Chapter 5
The Measles Virus Replication Cycle

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Contents

Abstract This review describes the two interrelated and interdependent processes of transcription and replication for measles virus. First, we concentrate on the ribonucleoprotein (RNP) complex, which contains the negative sense genomic template and in encapsidated in every virion. Second, we examine the viral proteins involved in these processes, placing particular emphasis on their structure, conserved sequence motifs, their interaction partners and the domains which mediate these associations. Transcription is discussed in terms of sequence motifs in the template, editing, co-transcriptional modifications of the mRNAs and the phase of the gene start sites within the genome. Likewise, replication is considered in terms of promoter strength, copy numbers and the remarkable plasticity of the system. The review emphasises what is not known or known only by analogy rather than by direct experimental evidence in the MV replication cycle and hence where

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additional research, using reverse genetic systems, is needed to complete our understanding of the processes involved.

Introductory Remarks

The focus of this chapter centres on the ribonucleoprotein (RNP) complex, which is the basic unit of infectivity of measles virus (MV) and deals with the two intimately linked processes of transcription and replication. The preceding chapters by Y. Yanagi, C. Kemper and J.P. Atkinson, and C. Navaratnarajah et al. in this volume on membrane receptor interactions have dealt with the processes of attachment and fusion, so we start our discussion and description of these processes immediately after fusion of the virion and host cell membrane, i.e. when the RNP containing the negative sense genome has entered the cell. The RNP has a density of 1.30 g/cm^3 in CsCl gradients and is comprised for more than 97% protein (Stallcup et al. 1979), which protects the RNA from RNAses (Andzhaparidze et al. 1987). In addition, the RNP is the template for transcription and replication processes.

In this volume, the structure of the nucleocapsid core is dealt with separately (see the chapter by S. Longhi, this volume), although for the sake of completeness occasionally we refer to structural aspects to illustrate mechanistic aspects of the two processes. Accompanying chapters have focussed on host factors involved in the replication of the virus (see the chapter by D. Gerlier and H. Valentin, this volume) and the ability of the virus to combat innate immune defence mechanisms of the cells and the host organism (see the chapter by D. Gerlier and H. Valentin, this volume), and again these aspects are only touched upon tangentially in this review of the MV replication cycle.

Ploidy and Particle-to-Plaque-Forming Unit Ratios

Early electron microscopy (EM) studies showed that MV populations are pleomorphic, being comprised of spherical particles with different diameters of 300–1000 nm (Casali et al. 1981; Lund et al. 1984). Interestingly, although this was an accepted part of MV biology, it took many years to demonstrate that this heterogeneity could be accompanied by incorporation of more than one RNP in a virion. Thus, many particles contain more than one functional RNP and are therefore functionally polyploid (Rager et al. 2002). Furthermore, the particle-to-plaque-forming unit (p.f.u.) ratio of MV is high (>10:1) (Afzal et al. 2003) and hence the numbers of defective RNPs introduced in a cell upon infection is not known. These defective RNPs can either be full-length or contain large internal deletions such as are found in defective interfering particles. The latter have been demonstrated to be present in both virus stocks and vaccines, where they affect replication of the standard virus significantly (Rima et al. 1977). Virions can also contain (+)RNPs, encapsidated, antigenomic, positive-sense full-length RNA molecules. These (+)RNPs

are essential intermediates in virus replication since they are required to generate progeny (–)RNPs. At present, it is thought that the budding process is unable to discriminate between (+) and (–) RNPs and genome containing RNPs are not preferentially packaged. This suggests that cells infected at a multiplicity of infection (MOI) of greater than 0.1 will contain a number of RNPs, many of which may be defective. Nothing is known about competition between multiple RNPs or whether there exists a defined, saturable set of intracellular replications site where the MV replication can progress. It remains unknown if the virus uses specific sites on the plasma membrane to enter a cell, and hence there is no information on potential coupling of entry and intracellular replication sites mediated, for example, by activities of the cytoskeleton.

The RNP

Although it is clear that more than one RNP can be found in a cell for the sake of simplicity, we will describe the ensuing processes of viral RNA synthesis, i.e. transcription and replication and mRNA translation assuming that a single RNP containing the full-length genome has entered the cell.

In addition to the genomic RNA, the RNP is comprised of the nucleocapsid (N) protein, the phospho- (P) protein and the large (L) protein (Table 1). The RNA-dependent RNA polymerase (RdRp) complex consists of the L protein and the P protein and functions both as the viral transcriptase and replicase. After negative staining and EM, the RNP appears as a helical nucleocapsid approximately 1 μm in length and 18–21 nm in diameter, with a central core of 5 nm (Waterson 1962). How the RNA and proteins are arranged in the RNP is not yet clear, but what is known is addressed in the chapter by S. Longhi, this volume. Three-dimensional reconstructions, calculated from cryo-negative stain transmission electron micrographs, indicate that the RNP exhibits extensive conformational flexibility (Bhella et al. 2004). As yet, X-ray crystallographic studies, such as those for lyssaviruses and Borna disease virus (Albertini et al. 2006; Green et al. 2006; Luo et al. 2007; Rudolph et al. 2003), have not yet been reported for MV. The MV RNP displays the characteristic herringbone structure of all paramyxovirus nucleocapsids. Electron microscopy studies of replicating intermediates have demonstrated that the herringbone points to the 5′ prime end of the genome (Thorne and Dermott 1977).

Transport of the RNP from the Plasma Membrane to the Intracellular Sites for Transcription and Replication

The first question that arises is: does replication occur diffusely throughout the cell cytoplasm or within dedicated viral factories? When the N and P proteins are localised by indirect immunofluorescence (IIF) both in acutely or persistently infected cells, the resulting intra-cytoplasmic staining pattern is punctuate (Duprex et al.

Table 1 Annotation of the measles virus genome sequence

Numbers in antigenome (positive sense)	RNA	Open reading frame	Number of codons	Protein
1–52	Leader	–	–	There is a small ORF of potentially 4 amino acids.
53–55	Leader N Ig			
56–1744	N gene	108–1682 (N)	525	Nucleocapsid protein: phosphorylated protein which encapsidates the RNA and protects it from RNAses.
1745–1747	N-P Ig			
1748–3402	P/V/C gene	1807–3327 (P)	507	Phosphoprotein associated with RNP in polymerase complex as well as a chaperone for the N protein.
		1807–2702 +1G (V)	231 + 69 = 300	Prevents interferon induced transcriptional responses in MV
		1807–2702 + 4G (V)	231 + 70 = 301	
		1829–2386 (C)	186	Prevents interferon-induced transcriptional responses; acts as an infectivity factor; inhibits transcription
3403–3405	P-M Ig			
3406–4872	M gene	3438–4442 (M)	335	Matrix protein: hydrophobic protein on inner leaflet of membrane; inhibits transcription
4873–4875	M-F Ig			
4876–7247	F gene	5458–7107 (F)	550	Fusion glycoprotein cleaved to a disulphide linked F2-F1 complex by furin-like proteases that are generally available; acylated
7248–7250	F-H Ig			
7251–9208	H gene	7271–9121 (H)	617	Haemagglutinin: attachment glycoprotein that interacts directly with entry receptors
9209–9211	H-L IG			
9212–15854	L gene	9234–15782 (L)	2183	Large protein in RdRp complex with P; RNA synthesis; capping and polyadenylation
15855–15857	L-tr Ig?			
15858–15894	Trailer			Transcript not identified

Ig nontranscribed intergenic sequence in positive sense

1999; Ludlow et al. 2005). These inclusions appear to be the sites where mRNAs encoding the N-, P- and matrix (M) proteins are localised using in situ hybridisation (ISH). Furthermore, it is clear that when we studied the distribution of L proteins that were tagged with enhanced green fluorescent protein (EGFP) or with a short c-Myc epitope tag, that these are also distributed unevenly and show a similar punctuate staining patterns (Duprex et al. 2002). Indirect immunofluorescence has been used to prove that the L protein co-localises with N and P and, more surprisingly, the M protein (M. Ludlow et al., unpublished observations). All these pieces of evidence suggest that specific viral factories exist in the cell. We have also observed an association of the L protein with the plasma membrane in cells acutely infected with the closely related rinderpest virus (Brown et al. 2005) and in persistently infected cells (M. Ludlow et al., unpublished observations).

The main site for localisation of these large viral factories is perinuclear, especially in the early stages of infection, and hence the question has arisen whether these factories are associated with mitochondrial organising centres and aggresomes, as is the case in other viral infections (Wileman 2007). Currently, it is not clear if the incoming RNP is actively transported to these sites due to an interaction with components of, for example, the cytoskeletal networks. MV can grow, albeit at reduced titres, in cells treated with cytochalasin B, which leads to enucleation of cells and severe effects on the cytoskeleton (Follett et al. 1976), suggesting that if there is a role for these components they are not absolutely required. In our study of MV infection in astrocytoma cell lines using a recombinant MV which expresses EGFP (MVeGFP), no alteration of the actin, tubulin, or vimentin components of the cytoskeleton was observed in either cell type, whereas a disruption of the glial-fibrillary-acidic protein filament network was noted in MVeGFP-infected U-251 cells (Duprex et al. 2000a).

The Genome and Viral Evolution

The MV genome is a nonsegmented RNA molecule of negative polarity, i.e. opposite polarity to viral messenger RNAs (mRNAs), with a length of 15,894 nucleotides (Blumberg et al. 1988). It contains six transcription units (genes), which are separated from each other by trinucleotide intergenic (Ig) sequences, and 3′ and 5′ terminal sequences containing genomic and anti-genomic promoters, respectively. The gene order was originally inferred from Northern blot analysis of mono- and polycistronic mRNA (Barrett and Underwood 1985; Richardson et al. 1985; Rima et al. 1986) and thereafter by genomic sequencing (Bellini et al. 1985, 1986). The transcription units encode at least eight proteins, six of which are components of the virion (Fig. 1 and Table 1). Protruding from the envelope are glycoprotein spikes consisting of the fusion (F) protein and the haemagglutinin (H) protein (see the chapter by C. Navaratnarajah et al., this volume). The inner leaflet of the virion membrane is coated with the hydrophobic M protein. At least two additional proteins are generated from the gene encoding the P protein. The C protein is translated

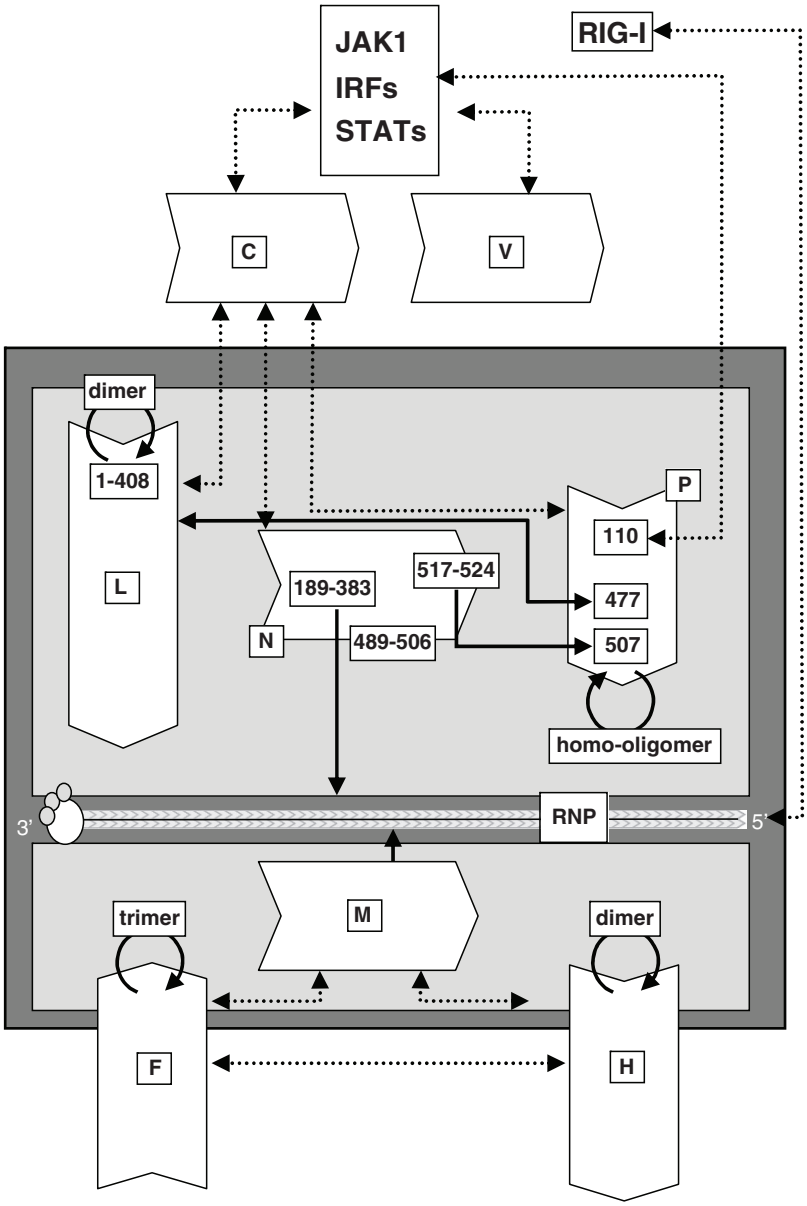

Fig. 1 The interactome of measles proteins with each other and selected host proteins. The interaction between viral proteins is depicted with a *broken arrow* if the domains have not been specified on both binding partners or when they are inferred from experimental observations, e.g. the effect of the C protein on the transcription–replication complex. A *solid arrow* indicates that the interacting domains on both partners have been determined by mutational analysis. Specific interactions of viral and host proteins are detailed in the chapter by D. Gerlier and H. Valentin, this volume

from the P mRNA from an overlapping open reading frame (ORF) (Bellini et al. 1985). The V protein is generated from an edited transcript of the P gene (Cattaneo et al. 1989a) (Table 1).

There are at least 22 known MV genotypes which are grouped in eight clades (Riddell et al. 2005; Rima et al. 1995). Seventeen genotypes are currently active in the world, and the remainder are probably extinct. So far, it has not been possible to link genotypic differences to specific biological phenotypes. There was early speculation that African B genotypes were responsible for the high levels of mortality associated with the infection on that continent, but, after more detailed epidemiological and sociocultural analysis, this has not been shown to be the case. No differences have been observed between genotypes in genome organisation or in the basic processes of transcription and replication. Furthermore, the lengths of all genes are conserved; there are no variations in editing, although this has been studied only for limited number of virus strains; the Ig sequences are conserved and, apart from subacute sclerosing panencephalitis (SSPE) strains, the transcription process is conserved. Morbilliviruses are unique within the *Paramyxoviridae* in that they contain a very long noncoding region of nearly 1 kb between the M and F ORFs (Bellini et al. 1985; Richardson et al. 1986; Table 1). The region is GC rich and is likely to fold into complex secondary RNA structures. The function of this region is not known, although the sequence is largely conserved in length and the position of the nontranscribed Ig trinucleotide spacer is also fixed since it is located almost in the middle of this region (Table 1). This long untranslated region (UTR) is a unique feature of morbilliviruses and, although dispensable for the growth of the virus in vitro (Takeda et al. 2005), the suggested functions include mRNA stabilisation and regulation of translation.

All MV strains appear to show the CpG and UpA suppression that is characteristic of most mammalian RNA viruses (Rima and McFerran 1997). In the case of MV, the CpG motifs that are most stimulatory for toll-like receptors (TLRs) are 4.3 times as infrequent as the ones that are least stimulatory. Recently, it has been shown that RNA oligonucleotides are able to stimulate the innate immune system, although the interacting partners are not well established and this may involve as yet unknown TLR-like molecules (Sugiyama et al. 2005). UpA suppression, which is also widespread throughout the RNA viruses, may be linked to the fact that in cells in which the innate immune responses are elicited, RNaseL may target RNA molecules containing this sequence for destruction (Washenberger et al. 2007).

In conclusion, it is clear that the basic processes for growth and replication of MV virus are conserved throughout all genotypes.

Transcription

In describing the processes of MV transcription and replication, it must be remembered that much of this is derived by analogy to the better studied paramyxoviruses and other members of the *Mononegavirales*, particularly vesicular stomatitis virus

(VSV) and Sendai virus (SeV) (for reviews see Lyles and Rupprecht 2006; Lamb and Parks 2006). The difficulty in reliably obtaining high MOIs, the relative low level of gene expression as well as the lack of a dependable in vitro transcription system has hindered the study of molecular biology of MV.

In the genome, the six transcription units are preceded by a region at the 3′-terminus coding for the 56 nucleotide leader RNA and are followed at the 5′-terminus by a trailer region of 40 nucleotides (Blumberg et al. 1988). The sizes of the various transcription units and the coding regions, and the derived sizes of the various proteins, are given in Table 1. The entire nucleotide sequence has been compiled for the measles prototype strain Edmonston (Radecke and Billeter 1995), which was isolated in 1954 and passaged in tissue culture by John Enders (Enders and Peebles 1954). In total approximately 30 vaccine and wild-type strains have been completely sequenced.

Virus and Host Proteins Associated with the RNP

The RNP consists of the genome or antigenome RNA and 2,649 copies of the N protein. This figure is based on the fact that one protein can protect six nucleotides (Calain and Roux 1993). Associated with this structure are two additional viral proteins: the P protein, in approximately one-tenth that number, and roughly 20–30 copies of the L protein (Lamb et al. 1976; Portner et al. 1988). Every virion must contain a nucleocapsid/L/P complex to ensure infectivity. The L protein contains the enzymatic activities of the RdRp, which acts as both the viral transcriptase and replicase. At least two additional nonstructural proteins are generated from the gene encoding the P protein. The C protein is translated from the P mRNA from an overlapping ORF (Bellini et al. 1985). The initiation codons for P and C are not in optimal context, and hence cap-dependent scanning appears to allow the ribosome to initiate translation at either the start of the P open reading frame at position 60 in the mRNA or that of the C ORF at position 82. The C protein is composed of 186 amino acids and it has been proposed to function both as an infectivity factor and an inhibitor of type I interferon-induced transcriptional responses and signalling (see the chapter by D. Gerlier and H. Valentin, this volume). Virus mutants lacking this protein can be propagated successfully in tissue culture (Radecke and Billeter 1996). However, more relevant to the present discussion, the C protein has been shown to inhibit expression of reporter genes in minigenome rescue assays (Bankamp et al. 2005). As these are combined replication and transcription assays, the actual step that is inhibited by the C protein of MV is not clear.

The V protein is generated from an edited transcript of the P gene (Cattaneo et al. 1989a). A recombinant virus which fails to express this protein has been generated (Schneider et al. 1997). Interference by with STAT-inducible transcription, which may lead to virus-induced cytokine inhibition in vivo, and promotion of STAT1 degradation have been observed (see the chapter by D. Gerlier and

H. Valentin, this volume). It is amino-co-terminal with the P protein for the first 231 amino acids and therefore shares the main phosphorylation sites of P, including the important tyrosine residue at position 110 (Devaux et al. 2007; Fontana et al. 2008). The role of tyrosine 110 in modulating interactions with the innate immune system is discussed in detail in the chapter by D. Gerlier and H. Valentin, this volume. A 69-residue cysteine-rich tail, which contains a zinc-binding finger domain, follows the common amino terminal 231 residues (Liston and Briedis 1994). This domain represents one of the most conserved products encoded by the P genes of paramyxoviruses. The V protein also has been implicated in affecting the expression of reporter genes in the minigenome expression assay (Parks et al. 2006; Witko et al. 2006), but this has not been confirmed in other studies. Thus, though the V and C proteins may affect the gene expression in these assays, viruses in which the expression of these proteins is ablated grow well in tissue culture, indicating that they are not essential components of the RdRp.

The RdRp can only use the RNP and not naked RNA as a template (Rozenblatt et al. 1979). Although the L/P complex contains the requisite catalytic activities for the RdRp, its activity may also involve or be modulated by host cell proteins and/or cytoskeletal components such as tubulin, which has been reported to stimulate in vitro RNA synthesis (Horikami and Moyer 1991). The stoichiometry of the complex is not known for MV and has been suggested to be L/P_4 for SeV and L/P_2 for VSV (Chen et al. 2006; Curran and Kolakofsky 2008). The P protein of the closely related morbillivirus RPV is a tetramer (Rahaman et al. 2004). Oligomerisation of the MV L protein has been inferred (Cevik et al. 2003), but this remains to be demonstrated directly. The interaction of the RdRp complex with the RNP probably involves a bridging function for the P protein, which has binding sites for both the N and L proteins. Direct L–N interactions have not been documented. In Fig. 1, we have attempted to synthesise the interactome of MV proteins with themselves (homo-oligomerisation), each other and with selected host factors (hetero-oligomerisation). The complexity of the diagram indicates the plethora of interactions exhibited by viral proteins even in a virus, which only expresses eight proteins. Apart from the N and P and L interactions, relatively few direct deletion analyses have been performed to locate the interacting sequences. The interactions of M with the F and H proteins and the oligomerisation of the glycoproteins themselves are described in the chapter by C. Navaratnarajah et al., this volume.

The original comparison by Poch et al. (1990) of five L proteins from *Mononegavirales* identified six (I–VI) conserved domains. Few have been linked to specific functions apart from domain III, which contains the canonical sequence GDNQ as a potential RdRp active site. For the morbilliviruses, we refined this analysis and found that there are two nonconserved hinges in the L protein separating three largely conserved domains (McIlhatton et al. 1997). The first (D1) contains the Poch domains I and II, the second (D2) domains III, IV and V, and the third (D3) containing conserved domain VI. The latter has recently been shown to be part of a structure involved in 2-O-methyl transferase activity (Ferron et al. 2002). Sequence comparison of the L proteins of vaccine and wild-type strains of MV confirmed that genotypic differences exist but no functional

differences were observed, and this study was not able to identify consistent changes between vaccine and wild-type strains (Bankamp et al. 1999).

In the *Mononegavirales* prototype, VSV, there is evidence that the transcriptase complex has a number of host proteins associated with it such as EF1α (Qanungo et al. 2004), which is not present in the replicase version of the complex. However, to date this has not been confirmed for paramyxoviruses, and the question of whether the transcriptase complex is the same as the replicase complex has not been resolved. Furthermore, although two forms of the RdRp complex may contain the same proteins, it is possible that they might differ in the phosphorylation state. Whilst the L protein of MV is not phosphorylated, both the N and P proteins are. Phosphorylation of the N protein, and whether this plays any role in the viral life cycle, has not been studied. It is known that threonine and serine but not tyrosine residues are involved. The same is the case for the P protein, where there are multiple phosphorylation sites. At least one of serine residues at positions 86, 151 and 180 are phosphorylated by casein kinase II. Tyrosine 110 is also phosphorylated (Devaux et al. 2007). Temporal analyses to gain insight in potential controlling roles for phosphorylation and function studies of the phosphorylation of viral proteins using reverse genetics remain to be done.

General Description of the Transcription Process

In the genome, the six transcription units are arranged in the order 3′-N-P(V/C)-M-F-H-L-5′ (Dowling et al. 1986; Rima et al. 1986). The sequence of the nontranscribed intergenic regions between the first five genes in the genome (negative strand) is 3′-GAA-5′. There is a single nucleotide difference in the sequence between the H and L genes which is 3′-GCA-5′ (negative sense). These Ig trinucleotides are not transcribed by the RdRp. The genome consists of a multiple of six nucleotides (6 × 2,649). A suggested explanation for the rule of six (Calain and Roux 1993), which is obeyed by MV (Sidhu et al. 1995), is that each N protein associates with exactly six nucleotides of RNA. The 2649 nucleocapsid proteins and the RNA form a helical RNP with 2649/13 = ~204 helical turns. The number of helical turns was determined to be 204 (Lund et al. 1984), remarkably accurately and long before the rule of six and the number of N proteins per helical turn were established.

In MV there two recognised sequence elements for the promoter sequence, the so-called A box which comprises the first 15 nucleotides at the 3′ end of the genome and antigenome, and the B box, which is represented by the sequence $GN_5GN_5GN_5$ at positions in the 14, 15 and 16th hexamers (see the chapter by S. Longhi, this volume). Structural studies of the RNP suggest this would place the B box immediately beside the A box in the first and second helical turn of the RNP, which has been proposed to contain approximately 13 N protein molecules per turn and hence 13 × 6 = 78 nucleotides (Lamb and Parks 2006). Mutagenesis has demonstrated that the three G residues in the B box are essential and this element

is conserved in all morbilliviruses (Rennick et al. 2007; Walpita 2004). Liu et al. (2006) analysed the genome termini of a large number of MV vaccine and wild-type strains and changes were only found in the 3′ genome terminus at positions 26 and 42. Minigenome expression assays indicate that these naturally occurring nucleotide variations in the 3′ leader region affected the levels of reporter protein synthesis (Liu et al. 2006).

It is not clear whether transcription always starts at the 3′ end of the template producing the 56 nucleotide leader or if a capped mRNA can be generated directly from the start of the N gene. The 56-nucleotide leader RNA has been found linked to the mRNA encoding the MV N protein (Castaneda and Wong 1990). This in itself should render the leader-N transcript untranslatable, as it would place an AUG codon and an ORF encoding four amino acids upstream of the major ORF encoding the N protein. Free leader RNA has been not been demonstrated, certainly not at the high levels one may expect in infected cells if the transcription process always involved the start of copying at the 3′ end of the genome, which should lead to at least equimolar amounts of the leader and N mRNA in the cells. However, there are caveats. Absence of evidence is not evidence of absence and the relative stability of such a small RNA molecule which has no 5′ cap or 3′ poly-A tail may make it prone to rapid degradation. Castaneda and Wong showed that there are a large number of leader-N and leader-N-P read-through RNA molecules which are polyadenylated but not capped in both acute and persistently infected cells. These are found in structures with the density of RNPs and not on polysomes (Castaneda and Wong 1990). This, and the fact that these molecules were not made when cells were treated with cycloheximide, indicates these leader-N-read-through products are probably derived from abortive replication processes rather than from transcription. They could not detect free leader and estimated their lower detection level to be 1/400th of the level expected if leader and N were expressed in equimolar amounts. Hence these data suggest that transcription starts not with a leader but with the beginning of the N gene. Recently proposed mechanisms for nonsegmented negative-strand RNA synthesis indeed suggest that the polymerase of well-studied *Mononegavirales* may be able to approach the 3′ end of the template and scan until it starts to copy the template at the N start site and that the synthesis of free leader is a relatively infrequent event as is found to be the case for MV (Curran and Kolakofsky 2008).

Modifications of mRNAs

Transcription starts, or restarts in the case of the second and subsequent transcription units, with a consensus gene start (GS) sequence of AGGRNNc/aARGa/t at the 5′ end of each transcript. Similar to what is observed in all *Mononegavirales*, the MV mRNAs have been shown to be capped (Hall and ter Meulen 1977). An O-methyl transferase motif has been identified in the D3 domain of the L protein of MV using bioinformatics-based approaches (Ferron et al. 2002). It is assumed that this would be involved in the formation of the methylated cap structure, which

contains an inverted guanosine residue. However, direct evidence from reverse genetics has not been published to support this. As MV is a strictly cytoplasmic virus with no known involvement of the nucleus, demonstrated by the fact that the virus grows equally well in enucleated cells (Follett et al. 1976), it is assumed that splicing of mRNAs does not take place. There is indeed no evidence for this from any cloning and sequencing studies that have been undertaken on mRNAs. However, there is evidence that the N and C proteins are translocated to the nucleus, although no associated functionality has been described (Huber et al. 1991; Nishie et al. 2007). Accumulation of RNP is particularly prominent in the nuclei in infected cells in cases of SSPE (Chui et al. 1986). All mRNAs are polyadenylated with the length of the tails similar to those of normal cellular mRNAs (Hall and ter Meulen 1977). The process used to generate the poly-A tail requires a signal sequence motif at each gene end (GE). When the RdRp complex encounters the GE sequence of 5′-RUUAUAAAACTT-3′ (positive sense), stutters or slips on the U-rich template sequence adding between 70–140-A residues to the 3′ end of the nascent mRNA (Hall and ter Meulen 1977). The GE signal is characterised by a conserved RUU motif in all morbilliviruses followed by an A-rich block of eight nucleotides interspersed with one or two pyrimidines. No mutational analysis has been performed to assess the functional importance of any of these sequence signals in MV. Mechanistically there is no information on the process by which the RdRp stops polyadenylation when the length of the tail reaches the desired length. Interestingly, although generated by entirely different mechanisms, this size range mirrors that of cellular mRNAs.

A similar signal to the GE is found in the middle of the P gene 5′AUUAAAAAGGG-CAC-AGA3′. In this case, this leads not to polyadenylation but to the nontemplated insertion of extra G residues due to RdRp slippage or stuttering on the UUUUUCCC template. This is referred to as co-transcriptional editing and leads to the generation of transcripts encoding the V and W proteins (Cattaneo et al. 1989a). Stuttering occurs to a very limited extent at the so-called editing site. The V protein is translated from an mRNA with one extra G, which shifts the ORF after the first 231 codons for P and gives rise to the unique carboxy terminal 69 amino acid extension. The protein (W) which would result from incorporation of two extra Gs has not been identified in morbillivirus-infected cells, possibly because editing is tightly controlled, meaning that in most cases editing leads to the insertion of only a single extra single G residue. In other members of the *Paramyxovirinae*, editing is less tightly controlled and a number of extra G residues (1–4) are incorporated during the process, giving rise to V-, W- and P-like proteins with extra glycine residues (Lamb and Parks 2006). The percentage of edited (V) as opposed to nonedited (P) mRNAs in acute MV-infected cells varies from 30% to 50%. This may depend on the cell type in which infection takes places. There is no evidence that other mRNAs of MV are similarly edited. The sequence motif of RYY followed by an A-rich stretch is found in several places in the genome, and we have analysed one of the at the 3′ end of the H mRNA, which through editing could access conserved ORFs in all MV strains, giving rise to different C terminal extensions of the H protein. However, at the RNA level no evidence was found that indicated that the process took place at this site (position 8943).

Phase and Transcription

A consequence of the fact that precisely six nucleotides associate with a single N protein is that each nucleotide has a specific phase (positions 1, 2, 3, 4, 5 or 6) with respect to the N protein and 3′ end of the genome. Minigenomes that do not obey the rule of six are neither replicated efficiently nor readily encapsidated (Sidhu et al. 1995). Comparative analysis of morbillivirus genomes has established that the phase of the transcription starts sites is conserved between the various viruses, although not between the genes (Iseni et al. 2002; Rima et al. 2005). Thus, the transcription start site for all N mRNAs of morbilliviruses is position 2, meaning that the U that resides in the template is the second one protected by the N protein molecule. The phase for the start sites of the P and L genes is also 2; for M it is 4 and for the H it is gene 3. The only variation appears to be in the phase of the start site for the F gene, as it is 3 in MV and 2 for rinderpest virus canine distemper virus (CDV), phocine distemper virus and the cetacean morbilliviruses. This raises the question: is phase important? Phasing is conserved but obviously not between genes in one genome. However, variations in morbillivirus genome lengths always involve the deletion or insertion of multiples of six nucleotides primarily, but not exclusively, within the UTRs. As such, this preserves the phasing of the genes. Hence, it appears there is a significant biological pressure for phase conservation. Furthermore, Iseni and coworkers demonstrated that phase affected co-transcriptional editing patterns in SeV (Iseni et al. 2002). This indicates that the RdRp complex has at least a potential to sense phase and encapsidation imposes a higher order structure on the genetic entity that is the RNP, similar to, for example, the histone code (Iizuka and Smith 2003).

The topology of the transcription, and for that matter the replication processes, is unknown. From first principles, the energy needed to have both the RdRp and the de novo RNA wind around the RNP as the RdRp copied the template would be significant. Topologically, this could be achieved with the least energy expenditure if the RdRp remained in a fixed position and helical RNP rotated around the axis of its length. Current models suggest that the interactions between the RNA and the N protein are either broken or that at least the RNA template is available for base recognition.

Dynamics of RNA Accumulation and Transcription Attenuation

When the RdRp reaches the GE, one of four processes may occur, the complex may:

1. Polyadenylate the mRNA, skip transcription of the intergenic sequence, commence transcription of the next gene, add an inverted guanosine residue to the 5′ end of the mRNA and finally methylate the newly formed cap;
2. Polyadenylate the mRNA and drop off the template;

3. Ignore the GE, Ig and GS sequences altogether, transcribe them all and thereby allow the formation of a read-through mRNA, which can contain as many as five ORFs (5′-N-P-M-F-H-3′ transcripts have been detected);
4. Start limited polyadenylation and then continue with the formation of a read-through mRNA. No evidence has been found for this latter process to occur in MV though it has been demonstrated to occur very occasionally in other *Paramyxovirinae* (Paterson et al. 1984).

The finite chance for the polymerase complex to leave the template gives rise to a gradient of gene expression in which the promoter proximal (e.g. N and P/V/C) genes are transcribed much more frequently into mRNA than the promoter distal H and especially the L gene.

Early studies examining RNA synthesis used ^{32}P orthophosphate labelling in the presence of actinomycin D to inhibit cellular but not viral RNA synthesis. Long labelling periods were required to achieve detectable amounts of incorporation, and hence it was not possible to carry our pulse chase experiments to assess rates of synthesis and degradation (Barrett and Underwood 1985). In the most successful experiments, 3- to 4-h labelling with carrier-free orthophosphate indicated that each of the viral mRNAs accumulated to levels that were in accordance with what would be expected from the transcription gradient. In one study in which cells were shifted to 39°C, at which little or no transcription takes place, it was clear from these and other experiments (Schneider-Schaulies et al. 1991) that none of the viral mRNAs decayed more quickly than the others and these experiments also showed a very low rate of turnover of the various viral RNA species in the infected cell (Ogura et al. 1987). The levels of the various mRNA species were analysed by Northern blots. Such approaches measure steady state levels at the time of harvesting the cells rather than dynamics.

Seminal studies were conducted by Cattaneo et al. (1987) and Schneider-Schaulies et al. (1989). Both used calibrated amounts of in vitro transcripts as internal controls but the two studies came to different conclusions about the steepness of the transcription gradient. The relative frequency of each of these events is approximate 0.7 for stop and restart, 0.2 for RdRp to leave the template and 0.05–0.1 for read-through in several of the *Mononegavirales*, where this process has been studied in detail, e.g. in VSV and Sendai virus (Gupta and Kingsbury 1985; Banerjee et al. 1991; Whelan et al. 2004). The paper by Cattaneo et al. indicates that in acutely infected Vero cells there are approximately 26,800 copies of the N mRNA but only approximately 9,100 copies of the P mRNA. After the P gene, the transcription gradient becomes less steep and the remainder of the mRNAs are present at amounts approximately 0.8 times the previous, e.g. 6,700 M mRNA copies, 5,400 F mRNA copies and 4,000 H mRNA copies. There appears to be a very high level of transcription attenuation at the H-L boundary, as only 400 copies of the L mRNA were detected. The Ig sequence between the H and L genes (3′-GCA-5′ in the template) is different from the consensus 3′-GAA-5′, and this has been suggested to be associated with very high levels of transcription termination. Alternatively, these results may reflect difficulties in the isolation of the very large L mRNA molecules during

polyA selection of mRNAs or double-stranded RNA formation due to the presence of defective interfering particles which may contain parts of the L gene during the RNA isolation. In the studies by Schneider-Schaulies et al., the copy numbers of the L mRNAs were not assessed (Schneider-Schaulies et al. 1989). In general, the transcription gradient was found to be less steep and the frequency of stop and restart events was approximately 0.75 ± 0.04 at the intergenic boundaries. Plumet et al. approached the enumeration of copy numbers of viral RNAs using a carefully calibrated real-time, reverse transcription-polymerase chain reaction (RT-PCR) using primer sets that gave as near equimolar products for each of the genes as was possible (Plumet et al. 2005). Their data support those from Cattaneo et al. and show a very severe attenuation after the N gene. Infected cells were found to contain 31,000 copies of N and only 4,800 copies of the P/V mRNA.

Re-analysing the older literature – ^{32}P orthophosphate-labelled mRNAs, Northern blots and protein synthesis experiments using pulse labelling with various labelled amino-acids including ^{35}S and ^{14}C amino acids – does not indicate that there is a large difference in the amount of the N and P mRNAs. However, these experimental approaches can obviously not prove this point. One would have to assume very large variations in rates of translation, specific activities of probes for different genes, etc., to reconcile the data with the presence of very significant attenuation after the N gene. Apart from the fact that this does not make much sense in terms of protein requirements, my own attempts (B.K. Rima, unpublished data) at quantifying the mRNAs in Northern blots using the same protocol as Cattaneo et al. did not support attenuation at this point in the genome to be larger than that observed at other intergenic boundaries. In contract, in several experiments we failed to observe any attenuation as the copy number of P mRNAs was the same as that of the N mRNAs. This is similar to the result obtained by Rennick et al. who used bi-cistronic minigenomes expressing fluorescent reporter proteins (Rennick et al. 2007). Thus the precise levels of mRNA and attenuation levels remain to be determined.

Plumet et al. described four phases in the dynamics of RNA accumulation (Plumet et al. 2005). In the first phase (0–6 h.p.i.) there is linear accumulation of mRNAs, probably resulting from RdRp molecules that were attached to the template in the incoming RNP. During this phase, transcription is not sensitive to inhibition by cycloheximide. From the accumulation of various mRNAs, the authors calculated an in vivo RNA synthesis rate of three nucleotides per second (nt/s) for the MV RdRp. This corresponds well with the rates for in vitro RNA synthesis determined for VSV and Sendai virus of 3.5 and 1.7 nt/s, respectively (Barr et al. 2002; Gubbay et al. 2001). In an MV strain in which the ORF of EGFP was inserted into the L protein (Edtag-L-MMEGFPM) the calculated rate of RNA synthesis was substantially reduced to 0.8 nt/s. This precipitates a problem since the current model assumes that initiation of RNA synthesis only occurs at the 3′ end of the template RNP. If this is the case at that rate of synthesis, the H and L mRNAs could only be completed after 3 and 7 h, respectively. The authors suggest that incoming RdRp already attached to the RNP template would be able to start (or continue) transcription starting at the location in the RNP where they had stalled. However, the

estimate may be incorrect as the growth curve of this virus indicated a delay of 4 h, which is not as much as would be expected from this calculation. Also, the activity in minigenome expression assays of the L protein of Edtag-L-MMEGFPM was between 40% and 60% (Duprex et al. 2002). In the second phase (6–12 h.p.i.), exponential accumulation of mRNAs takes place presumably as a result of the formation of new RdRp complexes. These start to contribute to replication in the third phase (12–24 h.p.i.). In the fourth phase (24–28 h.p.i.) the RNA synthesis slows down either due to an unknown mechanism or simply as a result of the widespread fusion, syncytium formation and associated cytopathology. There is no further accumulation of viral RNA in the fifth phase (>28 h.p.i.).

The slope of the transcription gradient varies from one cell type to another and appears especially steep (little L expression) in the human central nervous system (Cattaneo et al. 1987; Schneider-Schaulies et al. 1989). For example, in some neuronal cell lines and SSPE cases, which is a rare and invariably fatal late sequela of MV infection, the slope of this gradient of gene expression is so steep that very little F and H protein are produced (Cattaneo et al. 1987). This may aid the virus in escaping detection by the immune system by disposing the virus to a wholly intracellular existence. Consequently, the amounts of L protein synthesised should also be substantially reduced in these cells. Similar observations on the enhanced steepness of the gradient of gene expression have been made in in vitro infections of neuronal cells (Schneider-Schaulies et al. 1989). These cells also respond differently from others to the expression of the interferon inducible MxA protein, which is rapidly induced in these cells to high levels. After stable transfection with MxA into human glioblastoma cells (U-87-MxA), they released 50- to 100-fold less infectious virus and expression of viral proteins was highly restricted. The overall MV-specific transcription levels were reduced by up to 90% due to an inhibition of viral RNA synthesis and a steeper gradient of gene expression and not to decreased stability. However, this effect is cell-type-specific because in other cells MxA affects translation (Schnorr et al. 1993).

An additional transcription variation in SSPE has been observed in a number of cases where the transcriptase leads to frequent or exclusive generation of a P-M read-through transcript (Baczko et al. 1986; Yoshikawa et al. 1990). In the well-studied cases, this was found not to be due to mutations in the GE, Ig or GS sequences between the P and the M gene. Hence, other effects of mutations in the N or L proteins were invoked, but not analysed, to explain these data. To date, none of this has been subjected to analysis by reverse genetics analysis. To the best of our knowledge, the generation of such a read-through transcript means that no or little M protein will be expressed as it is in the second ORF and thereby probably unavailable for translation. Reduced expression of the M protein is a general feature of SSPE and many MV virus SSPE strains appear to have undergone hypermutation in this gene, testifying to the nonessential nature of the M protein in this CNS infection (Schneider-Schaulies et al. 1995). This may be related to the fact that M protein, though required for budding, has a general negative effect on RNA synthesis in several *Mononegavirales*, including MV (Suryanarayana et al. 1994).

In conclusion, there are still questions remaining about MV transcription. The lack of a reliable in vitro system has hampered studies, although more studies on transcription using real-time RT-PCR would be helpful.

Replication

During replication, the RdRp binds at the 3′ end of the genome and the nascent RNA molecule is immediately encapsidated by N protein. On the basis of the N protein requirement for replication, the level of this protein as a free N–P complex has been put forward as an important parameter which controls the relative levels of transcription and replication (Banerjee 1987; Kingsbury 1974). Signals in the GS, GE, the Ig trinucleotide and the editing site are ignored. This gives rise to full-length positive-stranded RNA molecules within an RNP, with Y forms of the RNP being observed as intermediates by EM (Thorne and Dermott 1977). This allowed identification of the 3′ and the 5′ ends of the herringbone as these images permit the template and the product RNP to be readily discriminated. The herringbone points to the 5′ end. The positive antigenome-containing RNPs have a strong promoter at their 3′ end, which allows for a generation of excess amounts of RNPs containing the genomic RNA. The ratio between antigenome and genome containing RNPs in infected cells was estimated to be 0.43 (300/700) by Cattaneo et al. (1989b) and 0.13 (130/1030) by Plumet et al. (2005), who also showed that the same ratio occurred in virions as in infected cells and thus that there was no selection for packaging in to virions between antigenome and genome containing RNPs. Udem and Cook (1984) estimated this ratio to be 0.40, e.g. 2.5 times more genome than antigenome RNPs (Udem and Cook 1984).

The genome, antigenome, leader and trailer RNAs are the only species in the replication process that have 5′ triphosphates at their end and hence are likely to activate the innate immune system through RIG-I (Plumet et al. 2007). Molecules with 5′ triphosphates are especially enriched in virus stocks that contain large numbers of defective interfering particles. These have been demonstrated to be present in vaccine stocks and to play a role in determining the levels of interferon induction.

The A and B boxes are all that is required for the MV replication process and knowledge of that has allowed the genome to be segmented (Takeda et al. 2006). Takeda et al. split the genome into three fragments: one encoding the N and P protein plus Lac-Z, a second one the M and F genes and DsRed and the third fragment the H and L genes and EGFP. Together, the three fragments were able to replicate as a segmented MV virus. Cells infected with only two of the three segments were observed at frequencies that were so low as to indicate that most MV particles contained at least one of each of the three (and more) segments, confirming the polyploid nature of MV particles. The system allowed the expression of up to six reporter genes and illustrates the stability and apparent unlimited packaging capacity of the particles, which make this system an excellent one for the concerted

delivery of multiple genes and proteins. It has also been possible to produce a single nonsegmented recombinant MV virus expressing three reporter genes (Lac-Z, EGFP and CAT) from separate additional transcription units (Zuniga et al. 2007). The fidelity of genome replication is somewhat an enigma. Mutations rates in vitro are estimated to be 9×10^{-5} (Schrag et al. 1999). However, *i*n vivo, i.e. circulating virus, the population appears to be much more stable with a mutation rate in the N gene of 5×10^{-4} per nucleotide per year (Rima et al. 1997). The genome is very stable and ORFs present within additional transcription units are very stably maintained (Duprex et al. 1999), even during passage in vivo (de Swart et al. 2007; Duprex et al. 2000b; Ludlow et al. 2008).

Translation

Surprisingly little is known about translational control in the RNA viruses, even though this is one of only two processes which offer opportunities for these viruses to fine-tune the expression of their proteins. Global effects on translation of host mRNAs have not been reported in MV-infected cells and there is no documented effect on the phosphorylation of eIF2α, which is a prominent mechanism for other viruses to suppress host mRNA translation (Williams 1999). Additionally, no activation of PKR has been demonstrated. Studies using the MxA protein, the type I interferon-inducible human protein, which confers resistance to VSV and influenza A virus in MxA-transfected mouse 3T3 cells, showed a 100-fold reduction of released infectious virus for MV from U937 clones that constitutively express MxA. In this cell line, but not in others MV rates of transcription or the levels of MV-specific mRNAs were not affected. However, a significant reduction in the synthesis of MV glycoproteins F- and H- but not other viral proteins was observed in U937/MxA cells, indicating differential translational effects on some specific viral mRNAs induced by MxA expression.

The absence of global effects of MV on host translation is not surprising as MV is very prone to establishing noncytolytic, persistent infections which allow for a balanced synthesis of viral and host mRNAs and their translation. However, in acutely infected Vero cells, suppression of host protein synthesis is observed (Rima and Martin 1979), although this may reflect competition for ribosomes as the detailed analysis of copy numbers of MV RNA indicates that roughly 52,000 copies of MV RNA would account for approximately 25% of the total RNA population in a cell.

MV has a demonstrated potential for controlling gene expression through translational controls. The initiation codons for the P and C ORFs are in suboptimal contexts in all morbilliviruses and hence cap-dependent scanning of the P/C or V/C mRNAs appears to allow the ribosome to initiate translation at either the start of the P/V ORF at position 60 in the mRNA or that of the C ORF at position 82. In principle, this offers an opportunity to modulate the ration of C proteins over the P and V proteins. The C protein has 186 amino acids, but its length and more importantly

its sequence differs in various morbilliviruses. It is not clear from the studies conducted so far if knocking out C expression by altering the initiation codon has an effect on P or V expression. Since the initiation codon for C is the second one, it is not clear whether the fact that the scanning ribosomes do not encounter the C initiation codon leads to formation of amino-terminally truncated P and V proteins or the expression of the 57 amino acid ORF starting at position 102 in the third frame, which contains a second methionine codon with a strong Kozak consensus sequence at position 165. Surprisingly altering the context of the start codon for the P ORF to a strong context from CCGAUGG to GAGAUGG did not alter the levels of P and C protein expression when the P/C gene was expressed in adenovirus vectors (Alkhatib et al. 1988). The authors interpreted this as indicative that the levels of C and P expression are not controlled by the leaky scanning of the ribosome.

One area that has been studied in relation to translational control in MV is the extended 3′ UTR of the M mRNA and the 5′ UTR of the F mRNA together forming a 1-kb-long unique untranslated region which is well conserved in morbillivirus genomes. Relatively little is known about the role of the 430-nt-long 3′ UTR of the M mRNA. Takeda et al. have shown that it promotes M protein synthesis and thereby stimulates replication, as M protein has been shown to be an inhibitor of transcription in most *Paramyxoviridae*, including MV (Takeda et al. 2005). The effect on translation of deleting this or mRNA stability has not been reported. In our study of gene expression in minigenomes that express two autofluorescent reporters, deletion of this sequence had no effect on gene expression (Rennick et al. 2007). The function of this 3′ UTR remains to be determined.

More work has been done on the 5′ UTR of the F mRNA. In CDV, the region appears to encode an extremely long signal sequence, but the details of its function are not known (von Messling and Cattaneo 2002). It would be expected that this long signal sequence (if that is what it is) must be co-translationally removed as no large precursors for the F protein have been identified in pulse-chase labelling experiments (Campbell et al. 1980). In MV, the initiation codon appears to be at position 572. Contradictory results have been published on the effect of the 5′ UTR. Removal of the this region from F mRNA transcripts leads to increased translation in vitro (Richardson et al. 1986), suggesting that the F-5′ UTR sequence may have a negative effect on translation of the mRNA. The region has an unusually high content of cytosine residues (44%, including a $G_7C_7A_7$ tract) with an overall GC content of 64% (de Carvalho et al. 2002) and a high degree of predicted secondary structure (Curran et al. 1986) and conservation between strains. It has been suggested that the sequence acts as a focusing factor, directing translation initiation to the second of three clustered in frame AUG codons (Cathomen et al. 1995). However, mRNA transcripts with a high potential to form stable secondary structure in the 5′ UTR tend to be translated inefficiently (Kozak 1991a, 1991b). It has also been shown that all known mRNA transcripts encoding ribosomal proteins have a short (5-14 nucleotide) oligopyrimidine tract at their 5′ end which has been associated with their underutilisation in translation (Pain 1996). The F-5′ UTR sequence contains multiple C_5 tracts towards the 5′ end, which could lead to a negative effect on translation of the F mRNA transcripts. A study of the phenotype of the recombinant MV (del5F),

which contains a 504-nt deletion in the F-5′ UTR in the SCID-human thymus and liver mouse model (Auwaerter et al. 1996), found that this deletion resulted in decreased peak virus production and a small change in the kinetics of growth (Valsamakis et al. 1998). The virus had an early growth advantage, but the infection led to increased thymocyte death. The authors speculated that high levels of F synthesis might lead to increased virus production early in infection but might also cause premature fusion and death of infected cells prior to peak virus production. Their conclusion was that the F-5′ UTR sequence was not absolutely required for MV replication in that model system, but that its deletion led to a reduction, but not the abrogation of F protein expression. These conclusions reflect observations using MV minigenomes expressing two fluorescent reporter genes where the presence of the F-5′ UTR led to decreased gene expression of the second reporter (Rennick et al. 2007). This effect was interpreted as indicating that the 5′ UTR of the F gene had a negative effect on translation of the gene. Interestingly a long 5′ UTR in the HN gene of human parainfluenza type I virus controls transcription read-through at the M-F gene border rather than translation (Bousse et al. 2002).

The translation of the M and F mRNAs is sensitive to elevated temperatures to a larger extent than that of the others, as its is abrogated immediately after a shift up to 39°C whilst the translation of the N, P and H protein mRNAs still occurs at this temperature, though at lower rates (Ogura et al. 1987). This effect is reversible because shift down immediately restores translation. The F mRNA also appears to be distributed on smaller polysomes than that of the H mRNA at the higher temperatures (Ogura et al. 1988), although it remains to be seen whether these effects are due to the presence of these extended UTRs.

The precise intracytoplasmic site of viral protein synthesis is essentially unknown. We have some preliminary evidence that the N, P and M mRNAs accumulate in the viral factories that have been identified earlier. Whether they are translated there or are more diffuse in the cytoplasm cannot be evaluated. It is also not clear whether transcription and translation are coupled. No information is available for the other mRNAs since their abundance is too low or too diffuse for detection by ISH. It is a reasonable assumption that the H and F mRNAs are localised at the rough endoplasmic reticulum and that these glycoproteins are translated there before processing through a pathway that involves the Golgi apparatus and vesicular transport to the cell membrane (see the chapter by C.K. Navaratnarajah et al., this volume).

Getting It All Together

Transport of the various viral components to the cell membrane and specific structures in these are processes that are not well understood, and this is exemplified best by considering what is known in SSPE. In this disease, MV is able to spread along neuronal anatomical pathways, which probably requires trans-synaptic transfer of the virus. This poses a remarkable problem for the virus. It is likely that replication

and transcription takes place in the cell bodies where most of the required host cell functions are localised. The RNP would then have to be transported to the end of the neuronal axons and or dendrites. Then, for budding to take place, somehow the two viral glycoproteins also need to arrive at the same location. Location and localisation are also important in cytopathology, as our study on persistence of MV in NT2 cells indicates (Ludlow et al. 2005). Here all viral proteins required for cell-to-cell fusion are present in the cells, and the cells express the receptor CD46, but through altered localisation of the receptor, cell-to-cell fusion does not occur, allowing the persistent state to continue in these cells. The complexity of these processes indicates that our understanding of the replication of MV is still in its infancy.

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