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Analysis of Multiple mRNAs from Pathogenic Equine Infectious Anemia Virus (EIAV) in an Acutely Infected Horse Reveals a Novel Protein, Ttm, Derived from the Carboxy Terminus of the EIAV Transmembrane Protein

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Transcription of pathogenic equine infectious anemia virus (EIAV) in an acutely infected horse was examined by using the polymerase chain reaction and nucleotide sequencing. Four spliced transcripts were identified in liver tissue, in contrast to the multiplicity of alternatively spliced messages reported for in vitro-propagated human immunodeficiency virus, simian immunodeficiency virus, and, to a lesser extent, EIAV. Nucleotide sequence analysis demonstrated that three of these mRNAs encode known viral proteins: the envelope precursor, the product of the S2 open reading frame, and the regulatory proteins Tat and Rev. The fourth transcript encodes a novel Tat-TM fusion protein, Ttm. Ttm is a 27-kDa protein translated from the putative *tat* CTG initiation codon and containing the carboxy-terminal portion of TM immediately downstream from the membrane-spanning domain. $p27^{ttm}$ is expressed in EIAV-infected canine cells and was recognized by peptide antisera against both Tat and TM. Cells transfected with *ttm* cDNA also expressed $p27^{ttm}$, which appeared to be localized to the endoplasmic reticulum or Golgi apparatus by indirect immunofluorescence. The carboxy terminus of lentiviral TM proteins has previously been shown to influence viral infectivity, growth kinetics, and cytopathology, suggesting that Ttm plays an important role in the EIAV life cycle.

Equine infectious anemia virus (EIAV) is a lentivirus, related to human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), bovine immunodeficiency virus, feline immunodeficiency virus, caprine arthritis-encephalitis virus, and visna virus. Clinical disease in susceptible equids depends on the virulence of the inoculum and ranges from asymptomatic to fatal, but typically involves fever, viremia, weight loss, and leukopenia. Surviving animals may experience a variable number of cyclic relapses and then chronic anemia and wasting or recovery. As is the case with other species infected by lentiviruses, the recovered horse is persistently infected for life, and blood remains infectious (22).

The study of the molecular events associated with the different states of EIAV-induced disease is complicated by the fact that pathogenic isolates of EIAV cannot be propagated readily in established cell lines, while tissue culture-adapted strains are generally attenuated (26, 28). As with other retroviruses, lentiviruses passed in tissue culture are frequently subjected to unique selective pressures that promote the emergence of variants which differ significantly from the original isolates in their genetic and biological properties (for a review, see reference 9). Furthermore, viral proteins expressed in immortalized cells may exhibit biological effects different from those in natural host cells (11, 25). Therefore, in order to define the molecular events important to EIAV-induced disease in vivo, we have been examining the biochemistry of viral replication and gene expression in horses infected with pathogenic EIAV (52).

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Lentiviral genomes encode multiple structural and regulatory proteins. Transcription initiates at a single site, and differential gene expression is accomplished in part by alternative splicing of the full-length primary transcript. Full-length RNA is translated to produce the Gag and Pol proteins, while a singly spliced transcript encodes the Env protein. In HIV and SIV, a large number of multiply spliced transcripts encoding the viral regulatory proteins have been identified (18, 53, 59). In EIAV, the *tat* gene is expressed on three spliced transcripts (13, 42, 44, 58, 62), while *rev* is expressed from one spliced transcript (44, 62). Tat and Rev coding sequences have also been found on several minor alternatively spliced transcripts (44). To date, these lentiviral transcripts have been identified only in infected cell lines; a complete understanding of their role in viral pathogenesis requires characterizing the mRNAs and translation products that are present in vivo.

In this study, we sought to define the EIAV transcripts and gene products that are expressed during acute pathogenic infection in a horse. To characterize viral transcription, we amplified reverse-transcribed liver mRNA by using the polymerase chain reaction (PCR) and analyzed the products by direct DNA sequencing. We identified three spliced transcripts encoding *env*, *tat*, and *rev*. We also found a fourth spliced transcript encoding a novel 27-kDa protein, Ttm, comprising the carboxy terminus of the EIAV transmembrane protein (TM), and characterized its expression in infected and transfected cells. This new protein is of particular interest, as passage of EIAV in certain cell lines selects for variants with premature stop codons that prevent translation of this region of TM and that exhibit enhanced growth or infectivity in vitro (51).

TABLE 1. EIAV oligonucleotides

Direction and oligonucleotide	Sequence (5′→3′)	Position[a]	
		5′	3′
Forward			
BS18A	CTAGTTTGTCTGTTCGAGATCC	297	318
$CB27^+$	CCAAGCTTGAACATGGCTGATCGTAGGATCC	368	392
$CB15^+$	GACAGCAGAGGAGAACTTACAGAAGTCTTC	397	426
Reverse			
$CB23^-$	GAGGTTGTCTTCCTTGTTGGATTGCCTTCAGTC	5262	5230
$CB26^-$	GGTTGAAACATTGTGTTCTCCTCACACTTAG	5416	5386
$CB24^-$	CTTGCTTCCTTCGATTCTGCCATGCTGTTC	5470	5441
$CB18^-$	CATACTGAGGCATTGATACATG	7887	7866
$CB28^-$	GGCTGCAGTTCTAAACATACTGAGGCATTG	7894	7873

[a] Numbering according to proviral sequence (GenBank accession number M16575).

MATERIALS AND METHODS

Virus and cells. A low-passage stock of the virulent Wyoming strain of EIAV ($EIAV_{Wyo}$) (34) was obtained from Louisiana State University and passed once in a horse. Serum from this horse had a titer of 10^6 infectious units per ml for equine leukocyte cultures and served as the virus stock for subsequent infections. Tissue culture-adapted EIAV, originally derived from $EIAV_{Wyo}$ (34), was grown in canine thymus cells (Cf2Th) and canine osteosarcoma cells (D17). Persistently infected and uninfected cell lines were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum.

Horse infection. An EIAV-seronegative quarter horse mare, number R1775, was inoculated intravenously with 10^6 infectious units of $EIAV_{Wyo}$ virus stock. Blood samples were drawn daily, and antibody to EIAV p26 was monitored by agar gel immunodiffusion (10). The horse was euthanized 16 days after infection, and both blood and 1-cm^3 pieces of major organs were collected and frozen immediately at −70°C.

RNA extraction. Polyadenylated RNA was prepared from frozen horse liver tissue by using a commercial kit (Invitrogen Corp.). Residual DNA was removed by DNase treatment in a reaction mixture containing 25 mM Tris (pH 7.8), 5 mM $MgCl_2$, 1 U of RNasin (Promega) per μl, and 1 to 10 U of DNase per μg of RNA. The RNA was then extracted with phenol and chloroform and ethanol precipitated.

Oligonucleotides. Oligonucleotide sequences are shown in Table 1. $CB27^+$ has a *Hin*dIII site added to its 5′ end and changes the putative *tat* CTG_{Leu} initiation codon to an ATG_{Met}. $CB28^-$ has a *Pst*I site added to its 5′ end. Melting temperatures (T_m) used to determine primer annealing and blot hybridization conditions were calculated by the nearest-neighbor thermodynamic method (35).

cDNA synthesis and PCR amplification. Approximately 1 μg of polyadenylated RNA was copied into cDNA in a 20-μl reaction mixture containing 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 500 μM each deoxynucleoside triphosphate, 50 ng of random hexamers, 20 U of RNasin (Promega), and 200 U of RNase H^- Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL). The reaction mixture was incubated at 43°C for 1 h and then diluted to 100 μl with 5 mM Tris (pH 7.8)–0.1 mM EDTA.

One to 5 μl of the diluted cDNA reaction was amplified by PCR in a 100-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 200 μM each deoxynucleoside triphosphate, 250 nM (each) forward and reverse primers, and 2.5 U of *Taq* polymerase (Perkin-Elmer Corp.). The incubation profile was 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1 to 3 min at 72°C; and 15 min at 72°C.

For each PCR reaction, 10 to 35 μl was electrophoresed on 5% nondenaturing polyacrylamide gels (acrylamide/bisacrylamide ratio, 29:1) in 1× Tris-borate-EDTA. DNA was visualized by staining with ethidium bromide, and the amplified fragments were either blotted or excised from the gel. Samples were also electrophoresed on 5% denaturing polyacrylamide gels (acrylamide/bisacrylamide ratio, 19:1) containing 8 M urea and blotted as described below.

Blotting and hybridization. Amplified DNA was transferred from polyacrylamide gels to positively charged nylon membranes (Boehringer Mannheim Biochemicals) by alkaline semidry electroblotting in 20 mM ethanolamine-glycine, pH 11.0 (60). Oligonucleotide probes were end labeled with [γ-^{32}P]ATP (56) and purified by chromatography on C_{18} Sep-Pak cartridges (Waters) (56). Hybridization solutions contained 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1 mM EDTA, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 100 μg of denatured salmon sperm DNA per ml, and 0.5 pmol of labeled oligonucleotide per ml. Hybridizations were overnight at approximately T_m − 20°C. The final wash was in 0.5× SSC–0.5% SDS at T_m − 5 to 10°C.

Sequencing and cloning. Individual amplified fragments were recovered from polyacrylamide gel slices by either diffusion (56) or electroelution (68). Nucleotide sequences of gel-purified fragments were determined directly by cyclic double-stranded chain termination sequencing with *Taq* polymerase, by using a commercial kit (GIBCO-BRL). Each reaction mixture contained 30 to 50 fmol of eluted DNA. Sequence data were assembled and analyzed with the GCG Sequence Analysis Software Package, versions 6.2 and 7.0 (12).

The pRSPA expression vectors consist of pBluescript-K/S (Stratagene Cloning Systems) with the Rous sarcoma virus promoter and the simian virus 40 polyadenylation signal inserted at the ends of the multiple cloning sites (13). Fragments amplified with primers $CB27^+$ and $CB28^-$ were digested with *Hin*dIII and *Pst*I, ligated into pRSPA-K (sense), and transformed into competent DH5α cells (GIBCO-BRL). Plasmid DNA was prepared from ampicillin-resistant colonies, and the insert size was verified by restriction digestion. Inserts of individual clones were sequenced to verify the absence of *Taq* polymerase-induced mutations.

Peptides and antisera. Synthetic peptide (SP) 185 represents the C terminus of the transmembrane protein and has been described previously (also called tm-3 [51]). SP1060 represents the N terminus of the *tat* protein (residues 2 to 16) and has the sequence Ala-Asp-Arg-Arg-Ile-Pro-Gly-Thr-Ala-Glu-Glu-Asn-Leu-Gln-Lys-Cys. The C-terminal cysteine does not occur in Tat and was added to allow coupling to keyhole limpet hemocyanin. Peptides were coupled and administered to rabbits as described previously (50).

Immunoprecipitation. Confluent T75 flasks of EIAV-infected and uninfected Cf2Th cells were lysed in TNT buffer (20 mM Tris [pH 7.2], 200 mM NaCl, 1% Triton X-100) and centrifuged at 10,000 × *g* for 10 min. The supernatant was divided into thirds and was immunoprecipitated with normal rabbit serum, anti-SP1060, or anti-SP185. The precipitate was collected on protein A-Sepharose (Pharmacia), washed in TNT, and then eluted from antibody by incubation with excess cognate peptide. The normal rabbit serum precipitate was incubated with SP1060. Aliquots of 10% of the eluate were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by immunoblotting.

Immunoblotting. Proteins were transferred from polyacrylamide gels to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp.) by semidry electroblotting (32). Immunodetection was with rabbit anti-SP1060 (1:5,000), peroxidase-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim) (1:10,000), and a chemiluminescent substrate (ECL Western blotting detection system; Amersham Corp.).

Transfections. Plasmid DNA was prepared by alkaline lysis and purified with pZ523 columns (5 Prime→3 Prime, Inc.). For transient transfections, a calcium phosphate precipitate (67) containing 500 ng of plasmid DNA and 7 μg of calf thymus DNA carrier was applied in 3 ml of medium to 30% confluent D17 cells in a T25 flask, incubated for 4 h, and then replaced with fresh medium. Cells were harvested 2 days later and analyzed by immunoblotting. Stable transfections were performed as above, with the addition of 50 ng of *Bam*HI-cut pSV2Neo DNA. After 24 h, the cells were split into medium containing 600 μg of G418 per ml. Colonies of resistant cells were isolated with cloning cylinders, and cell lines expressing Ttm were identified by immunoblot analysis with Tat peptide antiserum.

Indirect immunofluorescence. Cells transiently or stably expressing Ttm were seeded on sterile acid-etched coverslips in a 24-well culture dish and incubated overnight. Staining for immunofluorescence was essentially as described by Frangioni et al. (16). Cells were fixed in 2% paraformaldehyde and quenched in 50 mM glycine. The following immunostaining manipulations were in phosphate-buffered saline (PBS) containing 0.1% Nonidet P-40. Fixed cells were permeabilized, blocked with 5% goat serum, incubated with diluted preadsorbed primary antibody for 1 h at 37°C, washed three times, and incubated with a 1:20 dilution of fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G (Kirkegaard & Perry Laboratories, Inc.) for 45 min at 37°C. Stained cells were washed three times in PBS without Nonidet P-40, counterstained with 1% Evans blue, rinsed, and mounted on glass slides with Vectashield (Vector Laboratories). Cells were photographed with a Leitz Ortholux-II microscope with T-Max 400 black-and-white film (Kodak).

Nucleotide sequence accession number. The GenBank accession number for the sequence reported here is M93674.

RESULTS

Previous studies of tissue culture-adapted EIAV have identified several alternatively spliced transcripts, three of which are known to encode viral proteins. In order to provide the background for understanding the transcript mapping results described below, we will first briefly review the structures and gene products of the known EIAV mRNAs.

A singly spliced transcript (Fig. 1B, transcript a) contains three open reading frames (ORFs), encoding the envelope polyprotein as well as the Tat and S2 proteins (43, 58). Translation of EIAV *tat*, unlike that of HIV, begins in exon 1 and continues into exon 2, which encodes the transactivating domain. Relatively few of the envelope mRNAs are expected to express Tat, since translation initiates at a CUG codon in exon 1 (6). Immediately downstream from *tat* in this message is the S2 ORF. Translation from the first AUG predicts a protein of 65 amino acids which, although not yet detected in infected cells, is recognized by antibody from EIAV-infected horses (58). Translation of S2 is also expected to be somewhat inefficient, given the context of the first AUG (UxxAUGG). Thus, most ribosomes should reach the next AUG in the message, which is the initiator for the Env polyprotein. The singly spliced transcript may thus give rise to Tat, S2, or envelope.

Two multiply spliced messages, containing three exons and four exons (Fig. 1B, transcripts c and b, respectively), have been described (13, 44, 58, 62). Both of these messages have the same first splice as the envelope message and are thus also expected to translate Tat. The four-exon transcript (transcript b) contains two additional splices which remove S2 and most of Env, joining exons 2a (*tat*), 3, and 4. A small region from near the 5′ end of *env* in exon 3 is fused to the *rev* ORF in exon 4, encoding the putative Rev protein (as with *tat*, this arrangement is unlike that of HIV, where the first exon of *rev* is upstream from *env*). The portion of the *env* ORF in exon 4 has no obvious potential initiation codon, and thus the products of this message are expected to be Tat and Rev. In the three-exon transcript, the second splice bypasses exon 3 and joins exons 2a to 4 (Fig. 1B, transcript c). Neither the *rev* nor the *env* ORFs in exon 4 have suitable AUG codons, so the predominant product of this transcript is expected to be Tat.

Finally, a small singly spliced transcript containing only exons 1 and 4 has been observed as a single cDNA clone in a library of 75 but has not been demonstrated to encode a viral protein (44).

Infection with pathogenic EIAV. In order to examine the viral transcripts and gene products produced in vivo, a horse was infected with pathogenic $EIAV_{Wyo}$. The horse became febrile on day 8 postinoculation and was leukopenic from day 11 onward. On day 16 the animal seroconverted, became thrombocytopenic, and first demonstrated anorexia and depression. The horse was euthanized on day 16, and both blood and tissue samples were collected.

EIAV transcription in an acutely infected horse. To determine the structure of viral mRNAs in the acutely infected horse, we reverse transcribed and amplified polyadenylated RNA from liver tissue. The liver was selected as a source for RNA because, among tissues that we previously surveyed (52), it contains the highest concentration of viral DNA. Forward primers from the first exon were matched with a series of reverse primers from the downstream exons and introns of the alternatively spliced transcripts described above (Fig. 1B and C). Amplification with primers located in exons 1 and 2a produced a single 0.23-kb fragment (Fig. 2A,

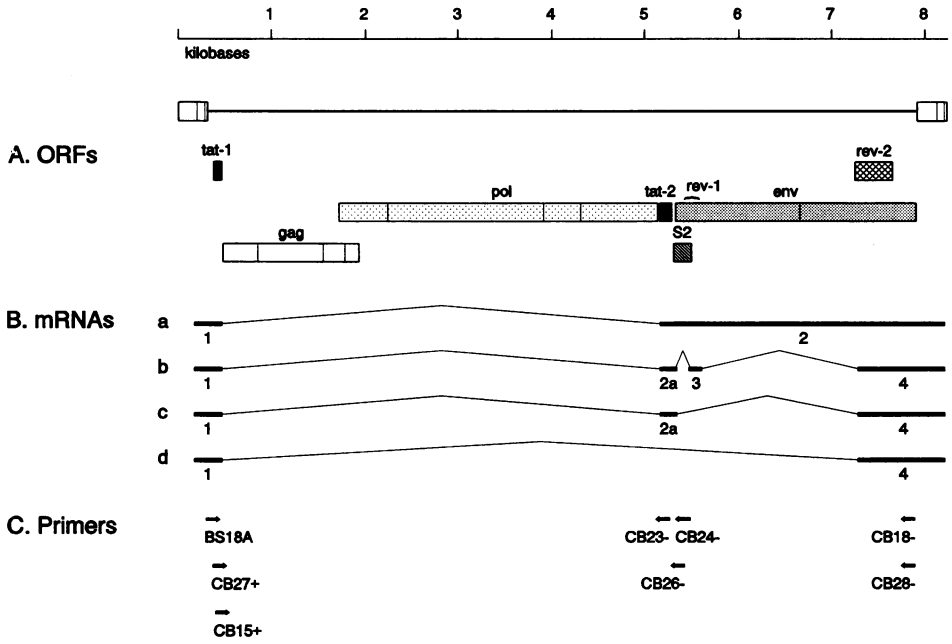

FIG. 1. Organization of the EIAV genome. (A) Known ORFs are shown as open or shaded boxes. (B) Principal transcripts from tissue culture-adapted virus as previously defined by ourselves (62) and others (13, 42, 44, 58). Minor alternative splice sites are not shown. Exons are identified by numbers. (C) Locations of oligonucleotide primers and probes (see also Table 1).

lane 1). A band of this size is predicted for three of the spliced transcripts described above (Fig. 1B, transcripts a, b, and c). A 4.9-kb band from full-length transcripts is not present, but a fragment of this size would not be expected to amplify efficiently. Primers from exon 1 and intron 2a/3 produced a single 0.38-kb band (Fig. 2A, lane 2), as expected from the envelope message (Fig. 1B, transcript a). Amplification with primers from exons 1 and 3 produced two bands (Fig. 2A, lane 3): a 0.50-kb band from the *env* message (Fig. 1B, transcript a), and a 0.34-kb band from the four-exon *tat/rev* message (Fig. 1B, transcript b). Finally, the amplification with primers from exons 1 and 4 produced four bands (Fig. 2A, lane 4): a 2.9-kb band from envelope, a 1.0-kb band from the four-exon message, a 0.91-kb band from the three-exon message, and a 0.77-kb band consistent with the transcript containing only exons 1 and 4 (Fig. 1B, transcript d).

Several additional amplified fragments were also detected on ethidium bromide-stained gels. Minor bands larger than the *env*-derived band were present in exon 1-3 amplifications, and to a lesser extent in exon 1-2a and exon 1-intron amplifications (Fig. 2A, lanes 1-3). Such bands could be produced by transcripts larger than *env* with splice acceptors upstream from exon 2/2a (43). Alternatively, they could be heteroduplexes between homologous amplified bands derived from overlapping transcripts (69). When the amplification reactions were electrophoresed on denaturing polyacrylamide gels, blotted, and hybridized with an oligonucleotide probe derived from exon 1 (Fig. 2B), the minor bands were not detected, as would be expected for heteroduplexes. These bands will not be considered further.

For comparative purposes, viral transcripts from Cf2Th cells persistently infected with cell culture-adapted EIAV were also amplified. Primers from exons 1 and 4 produced

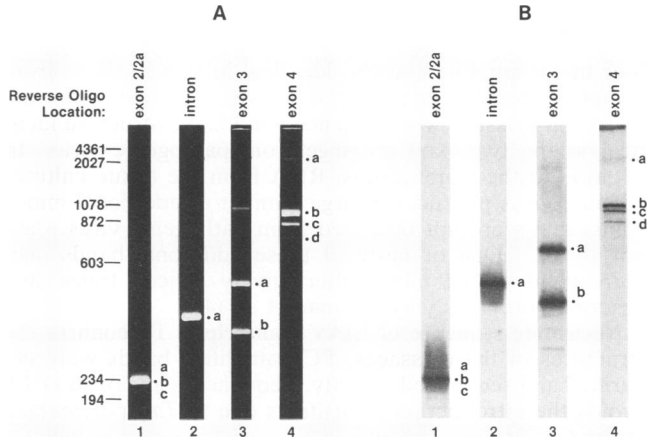

FIG. 2. PCR amplification of pathogenic EIAV mRNAs. Polyadenylated liver RNA from the acutely infected horse was amplified with a series of primer pairs. Forward primers, located in exon 1, were CB27$^+$ (lanes 1, 2, and 4) and BS18A (lane 3). The locations and designations of reverse primers were exon 2/2a, CB23$^-$ (lane 1); intron between exons 2a and 3, CB26$^-$ (lane 2); exon 3, CB24$^-$ (lane 3); and exon 4, CB28$^-$ (lane 4). The transcript(s) that produced each amplified fragment is indicated by letters corresponding to those used in Fig. 1B: a, exons 1 and 2 (*tat*, S2, and *env*); b, exons 1, 2a, 3, and 4 (*tat* and *rev*); c, exons 1, 2a, and 4 (*tat*); d, exons 1 and 4. (A) Ethidium bromide-stained 5% polyacrylamide gel of amplified fragments. The sizes of selected ϕX174-RF *Hae*III and λ *Hin*dIII fragments are shown at the left. (B) Southern blot of denatured amplified fragments. PCR mixtures were electrophoresed on a denaturing 5% polyacrylamide gel, transferred to nylon, and probed with the exon 1-specific oligonucleotide CB15$^+$.

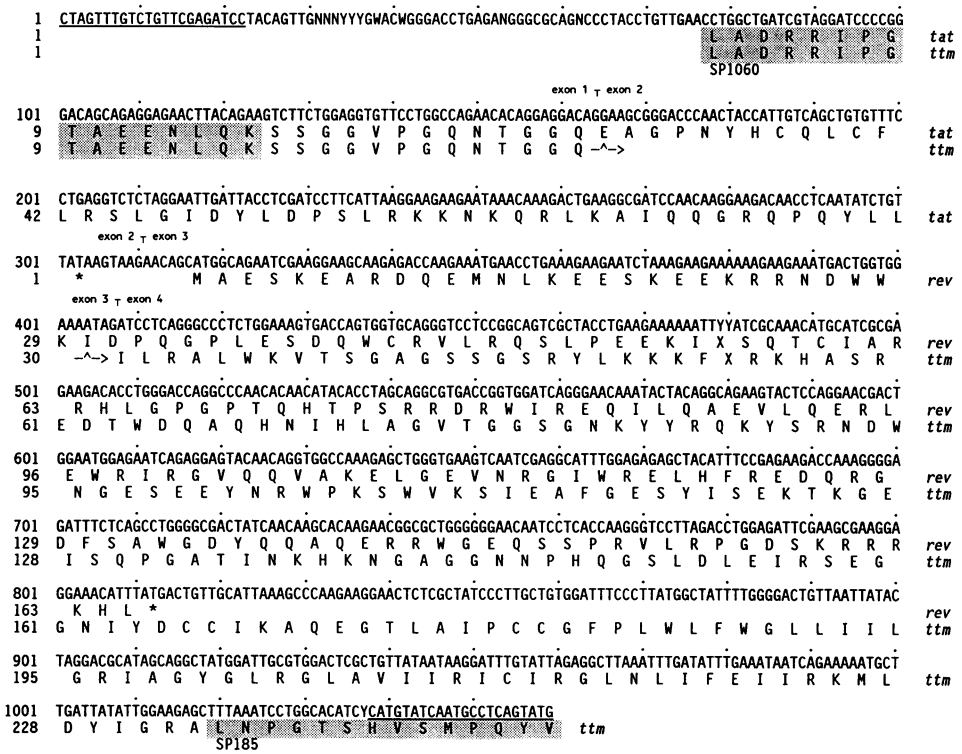

FIG. 3. Sequence of the four-exon transcript from pathogenic EIAV. The sequence is bounded by PCR primers BS18A and CB18$^-$ (underlined) and was compiled from direct sequencing of the uncloned PCR amplification products from the four-, three-, and two-exon transcripts. Bases near the primers could not be defined with certainty. IUB ambiguity codes are used as follows: W = A or T, Y = C or T, and N = any base. Splice junctions are indicated. The predicted translation products of the *tat*, *ttm*, and *rev* ORFs are shown below the sequence. Amino acid sequences used for synthetic peptides are in shaded boxes.

four major amplified bands, identical in size to those from acutely infected liver (data not shown). The band from the two-exon message was sequenced and had a structure identical to the two-exon message from pathogenic virus. In addition to the major bands, RNA from the tissue culture-adapted virus produced a large number of additional minor bands that were not observed from pathogenic virus (data not shown). One or more of these additional bands may correspond to the minor alternatively spliced transcripts described previously by Noiman et al. (44).

Nucleotide sequence of EIAV transcripts. To confirm the structures of the messages, PCR-amplified bands were gel purified and sequenced directly. Sequencing uncloned DNA avoids the introduction of artifacts due to *Taq* polymerase errors (14) or biased growth in *Escherichia coli* (see below). The three small bands from the exon 1-4 amplification were sequenced completely (Fig. 2A, lane 4, bands b, c, and d), and the splice junction for *env* was sequenced from the exon 1-3 and exon 1-4 amplifications (Fig. 2A, lanes 3 and 4, band a). Comparison of the sequences revealed complete identity between the common exons of all four transcripts (Fig. 3).

The PCR and nucleotide sequence data reveal that the structure and predicted gene products of pathogenic EIAV transcripts are essentially identical to those of the major transcripts from tissue culture-adapted virus. EIAV$_{Wyo}$ produces only four detectable spliced transcripts, all generated by using the same three donors and three acceptors that have been defined previously in nonpathogenic virus. The predicted translation products of three of these messages are also as previously characterized: the singly spliced transcript (Fig. 1B, transcript a) encodes Tat, the S2 protein, and the envelope precursor; the four-exon transcript (transcript b) encodes Tat and Rev; and the three-exon transcript (transcript c) encodes Tat. Unlike the *rev* ORFs characterized in some isolates of tissue culture-adapted virus (13, 62), EIAV$_{Wyo}$ *rev* does not contain deletions that prematurely terminate *rev* or shift the reading frame to the parallel *env* ORF. The remaining two-exon transcript (transcript d) has not been well characterized in nonpathogenic virus. Its sequence predicts a protein that has not been described previously in EIAV or other lentiviruses; the *tat* ORF in the first exon is joined in frame to the C-terminal end of the TM region of the *env* ORF, immediately downstream from the putative membrane-spanning region. We have designated this truncated transmembrane protein as Ttm. Only the exon 1-4 message has the potential to express the *ttm* protein; there are no suitably located AUG codons in either the TM ORF itself or in the upstream sequences in frame with TM in either the four-exon or three-exon messages.

Lentiviruses have been described as populations of variant viral genomes, or quasispecies. Any such subpopulation comprising a significant fraction of the EIAV$_{Wyo}$ genomes would be detected by direct nucleotide sequencing of PCR-amplified cDNA; base differences would appear as reproducible multiple bands at a single position on a sequencing gel. We observed only two such occurrences: at positions 479 to 480 (Fig. 3), the sequence read as $\frac{CT}{TC}$, with the intensity of the C and T in the first position approximately equal, and of the T in the second position significantly stronger than the C (data not shown). The actual representation in the viral

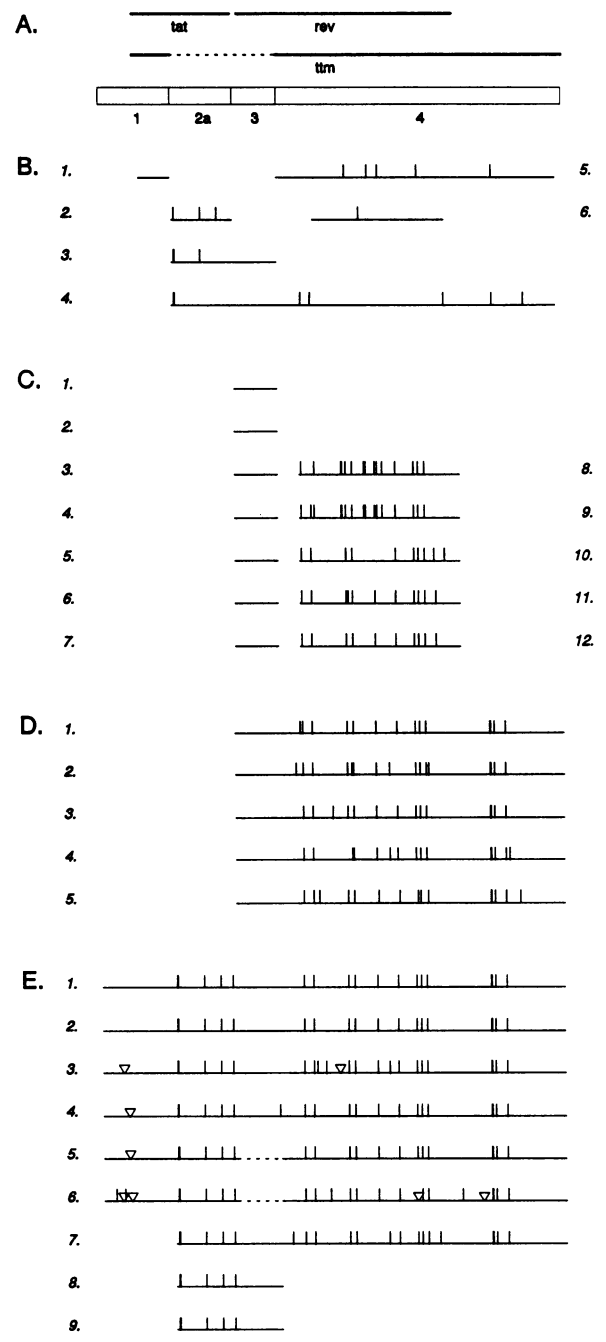

FIG. 4. Nucleotide sequence comparison between $EIAV_{Wyo}$ cDNA and other pathogenic and tissue culture-adapted isolates. EIAV sequences available on GenBank, release 00.2, are represented graphically as individually numbered lines. Introns have been removed where necessary to preserve alignment. Dashes indicate missing exons in cDNAs. The positions of nucleotide differences between each sequence and the $EIAV_{Wyo}$ cDNA are indicated by vertical lines. Deletions are indicated by triangles. (A) Location of *tat*, *rev*, and *ttm* ORFs with respect to the four exons of the $EIAV_{Wyo}$ cDNA. (B) PCR-amplified proviral DNA from $EIAV_{Wyo}$, accession numbers M87575, M87586, M87582, M87580, M87587, and M87593. (C) PCR-amplified proviral DNA from an independent pathogenic field isolate, $EIAV_{Th-1}$, accession numbers M62655 to M62661, M62679, M62676, M62677, M62678, and M62680. (D) cDNAs from tissue culture-adapted virus reintroduced into horses. Lines 1 to 4, cDNA to viral RNA of isolates from sequential febrile periods, accession numbers M18385 to M18388; line 5, cDNA from $EIAV_{WSU5}$, accession number X16988. (E) Tissue culture-adapted EIAV. All isolates are derived from original adaptation of $EIAV_{Wyo}$ to tissue culture (34) except line 7, which is derived from $EIAV_{Th-1}$. Line 1, GenBank version of complete proviral genome, accession numbers M61575, K03334, M11337, and M14855; line 2, infectious proviral molecular clone CL22, accession number M87581; lines 3 to 6, cDNAs to multiply spliced transcripts, accession numbers M54797, M36592, S55705, and M30138; line 7, proviral DNA from $EIAV_{MA-1}$, accession number M58038; line 8, cDNA to virion RNA, accession numberM21653; line 9, proviral DNA, accession number M33845.

population is unclear, as the relative abundance of each base may be affected by inconsistent termination during direct sequencing. Among five clones of the exon 1-4 cDNA, only one had the bases CT, while the rest had the bases TC (data not shown), perhaps due to selection in *E. coli* against the apparently more abundant nucleotide combination. Otherwise, the clones were 99.6 to 100% identical to the direct sequence. Two clones matched the direct sequence exactly. One clone had a G→A substitution at position 424 (Fig. 3) that introduced a stop into the *ttm* ORF. This may be derived from a naturally occurring variant, as a stop at this position has been seen previously in a cDNA derived from tissue culture virus (13). Four other base changes distributed among the remaining two clones were not evident by direct sequencing and could not be characterized as either viral or PCR derived.

Nucleotide sequence comparisons. In order to determine if there is a pattern to the nucleotide differences between pathogenic EIAV and virus adapted to growth in tissue culture, the nucleotide sequence of the four-exon $EIAV_{Wyo}$ cDNA was compared with previously published EIAV sequences representing both pathogenic and cell culture-adapted virus (Fig. 4). The sequences are all quite similar. Three areas are 100% identical among nearly all of the isolates: the entire exon 3 and bases 745 to 879 and 927 to 1059 in exon 4 (Fig. 3 and 4B to E). Recently described clones of PCR-amplified $EIAV_{Wyo}$ proviral DNA (49) are nearly identical to the $EIAV_{Wyo}$ cDNA sequence (0 to 2.1% divergence) (Fig. 4B). The remaining sequences (Fig. 4C to E) are all clearly distinct from $EIAV_{Wyo}$ and very similar to each other. Most of these sequences are derived from the original tissue culture adaptation of $EIAV_{Wyo}$ (34). Interestingly, the sequences of isolates derived by reintroducing tissue culture-adapted virus into horses do not revert to a form more similar to $EIAV_{Wyo}$ but remain essentially identical to the parental attenuated virus (Fig. 4D). This suggests that nucleotide differences between the four exons of tissue culture-adapted EIAV and pathogenic $EIAV_{Wyo}$ are not specifically related to the nonpathogenic phenotype. Furthermore, the independent pathogenic field isolate $EIAV_{Th-1}$ (5) is extremely similar to the Wyoming-derived tissue culture virus: exon 4 of $EIAV_{Th-1}$ (Fig. 4C, lines 8 to 12) is only 0.3 to 3.2% divergent from tissue culture-adapted EIAV and 3.2 to 4.9% divergent from pathogenic $EIAV_{Wyo}$. These observations are consistent with the recent finding that the in vitro host range of tissue culture-adapted virus is not altered by replacement of its envelope gene with *env* from pathogenic $EIAV_{Wyo}$ (49).

The exon 1-4 transcript expresses Ttm in EIAV-infected cells. The proposed *ttm* protein contains 247 amino acids, 29 from Tat and 218 from TM, and has a predicted molecular mass of 27 kDa. To determine whether the protein is actually

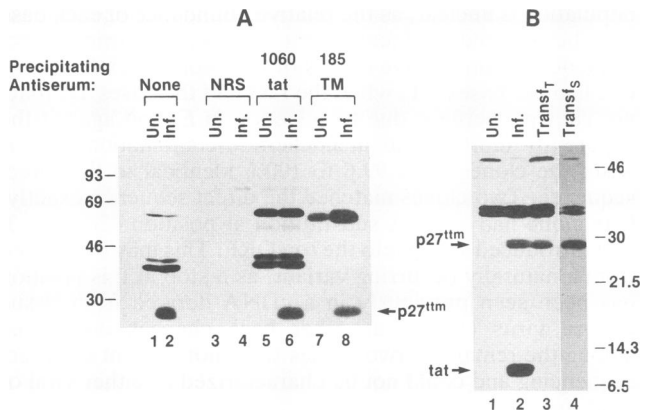

FIG. 5. Detection of *ttm* expression in persistently infected and transfected canine cells. (A) Uninfected and virus-producing Cf2Th cells. Untreated (lanes 1 and 2) or immunoprecipitated (lanes 3 to 8) lysates were fractionated on an SDS–10 to 20% polyacrylamide gel, blotted, and probed with Tat peptide antiserum (anti-SP1060). Immunoprecipitation was with anti-peptide sera specific for Tat (lanes 5 and 6) or TM (lanes 7 and 8) or with normal rabbit serum (lanes 3 and 4). (B) D17 cells were transfected with an expression plasmid containing *ttm* cDNA. Lysates of transiently and stably transfected cells (lanes 3 and 4), as well as EIAV-infected (lane 2) and uninfected D17 cells (lane 1), were electrophoresed on an SDS–15% polyacrylamide gel, blotted, and probed with Tat peptide antiserum (anti-SP1060).

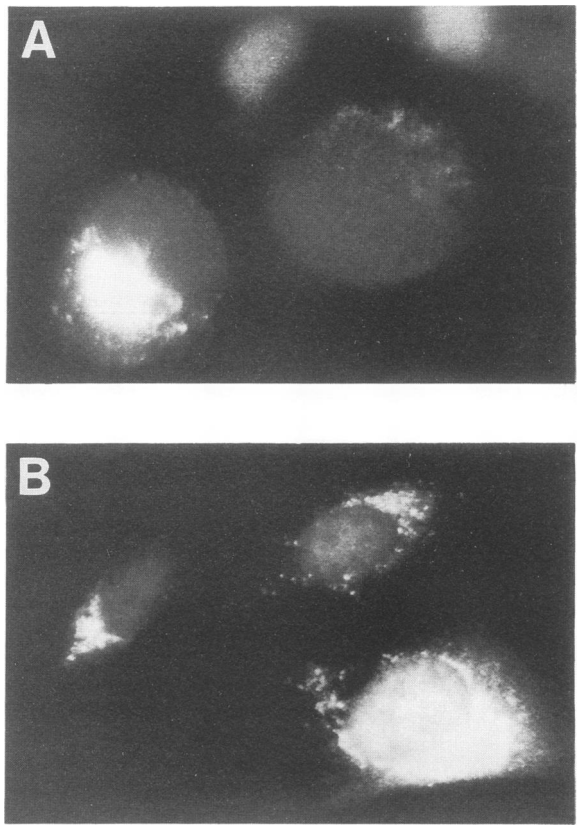

FIG. 6. Localization of Ttm in transfected cells. Transfected D17 cells expressing *ttm* were fixed and subjected to indirect immunofluorescence with TM peptide antiserum (anti-SP185). (A) Transient transfection; (B) stable transfection.

expressed in EIAV-infected cells, we probed an immunoblot with a peptide antiserum (anti-SP1060) directed against the N terminus of Tat. A 27-kDa protein was detected in infected cells but not in uninfected cells (Fig. 5A, lanes 1 and 2). Tat itself is 8 kDa and is not present on this blot. To test whether p27 also contains TM epitopes, a lysate from infected cells was immunoprecipitated with anti-SP185, which recognizes the extreme C terminus of TM. The precipitate was fractionated by SDS-PAGE and analyzed by immunoblotting with the anti-Tat serum. Once again a 27-kDa protein was seen in infected but not in uninfected cells (lanes 7 and 8). No protein was detected if the initial precipitation was performed with normal rabbit serum rather than anti-SP185 (lanes 3 and 4). This experiment demonstrates that there is a 27-kDa protein in EIAV-infected cells that has both Tat and TM reactivity. We conclude that p27 results from translation of the exon 1-4 transcript.

To test this conclusion, we expressed a cloned exon 1-4 cDNA in canine cells. Liver mRNA was amplified with primers that changed the *tat* CTG initiation codon to an ATG and then cloned in the expression vector pRSPA (13). Transiently and stably transfected D17 cells were examined for Ttm expression by immunoblotting and detection with Tat peptide antiserum (Fig. 5B). The 27-kDa Ttm protein was detected in both transiently and stably transfected cells (lanes 3 and 4) and in EIAV-infected cells (lane 2) but not in uninfected, mock-transfected cells (lane 1). Thus, the protein encoded by the transfected exon 1-4 cDNA comigrates with the p27 seen in infected cells, confirming that the latter is p27^{ttm}.

Ttm is localized in the endoplasmic reticulum or Golgi. Since Ttm lacks both the signal sequence and the putative membrane-spanning region of the envelope polyprotein, it may not have the same cellular or viral localization as the Env precursor or TM. To test this, about 25 μg of purified EIAV (double banded in sucrose density gradients) was fractionated by SDS-PAGE and analyzed by immunoblotting with the Tat antiserum (anti-SP1060). No p27^{ttm} was detected (data not shown). Thus, like the regulatory proteins Tat and Rev, Ttm may be predominantly intracellular. We next examined its intracellular localization by indirect immunofluorescence with TM antiserum. Transfected D17 cells expressing Ttm were used to avoid serum cross-reaction with the C terminus of Env or TM. Expression constructs with the initiation codon of Ttm changed to an AUG were used to ensure that sufficient protein would be synthesized for detection by immunofluorescence; previous experiments have shown that Tat expressed from the weakly initiating natural CUG codon cannot be detected by immunofluorescence. As shown in Fig. 6, both transiently and stably transfected cells showed a strong punctate perinuclear fluorescence consistent with localization of Ttm in the endoplasmic reticulum or Golgi apparatus. No staining was seen in untransfected D17 cells (data not shown).

DISCUSSION

In the present study, we used PCR and nucleotide sequencing to define the structure and predicted gene products of transcripts from pathogenic EIAV in a horse in the early stages of infection. To our knowledge, this is the first detailed characterization of transcripts from a pathogenic lentivirus in vivo. Transcripts from pathogenic EIAV are essentially the same as the major messages previously de-

fined in vitro for tissue culture-adapted virus. There is, however, no evidence for the minor alternatively spliced transcripts that have also been described for EIAV in culture and which are abundant in HIV- and SIV-infected cells. We also found that one of the EIAV transcripts expresses the C terminus of the EIAV TM protein as a distinct gene product in infected cells. The discovery of this novel protein, Ttm, is particularly significant; translation of this region of TM in EIAV and SIV is often selected against during passage in culture, suggesting that Ttm may play an important role in lentiviral growth or infectivity.

EIAV transcripts characterized previously have been derived only from tissue culture-adapted virus (13, 42, 44, 58, 62) and might be expected to differ from those of pathogenic virus that has not undergone adaptation. Because lentiviruses are subject to extensive genetic change during replication, modulation of selective pressures in vivo or in vitro can produce variants that are significantly altered in biological properties such as growth and pathogenicity. The cyclic recrudescence of EIAV following primary infection is associated with the emergence of *env* variants refractory to neutralization by previously induced antibody (5, 40). Adaptation of EIAV to growth in tissue culture results in a broadened host range in vitro but loss of pathogenicity in vivo (3, 4). Subsequent passage in culture can produce identifiable changes in viral gene expression, such as truncation of the C terminus of the transmembrane protein (51). Because of in vitro variations such as these, relating gene expression to pathogenesis requires in vivo corroboration of observations made in tissue culture.

The spliced messages transcribed from pathogenic EIAV in vivo are identical to the major transcripts described previously for tissue culture-adapted virus in vitro. We detected four spliced messages: a singly spliced transcript predicted to encode *env*, *tat*, and the putative product of the S2 ORF; a four-exon transcript encoding *tat* and *rev*; a three-exon transcript encoding *tat*; and a two-exon transcript encoding the novel *ttm* protein. The structure and gene products of the first three transcripts have been well characterized for tissue culture-adapted virus (13, 42, 44, 58, 62), and conservation of these transcripts in attenuated virus suggests an important role for each. For example, expression of *tat* from multiple messages may be necessary to compensate for reduced translational efficiency from the CUG initiation codon. The exon 1-4 transcript has been described previously only once, as a single cDNA clone in a library derived from cultured cells persistently infected with EIAV, but the message was not characterized further (44).

Although pathogenic and tissue culture-adapted EIAV produce the same four major spliced transcripts, the pathogenic virus does not appear to produce significant quantities of any minor alternatively spliced transcripts; in side-by-side amplifications, only RNA from tissue culture-adapted virus yielded detectable quantities of multiple minor, amplified bands. These results are consistent with previous reports of a number of minor alternatively spliced transcripts from EIAV in tissue culture. Prior descriptions of the three-exon *tat* message have identified two different splice acceptors for exon 4: one identical to that used by the 4-exon *tat/rev* transcript (13) and another located 8 bp downstream (44). In acutely infected liver, however, all detectable splices into exon 4 use the same acceptor, which is identical to the previously defined upstream site (13, 62). Alternatively spliced versions of the four-exon transcript have also been seen. A cDNA library from tissue culture-adapted EIAV RNA contained three forms of this message: a predominant species identical to the one produced by pathogenic virus and two minor forms with the exon 2a acceptor displaced downstream or the exon 4 acceptor displaced upstream (44). Although amplifications of tissue culture-adapted EIAV RNA produced a variety of minor bands, we do not know which, if any, correspond to the alternatively spliced transcripts that have been seen previously. Nevertheless, the difference between the amplification patterns of pathogenic and tissue culture-adapted EIAV leads us to suggest that alternative splice sites are arising as a result of passage in tissue culture. Cultured cells infected with HIV or SIV produce an abundance of alternatively spliced transcripts (1, 18, 47, 53, 59, 64), but it is not known to what extent similar splicing patterns occur in vivo. Conceivably, alternative splice sites may arise during passage in vitro and produce transcripts which are not necessary in vivo, but which either confer a selective advantage or simply persist nonselectively in culture.

The exon 1-4 transcript encodes a previously undescribed protein that we have designated Ttm. This is the first reported occurrence of the independent expression of the carboxy terminus of a lentiviral transmembrane protein. This region tends to be considerably longer in lentiviruses than in other retroviruses, suggesting a possible additional function. Transcripts have been described for most other lentiviruses that might encode a Ttm-like protein. In HIV-1, a three-exon transcript produces a 17-kDa Tat/TM hybrid protein when translated in vitro (17), but the protein has not been identified or characterized in infected cells. A singly spliced transcript resembling the EIAV *ttm* message has also been reported; the 5′ viral exon is joined to the *env* ORF immediately downstream from the TM membrane-spanning domain (17, 53). Neither the 5′ exon nor the TM ORF contains an AUG codon in good context (29), but initiation in the 5′ exon at a contextually suitable CUG codon (30, 38) would produce a 167-amino-acid, 19-kDa Ttm-like protein. For SIV_{mac}, two transcripts which place a GUG in good context in frame with the carboxy terminus of TM and predict a 158-residue, 19-kDa Ttm-like protein have been described (47). Although initiation of translation at non-AUG codons has not been documented in HIV or SIV, its occurrence in EIAV suggests that the possibility should be considered. In visna virus, transcripts splicing an N-terminal *env* exon to TM have been described (37, 57), and one sequence variant contains an AUG in the *env* exon from which a 135-residue, 17-kDa Ttm-like protein can be translated in vitro (37). Caprine arthritis-encephalitis virus transcripts join either the 5′ exon or a central exon to the carboxy terminus of TM (24, 55). No contextually acceptable AUG or non-AUG initiation codons exist in either of the exons joined to TM, but translation from an AUG 49 bp downstream from the splice acceptor would produce a 94-amino-acid, 11-kDa Ttm-like protein. Finally, in the three-exon *rev* transcript of bovine immunodeficiency virus (45), an AUG immediately downstream from the last splice could initiate translation of a 159-amino-acid, 18-kDa Ttm-like protein. The occurrence of these transcripts suggests that lentiviruses other than EIAV may also be expressing their TM carboxy termini as separate Ttm proteins. We are now testing this premise for HIV type 1 (HIV-1).

Although the biological properties of Ttm could be derived from either or both of its two exons, the TM sequences are likely to be the most important. Unlike HIV *tev*, which encodes fusions of Tat to Rev and/or Env (2, 17, 54), the *tat* exon of *ttm* does not encode sequences involved in transactivation (13) and has no other known functions. In contrast,

the C termini of lentiviral TM proteins, although not fully characterized, have been demonstrated to interact with cellular membranes. We have shown that the EIAV *ttm* protein localizes in the endoplasmic reticulum or Golgi in transfected cells, and others have found that the HIV-1 TM C terminus may also mediate intracellular retention. Both the HIV-1 envelope precursor gp160 and its 147-amino-acid C-terminal polypeptide associate with microsomal membranes and are retained intracellularly in transfected cells (19, 20). Removal of the C-terminal 106 amino acids from gp160 allows its export to the cell surface (20). This membrane association in the absence of the TM membrane-spanning domain has been proposed to be mediated by one or both of two predicted amphipathic alpha helices that are highly conserved among HIV-1 isolates (15, 61, 65). Two such helices are also predicted for EIAV (Ttm residues 100 to 122 and 205 to 234), SIV, and bovine immunodeficiency virus. Whether these structures can specifically mediate intracellular retention is not known, but Ttm contains none of the sequence motifs that have been implicated in endoplasmic reticulum retention in either cellular or viral proteins (31, 46, 48, 63).

Identification of the EIAV *ttm* protein adds yet another dimension to the intriguing problem of defining the role of the TM C terminus in lentiviral infection and pathogenesis. The context in which this region is expressed is complex, as it is included in several distinct viral proteins. In EIAV, the TM C terminus is present in at least four discrete proteins: the envelope precursor, its cleavage products gp45 (TM) and p20 (51), and Ttm. Further, these sequences in HIV and SIV have been associated with a wide spectrum of biological effects. A negative regulatory function for viral replication or infectivity has been proposed to account for the coincident appearance of carboxy-terminal TM truncations and enhanced growth kinetics in SIV passaged in certain human cell lines (7, 27, 41, 66). A similar relationship has been observed with EIAV in culture (51). In vivo, however, SIV appears to favor a complete C terminus, as truncated isolates revert to full length (21, 27) or replicate only transiently (36). The region may also play a role in cytopathology, as suggested by the reduced infectivity and cytopathogenicity of extreme C-terminal deletion mutants of HIV-1 (33) and by the prevalence of distal in-frame stops in TM of SIV from asymptomatic, infected macaques (23). Further, the amphipathic helices are structurally similar to naturally occurring cytolytic proteins and, when expressed as individual peptides, are cytolytic in vitro (39, 61). The region has also been implicated in the B-cell stimulatory activity of HIV-1 (8). Which, if any, of these properties may be attributable uniquely to Ttm is unknown, and it is likely that the biological effects of the TM C terminus will be specific to the context in which it is expressed.

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