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Identification of Latency-Associated Transcripts That Map Antisense to the ICP4 Homolog Gene of Marek's Disease Virus†

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Two small RNAs (0.9 and 0.75 kb), named Marek's disease virus (MDV) small RNAs (MSRs) and a 10-kb RNA, all of which map antisense to the MDV ICP4 homolog gene, have been readily detected in MDCC-MSB1 MDV-transformed T-lymphoblastoid cells. These RNAs were not detectable in reticuloendotheliosis virus-transformed T cells. When MDV was reactivated by treatment of lymphoblastoid cells with 25 μg of iododeoxyuridine per ml, the relative levels of the transcripts decreased. These RNAs were not detected by Northern (RNA) hybridization in productively infected chicken embryo fibroblasts 48 h postinfection; however, they were apparent 140 h postinfection. By using Northern hybridization, RNase protection assays, and primer extension analysis, the MSRs were determined to map antisense to the predicted translational start site of the ICP4 homolog gene. The conclusion most consistent with the data is that the two MSRs are overlapping, spliced RNAs. Both small RNAs contain a latency promoter binding factor consensus recognition sequence located toward their 5′ ends as well as two potential ICP4 recognition consensus sequences, one in each orientation. The region contains a number of small open reading frames on each side and within the MSRs. Although the exact endpoints are unknown, the large 10-kb species spans the entire ICP4 homolog region. We believe that this group of RNAs, which map antisense to the ICP4 homolog gene, are latency-associated transcripts of MDV.

Marek's disease virus (MDV) is a cell-associated herpesvirus that causes T-cell lymphomas following infection of susceptible chickens (7). MDV replicates in a productive-restrictive manner in B lymphocytes, some epithelial cells, and cells grown in tissue culture. Fully enveloped virus is produced only from feather follicle epithelium (5, 22). MDV establishes a latent infection primarily in T lymphocytes. MDV-induced lymphomas contain a variety of cell types, most notably transformed T cells. Lymphoblastoid cell lines, which are immortal cell lines derived from MDV-induced lymphomas, comprise transformed T lymphocytes. Therefore, it appears that in chickens only T lymphocytes are susceptible to transformation (7).

The relationship between latency and transformation is not clear. Some investigators have viewed MDV in transformed cells as latent (33), whereas others have viewed latency as a prerequisite to transformation (7). Among most lymphoblastoid cell lines, MDV antigens are expressed and virus is produced spontaneously to various degrees depending on the cell line (6). A few lymphoblastoid cell lines, termed "nonproducers," that have no or very limited expression of the MDV genome have been reported, and MDV cannot be rescued from at least one member of this group (7). In general, however, treatment of lymphoblastoid cell lines with agents such as iododeoxyuridine triggers the production of viral antigens and reactivates infectious virus (6, 12).

The MDV ICP4 homolog gene is 4,245 nucleotides long, maps to the *Bam*HI A fragment of the genome, and shows similarity to ICP4 homolog genes of alphaherpesviruses (1). The MDV ICP4 protein would have a predicted structure similar to that of ICP4-like proteins of other herpesviruses in that it should have five distinct regions, the second and fourth of which are conserved. In addition, the MDV protein would contain the characteristic run of serine residues located toward its amino terminus. The MDV ICP4 protein can transactivate the pp38 gene (23) which encodes a major phosphoprotein present in chicken embryo fibroblasts (CEF) infected with serotype 1 (oncogenic) strains of MDV (8, 11).

Latency-associated transcripts (LATs) which map antisense to immediate-early genes have been described for many herpesviruses. An antisense transcript that overlaps the ICP4 homolog has been reported for pseudorabies virus (9, 24). In bovine herpesvirus 1, the latency-related RNA is complementary to one of two transcripts derived by alternate splicing from a major immediate-early transcription unit and the alternate splicing variant is translated into the ICP4 homolog (16, 25, 34). In the case of herpes simplex virus (HSV), LATs map antisense to the ICP0 gene (14, 29–31). The situation appears to be different for varicella-zoster virus. In human trigeminal ganglia, varicella-zoster virus transcripts that arise from three separate regions of the genome have been detected (10). These transcripts are colinear with ORF29 and ORF62, ORF62 being homologous to HSV ICP4 (20). Therefore, varicella-zoster virus latency appears to result in selective transcription of some immediate-early and early genes. Most LAT transcripts are generally localized in the nuclei of latently infected cells and are nonpolyadenylated, although poly(A)-containing antisense transcripts have been reported for the Becker strain of pseudorabies virus (9) but not the Sullivan strain (24). In this article, we report the identification and characterization of RNAs that map antisense to the MDV ICP4 homolog gene.

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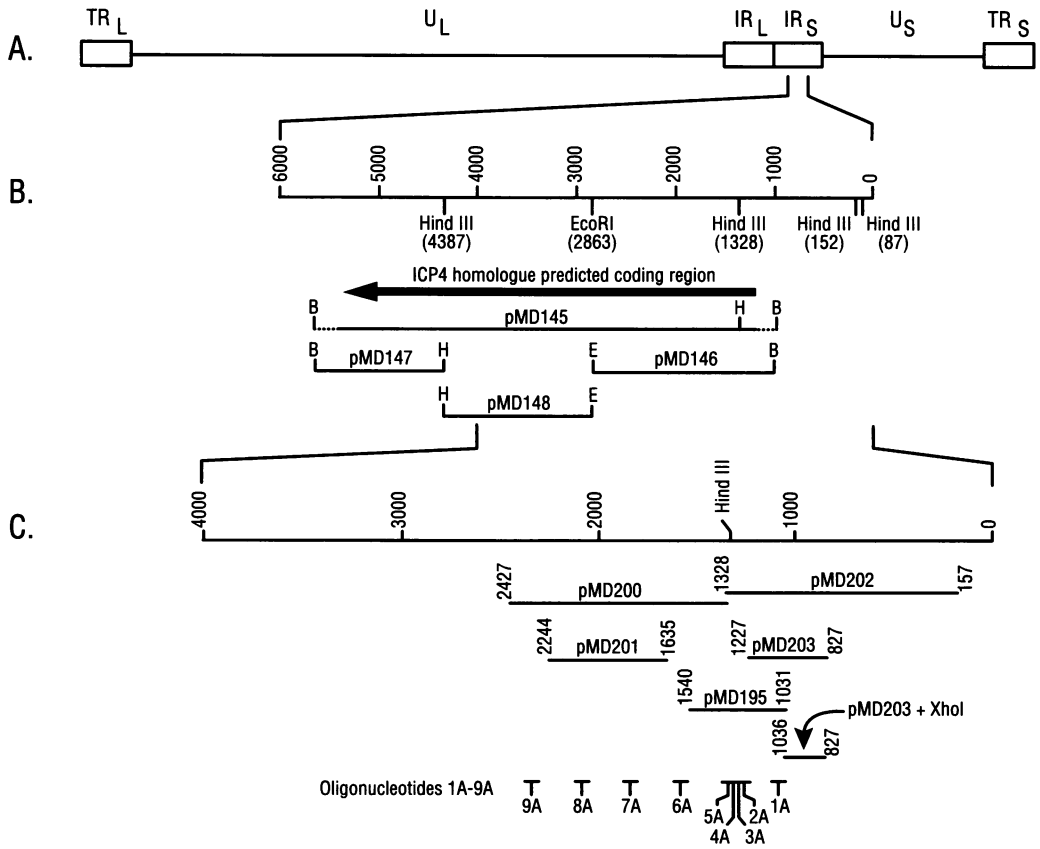

FIG. 1. (A) Diagram of the MDV genome showing the unique long region (U_L) and unique short region (U_S) flanked by repeat regions. TR_L, terminal repeat flanking U_L; IR_L, internal repeat flanking U_L; IR_S, internal repeat flanking U_S; TR_S, terminal repeat flanking U_S. (B) Expanded view of a portion of the IR_S. The position and polarity of the predicted coding region for the MDV ICP4 homolog gene (arrow) and the positions of MDV plasmid clones pMD145, pMD146, pMD147, and pMD148 are indicated. (C) Expanded view of the ICP4 homolog 5′ and upstream region. The positions of all MDV plasmid clones and oligonucleotides (1A to 9A) used in this study are indicated. The map positions correspond to previously published coordinates for the MDV ICP4 homolog gene (1).

MATERIALS AND METHODS

Cells and viruses. Virus was propagated in secondary CEF by using growth medium consisting of M199 medium (GIBCO, Grand Island, N.Y.) containing 3% calf serum (GIBCO), 100 IU of penicillin G per ml, and 100 μg of dihydrostreptomycin per ml. The GA strain of MDV was obtained from M. Nonoyama, Tampa Bay Research Institute, St. Petersburg, Fla., and passage level 75 of this strain was used. MDCC-MSB1 (MSB1) MDV-transformed T-lymphoblastoid cells and RECC-CU91 (CU91) reticuloendotheliosis virus-transformed T-lymphoblastoid cells were obtained from K. A. Schat (Cornell University, Ithaca, N.Y.) and propagated in RPMI medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO), 100 IU of penicillin G per ml, and 100 μg of dihydrostreptomycin per ml.

Plasmid constructions. Cloning was done by using standard methods (3, 26), and restriction enzyme digestions were carried out according to the recommendations of the suppliers. Plasmids used in this study are diagrammed in Fig. 1. The construction of plasmid pMD145, which contains the entire 4.2-kb ICP4 homolog coding region cloned into pGEM4Z, has been described previously (1). Plasmids pMD146, pMD148, and pMD147 are derivatives of pMD145 in which various subfragments were cloned into the pGEM3Z or pGEM4Z vector (Promega Biotec, Madison, Wis.). Plasmids pMD202 and pMD200 are two pGEM3Z-based plasmids derived from a set of directed progressive deletions of p*Bam*HI-A constructed during the sequencing of the ICP4 homolog gene (1). pMD201 was generated by PCR amplification of a 609-bp fragment from pMD200, and pMD203 was generated by PCR amplification of a 400-bp fragment from pMD202. The amplified fragments were cloned into the pCRII vector (In vitrogen Corp., San Diego, Calif.). pMD195 was generated by PCR amplification of a 509-bp fragment from pMD130 (5.8-kb *Eco*RI subfragment of p*Bam*HI-A) and cloning of the amplified DNA into pCRII.

Oligonucleotides. Oligonucleotides were obtained commercially (Operon Technologies Inc., Alameda, Calif.) and used as primers for RNA-directed PCR (RNA PCR) or primer extension analysis and as probes for Northern (RNA) blot analysis. Oligonucleotide sequences and positions were obtained from the previously published MDV ICP4 homolog gene sequence (1). Oligonucleotides numbered 1A to 9A consisted of DNA from the coding strand of the MDV ICP4 homolog gene and correspond to the following positions in the ICP4 sequence: 1033 to 1050 (1A), 1211 to 1230 (2A), 1234 to 1255 (3A), 1264 to 1281 (4A), 1291 to 1310 (5A), 1530 to 1546 (6A), 1783 to 1800 (7A), 2059 to 2077 (8A), and 2280 to 2297 (9A). Oligonucleotide 1B (positions 1395 to 1416) consisted of DNA

from the complementary strand of the MDV ICP4 homolog gene.

RNA purification. Cultures of CEF (2×10^7 cells per 75-cm^2 tissue culture flask) were mock infected or heavily infected with cell-associated GA (2×10^5 PFU per flask) and incubated for 48 h until cytopathic effects were evident. Dividing lymphoblastoid cells (5×10^7 to 10×10^7 per preparation) were harvested by centrifugation, and the pellet was used for RNA purification. Total RNA was purified by using the guanidinium method for RNA purification followed by centrifugation through a cesium chloride step gradient (26). RNA preparations were treated with 2.5 U of RNase-free RQ1 DNase (Promega) for 15 min at 37°C and quantitated by A_{260}. Poly(A) RNA was prepared by passage of total RNA over Poly(A)Quik columns (Stratagene, San Diego, Calif.) a total of six times or by using the Poly(A) Tract mRNA isolation system (Promega) according to the recommendations of the supplier.

Northern blot analysis. Electrophoresis and Northern hybridizations were carried out essentially as described elsewhere (3). RNA size markers were obtained commercially (Bethesda Research Laboratories, Gaithersburg, Md., and Promega). DNA probes were labelled by using [^{32}P]dCTP and a random-primed DNA labelling kit (United States Biochemical Corporation, Cleveland, Ohio) according to the recommendations of the supplier. Riboprobes were synthesized by using [^{32}P]CTP and a commercial in vitro transcription system according to the recommendations of the supplier (Promega). Hybridizations using DNA and RNA probes were done at 42°C for 16 h in 50% formamide (Fluka Chemical Corporation, Ronkonkoma, N.Y.), 25 mM potassium phosphate (pH 7.4), 150 μg of denatured salmon sperm DNA per ml, 5× Denhardt's solution, and 5× SSC (1× SSC contained 0.15 M sodium chloride and 0.015 M sodium citrate [pH 7]). Denhardt's solution contained 0.1% Ficoll 400 and 0.1% polyvinylpyrrolidone (Sigma Chemical Company, St. Louis, Mo.). Filters hybridized with the double-stranded probe were washed twice at room temperature with 1× SSC–0.1% sodium dodecyl sulfate (SDS) and two times at 65°C with 0.1× SSC–0.1% SDS and autoradiographed. In some cases, filters hybridized with riboprobes were washed once for 10 min at room temperature in 2× SSC containing 1.0 μg of RNase A per ml to eliminate nonspecific hybridization to rRNA (21) and twice at 65°C with 0.1× SSC–0.1% SDS. Oligonucleotide probes were labelled by incubating 50 pmol of [γ-^{32}P]ATP (6,000 Ci/mmol), 10 pmol of the oligonucleotide, and 16 U of T4 polynucleotide kinase (Promega) at 37°C for 90 min. The unincorporated isotope was removed by gel filtration through Sephadex G-25 (Sigma). Hybridizations using oligonucleotide probes were done at 38°C for 16 h in 6× SSC–10× Denhardt's solution–0.5% SDS–50 mM sodium phosphate (pH 7.0)–150 μg of denatured salmon sperm DNA per ml. Oligonucleotide-hybridized filters were washed in 3× SSC–0.1% SDS as follows: twice at room temperature for 30 min, once at 40°C for 30 min, and once at 10°C below the melting temperature [melting temperature = 69.3 + 0.41(G+C)% − 650/L, where L is probe length in nucleotides].

RNA PCR. RNA PCR was done by using a commercial GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, Conn.). Primer 3A, the reverse transcriptase primer (5′ GC CTTTATGGTCCAACAACTCG 3′), corresponded to positions 1234 to 1255 (Fig. 1), and the upstream primer, 1B (5′ GTCATCGTCCATATTGGCGTTG 3′), corresponded to positions 1395 to 1416. Prior to use in the RNA-driven reactions, RNA preparations were checked for the presence of contaminating DNA amplifiable by *Taq* polymerase. Reverse transcription primed by the reverse transcriptase primer and amplification of the resulting cDNA was done under conditions recommended by the supplier. Following the reverse transcription reaction, the sample was heated to 99°C for 5 min. Subsequent addition of the upstream primer, 1B, resulted in the amplification of a 182-bp segment of DNA within 35 cycles in a Perkin-Elmer Cetus thermocycler. All amplifications were demonstrated to be dependent on the presence of both the RNA template and reverse transcriptase.

RPA. RNase protection assays (RPA) were done with single-stranded [α-^{32}P]CTP-labelled riboprobes generated by in vitro transcription off a linearized plasmid template as suggested by the supplier of the in vitro transcription kit (Promega). Labelled riboprobes were ethanol precipitated with 20 μg of carrier yeast tRNA, and the pellet was resuspended in 100 μl of hybridization buffer (40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 6.4], 400 mM NaCl, 1 mM EDTA, 80% formamide). Approximately 500,000 cpm of the riboprobe was added to 7.5 μg of sample RNA in hybridization buffer to a total volume of 30 μl. The sample was heated to 85°C for 10 min and immediately transferred to 45°C overnight. Following hybridization, the samples were cooled to room temperature and 300 μl of RNase digestion buffer (10 mM Tris-HCl [pH 7.6], 300 mM NaCl, 5 mM EDTA) containing 10 U of RNase One (Promega) was added at 30°C for 30 min. The sample was precipitated by adding 10 μl of 10% SDS, 700 μl of 100% ethanol, and 10 μg of carrier yeast tRNA. The pellet was resuspended in 5 μl of loading buffer (80% formamide, 10 mM EDTA, 1 μg of xylene cyanol per μl, 1 μg of bromophenol blue per μl), heated to 95°C for 4 min, and electrophoresed through a 4 to 5% denaturing polyacrylamide gel. An RNA Century Marker template set (Ambion, Inc., Austin, Tex.) was prepared as recommended by the supplier.

Primer extension analysis. Primer extension analysis was carried out by using 20 pmol of the 5′-end-labelled oligonucleotide. Oligonucleotides were labelled as described previously. Approximately 10^6 cpm of the labelling reaction mixture was ethanol precipitated with 10 μg of total RNA, and the pellet was resuspended in 30 μl of hybridization buffer (400 mM NaCl, 40 mM PIPES [pH 6.4], 1 mM EDTA). The sample was heated to 90°C for 10 min, immediately transferred to 37°C, and allowed to hybridize overnight. Following hybridization, 170 μl of water and 400 μl of 100% ethanol were added to precipitate the sample. The pellet was resuspended in 19 μl of reverse transcription buffer (5 mM $MgCl_2$, 1× PCR buffer [Perkin-Elmer Cetus], 1 mM each deoxynucleoside triphosphate, 2.5 U of RNase inhibitor, 50 μg of actinomycin D per ml). Finally, 2.5 U of reverse transcriptase was added, and the sample was incubated at 37°C for 2 h. Next, 1 μl of 0.5 M EDTA and 1 μl of DNase-free RNase (5 μg/ml) were added, and the solution was incubated at 37°C for 30 min. One hundred fifty microliters of TEN (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 100 mM NaCl) was added, and the sample was phenol-chloroform extracted and ethanol precipitated. The pellet was resuspended in 5 μl of loading buffer and electrophoresed on an 8% denaturing polyacrylamide gel. The size of the extended product was determined by comparison with a DNA sequence ladder.

Time course analysis of RNA expression in MDV-infected CEF. Cultures of CEF (5.5×10^6 cells per 25-mm^2 tissue culture flask) were infected with 4×10^4 to 8×10^4 PFU of MDV strain GA. Infected CEF were harvested at 28, 72, and 140 h postinfection (p.i.). Total RNA from each time point was purified as described above. Ten micrograms of total RNA was divided into two 5-μg samples and electrophoresed through a 1.6% agarose–2.2 M formaldehyde gel and transferred to nitrocellulose. One sample was hybridized with a riboprobe

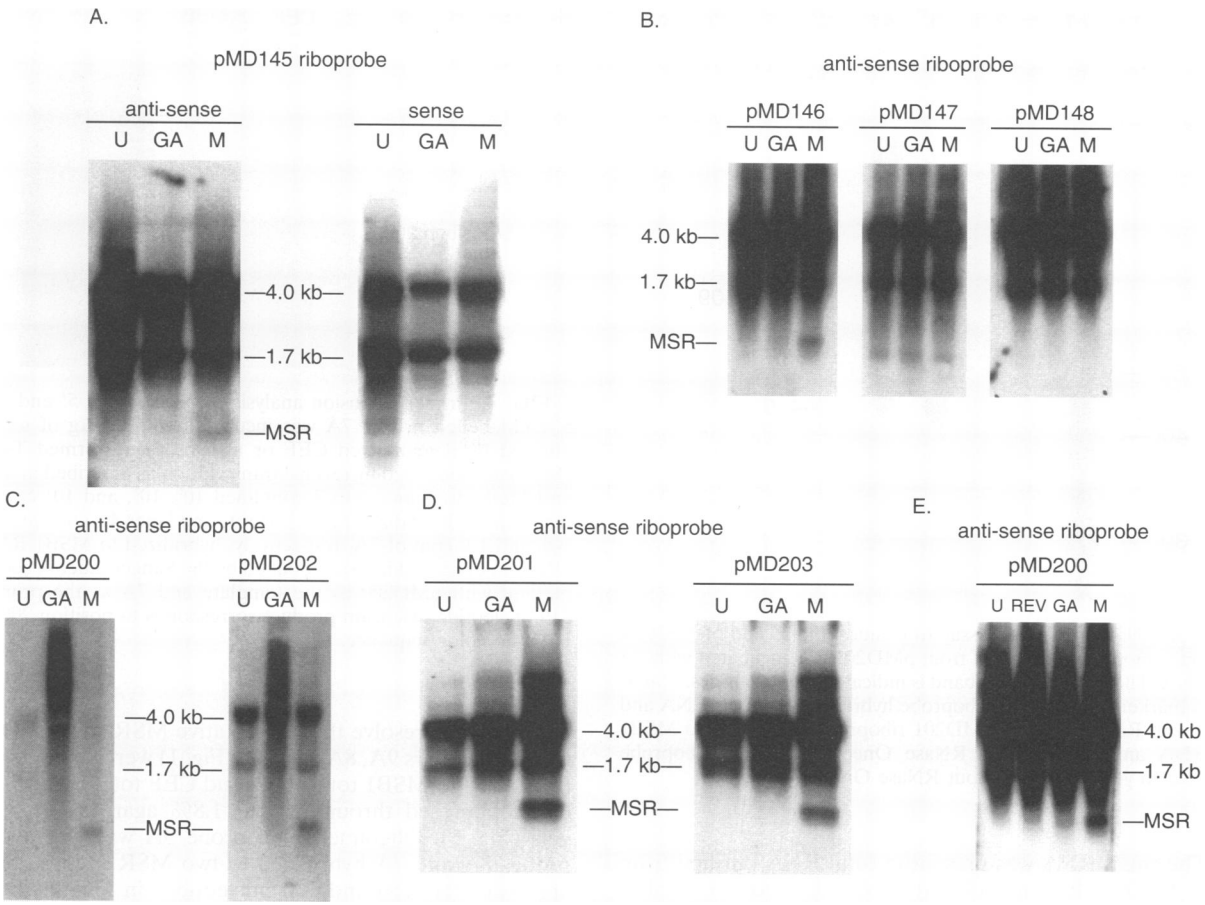

FIG. 2. Northern hybridization of total RNA probed with riboprobes corresponding to the ICP4 coding region. Blots contained 15 μg of total RNA purified from uninfected CEF (U), GA-infected CEF (GA), MDV-transformed T-lymphoblastoid cells (M), or reticuloendotheliosis virus-transformed T-lymphoblastoid cells (REV). All RNA samples were separated on 1% agarose-formaldehyde gels. The positions of 28 and 18S rRNAs (4.0 and 1.7 kb, respectively) and MSR are indicated. Blots were hybridized with riboprobes generated from pMD145, which would detect ICP4 sense RNA or ICP4 antisense RNA (A); pMD146, pMD147, or pMD148, which would detect ICP4 antisense RNA (B); pMD200 or pMD202, which would detect ICP4 antisense RNA (C); pMD201 or pMD203, which would detect ICP4 antisense RNA (D); or pMD200, which would detect ICP4 antisense RNA (E).

transcribed from pMD200 with SP6 RNA polymerase, while the other RNA sample was hybridized with a riboprobe transcribed from pMD200 with T7 RNA polymerase. The SP6-derived riboprobe was complementary to ICP4 antisense RNA, and the T7 riboprobe was complementary to ICP4 sense RNA. Blots were hybridized and washed as described above.

Treatment of MSB1 T-lymphoblastoid cells with iododeoxyuridine. MSB1 cells were seeded at 5×10^5/25-mm^2 tissue culture flask in RPMI 1640 medium or RPMI 1640 medium containing 25 μg of iododeoxyuridine per ml. Forty-eight hours later, total RNA from each treatment was purified from 2.5×10^7 cells. Twenty micrograms of total RNA from each treatment was divided into two 10-μg samples, electrophoresed through a 1.0% agarose–2.2 M formaldehyde gel, and transferred to nitrocellulose. The blots were hybridized with riboprobes derived from pMD200 by using SP6 or T7 RNA polymerase as described above.

RESULTS

Detection of a small RNA in MSB1 T-lymphoblastoid cells that mapped antisense to the predicted translational start site of the ICP4 homolog gene. A small RNA was detected in total RNA from MSB1 MDV-transformed T-lymphoblastoid cells but not in total RNA from uninfected CEF or productively infected CEF by hybridization of Northern blots with a ^{32}P-labelled double-stranded probe corresponding to the ICP4 homolog coding region (data not shown). This RNA was approximately 0.85 kb in size when electrophoresed through 1% agarose-formaldehyde gels. When strand-specific riboprobes prepared from pMD145 (Fig. 1) were used to detect this RNA, it was found to be complementary to the ICP4 homolog gene (Fig. 2A). Three subclones of pMD145 were constructed (Fig. 1), and riboprobes derived therefrom were used to probe Northern blots containing RNA from uninfected CEF, GA-infected CEF, and MSB1 cells. Only the pMD146 antisense riboprobe hybridized to the RNA, confirming that it was complementary to the ICP4 homolog gene and indicating that it overlapped the 5′ end of the ICP4 homolog coding region (Fig. 2B). Two more subclones, pMD200 and pMD202, that lie within and overlap pMD146, respectively, were constructed (Fig. 1). Both pMD200 and pMD202 riboprobes detected the RNA, indicating that the RNA traverses the *Hin*dIII site lying at the 5′ end of the coding region of the ICP4 homolog gene (Fig. 2C). In addition, riboprobes derived from pMD201 and pMD203 (Fig. 1) both detected the RNA (Fig.

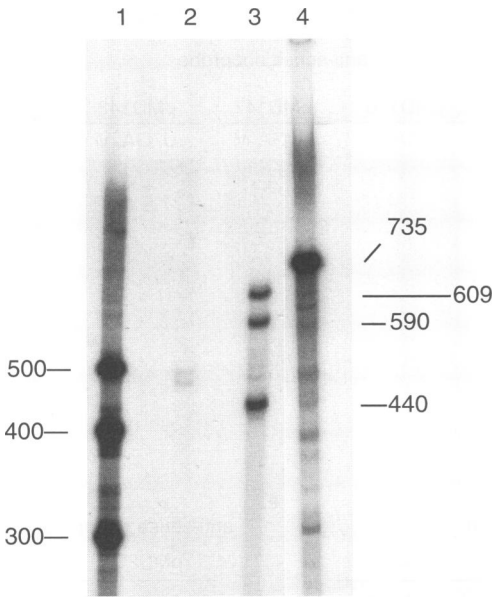

FIG. 3. RNase protection assay to position the 5′ end of MSR. A full-length riboprobe generated from pMD201 was incubated with 7.5 μg of RNA. The length of each band is indicated in nucleotides. Lanes: 1, RNA markers; 2, pMD201 riboprobe hybridized to yeast tRNA and treated with RNase One; 3, pMD201 riboprobe hybridized to MSB1 total RNA and treated with RNase One; 4, pMD201 riboprobe hybridized to yeast tRNA without RNase One treatment.

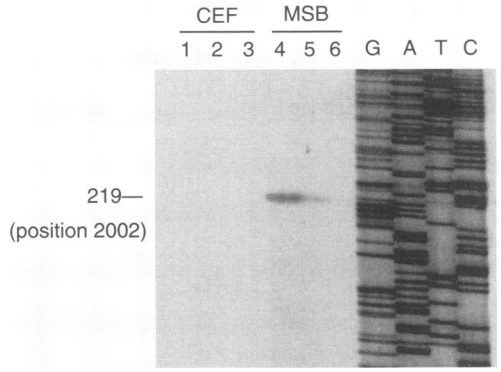

FIG. 4. Primer extension analysis to position the 5′ end of MSR. 5′-end-labelled primer 7A was incubated with 10 μg of total RNA purified from uninfected CEF or an MDV-transformed T-cell line (MSB) and extended by reverse transcription as described in Materials and Methods. Lanes 1 to 3 contained 10^6, 10^5, and 10^4 cpm of 7A, respectively, hybridized to CEF RNA, and lanes 4 to 6 contained 10^6, 10^5, and 10^4 cpm of 7A, respectively, hybridized to MSB1 RNA. The DNA sequence ladder was obtained by the Sanger dideoxy sequencing method with pMD200 as the template and 7A as the primer. The 219-nucleotide extension product corresponds to position 2002 in the ICP4 homolog sequence.

2D). The small RNA was present in total RNA purified from MDCC-UD02 cells, lymphoblastoid cells derived in our laboratory from an MDV-induced lymphoma (data not shown). This result indicated that the small RNA was not unique to MSB1 cells but was present in other MDV-induced lymphoblastoid cells. Finally, the RNA was not detected in RNA purified from CU91, a reticuloendotheliosis virus-transformed T-lymphoblastoid cell line (Fig. 2E), nor was it detected in poly(A) RNA purified from MSB1 cells (data not shown). The RNA was named "MDV small RNA" (MSR).

Map position of the 5′ end of MSR. RPA, primer extension, and Northern blot analysis using oligonucleotide probes were used to map the 5′ end of MSR (Fig. 3 to 5). To position the 5′ end of MSR, a full-length probe prepared from pMD201 was used for RPA. The pMD201 riboprobe (positions 1635 to 2244) consisted of 609 nucleotides of the MDV sequence and 126 nucleotides of the vector sequence. MSB1 total RNA protected the full-length probe and two smaller fragments approximately 590 and 440 nucleotides in length (Fig. 3). This protection pattern suggested that there might be two 5′ ends of MSR that map approximately to positions 2075 and 2225 in the ICP4 homolog gene. Primer extension analysis was used to examine the possibility of two 5′ ends for MSR and to more precisely pinpoint the 5′ end(s). Primer 7A (Fig. 1) was extended to position 2002 when hybridized to MSB1 total RNA but not when hybridized to CEF total RNA (Fig. 4). Although we did not observe another primer extension product using primer 7A, examination of the DNA sequence in this region revealed a 26-bp perfect palindrome at position 1999 (see below). We believe that this palindrome may have interfered with the primer extension, resulting in premature termination by the reverse transcriptase.

Northern blot analysis using oligonucleotide probes and higher-percentage agarose gels was also used to locate the 5′ end(s) and to resolve the two putative MSR species. Oligonucleotide primers 9A, 8A, and 7A (Fig. 1) were end labelled and hybridized to MSB1 total RNA and CEF total RNA that were electrophoresed through 1.6 to 1.8% agarose-formaldehyde gels (Fig. 5). Oligonucleotide probe 9A was negative, while probes 8A and 7A hybridized to two MSR species that were approximately 750 and 900 nucleotides in length. From this analysis, we could conclude that MSR was actually two species, and on the basis of the map positions of oligonucleotides 9A and 8A, the ends of the RNAs were tentatively placed between positions 2059 and 2297. These positions were consistent with the RPA results.

Map position of the 3′ end of MSR. RPA and Northern blot analysis using oligonucleotide probes or strand-specific riboprobes were used to map the 3′ ends of the MSRs. RPA using pMD203 was performed since previous Northern blot analysis

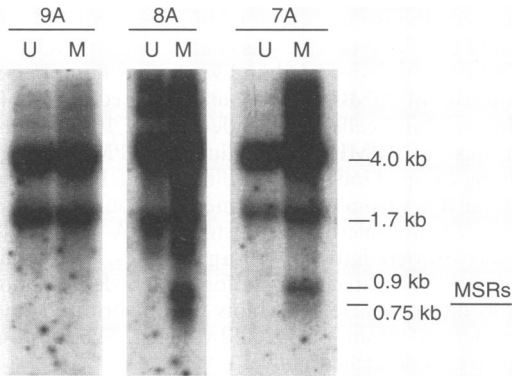

FIG. 5. Northern blot analysis to map the 5′ end of MSR. Fifteen micrograms of total RNA purified from uninfected CEF (U) or an MDV-transformed T-cell line (M) was separated on 1.6 to 1.8% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized with 5′-end-labelled oligonucleotides 9A, 8A, and 7A as described in Materials and Methods. The two MSR species are indicated.

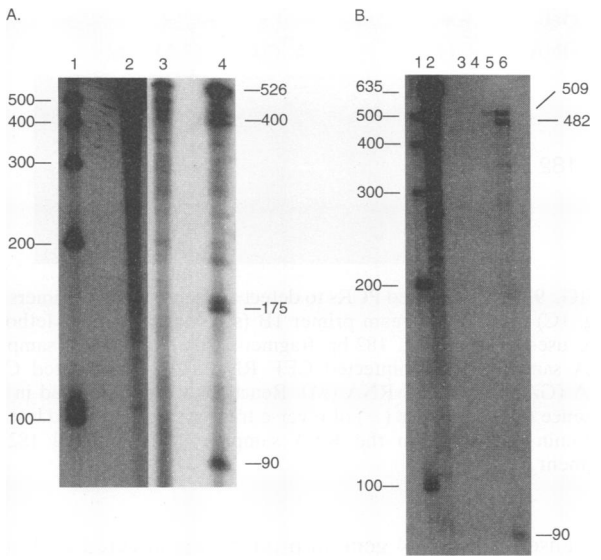

FIG. 6. RNase protection assay to position the 3′ end of MSR. (A) A full-length riboprobe generated from pMD203 was incubated with 7.5 μg of RNA purified from MSB1 cells. Lanes: 1, RNA markers; 2, pMD203 riboprobe hybridized to yeast tRNA without RNase One treatment; 3, pMD203 riboprobe hybridized to yeast tRNA and treated with RNase One; 4, pMD203 riboprobe hybridized to MSB1 total RNA and treated with RNase One. (B) A full-length riboprobe generated from pMD195 was incubated with 7.5 μg of RNA. Lanes: 1, RNA markers; 2, pMD195 riboprobe hybridized to yeast tRNA without RNase One treatment; 3, pMD195 riboprobe hybridized to yeast tRNA and treated with RNase One; 4, pMD195 riboprobe hybridized to RNA from uninfected CEF and treated with RNase One; 5, pMD195 riboprobe hybridized to RNA from GA-infected CEF and treated with RNase One; 6, pMD195 riboprobe hybridized to RNA from MSB1 cells and treated with RNase One. Band lengths in nucleotides are indicated.

demonstrated that a pMD203 riboprobe could hybridize to the MSR (Fig. 1 and 2D). The pMD203 riboprobe consisted of 400 nucleotides of the MDV sequence and 126 nucleotides of the vector sequence. MSB1 total RNA protected the full-length probe as well as two smaller fragments approximately 175 and 90 nucleotides in length (Fig. 6A). In addition, the pMD195 riboprobe (Fig. 1) was used for RPA. This probe consisted of 509 nucleotides of the MDV sequence and 126 nucleotides of the vector sequence. MSB1 total RNA protected the full-length pMD195 probe as well as two smaller fragments approximately 482 and 90 nucleotides in length (Fig. 6B). In addition, total RNA from GA-infected CEF protected the pMD195 riboprobe in the same manner as MSB1 total RNA. These results suggested multiple 3′ ends for MSR, with the ends lying between positions 917 and 1513.

Northern blot analysis using oligonucleotides or riboprobes was performed to resolve the complexity of the pMD203 and pMD195 RPA results (Fig. 7). Oligonucleotides 6A, 5A, 4A, 3A, 2A, and 1A (Fig. 1) were hybridized to MSB1 total RNA and CEF total RNA that were electrophoresed through a 1.6 to 1.8% agarose-formaldehyde gel. Only oligonucleotide 6A hybridized to both MSR species (Fig. 7A). Since pMD203 detected MSR by Northern hybridization (Fig. 2D) yet oligonucleotides 5A, 4A, 3A, 2A, and 1A did not, it appeared that the 3′ portion of MSR was spliced. One splice site should lie between positions 1546 and 1291 since these are the boundaries of oligonucleotides 6A and 5A, respectively. The other splice site should lie upstream of position 1033, the terminus of the 1A oligonucleotide. A riboprobe that contains the portion of pMD203 that maps upstream of oligonucleotide 1A was prepared by using gel-purified pMD203 restricted with *Xho*I (Fig. 1). This riboprobe spanned positions 827 to 1036 (Fig. 1) and hybridized to both MSR species (Fig. 7B). This result suggested that the 3′ splice site should lie within the region covered by this riboprobe. That the MSRs are spliced is supported by the distance between the 5′ ends of the MSRs (positions 2297 to 2059) and the map location of the *Xho*I-digested pMD203 riboprobe (827 to 1036) which detected both MSRs. The smallest possible intervening distance is 1,023 nucleotides, considerably more than the sizes of the MSRs.

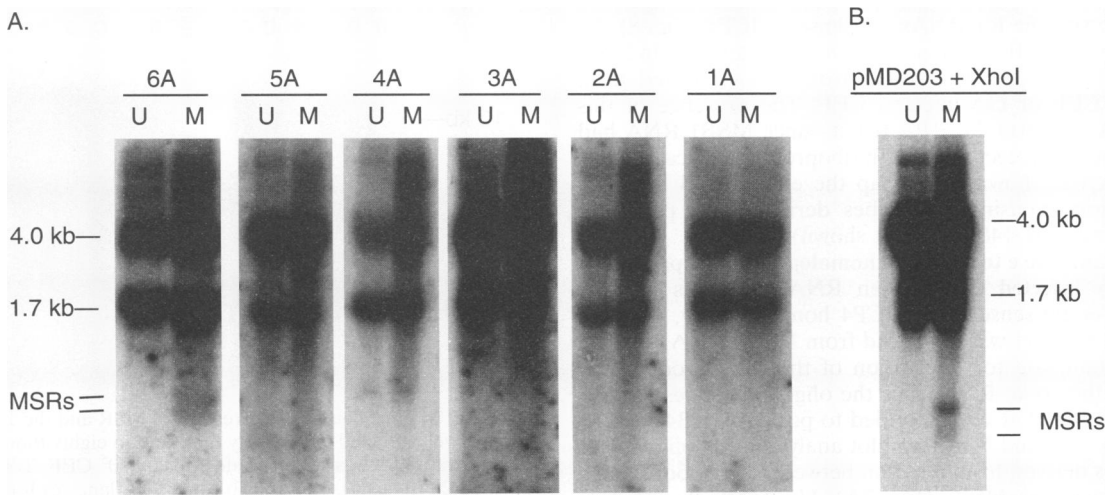

FIG. 7. Northern blot analysis to map the 3′ end of MSR. Fifteen micrograms of total RNA purified from uninfected CEF (U) or MSB1 cells (M) was separated on 1.6 to 1.8% agarose-formaldehyde gels and probed with 5′-end-labelled oligonucleotides (6A, 5A, 4A, 3A, 2A, and 1A) (A) or a riboprobe generated from an agarose gel-purified pMD203 that was linearized with *Xho*I (Fig. 1) (B). The two MSR species detected by oligonucleotide 6A and the pMD203 truncated riboprobe are indicated.

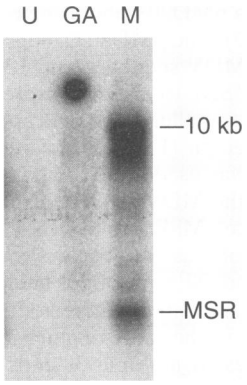

FIG. 8. Northern blot analysis to detect additional antisense transcripts. A riboprobe generated from pMD200 that should detect ICP4 antisense RNA was hybridized to total RNA purified from uninfected CEF (U), GA-infected CEF (GA), and MSB1 cells (M). The RNA was separated on a 1% agarose-formaldehyde gel. Following hybridization, the blots were rinsed in 2× SSC containing 1 μg of RNase A per ml to reduce nonspecific hybridization to rRNA. The positions of MSR and the 10-kb RNA are indicated.

Examination of the region within the predicted boundaries of the 5′ splice site (1546 to 1291) revealed a perfect AGGTA AGT splice donor consensus sequence at positions 1529 to 1522 as well as several other imperfect splice donor possibilities. Furthermore, at least three potential 3′ CAG splice acceptor sites are located within the boundaries of the predicted 3′ splice site (1036 to 827) at positions 984, 950, and 917.

A related 10-kb RNA was present in MSB1 cells. Our initial Northern hybridizations were to blots of total RNA, and in these blots nonspecific probe binding to rRNA was significant and could have obscured the identification of additional RNAs. The MSR was easily identified because it migrated at a position distant from that of rRNA. To eliminate the problem of nonspecific rRNA binding to the probe, in some cases Northern blots were rinsed once for 10 min at room temperature in 2× SSC containing 1.0 μg of RNase A per ml (21). With this modification of the Northern hybridization washing procedure, an additional transcript of 10 kb was detected with a riboprobe specific for RNAs antisense to ICP4 (Fig. 8). By Northern hybridization, this species was detected only in RNA from MSB1 cells and was absent from RNA purified from uninfected CEF or GA-infected CEF. The presence of this larger RNA supports the RPA results since MSB1 RNA had the capability to protect full-length riboprobes in all cases. This 10-kb RNA was shown to overlap the entire ICP4 homolog coding region by using riboprobes derived from pMD146, pMD147, and pMD148 (data not shown).

An RNA antisense to the ICP4 homolog gene was present in productively infected CEF. When RNA PCR was used to detect RNAs antisense to the ICP4 homolog gene, the predicted PCR product was produced from MSB1 RNA (Fig. 9). The RNA template for generation of the PCR product was most likely the 10-kb RNA since the oligonucleotides used to prime the RNA PCR corresponded to positions 1234 to 1255 and 1395 to 1416 and Northern blot analysis using oligonucleotide probes derived from a region between these coordinates failed to detect the MSRs (Fig. 7A). Unexpectedly, productively infected CEF but not uninfected CEF also contained an RNA that could serve as a template for RNA-driven PCR using primers that would detect RNAs antisense to the ICP4 homolog gene. This result suggested the presence of RNA(s)

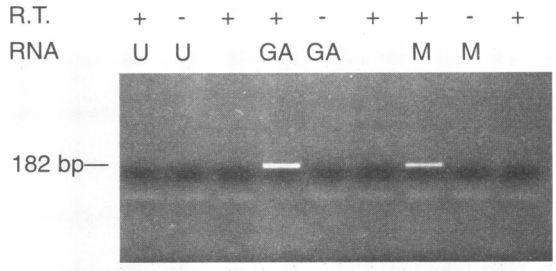

FIG. 9. RNA-directed PCRs to detect antisense RNAs. Primers 3A (Fig. 1C) and the upstream primer 1B (see Materials and Methods) were used to amplify a 182-bp fragment from total RNA samples. RNA samples were uninfected CEF RNA (U), GA-infected CEF RNA (GA), and MSB1 RNA (M). Reactions were performed in the presence (+) or absence (−) of reverse transcriptase (R.T.) to test for contaminating DNA in the RNA samples. The amplified 182-bp fragment is indicated.

antisense to the ICP4 gene in productively infected CEF at a level below that detectable in our Northern hybridizations. This result is consistent with the RPA which showed the presence of protected species from productively infected cells (Fig. 6B).

MSR and the 10-kb RNA were detected in GA-infected CEF during late times of infection. Up to this point, total RNA from productively infected CEF was prepared 48 h p.i. RNA prepared at this time point was negative for MSR and 10-kb RNA expression by Northern hybridization (Fig. 2). However, ICP4 RNA could be detected with ICP4-specific riboprobes (data not shown). The detection of ICP4 antisense RNA in productively infected CEF by RNA PCR (Fig. 9) and RPA (Fig. 6B) prompted us to examine MSR expression at various times p.i. in MDV GA-infected CEF (Fig. 10). Riboprobes derived from

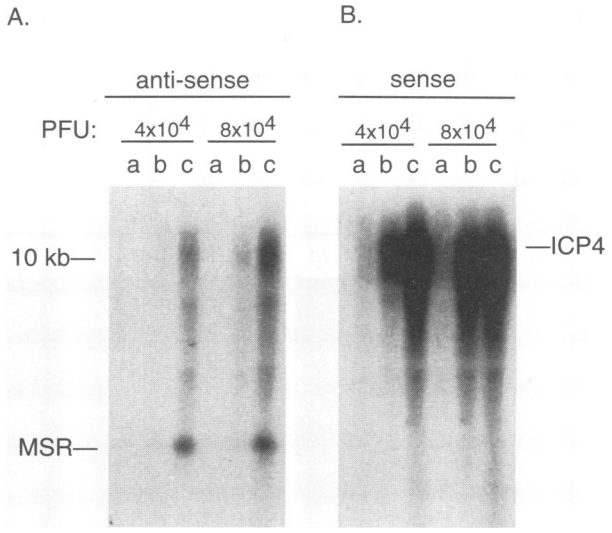

FIG. 10. Time course of expression of MSR and the 10-kb RNA in productively infected CEF. Forty thousand to eighty thousand PFU of MDV strain GA was used to infect 5.5×10^5 CEF. Total RNA was purified 28 (lanes a), 72 (lanes b), and 140 (lanes c) h p.i. Each lane contains 6 μg of RNA. Blots were probed with riboprobes generated from pMD200 which would detect ICP4 antisense RNA (A) or ICP4 sense RNA (B). Following hybridization, the blots were rinsed in 2× SSC containing 1 μg of RNase A per ml to reduce nonspecific hybridization to rRNA.

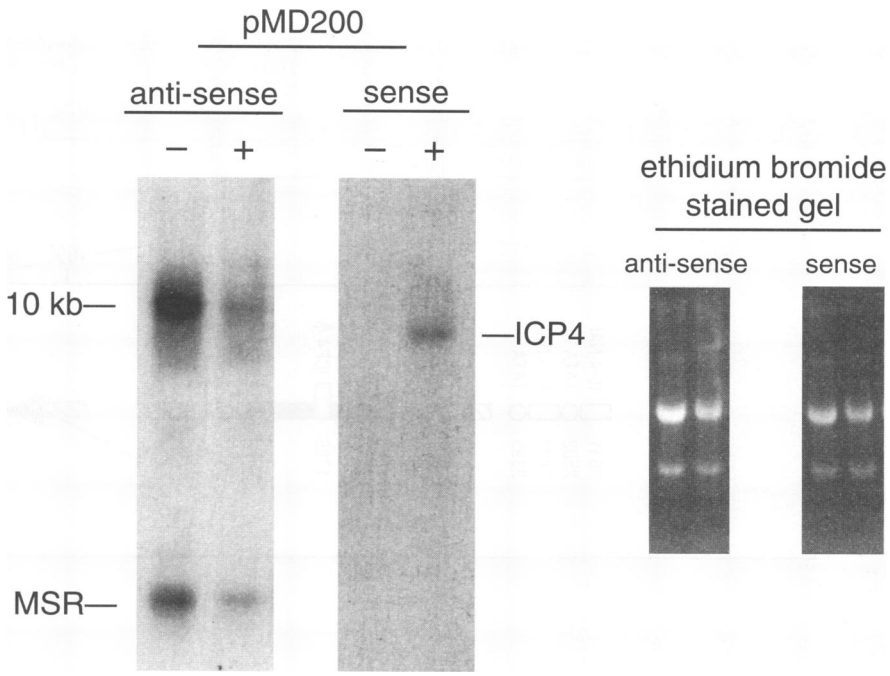

FIG. 11. Effect of iododeoxyuridine on expression of MSR and the 10-kb RNA in MSB1 cells. Total RNA was purified from 2.5×10^7 cells that were (+) or were not (−) treated with 25 μg of iododeoxyuridine per ml for 48 h. Each lane contains 10 μg of total RNA electrophoresed through a 1% agarose-formaldehyde gel. Following hybridization, the nitrocellulose blots were rinsed in 2× SSC containing 1 μg of RNase A per ml to reduce nonspecific hybridization to rRNA. The positions of MSR, the 10-kb RNA, and ICP4 RNA are indicated. Blots were hybridized with riboprobes generated from pMD200 that would detect ICP4 antisense RNA or ICP4 sense RNA. The ethidium bromide-stained gels are shown to demonstrate similar amounts of RNA in different lanes.

pMD200 by using SP6 RNA polymerase or T7 RNA polymerase were used to detect MSR and ICP4 RNA, respectively. At 28 and 72 h p.i., MSR and the 10-kb RNA were not detected in GA-infected CEF by Northern hybridization. However, at 140 h p.i. MSR and the 10-kb RNA were readily detected by Northern hybridization (Fig. 10A). In contrast, ICP4 RNA was detectable at all time points tested, with ICP4 RNA levels at 140 h p.i. being the highest (Fig. 10B). These results are consistent with RPA and RNA PCR and indicated that MSR and the 10-kb RNA are detectable in productively infected tissue culture cells 3 to 6 days p.i.

Expression of MSR and the 10-kb RNA was reduced in MSB1 cells treated with iododeoxyuridine. Treatment of MSB1 cells with iododeoxyuridine has been shown to reactivate MDV (6, 12). Treatment of MSB1 cells with 25 μg of iododeoxyuridine per ml resulted in a reduction in the relative quantity of both MSR and the 10-kb RNA, with an accompanying increase in ICP4 RNA (Fig. 11).

Notable sequence elements of the MSR. Notable sequence elements in the vicinity of the MSR are indicated in Fig. 12. A latency promoter binding factor (LPBF) consensus recognition sequence (35) was found at position 2008 (Fig. 12). LPBF is a palindromic sequence (CCACGTGG) that was first identified in the major HSV LAT promoter and that is recognized by a factor present in neuronal and nonneuronal cells. The sequence shows strong homology to upstream stimulating factor and major late transcription factor sites present in the adenovirus major late promoter and several other cellular and viral promoters (35). The MDV LPBF sequence lies in the middle of a 26-bp perfect palindrome with a ΔG of formation of −32.0 kcal (−134 kJ)/mol. The primer extension analysis indicated that the 5′ end of MSR was positioned within this palindrome. However, the palindromic structure may have interfered with this assay, complicating 5′-end mapping results. Another notable sequence element (ATCGTCCATATTGGC) located toward the 3′ portion of the MSRs (position 1399) is homologous to the ICP4 recognition consensus sequence, ATCGT Cn4YCGRC (13). This is the third such sequence in the vicinity of the ICP4 gene, with one other being located on the opposite strand 572 nucleotides into the predicted coding region of the ICP4 homolog (position 1789 in Fig. 1) (1). Both of these sites lie within the region encoding MSR. The third ICP4 autoregulatory site was located approximately 1.5 kb upstream of the ICP4 homolog gene (2). In addition, two interleukin-4-responsive elements (18) are located at positions 2402 and 2631, and two TATA boxes are located at positions 2395 and 2502.

DISCUSSION

Small RNAs (0.9 and 0.75 kb) complementary to the MDV ICP4 homolog gene, named MSRs, have been detected in MSB1 cells. These cells are considered by many investigators to be latently infected, although definitions of latency vary among herpesvirus systems and laboratories. Nevertheless, MDV-transformed T-lymphoblastoid cells harbor MDV in a relatively quiescent state. These RNAs were not detectable in CU91, a reticuloendotheliosis virus-transformed T-lymphoblastoid cell line, an observation consistent with the conclusion that they are MDV specific. In addition, a larger 10-kb RNA complementary to the ICP4 homolog gene was also present in MSB1 cells. The relationship between the larger species and the MSRs is not known, although it is possible that the MSRs are processing products of the 10-kb RNA.

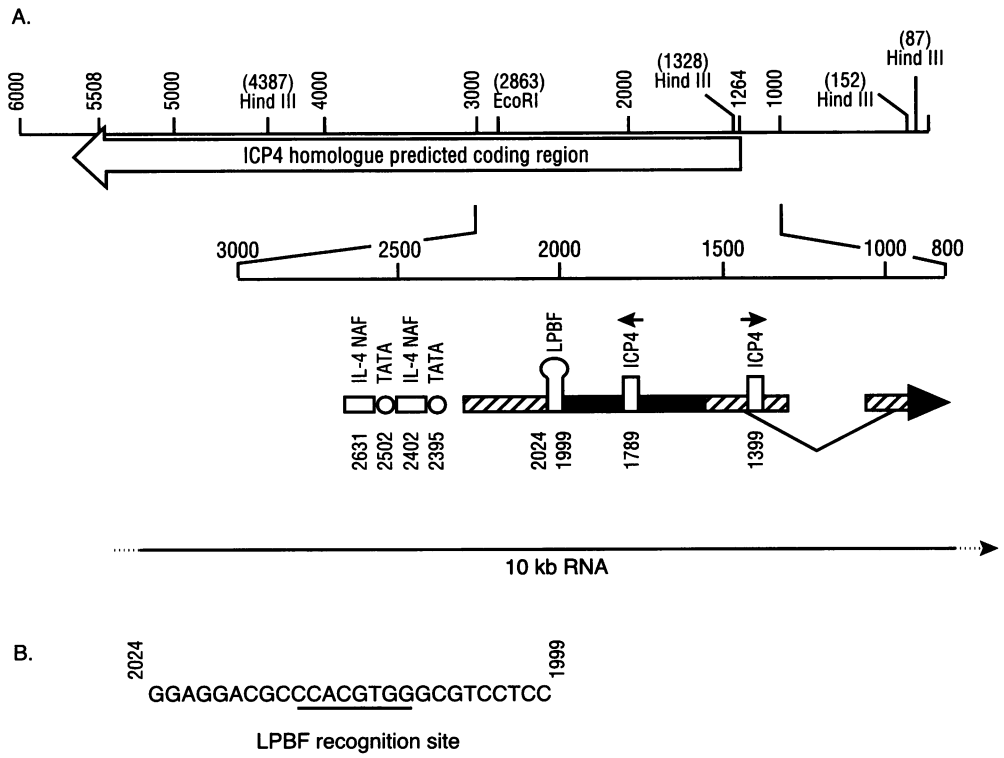

FIG. 12. (A) Positions of notable sequence elements within and upstream of MSR. Two TATA box sequences and consensus recognition sites for interleukin-4 nuclear activated factor (IL-4 NAF), LPBF, and ICP4 are indicated. Sites for which exact endpoints and splice junctions are unknown are hatched. The predicted 3′ splicing pattern and the position of the 10-kb RNA are indicated. (B) MDV 26-bp palindrome, with the LPBF recognition site homology underlined.

The 5′ ends of the MSRs have been difficult to pinpoint because of the presence of a perfect 26-bp palindrome located at positions 1999 to 2024. This palindrome may act as a barrier for reverse transcriptase or create an RNase-sensitive site, resulting in apparent 5′ ends that do not represent the true termini of the MSRs. Several possibilities exist with regard to the 5′ ends of the MSRs. First, it is possible that there are two MSR species with different 5′ ends that lie between positions 2059 and 2297. Second, there may be two MSRs that share a common 5′ terminus located between positions 2059 and 2297. The MSRs may then differ with regard to their 3′ termini. Third, the MSRs may be initiated upstream of the 9A oligonucleotide, and the resulting transcripts may be processed in a manner that involves a site or sites located between positions 2059 and 2297. All of these possibilities are consistent with Northern hybridization analysis using oligonucleotides 9A and 8A (Fig. 5).

The 3′ ends of the MSRs have been equally elusive. RPA using pMD203 and pMD195 as riboprobes suggested the presence of multiple 3′ ends. Northern hybridizations using oligonucleotides and riboprobes derived from pMD203 and gel-purified *Xho*I-digested pMD203 indicated that the MSRs were spliced. The proposed map positions of the MSR and the 10-kb RNA relative to the ICP4 homolog gene are shown in Fig. 12.

The pattern of MSR and 10-kb RNA expression relative to ICP4 RNA expression in different cellular environments is interesting. Studies on the replication cycles of various herpesviruses suggest that latent infections may result from an absence of host factors critical for the expression of viral early gene products. Activation of these host factors in response to extracellular stimuli can induce the expression of these viral proteins and lead to lytic replication (15). MSR and 10-kb RNA expression in MSB1 cells was relatively high compared with ICP4 RNA expression. However, upon virus reactivation by iododeoxyuridine treatment, steady-state ICP4 RNA levels increased and both MSR and 10-kb RNA levels decreased (Fig. 11). Likewise, during early times of productive infection in cell culture when CEF were freshly plated, MSR and 10-kb RNA levels were not detectable by Northern hybridization while ICP4 RNA was apparent. Later during productive infection (140 h p.i.) when CEF were aged and probably depleted of some necessary cellular factors required for productive infection, MSR and 10-kb RNA levels were relatively abundant (Fig. 10). These patterns of expression suggest that MSR and the 10-kb RNA may be involved with the molecular switches important for turning off MDV replication when the virus resides in a relatively nonpermissive environment.

The MSRs belong to a group of RNAs that are expressed in transformed lymphoblastoid cells. An early report indicated that 12 to 14% of the viral genome was transcribed in lymphoblastoid cells (28). Another report indicated that only a few transcripts (<10) were detectable in lymphoblastoid cell lines and that these were derived from immediate-early genes that map in the repeat sequences flanking the unique long and unique short regions as well as in the unique short region itself (27). Others have reported a larger number of transcripts, ranging from 29 to 32, that are derived from many sites on the genome (19) or that map to the repeat sequences flanking the unique long and unique short regions and the adjacent unique sequences (32). Taken together, these results indicate that transcription of the MDV genome in MDV-transformed lym-

phoblastoid cells is more extensive than that of the latent HSV genome but less extensive than that of the MDV genome in productively infected cells. The repeat regions of the genome are particularly active transcriptionally in these cells, and these RNAs are, in general, also present in cytolytically infected cells. In most cases, the gene products derived from these RNAs are unknown. However, a group of transcripts mapping to *Eco*RI Q and *Bam*HI I2 and ranging in size from 1.2 to 3.8 kb correspond to the *meq* gene (17). The *meq* gene is highly expressed in MDV-transformed lymphoblastoid cells. The *meq* protein should contain 362 amino acids and is predicted to share features with the *fos/jun* family of oncoproteins including a leucine zipper motif, an upstream region rich in basic amino acids, and a domain rich in prolines.

The MSRs have several features in common with LATs of alphaherpesviruses. First, they are most abundantly expressed under circumstances wherein the virus is not undergoing productive replication. Second, they map antisense to an immediate-early gene. As in the case of pseudorabies virus, the relevant immediate-early gene is the ICP4 homolog. Third, they contain an LPBF consensus recognition sequence, a feature shared with the HSV LAT. The presence of this recognition site suggests, by analogy to HSV, that a factor similar to LPBF can recognize the sequence. Fourth, MSRs contain an ICP4 regulatory site as does HSV LAT (4), suggesting that ICP4 can regulate LAT expression. Thus, these features imply that MSRs may be MDV LATs.

The fact that MSRs are abundant in cells in which the ICP4 homolog is not highly expressed suggests that MSRs may negatively regulate ICP4 homolog expression or vice versa. The function of MSRs is unknown, but several possibilities exist. First, MSRs may be naturally occurring antisense RNAs and interfere with the translation of ICP4 homolog mRNA. Second, MSRs may compete with the ICP4 homolog gene for factors that regulate gene expression. Third, MSRs may encode protein gene products. Fourth, MSRs may be stable introns. With regard to possibilities 3 and 4, there are a number of small open reading frames flanking and within MSR sequences, and it is possible that via RNA processing one or several of these open reading frames are expressed as a protein gene product.

Many questions remain with regard to MDV MSRs. It would be interesting to determine whether these RNAs are present in latently infected, nontransformed T cells or whether they are specific to transformed T cells. In addition, it would be interesting to assess the expression of these RNAs in neuronal and other tissues, to determine the primary intracellular location of these RNAs, to identify any protein gene products they might encode, and to assess the effect of ablation of expression of these RNAs on MDV latency and/or transformation. The significance of the expression of MDV ICP4 and its antisense RNAs relative to virus gene expression, establishment of and reactivation from latency, and transformation of T lymphocytes remains to be seen.

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REFERENCES

1. **Anderson, A. S., A. Francesconi, and R. W. Morgan.** 1992. Complete nucleotide sequence of the Marek's disease virus ICP4 gene. Virology **189**:657–667.
2. **Anderson, A. S., J. Goetz, J. L. Cantello, and R. W. Morgan.** Unpublished data.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, New York.
4. **Batchelor, A. H., and P. O'Hare.** 1990. Regulation and cell-type-specific activity of a promoter located upstream of the latency-associated transcript of herpes simplex virus type 1. J. Virol. **64**:3269–3279.
5. **Calnek, B. W., H. K. Adldinger, and D. E. Kahn.** 1970. Feather follicle epithelium: a source of enveloped and infectious cell-free herpesvirus from Marek's disease. Avian Dis. **14**:219–233.
6. **Calnek, B. W., W. R. Shek, and K. A. Schat.** 1981. Spontaneous and induced herpesvirus genome expression in Marek's disease tumor cell lines. Infect. Immun. **34**:483–491.
7. **Calnek, B. W., and R. L. Witter.** 1991. Marek's disease, p. 342–385. *In* B. W. Calnek, H. J. Barnes, C. W. Beard, W. M. Reid, and H. W. Yoder, Jr. (ed.), Diseases of poultry, 9th ed. Iowa State University Press, Ames.
8. **Chen, X., P. J. A. Sondermeijer, and L. F. Velicer.** 1992. Identification of a unique Marek's disease virus gene which encodes a 38-kilodalton phosphoprotein and is expressed in both lytically infected cells and latently infected lymphoblastoid tumor cells. J. Virol. **66**:85–94.
9. **Cheung, A. K.** 1989. Detection of pseudorabies virus transcripts in trigeminal ganglia of latently infected swine. J. Virol. **63**:2908–2913.
10. **Croen, K. D., J. M. Ostrove, L. J. Dragovic, and S. E. Straus.** 1988. Patterns of gene expression and sites of latency in human nerve ganglia are different for varicella-zoster and herpes simplex virus. Proc. Natl. Acad. Sci. USA **85**:9773–9777.
11. **Cui, Z., L. F. Lee, J. L. Liu, and H. J. Kung.** 1991. Structural analysis and transcriptional mapping of the Marek's disease virus gene encoding pp38, an antigen associated with transformed cells. J. Virol. **65**:6509–6515.
12. **Dunn, K., and K. Nazerian.** 1977. Induction of Marek's disease virus antigens by IdUrd in a chicken lymphoblastoid cell line. J. Gen. Virol. **34**:413–419.
13. **Faber, W. W., and K. W. Wilcox.** 1986. Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. Nucleic Acids Res. **15**:4491–4511.
14. **Feldman, L. T.** 1991. The molecular biology of herpes simplex virus latency, p. 233–243. *In* E. K. Wagner (ed.), Herpesvirus transcription and its regulation. CRC Press, Inc., Boca Raton, Fla.
15. **Garcia-Blanco, M. A., and B. R. Cullen.** 1991. The molecular basis of latency in pathogenic human viruses. Science **254**:815–820.
16. **Jones, C., G. Delhon, A. Bratanich, G. Kutish, and D. Rock.** 1990. Analysis of the transcriptional promoter which regulates the latency-related transcript of bovine herpesvirus 1. J. Virol. **64**: 1164–1170.
17. **Jones, D., L. F. Lee, J. L. Liu, H. J. Kung, and J. K. Tillotson.** 1992. Marek's disease virus encodes a basic leucine zipper gene resembling the *fos/jun* oncogene that is highly expressed in lymphoblastoid tumors. Proc. Natl. Acad. Sci. USA **89**:4042–4046.
18. **Kotanides, H., and N. C. Reich.** 1993. Requirement of tyrosine phosphorylation for rapid activation of a DNA binding factor by IL-4. Science **262**:1265–1267.
19. **Maray, T., M. Malkinson, and Y. Becker.** 1988. RNA transcripts of Marek's disease virus (MDV) serotype-1 in infected and transformed cells. Virus Genes **2**:49–68.
20. **Meier, J. L., R. P. Holman, K. D. Croen, J. E. Smialek, and S. E. Strauss.** 1993. Varicella-zoster virus transcription in human trigeminal ganglia. Virology **193**:193–200.
21. **Muesing, M. A., D. H. Smith, and D. J. Capon.** 1987. Regulation of mRNA accumulation by a human immunodeficiency virus *trans*-activator protein. Cell **48**:691–701.
22. **Nazerian, K., and R. L. Witter.** 1970. Cell-free transmission and in vivo replication of Marek's disease virus. J. Virol. **5**:388–397.
23. **Pratt, W. D., J. Cantello, R. W. Morgan, and K. A. Schat.** 1994. Enhanced expression of the Marek's disease virus-specific phosphoproteins after stable transfection of MSB-1 cells with the Marek's disease virus homolog of ICP4. Virology **201**:132–136.
24. **Priola, S. A., D. P. Gustafson, E. K. Wagner, and J. G. Stevens.** 1990. A major portion of the latent pseudorabies virus genome is

transcribed in trigeminal ganglia of pigs. J. Virol. **64**:4755–4760.
25. **Rock, D. L., S. L. Beam, and J. E. Mayfield.** 1987. Mapping bovine herpesvirus type 1 latency-related RNA in trigeminal ganglia of latently infected rabbits. J. Virol. **61**:3827–3831.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
27. **Schat, K. A., A. Buckmaster, and L. J. N. Ross.** 1989. Partial transcription map of Marek's disease herpesvirus in lytically infected cells and lymphoblastoid cell lines. Int. J. Cancer **44**:101–109.
28. **Silver, S., A. Tanaka, and M. Nonoyama.** 1979. Transcription of the Marek's disease virus genome in a nonproductive chicken lymphoblastoid cell line. Virology **93**:127–133.
29. **Spivack, J. G., and N. W. Fraser.** 1987. Detection of herpes simplex virus type 1 transcripts during latent infection in mice. J. Virol. **61**:3841–3847.
30. **Spivack, J. G., G. M. Woods, and N. W. Fraser.** 1991. Identification of a novel latency-specific splice donor signal within the herpes simplex virus type 1 2.0-kilobase latency-associated transcript (LAT): translation inhibition of LAT open reading frames by the intron within the 2.0-kilobase LAT. J. Virol. **65**:6800–6810.
31. **Stevens, J. G.** 1989. Human herpesviruses: a consideration of the latent state. Microbiol. Rev. **53**:318–332.
32. **Sugaya, K., G. Bradley, M. Nonoyama, and A. Tanaka.** 1990. Latent transcripts of Marek's disease virus are clustered in the short and long repeat regions. J. Virol. **64**:5773–5782.
33. **Wen, L.-T., A. Tanaka, and M. Nonoyama.** 1988. Identification of Marek's disease virus nuclear antigen in latently infected lymphoblastoid cells. J. Virol. **62**:3764–3771.
34. **Wirth, U. V., B. Vogt, and M. Schwyzer.** 1991. The three major immediate-early transcripts of bovine herpesvirus 1 arise from two divergent and spliced transcription units. J. Virol. **65**:195–205.
35. **Zwaagstra, J. C., H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 1991. Identification of a major regulatory sequence in the latency associated transcript (LAT) promoter of herpes simplex virus type 1 (HSV-1). Virology **182**:287–297.