

MicroRNA Profile of Marek's Disease Virus-Transformed T-Cell Line MSB-1: Predominance of Virus-Encoded MicroRNAs[∇]

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Research over the last few years has demonstrated the increasing role of microRNAs (miRNAs) as major regulators of gene expression in diverse cellular processes and diseases. Several viruses, particularly herpesviruses, also use the miRNA pathway of gene regulation by encoding their own miRNAs. Marek's disease (MD) is a widespread lymphomatous neoplastic disease of poultry caused by the highly contagious Marek's disease virus type 1 (MDV-1). Recent studies using virus-infected chicken embryo fibroblasts have identified at least eight miRNAs that map to the R_L/R_S region of the MDV genome. Since MDV is a lymphotropic virus that induces T-cell lymphomas, analysis of the miRNA profile in T-cell lymphoma would be more relevant for examining their role in oncogenesis. We determined the viral and host miRNAs expressed in MSB-1, a lymphoblastoid cell line established from an MDV-induced lymphoma of the spleen. In this paper, we report the identification of 13 MDV-1-encoded miRNAs (12 by direct cloning and 1 by Northern blotting) from MSB-1 cells. These miRNAs, five of which are novel MDV-1 miRNAs, map to the Meq and latency-associated transcript regions of the MDV genome. Furthermore, we show that miRNAs encoded by MDV-1 and the coinfecting MDV-2 accounted for >60% of the 5,099 sequences of the MSB-1 "miRNAome." Several chicken miRNAs, some of which are known to be associated with cancer, were also cloned from MSB-1 cells. High levels of expression of MDV-1-encoded miRNAs and potentially oncogenic host miRNAs suggest that miRNAs may have major roles in MDV pathogenesis and neoplastic transformation.

Marek's disease (MD) is a naturally occurring rapid-onset aggressive T-cell lymphoma of poultry. Named after the Hungarian veterinarian József Marek, who first reported the disease in 1907 (41), the disease is induced by Marek's disease virus type 1 (MDV-1), a highly contagious alphaherpesvirus belonging to the genus *Mardivirus* of the family *Herpesviridae* (31). Apart from being a major disease affecting poultry health and welfare, MD is considered to be an excellent biomedical model for virus-induced lymphoma (7, 14). Among the 100-plus genes predicted for the MDV genome (40, 47, 48), the gene for the basic leucine zipper protein Meq is considered to be the major oncogene (39, 44). Some of the functions of Meq associated with oncogenic properties, such as its interaction with CtBP, parallel those of other viral oncogenic sequences, such as adenovirus E1A and Epstein-Barr virus (EBV) nuclear antigens EBNA3A and -3C (6), highlighting the convergent evolution of oncogenic pathways in these viruses. Recent studies have also identified the role of other genes, such as the pp38 (23), viral interleukin-8 (vIL-8) (49), ICP4 (15, 38), R-LORF4 (33), UL36 (32), and MDV-encoded telomerase RNA (22, 63) genes, in pathogenesis.

Increasing evidence demonstrates that in addition to the direct role of protein-encoding genes, noncoding RNAs have profound effects in mediating neoplastic transformation (13).

Among these, the 22-nucleotide microRNAs (miRNAs) have emerged as a major regulatory tier of gene expression, with the potential of targeting up to 30% of genes in humans (17, 27, 37). Given their small size with the capability for regulating multiple genes, several viruses have adopted the miRNA machinery to manipulate the cellular and viral pathways of gene regulation by encoding their own miRNAs (19, 24, 26, 42). Among the different families of viruses, herpesviruses have exploited the miRNA-mediated gene regulation pathway most successfully, since 124 of the 127 virus-encoded miRNAs in miRBase release 10.1 (<http://microrna.sanger.ac.uk>) are encoded by herpesviruses. It has been suggested that the miRNA-mediated regulatory mechanisms are very suited for the herpesvirus life cycle, which is characterized by nuclear replication and latent periods with minimal antigen expression (19).

Specific miRNA signatures in different types of tumors have been identified using high-throughput microarray analysis of miRNA expression (60, 64, 67). Considering the aggressive nature and rapid onset of tumors induced by MDV-1, analysis of the miRNA profile of MDV-transformed tumor cells could provide further insights into MD oncogenesis. Previous studies using small RNAs from MDV-infected chicken embryo fibroblasts (CEF) have identified several miRNAs, including eight MDV-encoded miRNAs that mapped to the Meq and the latency-associated transcript (LAT) region of the genome (8, 9). Although identification of MDV and host miRNAs in lytically infected CEF is valuable, understanding the expression profiles of miRNAs in the lymphocyte target cells of MD lymphomas would be crucial to delineate their role in neoplas-

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TABLE 1. Sequences, chromosomal locations, and cloning frequencies of chicken miRNAs cloned from an MSB-1 library

Name ^a	Sequence	No. of hits in library	Chromosomal location	Start position	End position
gga-mir-7	TGGAAGACTAGTGATTTTGTG	3	Z_random	12717978	12718086
gga-mir-15a	TAGCAGCACATAATGGTTTGT	28	1	161540787	161540869
gga-mir-15b	TAGCAGCACATCATGGTTTGCA	9	9	21649291	21649381
gga-mir-16	TAGCAGCACGTAATATTGGTG	82	1	161540645	161540728
gga-mir-17-5p	CAAAGTGCTTACAGTGCAGGTAA	55	9	21649116	21649209
gga-mir-18a	TAAGGTGCATCTAGTGCAGATA	10	1	140631124	140631208
gga-mir-18b	TAAGGTGCATCTAGTGCAGTTA	10	1	140630969	140631061
gga-mir-19a	TGTGCAAATCTATGCAAACTGA	10	4	3781954	3782037
gga-mir-19b	TGTGCAAATCCATGCAAACTGA	71	1	140630835	140630915
gga-mir-20a	TAAAGTGCTTATAGTGCAGGTGA	71	1	140630526	140630612
gga-mir-20b	CAAAGTGCTCATAGTGCAGGTAG	18	1	140630649	140630746
gga-mir-21	TAGCTTATCAGACTGATGTTGA	16	4	3781773	3781857
gga-mir-23b	ATCACATTGCCAGGGATTAC	249	19	6933581	6933677
gga-mir-24	TGGCTCAGTTCAGCAGGAACAG	2	Z_random	14203199	14203284
gga-mir-26a	TTCAAAGTAATCCAGGATAGGC	6	Z_random	14203968	14204035
gga-mir-27b	TTCACAGTGGCTAAGTTCTGC	14	2	4034213	4034289
gga-mir-29b	TAGCACCATTTGAAATCAGTGTT	10	Z_random	14203435	14203531
gga-mir-30b	TGTAACATCCTACACTCAGCT	98	26	1280249	1280328
gga-mir-30c	TGTAACATCCTACACTCTCAGCT	1	1	204569	204649
gga-mir-30d	TGTAACATCCCCGACTGGAAGC	1	2	141145952	141146038
gga-mir-30a-5p	TGTAACATCCTCAGACTGGAAGCT	16	3	80699454	80699525
gga-mir-33	GTGCATTGTAGTTGCATTG	13	23	4431115	4431203
gga-mir-34a	TGGCAGTGTCTTAGCTGGTTGTT	6	2	141142201	141142264
gga-miR-92	TATTGCACTTGTCCCGCCTGT	7	3	80674840	80674911
gga-mir-101	TACAGTACTGTGATAACTGAAG	6	1	46203134	46203202
gga-mir-106	AAAAGTGCTTACAGTGCAGGTA	9	21	3118925	3119033
gga-mir-130a	CAGTGCAATATTAAGGGCA	9	1	140630413	140630490
gga-mir-140	AGTGGTTTTACCCTATGGTAG	9	Z	11651019	11651097
gga-mir-142-3p	TGTAGTGTTCCTACTTTATGGA	55	4	3782085	3782165
gga-mir-142-5p	CCCATAAAGTAGAAAGCACTAC	4	15	393029	393111
gga-mir-146b	TGAGAAGTGAATTCCATAGGCG	5	11_random	250924	251018
gga-mir-181a	AACATTCAACGCTGTCGGTGTAGTT	224	Un	130069949	130070036
gga-mir-181b	AACATTCAATGCTGTTCGGTGGGTTT	243	Un	130069949	130070036
gga-mir-221	AGCTACATTGCTGTCTGGGTTTC	44	6	22212586	22212690
gga-mir-222	AGCTACATCTGGCTACTGGGT	5	8	1957561	1957664
gga-mir-301	CAGTGCAATAATATTGTCAAAGCATT	17	17	945791	945881
gga-mir-456	CAGGCTGGTTAGATGGTTGTCCT	8	8	1957750	1957838
gga-let-7i	TGAGGTAGTAGTTTGTGCTGT	6	17	944157	944241
gga-let-7a	TGAGGTAGTAGTTTGTATAGTT	11	1	104369325	104369423
gga-mir-363*	AATTGCACGGTATCCATCTGTA	7	1	104368821	104368918
gga-mir-454*	TAGTGCATATTGCTTATAGGGT	3	15	391803	391895
gga-mir-425*	AATGACACGATCACTCCCGTTGA	4	3	27735429	27735540
gga-mir-191*	CAACGGAATCCCAAAAGCAGCTG	48	1	29988296	29988379
gga-mir-22*	AAGCTGCCAGTTGAAGAACTGT	12	12	6149732	6149821
gga-mir-739*	AAGGCCGAAGTGGAGAAGGGTTCCA	1	24	3263054	3263125
			1	67795667	67795742

^a *, novel miRNAs identified in chickens and assigned names on the basis of homology to miRNAs in other species.

tic transformation. Primary MD lymphomas are often heterogeneous mixtures of neoplastic T cells and nontransformed cells of other lineages (50), so analysis of the whole tumor may not provide the miRNA profile of the transformed target cell. However, the ability to establish homogeneous clonal populations of lymphoblastoid cell lines from primary tumors has helped to gain insights into the gene expression profiles of MD tumor cells (10). We reasoned that examination of the miRNA profiles of MDV-transformed lymphoblastoid cell lines could

help to analyze their roles in neoplastic transformation and in the maintenance of MDV latency in target T cells.

MSB-1 is an MDV-transformed CD4⁺ T-cell line derived from a spleen lymphoma induced by the BC-1 strain of MDV-1 (1, 30). The MSB-1 cell line, used in this study at passage level 13, has a CD4⁺ phenotype and has been shown to be coinfecting with MDV-1 and MDV-2 (66). The MSB-1 cell line has both integrated and circular copies of the MDV-1 genome (56) and induces tumors when it is inoculated into susceptible

TABLE 2. Sequences and genomic positions of MDV-1 miRNAs^a

Name	Sequence (5' to 3')	Length (nt)	No. of hits	Nucleotide position ^b
MDV1-miR-M1-5p	UGCUUGUACACUGUGCGGCA(UUAU)	20–24	339	136873–136896
MDV1-miR-M1-3p	(A)UGCUGCGCAUGAAAGAGCGA(A)	21–23	4	136913–136934
MDV1-miR-M2-5p	(G)UUGUAUUCUGCCCCGUAGUCC(GUUU)	22–26	16	134231–134256
MDV1-miR-M2-3p	(A)CGGACUGCCGCAGAAUAGC(UUU)	19–22	11	134270–134292
MDV1-miR-M3-5p	(CAUG)AAAAUGUGAAACCUCUC(CCGCU)	20–25	390	134079–134104
MDV1-miR-M4-5p	(UUUAA)UGCUGUAUCGGAACC(CUUCGUU)	19–26	341	134367–134393
MDV1-miR-M4-3p	(CGA)AUGGUUCUGACAGCAUGAC(CU)	20–22	50	134403–134426
MDV1-miR-M5-5p	AACCGUAUGCGAUCACAUUGAC	22	0	133606–133628
MDV1-miR-M5-3p	(U)GUGUAUCGUGGUCGUCUACU(GUU)	21–24	176	133647–133670
MDV1-miR-M6-5p	(UCU)GUUGUCCGUAGUGUUC(UC)	18–22	278	142335–142356
MDV1-miR-M6-3p	(GAG)AUCCUGCGAAAUGACAGU(U)	19–23	14	142370–142392
MDV1-miR-M7-5p	(UGU)UAUCUCGGGGAGAUCCC(GAU)	19–23	800	142508–142530
MDV1-miR-M8-5p	UAUUGUUCUGUGGUUGUUC(GA)	21–23	18	142216–142238
MDV1-miR-M8-3p	(GU)GACCUCUACGGAACAAUAG(U)	20–22	36	142258–142279
MDV1-miR-M9-5p	UUUUCUCCUCCCCCGGAGUU(CA)	22–24	45	133374–133397
MDV1-miR-M9-3p	AAACUCCGAGGGCAGGAAAAAG	22	1	133414–133435
MDV1-miR-M10-3p	(UCG)AAAUCUCUACGAGUAACA(GU)	20–23	6	142667–142690
MDV1-miR-M11-5p	UUUCCUUAACCGUGUAGCUUAGA	23	2	136053–136075
MDV1-miR-M11-3p	UGAGUACAUGGUCAGGGGAUU	22	0	136092–136113
MDV1-miR-M12-3p	(U)UGCAUAAUACGGAGGGUUCU(G)	21–22	35	133925–133946
MDV1-miR-13-3p	GCAUGGAAACGUCCUGGGAAA	21	0	142313–142333

^a Sequence variation surrounding the recovered BC-1 miRNAs is indicated by parentheses surrounding the variable nucleotides. miRNAs derived from a single primary miRNA stem-loop precursor are indicated by a “-5p” (5' arm) or “-3p” (3' arm) suffix.

^b Based on the Md5 sequence (GenBank accession no. AF243438).

chickens (21, 35). As reported for some MD tumors, these cells also showed truncated forms of p53 tumor suppressor protein (62). Northern blot analysis of the expression of MDV-induced miRNAs in MSB-1 cells showed that many of the miRNAs are expressed at much higher levels than those in infected CEF (8, 9). These results demonstrate that the MSB-1 lymphoblastoid cell line, which shares many properties of MD tumors, could be used as a model system for analyzing the molecular pathways and mechanisms of neoplastic transformation in MD tumors. We recently reported the construction of a library, using small RNAs fractionated from MSB-1 cells, to identify novel MDV-2-encoded miRNAs (66). In this paper, we describe the results of analysis of the MSB-1 “miRNAome” to examine the population of host and viral miRNAs expressed in this transformed cell line.

MATERIALS AND METHODS

Cells and viruses. CEF prepared from 10-day-old specific-pathogen-free embryos obtained from flocks maintained at the Institute for Animal Health were used for the propagation of viruses. Low-passage-number virus stocks of RB-1B (58) grown in CEF for 72 to 96 h were used for the preparation of RNA for Northern blotting analysis. The MDV-transformed lymphoblastoid cell line MSB-1 (1) and the REV-T-transformed (16) chicken CD4⁺ T-cell line AVOL-1 were grown at 38.5°C in 5% CO₂ in RPMI 1640 medium containing 10% fetal calf serum, 10% tryptose phosphate broth, and 1% sodium pyruvate.

Cloning and identification of miRNAs. We have previously described the construction of a cDNA library from small RNAs prepared from MSB-1 cells (66). Concatemered sequences of putative miRNAs from the pGEM-T Easy (Promega, Southampton, United Kingdom) library were determined using vector-specific primers. High-quality reads of small RNA sequences with both 5' and 3' adapters were analyzed for the characterization of miRNAs.

Northern blotting analysis. Total RNA was extracted from cultured cells by using TRIzol reagent (Invitrogen) according to standard methods described by the manufacturer. RNAs were also isolated from samples of MD lymphomas as well as from livers, brains, hearts, kidneys, ovaries, lungs, thymuses, and spleens of uninfected adult chickens, using TRIzol reagent. Samples of 20 µg total RNA were resolved in a 15% polyacrylamide-urea gel and blotted onto a GeneScreen Plus membrane (Perkin-Elmer). DNA oligonucleotides with the exact comple-

mentary sequence to selected miRNAs were end labeled with [γ -³²P]ATP by use of T4 polynucleotide kinase (New England Biolabs, Hertfordshire, United Kingdom) to generate high-specific-activity probes. Hybridization, washing, and autoradiography were carried out as previously described (36, 53).

RESULTS

Identification of miRNAs expressed in MSB-1 cells. The MDV-transformed lymphoblastoid T-cell line MSB-1 has been used extensively in different laboratories for various studies, particularly for the analysis of MDV latency programs (43). As a tumor cell line latently infected with MDV-1, we chose MSB-1 to analyze the miRNA profile of MD tumor cells. Sequence analysis of ~1,200 pGEM-T Easy clones of cDNA concatemers of small RNA sequences from the MSB-1 library identified a total of 5,099 high-quality reads. The sequences were scored as miRNAs on the basis that their flanking sequences could be predicted into a stem-loop structure with low free energyBlast (2). Homology searches of these sequences against the miRBase (25) and GenBank (5) databases were used to determine the identities of the different host- and virus-encoded small RNAs. Of the total reads, 1,641 (32.2%) matched known *Gallus gallus* miRNAs in the miRBase. The most abundant host miRNAs in the MSB-1 library were gga-miR-21, gga-miR-142-3p, gga-miR-142-5p, gga-let-7i, gga-miR-29b, gga-miR-16, gga-miR-17-5p, gga-miR-19a, gga-miR-19b, gga-miR-106, and gga-miR-146b. Sequence analysis of the clones from the MSB-1 library also identified six novel chicken miRNAs, which appeared to be homologs of hsa-miR-363, hsa-miR-454-3p, hsa-miR-425-5p, bta-miR-191, hsa-miR-22, and dre-miR-739. The number of reads of each host-encoded miRNA in the library and their chromosomal locations are shown in Table 1. In addition to these miRNAs and the virus-encoded miRNAs (see below), 128 (2.5%) were noncoding

RNA fragments, 76 (1.5%) were mRNA fragments, and 174 (3.4%) showed no matches to any known RNAs.

For the identification of the virus-encoded miRNAs, BLAST searches were carried out against the full-length sequences of MDV-1 (Md5 strain; GenBank accession number AF243438) and MDV-2 (HPRS-24 strain; GenBank accession number AB049735). We have previously shown that 518 (10.2%) sequences from the MSB-1 library are encoded by MDV-2 (66). However, the majority of the 2,562 (50.2%) sequences from this library showed perfect sequence identity to the genome sequence of the Md5 strain. These miRNA sequences, ranging in length from 18 to 26 nucleotides, belonged to 12 distinct MDV-1-encoded miRNAs. These included the eight miRNAs (mdv-miR-M1 to -M8) identified previously from MDV-infected CEF cultures (8) and four novel MDV-1-encoded miRNAs (mdv-miR-M9 to -M12). Additionally, we identified another novel miRNA, mdv-miR-M13, using Northern blotting analysis of RNAs extracted from MSB-1 cells (see below). The genomic location and cloning frequency of each of the MDV-1-encoded miRNAs are shown in Table 2.

MDV-1 miRNAs fold into distinct hairpin structures. For the validation of a sequence as a miRNA, demonstration of its expression as well as its processing through the miRNA biogenesis pathway is required. One of the distinct indicators of miRNA biogenesis is the presence of adjacent complementary sequences that are able to form stable hairpins. In order to analyze the potential precursor structures of miRNAs encoded by MDV-1, the sequences of the 13 miRNAs with their adjacent 60 to 80 nucleotides were analyzed by MFOLD calculation, and the secondary structures were drawn using RNADRAW software as described previously (66). All of the MDV-1 miRNAs showed a stable hairpin with long paired stems (Fig. 1), indicating that they are bona fide miRNAs. Of the two strands of the miRNA duplex generated during biogenesis, only one of the strands, the miRNA strand, is incorporated into the RNA-induced silencing complex and guides gene regulation (3). Although the non-miRNA strand is rapidly degraded, in many instances it is also captured during cloning and may sometimes be detected with a comparable frequency to that of the miRNA strand (66). Among the MDV-1 miRNAs in MSB-1 cells, two mature forms, representing both strands of the duplex, were demonstrated by cloning or Northern blotting for 8 of the 13 candidate miRNAs, increasing the total number of miRNAs to 21. The suffixes “-5p” and “-3p” were added to designations to indicate the 5' and 3' arms, respectively, of the stem-loop precursor from which the miRNAs were derived (Table 2).

MDV-1 miRNAs show differences in cloning frequencies. We then examined the cloning frequency of each of the MDV-1 miRNAs as a measure of their expression levels in MSB-1 cells. The most abundantly cloned miRNAs were mdv-miR-M7-5p (800 hits), mdv-miR-M3-5p (390 hits), mdv-miR-M4-5p (341 hits), mdv-miR-M1-5p (339 hits), mdv-miR-M6-5p (278 hits), and mdv-miR-M5-3p (176 hits). Compared to this, mdv-miR-M10, -M11, and -M13 were of very low abundance, while mdv-miR-M2, -M8, -M9, and -M12 showed moderate copy numbers in the library. For most miRNAs, the non-miRNA strand of the duplex was either not cloned or had a relative frequency much lower than that of the miRNA strand. However, for some of the miRNAs, such as mdv-miR-M2, the

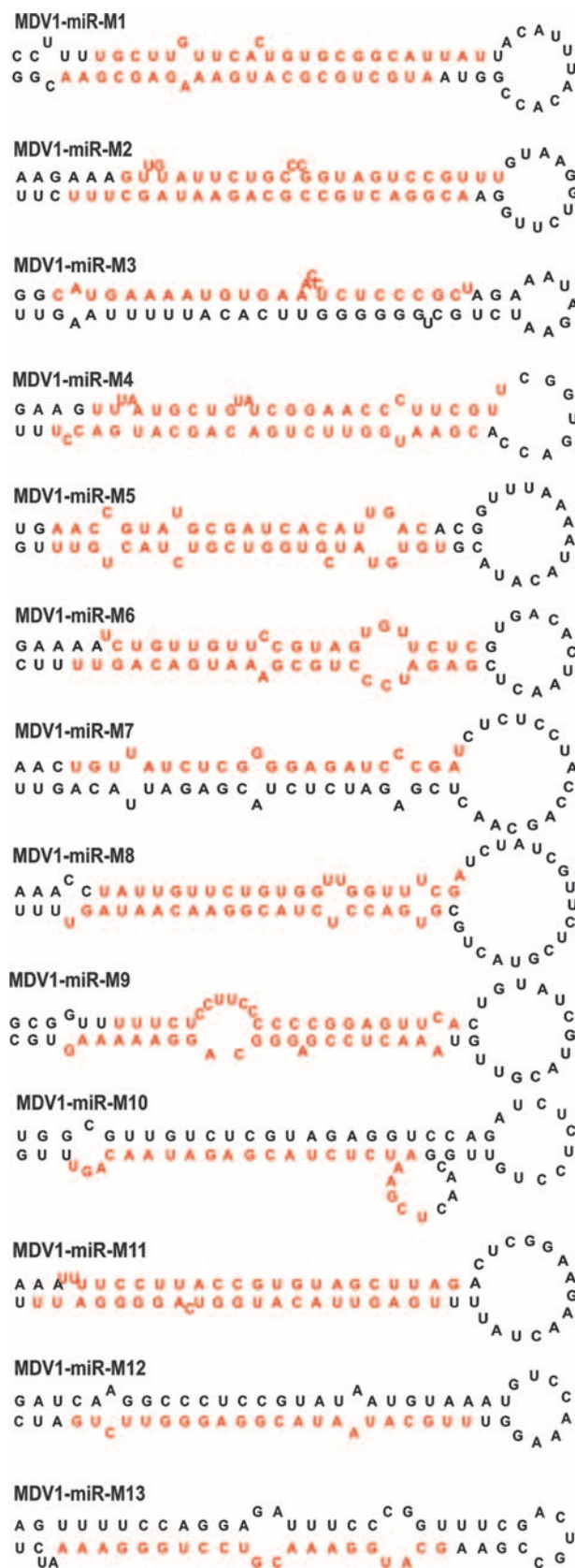


FIG. 1. Secondary structures of MDV-1 pre-miRNAs predicted using the MFOLD algorithm (68). The mature miRNA strands are indicated in red.

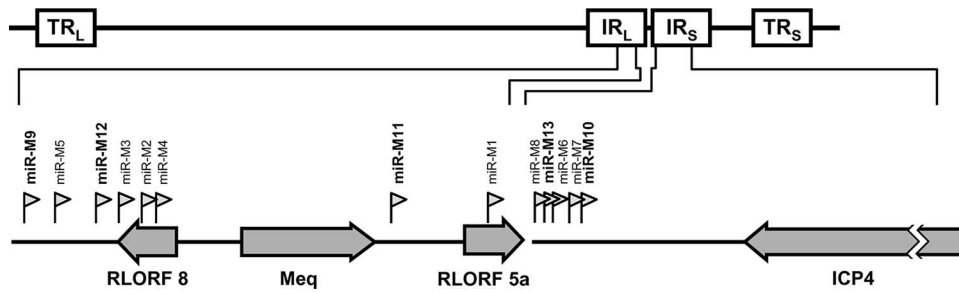


FIG. 2. Genomic locations of MDV-1 miRNAs. The schematic diagram shows where the MDV-1 miRNAs (small arrowheads) identified in this report map. The five novel miRNAs identified in this report are shown in bold. The TR_L and IR_L regions flanking the unique long region and the TR_S and IR_S regions flanking the unique short regions are shown. Genomic positions and orientations of MDV ORFs contained in the miRNA loci are indicated.

frequencies of both strands were very similar, suggesting that both may be functional and are incorporated into the RNA-induced silencing complex.

MDV-1 miRNAs are clustered in the repeat regions of the MDV-1 genome. The nucleotide sequence positions of the different miRNAs are shown in Table 2. All 13 MDV-1-encoded miRNAs are clustered in an ~9-kb region within the R_L/R_S sequences of the MDV genome (Fig. 2). The miRNAs mdv-miR-M2, -M3, -M4, -M5, -M9, and -M12 are located upstream of Meq and are antisense to the R-LORF8 transcript. The miRNAs mdv-miR-M1 and -M11 lie downstream of Meq and are embedded within the open reading frame (ORF) of the L1/LORF5a transcript (46, 59) as well as within the intron of the splice variant Meq-sp (51). MDV-1-encoded miRNAs mdv-miR-M6, -M7, -M8, -M10, and -M13 are located between the a-like sequence and the ICP4 sequence within the large intron of the LAT of MSR (15). Of this cluster, the miRNAs mdv-miR-M6 and mdv-miR-M13 were separated by only a single nucleotide. The occurrence of the miRNAs in distinct clusters in the same orientation strongly suggests that these miRNAs are likely to be processed as multicistronic pre-miRNA transcripts. Despite being processed from a single transcript, there are differences in the expression levels of the mature miRNAs, and these are thought to be due to differences in Drosha processing and/or miRNA stability.

Analysis of miRNA expression by Northern blotting. For further confirmation of the expression of miRNAs in MSB-1 cells, Northern blot hybridization with individual MDV-1 miRNA probes was carried out on RNAs extracted from MSB-1, AVOL-1 (an MDV-negative T-cell line), or uninfected or RB-1B virus-infected CEF cells and from samples of MD lymphoma. These studies confirmed that MDV-1-encoded miRNAs are expressed at high levels in MSB-1 cells and MD lymphomas and at low levels in infected CEF (Fig. 3). No signals were obtained from the RNAs extracted from AVOL-1 cells and uninfected CEF, validating the specificity of the miRNA probes. Based on the intensities of signals, the levels of expression of the majority of miRNAs were similar in both MSB-1 cells and lymphoma samples. Some of the most abundantly cloned miRNAs, such as mdv-miR-M3, -M4, -M5, -M6, and -M7, showed very strong signals by Northern blotting, validating the correlation between cloning frequency and expression level. Similarly, mdv-miR-M10, -M11, and -M13, cloned at very low frequencies from the library, gave weak

signals by Northern blotting. A previous study using Northern blotting of RNAs extracted from MD lymphomas reported that mdv-miR-M6 is expressed at low levels, and mdv-miR-M7 was not detected at all (8). However, our studies using probes specific for the mdv-miR-M6-5p and mdv-miR-M7-5p strands gave strong signals for all samples, including MD lymphomas, indicating that the -5p strand of the duplex is the functional miRNA strand. The failure to detect these miRNAs in Northern blots in the previous study was likely due to the use of the non-miRNA strand as the probe. In most cases, both pre-miRNAs and mature miRNAs could be detected by Northern blotting, with the former giving much lower signals. For some of the miRNAs, such as mdv-miR-M5-5p, mdv-miR-M9-3p, and mdv-miR-M13, the signals of pre-miRNAs were higher than those of the mature miRNAs, indicating less efficient processing.

Northern blotting was also carried out on RNAs extracted from eight different normal tissues from adult noninfected chickens to validate the expression of some of the miRNAs cloned from the MSB-1 library. Some of these miRNAs, including novel chicken miRNAs such as gga-miR-363, gga-miR-454, gga-miR-425, gga-miR-191, and gga-miR-22, could be detected by Northern blot analysis, albeit with differences in expression levels between tissues (Fig. 3b). While gga-miR-425 and gga-miR-22 showed high levels of expression in all tissues, gga-miR-454 was detected at very low levels. The expression of the miRNAs gga-miR-191, gga-miR-363, and gga-miR-425 in lymphoid organs, namely, the spleen, thymus, and the lungs (55), was at the levels observed for the lymphocyte-specific miRNA gga-miR-142 (54, 65), which gave strong signals with probes specific for either of the strands of the duplex in these tissues.

DISCUSSION

As efficient inducers of cancer, oncogenic viruses have helped to reveal several major pathways of oncogenesis. Most of these pathways involve the interactions of virus-encoded oncoproteins, such as simian virus 40 T antigen, adenovirus E1A, human papillomavirus E6/E7, and EBV EBNA5 (69). In MD tumors, MDV-encoded Meq is considered to be the major oncoprotein (39), although other proteins also contribute to oncogenesis (44). The discovery of virus-encoded miRNAs in several oncogenic viruses (53) has added yet another armory to

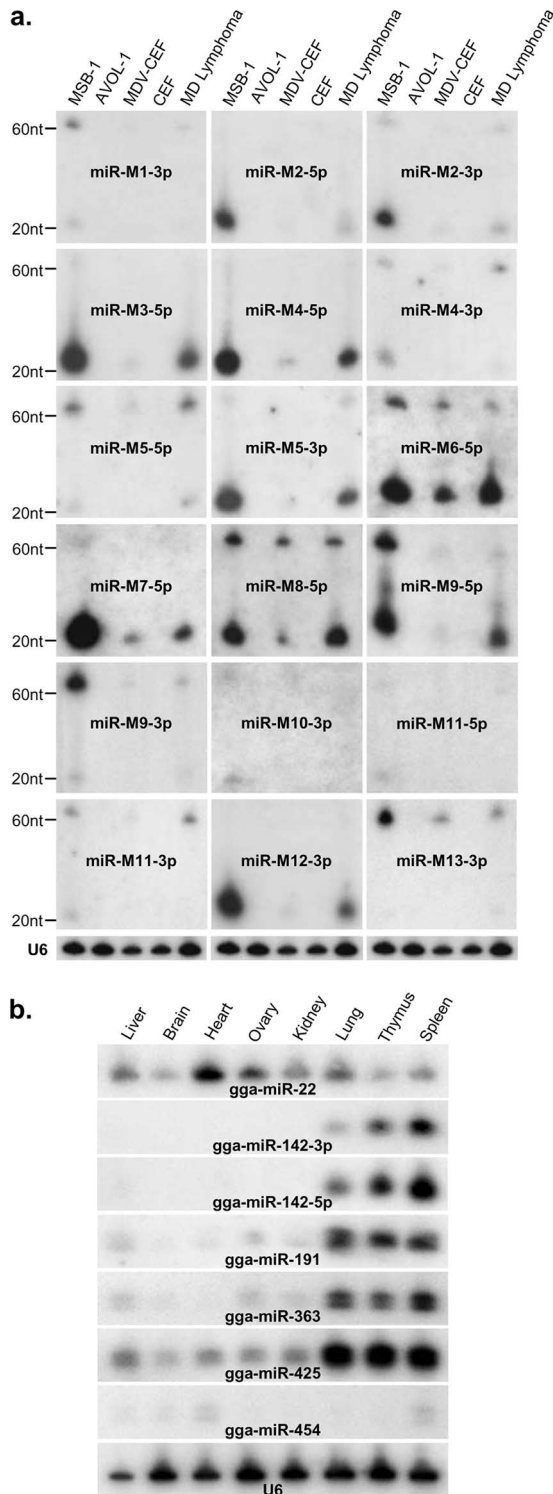


FIG. 3. (a) Northern blotting analysis for determining the expression of MDV-1 miRNAs. Twenty micrograms of total RNA from MSB-1 cells, the MDV-negative lymphoid cell line AVOL-1, MDV-1-infected CEF, uninfected CEF, or MD lymphoma tissues was separated in a 15% denaturing polyacrylamide gel, blotted, and probed with end-labeled antisense oligonucleotides to the indicated miRNAs. Size markers to indicate the positions of the pre-miRNA and the mature miRNA are shown. The cellular U6 snRNA served as the loading control, and a representative blot of this set is shown. (b) Analysis of tissue-specific expression of *Gallus gallus* (gga-) miRNAs

these viruses for regulating gene expression in cancer cells. Recent studies on small RNAs from infected CEF have identified eight MDV-1-encoded miRNAs that map to the Meq and LAT regions of the genome (8, 9). Furthermore, several recent studies have identified specific miRNA signatures in different tumors, providing insights into the different oncogenic pathways in these tumors (20, 64). In order to analyze the expression profiles of the host- and MDV-1-encoded miRNAs in MD tumor cells, we examined the miRNAs expressed in the MDV-transformed lymphoblastoid cell line MSB-1 by cloning and Northern blot analysis.

One of the conspicuous findings from the analysis of the miRNA sequences from the MSB-1 library was the very large proportion of MDV-1-encoded miRNAs (51%) in relation to the number of host miRNAs (Fig. 4). This level of expression of MDV-1 miRNAs is much higher than that identified from MDV-1-infected CEF, where only 0.6% of the nearly 172,000 reads were miRNAs encoded by MDV-1 (9). The low level of expression of MDV-1 miRNAs in CEF (also evident from the results of the Northern blotting analysis) could partly be explained by the smaller proportions of infected cells in CEF cultures in comparison to MSB-1 cell cultures, where each cell has multiple copies of the MDV genome. However, the increased expression of MDV-1 miRNAs in MSB-1 cells may also be related to the increased lymphocyte-specific expression of these miRNAs in these transformed target cells. An increased proportion of virus-encoded miRNAs over host-encoded miRNAs is not uncommon in transformed cell lines. For example, miRNAs encoded by Kaposi's sarcoma-associated herpesvirus and EBV accounted for >40% of the entire miRNA pool identified from the BC-1 cell line coinfecting with these two viruses (11). Once the 518 (10%) MDV-2-encoded miRNAs that we reported previously (66) were also considered, the total proportion of virus-encoded miRNAs in the MSB-1 library was 61%, compared to the 32.2% expression of host-encoded miRNAs. The reasons for the fivefold difference in the levels of miRNAs encoded by the two viruses are not known but may be connected with the differences in relative copy numbers of the two viruses. The precise copy numbers or replication rates of the two viruses in MSB-1 cells are not known. However, on CEF cocultivated with MSB-1 cells, MDV-2 produced 10-fold more plaques than did MDV-1, suggesting that MDV-2 is better adapted for faster replication on CEF (66).

Previous studies of MDV-1-infected CEF identified eight miRNAs that mapped to the Meq and LAT regions of the MDV genome (8, 9). We also cloned all eight of these miRNAs from the MSB-1 library. However, we also identified 5 new MDV-1 miRNAs, taking the total number of MDV-1-encoded miRNAs to 13. As in the case of the eight previously identified miRNAs, the five new MDV-1 miRNAs mapped to the Meq and LAT regions of the genome (Fig. 2). In the MDV genome,

identified in the MSB-1 library. Total RNAs (20 μ g) extracted from different tissues of chickens were separated in polyacrylamide gels and probed with end-labeled antisense oligonucleotides specific for the individual miRNAs indicated. The cellular U6 snRNA served as the loading control.

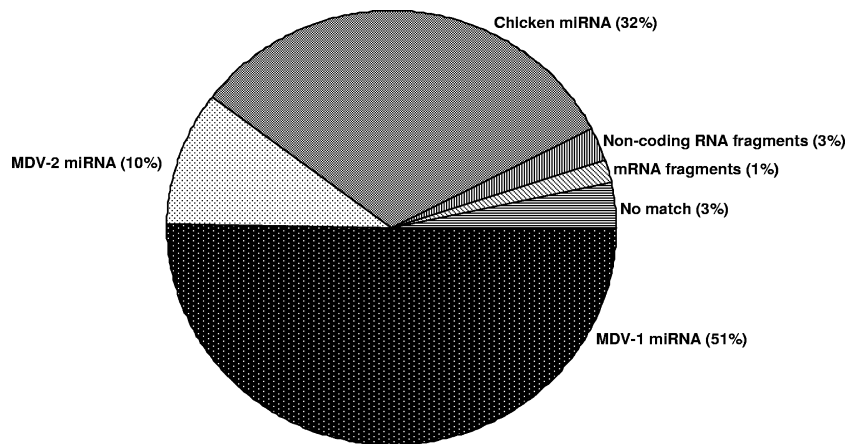


FIG. 4. Pie chart showing proportions of MDV-1, MDV-2, and chicken miRNAs and other molecules cloned from the MSB-1 library.

while most of the genes are transcriptionally silent in latently infected and tumor cells, the repeat (R_L/R_S) regions are generally active (34, 45, 61). Thus, it is not surprising that all of the miRNAs that are expressed at high levels in latently infected/tumor cells are located in a transcriptionally active region of the genome. The genomic locations of the eight previously reported MDV-1-encoded miRNAs have been described (8, 9). Two of the new miRNAs, mdv-miR-M9 and mdv-miR-M12, are also located upstream of the Meq promoter region, like the previously identified miR-M2 to miR-M5 miRNAs, suggesting that these six miRNAs are part of the same transcriptional unit in the same transcriptional orientation as Meq. The high levels of expression of all six of these miRNAs, demonstrated by strong signals in Northern blotting of RNAs from MSB-1 and tumor cells (Fig. 3), suggest that these miRNAs may have major roles in regulating the expression of viral and host genes in latently infected/transformed T cells. The transcription unit of these miRNAs is also antisense to another potential transcript, RLORF8, demonstrated in both CEF and lymphoblastoid cells (52), and hence has the potential to regulate the expression of RLORF8. A recent study demonstrated that EBV-encoded miR-BART2 can downregulate the viral DNA polymerase BALF5 via a similar mechanism (4). However, unlike miR-M2 and miR-M4, which are embedded in the RLORF8 ORF, the newly identified miRNAs miR-M9 and miR-M12 are located downstream of the ORF (Fig. 2).

One of the previously identified miRNAs, miR-M1, mapped downstream of Meq embedded in the ORF of the L1/RLORF5a transcript, although it is not clear whether it affects the expression of this transcript (33, 46, 59). We have identified a new miRNA, mdv-miR-M11, located just downstream of the Meq ORF (Fig. 2). The importance of this novel miRNA is not known, but it is expressed at only very low levels, as indicated by a low cloning frequency in the library and weak signals in Northern blot analysis.

In addition to the three miRNAs, miR-M6 to -M8, that mapped to the LAT region, our studies have revealed miR-M10 and miR-M13, two novel miRNAs encoded from this region. Because these miRNAs are located very close to miR-M6 to -M8 and are in the same transcriptional orientation, these two miRNAs are highly likely to be part of the same

cluster. However, compared to miR-M6 to -M8, which are expressed at very high levels (Table 2 and Fig. 3), the levels of expression of miR-M10 and miR-M13, shown by Northern blotting and cloning frequencies, are very low. Although the reasons for their low expression levels are not known, the efficiency in processing of the mature miRNAs could be a factor, especially because of their close proximity within the cluster. For example, miR-M13 is located between the highly expressed miR-M6 and miR-M8 miRNAs, with the mature miRNA sequence of miR-M6 being separated from that of miR-M13 by only a single nucleotide (Table 2). Similarly, the newly identified miR-M10 (only 6 hits in the library) is located just adjacent to the most highly expressed miRNA, miR-M7, which had 800 hits in the library.

Although the expression levels of MDV-1-encoded miRNAs in MSB-1 cells were generally similar to those in tumor tissues, there were clear differences in expression level between infected CEF and transformed MSB-1 cells, with the latter generally expressing higher levels of all miRNAs. However, it was also interesting to see clear differences between miRNAs in the specificity of the strand expressed in infected CEF and MSB-1 cells. The most striking example of strand-specific expression was noted for miR-M7, where the mature miRNA strand, miR-M7-5p, had 800 hits, accounting for 16% of the entire MSB-1 library. Northern blot analysis also revealed very strong expression of this miRNA in both MSB-1 cells and tumor tissues. Although weak signals for miR-M7-5p were detected in the infected CEF, this strand was not identified even once among the nearly 172,000 high-quality reads of small RNA sequences from infected CEF (9), suggesting that this miRNA strand is processed only at very low levels in lytically infected CEF. This cluster of miRNAs maps antisense to the ICP4 gene and to the large intron in the 5' end of the putative LAT, expressed at high levels in transformed cells/lymphomas as well as in the late stages of lytic infection of CEF (57). Although the reasons for the differences in processing of the two miRNA strands during miRNA biogenesis in this region between CEF and lymphocytes are not clear, it would be interesting to see whether any of the miRNAs play a role in switching between lytic replication and latency. Intriguingly, mdv-miR-M7 also showed evidence of RNA editing. However,

in the absence of knowledge on the targets of any of the MDV-1-encoded miRNAs, the significance of this remains unknown.

Analysis of the miRNA repertoire from MSB-1 cells also identified several host miRNAs, some of which were cloned at high frequencies indicating high levels of expression (Table 1). Some of the more abundant host miRNAs, such as those within the miR-17-92 cluster, have been shown to be amplified in several types of cancer (28, 29). Since these miRNAs have been shown to accelerate the formation of lymphoid malignancies in mouse models (29), the increased expression of these miRNAs in MSB-1 cells could be significant. Similarly, other highly expressed miRNAs, such as miR-21 (249 hits), let-7i (148 hits), the two strands of miR-142 (224 and 243 hits), miR-15a (28 hits), and miR-16 (82 hits), have also been associated with various malignancies, including chronic lymphocytic leukemia (12, 18), suggesting that these miRNAs may contribute toward MDV oncogenicity. Currently, we are examining the roles of different host miRNAs in the induction of lymphomas by MDV. Our studies on MSB-1 cells also revealed six novel chicken miRNAs. The expression of five of these novel miRNAs could be detected by Northern blotting of different chicken tissues, although the expression of miR-454 in all tissues was very weak (Fig. 3b). The expression of both strands of miR-142 appeared to be restricted to the lymphocyte-enriched lungs, thymus, and spleen (Fig. 3b), suggesting that it is likely to be a lymphoid cell-specific miRNA.

We have carried out a study to examine the miRNAome of a herpesvirus-induced lymphoma in chickens by determining the miRNAs expressed in a lymphoblastoid cell line derived from a lymphoma. This study is the first of its kind with an avian lymphoma and has demonstrated that the analysis of the miRNA repertoire would enable investigation of some of the potential pathways used by viruses in neoplastic transformation. A major challenge in the next stage would be the identification of potential targets for some of the miRNAs overexpressed in these cells to identify networks of molecular events regulated by the altered miRNAome in these cells. The present study has not identified miRNAs that are downregulated in transformed cells, whose profiles are also very important for understanding the global events involved in transformation. Currently, we are using microarray analysis of global viral/host miRNA expression in MD tumor cells in relation to that in normal lymphocytes to determine the entire repertoire of upregulated and downregulated miRNAs to identify the extent to which MDV exploits the cellular miRNA pathways to induce neoplastic transformation.

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