Human Cytomegalovirus microRNAs

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Contents

Abstract MicroRNAs (miRNAs) are approximately 22 nucleotide RNAs that mediate the posttranscriptional regulation of gene expression. miRNAs regulate diverse cellular processes such as development, differentiation, cell cycling, apoptosis, and immune responses. More than 400 miRNAs have been identified in humans and it is predicted that over 30% of human gene transcripts are regulated via miRNAs. Since 2004, many viral miRNAs have been described in several families of viruses. More than half of currently known viral miRNAs are encoded by viruses of the human Herepsviridae and 14 miRNAs have been found to be encoded by Human cytomegalovirus (HCMV). Thus far, HCMV is the only betaherpesvirus in which miRNAs have been described and these miRNAs possess many characteristics, including their genomic arrangement and temporal/spatial expression, which distinguish them from the other known herpesvirus miRNAs described. As a herpesvirus,

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HCMV establishes infection for the life of the host characterized by latent infection with periodic reactivation for production and spread of infectious progeny. This multifaceted life cycle of the herpesvirus requires an abundance of gene products and regulatory elements that makes cytomegalovirus genomes one of the most complex among human viruses. The defining characteristics of the cytomegalovirus and the minimal impact on genome size afforded by miRNAs inform the logic of virus-encoded miRNAs.

Abbreviations miRNA: MicroRNA; HCMV: Human cytomegalovirus; EBV: Epstein Bar Virus; HSV: Herpes simplex virus; miRISC: MicroRNA-induced silencing complex; MHV 68: Murine gammaherpesvirus 68; RRV: Rhesus macaque rhadinovirus; rLCV: Rhesus lymphocryptovirus; MDV: Marek's disease virus

Introduction

In 1993, the discovery of an approximately 22-nucleotide RNA (*lin-4*) responsible for the posttranscriptional regulation of LIN-14 protein levels in *caenorhabditis elegans* represented the beginnings of a paradigm shift in molecular biology (Lee et al. 1993; Wightman et al. 1993). While *lin-4* was discovered in 1993, it was not until 1999 that it was shown to inhibit protein synthesis after the initiation of translation (Olsen and Ambros 1999). *Lin-4* is the founding member of a family of small RNAs, termed microRNAs (miRNA), that now number over 400 in humans (Bentwich et al. 2005). Not 15 years after *lin-4*'s discovery, it has been predicted that over 30% of human gene products are subject to miRNA-mediated regulation (Lewis et al. 2005).

miRNAs are endogenously encoded approximately 22-nt RNAs that are responsible for the temporal and spatial regulation of gene products involved in diverse cellular processes including development, apoptosis, differentiation, cell cycle regulation, and immune response (Ambros 2004; Taganov et al. 2006; O'Connell et al. 2007; Rodriguez et al. 2007; Taganov et al. 2007). Functionally, miRNAs mediate gene silencing by guiding the miRNA-induced gene silencing complex (miRISC) to target mRNAs (Tang 2005). Targeting of mRNA by miRISC leads to translational inhibition or cleavage of the targeted mRNA. The specificity of most animal miRNA–target mRNA complexes is determined by complementarity of a seed region, namely nucleotides 2–7 of the miRNA (Brennecke et al. 2005). When recognition of mRNA by miRNA is mediated primarily by the seed region, translational inhibition is typically the end result, while extensive sequence complementarity between the miRNA and target mRNA results in cleavage of the target mRNA (Ambros 2004). Complementary sequences in the target mRNA usually reside in the 3′ UTR (Lewis et al. 2005). Due to the size of the seed region, miRNAs are predicted to target as many as ten mRNAs. Additionally, multiple miRNAs may target the same mRNA with the 3′-UTRs containing target sites for multiple miRNAs,

and in situ adenosine to inosine substitutions in mature miRNAs can also alter targeting (Wightman et al. 1993; Doench and Sharp 2004; Kawahara et al. 2007). Finally, the miRNA sequence outside the seed region has been found to play a role in subcellular localization of miRNAs (Hwang et al. 2007).

Eleven years after the discovery of miRNAs, the first virally encoded miRNAs were reported (Pfeffer et al. 2004). As most algorithms for the identification of miRNAs rely on conservation of sequence, viral miRNA prediction was particularly difficult due to the absence of significant homology to known miRNAs. By sequencing of a small RNA library from a Burkitt's lymphoma cell line latently infected with Epstein Bar Virus (EBV), Tuschl and colleagues identified five miRNAs that originated from the EBV (Pfeffer et al. 2004). Subsequently, many groups, including our own, have contributed to the identification of miRNAs encoded by other human herpesviruses. Additionally, many nonhuman herpesvirus miRNAs have been identified, providing animal models in which to study the function of virally encoded miRNAs. Thus far, 106 of the 108 mature viral miRNAs species in the miRNA registry are encoded by herpesviruses (Griffiths-Jones 2004, 2006; Griffiths-Jones et al. 2006). Currently, it is known that HCMV expresses 14 mature miRNAs from 11 precursor miRNAs (Fig. 1).

A herpesvirus is a large dsDNA virus that replicates in the nucleus of the host cell, and after an initial lytic replication cycle establishes latent infection for the life of the host. Reactivation from latency and initiation of secondary lytic replication occurs periodically. Betaherpesviruses, which include HCMV and herpesvirus-6 and -7, can replicate in a wide variety of cell types, but exhibit strict species specificity. The complex life cyle of herpes viruses illustrates the need for distinctive gene regulation mechanisms that viral miRNAs provide. Utilization of miRNA offers the virus, which possesses limited coding capacity, a means to alter gene expression with relatively minimal impact on genome size; maintenance of latent infection requires limited, nonimmunogenic gene expression; tissue tropism implies an array of gene products suited to the exploitation of specific host-cell types. As such, it is not surprising that the first virally encoded miRNAs were discovered in a herpesvirus, EBV.

miRNA Biogenesis

The progress made in understanding miRNA biogenesis (Fig. 2) stands in stark contrast to the limited understanding of miRNA-mediated regulatory networks. While many fundamental questions about regulation of miRNA biogenesis still need to be addressed, significant progress has been made concerning the process of miRNA maturation.

Many of the subtleties and details concerning miRNA biogenesis are beyond the scope of this review. Rather, it is our aim to provide an overview of the biogenesis process as an aid to understanding HCMV miRNA. There is little evidence to suggest that viral miRNA biogenesis differs from that of cellular miRNAs. Recently, however, aspects of miRNA biogenesis such as substrate

microRNA	Evidence	Expression Kinetics	Position	Reference
hcmv-mir-UL22A-5p / hcmv-mir-UL22A-3p	5p - Cloned 3p - Cloned	IE	16820-16884 (Between UL22-UL23)	Dunn et al. 2005 Pfeffer et al. 2005
hcmv-mir-UL36	Cloned ; Northern	IE	38676-38750 (Between UL36-37)	Grey et al. 2005 Pfeffer et al. 2005
hcmv-mir-UL70-5p / hcmv-mir-UL70-3p	5p - Northern 3p - Northern	IE	92890-92955 (Antisense UL70)	Grey et al. 2005
hcmv-mir-UL112	Cloned	unknown	152992-152926 (Antisense UL114)	Pfeffer et al. 2005
hcmv-mir-UL148D	Cloned	unknown		Pfeffer et al. 2005
hcmv-mir-US4	Northern	E	195149 - 195232 (Partial US4)	Grey et al. 2005
hcmv-mir-US5-1	Cloned ; Northern	E	196060 - 196125 (Between US6-US7)	Grey et al. 2005 Pfeffer et al. 2005
hcmv-mir-US5-2	Cloned	E	196189 - 196253 (Between US6-US7)	Pfeffer et al. 2005
hcmv-mir-US25-1	Cloned	E	208315-208384 (Between US24-25)	Dunn et al. 2005 Pfeffer et al. 2005
hcmv-mir-US25-2-5p / hcmv-mir-US25-2-3p	5p - Cloned 3p - Cloned	E	208517-208606 (Partial antisense US25)	Pfeffer et al. 2005
hcmv-mir-US33	Cloned	unknown	213547-213616 (Antisense US29)	Pfeffer et al. 2005

Fig. 1 HCMV miRNAs. The eleven HCMV pre-miRNAs found in the miRNA registry and their predicted foldback structures are presented. Mature miRNA sequences are *highlighted*. Information regarding evidence for the indicated miRNA its expression, position and potential targets are given

specificity and Dicer activity have been found to differ during stages of development (Eis et al. 2005; Kim 2005; Lund and Dahlberg 2006). Finally, unique features of the murine gammaherpesvirus-68 (MHV68) miRNAs such as transcription by pol III and short hairpin structure suggest distinct mechanisms of biogenesis

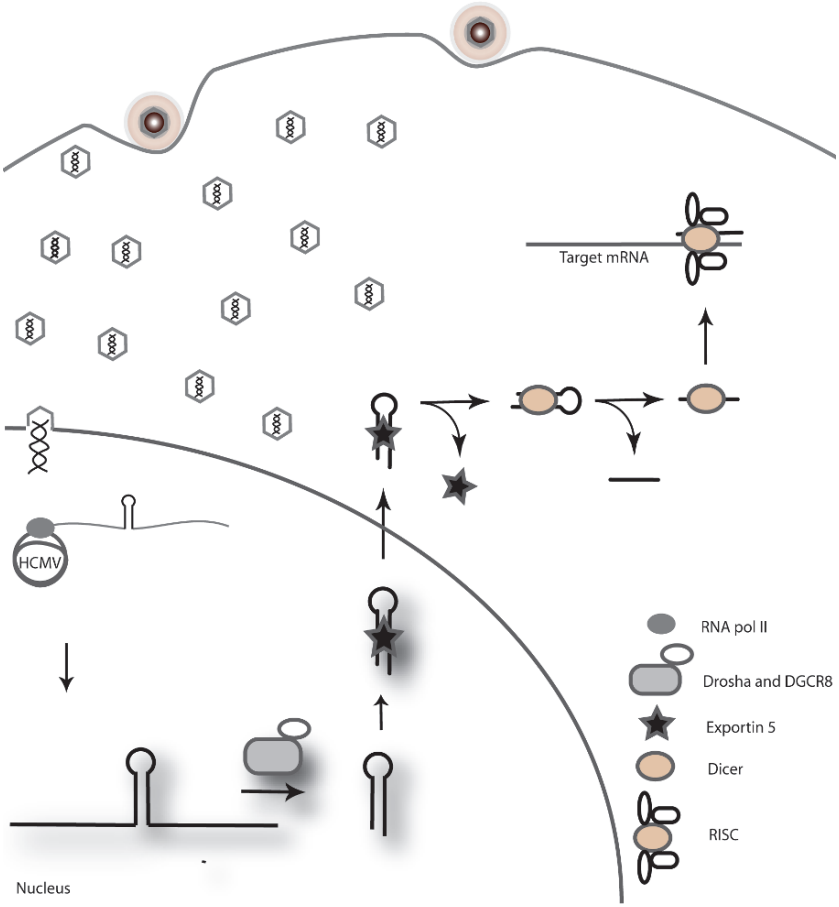

Fig. 2 Biogenesis of miRNAs. Transcription of viral pri-miRNA occurs most often by RNA polymerase II. Pri-miRNAs are then capped and polyadenylated. Nuclear RNase type III endonuclease Drosha then processes the pri-miRNA to pre-miRNA. Pre-miRNAs contain 2-nt 3′ overhangs characteristic of processing by RNase type III nucleases. Pre-miRNA is then exported via exportin-5 from the nucleus. Cytoplasmic type III endonuclease Dicer then cleaves the stem loop of the pre-miRNA to yield a 22-nt dsRNA. The strand with lowest thermodynamic stability at its 5′ end is chosen as the guide strand and incorporated into the RISC complex. The strand not chosen for incorporation is typically degraded. Guide strand miRNA enables recognition of target mRNA by RISC. Target mRNAs are then either cleaved or translation is inhibited

(Pfeffer et al. 2005). As such, the reader is encouraged to pursue the topics included in this section with the host–microbe interaction in mind.

The miRNA transcriptional unit is termed a primary miRNA (pri-miRNA). The pri-miRNA may possess multiple hairpins or single hairpins (Kim 2005). While most miRNAs are located within an intron of a protein-coding transcript, miRNAs can be found in the exons as well as the protein coding potential of the transcriptional

unit (Rodriguez et al. 2004). Finally, some pri-miRNAs are found in intergenic regions whereby they contain their own promoters and are independently transcribed (Zeng 2006).

Transcription of most pri-miRNAs is RNA polymerase II-dependent (Lee et al. 2004). Notable exceptions include the MHV68 miRNAs, whose expression is believed to be driven by a pol III promoter (Pfeffer et al. 2005). Pol II-dependent pri-miRNAs possess 5′ cap structures and are polyadenylated (Cullen 2004). Due to the enormity and diversity of pol II-associated transcription factors, pol II-dependent transcription allows for the exquisite temporal and spatial control characteristic of miRNAs.

Following transcription, an approximately 65-nt hairpin with a 2-nt 3′ overhang is excised from the pri-miRNA by the nuclear Microprocessor (Gregory et al. 2004). Microprocessor consists of the RNase III type endonuclease Drosha in complex with the cofactor DGCR8 (Han et al. 2004). These two components of Microprocessor are both necessary and sufficient to affect cleavage in vitro. Using mutagenesis and in vitro processing assays, it was determined that Microprocessor recognition of pri-miRNA is through the ssRNA flanking strands and the structural motif of the hairpin (Han et al. 2006). Recent studies suggest that pri-miRNA processing to mature miRNAs is a regulated step in miRNA biogenesis. Mature miRNAs have been found in fully differentiated cells, while the pri-miRNAs accumulate in undifferentiated cells (Thomson et al. 2006). Additionally, in human tumors pri-miRNAs are expressed at high levels while the formation of mature miRNA is downregulated (Thomson et al. 2006). This suggests that there may be additional cofactors modulating either substrate specificity and/or activity of Microprocessor.

Following conversion of pri-miRNA to pre-miRNA, Exportin-5, in a Ran-GTP-dependant manner, exports the pre-miRNA from the nucleus to the cytoplasm (Lund et al. 2004). A cytoplasmic RNase III type endonuclease, Dicer, then removes the stem loop from the pre-miRNA converting it to a 22-bp dsRNA with 2-nt 3′ overhangs on each strand (Carmell and Hannon 2004). Evidence suggests that pre-miRNA processing to mature miRNAs is also subject to regulation. It is unclear whether this is due to regulation of pre-miRNA export from the nucleus, or due to the presence of cofactors, which may influence substrate specificity, and/or activity of Dicer cleavage (Lund and Dahlberg 2006; Obernosterer et al. 2006).

Typically, one strand of the 22-bp dsRNA, designated the guide strand, is fated to pair with a target mRNA to facilitate translation inhibition or cleavage of the target mRNA. The guide strand is chosen based on lower thermodynamic stability at its 5′ end in the duplex RNA (Khvorova et al. 2003; Schwarz et al. 2003). Incorporation of the guide strand in the miRISC enables identification of target mRNA for either translation inhibition or cleavage.

Mammalian miRISC is at minimum comprised of Dicer, Argonaute proteins, TRBP, and PACT (Rana 2007). In general, translation inhibition occurs if there is imperfect complementarity of the guide strand with the target mRNA. Cleavage occurs if the complementarity between the guide strand and the target mRNA is perfect. Nucleotides 2–7 of the miRNA constitute the seed region of the miRNA

and appear to be critical for miRNA-target mRNA recognition (Jackson and Standart 2007).

The major functional consequence of miRNA biogenesis is alteration of protein concentration through either translation inhibition or cleavage of mRNA. It is important to note that operationally miRNA-mediated inhibition of gene expression is not necessarily an all-or-nothing mechanism. Rather, the present consensus is that miRNAs allows a fine tuning of gene expression (Bartel 2004). miRNAs may be seen as a means by which the cell or virus can achieve a spectrum of gene expression levels appropriate to differentiation or developmental states, proliferative signals, and most recently in response to infection. As such, it is not difficult to imagine a role for viral miRNAs, particularly in those viruses that find persistence and/or latency part of their life cycle.

Location and Conservation of HCMV miRNAs

Mapping of HCMV miRNAs

Using a small RNA cloning and sequencing approach, a total number of 14 mature HCMV miRNAs were identified, which arise from 11 pre-miRNAs (Fig. 1) (Dunn et al., 2005: Grey et al., 2005; Pfeffer et al., 2005). In order to correlate our previous work (Dunn et al. 2003), where we functionally profiled the HCMV genome by constructing a deletion mutant library, with the discovery of HCMV miRNAs, we have mapped the HCMV miRNAs to the genome of the HCMV Towne (HCMV Towne-BAC) strain that was cloned as a bacterial artificial chromosome (Fig. 3). Our annotation of open reading frames (ORFs) within the HCMV Towne-BAC reveals that five HCMV pre-miRNAs are intergenic, four are found within ORFs and two partially overlap annotated ORFs. The vector for bacterial propagation of the Towne-BAC replaces ORFs US1–US12. Additionally, a well-characterized spontaneous deletion of UL150 has occurred on passaging of Towne in tissue culture. To map miRNAs in these regions, we have deferred to other publications for their annotation (Grey et al. 2005; Pfeffer et al. 2005). From the map and data generated by the deletion mutant library, we have determined that six of the miRNAs (mir-US4, mir-US5-1, mir-US5-2, mir US25-2, mir-US33 and mir-UL148D) are not essential for growth in tissue culture. These six miRNAs along with their associated ORFs have been deleted from the viral genome with no apparent resulting defect in viral replication in tissue culture. The possibility remains, however, that these miRNA targeted the ORFs in which they are found, and that concomitant deletion of both the miRNA and its target ORF precluded any phenotype we may have observed if only the miRNA had been disrupted. The two deletions that did result in a phenotype (UL70 and UL114) are in ORFs whose gene products are known to be important for viral infection (Pari and Anders 1993; Courcelle et al. 2001). This precludes determining the contribution that the deletion of miRNAs in these ORFs makes to viral growth

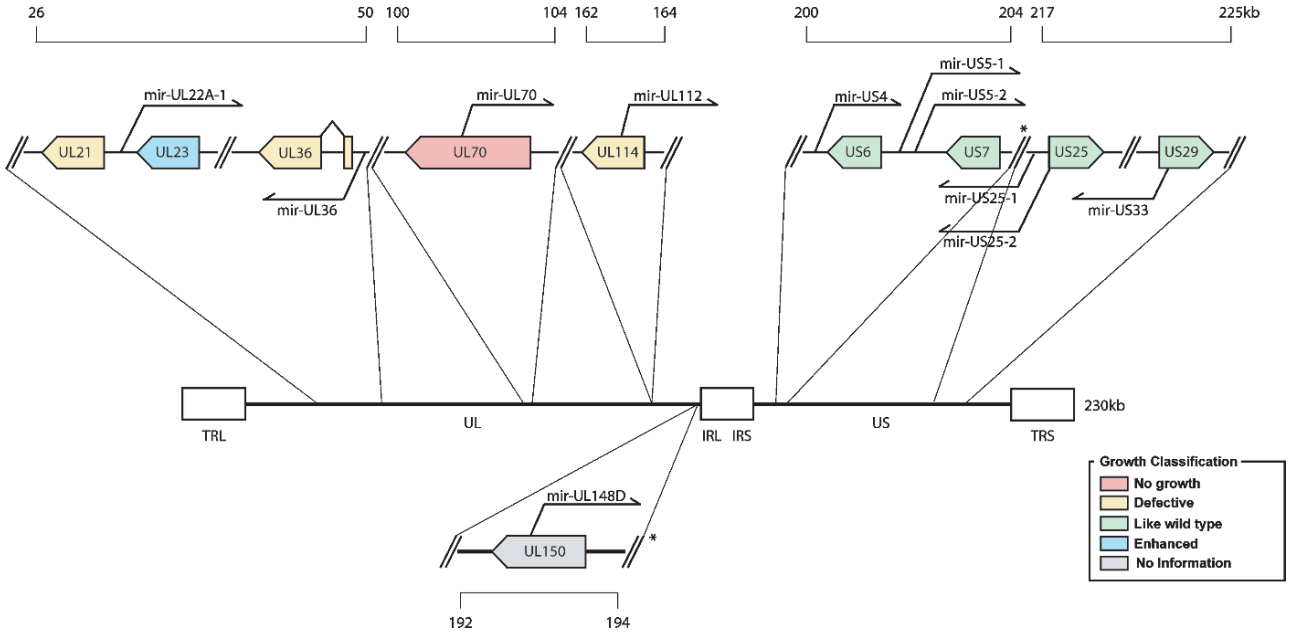

Fig. 3 Map of HCMV miRNAs. Relative positions of HCMV miRNAs were mapped by aligning the sequence of 11 miRNAs found in the miRNA registry with the Towne BAC sequence. The Towne BAC genome (illustrated in the *middle*) consists of two unique regions bracketed by repeat regions. Regions of the genome containing miRNAs have been expanded. The Towne BAC is missing ORFs US1–11 and UL150. To accommodate HCMV miRNAs in these regions, we have used the HCMV Merlin sequence, which possesses a similar arrangement of ORFs as Towne BAC. Growth phenotype of deletion mutants is indicated for ORFs that either contain or immediately surround HCMV miRNAs. Scale in kilobase pairs

in tissue culture. Characterizing the contribution that the remaining HCMV miRNAs (mir-UL22A-1, mir-UL36 and mir-US25-1) make to virus growth in tissue culture awaits the generation of novel recombinant virus.

HCMV is the only betaherpesvirus in which miRNAs have been described, and several aspects of HCMV miRNAs are unique. Firstly, unlike the miRNAs identified in alpha- and gammaherpesviruses, HCMV miRNAs are not found clustered in small regions of the genome, but can be found up to 195 kb apart (Fig. 3). Secondly, due to the diversity of cells permissive to HCMV infection, it is possible to study the expression of viral miRNAs in a number of cell types. Thirdly, because of the lack of a simple in vitro model for HCMV latency, all of the HCMV miRNAs identified in the experiments above have been in cells lytically infected with HCMV. Thus far, all miRNAs identified in prototypic human alpha-(herpes simplex virus 1 [HSV-1] and gamma-Kaposi's sarcoma associated herpesvirus [KSHV] and EBV) herpes viruses have been found in regions of the genome that are transcriptionally active during latent infection. The similarities and differences between HCMV miRNAs and other herpesvirus miRNAs, in terms of their genomic arrangement, expression and conservation will be discussed below.

HCMV miRNA Conservation

Multiple ORFs are conserved throughout the herpesvirus family, which are referred to as core herpesvirus ORFs. As none of the miRNAs identified thus far are conserved among the human herpesviruses, it can be inferred, just as it is for the non-core herpesvirus ORFs, that these miRNAs impart functions unique to the identity and lifestyle of each virus.

Our lab was the first to show the conservation of miRNAs between primate herpesviruses of the same genera, HCMV and chimpanzee cytomegalovirus (CCMV) (Dunn et al. 2005). For purposes of this review, we have extended our analysis to include all HCMV miRNAs identified to date and we report here that at least five of the HCMV miRNAs are 100% conserved in CCMV (Fig. 4). Related herpesviruses are expected to have diverged with their host species. Chimpanzee and human are thought to have diverged approximately 5 million years ago and as such, CCMV is the closest relative of HCMV (Davison et al. 2003).

Similar analysis of the conservation of miRNAs between EBV and the closely related rhesus lymphocryptovirus (rLCV) revealed that at least seven of the EBV miRNAs are conserved in rLCV (Cai et al. 2006). The strength of this report lies in the fact that bioinformatics were not used to identify rLCV miRNAs. Rather, rLCV miRNAs were cloned from infected cells prior to sequence analysis. Interestingly, the rLCV miRNAs that have sequence identity with EBV BART miRNAs possess identical synteny with EBV BART miRNAs (Cai et al. 2006).

Two human herpesviruses, HSV-1 and HSV-2, exhibit close homology. Eight of the miRNAs predicted for HSV-1 were conserved in HSV-2 (Pfeffer et al. 2005; Cui et al. 2006). Of the eight, two of the precursor miRNAs were conserved in HSV-2 (Pfeffer

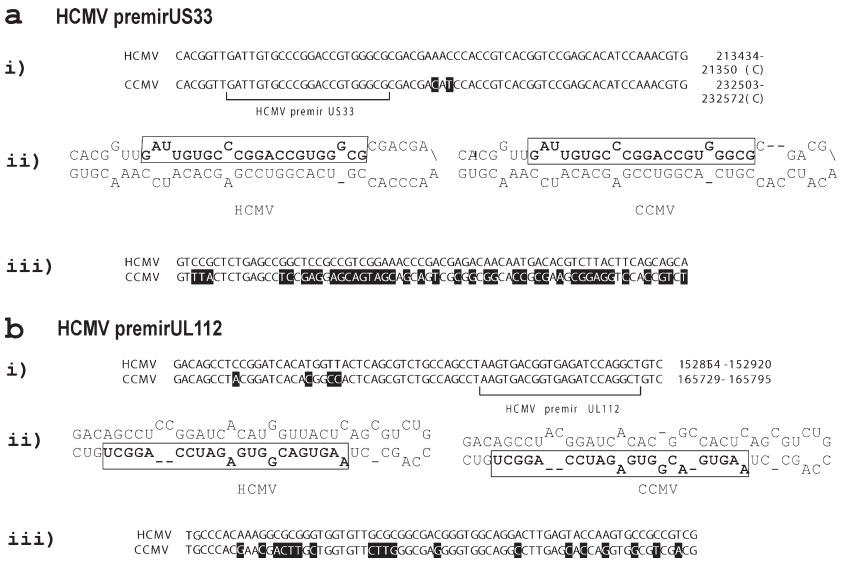

Fig. 4 Conservation of HCMV miRNAs in chimpanzee cytomegalovirus (CCMV). Alignment and secondary structure of two HCMV pre-miRNAs predicted to be conserved in CCMV. HCMV miRNA sequences found in the miRNA registry were aligned with the publicly available CCMV genome sequence (Davison et al. 2003). Sequences for HCMV pre-miRNAs and predicted CCMV pre-miRNAs are shown in (i), secondary structures are compared in (ii), finally alignment of sequences flanking the respective premiRNAs are compared in (iii). Mismatched bases are *highlighted*

et al. 2005; Cui et al. 2006). However, neither of these conserved miRNAs has been demonstrated to be expressed by either virus. Finally, Marek's disease viruses one and two (MDV-1, -2), closely related avian alpha herpesviruses, have been shown to express miRNAs (Burnside et al. 2006; Yao et al. 2007). Interestingly, while these miRNAs are conserved in their genomic location, none of the miRNAs possess sequence homology (Yao et al. 2007). It will be interesting to reevaluate the relatedness of viral miRNAs in terms of their function, as more targets of these miRNAs are identified.

Genomic Arrangement of HCMV miRNAs

As mentioned above, one of the distinguishing features of HCMV miRNAs is that they are widely distributed across the genome (Fig. 3). In contrast, miRNAs identified in all other herpes viruses to date are clustered in short regions of the genome, usually less than 5 kbp. An additional important difference is that all herpesvirus-encoded miRNAs are encoded in regions of the genome that are transcriptionally active during latency. Due to the lack of a simple tissue culture model for HCMV latency, transcriptionally active regions of the viral genome during latency remain controversial.

Genomic arrangement has important implications for miRNA expression. Clustering of miRNAs implies the ability to coordinate miRNA expression. It has recently been shown that the 14 EBV *BART* miRNAs are indeed coordinately expressed, and that expression of the *BART* miRNAs, which lie in the intron(s) of the *BART* mRNA, was correlated with the expression of the *BART* mRNA (Cai et al. 2006). The three *BHRF1* miRNAs are temporally and coordinately expressed (discussed Sect. 4.1 below) predominately by the usage of alternate promoters active in stage III EBV latency (Cai et al. 2006). All twelve KSHV miRNAs identified to date originate from a single cluster approximately 4 kb in length (Gottwein et al. 2006). Of the 12, ten of these miRNAs are intronic, one is in the kaposin ORF, and one is found in the 3′-UTR of the kaposin gene (Cai et al. 2005; Pfeffer et al. 2005). Transcript mapping has identified four different transcripts from which all or only some of the KSHV miRNAs are derived (Cai and Cullen 2006). Interestingly, rLCV and rhesus macaque rhadinovirus (RRV), primate herpesviruses that are distantly related to EBV and KSHV, respectively, possess miRNA clusters that are in similar genomic positions (Schafer et al. 2007) .

HCMV pre-miRNAs are mostly intergenic (5) or are coded for on the anti-sense strand of ORFs (4), while the remaining two HCMV pre-miRNAs partially overlap known ORFs (Figs. 1, 3). The distribution of HCMV miRNAs has likely implications for their transcriptional regulation. The clustering of other herpesvirus miRNAs implies their coordinated expression under the control of a master regulatory sequence, while the broad distribution of HCMV miRNAs across the genome implies different mechanisms of transcriptional control. Most likely, each spatially isolated miRNA may have its own regulatory sequence dedicated solely to its transcriptional control and would be operating independently of other miRNAs. Alternatively, expression of HCMV miRNAs may also be coordinated with each other, with an enhancer-like element coordinating expression of multiple miRNAs found far apart on the genome. It will be important to identify the promoter elements and signaling events associated with expression of HCMV miRNAs.

HCMV miRNA Expression

Owing to the complex life cycle exhibited by HCMV, multiple issues regarding the expression of viral miRNAs should be addressed, including expression kinetics, miRNA expression in different cell types and latent vs lytic expression.

Kinetic Classes of HCMV miRNAs

Herpesvirus lytic gene expression is characterized by cascade regulation (Roizman 2001; Roizman and Pellett 2007). As such, there are three kinetic classes of HCMV genes characterized by their dependence on viral protein synthesis and/or viral genome replication. Viral genes that are expressed prior to de

novo viral protein synthesis are defined as immediate early genes. Early gene expression requires de novo protein synthesis but not viral genome replication. All other genes are designated as late genes and their expression occurs after viral genome replication.

Work in our lab and the Nelson lab has addressed the kinetics of HCMV miRNA expression. In general, HCMV miRNA levels within the infected cell are observed to increase over the course of the viral replication cycle. Specifically, HCMV miRNA expression is dependent on protein synthesis but independent of viral genome replication and as such, HCMV miRNAs are characterized as early genes (Dunn 2005; Grey 2005). Exceptions include mir-UL22A* (referred to as mir-UL22A-3p in Fig. 1) and mir-UL70. Our experiments show that mir-UL22A* is expressed in cells treated with a protein synthesis inhibitor (Dunn et al. 2005; Grey et al. 2005). This suggests that mir-UL22A* is expressed with immediate early kinetics. Mir-UL70, like mir-UL22A*, is expressed in the absence of protein synthesis (Grey et al. 2005). Curiously however, it was not expressed in the absence of viral genome replication and so does not fall into any of the previously described kinetic classes. miR-UL148D kinetics have not been examined. Mir-US33, reported by Tuschl and colleagues, could not be confirmed by experiments (Grey et al. 2005). A caveat about ascribing kinetics to HCMV miRNAs is that pre-mir-UL36 was shown to accumulate in cells treated with a protein synthesis inhibitor (Grey et al. 2005). Data on other pre-miRNAs in the presence of a protein synthesis inhibitor was not presented. The accumulation of pre-miRNA, and thus the absence of mature miRNA, may be due to the paucity of components in the miRNA biogenesis pathway due to inhibition of protein synthesis. Alternatively, the accumulation of pre-miRNA in cells treated with protein synthesis inhibitors may be due to the absence of host/viral cofactors important for the processing of pre-miRNAs.

Another issue of interest is that most of the HCMV strains used in the studies of HCMV encoded miRNAs were laboratory strains that had been passaged multiple times. Thus, to examine the possibility that miRNA expression from laboratory strains of HCMV may have been altered by their history of extensive passaging, we performed a limited comparison of miRNA expression between the laboratory Towne strain and a HCMV clinical isolate that had undergone very limited passaging in tissue culture (Dunn et al. 2005). Qualitatively speaking, the miRNA expression from the laboratory strain is comparable to that observed in the clinical strain.

Tissue-Specific HCMV miRNA Expression

In animals, miRNA expression is characterized by tissue specific expression patterns. HCMV lytically replicates in a wide variety of cell types both in vivo and in vitro. Viral gene expression has been shown to vary depending on host cell type (Yang et al. 2006; Goodrum et al. 2002, 2007) and work in our lab has identified a

number of HCMV gene products that contribute differentially to the replication success of the virus in different cell types (Dunn et al. 2003). Thus, HCMV miRNA expression might also be expected to vary in different cell types.

We have examined HCMV miRNA expression in multiple, clinically relevant cell types (Dunn et al. 2005). These cell types were of epithelial, microglial, and fibroblast origin. We found that at least in these three cell types, all of the HCMV miRNAs tested were expressed (Dunn et al. 2005). Generally, all HCMV miRNA examined were found to be expressed among all the cell types tested. Qualitatively, expression levels of individual HCMV miRNAs did vary between cell types. Specifically, they appeared to be expressed most highly in an astroglial cell line. In infected retinal pigment epithelial cell lines, viral miRNA expression appeared to be the lowest among the cell lines examined. Whether this was due to differential susceptibility of these cell types to viral infection, or in fact a result of cell type-specific differential HCMV miRNA expression is under investigation.

To aid in extending the discussion of tissue-specific expression of herpes virus miRNAs, interesting data have been recently reported concerning the tissue-specific control of viral miRNA expression in EBV. As noted above, EBV has been found to express at least 17 miRNAs. These miRNAs are encoded in two distinct clusters, denoted *BART* and *BHRF1*. While miRNAs derived from the *BART* cluster were detected in all cell lines studied, they were expressed at much higher levels in epithelial cells (Cai et al. 2006). In contrast, the *BHRF1* miRNAs were expressed predominantly in B cells exhibiting stage III EBV latency (Cai et al. 2006). Finally, it was shown that gastric carcinomas harboring EBV expressed the BART miRNA cluster but not the BHRF1 miRNAs (Kim do et al. 2007). Presently, however, it is not clear what role if any the EBV miRNAs play in different cell types and at different stages of viral infection. The impact of specific EBV-encoded miRNAs in infection awaits interference studies, miRNA target identification and analysis of recombinant virus deficient in one or more viral miRNAs.

Prior to the discovery of the MHV68 miRNAs, Stewart and colleagues examined the growth phenotype of a naturally occurring MHV68 variant, MHV-76 (Macrae et al. 2001). MHV76 was isolated from the yellow necked wood mouse (Blaskovic et al. 1980). MHV76 is identical to MHV68 except for a deletion of approximately 10 kbp of genomic DNA from the left end of the unique region (Macrae et al. 2001). This region includes four genes (M1–M4) and all nine MHV68 miRNAs. Identical growth of MHV68 and MHV76 were observed in vitro. However, in vivo growth phenotypes were distinguishable, most notably by a decrease in MHV76 pathogenicity in the spleen and more rapid clearance from the lung (Macrae et al. 2001; Clambey et al. 2002). It is difficult to ascribe the MHV76 in vivo growth to the loss of miRNA and analysis is confounded by the absence of four genes also found in the deleted region. Viruses with deletions in either M2, M3 or M4 were shown to have similar in vivo growth phenotypes (Parry et al. 2000; van Berkel et al. 2000; Jacoby et al. 2002; Geere et al. 2006). Nevertheless, it is interesting that a naturally occurring variant of MHV68, which is lacking the nine MHV68 miRNAs, exists.

Latent Versus Lytic Infection

The finding that, with the exception of HCMV, all human herpesvirus miRNAs originate from regions of the viral genome that are transcriptionally active during latency suggests a role for herpesvirus miRNAs in latency. Indeed, recent data suggest that miRNAs are potentially responsible for maintenance of a latent HSV-1 infection (Gupta et al. 2006). The HSV-1 mir-LAT has been shown to inhibit apoptosis in infected neuronal cells. However, as mir-LAT has not been shown to be expressed during latency, its role in latent infection has yet to be proven.

As mentioned above, there is no simple tissue-culture model in which to study HCMV latency. As such, there are no widely accepted HCMV gene products known to contribute to the establishment and/or maintenance of a latent infection. However, a promising recent cell culture model in which to study HCMV latency has been reported recently(Goodrum et al. 2007). We hope that this will allow future study of a potential role for HCMV miRNAs in latent infection.

Gammaherpesviruses, EBV and KSHV, establish latent infection in B cells. Both can be induced to undergo lytic infection after treatment of infected cells with either TPA or n-butyrate. The expression of EBV and KSHV miRNAs in latent vs lytically infected cells has been studied in such systems.

For KSHV, there was little change in the expression of miRNAs after the switch to lytic infection (Pfeffer et al. 2004, 2005; Cai et al. 2005). The exception from these studies appears to be mir-K12–10, which showed an increase after induction of lytic infection. Mir-K12–10 is located in the 3′-UTR of K12 (Cai et al. 2005; Pfeffer et al. 2005). K12 mRNA expression is increased during lytic infection and thus increased levels of mir-K12–10 are consistent with upregulated expression of K12 mRNA. As mir-K12–10 is the only KSHV miRNA not located in the intron (see Sect. 3.3 above), it is interesting to speculate about whether mir-K12–10's location in the 3′ UTR of K12 and its coincident expression are important to lytic replication.

Potential Function of HCMV miRNAs

Currently, miRNA target prediction lags far behind miRNA identification. As such, just two of the 80 or so viral miRNAs in the miRNA registry have validated targets. Simian Virus 40 (SV40), a polyomavirus, encodes one identified pre-miRNA that yields mature miRNA(s) from each arm of the hairpin (Sullivan et al. 2005). These miRNAs are perfectly complementary to early viral mRNAs and mediate cleavage of transcripts leading to a decrease in viral T antigens (Sullivan et al. 2005). Ganem and co-workers used a mutant virus deficient in the ability to form a pre-miRNA to demonstrate viral miRNA function. Thus, the differential contribution of each arm was not established and we refer to it as a single miRNA with a single function. Interestingly, mutant virus lacking functional miRNAs showed no replicative impairment in vitro and grew to wild type levels (Sullivan et al. 2005). Cells infected with these mutant virus were, however, more susceptible to lysis by

cytotoxic T lymphocytes (Sullivan et al. 2005), and thus we would expect that the miRNA mutant virus would be replicatively attenuated in vivo.

Two targets of the HSV-1 mir-LAT were recently identified. Mir-LAT targets the 3′-UTRs of TGF beta 1 and SMAD3 (Gupta et al. 2006), resulting in the disruption of pro-apoptotic pathways (Gupta et al. 2006). Expression of a miRNA from the LAT prompted many to attribute a role for mir-LAT in the maintenance of a latent infection. However, to date mir-LAT has not been studied in the context of a latent infection and its role in the maintenance of HSV-1 latency awaits further verification.

HCMV mir-UL112 has been found to target two viral open reading frames (Grey 2006). Nelson and co-workers reported that mir-UL112 targeted viral immediate early (IE) proteins-1 and -2. IE1 and IE2 are essential for viral infection and possess numerous reported functions, including transactivation and cell cycle inhibition (Mocarski et al. 2007). IE1 is not essential, but a deletion mutant shows a severe growth defect (Mocarski et al. 2007). Mir-UL112 is expressed with immediate early kinetics. The regulation of these two viral proteins is likely to have an important role in viral infection. Further studies are needed to address the functional importance of mir-UL112's negative regulation of IE1 and IE2.

Future Directions

The identification of viral miRNA targets is currently the bottleneck in further understanding the role and function of virally encoded miRNAs. It may be expected that an understanding of HCMV miRNA function will reveal novel mechanisms contributing to the unique replicative success of this herpesvirus. Further, identification of HCMV miRNA targets may well illuminate fundamental and universal components of the host response to microbial infection. Microbes have provided the foundation for many important discoveries in molecular biology. It is our hope that, in addition to expanding therapeutic and prevention strategies, probing the functions of miRNA in the context of viral infection will illuminate fundamental aspects of miRNA-mediated control of gene expression.

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