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MicroRNAs of Gallid and Meleagrid herpesviruses show generally conserved genomic locations and are virus-specific

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ABSTRACT

Many herpesviruses, including Marek's disease viruses (MDV1 and MDV2), encode microRNAs. In this study, we report microRNAs of two related herpesviruses, infectious laryngotracheitis virus (ILTV) and herpesvirus of turkeys (HVT), as well as additional MDV2 microRNAs. The genome locations, but not microRNA sequences, are conserved among all four of these avian herpesviruses. Most are clustered in the repeats flanking the unique long region (I/TR_L), except in ILTV which lacks these repeats. Two abundant ILTV microRNAs are antisense to the immediate early gene ICP4. A homologue of host microRNA, gga-miR-221, was found among the HVT microRNAs. Additionally, a cluster of HVT microRNAs was found in a region containing two locally duplicated segments, resulting in paralogous HVT microRNAs with 96–100% identity. The prevalence of microRNAs in the genomic repeat regions as well as in local repeats suggests the importance of genetic plasticity in herpesviruses for microRNA evolution and preservation of function.

Introduction

Gallid and Meleagrid herpesviruses (GaHV, MeHV) are double-stranded DNA viruses that infect poultry. These herpesviruses include GaHV1, more commonly known as infectious laryngotracheitis virus (ILTV); GaHV2 or oncogenic Marek's disease virus (MDV or MDV1); GaHV3 or non-oncogenic MDV (MDV2); and MeHV or herpesvirus of turkeys (HVT). ILTV belongs to the genus *Iltoviridae*, while the others are members of the *Mardiviridae* genus (Table 1). These viruses all have a general genome structure that resembles the herpes simplex virus (HSV) genome. Unique long (U_L) and unique short (U_S) regions are each flanked by terminal and inverted repeat regions. The U_S and U_L regions of these herpesviruses are generally conserved and appear collinear with the corresponding regions of other alphaherpesviruses, and gene annotation has relied on this similarity (Cebrian et al., 1982). The repeat regions are much less conserved and presumably contain some of the key information that distinguishes each virus. ILTV does not contain the internal and terminal repeats (I/TR_L) flanking the U_L. Recombination between viruses, especially in the repeat regions, has been demonstrated to be important in the evolution of herpesviruses and can lead to recovery or emergence of virulence (Hughes and Rivailler, 2007).

The evolution of herpesviruses is often measured by analysis of genes in the unique regions. Phylogenetic analyses of genes in the U_L region of the avian herpesviruses suggest that ILTV appeared earlier than HVT, MDV1 and MDV2 (Hughes and Rivailler, 2007; Kingham et al., 2001; McGeoch et al., 2006), but all of these avian herpesviruses evolved later than the branch point of avian-mammalian divergence, approximately 310 million years ago (mya) (McGeoch, Rixon, and Davison, 2006). ILTV and another Iltovirus, Psittacid herpesvirus of parrots, are more deeply branching than the Mardiviruses HVT and MDV, evolving earlier than HSV1 and its relatives, about 201 mya (McGeoch et al., 2006). MDV1 and MDV2 are thought to have separated about 26 mya, and HVT diverged from MDV1 and MDV2 approximately 38 mya (D. McGeoch, personal communication). Of the Gallid and Meleagrid herpesviruses, the youngest species is MDV1, which is also the most pathogenic.

Gallid and Meleagrid herpesvirus infections result in a variety of conditions in poultry, ranging from innocuous to severe terminal disease. These viruses are of economic significance, either as agents of disease or as tools for vaccination. ILTV causes a respiratory disease (laryngotracheitis or LT) in chickens and pheasants, with the severity of the disease varying from decreased egg production to 80%

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Table 1
Overview of Gallid and Meleagrid herpesviruses described in this study.

Genus	Species	Common name	Genome size (bp)	# of Predicted ORFs	Reference strain	Accession number
Iltovirus	GaHV1	ILTV	148,687	79	Composite	NC_006623
Mardivirus	GaHV2	MDV1	177,974	85	Md5	AF243438
Mardivirus	GaHV3	MDV2	164,270	76	HPRS24	NC_002577
Mardivirus	MeHV1	HVT	159,160	79	FC126	AF291866

morbidity (Bagust et al., 2000; Garcia and Riblet, 2001; Kirkpatrick et al., 2006). The three species of *Mardivirus* (MDV1, MDV2, and HVT) all infect chickens, but only MDV1 is oncogenic, inducing T-cell lymphomas in two to six weeks after infection of susceptible chickens (Witter and Schat, 2003). MDV2 and HVT are non-pathogenic, and both are used as live vaccines to prevent lymphomagenesis by MDV1 (Witter and Schat, 2003). As with other herpesviruses, the Gallid and Melagrid herpesvirus life cycles include both lytic and latent stages, and the latent virus can reactivate and re-infect exposed flocks.

MicroRNAs are small (~22 nt) non-coding RNAs found in plant, animal, and viral genomes and are now recognized as important contributors to regulation of cellular processes. MicroRNAs control gene expression in animals by partial base complementarity, typically in the 3′ untranslated region (3′ UTR) of mRNAs. This pairing targets mRNAs for translation inhibition or degradation (Filipowicz et al., 2008; Nilsen, 2007). A high level of conservation across phylogeny implies important function, and it has been suggested that at least 30% of vertebrate transcriptomes are regulated by microRNAs (Filipowicz et al., 2008; Lewis et al., 2005; Lim et al., 2003; Nilsen, 2007). Over 500 microRNAs have been discovered in the human genome (Griffiths-Jones et al., 2008), and genes encoding microRNAs have distinct expression profiles during development, in specific tissues, in disease, and in response to stimuli (for a recent review, see Stefani and Slack, 2008).

Among viruses, microRNAs have been described for viruses that replicate in the nuclei of animal cells; i.e., herpesviruses, retroviruses, and papovaviruses (Cullen, 2006; Pfeffer et al., 2005). Epstein-Barr Virus (EBV) (Pfeffer et al., 2004), Kaposi's sarcoma-associated herpesvirus (KSHV) (Cai et al., 2005), murine herpesvirus 68 (MHV68) (Pfeffer et al., 2005), human cytomegalovirus (hCMV) (Grey et al., 2005), HSV1 (Umbach et al., 2008), HSV2 (Tang et al., 2008), murine CMV (Buck et al., 2007), and Rhesus Money Rhadinovirus (RRV) (Schafer et al., 2007) are among the mammalian herpesviruses known to encode microRNAs. Our lab and the Nair lab have reported on microRNAs encoded by the avian herpesviruses, MDV1 and MDV2 (Burnside et al., 2006, 2008; Yao et al., 2007, 2008). There is no conservation of sequence among the microRNAs of these herpesviruses, but in many cases, the genomic location is conserved. For instance, microRNAs of MDV1 (Burnside et al., 2006), MDV2 (Yao et al., 2007), EBV (Grundhoff et al., 2006; Pfeffer et al., 2004), KSHV (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005), HSV 1 (Umbach et al., 2008), RRV (Schafer et al., 2007), and MHV68 (Pfeffer et al., 2005) are clustered at predominantly one or two sites in the genome, and one of the clusters lies in a latency-active region.

Assigning function to viral microRNAs has been challenging. In the case of herpesviruses, which are characterized by latent infections, it has been proposed that viral microRNAs repress host protein production in order to enhance viral effects on the host cell (Grey et al., 2008). Host genes predicted to be targets of viral microRNAs are implicated in a number of cellular and viral processes, including cell proliferation, apoptosis, latency, and immunoevasion (Cai et al., 2005; Grey et al., 2005; Murphy et al., 2008; Pfeffer et al., 2005, 2004; Samols et al., 2005; Stern-Ginossar et al., 2007). It has also been suggested that viral genes, particularly those involved in replication, are potential targets for viral microRNA action and that such a process might be important in the regulation of latency (Cullen, 2006; Grey et al., 2007; Murphy et al., 2008; Tang et al., 2008).

Although gene sequences are unique to each virus, all of the *Mardiviridae* and ILTV share many protein-encoding genes, including those for glycoproteins and ICP4, an immediate early gene and transcriptional activator required for all viral gene expression (Dixon and Schaffer, 1980; Preston, 1979; Watson and Clements, 1980). Only MDV1 encodes the putative oncogene, *meq* (Lupiani et al., 2004), which maps within the I/TR_L regions of the genome. MDV1 encodes 14 mature microRNAs (miRBase), the majority of which are clustered in the two repeat regions of the genome; namely, flanking the *meq* gene and within the LAT region in a location that is antisense to and just downstream of the 3′ UTR of ICP4. MDV2 encodes 17 microRNAs (miRBase), one of which maps antisense to the coding region of ICP4 and 16 that map within the I/TR_L. Here, we have sequenced small RNA libraries from chicken embryo kidney cells (CEK) infected with ILTV, and chicken embryo fibroblasts (CEF) infected with HVT, as well as MSB1 cells, a lymphoblastoid cell line which harbors both MDV1 and MDV2 (Hirai et al., 1990). In this study, we present the sequence and characterization of the microRNAs from these related viruses. As noted previously for MDV1 and MDV2 microRNAs (Yao et al., 2007), the genomic position, but not the sequence, of all of these microRNAs tends to be conserved.

Results

Deep sequencing

We used Solexa's deep sequencing SBS method to identify microRNAs of several avian herpesviruses representative of all Gallid and Meleagrid herpesviruses. The nomenclature and accession numbers for these viruses are shown in Table 1. An overview of the sequencing results for the three libraries are listed in Table 2. For each library, greater than 2 million sequences were obtained. In the ILTV-infected cell sample, a total of 2,414,012 sequence reads were obtained; of these, approximately 66% of the sequences matched the chicken genome and less than 1% matched the ILTV genome. For MSB1 cells, 43%, 9.4% and 13% matched the chicken, MDV1 and MDV2

Table 2
Small RNA library composition and matches to host and viral genomes.

Library[a]	Total sequence reads	Matches to chicken genome		Matches to viral genomes			
				ILTV	MDV1	MDV2	HVT
		Matches[b]	Other[c]	Matches[b]	Matches	Matches	Matches
ILTV	2,414,012	1,602,619	108,208	2029	2	0	0
MSB1	2,602,161	1,119,779	51,841	0	245,475	326,694	18
HVT	2,717,356	2,032,229	82,372	0	72	82	137,729

[a] Libraries were constructed from the following cell preparations: MSB1, a lymphoblastoid cell line harboring MDV1 and MDV2; HVT, CEF infected with HVT; ILTV, CEK cells infected with ILTV.
[b] Total number of matches indicates the number of sequence reads with an exact match to the chicken or viral genome.
[c] Other small RNAs, e.g. tRNA, rRNA, snRNA, snoRNA and mtRNA.

Table 3
Sequence and frequency of unique sequence reads matching the ILTV genome.[a]

Name	Signature[b]	Frequency	Position
iltv-miR-I1-5p	AGACTGATTGGGGAATGATTGG	4	534
iltv-miR-I2-5p	GGAAGGCTGTGCGATAGGAGCCGA	3	1425
iltv-miR-I3-3p	TCTTGTCTCTGGGTGGGTTCGGA	16	1634
iltv-miR-I4-5p	ATGTATAGCGAGCAATGACCGTG(T)	6	1721
iltv-miR-I5-5p	(C)TTCTCGTCCCCGTCTTCTTCA(GA)	23	115,047[c]
iltv-miR-I5-3p	(TG)AAGAAGACGACGACGAGGAG(CAT)	1103	115,084[c]
iltv-miR-I6-5p	GTCTCCTGTACCCTCATCGTCG	4	115,346[c]
iltv-miR-I6-3p	(AC)GCTGAGGGGCCATGAGACAG(T)	863	115,385[c]

[a] Accession number NC_006623.
[b] Sequence variations are indicated by terminal nt in parentheses (see Supplemental Table S2).
[c] A second copy is located in terminal repeat (TR_S), see Fig. 6.

genomes, respectively. For the HVT-infected cell sample, approximately 75% matched the chicken genome, and 5% matched the HVT genome. The efficiency of obtaining exact matches was similar to those of other deep sequencing projects (Morin et al., 2008). Sequences not matching the chicken or viral genomes most likely represented errors in sequencing, contained sequence polymorphisms not detected by this analysis, or were derived from unsequenced regions of the chicken genome.

ILTV microRNAs

We identified sequences that map to eight different loci on the ILTV genome (Table 3) and fold into typical stem loop structures (Supplemental Fig. S1), suggesting that these are microRNAs. Only a single strand of the 70-nt region surrounding iltv-miRs-I1 to 4 was represented in the library, suggesting that the small RNAs from these microRNAs were derived only from the mature strands. For iltv-miRs I5 and I6, secondary structure and differential abundances indicated that the "star" strand was also cloned (Table 3 and Supplemental Fig. S1). The ILTV microRNAs are clustered in three regions of the genome; namely, the U_L, IR_S, and TR_S. MicroRNAs in the I/TR_S (iltv-miRs 15 and 6) map antisense to the 3′ end of the ICP4 coding region. The microRNAs in the U_L (iltv-miRs I1 to I4) are located at terminus of the genome, and are located outside of any annotated open reading frames. This region contains no other features except for local 102-bp inverted repeats (nt3:104 and 848:949, see accession number NC_006623).

Sequence variability was noted at both the 5′ and 3′ ends of the small RNA reads, generating families of isomiRs for each microRNA. All isomiRs for iltv-miR-15-3p and iltv-miR-16-3p are listed in Supplemental Table S2. The pattern of sequence reads for these microRNAs is typical of results obtained with deep sequencing of highly expressed species and is attributed to imprecision in precursor processing by Dicer or Drosha (Morin et al., 2008). However, in general, one species of small RNA predominates (bold type in Table S2), and the variants can make up 20% or more of the total. Whether or not all small RNA species function similarly is not clear at this time. However, it is probable that the seed sequence is comprised of bases 2–7 of the most abundant isomiR sequence, and any extra nucleotides on the 5′ end might serve to anchor the microRNA on the target molecule.

We compared the sequence of the ILTV microRNAs to the genome of another Iltovirus, Psittacid herpesvirus (PsHV-1) and found that the sequence of iltv-miR-I5-3p matches well (18/22 nt) with the PsHV-1 genomic sequence in an analogous position (antisense to ICP4). However, its star strand, iltv-miR-I5-5p, is not conserved within PsHV-1, and the PsHV-1 sequence surrounding the conserved region does not fold into a stem loop structure, making it unlikely that this sequence encodes a functional microRNA in PsHV-1.

The genomic DNA sequence encoding iltv-miRs I5 and I6 was compared to sequences from 22 strains of ILTV (obtained from GenBank); there are no sequence variations in the microRNAs among strains (Supplemental Table S3). However, there are single nucleotide polymorphisms (SNPs) in the 560-nucleotide sequence upstream of the microRNA region, which is antisense to the ICP4 gene. These microRNAs could be processed from the putative ILTV latency associated transcript (LAT) (Johnson et al., 1995), and the promoter for the primary microRNA transcript could be within the region containing the SNPs. It is possible that differences in promoter sequences result in differential expression of the pri-microRNAs among strains. We also sequenced 150 nucleotides of the region upstream of the iltv-miR-I3, which maps to the U_L, and found three SNPs between the virulent USDA strain and the non-pathogenic tissue culture origin vaccine strain, IVAX (A to G, position 446; C to A, position 476; and T to A, position 514).

Expression of ILTV microRNAs

We were able to detect the most frequently sequenced ILTV microRNAs by northern blot analysis (Fig. 1). The mature strand of miR-I5, iltv-miR-I5-3p, was expressed in chicken kidney (CK) infected with either the USDA or 63140 strains of ILTV (Fig. 1A). However, specific hybridization to the star strand or the precursor was not detected using a probe to I5-5p (not shown). A low signal corresponding to the mature strand of iltv-miR-I3-3p was detected in LMH cells infected with the 63140 strain of ILTV, but not in uninfected cells (Fig. 1B). iltv-miR-I3-3p is also detected in CK cells infected with the USDA strain, but the signal is very low. The signal from this probe binding the precursor is also low in USDA strain-infected cells, but it is apparent just below a non-specific hybridization signal that is slightly larger than the precursor (Fig. 1B). iltv-miR-I6 was also expressed in infected LMH cells (Fig. 1B) and CK cells (data not shown). Higher expression of the mature strand (iltv-miR-I6-3p) is apparent. ILTV miR's I1, I2 and I4, found by deep sequencing in low abundance, were not detected in any infected cell samples by

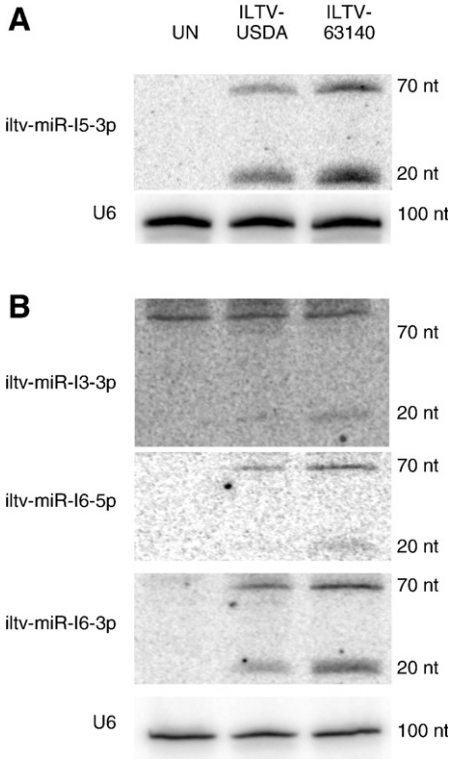

Fig. 1. ILTV microRNAs. Northern blotting analysis of RNA from (A) chicken kidney cells or (B) LMH cells. Cells were uninfected or infected with USDA or 63140 strains of ILTV. RNA was probed with ^{32}P-labelled oligonucleotides antisense to ILTV microRNAs indicated on the left. The cellular small RNA U6 was also probed on each blot as a loading control. Approximate nucleotide sizes are indicated.

northern blot analysis. Compared to other Gallid herpesviruses, relatively few ILTV-encoded microRNAs were identified. This could be due to low infectivity, and others might be discovered if heavier infections could be obtained.

HVT microRNAs

In the HVT library, we identified 34 small RNAs that match the HVT genome (Table 4). Sixteen of these represent the mature strand for HVT microRNAs, and in most cases, the star strands were also sequenced. With the flanking genomic sequences, these regions fold into stem loop structures, characteristic of *bona fide* microRNAs (Supplemental Fig. S2). As was found with ILTV small RNAs, sequence variability in the 5′ and 3′ ends was also apparent, as indicated by parentheses in Table 4. Additionally, several non-canonical short discrete small RNA species that are immediately adjacent to the mature and/or star strands were also sequenced multiple times (Table 4, designated with suffix 'a'). These likely represent the extensions of the pri-miRNA stem loop structure. In northern blot analysis, probes for these sequences hybridized only to RNA greater than 70 nt (data not shown). A cluster of 10 HVT microRNAs is found in a region of HVT I/TR_L that contains two local tandem repeats (Fig. 2). There are small sequence variations between the duplicates, some of which alter the seed sequence (nt 2–7). The most frequently sequenced HVT microRNA, hvt-miR-H15-3p, maps within the small predicted open reading frame (ORF) HVT074 (122069–122394), and hvt-miR-H18 is antisense to the predicted ORF HVT075 (122917–123423). SNP analysis among strains of HVT was not possible, since the genomic sequence of only one strain of HVT (FC126) is available.

One of the HVT microRNAs, hvt-miR-H14-3p, is a homologue of gga-miR-221 (Fig. 3A), with 21/23 nucleotide identity and 100% conservation of the seed/family sequence. In the region downstream of the mature microRNAs, the flanking region in HVT has sequence similarity to the chicken genome sequence (Fig. 3B), but the upstream region, containing the star or 5p strand, is not conserved. gga-miR-221 is very abundant in CEFs and other tissues (Burnside et al., 2006; Burnside et al., website; Burnside et al., 2008), and it is possible that this HVT homologue was captured from the chicken genome. Although the primary host for HVT is the turkey, the sequence of the genome is not yet available, and we cannot determine if there is additional conserved sequence with the turkey genome.

Expression of HVT microRNAs

We examined expression of several HVT microRNAs by northern blot analysis of RNA prepared from CEF infected with HVT (Fig. 4). For HVT microRNAs H7, H9, H10, H12, H13, H15 and H18, characteristic 20–22 nt mature strand bands and ~70 nt precursor bands were present in infected, but not uninfected cells; and the signal strength of the 5p and 3p strands generally corresponded to the numbers of sequence reads for each small RNA species. The hvt-miR-H7-5p and hvt-miR-H17-5p probes cross-hybridized with small RNA (>20 nt) bands in uninfected CEF, but there is no strong similarity of sequence between these probes and the chicken genome. In uninfected cells, H10-3p shows hybridization to a small RNA that is slightly larger and much less abundant than the specific hybridization seen in infected cells. The precursor for hvt-miR-H17-5p could not be discriminated from non-specific hybridization to a similarly sized fragment present in uninfected CEF. hvt-miR-H18-5p also hybridized to small RNAs in CEF; this sequence has a long series of G's ($n=7$), that could account for the high background signal. Expression of HVT microRNAs H1–H5 was not examined by northern blot analysis.

MDV1 and MDV2 microRNAs in MSB1 cells

The library prepared from the RNA of MSB1 cells had a high level of expression of viral microRNAs. Greater than 500,000 sequence reads matched viral genomes (Table 2). This was expected, as high viral microRNA expression was also noted in recent published studies of small RNAs from MSB1 cells (Yao et al., 2007, 2008). All MDV1 microRNAs sequenced in our study had been previously identified (Burnside et al., 2006, 2008; Yao et al., 2008), although some differences in relative frequency were noted (Morgan et al., 2008). Of the 36 MDV2 microRNAs found in this study, nine were not included among the 27 previously reported (Yao et al., 2008). A complete list of sequenced MDV2 microRNAs with sequencing read frequencies is in Supplemental Table S1. The new MDV2 microRNAs presented here are comprised primarily of star (*) strands of known mature strands and contain the expected 2-nucleotide 3′ overhang. A novel mature MDV2 microRNA, mdv2-miR-M32, was found; it folded into a typical stem loop structure (Fig. 5A) and was detected by northern blot analysis (Fig. 5B). The relative frequency of MDV2 microRNAs differed from those of a previous study (Yao et al., 2008), and is likely due to use of a different population of MSB1 cells and/or a different approach to sequencing and analysis. mdv2-miR-M16, and mdv2-miR-M17 were among the most abundant in both studies.

A comparison of the seed sequences for MDV2 microRNAs to known microRNAs revealed that mdv2-miR-M21 (GAGCACCACGCC-GATGGACGGAGA) and gga-miR-29 (TAGCACCATTTGAAATCAGTGTT)

Table 4
Sequence and frequency of small RNA reads matching the HVT genome.[a]

Name	Signature[b]	Frequency	Position[c]
HVT-miR-H1	GCGCAGGGATGTCGGGCG(CGCC)	43	118,146
HVT-miR-H2	CCCGTTCGGACGTTTCGGT(TT)	65	118,230
HVT-miR-H3-5p	CGTCATGGATCGCGGGGGGA(CG)	5	118,250
HVT-miR-H3-3p	CCCGACGGAGGCTCGGCG	3	118,300
HVT-miR-H4-5p	(G)GCGGAGGTGTGATATGGTG(T)	17	118,679
HVT-miR-H4-3p	GGGCGGTGGCACCCGCCGTCGGAGTA	2	118,725
HVT-miR-H5-5p	GCTGGTGCCGACGATCGCCGGGA	3	119,733
HVT-miR-H5-3p	GGTAACCTCTGCGATCGTTCGGT	13	119,778
HVT-miR-H7-5p	TTTTTCCTAAAGCGTGCCCGGGTT(A)	286	120,865
HVT-miR-H7-3p	(A)CCGGTCCGTTGTAGGAATTAGG(AA)	128	120,902
HVT-miR-H8	(GCATGG)ATCTCTCGCGGGGTGCGTAGGCGT	4	121,237
HVT-miR-H9-5p	(TT)TCTCATGGACCCCCGAGTTGTG(T)	340	121,408
HVT-miR-H9-3p[d]	(CA)GCTAGGCCGTCCATGAGGTCGT(A)	390	121,443
HVT-miR-H10-5p	TCCTTCTCGCGGATAGCGTAGATGT(C)	152	121,555
HVT-miR-H10-3p	(C)AGCTACATTGTCCGCAGGGATC	34	121,592
HVT-miR-H12-5p	TTTCTTATGGACCGCCGAGGTG(TGTC)	1126	121,726
HVT-miR-H12-3p	(CA)GCTAGGCCGTCCATGAGGTCGT(A)	390	121,764
HVT-miR-H13-5p	TTTTCTCCGGTGAGCGTAGCTG(C)	847	121,872
HVT-miR-H13-3p	(A)GCTACGTTGTCCGCGGGATTCTA	39	121,910
HVT-miR-H14-5p	(AC)TCATTCAGCGGGCAATGTAGACTGT	967	122,100
HVT-miR-H14-3p[e]	AGCTACATTGCCCGCTGGGTTT(C)	457	122,135
HVT-miR-H15-5p	(T)CTCGGAACAGGCGTATTTTTTGC(G)	17610	122,224
HVT-miR-H15-3p	(C)AAAGATACGCATGGGCTGAGGG(AAT)	82174	122,266
HVT-miR-H16-5p[f]	(CC)TTCATGTCGGGGGTGTTTGTGT(GT)	15367	122,510
HVT-miR-H16-3p[f]	(A)TAAATAGCCCCGATTTGACAG(GT)	65	122,546
HVT-miR-H17-5p	(CC)TTCACGTCGGGGGTGTTTGTGT(GT)	10013	122,672
HVT-miR-H17-3p	(A)TAAACAGCCCCGATTTGACAGG(TT)	762	122,710
HVT-miR-H18-5p	ATCAAACCGGGGGGGATTTCAGT(A)	1109	122,916
HVT-miR-H18-3p	(C)TGAGATACGCCCGATTTGACGA(A)	959	122,952
H9-5pa[g]	TGGACAGTTGGGGCATACTT	37	121,386
H9-3pa[g]	GTAGGCACCCAGTAATGATTCGC	2	121,464
H12-5pa[g]	CTGGACAGTTTGGGCATACTTT	2	121,707
H14-5pa[g]	GACAGATCAGACTAATGCACGGAC	95	122,074
H14-3pa[g]	CTGGCTGTCTGGAAATGCAAGAG	3	122,157

[a] Accession number AF291866.
[b] Sequence variations are indicated by terminal nt in parentheses.
[c] A second copy is located in terminal repeat flanking the unique long region (TR_L).
[d] A paralogue microRNA sequence is located in a tandem repeat region 321 base pairs downstream and is named miR H-12-3p.
[e] Orthologue of gga-miR-221 (miRBase accession# MIMAT0001108) (Fig. 3).
[f] Paralogue microRNAs with one or three nucleotide differences in sequence are located in a tandem repeat region 162 base pairs downstream and are named miR-H17-5p and miR-H17-3p.
[g] Read sequences that are short, discrete RNA species immediately adjacent to mature and star strands of microRNAs.

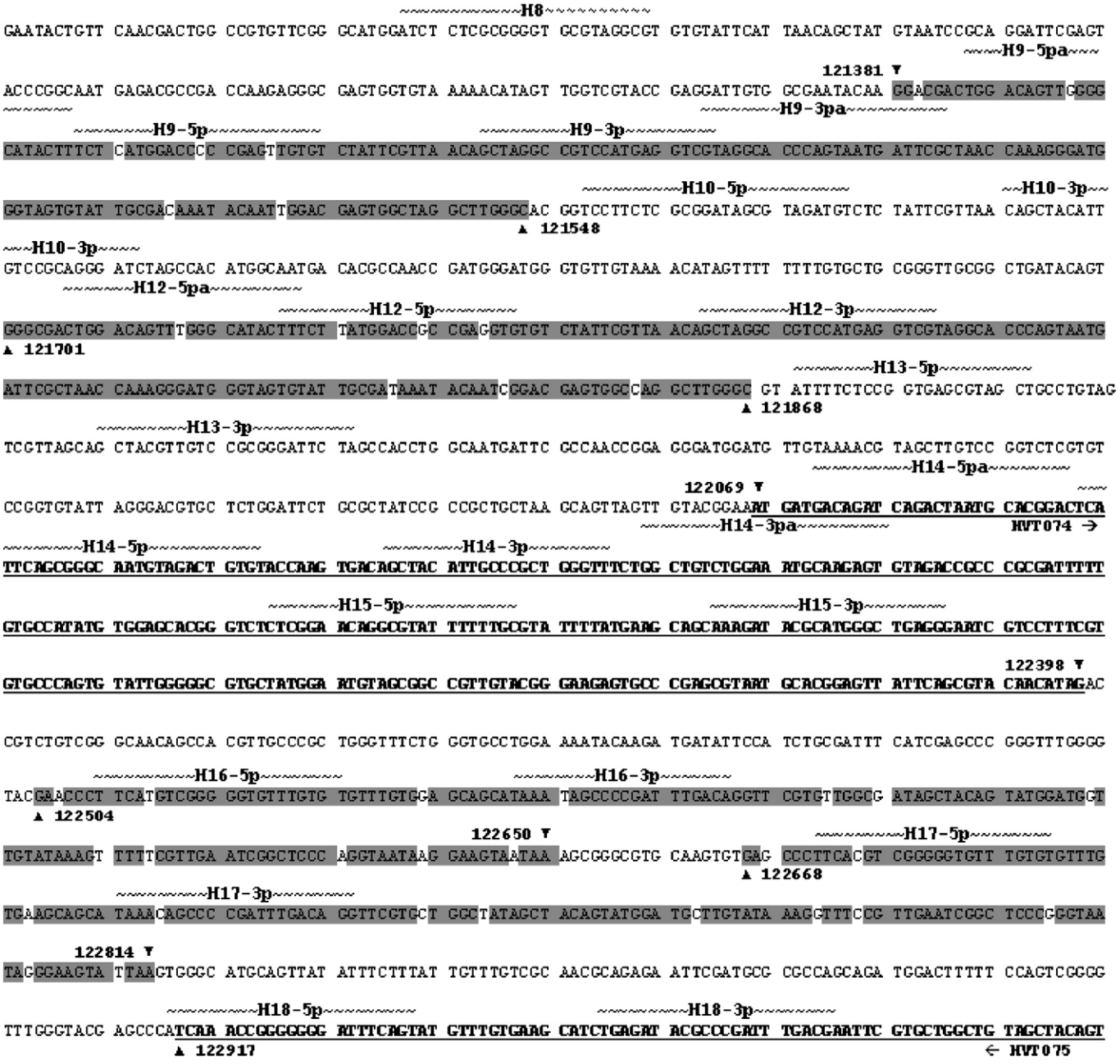

Fig. 2. Fragment of HVT genome (AF291866) containing locally duplicated sections and a cluster of HVT microRNAs. Duplicated sections are shown highlighted in grey. Letters without highlighting indicate non-matching nucleotides between the duplicated regions. Predicted HVT microRNAs are annotated above the sequence as H-(miR name). Non-canonical short, discrete RNA species immediately adjacent to mature and star strands of microRNAs are designated with suffix 'a' as indicated in Table 4. Nucleotides encoding annotated open reading frames HVT074 and HVT075 are in bold and underlined.

A

```
hvt-miR-H14-3p  1   AGCTACATTGCCCGCTGGGTTTC  23
                    |||||||||| | ||||||||||
gga-miR-221     1   AGCTACATTGTCTGCTGGGTTTC  23
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B

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HVT   122133    ACAGCTACATTGCCCGCTGGGTTTCTGGCTGTCTGGAAATGCAA
                |||||||||||| | ||||||||||  |||| |||||||| |||
Chr1 114218987 ACAGCTACATTGTCTGCTGGGTTTCCAGCTGCCTGGAAATACAA
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Fig. 3. HVT-miR-H14-3p is a homologue of gga-miR-221. hvt-miR-14-3p was aligned with (A) gga-miR-221; (B) HVT genomic sequence surrounding hvt-miR-14-3p (bold) was aligned with a portion of chicken chromosome 1 using BLAST-N.

share 44% nucleotide similarity and 100% similarity in the seed region (underlined). As with HVT, the sequence of other strains of MDV2 is not available and SNP analysis was not conducted.

Comparison of microRNAs from Gallid and Meleagrid herpesviruses

We compared all viral microRNA precursors to each other and to known chicken microRNA precursors using BLAST-N with an *E* value cutoff of $E<0.1$. Other than hvt-miR-H14-3p and miR-221, no matches were found, suggesting there is no phylogenetic relationship among the avian herpesvirus microRNAs. Using neighbor-joining analysis of a nucleotide alignment in order to identify distant phylogenetic relationships among viral microRNA seeds, we found that the hvt-

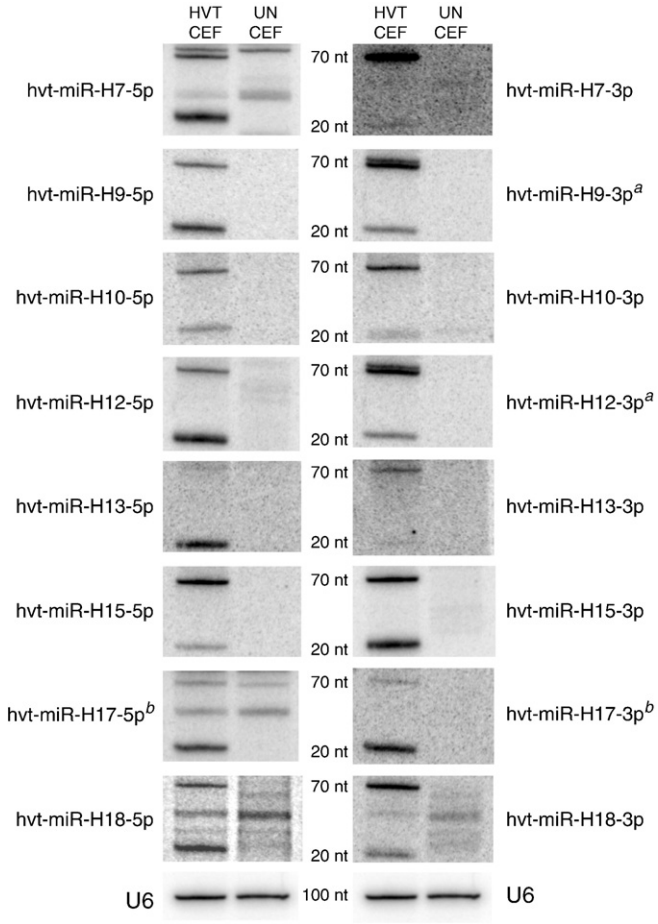

Fig. 4. HVT micro RNAs. Northern blot analysis of RNA from HVT-infected and uninfected chicken embryo fibroblasts (CEF). RNA was probed with ^{32}P-labelled oligonucleotides antisense to the hvt-miRs. The cellular small RNA U6 was probed on the same blots as a loading control. Representative U6 hybridizations are shown. Approximate nucleotide sizes are indicated. [a]hvt-miR-H9-3p and hvt-miR-H12-3p are detected by a single probe since the microRNA sequences are identical. [b]The probes for hvt-miR-H17-5p and hvt-miR-H17-3p should also detect hvt-miR-16-5p and hvt-miR-H16-3p, respectively, since their sequences differ by one or three nucleotides (Table 4). Since specific activities of the probes and exposure times varied, relative abundances across the various northern blots should not be compared to sequencing read frequencies.

miR-H13-5p and mdv1-miR 9-5p are 48% homologous, with a 100% match at the seed/family positions (nucleotides 2–8), indicating there may be some overlapping conservation of function.

A diagrammatic comparison of the genomes of the Gallid and Meleagrid herpesviruses with the location of their microRNAs is shown in Fig. 6. Despite the lack of similarity among sequences of the different viral microRNAs, it is clear that Gallid and Meleagrid microRNAs are all located in similar regions of the viral genomes. MDV1 microRNAs are clustered primarily in two regions: in the I/TR$_L$ and at the 5′ end of IR$_S$ (3′ end of TR$_S$). MDV2 microRNAs are found in similar positions, with the majority located in the I/TR$_L$ and one in the I/TR$_S$. All HVT microRNAs cloned map to the I/TR$_L$. ILTV does not contain the I/TR$_L$, but several microRNAs are found at the 5′ end of the I/TR$_S$ and in a similar position as one MDV2 microRNA, antisense to the coding region of ICP4. Very few of the microRNAs were located in unique regions of any of the viruses studied. Thus, the majority of these viral microRNAs are located in highly plastic and evolving regions of the genomes.

Discussion

The Gallid and Meleagrid herpesviruses all express microRNAs. These herpesviruses resemble mammalian herpesviruses in both genome organization and content in the U$_L$ and U$_S$, but the flanking repeats are unique for each virus. Tissue tropisms and host ranges vary among these viruses and the outcomes of infection span a wide range of pathologies. Some of these differences might be attributed, at least in part, to the difference in genes in the repeat regions. For example, HVT and MDV2 I/TR$_L$ regions do not resemble each other and contain genes of unknown function. Neither contains the homologues of the MDV1 genes in this region that are associated with virulence (*meq*, pp24, pp38, CxC cytokine) and, in the case of the *meq* gene, with oncogenicity. ILTV lacks the repeats flanking the unique long region, but the corresponding region of the ILTV U$_L$ contains a series of local repeats and is not gene rich (see NC_006623 gene record). The I/TR$_S$ of MDV1, MDV2, HVT, and ILTV all contain an ICP4 homolog, which is an essential immediate early gene, but other genes in the I/TR$_S$ are not conserved. These virus-specific repeat regions are where we generally find the microRNAs localized.

Sequence comparison of all Gallid and Meleagrid herpesviral microRNA precursors revealed no distant homology among themselves, with other herpesvirus sequences, or with host microRNAs (except for miR-221, see below). Phylogenetic analyses of 31 orthologous genes shared by all avian herpesviruses (Hughes and Rivailler, 2007) revealed that ILTV, an Iltovirus, was not closely related to the Mardiviruses (MDV1, MDV2, or HVT). In fact, Mardiviruses appear to be more closely related to mammalian alphaherpesviruses (McGeoch et al., 2006), supporting the hypothesis that the host does not drive the evolution of avian viruses (Hughes and Rivailler, 2007; McGeoch et al., 2006). It is clear that the evolution of genes in the repeats of herpesviruses is faster than that of genes in the unique regions (McGeoch et al., 2006), and it is likely that viral microRNAs evolved along with or after the repeat regions. We hypothesize, therefore, that the presence of microRNAs in repeated parts of the viral genome would facilitate gain-of-function mutations. Any loss of function would be complemented by sequences in the other repeat, while advantageous mutations would be maintained and eventually duplicated. However, it should be noted that the canonical function of

Fig. 5. Novel MDV2 microRNA precursor molecule. (A) Predicted secondary structure of mdv2-miR-M32 precursor molecule. The mature strand (61 sequence reads) is in bold and underlined. The star strand (4 sequence reads) is in bold italics text. (B) Northern blot validation of mdv2-miR-M32 expression. Lanes, MSB1 cells, uninfected CEF cells, CEF infected with MDV2 strain SB1, CEF infected with MDV1 strain RB1B.

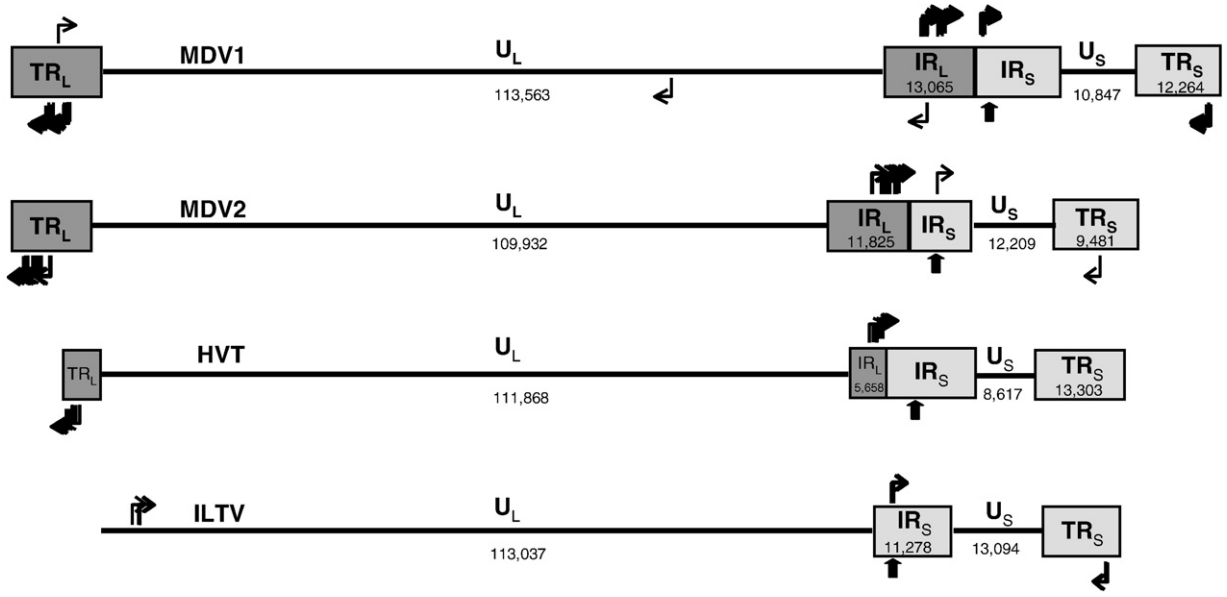

Fig. 6. Overview of Gallid and Meleagrid herpesvirus genomes and location of microRNAs. Terminal and internal (short and long) repeats are abbreviated TR_S, IR_S, TR_L, and IR_L, with lengths (base pairs) of each region listed below. Lengths of each region are derived from annotations of reference sequences listed in Table 1. Direction and location of microRNAs are denoted with small arrows. The ICP4 gene in all four viruses is located in the IR_S and IR_L repeat regions, denoted with a vertical arrowhead in the IR_S.

a microRNA action is to bind to the 3′ UTR of target genes, with binding mediated through the 'seed' sequence (nt 2–7) of the microRNA (Bartel, 2004). The same gene could be targeted by different microRNAs binding to different sites in the 3′ UTR, and we cannot rule out the possibility that some of these divergent avian herpesviral microRNAs share targets, be they viral or host.

Some of the microRNAs in HVT and MDV1 (Morgan et al., 2008) may have arisen by local duplication of a portion of the viral genome, and indeed, duplication is a common theme for development of microRNAs. For example, the microRNAs of the human miR17 clusters are thought to have evolved through duplication of individual microRNAs and clusters of microRNAs, and it is thought that these duplications arose simultaneously with the evolution of vertebrates (Tanzer and Stadler, 2004). Additionally, X-linked microRNA clusters in primates, like protein-encoding genes involved in male development, have been subjected to evolutionarily rapid duplication and sequence substitutions (Zhang et al., 2007). This has been well documented in *Arabidopsis*, as well (Maher et al., 2006). However, the role of duplication in the evolution of viral microRNAs has not been studied.

We found that one HVT microRNA, hvt-miR-H14-3p, is homologous to the cellular microRNA miR-221. This cellular microRNA is highly expressed in many tissues (Burnside et al., 2006; Burnside et al., website; Burnside et al., 2008). Deep sequencing analyses indicate a high abundance of miR-221 in MSB1 cells, CEF, CEK, spleen, and T cells, but not in MDV-induced tumors (Burnside et al., website; Burnside et al., 2008). One candidate target of miR-221 is p27Kip1, a cell cycle inhibitor (Galardi et al., 2007). Expression of a miR-221 mimic by HVT could enhance growth and proliferation of infected cells, thereby increasing the viral reservoir. We speculate that the HVT genomic region containing hvt-miR-14-3p was pirated from a host genome. Other herpesviruses, including MDV, acquire genes by capture from the host cell (McGeoch et al., 2006; Niikura et al., 2006), but this is the first report of the capture of a host microRNA. We also noted in this study that one MDV2 microRNA shares a seed sequence with gga-miR-29b. miR-29 is thought to promote apoptosis by down regulating Mcl1, an antiapoptotic protein (Mott et al., 2007). Previously, we reported that one MDV1 microRNA shares a seed with miR-155 and another shares a seed with miR-221 (Morgan et al., 2008). Some viral microRNAs may have evolved to augment the function of host microRNAs in order to poise the cell to support viral replication.

Several recent studies highlight the role of viral microRNAs in the down regulation of viral immediate early proteins and propose that these microRNAs function in the establishment and maintenance of latency (Grey et al., 2007; Murphy et al., 2008; Tang et al., 2008; Umbach et al., 2008). MDV2 and ILTV microRNAs map antisense to the coding region of ICP4 and could act as siRNAs to decrease expression of ICP4. Expression of these microRNAs and the consequent decrease in ICP4 activity would facilitate the latent stage of infection for these viruses. However, other viral microRNAs that are antisense to the coding regions of viral genes regulate distant targets and do not act as siRNAs (Grey and Nelson, 2008). Herpesviral microRNAs can target both viral and host genes (Grey et al., 2008), and functional studies are required to determine the role of microRNAs mapping to ICP4.

In summary, these results add to the list of herpesviruses that show conservation of microRNA sequence within a viral strain (Marshall et al., 2007; Morgan et al., 2008), but no apparent sequence conservation of microRNAs among related viruses (Morgan et al., 2008; Schafer et al., 2007). As with RRV and KSHV microRNAs (Schafer et al., 2007), we show that microRNAs share genomic localization among related Gallid and Meleagrid herpesviruses. The microRNAs examined in this study were located mostly in genomic repeat regions as well as in short tandem repeats, suggesting a possible role of genomic duplication in the evolution of microRNAs in herpesviruses.

Materials and methods

Cell cultures and viruses

CEF, CK and CEK were prepared as described previously (Schat and Purchase, 1998; Tannock et al., 1985). HVT Fc126 was from the University of Delaware collection, ILTV USDA was from ATCC via the University of Delaware collection; IVAX, ILTV USDA and ILTV 63140 strains and the LMH cell line (Kawaguchi et al., 1987) were obtained from the University of Georgia and University of Delaware collections.

Deep sequencing and sequence analysis

Total RNA was purified using Trizol (Invitrogen, Carlsbad, CA) and submitted to Illumina (Hayward, CA) for library preparation and

sequencing via their sequencing-by-synthesis (SBS) method (Illumina; Lu et al., 2006, 2007, 2005). Sequence reads with high base quality scores were trimmed of the 3′ adapter sequences and compared to the chicken and viral genomes, allowing only exact matches. Sequences mapping to unique loci were tallied to determine sequencing read frequency. Canonical microRNA folding was tested using Mfold (Zuker, 2003).

RNA preparation and northern blotting

RNA was purified using Trizol, according to the manufacturer's instructions (Invitrogen). Total RNA (10 μg) was electrophoresed on a denaturing polyacrylamide gel (0.5× TBE, 7 M urea, 15% acrylamide (19:1:acryl:bis-acryl), electroblotted to charged nylon, and hybridized to ^{32}P-labeled antisense primers complementary to the microRNAs at 42 °C in ULTRAhyb™-Oligo (Ambion). Blots were washed at 42 °C in 2× SSC, 0.5% SDS and exposed to phosphorimager screens for varying times; screens were developed using a Typhoon Phosphorimager. Blots were stripped in 0.1× SSC, 0.1% SDS at 65 °C for 30 min and re-exposed to phosphorimager screens for 20 h. Hybridization to an antisense primer for U6 was used as a loading control. The 10-bp DNA step ladder (Promega) was used to approximate size.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.02.043.

References

Bagust, T.J., Jones, R.C., Guy, J.S., 2000. Avian infectious laryngotracheitis. Revue Scientifique Et Technique De L Office International Des Epizooties 19 (2), 483–492.
Bartel, D.P., 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116 (2), 281–297.
Buck, A.H., Santoyo-Lopez, J., Robertson, K.A., Kumar, D.S., Reczko, M., Ghazal, P., 2007. Discrete clusters of virus-encoded microRNAs are associated with complementary strands of the genome and the 7.2-kilobase stable intron in murine cytomegalovirus. J. Virol. 81 (24), 13761–13770.
Burnside, J., Bernberg, E., Anderson, A., Lu, C., Meyers, B.C., Green, P.J., Jain, N., Isaacs, G.K., Morgan, R.W., 2006. Marek's disease virus microRNAs map to meq and LAT. J. Virol. 80, 8778–8786.
Burnside, J., Ouyang, M., Anderson, A., Bernberg, E., Lu, C., Meyers, B.C., Green, P.J., Markis, M., Isaacs, G.K., Huang, E., Morgan, R.W., 2008. Deep sequencing of chicken microRNAs. BMC Genomics 9 (185), doi:10.1186/1471-2164-9-185.
Burnside, J., Morgan, R.W., Green, P.J., Meyers, B.C.N., Kan, Kamboj, S., Gupta, R., McCormick, K., Shridhara, G., Nakano, M., Hetawal, A., Valliappan, R.M. (website). *Gallus gallus* sbs. *In* "http://mpss.udel.edu/gga/".
Cai, X., Lu, S., Zhang, S., Gonzalez, C.M., Damania, B., Cullen, B.R., 2005. Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. PNAS 102 (15), 5570–5575.
Cebrian, J., Kaschkadierich, C., Berthelot, N., Sheldrick, P., 1982. Inverted repeat nucleotide sequences in the genomes of Marek's disease virus and the herpesvirus of the turkey. PNAS 79 (2), 555–558.
Cullen, B.R., 2006. Viruses and microRNAs. Nat. Genet. 38, 525–530.
Dixon, R.A.F., Schaffer, P.A., 1980. Fine-structure mapping and functional-analysis of temperature-sensitive mutants in the gene encoding the herpes-simplex virus type-1 immediate early protein vp175. J. Virol. 36 (1), 189–203.
Filipowicz, W., Bhattacharyya, S.N., Sonenberg, N., 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat. Rev. Genet. 9 (2), 102–114.
Galardi, S., Mercatelli, N., Giorda, E., Massalini, S., Frajese, G.V., Ciafre, S.A., Farace, M.G., 2007. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. J. Biol. Chem. 282 (32), 23716–23724.
Garcia, M., Riblet, S.M., 2001. Characterization of infectious laryngotracheitis virus isolates: demonstration of viral subpopulations within vaccine preparations. Avian Dis. 45 (3), 558–566.
Grey, F., Nelson, J., 2008. Identification and function of human cytomegalovirus microRNAs. J. Clin. Virol. 41 (3), 186–191.
Grey, F., Antoniewicz, A., Allen, E., Saugstad, J., McShea, A., Carrington, J.C., Nelson, J., 2005. Identification and characterization of human cytomegalovirus-encoded microRNAs. J. Virol. 79 (18), 12095–12099.
Grey, F., Meyers, H., White, E.A., Spector, D.H., Nelson, J., 2007. A human cytomegalovirus-encoded microRNA regulates expression of multiple viral genes involved in replication. PLoS Path. 3 (11), 1593–1602.
Grey, F., Hook, L., Nelson, J., 2008. The functions of herpesvirus-encoded microRNAs. Med. Microbiol. Immunol. 197 (2), 261–267.
Griffiths-Jones, S., Saini, H.K., van Dongen, S., Enright, A.J., 2008. miRBase: tools for microRNA genomics. Nucleic Acids Res. 36, D154–D158.
Grundhoff, A., Sullivan, C.S., Ganem, D., 2006. A combined computational and microarray-based approach identifies novel microRNAs encoded by human gamma-herpesviruses. RNA 12 (5), 733–750.
Hirai, K., Yamada, T., Arao, Y., Kato, S., Nii, S., 1990. Replicating Marek's disease virus (MDV) serotype 2 DNA with inserted MDV serotype 1 DNA sequences in a Marek's disease lymphoblastoid cell line MSB1-41C. Arch. Virol. 114 (3–4), 153–165.
Hughes, A.L., Rivailler, P., 2007. Phylogeny and recombination history of gallid herpesvirus 2 (Marek's disease virus) genomes. Virus Res. 130 (1–2), 28–33.
Illumina http://www.illumina.com/.
Johnson, M.A., Tyack, S.G., Prideaux, C., Kongsuwan, K., Sheppard, M., 1995. Nucleotide sequence of infectious laryngotracheitis virus (Gallidherpesvirus-1) ICP4 gene. Virus Res. 35 (2), 193–204.
Kawaguchi, T., Nomura, K., Hirayama, Y., Kitagawa, T., 1987. Establishment and characterization of a chicken hepatocellular carcinoma cell line, LMH. Cancer Res. 47, 4460–4464.
Kingham, B.F., Zelnik, V., Kopacek, J., Majerciak, V., Ney, E., Schmidt, C.J., 2001. The genome of herpesvirus of turkeys: comparative analysis with Marek's disease viruses. J. Gen. Virol. 82, 1123–1135.
Kirkpatrick, N.C., Mahmoudian, A., Colson, C.A., Devlin, J.M., Noormohammadi, A.H., 2006. Relationship between mortality, clinical signs and tracheal pathology in infectious laryngotracheitis. Avian Pathol. 35 (6), 449–453.
Lewis, B.P., Burge, C.B., Bartel, D.P., 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15–20.
Lim, L.P., Glasner, M.E., Yekta, S., Burge, C.B., Bartel, D.P., 2003. Vertebrate microRNA genes. Science 299, 1540.
Lu, C., Tej, S.S., Luo, S., Haudenschild, C.D., Meyers, B.C., Green, P.J., 2005. Elucidation of the small RNA component of the transcriptome. Science 309, 1567–1569.
Lu, C., Kulkarni, K., Souret, F.F., MuthuValliappan, R., Tej, S.S., Poethig, R.S., Henderson, I.R., Jacobsen, S.E., Wang, W.Z., Green, P.J., Meyers, B.C., 2006. MicroRNAs and other small RNAs enriched in the *Arabidopsis* RNA-dependent RNA polymerase-2 mutant. Genome Res. 16 (10), 1276–1288.
Lu, C., Meyers, B.C., Green, P.J., 2007. Construction of small RNA cDNA libraries for deep sequencing. Methods 43 (2), 110–117.
Lupiani, B., Lee, L.F., Cui, X., Gimeno, I., Anderson, A., Morgan, R.W., Silva, R.F., Witter, R.L., Kung, H.-J., Reddy, S.M., 2004. Marek's disease virus-encoded Meq gene is involved in transformation of lymphocytes but is dispensable for replication. PNAS 101, 11820–11875.
Maher, C., Stein, L., Ware, D., 2006. Evolution of *Arabidopsis* microRNA families through duplication events. Genome Res. 16 (4), 510–519.
Marshall, V., Parks, T., Bagni, R., Wang, C.D., Samols, M.A., Hu, J.H., Wyvil, K.M., Aleman, K., Little, R.F., Yarchoan, R., Renne, R., Whitby, D., 2007. Conservation of virally encoded MicroRNAs in kaposi sarcoma-associated herpesvirus in primary effusion lymphoma cell lines and in patients with kaposi sarcoma or multicentric Castleman disease. J. Infect. Dis. 195 (5), 645–659.
McGeoch, D.J., Rixon, F.J., Davison, A.J., 2006. Topics in herpesvirus genomics and evolution. Virus Res. 117 (1), 90–104.
miRBase http://microrna.sanger.ac.uk/cgi-bin/sequences/query.pl?terms=mdv1.
Morgan, R., Anderson, A., Bernberg, E., Kamboj, S., Huang, E., Lagasse, G., Parcells, M., Isaacs, G., Burnside, J., 2008. Sequence conservation and differential expression of Marek's disease virus microRNAs. J. Virol. 82 (24), 12213–12220.
Morin, R.D., O'Connor, M.D., Griffith, M., Kuchenbauer, F., Delaney, A., Prabhu, A.L., Zhao, Y., McDonald, H., Zeng, T., Hirst, M., Eaves, C.J., Marra, M.A., 2008. Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells. Genome Res. 18 (4), 610–621.
Mott, J.L., Kobayashi, S., Bronk, S.F., Gores, G.J., 2007. mir-29 regulates Mcl-1 protein expression and apoptosis. Oncogene 26 (42), 6133–6140.
Murphy, E., Vanicek, J., Robins, H., Shenk, T., Levine, A.J., 2008. Suppression of immediate-early viral gene expression by herpesvirus-coded microRNAs: implications for latency. PNAS 105 (14), 5453–5458.
Niikura, M., Dodgson, J., Cheng, H., 2006. Direct evidence of host genome acquisition by the alphaherpesvirus Marek's disease virus. Arch. Virol. 151 (3), 537–549.
Nilsen, T.W., 2007. Mechanisms of microRNA-mediated gene regulation in animal cells. Trends Genet. 23 (5), 243–249.
Pfeffer, S., Zavolan, M., Grasser, F.A., Chien, M., Russo, J.J., Ju, J., John, B., Enright, A.J., Marks, D., Sander, C., Tuschl, T., 2004. Identification of virus-encoded microRNAs. Science 304, 734–736.
Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F.A., vanDyk, L.F., Ho, C.K., Shuman, S., Chien, M., Russo, J.J., Ju, J., Randall, G., Lindenbach, B.D., Rice, C.M., Simon, V., Ho, D.D., Zavolan, M., Tuschl, T., 2005. Identification of microRNAs of the herpesvirus family. Nat. Methods 2 (4), 269–276.
Preston, C.M., 1979. Abnormal properties of an immediate early polypeptide in cells infected with the Herpes Simplex Virus Type-1 mutant *tsK*. J. Virol. 32 (2), 357–369.
Samols, M.A., Hu, J., Skalsky, R.L., Renne, R., 2005. Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. J. Virol. 79 (14), 9301–9305.
Schafer, A., Cai, X.Z., Bilello, J.P., Desrosiers, R.C., Cullen, B.R., 2007. Cloning and analysis of microRNAs encoded by the primate gamma-herpesvirus rhesus monkey rhadinovirus. Virology 364 (1), 21–27.

Schat, K.A., Purchase, H.G., 1998. Isolation and Identification of Avian Pathogens, American Association of Avian Pathologists, pp. 223–234. Kennett Square, Pennsylvania, USA.
Stefani, G., Slack, F.J., 2008. Small non-coding RNAs in animal development. Nat. Rev. Mol. Cell Biol. 9 (3), 219–230.
Stern-Ginossar, N., Elefant, N., Zimmermann, A., Wolf, D.G., Saleh, N., Biton, M., Horwitz, E., Prokocimer, Z., Prichard, M., Hahn, G., Goldman-Wohl, D., Greenfield, C., Yagel, S., Hengel, H., Altuvia, Y., Margalit, H., Mandelboim, O., 2007. Host immune system gene targeting by a viral miRNA. Science 317 (5836), 376–381.
Tang, S., Bertke, A.S., Patel, A., Wang, K., Cohen, J.I., Krause, P.R., 2008. An acutely and latently expressed herpes simplex virus 2 viral microRNA inhibits expression of ICP34.5, a viral neurovirulence factor. PNAS 105 (31), 10931–10936.
Tannock, G.A., Bryce, D.A., Paul, J.A., 1985. Evaluation of chicken kidney and chicken embryo kidney cultures for the large-scale growth of attenuated influenza virus master strain A/Ann/Arbor/6/60-ca. Vaccine 3 (3), 333–339.
Tanzer, A., Stadler, P.F., 2004. Molecular evolution of a microRNA cluster. J. Mol. Biol. 339 (2), 327–335.
Umbach, J.L., Kramer, M.F., Jurak, I., Karnowski, H.W., Coen, D.M., Cullen, B.R., 2008. MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. Nature 454 (7205), 780–783.
Watson, R.J., Clements, J.B., 1980. A herpes simplex virus type-1 function continuously required for early and late virus RNA synthesis. Nature 285 (5763), 329–330.
Witter, R.L., Schat, K., 2003. Marek's disease, In: Saif, Y.M. (Ed.), Diseases of Poultry, 11th ed. Iowa State Press, Ames, Iowa, pp. 407–464.
Yao, Y., Zhao, Y., Xu, H., Smith, L.P., Lawrie, C.H., Sewer, A., Zavolan, M., Nair, V., 2007. Marek's disease virus type 2 (MDV-2)-encoded microRNAs show no sequence conservation with those encoded by MDV-1. J. Virol. 81 (13), 7164–7170.
Yao, Y., Zhao, Y., Xu, H., Smith, L.P., Lawrie, C.H., Watson, M., Nair, V., 2008. MicroRNA profile of Marek's disease virus-transformed T-cell line MSB-1: predominance of virus-encoded microRNAs. J. Virol. 82, 4007–4015.
Zhang, R., Peng, Y., Wang, W., Su, B., 2007. Rapid evolution of an X-linked microRNA cluster in primates. Genome Res. 17 (5), 612–617.
Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31 (13), 3406–3415.