Structure of the Marek's Disease Virus *Bam*HI-H Gene Family: Genes of Putative Importance for Tumor Induction

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The *Bam*HI H fragment of Marek's disease virus (MDV), which has been hypothesized to contain a tumor-inducing gene, was partially sequenced and analyzed for transcriptional activity. Transcription of several RNAs was found to initiate within *Bam*HI-H, with the major transcripts initiating on either side of a putative MDV origin of lytic replication. A 1.8-kilobase gene family found to be produced only by pathogenic MDV was detected, and its transcription map was produced. It is hypothesized that the 1.8-kilobase gene family is directly associated with the tumorigenic potential of MDV.

The association of herpesviruses with neoplastic diseases has long been recognized, but the mechanisms by which a specific herpesvirus induces neoplasia require further clarification. Marek's disease virus (MDV) infection of primary chicken embryo fibroblast (CEF) cells is an in vitro system that can be exploited to elucidate the relationships among herpesvirus DNA, its transcription, and the development of the disease state.

MDV is an avian herpesvirus which induces lymphoproliferative disease and demyelination of peripheral nerves in chickens. The virus-induced disease, Marek's disease, was one of the first neoplastic diseases found to be caused by a herpesvirus and subsequently became the first naturally occurring lymphomatous disorder to be effectively controlled by vaccination (28).

The viral DNA is a linear double-stranded molecule of 180 kilobase pairs (kbp) (20) whose *Bam*HI, *Sma*I, and *Bgl*II restriction endonuclease maps have been established (16). Analysis of MDV DNA by electron microscopy and by restriction enzyme digestion indicate it to be structurally similar to two human herpesviruses, herpes simplex virus type 1 (HSV-1) and HSV-2. Infection with both of these human herpesviruses results in the oncogenic transformation of rodent cells in vitro (11, 15).

Attenuated strains of MDV have been produced by repeated passage of the virus in cell culture. These strains have been shown to induce protective immunity against Marek's disease in chickens (7, 13, 26). The attenuation of MDV, seen as the loss of pathogenicity and tumorigenicity, results in an increased rate of virus replication in cell culture and alterations in the structure of the virion DNA.

Fukuchi et al. (17) demonstrated that serial in vitro passage of virulent MDV in primary CEF resulted in a loss of virion tumorigenicity. This loss of tumorigenicity strongly correlated with an expansion in two particular regions of the viral genome. Subsequent experiments comparing the structures of viral DNA from pathogenic and nonpathogenic strains of MDV revealed that a 1.5-kbp *Bgl*II-*Pst*I subfragment of *Bam*HI-D and -H present in the TR_L and IR_L, respectively (Fig. 1), exhibited size heterogeneity (expansion) in only the nonpathogenic strains (17). Other investi-

gators have reported a similar heterogeneity in this region of the DNA (33). Maotani et al. (23) reported that the heterogeneity was due to the amplification of a 132-base-pair (bp) repeat sequence found within the *Bam*HI D and H fragments. Since amplification of this region was only detected within nonpathogenic strains of MDV, the data suggest that the portion of *Bam*HI-D and -H included within TR_L and IR_L, respectively, may contain one or more genes responsible for the pathogenicity of the virus. Tumor induction studies in chickens showed that cloned virus populations which exhibit an amplification in this region of the viral DNA had decreased tumorigenic capability (17). By contrast, viruses which did not contain amplified *Bam*HI-D or -H regions efficiently induced tumors in chickens (17). These results suggest that the oncogenic potential of MDV is associated with a gene(s) within this region of the genome. We now report that this region of the MDV DNA is transcriptionally active and present a map delineating the transcripts produced.

MATERIALS AND METHODS

Cells and virus strains. The pathogenic strains of MDV used in this study were RBIB (provided by L. N. Schierman, University of Georgia, Athens) and GA clone 21. The nonpathogenic strains utilized were CVI-988 (CVI) (31) and GA clone 19. Both GA clones 21 and 19 were plaque purified from oncogenic GA MDV virus (14).

The lymphoblastoid cell line MDCC-MSB-1 (MSB-1), which can be induced to produce MDV, was provided by S. Kato (Osaka University, Osaka, Japan). This cell line was cultivated in RPMI 1640 medium with 10% newborn calf serum in an atmosphere of 5% CO₂ at 37°C. Prior to isolation of RNA from MSB-1, the cells were treated with iododeoxyuridine (IUdR) (50 µg/ml) for 48 h. The medium was then removed, and the cells were washed and incubated at 37°C for 48 h in RPMI 1640 containing 10% newborn calf serum.

Preparation of cellular DNA and cytoplasmic RNA from CEF infected with MDV. Primary CEF cell cultures prepared from specific-pathogen-free embryos (SPAFAS, Inc., Norwich, Conn.) were infected by cocultivation with MDV-infected CEF at a ratio of 10 parts of CEF to 1 part of virus-infected CEF. The cells received Eagle minimal essen-

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FIG. 1. Genomic map of MDV and detailed restriction maps of *Bam*HI-D and -H. The structure of MDV is shown along with the restriction maps of the *Bam*HI D and H fragments. The double-headed arrows above the restriction maps indicate the location of the genomic expansion, and the single-headed arrows locate the junction of the unique and repeat regions. TR, Terminal repeats; DR, direct repeats; U, unique region; IR, inverted repeats; subscripts L and S represent long and short, respectively. Open arrowheads locate 132-bp repeats. The restriction sites for *Sma*I (S), *Taq*I (T), *Acc*I (A), *Pst*I (P), and *Bam*HI (B) are shown. M.W., Molecular weight.

tial medium containing 1% newborn calf serum and were harvested when 80% of the cells showed cytopathic effect.

Total cellular DNA was extracted with pronase-sodium dodecyl sulfate followed by phenol extraction as previously described (16). Total cytoplasmic RNA was prepared by the method of Chirgwin et al. (6). Poly(A)⁺ RNA was isolated from total cytoplasmic RNA by oligo(dT)-cellulose chromatography (1).

Southern and Northern (RNA) blot hybridizations. Total infected cell DNA or *Bam*HI-cloned MDV DNA was digested with restriction enzymes following the recommendations of the manufacturer (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). DNA fragments were electrophoresed in agarose gels and then transferred to nitrocellulose paper by the method of Southern (34). Hybridizations were done under conditions described previously (16) in $6\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)– $6\times$ Denhardt solution ($1\times$ Denhardt solution is 0.05% bovine serum albumin, 0.05% Ficoll, 0.05% polyvinylpyrrolidone [10])–50% formamide at 45°C for ³²P-labeled nick-translated DNA probes (30) and at 55°C for ³²P-labeled RNA probes. Autoradiography was performed at –80°C with Cronex X-ray film and an intensifying screen.

Electrophoresis of poly(A)⁺ RNA after denaturation with 1 M glyoxal in aqueous 50% dimethyl sulfoxide was done by the method of McMaster and Carmichael (24). Chicken rRNAs of 28S and 18S were used as size markers.

Synthesis of ³²P-labeled RNA probes required cloning of the MDV *Bam*HI H fragment into pGem-1 (Promega Biotec, Madison, Wis.). The resulting plasmid, pGem-H, was linearized by digestion with *Pvu*II or *Bgl*II. Linear pGem-H DNA template (1 µg) was transcribed with 10 U of SP6 or T7 RNA polymerase in 40 mM Tris hydrochloride (pH 7.5)–6 mM

MgCl₂–10 mM NaCl–10 mM dithiothreitol–2 mM spermidine–0.5 mM each ATP, GTP, and CTP–12 µM UTP–50 µCi of [α -³²P]UTP (800 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.)–20 units of RNasin (Promega Biotec) at 37°C for 60 min.

Radiolabeled cDNA was synthesized in a 50-µl reaction mixture which contained 5 µg of poly(A)⁺ RNA, 0.01 µg of synthetic dT₁₅, 100 mM Tris hydrochloride (pH 8.3), 4 mM dithiothreitol, 10 mM MgCl₂, 140 mM KCl, 20 µg of actinomycin D per ml, 200 µM each dTTP, dGTP, and dATP, 20 µM dCTP, 80 U of avian myeloblastic leukemia virus reverse transcriptase (Seikagaku America, Inc., St. Petersburg, Fla.), and 100 µCi of [α -³²P]dCTP (3,000 Ci/mmol). The reaction mixture was heated briefly at 90°C and then transferred to 45°C for 2 h.

S1 analysis of viral mRNA. Viral poly(A)⁺ RNA from IudR-treated MSB-1 was analyzed by the method of Berk and Sharp (2). DNA restriction fragments were labeled at the 5' termini following removal of the terminal phosphate with bacterial alkaline phosphatase. The terminal phosphate was subsequently replaced with T4 polynucleotide kinase and [γ -³²P]ATP (3,000 Ci/mmol). DNA fragments were labeled at the 3' termini with the appropriate [α -³²P]deoxynucleotide triphosphate (depending on the restriction endonuclease used to generate the fragment) and the Klenow fragment of *Escherichia coli* DNA polymerase I (22). Specific DNA probes generated for S1 mapping are described in the figure legends. Approximately 50 ng (2×10^4 to 1×10^5 cpm) of the DNA probe was precipitated with 10 to 50 µg of poly(A)⁺ RNA combined with enough *E. coli* tRNA to achieve a total of 200 µg of nucleic acid. As a negative control, the probe was precipitated along with 200 µg of *E. coli* tRNA. The resulting pellets were dried and suspended in 30 µl of

hybridization buffer (40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [pH 6.4], 1 mM EDTA, 0.4 M NaCl, 80% formamide). The mixture was placed in a water bath at 80°C for 10 min and then immediately transferred to a water bath at 42 to 48°C and allowed to hybridize for 3 h. After hybridization, 0.3 ml of ice-cold S1 nuclease buffer (280 mM NaCl, 50 mM sodium acetate [pH 4.6], 4.5 mM ZnSO₄, 1,000 U of S1 nuclease per ml, 20 µg of heat-denatured salmon sperm DNA per ml) was added, mixed, transferred to a 45°C water bath, and incubated for 1 h. The reaction was terminated by the addition of 66 µl of 3.0 M ammonium acetate (pH 5.2). The sample was extracted once with phenol-chloroform and once with chloroform, and the protected fragments were precipitated with an equal volume of isopropanol. The precipitate was pelleted, dried, and then suspended in 5 to 10 µl of buffer (90% formamide, 1× TBE [pH 8.3; 50 mM Tris hydrochloride, 50 mM boric acid, 1 mM EDTA], 0.02% bromophenol blue, 0.02% xylene cyanol) and heated at 80°C for 5 min. The samples were subjected to electrophoresis through a 5% polyacrylamide-7 M urea gel for identification of S1-protected fragments.

S1 analysis was also done utilizing unlabeled (cold) DNA fragments. The procedure was as described above except the resulting S1-protected fragments were electrophoresed through neutral agarose gels, blotted to nylon filters, and then hybridized with a radiolabeled copy of the cold DNA probe.

DNA sequencing. DNA sequencing was performed on the GA clone 21 *Bam*HI H fragment from the MDV *Bam*HI library constructed by Fukuchi et al. (16). After the MDV DNA of interest was subcloned into an appropriate M13 sequencing vector (Cyclone system DNA sequencing kit; International Biotechnologies, Inc., New Haven, Conn.), the DNA sequence was determined by the dideoxy-chain termination method of Sanger et al. (32).

RESULTS

DNA sequencing and analysis of heterogeneous region. The *Eco*RI-*Bam*HI subfragment of *Bam*HI-H, having been previously identified as containing the region of DNA whose amplification is associated with loss of tumorigenicity, was sequenced (Fig. 2). Analysis of the results identified five direct repeats of 132 bp whose sequence was found to be identical to that previously reported (23). Computer analysis of the sequence with Mount and Conrad's Genetic DNA Sequencing Analysis Software (24a) (produced by Westico, Norwalk, Conn.) and PCGENE (IntelliGenetics, Mountain View, Calif.) identified several *Taq*I restriction sites throughout the sequence. Since two of the *Taq*I restriction sites were located 94 bp 5' and 54 bp 3', respectively, of the region containing the 132-bp repeats, *Taq*I digestion would generate a fragment whose size would depend on the number of repeats in this region of the viral genome. Therefore, *Taq*I digestion was utilized to determine the extent of amplification occurring within different strains of MDV and within MDV-positive lymphoblastoid cell lines.

Total cellular DNA isolated either from CEF infected with pathogenic or nonpathogenic MDV or from MDV-positive lymphoblastoid cell lines was digested with *Sma*I and *Taq*I, electrophoresed in agarose, blotted to a nylon membrane, and hybridized with the *Sma*I-*Pst*I subfragment of *Bam*HI-H (Fig. 3). The results demonstrated that the pathogenic strains of MDV and the lymphoblastoid cell lines contained only two 132-bp repeats, while the CEF infected with nonpathogenic strains of MDV produced a heterogeneous population

of virus containing from 4 to >30 repeat units. These results confirmed previous observations that the region of DNA amplified in the nonpathogenic virus is the 132-bp repeats (17, 23).

Detection of transcriptional activity within *Bam*HI-H. The transcriptional activity of the *Bam*HI-H region of the genome was examined. Poly(A)⁺ RNA (10 µg) isolated from IUDR-treated MSB-1 cells was denatured with glyoxal and dimethyl sulfoxide at 50°C, electrophoresed in a 1% agarose gel, and blotted to nylon membranes. Membrane strips containing the RNA were then hybridized to five radiolabeled subfragments of *Bam*HI-H, which together spanned the entire *Bam*HI H fragment (Fig. 4). There appeared to be no significant difference between the hybridization signals detected when RNA from CEF infected with RBIB or RNA from the lymphoblastoid cell line MSB-1 was used (data not shown).

The 1.6-kbp *Bam*HI-*Eco*RI fragment, which contains the 5' portion of *Bam*HI-H and lies within the U_{L2} region, hybridized to an RNA of 1.7 kilobases (kb) and very weakly to an RNA of 1.8 kb (Fig. 4, lane I), while the 0.8-kbp *Eco*RI-*Eco*RI fragment, which spans the U_{L2}-IR_L junction, hybridized weakly to an RNA of 3.8 kb and strongly to an RNA of 1.8 kb (Fig. 4, lane II).

When the 0.95-kbp *Eco*RI-*Sma*I fragment was used as a probe, RNAs of 3.8 and 1.8 kb were detected, with the signal from the 3.8-kb RNA being the weaker of the two (Fig. 4, lane III).

The 1.2-kbp *Sma*I-*Pst*I fragment, which contains the 132-bp repeats, was hybridized to the RNA from IUDR-treated MSB-1 and produced a smear hybridization pattern of approximately 0.4 to 10.0 kb (Fig. 4, lane IV). Northern blot hybridization with the 0.74-kbp *Pst*I-*Bam*HI fragment as a probe revealed a weak band of 3.8 kb and a strong band of a size similar to the 1.4- to 1.8-kb band detected with the *Eco*RI-*Sma*I probe (Fig. 4, lane V).

To complete the Northern hybridization studies, strand-specific RNA probes were generated and hybridized to filters containing poly(A)⁺ RNA from RBIB, MSB-1, GA clone 19, and CVI (Fig. 5). The SP6 polymerase-generated probe detected the 1.7- and 0.8-kb RNAs that had hybridized to the *Bam*HI-*Eco*RI probe (Fig. 4, lane I), indicating that these two RNAs were transcribed in a leftward direction. Both of these transcripts were produced by pathogenic (RBIB and MSB-1) and attenuated (GA clone 19 and CVI) viruses, suggesting that the attenuation process did not effect transcription of these genes. However, the T7 polymerase-generated probe, which would detect rightwardly transcribed RNAs, hybridized to RNAs of 3.8 and 1.8 kb produced by RBIB and MSB-1 and failed to hybridize to any RNAs produced by the attenuated viruses. These results suggested that these two RNAs are transcripts specifically produced only in cells infected with oncogenic MDV.

S1 nuclease mapping of transcripts within *Bam*HI-H. A series of S1 nuclease protection studies were performed to precisely locate and map the RNAs observed in the Northern blot hybridizations. The S1 nuclease studies were performed utilizing a series of labeled and nonlabeled (cold) DNA fragments and poly(A)⁺ RNA isolated from IUDR-treated MSB-1. The reasons for using the RNA from these cells to map the transcriptional activity from the *Bam*HI-H region of the MDV genome were as follows. (i) There appeared to be no significant difference between the Northern blot hybridizations of RNA from IUDR-treated MSB-1 and CEF infected with RBIB; (ii) virus produced by these cells after cocultivation with CEF is oncogenic; (iii) upon

FIG. 2. Sequence of the *EcoRI*-*BamHI* subfragment of *BamHI*-H. The sequence is shown with several restriction sites located. Two of the five identical 132-bp direct repeats are shown, with each repeat delineated by brackets.

injection into the chicken, MSB-1 cells release pathogenic virus; (iv) the MSB-1 genome does not contain the amplification of the 132-bp repeat sequences, whereas this region of RBIB undergoes amplification after only 15 to 20 passages in CEF; and (v) MSB-1, a lymphoblastoid cell line easily maintained in culture, provides a ready source for the RNA in the absence of extraneous tissue or cells.

S1 nuclease analysis was performed with the 5'-end-labeled *BamHI*-*EcoRI* subfragment of *BamHI*-H and the 3'-end-labeled *BamHI* K1 fragment (the *BamHI* fragment immediately 5' of *BamHI*-H). Protection of these end-labeled fragments revealed that the 1.7-kb RNA (detected with probe I, Fig. 4) initiates approximately 900 bp from the left end of *BamHI*-H and continues in a leftward direction 560 nucleotides into the adjacent *BamHI* K1 fragment (data not

shown) (manuscript in preparation). The 1.8-kb RNA which hybridized weakly to probe I and moderately to probe II (Fig. 4, lanes I and II) has been characterized by L. Velicer (personal communication) to code for the MDV B antigen. This RNA is a leftward transcript initiating approximately 380 nucleotides 3' of the second *EcoRI* site within *BamHI*-H and terminating approximately 1,150 nucleotides from the left end of *BamHI*-H.

Since the 1.8-kb RNA detected by probe V (Fig. 4) and the strand-specific probe generated by T7 polymerase (Fig. 5) were the major transcripts affected by the attenuation-amplification process, S1 nuclease protection assays were performed to map these transcripts. A schematic representation of the probes utilized and the protected fragments detected in the S1 nuclease protection assays is shown in

FIG. 3. Southern blot hybridization demonstrating the amplification of the 132-bp repeats. Total cell DNA from CVI- or RBIB-infected CEF, or from uninfected CEF or total cell DNA from the lymphoblastoid cell lines MSB-1 and MKT-1 was hybridized with the *SmaI-PstI* subfragment of *BamHI*-H. Arrows indicate positions of repeat-containing fragments, with the numbers above the arrows indicating the number of 132-bp repeats contained within the fragment. HET., Range of heterogeneity observed in CVI.

Fig. 6C. The 3.1-kbp *BamHI-SmaI* fragment was 5' end labeled and cut with *EcoRI*, and the resulting 0.95-kbp *EcoRI-SmaI* fragment was isolated. When the *EcoRI-SmaI* probe was utilized in the S1 nuclease protection assay, a protected fragment of 260 nucleotides was detected (Fig. 6A, probe a). The 0.26-kbp *SmaI-TaqI* fragment was then isolated, 5' end labeled, hybridized to MSB-1 poly(A)⁺ RNA, and digested with S1 nuclease. The RNA was observed to protect a fragment the total length of the probe (260 nucleotides) (Fig. 6A, probe b), suggesting that the transcript detected with the *EcoRI-SmaI* probe continued through the *TaqI* site. When the 5'-end-labeled 0.48-kbp *SmaI-AccI* fragment was used as a probe, two faint bands of 150 and 130 nucleotides were detected (Fig. 6A, probe c). The 480-nucleotide protected fragment was judged to be due to self-annealing of the 480-bp probe since the same fragment was observed when the probe was hybridized to tRNA (Fig. 6A, probe c, lane T). To complete the transcription mapping within the *SmaI-AccI* region of *BamHI*-H, we used the cold 0.48-kbp *SmaI-AccI* fragment in an S1 protection assay. The RNA protected fragments of 360, 260 and 140 nucleotides, with the 360- and 260-nucleotide bands generating stronger hybridization signals compared with the signal generated

FIG. 4. Northern blot hybridization of poly(A)⁺ RNA from IUDR-treated MSB-1. Lanes I to V were hybridized with the *BamHI* H subfragments I to V, respectively. The sizes of the RNAs detected are shown in kilobases. Chicken 28S and 18S rRNAs were used as size markers (not shown).

from the 140-nucleotide band (Fig. 6A, probe d). The 140-nucleotide band was believed to contain the 150- and 130-nucleotide fragments previously detected, but owing to the low resolution of the gel utilized, these two fragments comigrated as a single band of 140 nucleotides. Since no exon of 480 nucleotides was detected, these results reinforce the interpretation that the 480-nucleotide fragment detected with probe c was due to self-annealing.

To determine whether there existed any other exons within this region of *BamHI*-H, we used the unlabeled *EcoRI-SspI* fragment in a cold S1 nuclease protection assay. This assay verified the existence of two major exons of 620 and 520 nucleotides (Fig. 6A, probe e).

When the results from the S1 nuclease protection assays for the region within the *EcoRI-AccI* fragment were combined, two major exons sharing the same initiation site were located. The 260-nucleotide fragment, protected by the 5'-end-labeled *EcoRI-SmaI* probe, represented the 5' end of the exons, while the 360- and 260-nucleotide fragments represented the 3' ends of the exons. Owing to the weakness of the hybridization signals created by the other protected fragments and the strength of the signal generated by the 1.8-kb RNA in the Northern blots, it was concluded that the protected fragments generating the weaker signals likely represented minor species of RNAs and probably were not involved in the production of the 1.8-kb RNA.

The 0.6-kbp *AccI-PstI* fragment was isolated, 3' end labeled, and hybridized to poly(A)⁺ RNA from IUDR-treated MSB-1. S1 nuclease-protected fragments of 300 and 170 nucleotides were detected (Fig. 6B, probe f). The weakness of the signals detected suggested that these were low-abundance transcripts and probably not involved in the production of the 1.8-kb RNA. When the same *AccI-PstI* fragment was 5' end labeled, five strong signals representing

FIG. 5. Strand-specific hybridization to Northern blots of MDV RNA. Equivalent amounts of poly(A)⁺ RNA produced by pathogenic (RBIB and MSB-1) or attenuated (GA clone 19 and CVI) strains of MDV were hybridized with SP6- or T7 polymerase-generated RNA probes. Numbers at the left represent sizes of the hybridizing RNAs (in kilobases). Chicken 28S and 18S rRNAs were used as size markers.

protected fragments of 420, 350, 220, 180, and 100 nucleotides were detected (Fig. 6B, probe g).

The adjoining 0.79-kbp *PstI*-*BamHI* fragment was 3' end labeled and utilized in an S1 nuclease protection assay. MSB-1 poly(A)⁺ RNA was observed to protect fragments of 400 and 370 nucleotides (Fig. 6B, probe h).

To determine whether transcriptional activity was present at the right end of *BamHI*-H, the 1.38-kbp *AccI*-*BamHI* fragment was 5' end labeled, hybridized to MSB-1 poly(A)⁺ RNA, and subsequently digested with S1 nuclease. The RNA was observed to protect a single fragment of 150 nucleotides (Fig. 6B, probe i). This transcript was found to continue approximately 900 nucleotides into the adjacent *BamHI* I2 fragment when the 3'-end-labeled *BamHI* I2 fragment was used in the S1 nuclease protection assay (data not shown) (G. Bradley, A. Tanaka, and M. Nonoyama, manuscript in preparation).

To complete the S1 mapping of the *AccI*-*BamHI* region of the *BamHI* H fragment, we used the unlabeled 1.38-kbp *AccI*-*BamHI* subfragment to aid in the identification of all the exons lying within its sequences. Nine bands of 1,380, 820, 720, 610, 530, 450, 300, 170, and 150 nucleotides were detected upon hybridization (Fig. 6B, probe j). The 1,380-nucleotide band represented self-annealing of the 1,380-bp probe since the same band was produced upon hybridization of this probe to tRNA (Fig. 6B, probe j, lane T). The remaining eight bands accounted for all the previously protected fragments detected and residing within the *AccI*-*BamHI* region. Combining the S1 studies on transcription from this region of *BamHI*-H, six major exons of 820, 720,

610, 530, 450, and 150 nucleotides were found. The 150-nucleotide protected fragment (Fig. 6B, probes i and j) was found to belong to an exon stretching into the adjoining *BamHI* I2 fragment (data not shown) (manuscript in preparation). The remaining five major protected fragments, which were found to share the same 3' end, are alternative exons of the 1.8-kb RNA since addition of the lengths of these exons with the lengths of the two exons detected within the *EcoRI*-*AccI* region accounted for the size of the bands (1.8 to 1.4 kb) detected upon hybridization with probes III to V (Fig. 4) or the T7 polymerase-generated strand-specific probe (Fig. 5). Also, these RNAs were found to terminate just downstream from a polyadenylation signal AATAAA (nucleotide 2439) within a region containing G+T-rich transcription termination signals. Therefore, the rightwardly transcribed 1.8-kb RNA was found to be a family of RNAs that shared overlapping exons.

DISCUSSION

Previous studies implicate the amplification of regions contained in *IR_L* and *TR_L* within the *BamHI* H and D fragments, respectively, as a critical event associated with the loss of viral pathogenicity (14, 17, 26). These previous reports all anticipated the existence of a gene (or genes) within this region that was responsible for initiation or maintenance of the tumorigenic state.

In an attempt to gain insight into the mechanism(s) by which amplification within *BamHI*-H results in attenuation of the virus, we sequenced and then studied the transcriptional activity of the *BamHI*-H region. The sequencing data of the heterogeneous region of *BamHI*-H (the *EcoRI*-*BamHI* fragment) confirmed the presence of the 132-bp repeats previously reported (23) and confirmed that this repeat sequence was responsible for the observed genomic expansion.

The transcriptional activity was examined to relate the effect of the DNA expansion with loss of MDV oncogenic activity. Several RNAs detected by the Northern blot hybridization were found to originate within the *BamHI* H region. A leftward transcript of 1.7 kb initiates within the *U_{L2}* region of *BamHI*-H (Fig. 7) and continues into the adjoining *BamHI* K1 fragment (data not shown). A second leftwardly transcribed RNA of 1.8 kb initiates within *IR_L* approximately 380 nucleotides 3' of the second *EcoRI* site and within a region 30 to 40 nucleotides downstream of the TATA sequence TATAA. This 1.8-kb message continues into the *U_L* sequences and terminates approximately 450 nucleotides 5' of the first *EcoRI* site within *BamHI*-H (Fig. 7). This transcript is the message for the MDV B antigen previously described by L. Velicer (personal communication).

The 132-bp repeat region was found to be a region of high transcriptional activity producing a diffuse signal upon hybridization with the 132-bp sequence or the 1.2-kbp *SmaI*-*PstI* fragment. The large and diverse size of this signal and the absence of smear hybridization when probes flanking this region were used suggest the presence of an exon that is common to many viral transcripts. This exon, it is hypothesized, would be present in one or both of the 132-bp repeats. Two sequences were found, CCCTTGCTAG, 46 bp into each repeat, and ATGTGAGT, 75 bp into each repeat, that are strongly homologous to the splice acceptor [Py₆NCAGN] and splice donor (N/GTAAGT) sequences, respectively (5, 25). If these sites are utilized, an exon of 31 bp would be produced. Since this exon would not contain the

FIG. 6. S1 nuclease fine mapping of the pathogenicity-associated 1.8-kb RNA. (A and B) Results of the S1 nuclease protection assays. The sizes (in nucleotides) of the protected fragments were calculated with *Hae*III-digested ϕ X175 DNA as a marker (not shown). Lanes: M, MSB-1 poly(A)⁺ RNA; T, tRNA. The locations of the probes (a to j) are shown in panel C. (C) Schematic of the *Bam*HI H fragment, with the location of probes (a to j) and the fragments each probe protected. Solid lines represent probes; an asterisk (*) on the right of the probe represents 5' end labeling; an asterisk (*) on the left of the probe represents 3' end labeling; no asterisk represents a nonlabeled probe used for cold S1 analysis. Restriction enzyme sites are shown: B, *Bam*HI; P, *Pst*I; E, *Eco*RI; Sm, *Sma*I; T, *Taq*I; S, *Ssp*I; and A, *Acc*I. The solid boxes represent protected fragments which generated strong signals; open boxes represent protected fragments which generated weak signals. Numbers show the sizes of the protected fragments (in nucleotides).

cluded within the 1.8-kb RNA family. This question will be resolved by analyzing cDNAs from this region. Splicing of small exons linked to distant sequences in the MDV genome is similar to what is observed within the *Bam*HI W repeats of the Epstein-Barr virus (4, 27).

Strand-specific hybridization studies revealed significant differences in the transcriptional patterns of the pathogenic and attenuated viruses. An RNA of 3.8 kb and an abundant RNA of 1.8 kb were produced by the pathogenic viruses but not by the attenuated viruses. This clearly indicated that the attenuation-amplification process altered the transcriptional activity of this region.

Since the 1.8-kb RNA was found to be the most abundant transcript to be affected by the attenuation-amplification

*Acc*I restriction site within the repeat, the S1 nuclease protection assays that were performed would have and indeed did fail to identify it.

At this time, it cannot be concluded whether any of the RNAs generating the smear hybridization signals are in-

FIG. 7. Transcriptional map of *BamHI*-H. The results of the Northern blot hybridization and S1 nuclease protection assays were combined to produce the transcriptional map. The 1.8-kb gene family promoter region has been expanded to show the location of sequences homologous with known transcription-enhancing elements. The dark circles (●) locate these enhancing elements. The crosshairs (⊕) locate the A+T-rich putative MDV origin of lytic replication. The following restriction sites are shown: A, *AccI*; B, *BamHI*; E, *EcoRI*; P, *PstI*; S, *SmaI*; and T, *TaqI*.

process, we focused the transcriptional mapping studies on this RNA. However, the transcription of the less abundant 3.8-kb RNA is also under investigation. The 1.8-kb RNA was found actually to be a family of spliced RNAs of 1.4 to 1.8 kb that are transcribed from within the IR_L region of *BamHI*-H. Collectively, the S1 protection assays, Northern blot hybridizations, and nucleotide sequence indicate that the majority of the transcripts involved in the production of this 1.8-kb gene family shared a 5' initiation site and a 3' termination site (Fig. 7). Exon 1 continued to a locus 260 bp 3' of the *SmaI* site in a region containing a donor splice sequence, AA/GTATGT, or to a locus 380 bp 3' of the *SmaI* site containing the donor splice sequence CT/GTAACT. Therefore, two different exons arose from the same initiation site.

The family of 3' coterminal exons composing exon 2 each contained a sequence strongly homologous to the splice acceptor consensus sequence [Py₍₆₎NCAG] at their 5' ends. The 3' ends contained a sequence homologous to the polyadenylation signal, AATAAA (nucleotide 2439), and were terminated just upstream of a G+T-rich sequence (nucleotides 2529 and 2564) analogous to the transcription termination signal (3, 8). Calculation of the sizes of RNAs that could occur upon combination of the two possible lengths of exon 1 with the five possible lengths of exon 2 was able to account for the different-sized RNAs observed in the Northern blots.

The initiation site for this family of RNAs was located 30 to 40 nucleotides downstream of the sequence TATAAAA (nucleotide 654). Analysis of the sequence surrounding this TATA region revealed the presence of two CCAAT sequences (nucleotides 499 and 557) (18), two Sp1 (GGGCGG and CCGCCC) sequences (nucleotides 595 and 635) (12), an

immunoglobulin enhancer core sequence, ATGCAAATG, at nucleotide 456 (36), and an A+T-rich palindromic sequence (nucleotide 506) containing two 10-bp direct repeats (TATATTATAT) capable of forming a loop-stem structure. This promoter structure is similar to those found in the immediate-early genes of the simian virus 40 large T antigen, adenovirus type 2 E1A, HSV IE 175, and the cytomegalovirus IE 68 (19). This group of sequences was found within 200 bp 5' of the TATA sequence (Fig. 7).

Upon further analysis of the nucleotide sequence of this promoter region, a strong consensus sequence homologous to the HSV-1 and Varicella-zoster virus origins of replication was found (Fig. 8) (9, 21, 35). Preliminary data from our laboratory suggest that this region of the *BamHI* H fragment of MDV functions as an origin of DNA replication during lytic infection.

The 1.8-kb family of transcripts contains only one small open reading frame, which may encode a protein of 63 amino acids. Analysis of the sequence for homology to known proteins in the IntelliGenetics protein library database revealed the open reading frame to exhibit strongest homology to the retrovirus-related gag polyprotein (37.5% identity in a 24-amino-acid overlap) and a kinase-related transforming protein (fps-avian) (41.2% identity in a 17-amino-acid overlap). Analysis of the region exhibiting homology to the transforming protein kinase and comparison of this protein sequence with the conserved sequences and catalytic domains of several protein kinases revealed this region to reside within the highly conserved catalytic region, IX (29). Whether this observed homology with the transforming protein kinase is biologically significant and relates to the

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MDV: ACGCGTCAGCGTTTCGCACTGCGAACCAATAATATATTATATTATATTATATTGGACTCTGGTGCGAA
      *          *          *          *          *          *          *          *          *
HSV-1: AACGCGAAGCGTTTCGCACTTCGTCCTCAATA-TATA-----TATATTATTAGGCGAAGTGCGAG
      *          *          *          *          *          *          *          *          *
VZV:   CGTTTCGCACTTC          ATA-TATA          TATAT

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FIG. 8. MDV putative origin of lytic replication (Ori_{lyt}). The homology of the putative MDV Ori_{lyt} with the Ori-s from HSV-1 and varicella-zoster virus (VZV) is shown. *, Homologous regions.

pathogenicity and oncogenicity of MDV is unknown at this time.

The mechanism(s) by which amplification of the 132-bp repeat sequences interferes with the pathogenicity of MDV is still unknown. However, the differential transcriptional activity detected between the pathogenic and attenuated strains of MDV may be an integral part of the mechanism. Recent experiments in our laboratory have demonstrated that premature transcription termination leads to the disappearance of the 1.8-kb family of transcripts within *Bam*HI-H (manuscript in preparation). Therefore, the biological activity of the 1.8-kb gene family and its association with the pathogenicity-oncogenicity of MDV are areas requiring further attention.

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LITERATURE CITED

- Aviv, H., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA* **69**:1408-1412.
- Berk, A. J., and P. A. Sharp. 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease digested hybrids. *Cell* **12**:721-732.
- Birnstiel, M. L., M. Busslinger, and K. Strub. 1985. Transcription termination and 3' processing: the end is in site! *Cell* **41**:349-359.
- Bodescot, M., and M. Perricaudet. 1986. Epstein-Barr virus mRNAs produced by alternative splicing. *Nucleic Acids Res.* **14**:7103-7114.
- Breathnach, R., and P. Chambon. 1981. Organization and expression of eucaryotic split genes coding for proteins. *Annu. Rev. Biochem.* **50**:349-383.
- Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294-5299.
- Churchill, A. E., R. C. Chubb, and W. Baxendale. 1969. The activation, with loss of antigenicity of the herpes-type virus of Marek's disease (strain HPRS-16) on passage in cell culture. *J. Gen. Virol.* **4**:557-564.
- Connelly, S., and J. L. Manley. 1988. A functional mRNA polyadenylation signal is required for transcription termination by RNA polymerase II. *Genes Dev.* **2**:440-452.
- Deb, S., and M. Doelberg. 1988. A 67-base-pair segment from the Ori-S region of herpes simplex virus type I encodes origin function. *J. Virol.* **62**:2516-2519.
- Denhardt, D. 1966. A membrane filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* **23**:641-652.
- Duff, R., and F. J. Rapp. 1973. Oncogenic transformation of hamster embryo cells after exposure to inactivated herpes simplex virus type I. *J. Virol.* **12**:209-217.
- Dynan, W. S., and R. Tjian. 1983. Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. *Cell* **32**:669-680.
- Edison, C. S., and D. P. Anderson. 1971. Immunization against Marek's disease. *Avian Dis.* **15**:68-81.
- Edison, C. S., and S. C. Schmittle. 1968. Studies on acute Marek's disease. I. Characteristics of isolate GA in chickens. *Avian Dis.* **12**:467-476.
- Frenkel, N., B. Roizman, E. Cassai, and A. J. Nahmias. 1972. DNA fragment of herpes simplex virus type 2 and its transcription in human cervical cancer tissue. *Proc. Natl. Acad. Sci. USA* **69**:3780-3784.
- Fukuchi, K., K. Sudo, Y. Lee, A. Tanaka, and M. Nonoyama. 1985. The structure of Marek's disease virus DNA: detailed restriction enzyme map. *J. Virol.* **51**:102-109.
- Fukuchi, K., A. Tanaka, L. W. Schierman, R. L. Witter, and M. Nonoyama. 1985. The structure of Marek's disease virus DNA: the presence of unique expansion in nonpathogenic viral DNA. *Proc. Natl. Acad. Sci. USA* **82**:751-754.
- Graves, B. J., P. F. Johnson, and S. L. McKnight. 1986. Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. *Cell* **44**:565-576.
- Hayward, G. S. 1986. Herpesviruses. I. Genome structure and regulation, p. 59-77. In M. Botchan, T. Grodzicker, and P. Sharp (ed.), *DNA tumor viruses—control of gene expression and replication*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Lee, L. F., E. D. Kieff, S. L. Bachenheimer, B. Roizman, P. G. Spear, B. R. Burmester, and K. Nazarian. 1971. Size and composition of Marek's disease virus deoxyribonucleic acid. *J. Virol.* **7**:289-294.
- Lockshon, D., and D. A. Galloway. 1986. Cloning and characterization of *ori*_{L2}, a large palindromic DNA replication origin of herpes simplex virus type 2. *J. Virol.* **58**:513-521.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Maotani, K., A. Kanamori, K. Ikuta, S. Ueda, S. Kato, and K. Hirai. 1986. Amplification of a tandem direct repeat within inverted repeats of Marek's disease virus DNA during serial in vitro passage. *J. Virol.* **58**:657-659.
- McMaster, G. K., and G. G. Carmichael. 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA* **74**:4835-4838.
- Mount, D. W., and B. Conrad. 1984. Microcomputer programs for back translation of protein to DNA sequences and analysis of ambiguous DNA sequences. *Nucleic Acids Res.* **12**:819-823.
- Mount, S. M. 1982. A catalogue of splice junction sequences. *Nucleic Acids Res.* **10**:459-472.
- Nazarian, K. 1970. Attenuation of Marek's disease virus and study of its properties in two different cell cultures. *J. Natl. Cancer Inst.* **44**:1257-1267.
- Nonoyama, M. 1982. The molecular biology of Marek's disease herpesvirus, p. 333-346. In B. Roizman (ed.), *The herpesviruses*. Plenum Publishing Corp., New York.
- Okazaki, W., W. G. Purchase, and B. R. Burmester. 1970. Protection against Marek's disease by vaccination with a herpesvirus of turkeys (HVT). *Avian Dis.* **14**:413-429.
- Quinn, A. M., and T. Hunter. 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**:42-52.
- Rigby, P. W. J., M. Dieckman, C. Rhodes, and P. Berg. 1977. Labelling deoxyribonucleic acid to high specific activity by nick translation with DNA polymerase. *J. Mol. Biol.* **113**:237-251.
- Rispe, B., H. van Vloten, N. Mastenbroek, and K. A. Schat. 1972. Control of Marek's disease in the Netherlands. II. Field trials on vaccination with an avirulent strain (CVI 988) of Marek's disease. *Avian Dis.* **16**:108-125.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain termination inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.
- Silva, R. F., and R. L. Witter. 1985. Genomic expression of Marek's disease virus DNA is associated with serial in vitro passage. *J. Virol.* **54**:690-696.
- Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **113**:237-251.
- Stow, N. D., and A. J. Davison. 1986. Identification of a varicella-zoster origin of DNA replication and its activation by herpes simplex virus type 1 gene products. *J. Gen. Virol.* **67**:1613-1623.
- Wirth, T., L. Staudt, and D. Baltimore. 1987. An octamer oligonucleotide upstream of a TATA motif is sufficient for lymphoid-specific promoter activity. *Nature (London)* **329**:174-178.