alone for a duration that depends on the virological response (Ghany et al. 2011).

HCV RNA levels should be determined at baseline, week 4, 12, and 24, at the end of treatment, and 24 weeks after the end of treatment (Ghany et al. 2011; Chevaliez et al. 2012). According to the European and American labels, treatment-naïve patients and responder-relapsers must stop therapy at week 24 if they achieve an eRVR; if not, they should continue on pegylated IFN-α and ribavirin until week 48. Treatment-naïve patients with cirrhosis who have undetectable HCV RNA at weeks 4 and 12 may benefit from an additional 36 weeks of pegylated IFN-α and ribavirin (48 weeks total). Partial responders and null responders to a first course of pegylated IFN-α and ribavirin should receive 12 months of the triple combination followed by 36 weeks of pegylated IFN-α and ribavirin alone, regardless of their virological response. In all instances, it is recommended to halt treatment if the HCV RNA level is >1,000 IU/mL at week 4 or 12, or if HCV RNA remains detectable at week 24 (Chevaliez et al. 2012). HCV resistance testing based on sequence analysis of the protease region has no utility in clinical practice, because the results will have no impact on subsequent treatment decisions (Pawlotsky 2011).

2.2.2 Boceprevir-Based Therapy

Phase III Trials in Treatment-Naïve Patients

SPRINT-2 was a randomized, double-blind, and placebo-controlled Phase III trial designed with a 4-week "lead-in" period with pegylated IFN-α and ribavirin alone in all patients prior to the start of boceprevir administration (Table 3) (Poordad et al. 2011). Following the 4 week lead-in period with pegylated IFN-α2b (1.5 μg/kg/week) and ribavirin (600–1400 mg/day according to body weight), boceprevir was started at the dose of 800 mg tid, whereas pegylated IFN-α2b and ribavirin were continued at the same doses. Total treatment duration was 48 weeks in the fixed treatment duration arm. In the response-guided treatment (RGT) arm, the triple combination of boceprevir, pegylated IFN-α, and ribavirin was administered for 24 weeks (i.e., until week 28); patients with undetectable HCV RNA (<9.3 IU/mL) at week 8 through week 24 stopped therapy at week 28, whereas patients with detectable HCV RNA at week 8 or at any visit up to week 24 continued with pegylated IFN-α and ribavirin only until week 48. Patients with detectable HCV RNA at week 24 discontinued all study drugs. Patients in the control arm received pegylated IFN-α2b and ribavirin at the same doses for 48 weeks.

The SVR rates in SPRINT-2 were significantly higher in patients receiving a boceprevir-based regimen than in those receiving pegylated IFN-α and ribavirin alone: 63 % in the RGT arm and 66 % in the fixed treatment duration arm versus 38 % in

Table 3 Summary of virological results in the SPRINT-2 trial

	BOC/RGT N = 368 (%)	BOC/PR48 N = 366 (%)	48P/R N = 363 (%)
SVR			
Non-black	67	68	40
Black	42	53	23
Relapse			
Non-black	9	8	23
Black	12	17	14
SVR according to lead-in (*week* 4)			
Non-black			
≥1 Log_{10} HCV RNA decline	82	82	52
<1 Log_{10} HCV RNA decline	29	39	5
Black			
≥1 Log_{10} HCV RNA decline	67	61	46
<1 Log_{10} HCV RNA decline	25	31	0
SVR according to RVR (*week* 8)[a]			
Non-black			
RVR (HCV RNA undetectable)	89	91	86
No RVR (HCV RNA detectable)	37	43	31
Black			
RVR (HCV RNA undetectable)	78	82	75
No RVR (HCV RNA detectable)	32	28	21
Discontinuations due to stopping rule (*detectable HCV RNA at week* 24)			
Non-black	8	9	27
Black	17	15	46

SVR sustained virological response; *RVR* rapid virological response, i.e., undetectable HCV RNA at week 4 of boceprevir administration (week 8 of therapy). *BOC/RGT* lead-in with pegylated IFN-α and ribavirin followed by response-guided triple therapy with boceprevir, pegylated IFN-α and ribavirin (until week 28 or 48); *BOC/PR48* lead-in with pegylated IFN-α and ribavirin followed by 44 weeks of triple therapy with boceprevir, pegylated IFN-α and ribavirin; *48/PR* (*control arm*) pegylated IFN-α and ribavirin 48 weeks

[a] >3 times more patients (60 %) receiving boceprevir achieved an RVR compared to controls

the control arm ($p < 0.0001$ for both comparisons with the control arm) (Table 3) (Poordad et al. 2011). SVR rates were lower in black than in nonblack patients, but they remained significantly higher in the boceprevir arms than in the control arm in both groups: 42 % in the RGT arm and 53 % in the fixed treatment duration arm versus 23 % in the control arm in the black cohort ($p = 0.044$ and $p = 0.004$, respectively); 67 % in the RGT arm and 68 % in the fixed treatment duration arm versus 40 % in the control arm in the nonblack cohort ($p < 0.0001$ for both comparisons). In the RGT arm, 44 % of the patients achieved undetectable HCV RNA from week 8 through week 24 and were eligible to stop therapy at week 28. SVR was achieved in 96 % of them, including 97 % of the nonblack and 87 % of the black patients. In patients who did not meet criteria for early stopping and continued with pegylated IFN-α and ribavirin only until week 48, SVR was 72 %. Relapses were less frequent in the boceprevir arms than in the control arm (9 vs. 22 %) (Poordad et al. 2011).

Table 4 Summary of virological results in the RESPOND-2 trial

	BOC/RGT N = 162 (%)	BOC/PR48 N = 161 (%)	48P/R N = 80 (%)
SVR (overall)	59	66	21
Relapse (overall)	15	12	32
SVR according to previous response			
Nonresponders	40	52	7
Relapsers	69	75	29
SVR according to lead-in (week 4)			
$\geq$1 Log_{10} HCV RNA decline	73	79	25
<1 Log_{10} HCV RNA decline	33	34	0
SVR according to RVR (week 8)[a]			
RVR (HCV RNA undetectable)	86	88	100
No RVR (HCV RNA detectable)	40	43	12

SVR sustained virological response; *RVR* rapid virological response, i.e., undetectable HCV RNA at week 4 of boceprevir administration (week 8 of therapy). *BOC/RGT* lead-in with pegylated IFN-α and ribavirin followed by triple therapy with boceprevir, pegylated IFN-α and ribavirin until week 36, followed by response-guided additional 12 weeks of pegylated IFN-α and ribavirin; *BOC/PR48* lead-in with pegylated IFN-α and ribavirin followed by 44 weeks of triple therapy with boceprevir, pegylated IFN-α and ribavirin; *48/PR* (*control arm*) pegylated IFN-α and ribavirin 48 weeks

[a]46 % of patients in BOC/RGT achieved an RVR and were eligible for shorter therapy, 52 % in BOC/PR48, and 9 % in 48P/R

Phase III Trials in Treatment-Experienced Patients

The Phase III RESPOND-2 trial enrolled 403 treatment-experienced patients infected with HCV genotype 1, including prior partial responders and responder-relapsers (Table 4) (Bacon et al. 2011). Prior null responders were excluded from this trial. All patients received a 4 week lead-in with pegylated IFN-α2b (1.5 μg/kg/week) and ribavirin (600–1,400 mg/day according to body weight). Patients included in the control arm received pegylated IFN-α and ribavirin for 48 weeks. Boceprevir (800 mg tid) was added for 44 weeks in the fixed treatment duration arm. In the RGT arm, patients with undetectable HCV RNA (<9.3 IU/mL) at week 8 (i.e., at week 4 of boceprevir administration) completed all drugs at week 36, while those with detectable HCV RNA at week 8 that became undetectable at week 12 received the triple combination up to week 36, followed by an additional 12 weeks of pegylated IFN-α and ribavirin. In all treatment arms, patients with detectable HCV RNA at week 12 discontinued all study drugs.

SVR rates were significantly higher in patients receiving boceprevir than in the control arm: 59 % in the RGT arm and 66 % in the fixed treatment duration arm verses 21 % in the control arm ($p < 0.0001$ for both comparisons) (Table 4) (Bacon et al. 2011). The proportion of patients with undetectable HCV RNA at week 8 (i.e., week 4 of boceprevir administration) was 46 % in the RGT arm and 52 % in the fixed treatment duration arm. Their SVR rates were 86 and 88 % after 32 and 44 weeks of the triple combination, respectively, suggesting that patients with a RVR benefit from shorter therapy. SVR was strongly influenced by prior

treatment response, as responder-relapsers responded better than partial responders: 69 versus 40 % in the RGT arm, 75 versus 52 % in the fixed treatment duration arm, respectively (Bacon et al. 2011).

Safety with Boceprevir

Hemolytic anemia due to ribavirin is aggravated by the addition of boceprevir, as a result of bone marrow suppression. Patients treated with the triple combination of pegylated IFN-α, ribavirin, and boceprevir more often had severe anemia, defined by hemoglobin ≤10.0 g/dL, than those receiving pegylated IFN-α and ribavirin alone (50 versus 30 %, respectively) (Poordad et al. 2011; Bacon et al. 2011). Treatment was discontinued due to anemia in 2 % of patients receiving boceprevir compared to 1 % of those treated with pegylated IFN-α and ribavirin, while erythropoietin was used in 43 % of patients to maintain ribavirin dosing. The utility of erythropoietin has, however, been challenged in a recent Phase III trial showing no difference in SVR in two groups of patients who experienced anemia under the triple combination of pegylated IFN-α, ribavirin, and boceprevir who were randomized to receive EPO or dose-reduced ribavirin (Poordad et al. 2012). In addition, mild or moderate dysgeusia was more frequently reported in boceprevir-containing arms than in the control arm (40 versus 18 %, respectively) (Poordad et al. 2011; Bacon et al. 2011).

Practical Use

The triple combination of pegylated IFN-α2a or -α2b, ribavirin, and boceprevir is indicated in patients infected with HCV genotype 1, who qualify for therapy. The European and American labels indicate that boceprevir must be administered after a 4-week "lead-in" period with pegylated IFN-α and ribavirin alone (Ghany et al. 2011). Like with telaprevir, the duration of treatment depends on the virological response. HCV RNA levels should be determined at baseline, weeks 8 (i.e., week 4 of boceprevir administration), 12, and 24, at the end of treatment, and 24 weeks after the end of treatment (Chevaliez et al. 2012). Noncirrhotic, treatment-naïve patients should receive the triple combination of pegylated IFN-α, ribavirin, and boceprevir until week 28 if they have undetectable HCV RNA at week 8, or otherwise until week 36 and continue on pegylated IFN-α and ribavirin until week 48 (Ghany et al. 2011). The European label recommends that noncirrhotic responder-relapsers and partial responders receive the triple combination of pegylated IFN-α, ribavirin, and boceprevir until week 36 and then continue on pegylated IFN-α and ribavirin until week 48, whereas the American label recommends that patients receive the triple combination until week 36 and continue on pegylated IFN-α and ribavirin only if HCV RNA is still detectable at week 8. The European and American labels agree that null responders to a first course of pegylated IFN-α and ribavirin (whatever their fibrosis score) and cirrhotic patients should receive the

triple combination until week 48. Treatment should be stopped if the HCV RNA level is >100 IU/mL at week 12 or if HCV RNA is detectable at week 24. HCV resistance testing based on sequence analysis of the protease region has no utility in clinical practice, because the results will have no impact on subsequent treatment decisions (Pawlotsky 2011).

3 New HCV Drugs in Development

Virtually, every step of the HCV life cycle can be the target for one or several families of drugs that block virus production. As a result, a number of HCV DAAs and HTAs are at the preclinical and clinical developmental stage (for HCV and host cell proteins, see chapters "Hepatitis C Virus Proteins: From Structure to Function" and "Hepatitis C Virus RNA Replication", this volume). Nevertheless, only molecules that target polyprotein processing (i.e., NS3-4A protease inhibitors) and inhibitors of HCV replication through various targets and mechanisms have reached clinical development. The latter include nucleoside/nucleotide analogue inhibitors of HCV RNA-dependent RNA polymerase (RdRp), non-nucleoside inhibitors of RdRp, NS5A inhibitors, cyclophilin inhibitors (details about the targets are given in chapters "Hepatitis C Virus Proteins: From Structure to Function" and "Hepatitis C Virus RNA Replication", this volume), and a microRNA-122 antagonist. Table 5 shows drugs that have reached clinical development.

3.1 Direct-Acting Antivirals

3.1.1 NS3-4A Protease Inhibitors

A large number of so-called "second-wave, first-generation" NS3-4A protease inhibitors have reached clinical development. Simeprevir (Janssen) and faldaprevir (Boehringer-Ingelheim) are currently being assessed in Phase III trials in combination with pegylated IFN-α and ribavirin. Several other compounds are in Phase II (Table 5). Like telaprevir and boceprevir, NS3-4A protease inhibitors are peptidomimetic compounds that target the catalytic site of the enzyme and block post-translational processing of the viral protein that gives birth to the mature nonstructural proteins (see chapter "Hepatitis C Virus Proteins: From Structure to Function", this volume). NS3-4A protease inhibitors inhibit viral replication by 3.5–4.5 Log IU/mL when administered alone for a few days (Table 5) (Reesink et al. 2009; Detishin et al. 2010; Forestier et al. 2011; Manns et al. 2011; Manns et al. 2008; Lawitz et al. 2008; Goldwater et al. 2010; Hotho et al. 2009; Lawitz et al. 2010a; Lawitz et al. 2010b). Telaprevir and boceprevir are active against genotypes 1 and 2 only. Other protease inhibitors have broader genotype coverage;

Table 5 DAAs and HTAs in clinical development

Drug	Manufacturer	Current Phase	Dose	Duration	Mean/median log HCV RNA reduction
NS3-4A protease inhibitors					
Telaprevir	Vertex & Janssen	Approved	750 mg q8 h	14 days	−4.4
Boceprevir	Merck	Approved	400 mg tid	7 days	−1.6
Simeprevir	Janssen	III	200 mg qd	7 days	−4.1
Faldaprevir	Boehringer-Ingelheim	III	240 mg qd	14 days	−4.0
Danoprevir/r	Roche/Genentech	II	200 mg q8 h	14 days	−3.8
Vaniprevir	Merck	II	700 mg bid	8 days	−4.7
Narlaprevir/r	Merck	II	400 mg bid	7 days	−4.2
Asunaprevir	Bristol-Myers Squibb	II	300 mg bid	3 days	−3.3
Sovaprevir	Achillion	II	600 mg qd	5 days	−4.2
GS-9256	Gilead	II	450 mg qd	1 day	−2.7
GS-9451	Gilead	II	400 mg qd	3 days	−3.5
ABT-450/r	Abbott	II	200 mg qd	3 days	−4.1
MK-5172	Merck	II	400 mg qd	7 days	−5.4
Nucleoside/nucleotide analogue inhibitors of HCV RNA-dependent RNA polymerase					
Sofosbuvir	Gilead	III	400 mg qd	3 days	−3.7
Mericitabine	Roche/Genentech	II	1500 mg bid	14 days	−2.7
IDX184[a]	Idenix	II	100 mg qd	3 days	−0.7
Non-nucleoside inhibitors of HCV RNA-dependent RNA polymerase					
Tegobuvir	Gilead	II	40 mg bid	8 days	−1.4
Filibuvir	Pfizer	II	300 mg bid	8 days	−2.1
Setrobuvir	Roche/Genentech	II	800 mg bid	3 days	−2.9
BI207127	Boehringer-Ingelheim	II	800 mg q8 h	3 days	−3.1
ABT-333	Abbott	II	600 mg bid	2 days	−1.5
ABT-072	Abbott	II	600 mg qd	3 days	−1.6
VX-222	Vertex	II	750 mg bid	3 days	−3.7
NS5A inhibitors					
Daclatasvir	Bristol-Myers Squibb	II	10 mg qd	1 day	−3.2

(continued)

Table 5 (continued)

Drug	Manufacturer	Current Phase	Dose	Duration	Mean/median log HCV RNA reduction
PPI-461	Presidio	II	100 mg qd	3 days	−3.7
GS-5885	Gilead	II	30 mg qd	3 days	−3.3
BMS-824393	Bristol-Myers Squibb	II	50 mg qd	3 days	−3.9
ACH-2928	Achillion	II	60 mg qd	3 days	−3.7
Cyclophilin inhibitors					
Alisporivir[a]	Novartis	III	1200 mg bid	14 days	−3.6
SCY-465	Scynexis	II	900 mg qd	15 days	−2.2
microRNA-122 antagonist					
Miravirsen	Santaris	Ib	7 mg/kg/wk SC	4 weeks	−3.0

The table shows the mean or median HCV RNA level reduction in Phase Ib trials. The dosages in these trials could be different from those in subsequent Phase II or III clinical trials. Durations of administration differed between the different drugs

[a]Drugs on clinical hold due to safety concerns

however, none of the first-generation NS3-4A protease inhibitors is fully active against genotype 3. With some of these drugs, dosing intervals have been extended while enhancing patient exposure and reducing side effects by means of ritonavir boosting (100 mg/day) (Reesink et al. 2009; Lawitz et al. 2010a; Rouzier et al. 2011).

First-generation protease inhibitors have a low barrier to resistance. Indeed, a large number of amino acid substitutions conferring resistance to protease inhibitors have been shown to preexist at generally low levels in infected patients. They are selected within a few days to weeks on drug monotherapy (Kieffer et al. 2007; Sarrazin et al. 2007; Chevaliez et al. 2011). Different resistance profiles have been reported for subtypes 1a and 1b. The profiles also slightly differ between different members of the protease inhibitor family. However, cross-resistance is conferred by most of these amino acid substitutions.

Second-generation NS3-4A protease inhibitors, such as MK-5172, have pangenotypic coverage (including genotype 3) and a higher barrier to resistance (Brainard et al. 2010).

3.1.2 Nucleoside/Nucleotide Analogue Inhibitors of HCV RdRp

Nucleoside/nucleotide analogues target HCV RNA chain formation within the catalytic site of the HCV RdRp (see chapters "Hepatitis C Virus Proteins: From Structure to Function" and "Hepatitis C Virus RNA Replication", this volume). They act as false substrates for the RdRp, leading to chain termination after incorporation into the newly synthesized RNA chain. Nucleoside analogues need three phosphorylation steps to be activated. In contrast, nucleotide analogues are already phosphorylated and need only two additional phosphorylations, making them more rapidly active at the target site. Several drugs have reached clinical development, including purine and pyrimidine analogues (Table 5) (Reddy et al. 2007; Lawitz et al. 2011a). They are active on all known genotypes and subtypes. One single amino acid substitution is sufficient to confer resistance to C-methyl nucleosides. However, due to the mutational bias of HCV RdRp in favor of transitions over transversions (Powdrill et al. 2011), this mutation is less likely to occur than mutations conferring resistance to other drug families. In addition, variants bearing this amino acid substitution exhibit extremely low fitness and are thus unlikely to grow and fill in the replication space and subsequently become clinically meaningful. Therefore, nucleoside/nucleotide analogues have a high "barrier" to resistance.

The most advanced nucleotide analogue, now in Phase III in combination with pegylated IFN-α and ribavirin and in IFN-free regimens, is sofosbuvir (GS-7977, Gilead) (Gane et al. 2012). Mericitabine (Roche) is less potent. Recently, the development of another promising agent, BMS-986094 (Bristol-Myers Squibb), formerly known as INX-189, has been halted due to the occurrence of heart failures, including one fatal case, in a Phase II trial. For the same reason, a close compound, IDX184 (Idenix) is on clinical hold. These events raise concern

as to the overall safety of nucleoside/nucleotide analogues, which has been questioned in the past in human immunodeficiency virus and hepatitis B virus drug development.

3.1.3 Non-nucleoside Inhibitors of HCV RdRp

Non-nucleoside inhibitors of HCV RdRp are a heterogeneous group of drug families targeting one of four allosteric sites at the surface of the viral enzyme, including "thumb" domains I and II and "palm" domains I and II (Table 5) (Erhardt et al. 2009; Wagner et al. 2011; Larrey et al. 2009; Rodriguez-Torres et al. 2010; Lawitz et al. 2009; de Bruijne et al. 2010) (see chapters "Hepatitis C Virus Proteins: From Structure to Function" and "Hepatitis C Virus RNA Replication", this volume). Their binding alters the three-dimensional structure of the RdRp, thereby altering its catalytic function and blocking RNA replication. Their antiviral action is thus far restricted to HCV genotype 1 in most instances. Different non-nucleoside inhibitors have different antiviral potencies (Table 1) (Erhardt et al. 2009; Wagner et al. 2011; Larrey et al. 2009; Rodriguez-Torres et al. 2010; Lawitz et al. 2009; de Bruijne et al. 2010). They select amino acid substitutions conferring resistance that are generally, but not always, located in close vicinity to their target site. They have a low genetic barrier to resistance, and selected variants are generally fit. Extensive cross-resistance has been reported between drugs targeting the same site, and cross-resistance can also occur between drugs targeting different sites. Non-nucleoside inhibitors of HCV RdRp such as tegobuvir (Gilead) or filibuvir (Pfizer) have been disappointing when combined with pegylated IFN-α and ribavirin. Other compounds, such as setrobuvir (Roche), BI207127 (Boehringer-Ingelheim), ABT-333 and ABT-072 (Abbott), or VX-222 (Vertex) are currently being tested in all-oral, IFN-free treatment strategies.

3.1.4 NS5A Inhibitors

NS5A inhibitors bind to domain I of the NS5A protein and block its ability to regulate HCV replication within the replication complex, through an as-yet-unclear mechanism (for details about NS5A, see chapters "Hepatitis C Virus Proteins: From Structure to Function" and "Hepatitis C Virus RNA Replication", this volume). They could inhibit both the *cis* and *trans* functions of NS5A and perturb the function of newly formed replication complexes through redistribution of this viral protein from the endoplasmic reticulum to lipid droplets (Fridell et al. 2011; Targett-Adams et al. 2011). NS5A inhibitors are potent and have pangenotypic coverage, but the barrier to resistance of first-generation molecules is low for HCV subtype 1a (Table 5) (Gao et al. 2010; Nettles et al. 2008; Brown et al. 2010; Nettles et al. 2010; Lawitz et al. 2011b). The most advanced NS5A inhibitor, now in Phase III, is daclatasvir (Bristol-Myers Squibb).

3.2 Host-Targeted Agents

3.2.1 Cyclophilin Inhibitors

Cyclophilin A plays an important role in the HCV replication cycle by binding to both NS5A and the RdRp within the viral replication complex (see chapter "Hepatitis C Virus RNA Replication", this volume). Blocking its peptidyl-prolyl *cis–trans* isomerase enzyme activity is associated with a significant inhibition of HCV replication, through mechanisms that remain unclear (Coelmont et al. 2008). Because their target is a host protein, HTA, cyclophilin inhibitors have pangenotypic coverage and a high barrier to resistance (Coelmont et al. 2009). Variants bearing amino acid substitutions in the NS5A protein have been selected after numerous passages in cell culture. However, these substitutions confer low-level resistance and the corresponding variants exhibit low fitness both in vitro and in vivo. Alisporivir (Novartis) has reached Phase III clinical development in combination with pegylated IFN-α and ribavirin. However, the drug is on clinical hold due to cases of acute pancreatitis, including one fatal case, in patients also receiving pegylated IFN-α and ribavirin.

3.2.2 MiR-122 Antagonist

MiR-122 binding to two specific sites in the 5′ untranslated region of HCV genome is required for efficient RNA replication (see chapters by "Hepatitis C Virus RNA Translation" and "Hepatitis C Virus RNA Replication", this volume). Miravirsen is a miR-122 antagonist with potent anti-HCV activity in vitro and in vivo (Reesink et al. 2012). Unlike other DAAs and HTAs, this compound must be injected.

4 Future Treatment Strategies

4.1 IFN-Containing DAA/HTA Regimens

IFN-containing DAA/HTA regimens include both triple and quadruple combinations with pegylated IFN-α and ribavirin, which are currently being evaluated in clinical trials.

4.1.1 Triple Combination Treatment Regimens

A number of new triple combinations including pegylated IFN-α, ribavirin, and a DAA are expected to reach the market over the next 2–4 years. They will include second-wave, first-generation NS3-4A protease inhibitors, nucleotide analogues, and

non-nucleoside inhibitors of HCV RdRp or NS5A inhibitors. Evaluation of triple combination with the cyclophilin inhibitor alisporivir is currently on clinical hold.

In treatment-adherent patients, eradication of HCV infection with the triple combination of pegylated IFN-α, ribavirin, and a protease inhibitor is frequent in good responders to IFN and ribavirin. Conversely, failure to eradicate HCV results primarily from an inadequate response to pegylated IFN-α and ribavirin, which leads to uncontrolled outgrowth of resistant variants selected by the protease inhibitor (Zeuzem et al. 2011; Poordad et al. 2011; Bacon et al. 2011). Indeed, in the clinical trials that included a "lead-in" phase, consisting of pegylated IFN-α plus ribavirin for 4 weeks before adding the protease inhibitor, the probability of achieving an SVR during triple combination therapy was of the order of 80 % when the HCV RNA level had been reduced by more than 1.0 Log_{10} IU/mL, and of the order of 30 % when it had been reduced by less than 1.0 Log_{10} IU/mL at week 4 of the lead-in phase, regardless of the total treatment duration (Zeuzem et al. 2011; Poordad et al. 2011; Bacon et al. 2011). It is likely that the effect of IFN responsiveness on the outcome of triple combination therapy will be attenuated when drugs with a high barrier to resistance are used in combination with pegylated IFN-α and ribavirin, probably because sustained inhibition of virus production restores some extent of IFN responsiveness in IFN-refractory patients.

4.1.2 Quadruple Combination Treatment Regimens

Interest was recently raised about quadruple combination treatment regimens that combine pegylated IFN-α, ribavirin, and two DAAs belonging to different drug classes without cross-resistance. In a study in patients who previously experienced a null response to pegylated IFN-α and ribavirin, 10 out of 10 patients receiving a quadruple combination including an NS5A inhibitor and an NS3-4A protease inhibitor achieved an SVR (Lok et al. 2012). These results have been recently confirmed in a larger series of patients infected with HCV subtype 1b (Suzuki et al. 2012). It remains to be established whether two drugs with a low barrier to resistance do better in combination with pegylated IFN-α and ribavirin than one drug with a high barrier to resistance, or whether quadruple combinations may be further improved by including at least one drug with a high barrier to resistance.

4.2 All-Oral, IFN-Free DAA Regimens

High SVR rates can be achieved with all-oral, IFN-free drug regimens if the drug or drug combination: (1) is potent enough to efficiently shut down virus production, (2) has a high enough barrier to resistance to maintain viral inhibition throughout treatment, and (3) is able to induce a steep and consistent second-phase decline that will ultimately lead to the definitive clearance or cure of HCV-infected liver cells.

The first-phase decline is rapid and profound with most available DAAs used as monotherapies. Indeed, antiviral effectiveness of drugs belonging to the four families of DAAs having reached clinical development can reach −3.0–4.0 Log_{10} IU/mL as early as at day 3. Similar antiviral effectiveness can be achieved after more days of administration with DAAS that need to be activated or with HTAs.

HCV drugs in development can be split into two groups according to their barrier to resistance. HCV DAAs with a low barrier to resistance include first-generation NS3-4A protease inhibitors, non-nucleoside inhibitors of HCV RdRp and, for subtype 1a, first-generation NS5A inhibitors. HCV drugs with a high barrier to resistance include nucleoside/nucleotide analogues, second-generation protease inhibitors, NS5A inhibitors in subtypes other than 1a, and HTAs such as cyclophilin inhibitors (Pawlotsky 2011). The combination of two oral drugs with a low barrier to resistance was recently shown to result in early virological breakthroughs due to the selection of viral populations that were resistant to both drugs (Lok et al. 2012; Zeuzem et al. 2012). In contrast, the use of combinations including at least one drug with a high barrier to resistance, such as a nucleoside/nucleotide analogue regardless of the HCV genotype/subtype, or an NS5A inhibitor in patients infected with subtype 1b, was shown to raise a high barrier to resistance (Gane et al. 2010; Gane et al. 2011; Chayama et al. 2011).

The second-phase decline is the combined result of the natural death rate of infected cells and the rate of loss of the ability of the remaining infected cells to produce virus as their intracellular RNA degrades (cell cure) (Dahari et al. 2011). The second-phase decline is under the influence of several parameters, including antiviral treatment effectiveness, i.e., the first-phase decline (a more potent blocking of virus production is associated with a steeper second-phase decline) (Guedj and Perelson 2011); the genetic background of the host [the IL28B genotype (see chapter "Innate Immune Responses to Hepatitis C Virus" this volume) was recently shown to influence the second-phase slope without altering the initial antiviral response to a combination of DAAs (Chu et al. 2012)]; the severity of liver disease (extensive fibrosis and cirrhosis are associated with a slower clearance of infected cells, through mechanisms that remain to be elucidated). Therefore, the duration of treatment required to eradicate infection varies from one patient to another, and a fixed duration of treatment will not fit all patients. Further studies are needed to establish the appropriate treatment duration in different groups of patients receiving all-oral, IFN-free treatment regimens.

In combination with pegylated IFN-α, ribavirin has been shown to accelerate the second-phase decline and shorten the needed treatment duration (Bronowicki et al. 2006; Herrmann et al. 2003; Pawlotsky et al. 2004). Recent findings suggest that this effect is not IFN-dependent and is obtained when ribavirin is combined with potent DAAs (Zeuzem et al. 2012; Gane et al. 2011). Therefore, ribavirin addition is likely to be useful to shorten treatment duration with future all-oral, IFN-free regimens. The mechanisms by which ribavirin exerts its effects in HCV therapy remain debated (Thomas et al. 2012). They are not related to a direct antiviral effect.

5 Conclusion

With the approval of telaprevir and boceprevir in Europe and the United States, SVR rates will improve in patients infected with HCV genotype 1, while response-guided therapy will result in shortening treatment duration down to 24–28 weeks in a substantial proportion of them. However, triple combination treatment with telaprevir or boceprevir has limitations. New therapeutic approaches using combinations of DAAs with or without IFN-α and/or ribavirin are currently under study. Rapid and profound HCV RNA level declines have been observed and the concept that HCV can be eradicated by an IFN-free regimen has been proven. The ideal oral combination for universal HCV cure has not been found yet, but one can reasonably expect considerable progress in this direction over the next 5–10 years.

Conflicts of interest The author has received grant/research support from Gilead and Roche; he has served as an advisor for Abbott, Achillion, Anadys, Biotica, Boehringer-Ingelheim, Bristol-Myers Squibb, Gilead, Glaxo-SmithKline, Janssen, Merck, Novartis, Pfizer, and Roche.

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