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Isolation and Characterization of cDNAs from *Bam*HI-H Gene Family RNAs Associated with the Tumorigenicity of Marek's Disease Virus

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It has been reported that loss of the tumorigenic potential of attenuated Marek's disease virus (MDV) is strongly associated with amplification of the 132-bp repeat sequences found within the *Bam*HI-D and *Bam*HI-H fragments contained within the long terminal repeat and the long internal repeat, respectively. The expansion of this region results in loss of transcripts that are 3.8, 3.0, and 1.8 kbp long that are produced by tumorigenic strains of MDV. This evidence suggests that production of one or more of these three RNAs is strongly associated with the tumorigenic potential of the virus. In this study, we have cloned and sequenced 1.69-, 1.5-, 1.9-, and 2.2-kbp cDNAs from the *Bam*HI-H gene family RNAs associated with tumorigenicity. The 1.69- and 2.2-kbp cDNAs are derived from nonspliced transcripts, whereas the 1.5- and 1.9-kbp cDNAs are from single spliced mRNAs spanning the *Bam*HI-H and *Bam*HI-I2 fragments of MDV DNA. Sequence analysis has shown two potential open reading frames in each of the cDNAs. The putative 63-amino-acid protein encoded by the first open reading frame in the 1.69-kbp cDNA and a putative 75-amino-acid protein encoded by the first open reading frame in the 1.5-kbp cDNA showed limited homology with the mouse T-cell lymphoma oncogene and the *fes/fps* family of kinase-related transforming proteins.

Marek's disease virus (MDV) is an oncogenic avian herpesvirus which induces lymphoma in its natural host, the chicken. It has been demonstrated that serial passage of virulent MDV in primary chicken embryo fibroblasts (CEF) in vitro results in viral attenuation and loss of tumorigenicity (6, 19). The loss of viral tumorigenicity strongly correlates with amplification of 132-bp direct repeat sequences located within the homologous *Bam*HI-D and -H fragments contained within the long terminal repeat and long internal repeat, respectively (6, 14, 19). The ability of the virus to induce tumors in infected chickens is severely compromised if its DNA has undergone amplification of the 132-bp repeats.

Analyses of viral transcription in lymphoma tissue obtained from infected chickens or from MDV-induced lymphoma cell lines have revealed transcriptional activity which is limited to approximately 20% of the viral genome (15, 18, 20, 21). Transcriptional activity in the transformed lymphoid cells is detected within the long and short terminal repeats and the long and short internal repeats, while little activity is detected within either the long unique region or the short unique region (21). Recent studies have demonstrated the presence of a *Bam*HI-H gene family producing 5′ coterminal transcripts of 1.8, 3.0, and 3.8 kb from the *Bam*HI-D and -H regions that strongly correlated with the tumorigenicity of the virus (1, 2). These RNAs could be detected in primary CEF infected with oncogenic strains of MDV, tissue from MDV-induced lymphoma, and lymphoma-derived cell lines (2, 21). It has been reported that the *Bam*HI-H 1.8-kbp gene family is produced only by oncogenic MDV, and its transcription map was proposed according to Northern (RNA) blot and S1 mapping analyses (1). However, it will be essential to isolate and characterize the cDNAs derived from these MDV transcripts to determine their precise locations in the MDV genome. Availability of these cDNA clones can be used to identify the biological roles of these oncogenic virus-specific RNAs in tumor induction by MDV. We report here the isolation and characterization of cDNAs derived from the *Bam*HI-H gene family RNAs associated with tumorigenicity of MDV.

MATERIALS AND METHODS

Cells and viruses. The oncogenic strain of MDV, RBIB, was provided by L. N. Schierman (University of Georgia, Athens). The attenuated strain of MDV is a high-passage-number clone of RBIB developed by serial passage in CEF to passage 64. The preparation, propagation, and infection of primary CEF with oncogenic and attenuated MDV were performed as previously described (1).

Isolation of RNA and construction of cDNA libraries. Total cellular RNA was isolated from RBIB-infected CEF according to the method described by Chomczynski and Sacchi (4). Poly(A)$^+$ RNA was isolated from total cellular RNA by use of the streptavidin–biotin–magnetic-bead technique provided in the Promega PolyAtract kit (Madison, Wis.) according to the manufacturer's instruction. Construction of cDNA libraries from poly(A)$^+$ RNA utilized the cDNA synthesis kits obtained from Pharmacia (Piscataway, N.J.) and Promega. The resultant cDNAs were ligated into *Eco*RI-digested, dephosphorylated lambda gt10 vector DNA (Stratagene, La Jolla, Calif.) or *Eco*RI-*Xba*I-digested lambda GEM4 vector DNA (Promega).

Isolation of oncogenic virus-specific cDNAs and cDNA sequencing. Virus-specific cDNAs from cDNA libraries were screened by the standard plaque assay (7). Briefly, each

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library was plated at 10,000 PFU per plate with *Escherichia coli* C600 (*hfl* mutant) (Pharmacia) or LE393 (Promega). After being transferred to filters, the plaques were hybridized with appropriate ^{32}P-labeled DNA probes synthesized by the method of Feinberg and Vogelstein (5). Each positive plaque isolated was subject to rescreening at 1,000, 100, and fewer than 10 PFU per plate. Repeated isolation of single plaques giving positive signals yielded pure virus-specific cDNA clones. The cloned cDNA was subcloned into the pBluescript KS$^+$ plasmid vector (Stratagene), which was used to transform *E. coli* DH5α (F'). cDNAs cloned into the lambda GEM4 vector were rescued by cutting the phage DNA with *Spe*I and adding T4 DNA ligase to assemble the pGEM4-cDNA plasmid construct and then used to transform *E. coli* DH5α (F'). Double-stranded DNA sequencing of the cloned cDNA was carried out by the Sanger dideoxy-chain termination method (17) using the Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, Ohio). Both strands of the cDNA were sequenced.

PCR. Oligonucleotide primers were synthesized with the Applied Biosystems 380B DNA synthesizer (Foster City, Calif.). The polymerase chain reaction (PCR) was performed with a GeneAmp Kit (Perkin Elmer Cetus, Norwalk, Conn.) and a Perkin Elmer Cetus DNA Thermal Cycler (Cetus Corp., Emeryville, Calif.).

Northern blot hybridization. Electrophoresis of poly(A)$^+$ RNA after denaturation with 1 M glyoxal in aqueous 50% dimethyl sulfoxide was performed as described by McMaster and Carmichael (16). An RNA ladder from 0.24 to 9.5 kb (GIBCO-Life Technologies, Inc., Grand Island, N.Y.) was used as the size marker. Hybridization was performed as previously described (1).

Computer analysis of cDNA sequence. Analyses of the cDNA and amino acid sequences of the polypeptides encoded by the potential open reading frames (ORF) contained in the cDNAs were performed by using the IntelliGenetics Suite release 5.4 and PC/Gene Software release 6.01 (Intelligenetics, Inc., Mountain View, Calif.).

Nucleotide sequence accession number. The sequences of the 1.69-, 1.5-, 1.9-, and 2.2-kbp cDNAs were submitted to GenBank and have been assigned accession numbers M62573, M77342, M77343, and L01618, respectively.

RESULTS

Isolation of cDNAs derived from oncogenic virus-specific *Bam*HI-H gene family transcripts. Several cDNAs ranging in size from 1.5 to 2.2 kbp were isolated when the cDNA library made from CEF infected with oncogenic MDV RBIB was screened with the 2.9-kbp *Eco*RI-*Bam*HI subfragment of the *Bam*HI-H DNA fragment and the 1.0-kbp *Sst*II-*Xba*I subfragment of *Bam*HI-I2 DNA fragment. On the basis of the sequences of the 5' and 3'-end sequences of each clone and their sizes and locations on the *Bam*HI-H and *Bam*HI-I2 fragments of the MDV genomic DNA, three classes of cDNA were identified (Fig. 1). For complete sequencing, cDNAs 1 (class I, 1.69 kbp), 4 (class I, 1.5 kbp), 3 (class II, 1.9 kbp), and 6 (class III, 2.2 kbp) were chosen as representatives of the classes of cDNAs (Fig. 2). The sequences of these representative cDNAs showed that the cDNAs were derived from rightward-transcribed mRNAs of the *Bam*HI-H gene family (Fig. 1 and 2).

The 1.69-kbp cDNA was derived from a nonspliced mRNA containing two copies of the 132-bp repeat sequence. The initiation site of the 1.69-kbp cDNA was at nucleotide 741 of the previously reported *Bam*HI-H sequence (1), a position 87 nucleotides downstream from the TATAAA sequence. The 3' end of the 1.69-kbp cDNA was located at nucleotide 2437 of the *Bam*HI-H sequence (1), a position 2 nucleotides downstream from a potential polyadenylation signal sequence (AATAAA) (1). The 1.5-kbp cDNA was derived from a spliced transcript sharing the same initiation site with the transcript represented by the 1.69-kbp cDNA but terminating at nucleotide 1449 of the *Bam*HI-I2 fragment (unpublished *Bam*HI-I2 fragment sequence). This spliced transcript utilized a splice donor, GTATGG, beginning at nucleotide 988 of the previously reported *Bam*HI-H fragment (GenBank accession number M26392) and a splice acceptor (ATCGTTGCAG) located 226 bp into the *Bam*HI-*Sst*II subfragment of the *Bam*HI-I2 fragment (see Fig. 4, unpublished I2 sequence).

The 1.9-kbp cDNA of class II was derived from another spliced transcript. The 5' end of this cDNA was located 113 bp downstream from the 5' end of the first 132-bp repeat. Analysis of the cDNA sequence showed that this spliced transcript utilized a splice donor, GTATGC, beginning at position 2088 of the *Bam*HI-H fragment and the same splice acceptor site utilized by the spliced RNA from which the class I 1.5-kbp cDNA was derived (see Fig. 4). This 1.9-kbp cDNA also had the same termination site as the 1.5-kbp cDNA. The 1.9-kbp cDNA is thought to be a partial cDNA clone of the 3.0-kbp transcript for the following reasons. (i) The 3.0-kbp transcript could be detected by hybridization with the PCR-generated DNA probe representing the 5' end of the *Bam*HI-H gene family (Fig. 3, lane 1) and the *Sst*II-*Xba*I DNA subfragment (Fig. 3, lane 3) of the *Bam*HI-I2 fragment but not with the PCR-generated probe which detected only the 3.8-kbp transcript (Fig. 3, lane 3). (ii) If 1.9-kbp cDNA is a full-length cDNA, the *Sst*II-*Xba*I DNA subfragment of the *Bam*HI-I2 fragment used as probe should detect the 2.0- to 2.1-kbp transcripts upon Northern blot hybridization of poly(A)$^+$ RNA of CEF infected with oncogenic MDV. However, no 2.0- to 2.1-kbp transcripts were detected in Northern blot hybridization of poly(A)$^+$ RNA isolated from oncogenic-MDV-infected CEF with the *Sst*II-*Xba*I DNA subfragment of the *Bam*HI-I2 fragment as probe (Fig. 3, lane 5). (iii) Extension of the 1.9-kbp cDNA linearly from the 5' end of this cDNA to the transcription start site of the *Bam*HI-H gene family makes a 2.72-kbp cDNA, which is close to the expected size of the full-length cDNA derived from the 3.0-kbp transcript.

Class III cDNAs are represented by the 2.2-kbp cDNA which was derived from a nonspliced mRNA joining the *Bam*HI-H and *Bam*HI-I2 fragments of MDV DNA. The 5' end of this cDNA is located at nucleotide 2161 of the previously reported *Bam*HI-H sequence (1), and it shares its 3' termination site with the class I 1.5-kbp cDNA and the class II 1.9-kbp cDNA. The 2.2-kbp cDNA (class III, cDNA 6) is likely a partial cDNA clone of the 3.8-kbp transcripts because (i) when aligned with the MDV genomic sequence, no promoter-like sequence within the appropriate context was found upstream of the 5' end of this cDNA; and (ii) a PCR-generated probe prepared from nucleotides 736 to 957 of this 2.2-kbp cDNA sequence, which represented the first 221 bp of the *Bam*HI-I2 fragment (unpublished *Bam*HI-I2 sequence), detected only a 3.8-kbp band upon Northern blot hybridization of poly(A)$^+$ RNA isolated from CEF infected with oncogenic MDV (Fig. 3, lane 3).

Northern blot analysis of oncogenic virus-specific transcripts. To confirm the origins of the cDNA clones, we used PCR-generated DNA fragments prepared from these cDNAs and cloned subfragments of MDV genomic DNA as probes to hybridize with poly(A)$^+$ RNA isolated from CEF infected with oncogenic RBIB or attenuated RBIB, respectively. A

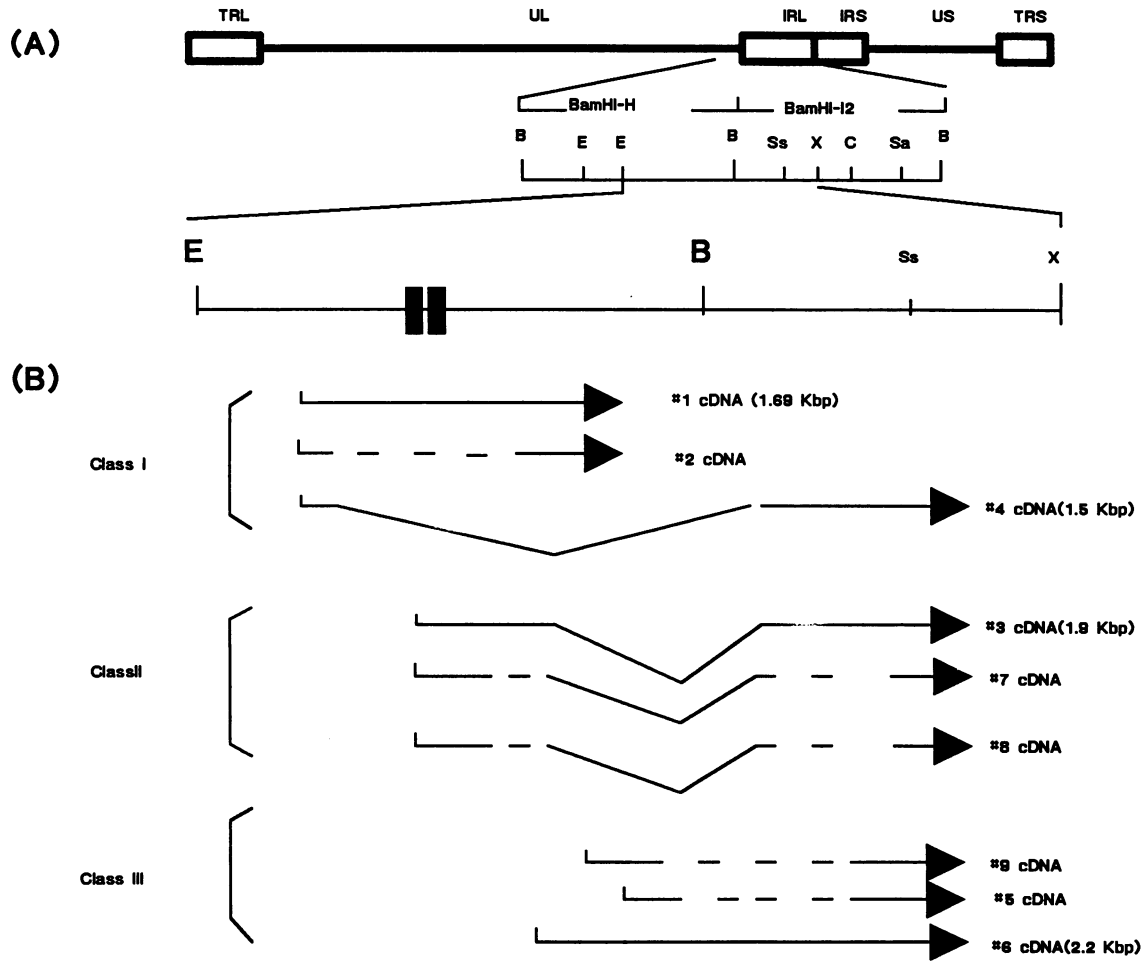

FIG. 1. Genomic structure of MDV and locations of the three classes of oncogenic virus-specific cDNAs from the *Bam*HI-H gene family. (A) Genomic structure of MDV with restriction map of the *Bam*HI-H and *Bam*HI-I2 fragments and the region where the oncogenic virus-specific cDNAs are located. Two vertical bars indicate the 132-bp direct repeats. (B) Three classes of oncogenic virus-specific cDNAs are shown, with curves indicating introns and dashed lines representating unfinished sequences. TRL, long terminal repeat; UL, long unique region; IRL, long internal repeat; IRS, short internal repeat; US, short unique region; TRS, short terminal repeat. The restriction sites shown are *Bam*HI (B), *Cla*I (C), *Eco*RI (E), *Sal*I (Sa), *Sst*II (Ss), and *Xba*I (X).

PCR-generated probe prepared from the 1.69-kbp cDNA and located at the 5′ end of the *Bam*HI-H gene family (nucleotides 611 to 858) (2) hybridized with the 3.8-, 3.0-, and 1.8-kbp transcripts produced by oncogenic RBIB in CEF (Fig. 3, lane 1). On the other hand, this probe did not detect those 3.8-, 3.0-, and 1.8-kbp transcripts, which could be detected in poly(A)$^+$ RNAs isolated from CEF infected with oncogenic MDV, in Northern blot hybridization of poly(A)$^+$ RNAs isolated from CEF infected with attenuated MDV.

A PCR-generated probe derived from the 2.2-kbp cDNA and representing the first 220 bp of the *Bam*HI-I2 fragment was found to hybridize specifically to the 3.8-kbp transcripts but not to 3.0- and 1.8-kbp transcripts (Fig. 3, lane 3). This indicated that this DNA probe was specific for the 3.8-kbp RNA and could be used to screen for cDNAs representative of the 3.8-kbp RNA. No 3.8-kbp RNA was detected by this probe when it was hybridized to RNAs from CEF infected with attenuated RBIB (Fig. 3, lane 4).

To confirm the locations of the 3′ termination sites of the *Bam*HI-H gene family transcripts spanning the *Bam*HI-H and *Bam*HI-I2 region, subfragments of *Bam*HI-I2 were used as probes in Northern blot analyses of poly(A)$^+$ RNA isolated from CEF infected by oncogenic or attenuated MDV. The *Sst*II-*Xba*I subfragments detected the 3.8- and 3.0-kbp transcripts from RNAs isolated from CEF infected by oncogenic RBIB (Fig. 3, lane 5), but not from RNAs isolated from CEF infected with attenuated RBIB (Fig. 3, lane 6). The 1.7-kbp transcripts were detected in Northern blots containing poly(A)$^+$ RNAs isolated from CEF infected with oncogenic or attenuated MDV. Since probe I failed to detect the 1.7-kbp transcript from poly(A)$^+$ RNAs isolated from CEF infected with attenuated MDV (Fig. 3, lane 2), the 1.7-kbp transcripts detected in CEF infected with attenuated MDV is different from the oncogenic virus-specific 1.7-kbp transcript of the *Bam*HI-H gene family represented by class I 1.5-kbp cDNA (Fig. 1, cDNA 4). In contrast, the *Xba*I-*Cla*I subfragment of *Bam*HI-I2 failed to detect these transcripts (Fig. 3, lane 7 and 8). Taken together, the results from cDNA sequencing and Northern blot hybridization suggest that the *Sst*II-*Xba*I subfragment contains the 3′ end of those rightward transcripts of the *Bam*HI-H gene family spanning the *Bam*HI-H and *Bam*HI-I2 regions.

Computer analysis of putative polypeptides encoded by ORFs within the oncogenic virus-specific cDNAs. Computer-

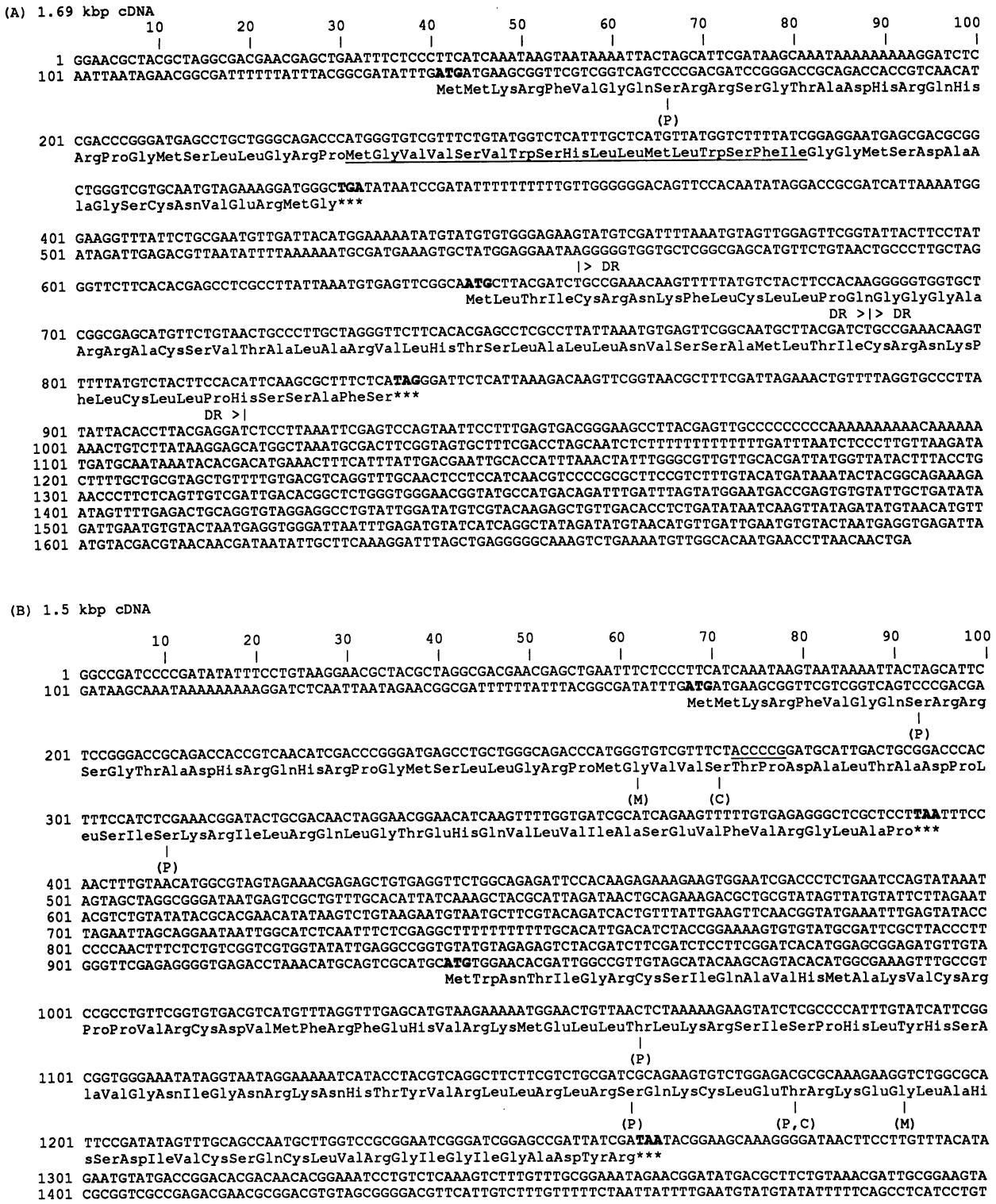

FIG. 2. Sequences of oncogenic virus-specific 1.69-kbp cDNA (A), 1.5-kbp cDNA (B), 1.9-kbp cDNA (C), and 2.2-kbp cDNA (D). Underlining below nucleotide sequences marks the first 6 nucleotides of the second exon of the 1.5- and 1.9-kbp cDNAs. Boldface letters indicate start and stop codons of the ORFs. Amino acid sequences of these ORFs are indicated with the three-letter code. Underlining of the ORF-A amino acid sequence marks the transmembrane domain predicted by computer analysis. (P), potential protein kinase C phosphorylation site; (C), potential casein kinase II phosphorylation site; (M), myristylation site; DR, direct repeat.

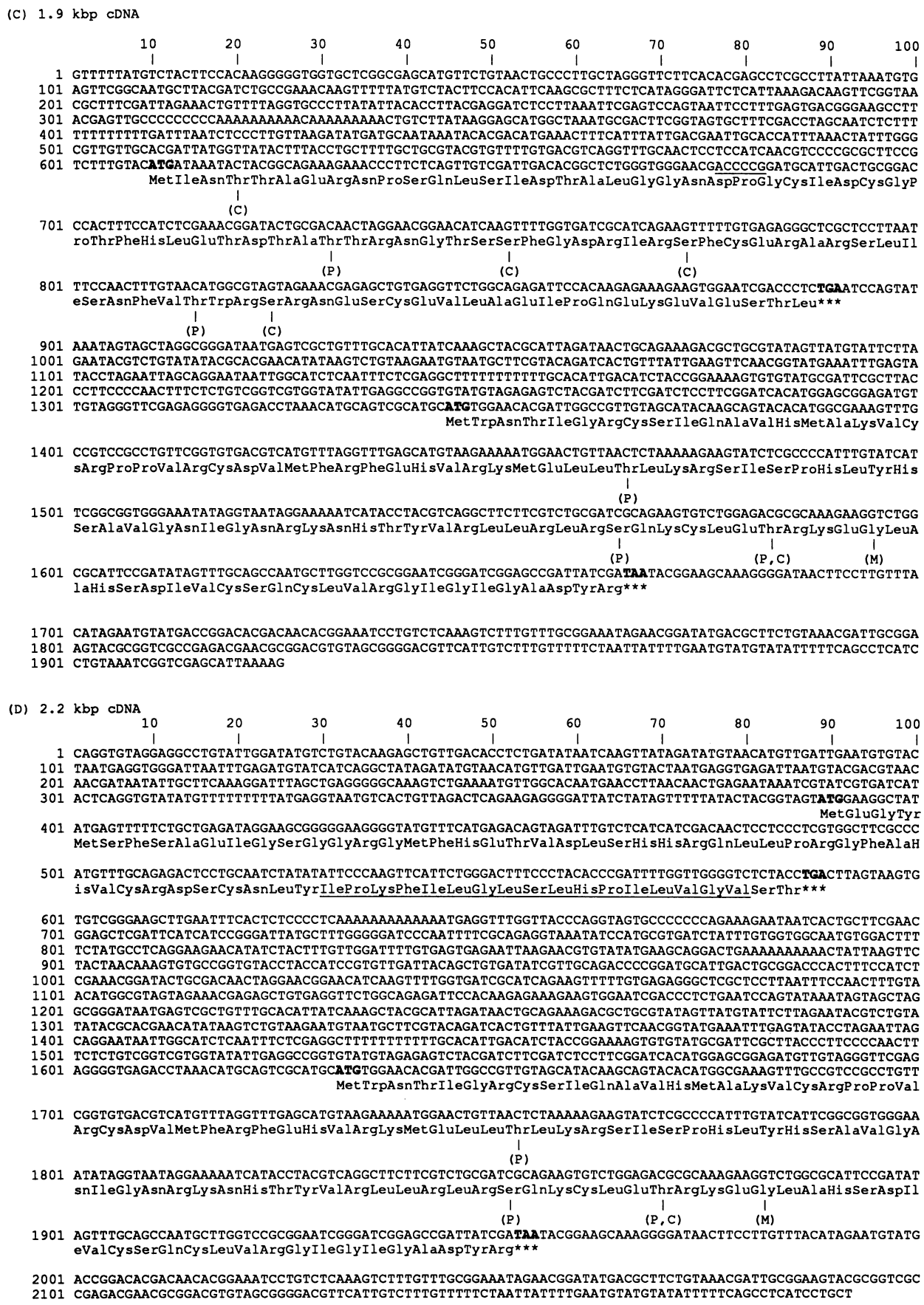

FIG. 2—*Continued.*

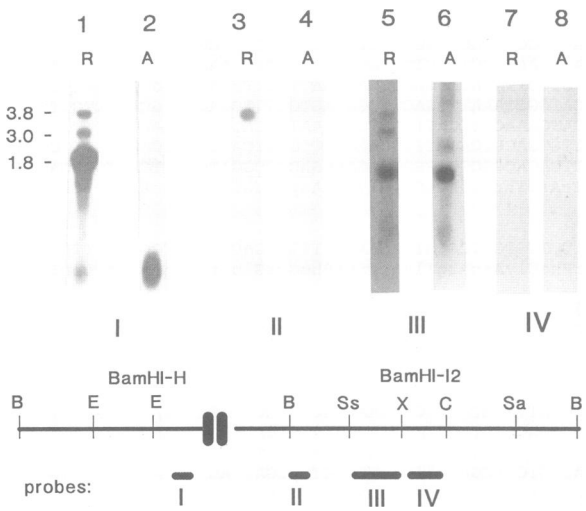

FIG. 3. Northern blot hybridization to confirm the origins of cDNAs. Poly(A)$^+$ RNA isolated from CEF infected with oncogenic or attenuated MDV was hybridized with the PCR-generated probe representing the 5′ end of the 1.69-kbp cDNA (probe I, lanes 1 and 2), a 3.8-kbp RNA-specific PCR-generated probe (probe II, lanes 3 and 4), an *Sst*II-*Xba*I subfragment of the *Bam*HI-I2 fragment DNA probe (probe III, lanes 5 and 6), and a *Xba*I-*Cla*I subfragment of the *Bam*HI-I2 fragment DNA probe (probe IV, lanes 7 and 8). Locations of the probes within the *Bam*HI-H and -I2 fragments are shown. Sizes of the hybridizing RNAs are given at the left in kilobases. R, poly(A)$^+$ RNA from RBIB; A, poly(A)$^+$ RNA from attenuated strain of RBIB. The restriction sites shown are *Bam*HI (B), *Cla*I (C), *Eco*RI (E), *Sal*I (Sa), *Sst*II (Ss), and *Xba*I (X).

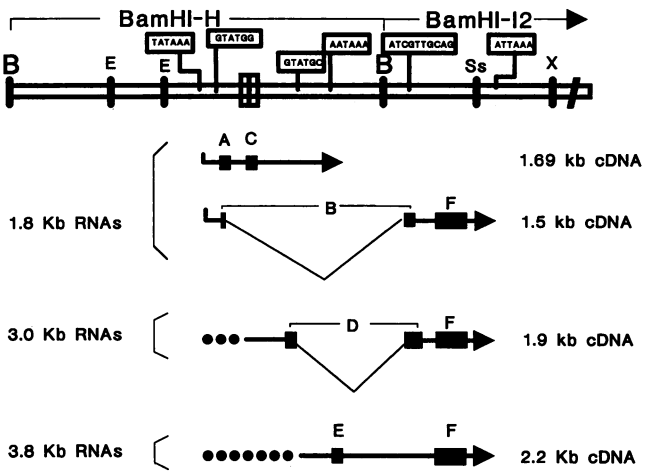

FIG. 4. Schematic representation of rightward transcriptional units within the *Bam*HI-H and -I2 regions of MDV shown with pertinent transcription-associated signals and locations of oncogenic virus-specific RNAs. Transcriptional signal sequences are shown within horizontal boxes that connect the sequences to their locations on the genome. The two vertical boxes indicate the positions of the two copies of the 132-bp repeat. Locations of the three classes of rightward transcribed RNAs are indicated. Curves represent intron regions not present in the mature RNAs. Black boxes represent locations of the potential ORFs labeled A through F according to the locations of their start codons on the MDV genome. Box A represents ORF-A, encoding 63 amino acid residues; interrupted box B represents ORF-B, encoding 75 amino acid residues; box C represents ORF-C, encoding 64 amino acid residues; interrupted box D represents ORF-D, encoding 93 amino acid residues; box E represents ORF-E, encoding 66 amino acid residues; and box F represents ORF-F, encoding 107 amino acid residues. The dotted line indicates the predicted cDNA sequence from 3.0- and 3.8-kb RNAs. Several restriction sites are located on the map: *Bam*HI (B), *Eco*RI (E), *Sst*II (S), and *Xba*I (X).

assisted translation of the class I 1.69-kb cDNA identified two ORFs, one encoding 63 amino acid residues (Fig. 4, ORF-A) and another encoding 64 amino acid residues (Fig. 4, ORF-C). ORF-A (nucleotides 141 to 329) encodes a putative protein with a molecular size of 6,962 Da. Analysis of the putative protein with the SOAP program as described by Klein et al. (11) suggested that ORF-A encodes an integral membrane protein which contains a transmembrane domain from residue 31 to 47 (MGVVSVWSHLLMLW SFI). A potential protein kinase C phosphorylation site was found at position 9 of ORF-A. Protein sequence homology searches did not reveal highly significant homology with the protein sequences deposited within the Protein Identification Resource (PIR) and Swiss-Prot data bases. However, limited homology of the ORF-A was found with the mouse T-cell lymphoma (TLM) oncogene protein. The N terminus of ORF-A exhibits a 50% homology (17 of 34 residues from residue 8 to residue 41) with the N terminus of the mouse TLM oncogene protein (Fig. 5). Limited homology of ORF-A with domain IX of the family of kinase-related transforming proteins (7) exemplified by the feline c-*fes*/*fps* proto-oncogene, feline sarcoma virus v-*fes* oncogene, and avian sarcoma virus v-*fps* oncogene was also found (Fig. 5).

ORF-C (nucleotides 644 to 835) encodes a putative protein with a molecular size of 6,891 Da. No significant homology between ORF-C and the protein sequences currently deposited in the PIR and Swiss-Prot data bases was found.

Analysis of the 1.5-kbp cDNA sequence indicated that this spliced mRNA contains an ORF encoding 75 amino acid residues and another ORF encoding 107 amino acid residues (Fig. 4, ORF-B and ORF-F, respectively). ORF-B utilizes the same start codon as ORF-A (contained in the 1.69-kb cDNA), but because of the splicing, ORF-B contains a different carboxyl terminus. ORF-B encodes a putative protein of 8,220 Da with two potential protein kinase C phosphorylation sites (positions 9 and 48), a myristylation site (position 32), and a casein kinase II phosphorylation site (position 35). ORF-B was predicted not to be a transmembrane protein. Since ORF-B shares some sequences with ORF-A, it also exhibits limited homology with the mouse TLM oncogene (Fig. 5). ORF-F (107 codons) is contained entirely within the second exon of the 1.5-kbp cDNA located in the *Bam*HI-I2 region. ORF-F codes for a putative protein with a molecular size of 12,332 Da which contains three protein kinase C phosphorylation sites (positions 41, 74, and 80), a casein kinase II phosphorylation site (position 80), and a myristylation site (position 84). Limited homology (39%; 13 of 33 amino acid residues) of ORF-F with the feline c-*fes*/*fps* proto-oncogene and the feline sarcoma virus v-*fes* oncogene was found within domain VII of the kinase family of transforming proteins (Fig. 5).

Analysis of the 1.9-kbp cDNA sequence suggested that this class II spliced mRNA contained two ORFs: ORF-D, encoding 93 amino acid residues (Fig. 4), and ORF-F, which was also contained in the 1.5-kbp cDNA. ORF-D spans two exons, while ORF-F is contained entirely within the second exon.

Two potential ORFs were found within the 2.2-kbp cDNA: ORF-E, encoding 66 amino acid residues (Fig. 4), and ORF-F, which is present in both the 1.5- and the 1.9-kbp cDNAs. No significant homology between ORF-D or ORF-E

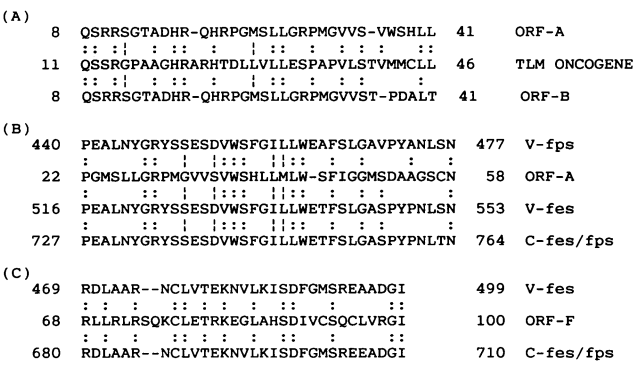

FIG. 5. Homology of potential ORFs with the transforming protein. (A) Homology of ORF-A (1.69-kbp cDNA) and ORF-B (1.5-kbp cDNA) with mouse TLM oncogene protein. (B) Homology of ORF-A (1.69-kbp cDNA) with domain IX of the family of kinase-related transforming proteins, feline c-*fes*/*fps* proto-oncogene, and avian sarcoma virus v-*fes* and v-*fps* oncogenes. (C) Homology of ORF-F (1.5-, 1.9-, and 2.2-kbp cDNAs) with domain VII of the family of kinase-related transforming proteins, feline c-*fes*/*fps* proto-oncogene, and feline sarcoma virus v-*fes* oncogene. The amino acid sequence is shown with the single-letter code, with sequence numbers of the residues on each end of the sequences. Double dots indicate identical amino acid homology, and double vertical lines indicate conserved amino acid homology.

and the protein sequences contained in the PIR and Swiss-Prot data bases was found.

DISCUSSION

Previous results have shown that the 3.8-, 3.0-, and 1.8-kbp transcripts of the *Bam*HI-H gene family are expressed by oncogenic MDV. These RNAs are not produced by attenuated MDV that has undergone amplification of the 132-bp repeat sequences located in the *Bam*HI-D and -H regions (1). It has been reported on the basis of S1 mapping that the *Bam*HI-H 1.8-kbp gene family transcripts produced in MSB-1 cells are products of splicing within the *Bam*HI-H region, contain two exons, and exclude the 132-bp repeats (1). The nonspliced transcripts were not included in that report because S1 protection of nonspliced RNA could not be distinguished from S1 protection due to self-annealing of the probe. The existence of nonspliced transcripts within the *Bam*HI-H region remains to be clarified by the cloning of cDNAs derived from *Bam*HI-H gene family transcripts. In this study, we have undertaken the cloning of cDNAs derived from these oncogenic virus-specific transcripts in order to identify their precise transcription patterns and locations on the MDV genome. Also, availability of the cDNAs derived from these oncogenic virus-specific transcripts is useful for studying their biological significance in tumor induction by MDV. We have chosen CEF lytically infected with RBIB to construct the cDNA library for cloning cDNAs representative of the *Bam*HI-H gene family because (i) transcription and the splicing pattern of the *Bam*HI-H gene family in CEF lytically infected with RBIB are similar to those in iododeoxyuridine-treated MSB-1 cells according to S1 mapping analysis (unpublished data), and (ii) an abundance of *Bam*HI-H gene family transcripts was produced in CEF infected with RBIB.

Our data from cDNA analysis indicated that two kinds of transcripts belong to the 1.8-kbp RNAs of the *Bam*HI-H gene family: (i) a nonspliced transcript (1.69-kbp cDNA) within the *Bam*HI-H fragment which contains two copies of the 132-bp repeats and (ii) a spliced RNA (1.5-kbp cDNA) with exon 1 at the *Bam*HI-H region and exon 2 at the *Bam*HI-I2 fragment. The 1.69- and 1.5-kbp cDNAs share the same initiation site at nucleotide 741 of the *Bam*HI-H sequence, as was previously predicted by S1 mapping analysis (1). The 1.69-kbp cDNA terminated at nucleotide 2437 of the *Bam*HI-H sequence, as was previously predicted by S1 mapping (1), whereas the 1.5-kbp cDNA terminated at nucleotide 1449 of the *Bam*HI-I2 fragment (unpublished I2 sequence) through use of the polyadenylation signal (ATTAAA) located at nucleotides 1463 to 1468 of the *Bam*HI-I2 fragment.

The 1.69-kbp cDNA derived from nonspliced RNA is identical to those cDNA clones of linear transcripts reported recently by Iwata et al. (9). A comparison of sequence data indicated a difference between amino acid 23 of ORF-C (Cys) and amino acid 23 of ORF-2 reported by Iwata et al. (9). This difference is likely due to the different MDV strains (RBIB versus Md5) used to isolate cDNAs. Cloning of these cDNAs demonstrated the existence of nonspliced transcripts within the *Bam*HI-H 1.8-kbp gene family. So far, cDNAs which are derived from the spliced transcripts and have exon 2 located within the *Bam*HI-H fragment have not been identified. However, S1 mapping analysis using a 5′-end-labeled single-stranded probe (as previously described in reference 1) prepared from 1.69-kbp cDNA to hybridize with poly(A)$^+$ RNA isolated from iododeoxyuridine-treated MSB-1- and RBIB-infected CEF did confirm the presence of spliced transcripts of the *Bam*HI-H 1.8-kbp gene family in both type of cells (data not shown). This S1 mapping analysis also excluded the possibility that the probes used in a previous study (1), which were prepared from the *Bam*HI-H fragment of DNA from the GA strain of MDV that had been cloned and maintained for a long time in bacteria, had significant base changes which might give errors in S1 mapping. The cloning and sequencing of cDNAs derived from the spliced mRNAs which contain two exons located within the *Bam*HI-H region are in progress. It should be noted that both spliced mRNAs (predicted by previous S1 mapping) and nonspliced mRNAs transcribed entirely within the *Bam*HI-H region (identified by cDNA cloning) of the *Bam*HI-H 1.8-kbp gene family contain the 63-amino-acid ORF (ORF-A).

Kawamura and colleagues (10) prepared sense and antisense oligonucleotides directed against the first splice donor sequence (nucleotide 1178 of GenBank sequence M26392) of the *Bam*HI-H 1.8-kbp gene family and added the oligonucleotides to MSB-1 cultures. They observed a rapid decrease in cell proliferation only when the antisense oligonucleotide, A1, was used. They also observed that antisense oligonucleotide A1 inhibited transcription of the *Bam*HI-H gene family, suggesting that A1 inhibition may be caused by some other novel mechanisms of antisense RNA.

Chen and Velicer (3) have described the isolation of several cDNAs derived from duck embryo fibroblasts infected with the GA strain of MDV. Their analysis revealed multiple bidirectional initiations and terminations of transcription in the *Bam*HI-D and *Bam*HI-H regions. It is therefore possible that transcripts from the *Bam*HI-H fragment of MDV DNA include mRNAs transcribed in both directions.

Since the 3.0-kbp mRNA could be detected by hybridization with the PCR-generated DNA probe representing the 5′ end of the *Bam*HI-H gene family, the initiation site of the 3.0-kbp transcript is possibly identical to those of the transcripts from which class I cDNAs are derived. Precise genomic mapping of the 3.0-kbp transcripts awaits the iso-

lation and characterization of full-length cDNAs. Meanwhile, we could not exclude a possibility that some class II cDNA clones are full-length cDNAs derived from the transcripts initiated at the first unit of the 132-bp repeats, because a TATAA-like sequence (TATTAAA) was found within the proper context of the MDV genomic DNA sequence upstream of the 5′ end of this cDNA.

Sequence analysis of the putative proteins translated from ORF-A and ORF-B show a 50 or 44% homology, respectively, with the N terminus of the mouse TLM oncogene protein (12, 13). The TLM oncogene is a stage-specific transforming gene which is frequently altered during mouse T-cell leukemia and lymphoma by a single base mutation or by a more-complex gene rearrangement. MDV could induce T-cell lymphoma within 6 to 8 weeks in chickens. The significance of the limited homology of ORF-A and ORF-B with TLM oncoprotein in tumor induction by MDV is not clear, because no information about the functional domain of the TLM oncoprotein is available. It should be noted that ORF-A is likely a transmembrane protein, whereas ORF-B is predicted to be intracytoplasmic. Thus, the locations of these two proteins in MDV-infected cells would be different. The computer-predicted location and structural characteristics of ORF-A suggest that it might encode a receptor-like protein.

Protein sequence homology searches have shown 48% homology (15 of 31 residues) between ORF-A and domain IX of the *fes/fps* kinase-related transforming protein. However, the significance of this homology is unclear because (i) domain IX is not a catalytic domain of the family of kinase-related transforming proteins, and (ii) the invariable residues SDVMxxGxxxxE, which are characteristic of all kinase family transforming proteins and are present in domain IX, are not conserved within ORF-A. It should be noted that we cannot find 70% homology (23 amino acids) of ORF-A with domain IX of v-*fms*, a kinase-related transforming protein, reported recently by Iwata et al. (9).

ORF-F, in 1.5-, 1.9-, and 2.2-kbp cDNAs, shows 39% homology with domain VII of the *fes/fps* family of kinase-related transforming proteins. Domain VII is a catalytic domain of the kinase-related transforming protein. However, the biological significance of this homology between domain VII of *fes/fps* transforming protein and ORF-F is uncertain because of the absence of homology of ORF-A with the invariable residues DFGxxR of domain VII.

In this report, we have described the isolation and characterization of several cDNA clones from CEF infected with oncogenic MDV RBIB. These cDNA clones are representatives of rightward transcripts of the *Bam*HI-H gene family. A number of ORFs in these cDNA clones have been identified; they may encode polypeptides which have homology with mouse TLM oncogene and the kinase family of transforming proteins. It is not known whether one or more genes of these transcripts, which are specifically expressed by oncogenic RBIB in lytically infected CEF, are expressed in MDV-induced chicken T-cell lymphoma cells, although transcripts of similar sizes have been detected in MDV-induced tumor cells. The isolation and characterization of these oncogenic virus-specific cDNAs will facilitate identification of the biological function of the *Bam*HI-H gene family and aid our investigations into the oncogenic mechanism of MDV.

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