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The Transcripts from the Sequences Flanking the Short Component of Marek's Disease Virus during Latent Infection Form a Unique Family of 3′-Coterminal RNAs

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We have constructed a cDNA library using poly(A)$^+$ RNA from the stably transformed Marek's disease virus cell line MKT-1 and isolated cDNAs specific to the short internal repeat region of the *Bam*HI-A fragment of the viral genome. Four distinct classes of cDNA were identified through sequence analysis of the 5′ and 3′ termini of each clone isolated, and a representative of each class was chosen for complete sequencing. These cDNAs were mapped on the basis of the genomic nucleotide sequence of this region, and a family of 3′-coterminal overlapping transcripts, consisting of several highly spliced species, was identified. PCR was used to amplify specific regions of each cDNA, which were subcloned and used to generate riboprobes. These riboprobes hybridized to a variety of transcripts in poly(A)$^+$ RNA fractions isolated from cells either lytically or latently infected with Marek's disease virus.

Marek's disease virus (MDV) is an avian herpesvirus which causes lymphoproliferative disease in its natural host, the chicken. The viral DNA is a linear double-stranded molecule of 180 kbp (14) which is similar in structure to the DNAs of the alphaherpesviruses herpes simplex virus types 1 and 2. It consists of a long unique (U_L) sequence and a short unique sequence (U_S), which are flanked by the inverted repeat sequences TR_L/IR_L and TR_S/IR_S, respectively (9). Lymphoblastoid cell lines established from tumors contain multiple copies of the viral genome, which are maintained as circular episomes (29) or integrated into host chromosome (7). Analysis of viral transcription in these cell lines and in lymphoma tissue obtained from chickens has demonstrated that transcriptional activity is restricted to the reiterated sequences flanking U_S and U_L (24, 28).

Transcription within the *Bam*HI-H region of the viral genome during latent infection has been intensively studied (2, 3, 10, 12, 20, 21, 22). Analysis of genomic DNAs of attenuated and pathogenic strains of MDV demonstrated that attenuation of oncogenic MDV following serial passage in cell culture is due to the amplification of a 132-bp direct repeat sequence (16), which causes premature termination of transcription of the *Bam*HI-H gene family (3). In contrast, other regions of the viral genome which are expressed in latently infected cells, in particular the *Bam*HI-A, -L, and -I_2 genomic fragments, have been less well characterized. Recently, several cDNA clones derived from the poly(A)$^+$ RNA fraction of the MDV-transformed nonproductive cell line MDCC-CU41 (5) have been isolated and characterized (18, 19). Several cDNAs derived from mRNA species specific for the *Bam*HI-L and adjacent Q_2 regions were shown to originate from mRNAs of 0.6, 0.8, and 2.5 kb transcribed from this region (19). cDNA clones specific to the *Bam*HI-A region of the MDV genome were mapped by Southern blot analysis, and three distinct groups of cDNA were isolated, all of which were found to hybridize to sequences within IR_S (18).

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Previous studies in our laboratory demonstrated the existence of a number of transcripts which were detected by using *Bam*HI-A-specific probes for Northern (RNA) blot analysis (28). On the basis of size, these transcripts were identical to those isolated from kidney lymphoma cells obtained from 5-week-old chickens which had been injected intra-abdominally with GA, a pathogenic strain of MDV (28). Therefore, the MKT-1 cell line provides an excellent in vitro model for studies of MDV latency and oncogenicity. To further characterize MDV latency-associated transcripts, a cDNA library was constructed from poly(A)$^+$ RNA isolated from the T-lymphoblastoid cell line MKT-1, which is latently infected with MDV. Total cellular RNA was prepared essentially as described by Chomczynski and Sacchi (6), and the poly(A)$^+$ fraction was subsequently isolated by using the poly(A) Tract mRNA isolation system (Promega, Madison, Wis.) in accordance with the manufacturer's recommended instructions. The cDNA library was screened using the 1.8-kb *Eco*RI-e subfragment of *Bam*HI-A (nucleotide positions [np] 11183 to 13015), previously used to detect all *Bam*HI-A-specific transcripts in the MKT-1 cell line (28).

In order to map the cDNA transcripts corresponding to specific regions of the *Bam*HI-A fragment, it was necessary to determine the total sequence of the IR_S region of the MDV genome. *Bam*HI-A subfragments *Eco*RI-a, -b, and -e span this entire region and were thus cloned from genomic DNA into Bluescript SK +/−. To facilitate our sequence analysis, these fragments were further subcloned to generate seven subfragments: *Eco*RI-*Hin*dIII (np 1 to 1599), *Hin*dIII-*Hin*dIII (np 1599 to 3912), *Hin*dIII-*Eco*RI (np 3912 to 5441), *Eco*RI-*Hin*dIII (np 5441 to 6972), *Hin*dIII-*Hin*dIII (np 6972 to 8213), *Hin*dIII-*Eco*RI (np 8213 to 11183), and *Eco*RI-*Eco*RI (np 11183 to 13015). DNA sequences were assembled and analyzed by using the Gened and Translate programs of PC-Gene.

A family of 3′-coterminal cDNAs ranging in size from 0.5 to 2.2 kb were isolated when the cDNA library constructed from MKT-1 poly(A)$^+$ RNA was screened with the 1.8-kb *Eco*RI subfragment of *Bam*HI-A DNA (Fig. 1). On the basis of size and sequence of the 5′ and 3′ termini of each clone, four

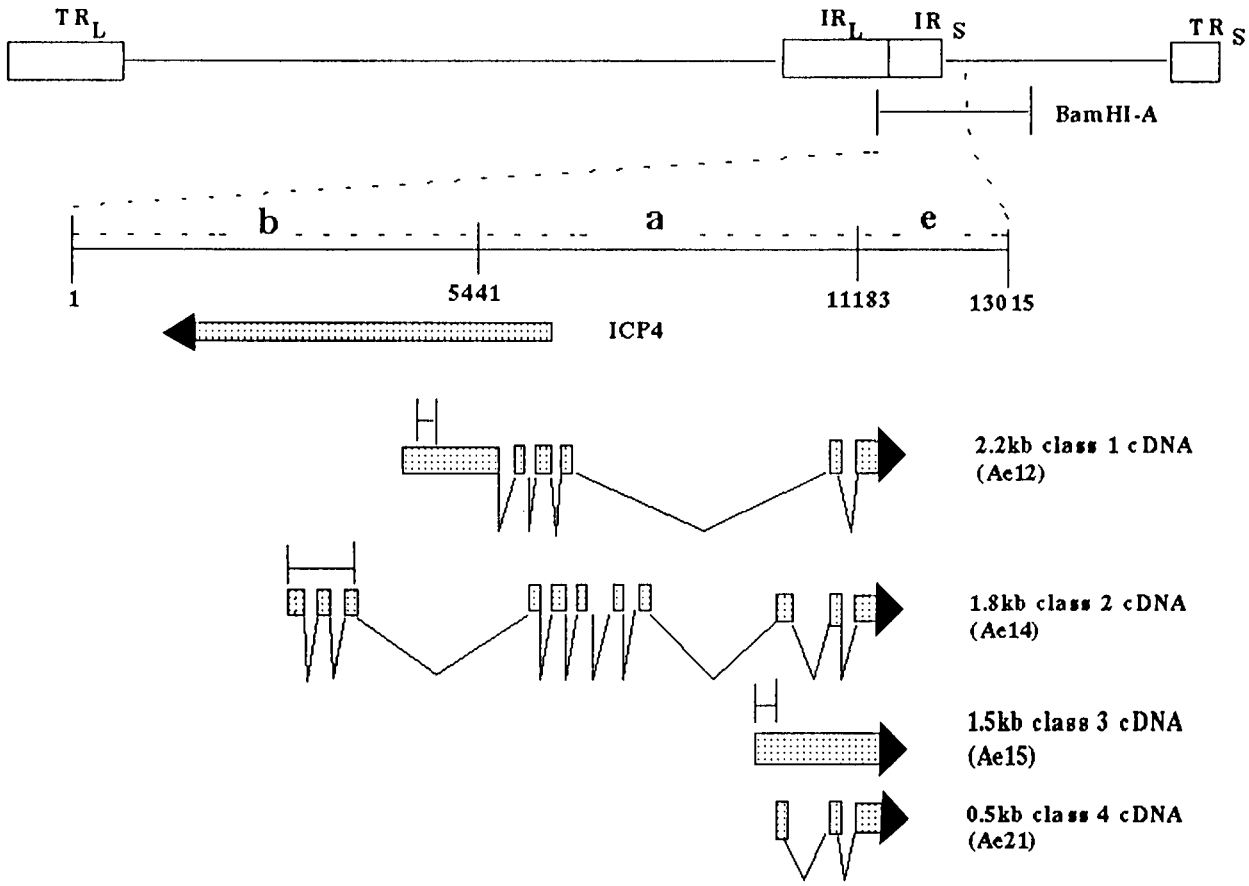

FIG. 1. Diagrammatic representation of the MDV genome showing an enlargement of the *Bam*HI-A *Eco*RI-a, -b, and -e subfragments, which are 5.4, 5.7, and 1.8 kb, respectively. The locations of the *Bam*HI-A-specific cDNA clones are shown, and their sizes are indicated on the right. Stippled boxes, exons; lines, introns. The regions of Ae12 (probe A), Ae14 (probe B), and Ae15 (probe C) cDNAs which were amplified by PCR and subcloned into pGEM11zf(+) for production of strand-specific riboprobes are indicated (⊢⊣).

distinct classes of cDNA were initially identified. As multiple clones of the same transcript were isolated, a representative of each class was chosen for complete sequencing: Ae12 (class 1; 2.2 kb), Ae14 (class 2; 1.8 kb), Ae15 (class 3; 1.5 kb), and Ae21 (class 4; 0.5 kb).

Ae12 cDNA was derived from a highly spliced mRNA containing six exons (Fig. 1; Table 1). The 5′ end of the 2.2-kb cDNA was at np 4868 of the *Bam*HI-A sequence, 358 to 428 bp downstream from the nearest TATAA sequence (4), CCAAT homology box (11), and SP1 binding site (GGCGGG) (8). The 3′ end of the 2.2-kb cDNA was located at np 11593 of the *Bam*HI-A sequence within IR_S, a position 35 bp downstream from a potential polyadenylation signal, AATAAA, common to the 3′-terminal regions of all the cDNA clones isolated.

TABLE 1. Locations of exons on 3′-coterminal cDNAs corresponding to the *Bam*HI-A sequence[a]

Exon	np		
	Ae14	Ae12	Ae21
1	3382–3464	4868–6599	10069–10138
2	3595–3708	6671–6777	11195–11264
3	3987–4228	7147–7236	11372–11593
4	6051–6113	7322–7397	
5	6200–6599	11159–11264	
6	6671–6777	11359–11593	
7	7147–7236		
8	7322–7397		
9	10013–10138		
10	11159–11264		
11	11372–11593		

[a] Ae15: exon 1, np 9807 to 11593.

The 1.8-kb cDNA, represented by Ae14, was composed of 11 exons (Fig. 1; Table 1). The 5′ end of this cDNA was located at np 3382 of the *Bam*HI-A sequence. A CCAAT box and TATAA-like sequence (TAATAA) were located 46 and 34 bp, respectively, upstream from the 5′ end of this 1.8-kb cDNA. We noted that exons 2 to 6 of class 1 cDNAs were identical to exons 6, 7, 8, 10, and 11, respectively, of the class 2 cDNAs. The last exon of each of the three classes of spliced cDNAs had a common splice donor site (CAG/GCAAGT) (17) at np 11264 of *Bam*HI-A. Class 2 and class 4 cDNAs utilized the same splice acceptor site (CACAG/C), whereas class 1 cDNAs utilized a splice acceptor site which was further upstream (TCAAG/G). These splice acceptor and splice donor sites, as well as others utilized by our cDNAs, showed good homology to the published consensus sequence (17).

The majority of the cDNAs which we isolated belonged to the class 3 cDNAs. These were represented by Ae15 cDNA which was derived from a nonspliced mRNA corresponding to np 9807 to 11593 of the *Bam*HI-A sequence (Fig. 1). It is likely that our class 3 cDNA is equivalent to the group 3 cDNAs recently reported by Ohashi et al. (18). The TATAA box clos-

est to the 5′ end of this cDNA is located at np 9337; however, a CCAAT homology and SP1 binding site are located at np 9727 and 9747, respectively.

Class 4 cDNA, represented by Ae21 cDNA, was derived from a spliced message containing three exons (Fig. 1; Table 1). The 5′ end of this cDNA is located at np 10076 of the *Bam*HI-A sequence. There is a potential TATAA sequence for this transcript at np 9974 and a CCAAT homology box at np 10019.

Among the cDNAs which we isolated, class 1 and 2 cDNAs were derived from highly spliced messages. Although the biological significance of this splicing is unclear, it has recently been demonstrated (26) that cDNAs derived from an immediate-early locus on the human herpesvirus 6 genome are similarly highly spliced and it is becoming apparent that many herpesvirus immediate-early genes are derived from spliced transcripts. Further analysis will be required to determine whether the cDNAs which we have isolated are similarly derived from immediate-early transcripts.

Computer analysis was carried out to search for potential open reading frames (ORFs). Several were identified within our cDNAs which had optimal translation initiation environments (13). However, although extensive efforts have been made to determine whether these are actually protein coding, through the use of in vitro transcription/translation systems and the generation of polyclonal antiserum, we are at present unable to provide evidence that any of these ORFs are actually translated in vivo. As with other reports of ORFs encoded by MDV latency-associated transcripts (18–20), these were generally small, with the largest ORF identified being only 114 amino acids in size. Analysis of Ae15 cDNA identified a small ORF (A41 ORF), recently described by Ohashi et al. (18), which could potentially encode a 10.6-kDa polypeptide. In their study, they expressed this protein in bacteria, as a glutathionine *S*-transferase fusion, which they purified and used to produce polyclonal antiserum. By using indirect immunofluorescence, this protein was localized to the cytoplasm of lytically infected cells; however, it could not be detected in latently infected cells, presumably because it was produced in smaller quantities in this cell line.

The sequence of the ICP4 gene, which is located in IR_S, has been previously reported (1). Our ORF analysis identified this gene as the major leftward transcript in IR_S. Comparison of the previously reported sequence with that generated in this study showed almost 100% identity with no amino acid substitutions.

To determine whether the cDNAs which we isolated represented a full complement of the cDNAs transcribed from the *Bam*HI-A region of the genome, specific regions of the class 1 to 3 cDNAs were amplified by PCR (Fig. 1; Table 2) and subcloned into pGEM11zf(+). These were subsequently used as templates to generate riboprobes (Promega) which could specifically detect rightward transcripts and hybridized (10^7 cpm/ml) to poly(A)$^+$ RNA (2 μg per well) which had been transferred to nylon membranes following electrophoresis through 1% agarose gels. In addition, we wished to determine which mRNAs encoded by the *Bam*HI-A fragment were latency/oncogenicity specific and were present exclusively in transformed cells harboring latent virus. Thus, we compared transcripts accumulating in cells latently and lytically infected with MDV. As a representative of cells productively infected with MDV, the MDV nonpathogenic vaccine strain CVI-988 (23) was used at a passage level greater than 100. This virus was propagated in primary chicken embryo fibroblasts (CEF) as described previously (9). Cells were harvested when 80% of cells showed cytopathic effect.

TABLE 2. *Bam*HI-A cDNA-specific probes used for Northern blot analysis

Probe	Representative cDNA	Corresponding MDV *Bam*HI-A np	Size (bp)
A	Ae12	5246–5442	197
B	Ae14	3382–3462, 3592–3711, 3396–4164	376
C	Ae15	9801–11058	268

The locations of the probes used for Northern blot analysis are listed in Table 2, and the results are shown in Fig. 2. In each case, the cDNAs were used as templates to amplify specific regions of DNA for probe production rather than genomic DNA. For example, in the case of Ae14 cDNA, the region amplified covers two regions which are spliced from total RNA to produce the mature Ae14 poly(A)$^+$ transcript. All probes were hybridized to the poly(A)$^+$ fractions obtained from each cell line. Hybridizations were carried out at 65°C overnight in 50% formamide–5× SSPE (1× SSPE contains 0.15 M NaCl, 0.01 M NaH_2PO_4, and 1 mM EDTA [pH 7.4])–5× Denhardt's solution (1× Denhardt's solution contains 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin)–0.1% sodium dodecyl sulfate–100 μg of herring sperm DNA per ml. For each hybridization larger transcripts of >3 kb were observed in addition to those of the expected size. In general, the patterns of transcription in MSB-1-, MKT-1-, and CVI-988-infected CEF cells were very similar.

Probe A, unique to Ae12 cDNA, did not detect a band of 2.2 kb, the expected size of Ae12 mRNA, although it did detect larger bands of 4.8 and 8.4 kb. An additional band of 4.2 kb was apparent in CEF lytically infected with MDV. Several results would therefore indicate that Ae12 cDNA is not full length: (i) the nearest TATAA box which could potentially be utilized by Ae12 cDNA is over 350 bp from the 5′ end of this cDNA; (ii) when we used primer extension analysis to map the 5′ end of Ae12 cDNA, the product of this reaction was much larger than expected, although the actual size could not be determined, as the band migrated close to the top of the gel (data not shown); and (iii) no transcript equivalent in size to Ae12 cDNA was detected in MKT-1 cells with the Ae12-specific riboprobe. From the result of Northern blot analysis we can hypothesize that Ae12 cDNA is possibly an incomplete copy of the 4.8- or 8.4-kb transcripts detected with this probe.

Probe B, unique to Ae14 cDNA, detected a band of 1.8 kb, which is the expected size of Ae14 cDNA in both latently infected cells and lytically infected cells. Transcripts of 2.8, 4.8, and 8.4 kb were detected in all cell lines tested, whereas a 2.0-kb transcript was detected only in lytically infected cells.

Probe C, specific for Ae15 cDNA, detected a 1.5-kb transcript, the expected size of Ae15 mRNA in all cell lines tested. The 2.0-kb transcript detected by probe B was also detected by probe C. If this is the same mRNA, it would indicate that this is a spliced message, as the two probes used are more than 2 kb apart on the viral genome. The 2.8- and 3.9-kb transcripts detected by this probe were common to all cell lines.

To demonstrate that the cDNAs which we isolated shared a common 3′ terminus, Ae21, which contains 3′ sequences common to each cDNA, was used as a probe for Northern blotting (Fig. 3). As expected, bands of 1.5 and 1.8 kb, corresponding to Ae15 and Ae14 cDNAs, respectively, were detected. The band running just below 0.7 kb is probably the mRNA corresponding to Ae21 cDNA. As expected, no transcript of 2.2 kb, corresponding to Ae12 cDNA, was detected, thus providing further evidence that this cDNA is not full length.

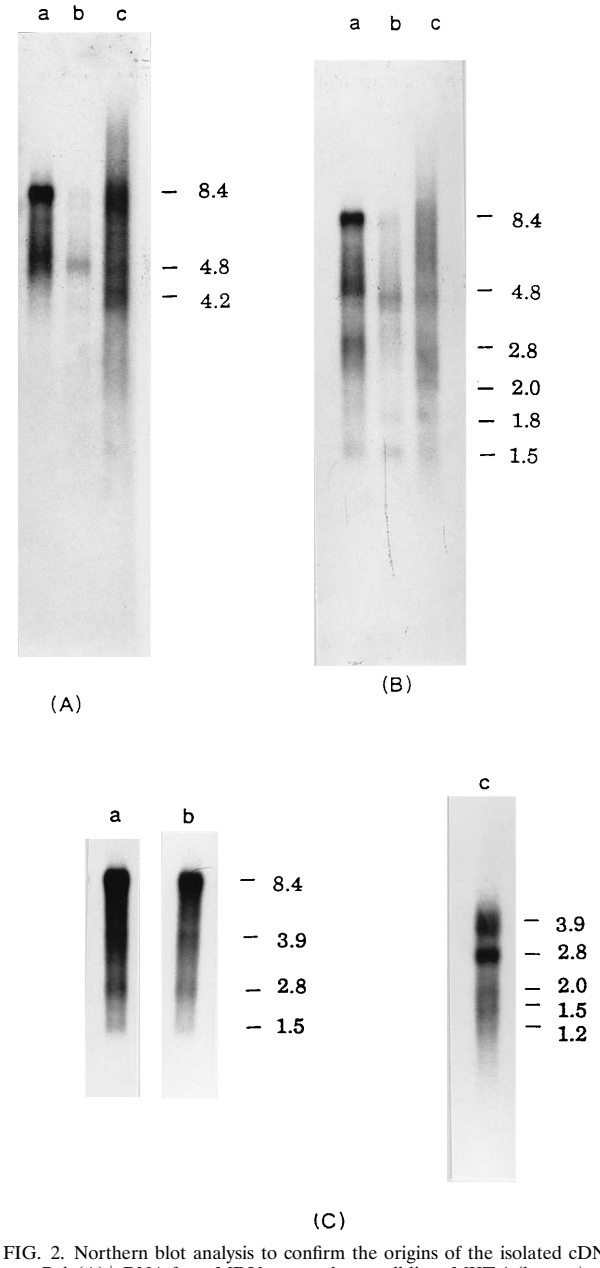

FIG. 2. Northern blot analysis to confirm the origins of the isolated cDNA clones. Poly(A)$^+$ RNA from MDV nonproducer cell lines MKT-1 (lanes a) and MSB-1 (lanes b) and CEF infected with the nonpathogenic strain CVI-988 (lanes c) were hybridized (see the text) to strand-specific riboprobes synthesized from short specific regions of *Bam*HI-A which had been cloned under the control of the SP6 promoter. These riboprobes were specific for Ae12, a representative of class 1 cDNAs (2.2 kb) (A); Ae14, a representative of class 2 cDNAs (1.8 kb) (B); and Ae15, a representative of class 3 cDNAs (1.5 kb) (C). Sizes of transcripts were estimated with an RNA ladder (Gibco-BRL) and are given in kilobases.

Originally, we had hypothesized that all the rightward transcripts in the IR_S region of the MDV genome, which are produced during latent infection, share a common 3′ terminus. However, in a recent publication, Li et al. (15) described the isolation of a cDNA clone, constructed by using poly(A)$^+$ RNA from MSB-1 cells, which was antisense to the ICP4 gene but terminated at a polyadenylation signal located at np 10524, 1,070 bp upstream from the polyadenylation signal common to all the cDNAs which we isolated. However, we have aligned their cDNA sequence with the genomic sequence of this region

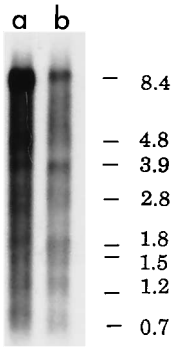

FIG. 3. Northern blot analysis to confirm that the cDNAs which were isolated had common 3′ termini. Poly(A)$^+$ RNA from the MDV nonproducer cell lines MKT-1 (lane a) and CEF infected with the nonpathogenic strain CV1-988 (lane b) were hybridized to Ae21 cDNA which had been linearized and used to produce a strand-specific riboprobe. Hybridization conditions are described in the text. Sizes of transcripts were estimated by using an RNA ladder (Gibco-BRL) and are given in kilobases.

and found that the region they hypothesized to be a poly(A)$^+$ tail actually corresponds to a stretch of 15 A residues in the genomic sequence. Furthermore, in their Northern blot analysis of transcripts which were antisense to the ICP4 gene, they detected two major transcripts of 15 and 1.32 kb, neither of which correlated with the size of the cDNA which they had isolated (2,756 bp). The authors neither presented any data to explain this discrepancy nor confirmed the 5′ and 3′ origins of their cDNA. Thus, the precise derivation of this cDNA has yet to be confirmed.

Prior to construction of the MKT-1 cDNA library, our goal had been the isolation and characterization of cDNAs which were specific to MDV latency/oncogenicity. It is apparent from our results that the cDNAs isolated are not derived from mRNAs specifically expressed during MDV latency. However, it would be unreasonable to simply dismiss the potential importance of these transcripts in the establishment and maintenance of MDV latency, since as has previously been demonstrated for herpes simplex virus type 1 (27), the major latency-associated transcript is similarly detectable during lytic infection although the minor species are observed only during latency. Furthermore, from our Northern blot analysis it is apparent that there are several longer mRNA species in this region from which we have failed to obtain cDNA clones, probably because of the inefficiency of the reverse transcriptase used for first-strand synthesis. Construction of a new cDNA library with the specific aim of isolating these longer cDNA clones is in progress.

At present the functional significance of these cDNAs, specific to the *Bam*HI-A region of the MDV genome, remains unclear. Whether they are regulatory RNAs or encode viral proteins involved in specific virus-host interactions such as the maintenance of viral latency or suppression of viral replication has yet to be determined.

Nucleotide sequence accession numbers. The sequences of *Bam*HI-A subfragments a, b, and e have been submitted to GenBank and given accession numbers U17705, U17704, and U17706, respectively. cDNAs Ae12, Ae14, Ae15, and Ae21 have been given GenBank accession numbers U17700, U17701, U17702, and U17703, respectively.

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