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Simian Immunodeficiency Virus Displays Complex Patterns of RNA Splicing

GREGORY A. VIGLIANTI,†* PREM L. SHARMA,‡ AND JAMES I. MULLINS§

Department of Cancer Biology, Harvard University School of Public Health, Boston, Massachusetts 02115

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The human and simian immunodeficiency viruses encode at least six gene products that apparently serve regulatory functions. To evaluate the regulation of simian immunodeficiency virus gene expression at the level of RNA splicing, we used the polymerase chain reaction to amplify and clone cDNAs corresponding to a large array of mRNAs from infected cells. We identified mRNAs that used splice acceptor sites upstream of the initiator codons for *tat*, *rev*, *vpr*, *nef*, *vif*, and *vpx*, suggesting that these proteins may be expressed from different mRNAs. We also provide hybridization data suggesting that the same splice acceptor site may be used for both *rev* and *env* mRNAs. Furthermore, we isolated both *tat* and *rev* cDNAs that utilized three alternative splice acceptor sites at the start of coding exon 2, indicating that different versions of these proteins may be encoded. Finally, approximately 10 to 20% of simian immunodeficiency virus mRNAs spliced an intron from their untranslated 5′ ends, and sequences contained within this intron constituted a portion of the *tat*-responsive TAR element. Thus, alternative pre-mRNA splicing adds a level of complexity to simian immunodeficiency virus expression, which may affect several levels of gene regulation.

The genomes of human (HIV-1 and HIV-2) and simian (SIV) immunodeficiency viruses are more complex than the genomes of most other retroviruses. In addition to encoding *gag*, *pol*, and *env*, which are found in all replication-competent retroviruses, the primate immunodeficiency viruses each encode at least six other proteins, *tat*, *rev*, *vif*, *vpr*, and *nef* and either *vpx* (HIV-2 and SIV) or *vpu* (HIV-1). Most of these proteins have been shown to be either positive or negative regulators that affect various aspects of viral expression or infectivity (reviewed in references 8, 18, and 39).

Each of these regulatory proteins is believed to be expressed from spliced, subgenomic-length mRNAs. Furthermore, there is evidence indicating that the temporal accumulation of at least some of these mRNAs is regulated and may be important in the viral life cycle. In the early stages of HIV-1 infection of H9 cells, small, multiply spliced mRNAs, presumably encoding the regulatory proteins, accumulate preferentially; in the later stages, there is an increase in the accumulation of both singly spliced and unspliced mRNAs (23). These studies support a model (1, 8, 10, 11, 18, 36, 41) of the HIV-1 life cycle in which *tat* and *rev* are proposed to modulate early and late phases of viral infection. According to this model, the early phase is marked by the expression of the *trans*-activator protein, *tat*, which is a positive regulator of long terminal repeat (LTR)-directed gene expression (3, 37). *tat* stimulates viral gene expression, which in the absence of *rev* results in the accumulation of multiply spliced mRNAs, including those encoding *tat* and *rev*. The transition to the late phase is marked by the action of the *rev* protein, which is a positive regulator thought to facilitate the generation or nuclear export, and therefore the cytoplasmic accumulation of viral mRNAs containing a *rev*-responsive element located within *env* coding sequences (2, 9, 11, 17, 26). Included among these *rev*-responsive mRNAs are the unspliced and singly spliced mRNAs encoding the virion-associated *gag*, *pol*, and *env* proteins. Late expression of *rev* is also proposed to downregulate the accumulation of the multiply spliced mRNAs encoding the regulatory proteins. This later point is supported by recent evidence indicating that *rev* expression may downregulate the accumulation of at least some of the multiply spliced mRNAs (2).

These temporal changes in the accumulation of viral mRNAs are apparently important; however, insufficient information concerning the splicing patterns of individual mRNAs hinders thorough evaluation of their significance. For example, it is not known whether each regulatory protein is encoded by an individual mRNA or whether some are encoded by multifunctional mRNAs. Nor is it known whether some proteins can be encoded by more than one mRNA. Here we examine the splicing patterns of mRNAs expressed by SIV (isolate BK28) from a rhesus macaque (SIV_{mac}) (25). This analysis provides the opportunity to define the molecular functioning of a virus quite similar to HIV-1 and especially HIV-2 and provides the eventual possibility of examining the significance of these features in an in vivo animal model of lentivirus infection and pathogenesis. We demonstrate that pre-mRNA splicing in SIV_{mac} is more complex than previously thought, and these data add a level of complexity to SIV_{mac} expression which affects several levels of gene regulation.

MATERIALS AND METHODS

RNA isolation and the synthesis of cDNAs. Total cellular RNA was isolated from SIV_{mac} (isolate BK28)-infected HuT78 T cells (31 days postinfection) as previously described (40). Single-stranded cDNAs were synthesized by dissolving 15 μg of total RNA in a volume of 6.5 μl

* Corresponding author.
† Present address: Program in Molecular Medicine and Department of Molecular Genetics and Microbiology, University of Massachusetts Medical Center, 373 Plantation St., Worcester, MA 01605.
‡ Present address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Dana Farber Cancer Institute, Boston, MA 02115.
§ Present address: Department of Microbiology and Immunology, Stanford University Medical School, Stanford, CA 94305-5402.

Oligo	Sequence	Position in genome: Nucleotides	Region
PCRAR	gctccgaattcGGGAGGTTCTCTCCAGCACTAGC	542-564	R-LTR
PCR25	gaatagcatgcCGATAGCAAGGTTCTGCAGTTGCTG	8961-8937	3' *rev*
PCRTAT5'	gaatagcatgcATGAGTTCTCCTGCTCCCTCAAGG	6311-6288	5' *tat*
PCRSTAT	gctccgaattcAGGCCTCCGGTTGCAG^ACATGG	957-972^6276-6281	5' *tat* splice
PBA	GCATCAAACAA^TAGACTCATACCC	6563-6573^8785-8797	splice A probe
PBB	GCATCAAACAA^ACTCATACCCAAC	6563-6573^8788-8800	splice B probe
PBC	GCATCAAACAA^GACCCGGCACTGC	6563-6573^8803-8815	splice C probe
PBTAT	GTGTCTCCATGT^CTGCAACCGGAG	6287-6276^972-961	*tat* probe
PBREV	TATCCCCAATCC^CTGCAACCGGAG	6499-6488^972-961	*rev* probe
PBVPR	TTTATGCTAGTC^CTGCAACCGGAG	6127-6116^972-961	*vpr* probe
PBW	GTGTCTCCATGT^CTATGAGCTCTC	6287-6276^5740-5729	*tat* with upstream exon 1
PBX	GTGTCTCCATGT^CTGGTACCTATG	6287-6276^5747-5736	*tat* with upstream exon 2
PBY	TCTCTGCCTTCT^CTGCAACCGGAG	5199-5188^972-961	*pol* region splice
PBZ	TCACTTCTCCCG^CTGCAACCGGAG	5682-5671^972-961	*vif* region splice
PBS	ACCAGGCGGCGA^CTGCTAGTGCTG	722-711^566-555	spliced LTR
PBU	CCCAGGCTCTACCTGCTAGTGCTG	578-555	unspliced LTR

FIG. 1. PCR primer and hybridization probe sequences. Lowercase letters refer to restriction enzyme cleavage sites added to the PCR primers to facilitate subsequent cloning of the amplified cDNA fragments. Numbers represent the positions of the oligonucleotides relative to the SIV_{mac} BK28 provirus. Sequences and numbers are separated by a carat (^) to indicate juxtaposition of two noncontiguous regions of the viral genome.

containing 1 μg of $p(dT)_{15}$ primer and 2 μl of 5× reverse transcriptase buffer (5× buffer is 250 mM Tris [pH 8.3 at 42°C]–250 mM KCl–30 mM $MgCl_2$). The tubes were heated to 65°C for 10 min and then allowed to cool to room temperature. The samples were brought to a final volume of 10 μl by the addition of dithiothreitol to 10 mM, deoxynucleoside triphosphates to 0.5 mM each, RNasin to 2 U/μl, and avian myeloblastosis virus reverse transcriptase to 2 U/μl. The reaction mixtures were incubated at 42°C for 1 h and then heat inactivated at 75°C for 15 min. One half of each reaction mixture (5 μl) was then directly used in the polymerase chain reaction (PCR) amplification (30, 25) of single-stranded cDNAs with specific primers. Each PCR reaction mixture contained 2.5 U of *Taq* polymerase (Cetus-Perkin Elmer Corp.), 100 pmol of each primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3 at 25°C), and 0.01% gelatin. All amplifications were performed for 35 cycles in a DNA thermal cycler (Cetus-Perkin Elmer), with each cycle consisting of a 45-s denaturation step at 94°C, a 1-min annealing step at 55°C, and a 2.5-min extension step at 72°C. The final extension step was prolonged to 10 min to complete the synthesis of any partial amplification products. Immediately after the last cycle, the amplification products were extracted two times with phenol-chloroform and then precipitated with ethanol. The amplified fragments were digested with appropriate restriction enzymes, gel purified, and cloned into pUC18.

Oligonucleotides. Oligonucleotides were synthesized on a BioSearch model 8750 DNA synthesizer (Fig. 1).

Colony hybridizations. Colonies were grown directly on nitrocellulose filters and processed for hybridization as described previously (15). The filters were prehybridized at 42°C in 6 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1× Denhardt solution–0.05% sodium pyrophosphate–0.25% sodium dodecyl sulfate–9% formamide–100 μg of yeast tRNA per ml. Hybridizations were performed for 14 to 18 h in the same solution containing 5 ng of ^{32}P-end-labeled oligonucleotide probe per ml. The filters were washed three times for 5 min each at 65°C in 6× SSC–0.05% sodium pyrophosphate and then exposed to X-ray film with an intensifying screen at −70°C.

DNA sequencing. Double-stranded plasmid DNA (4 μg) was dissolved in distilled water (20 μl), 2 M NaOH (2 μl) was added, and the samples were kept at room temperature for 5 min. The samples were neutralized by the addition of 0.9 M sodium acetate (pH 5.2) (10 μl) and then precipitated by the addition of 95% ethanol (80 μl). The precipitated DNA was dissolved in Sequenase (U.S. Biochemical Corp.) sample buffer (10 μl) containing 60 ng of sequencing primer. Sequencing reactions were then carried out with Sequenase and [α-^{35}S]dATP according to the protocols of the manufacturer (U.S. Biochemical Corp.).

RNA analysis. Oligo(dT)-cellulose-selected (27) RNA (40 μg) was loaded into a single gel-wide lane of an agarose-formaldehyde gel (27) and electrophoretically separated. RNAs were transferred to a nylon-backed nitrocellulose filter (Stratagene), and the filter was cross-linked with UV light and cut vertically into strips. Each strip was hybridized separately with oligonucleotide probes specific for different spliced mRNAs as follows. The filters were prehybridized at 42°C in 6× SSC, 1× Denhardt solution, 0.05% sodium pyrophosphate, 2.5% sodium dodecyl sulfate, 25% formamide, 100 μg of yeast tRNA, and 75 ng of a nonspecific 24-nucleotide oligonucleotide per ml. Hybridizations were carried out for 14 to 18 h in the same solution containing 5 ng of ^{32}P-end-labeled oligonucleotide probe per ml. The filters were washed three times for 5 min each at 65°C in 1× SSC–0.05% sodium pyrophosphate–0.1% sodium dodecyl sulfate. All hybridizations also included filters containing oligo(dT)-cellulose-selected RNA from uninfected cells and filters containing known amounts of in vitro-synthesized, cDNA-derived RNA. These latter filters controlled for the specificity of hybridization with the oligonucleotide probes. cDNA-derived RNAs were synthesized in vitro as follows.

Restriction fragments containing various cDNAs isolated by PCR amplification were cloned between the T7 and SP6 promoters contained within the vector pSP72 (Promega-Biotec Co.). The resultant plasmids were linearized at the 3′ end of their cDNA inserts with appropriate restriction enzymes and then used as templates for in vitro transcription with either T7 or SP6 RNA polymerase according to the protocols of the manufacturers (Promega-Biotec).

Plasmid constructions. Plasmid pSHCAT (40) contains a portion of an LTR from SIV_{mac} isolate BK28, including the entire U3 region and extending through to nucleotide position +218 (relative to the start site of transcription) within U5, placed upstream of the chloramphenicol acetyltransferase (CAT)-coding region of pSV0CAT (14). p60CAT was derived from pSHCAT by replacing LTR sequences from positions +34 (*Ban*II) through +218 (*Hin*cII) with a double-stranded oligonucleotide corresponding to nucleotides +34 through +60. Therefore, p60CAT contains LTR sequences including the entire U3 region and extending to the splice donor site at the beginning of the 5′ intron within R. The *tat*-expressing plasmids pSCT8785, pSCT8788, and pSCT8803 were constructed by inserting the *tat*-coding region from different cDNA clones between the simian virus 40 early promoter and large-T polyadenylation sequences of pSV2CAT (14). *Hin*dIII-*Xmn*I restriction fragments from the cDNA clones were inserted between the *Hin*dIII and *Hpa*I sites of pSV2CAT. The numbers used in these clone designations refer to the splice acceptor sites used in the parental cDNA.

Transfections and CAT assays. Transfections into HeLa cells and CAT assays were performed as described previously (40).

RESULTS

Isolation of cDNA clones representing *rev* mRNAs. We previously demonstrated that the existence of alternative splice acceptor sites located at nucleotides 8788 and 8803 at the beginning of coding exon 2 allows the production of different forms of the SIV_{mac} *tat* and *rev* mRNAs (40). Examination of the nucleotide sequence in this region revealed a third potential splice acceptor site at nucleotide 8785. To determine which of the three splice acceptor sites at the beginning of *tat*/*rev* coding exon 2 is used by the virus, we cloned and characterized SIV_{mac} *tat* and *rev* cDNAs by PCR amplification (30, 35). A 5′ primer (PCRAR) homologous to the R region of the LTR and a 3′ primer (PCR25), homologous to the 3′ end of the *rev* reading frame (Fig. 1) were used to amplify single-stranded cDNA synthesized from total RNA from chronically SIV_{mac}-infected HuT78 T cells. This primer pair was expected to amplify both *tat* and *rev* cDNAs.

A large number of clones representing the predominant 550-base-pair (bp) (314 colonies) and 750-bp (261 colonies) products of this amplification were analyzed by hybridization with oligonucleotide probes (PBA, PBB, and PBC) specific for the three predicted splice junctions, referred to as A (nucleotide 8785), B (nucleotide 8788), and C (nucleotide 8803) (Fig. 1 and 2). These probes hybridized to different subsets of colonies, indicating that all three splice acceptor sites were used. The A site was the one most commonly detected (59 and 63% of the 550- and 750-bp clones, respectively), and the C site was the least commonly detected (2% of the 550- and 750-bp clones). In addition, 26 colonies (~4%) did not hybridize with either oligonucleotide probe.

To further characterize these cDNAs, 33 clones each of the 550- and 750-bp fragments that hybridized with the oligonucleotide probes were analyzed by restriction enzyme digestion. Representative clones were then sequenced.

Although we expected that the two major classes (550 and 750 bp) would be the cDNAs of *tat* and *rev* mRNA, they both proved to be *rev*, since they used a splice acceptor (position 6488) just before the *rev* initiator codon and therefore beyond the *tat* initiator codon (Fig. 2). These results indicate that, like HIV-1 (34), SIV_{mac} encodes different *tat* and *rev* mRNAs. Furthermore, since these *rev* mRNAs have three splice acceptor sites at the beginning of coding exon 2, SIV_{mac} can encode three forms of *rev* (Fig. 2 and 3B). The difference between the 750- and 550-bp clones was found to be the presence or absence of an intron located near the 5′ end of these cDNAs (between positions 566 and 712; Fig. 2, pREV6I2 versus pREV4I1). Therefore, *rev* mRNA exists in at least six forms. In addition, restriction digestion and sequencing data showed that 2 of 33 clones of the 550-bp species contained an extra noncoding exon with borders at nucleotides 5188 and 5220 (Fig. 2, pREV4I21). So the total number of *rev* mRNA species is at least seven.

Identification of cDNA clones representing the 5′ end of *nef* mRNAs. The 26 clones that did not hybridize to probes for the *tat*/*rev* splice junctions fell into three classes based on the restriction enzyme and DNA sequencing analyses (Fig. 2). These classes are represented by clones pNEF4I32, pNEF4I25, and pNEF6II42. Each of these cDNAs contained the same untranslated exons at their 5′ ends as found in the *rev* clones; however, these exon(s) were spliced directly to the most 3′ splice acceptor sites of *tat*/*rev* coding exon 2. The first initiator codon in these mRNAs will not occur until the beginning of the *nef* reading frame. It therefore seems likely that these 26 clones represent the 5′ untranslated portion of *nef* mRNA.

Isolation of cDNA clones representing the 5′ end of *tat* and *vpr* mRNAs. None of the cDNA clones in our initial analysis included the *tat* initiator codon, suggesting that *tat* mRNAs are rare relative to *rev* mRNAs in chronically infected cells. To identify cDNAs derived from the 5′ end of *tat* mRNAs, we synthesized a primer (PCRTAT 5′) homologous to a region just downstream of the *tat* initiator codon. The primer pair PCRAR-PCRTAT 5′ allowed amplification of a major fragment of approximately 230 bp and some less abundant larger fragments. Plasmid clones of each size class were analyzed by restriction enzyme digestion and DNA sequencing. The 230-bp cDNAs contain a junction that joins a small untranslated 5′ exon to an exon that starts just before the *vpr* open reading frame (Fig. 2, p5′.1). Interestingly, these clones may represent an unusual mRNA species, since they do not use conventional splice donor and/or acceptor sites (see Discussion). That they exist as RNA in infected cells was demonstrated by S1 nuclease protection analysis (data not shown). Therefore, these cDNAs are not an artifact of the PCR; however, their significance is unclear.

Restriction digestion of 88 clones representing the collection of larger fragments showed that they fell into seven classes (Fig. 2). These classes are represented by p5′L33, p5′L3, pTAT5′20, pTAT5′2, pTAT5′48, pTAT5′35, and pTAT5′43, and they most likely correspond to the 5′ ends of either *tat* or *vpr* mRNAs (Fig. 2). The 5′ ends of the putative *tat* messages use a splice acceptor (position 6276) 2 nucleotides upstream of the *tat* initiator codon, whereas the 5′ ends of the putative *vpr* messages use a splice acceptor (position 6116) 11 nucleotides upstream of the *vpr* initiator codon (Fig. 2 and 3A). The 5′ untranslated exons of the *tat* and *vpr* cDNAs are identical to those identified for *rev* and what we

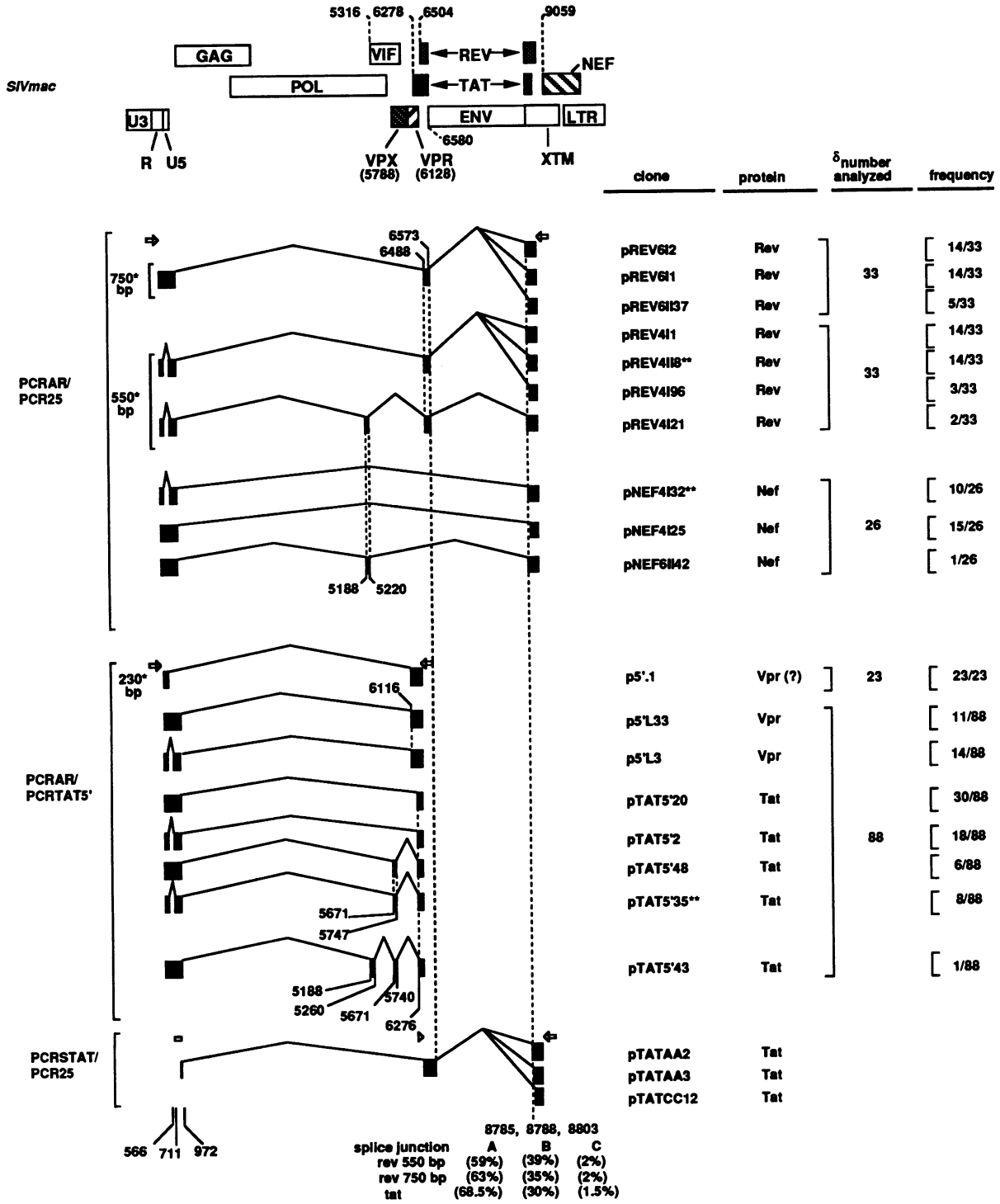


FIG. 2. SIV_{mac} cDNA clones isolated by PCR amplification. The genome organization of SIV_{mac} BK28 is shown at the top. The numbers here refer to the positions of initiator codons for the various genes. The start of transcription is at nucleotide 506. The structures of representative SIV_{mac} cDNA clones isolated by PCR amplification are shown below. The filled boxes represent exons, and the lines represent introns, deduced from these cDNA sequences. The nucleotide positions of splice sites relative to the provirus are shown. The PCR primers used are represented by arrows. Colonies representing the PCR amplification products of the primer pairs PCRAR-PCR25 and PCRSTAT-PCR25 were initially screened by hybridization with oligonucleotide probes specific for splice junctions with three different splice acceptor sites located at the beginning of *tat/rev* coding exon 2. The frequencies with which these alternative splice acceptors were used are shown at the bottom of the figure beneath their corresponding nucleotide positions. The number of clones from each amplification that were analyzed by restriction enzyme digestion is shown (δ). Representative clones of each distinct restriction digestion and oligonucleotide hybridization class were sequenced. The frequencies with which these distinct classes were found are indicated. Numbers with asterisks (*) refer to the approximate sizes of the amplified products. Similar cDNAs (**) were identified by Colombini et al. (7).

A.

NUCLEOTIDE POSITION	SEQUENCE	COMMENTS
566	GCAG/GUAGAGCC	5' intron splice donor
972	GCAG/GUAAGUGC	major subgenomic splice donor
5220	CCCG/GUGAGCUA	rare
5260	AAAG/GUAGGGAC	rare
5740	AUAG/GUACCAGG	rare
5747	CCAG/GUACCAAG	rare
6573	ACAA/GUAAGUAU	tat/rev coding exon 1 splice donor
	C/A AG/GU C/A AGU	consensus splice donor (27)
711	UCCCAUCUCUCCUAG/UCG	5' intron splice acceptor
5188	UCGGGUCUAUUACAG/AGA	rare; possibly vif
5671	UCCCUUGCUUUACAG/CGG	alternative tat; possibly vpx
6116	CUCCUCCCCCUCCAG/GAC	vpr splice acceptor
6276	UCAUAUCUAUAAUAG/ACA	tat splice acceptor
6488	UGUUUUCUUAAAAAG/GGA	rev/env splice acceptor
8785	CCCUCUUAUUUCCAG/UAG	tat/rev coding exon 2; nef
8788	UCUUAUUUCCAGUAG/ACU	tat/rev coding exon 2; nef
8803	ACUCAUACCCAACAG/GAC	tat/rev coding exon 2; nef
	(U/C)$_n$ N C/U AG/G	consensus splice acceptor (27)

B.

		Splice acceptor site
GCATCAAACAAtagactcatacccaacaggacccggcactgc		
AlaSerAsnAsnArgLeuIleProAsnArgThrArgHisCys	TAT	8785
HisGlnThrValAspSerTyrProThrGlyProGlyThr	REV	
GCATCAAACAAac---tcatacccaacaggacccggcactgc		
AlaSerAsnLysL---euIleProAsnArgThrArgHisCys	TAT	8788
HisGlnThrAsn---SerTyrProThrGlyProGlyThr	REV	
GCATCAAACAAga----------cccggcactgc		
AlaSerAsnLysP----------hrArgHisCys	TAT	8803
HisGlnThrArg----------ProGlyThr	REV	

FIG. 3. (A) SIV$_{mac}$ splice donor and splice acceptor sites. The nucleotide sequences of the various cDNA clones isolated by PCR amplification were determined, and the splice sites used were deducted by comparison with the proviral sequence. The nucleotide positions indicated refer to the positions of the splice donor and splice acceptor sites. (B) Predicted amino acid changes in the different forms of *tat* and *rev*. The nucleotide sequences of cDNAs representing the various alternatively spliced *tat* and *rev* mRNAs are shown in the top lines. Uppercase letters refer to nucleotides from the first coding exon, and lowercase letters refer to nucleotides from the various second coding exons. The deduced amino acid sequences of *tat* and *rev* are shown below the nucleotide sequences. The dashes indicate either nucleotides or amino acids missing from the alternatively spliced mRNAs or proteins. The amino acids highlighted by a box represent substitutions relative to the proteins shown at the top. The numbers to the right refer to the position of the various splice acceptor sites used.

think are *nef* cDNAs, except that approximately 24% of the *tat* cDNAs contained an additional noncoding exon from within the *vif* region (nucleotides 5671 through 5747) (Fig. 2, pTAT5'35 and pTAT5'48). Furthermore, one clone, pTAT5'43, contained two additional noncoding exons from within the *pol* (nucleotides 5188 through 5260) and *vif* (nucleotides 5671 through 5740) regions.

Isolation of *tat* cDNAs including both coding exons. We next isolated cDNA clones corresponding to mRNAs that used the splice acceptor site (position 6276) immediately 5' to the *tat* initiator codon and included both coding exons of *tat*. Primer PCRSTAT, whose sequence spans the splice junction created by joining the splice donor at position 972 with the splice acceptor at 6276, and primer PCR25, which is complementary to a sequence just downstream from the *tat* reading frame, allowed amplification of two major fragments of approximately 300 and 500 bp. The 500-bp fragments hybridized to an oligonucleotide probe (PBTAT) that was specific for the predicted *tat* splice junction (joining nucleotides 972 and 6276) (data not shown). This probe overlaps and extends beyond the ends of primer PCRSTAT; therefore, it will not hybridize to nonspecific amplification products under the conditions used (data not shown). The 500-bp fragments were cloned into pUC18 and hybridized with probes PBTAT, PBA, PBB, and PBC, and representative clones were sequenced. This analysis demonstrated that *tat* cDNAs used all three splice acceptor sites at the beginning of coding exon 2 with frequencies roughly equivalent to those found for *rev* (Fig. 2; pTATAA2, pTATAA3, and pTATCC12).

Northern blot analysis of alternatively spliced mRNAs. The above results indicate that alternative splicing is used by SIV$_{mac}$ to produce a wide variety of mRNAs with the capacity to encode several different regulatory proteins. However, since PCR amplification may be subject to size and abundance biases, these cDNAs may not reflect the actual abundances of the different mRNA species nor the complete catalog of species. We therefore used Northern blot analysis of poly(A)$^+$ RNA from chronically infected cells to quantitate directly the different mRNA species.

A series of oligonucleotide probes was synthesized that spanned various splice junctions identified in the cDNA clones. Each probe was homologous to the last 12 nucleotides of a 5' exon and the first 12 nucleotides of its corresponding 3' exon. Hybridization and wash conditions for the specificity of these probes were established by using RNAs synthesized in vitro from the various cDNAs cloned into SP6 or T7 promoter-containing vectors. Oligo(dT)-selected RNAs isolated from HUT78 cells chronically infected with SIV$_{mac}$ were separated on agarose-formaldehyde gels and

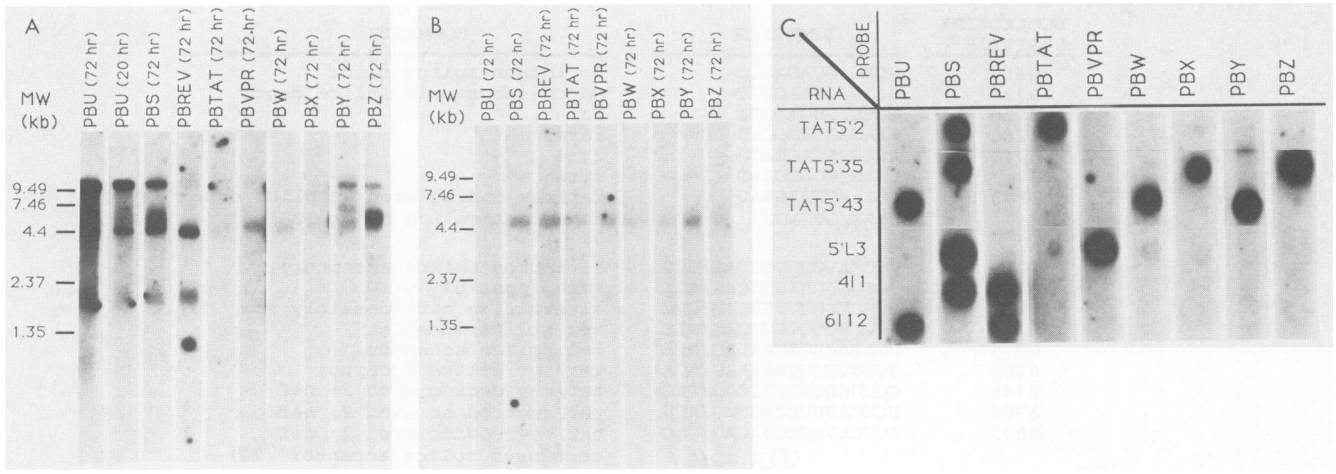


FIG. 4. Blot analysis of SIV_{mac} mRNAs from chronically infected Hut78 T cells. (A and B) Oligo(dT)-cellulose-selected RNA (40 μg) was loaded into a single-gel-wide lane of an agarose-formaldehyde gel and electrophoretically separated. RNAs were transferred to a nylon-backed nitrocellulose filter, and the filter was cut vertically into strips. Each strip was hybridized separately with oligonucleotide probes specific for different splice junctions. The probes used are indicated above each filter. Exposure times are also indicated. (A) SIV_{mac}-infected HuT78 cell RNA. (B) Uninfected HuT78 cell RNA. (C) In vitro-synthesized RNAs (10 ng) corresponding to the various cDNAs indicated at the left were immobilized on nylon-backed nitrocellulose filters. Individual filters were hybridized with oligonucleotide probes specific for different splice junctions. The probes are indicated at the top. Hybridizations with each specific probe included filters containing RNA from (A) SIV_{mac}-infected cells, (B) uninfected cells, and (C) in vitro-synthesized RNAs.

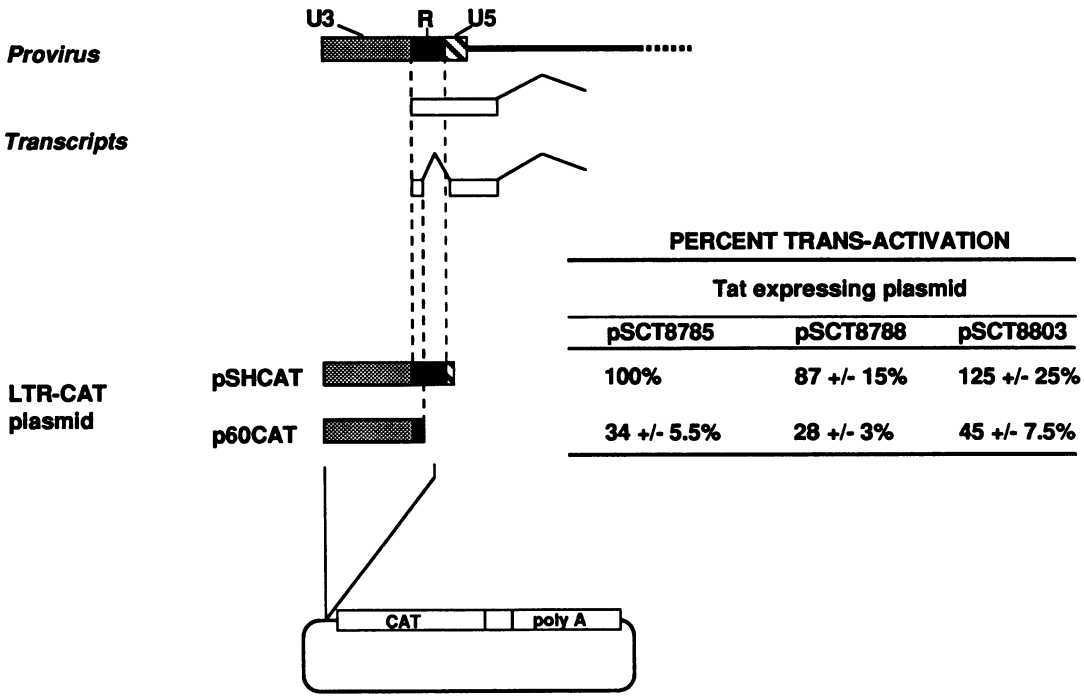
transferred to nylon-backed nitrocellulose filters. The filters were cut vertically into strips, and each strip was hybridized with a different oligonucleotide probe. Also included in each hybridization were filters containing RNA from uninfected cells and filters containing known amounts of in vitro-synthesized cDNA-derived RNAs. These latter filters controlled for the specificity of the oligonucleotide hybridizations.

Northern blots were first hybridized with oligonucleotide probes specific for the *rev* (nucleotides 972 and 6488) or *tat* (nucleotides 972 and 6276) splice junction (Fig. 4). The *rev* probe (PBREV) hybridized to both 4.5- and 2.0-kilobase (kb) mRNAs, suggesting that the same splice acceptor site is used for most *env* and *rev* mRNAs. In contrast, the *tat* probe (PBTAT) failed to hybridize detectably to any mRNA species, suggesting that *tat*-specific mRNAs are of low abundance in chronically infected cells. These results are consistent with our failure to detect *tat* mRNAs in the initial *rev* and *nef* amplifications. However, *tat* may also be encoded by bifunctional mRNAs, perhaps like those identified as encoding *vpr* and that would not be detected by probe PBTAT. We therefore hybridized Northern blots with a *vpr*-specific probe (junction of nucleotides 972 and 6116 [PBVPR]) and with probes (nucleotides 5740 and 6276 [PBW] and nucleotides 5747 and 6276 [PBX]) specific for *tat* mRNAs containing extra upstream noncoding exons. All three failed to hybridize specifically with any mRNA species. Taken together, these results suggest that *tat* mRNA is not abundant at late stages of infection. Some probes hybridized nonspecifically to residual 28S rRNA contained within the RNA sample at approximately 4.5 kb (Fig. 4B). However, this did not interfere with the interpretation of the PBREV probe or with probe PBU, PBS, PBY, or PBZ (see below).

We next hybridized Northern blots with probes specific for some of other splice junctions we detected in cDNAs. Probes PBY (junction of nucleotides 972 and 5188) and PBZ (nucleotides 972 and 5671), which detect mRNAs with splice acceptors located within the *pol* and *vif* regions, hybridized specifically with 6- and 5-kb RNAs, respectively. Since the *vif* and *vpx* initiator codons are the first ones present downstream of those splice acceptor sites, these mRNAs are tentatively identified as encoding *vif* and *vpx*. It is interesting to note that these mRNAs are large enough to contain the *rev*-responsive element located within the common *tat*/*rev* intron (9, 11, 17, 26). Therefore, their expression may be regulated by *rev*. Probe PBY also hybridized nonspecifically with 28S rRNA. Both PBY and PBZ also hybridized with an RNA of approximately 9.4 to 9.5 kb that was present in infected cells. Hybridization to this band was not consistent, and in some instances other probes also hybridized with it. The identity of this larger band is not known.

Northern blots were also hybridized with probes specific for either the spliced (PBS) or unspliced (PBU) 5′ ends of SIV_{mac} mRNAs (Fig. 4). Both probes hybridized to 9.6-, 4.5-, and 2.0-kb mRNAs; however, the unspliced mRNAs were about 5- to 10-fold more abundant than the spliced mRNAs. These results indicate that, for each of the three major classes of SIV_{mac} mRNA, about 10 to 20% of the messages from chronically infected cells have the small intron within the 5′ LTR removed.

The 5′ intron of SIV_{mac} mRNAs overlaps the TAR element. The intron spliced from the 5′ end of SIV_{mac} mRNAs extends from nucleotide positions +60 through +206, relative to the start of transcription. Its location suggested that it might overlap the TAR element, required for response to *tat*-mediated *trans* activation (19, 29, 33). To determine whether sequences contained within this intron constitute a portion of TAR, we constructed a plasmid (p60CAT) containing SIV_{mac} LTR sequences encompassing the entire U3 region and extending to the splice donor site for the 5′ intron. These viral sequences were placed upstream of the bacterial gene for CAT (Fig. 5). p60CAT and another LTR-CAT fusion gene, pSHCAT (40), containing the entire SIV_{mac} TAR element, were independently cotransfected into HeLa cells along with various *tat*-expressing plasmids. These latter plasmids, pSCT8785, pSCT8788, and pSCT8803, were constructed by placing cDNAs representing the three spliced



PERCENT TRANS-ACTIVATION

Tat expressing plasmid		
pSCT8785	pSCT8788	pSCT8803
100%	87 +/- 15%	125 +/- 25%
34 +/- 5.5%	28 +/- 3%	45 +/- 7.5%

FIG. 5. *Trans* activation of SIV_{mac} LTRs by three forms of *tat*. Plasmid pSHCAT (40) contains a portion of an LTR from SIV_{mac} isolate BK28, including the entire U3 region and extending through to nucleotide position +218 (relative to the start site of transcription) within U5, placed upstream of the CAT-coding region of pSV0CAT. p60CAT was derived from pSHCAT and contains LTR sequences including the entire U3 region and extending to the splice donor site at the beginning of the 5′ intron within R. The *tat*-expressing plasmids pSCT8785, pSCT8788, an pSCT8803 were constructed by inserting the *tat* coding region from different cDNA clones between the simian virus 40 early promoter and large-T polyadenylation sequences of pSV2CAT. The numbers in the clone designations refer to the splice acceptor site used in the parental cDNA. pSHCAT and p60CAT were independently transfected into HeLa cells either by themselves or with the various *tat*-expression plasmids. The levels of expression of pSHCAT and p60CAT were determined by an enzymatic assay for CAT activity. Levels of *trans* activation were calculated as the ratio of LTR-directed CAT activity in the presence of *tat* to that in the absence of *tat*. The numbers shown are the percent *trans* activation of various LTR and *tat* combinations compared with the level of *trans* activation of pSHCAT induced by pSCT8785 and were calculated from four independent experiments. All comparisons are from parallel transfections. The standard errors of the means are also shown.

forms of *tat* under the transcriptional control of the simian virus 40 early promoter (Fig. 5). The levels of expression of pSHCAT and p60CAT were measured by an enzymatic assay for CAT activity (Fig. 5) and by S1 nuclease protection assays (data not shown). In both assays, expression of pSHCAT was activated approximately 40-fold in the presence of *tat* expression, whereas expression of p60CAT was activated approximately 14-fold (Fig. 5). Thus, sequences located within the 5′ intron are required for full responsiveness. Whether this requirement is at a transcriptional or posttranscriptional level is not known.

The three spliced forms of *tat* examined in these transfection experiments did not differ significantly in their ability to *trans* activate SIV_{mac} LTRs (Fig. 5). We have not yet tested the activities of the various forms of *rev*.

DISCUSSION

PCR amplification was used to isolate cDNA clones representing a variety of mRNAs for the SIV_{mac} regulatory proteins. The results obtained with PCR were corroborated by Northern blot analyses with oligonucleotide probes specific for the different splice junctions identified in the cDNA clones. These analyses demonstrated that pre-mRNA splicing in SIV_{mac} is highly complex.

The variety of spliced mRNAs identified in these analyses implies that alternative splicing affects a number of aspects of SIV_{mac} gene expression. We identified mRNAs with splice acceptor sites 5′ to the initiator codons for *tat*, *rev*, *vpr*, *nef*, *vif*, and *vpx*. In all cases, these are the first initiator codons present in each mRNA, suggesting that these proteins can each be encoded by a separate message. Furthermore, RNA blot analysis with a splice junction-specific probe indicated that *rev* and *env* mRNAs may use the same splice acceptor site. Finally, the *vif* and *vpx* mRNAs identified by RNA blot analysis are large enough to contain the entire *env* coding sequence and should therefore contain the *rev*-responsive element. The expression of these mRNAs, as well as *env*, may therefore be regulated by *rev*.

We also identified mRNAs with the capacity to encode three different versions of two essential regulatory proteins, the *tat* and *rev* products. Three different splice acceptor sites, located at nucleotide positions 8785, 8788, and 8803, were used at the beginning of *tat*/*rev* coding exon 2. The 5′ sites were used most frequently (8785 site, ~69 and ~61% for *tat* and *rev*, respectively; 8788 site, ~30 and 37% for *tat* and *rev*, respectively), whereas the 3′ site was used least frequently (~2% for both *tat* and *rev*). It is worth noting that even though the two most 5′ sites are only three nucleotides apart, they are, quite surprisingly, both used to significant extents. These splice acceptor sites are located within the same reading frame(s); therefore, proteins translated from mRNAs with the two downstream sites will differ from

proteins translated from mRNAs with the upstream site by single amino acid substitutions and the deletion of either one or six amino acids (Fig. 3B).

The *tat* proteins expressed by the three alternatively spliced mRNAs did not differ significantly in their ability to *trans* activate SIV_{mac} LTRs. Thus, either our assay is not measuring some important difference in their behavior, or the three forms have the same activity. Whether the three forms of *rev* generated by alternative splicing differ in their activities has not yet been addressed. It is perhaps significant that the middle splice acceptor site at beginning of *tat/rev* coding exon 2 precisely coincides with a stop codon that prematurely truncates the SIV_{mac} *env* gene in the region encoding the transmembrane protein gp41 (20). Similar stop codons have been identified in other isolates of SIV_{mac} (6, 13) and in HIV-2 (16). In SIV_{mac}, these stop codons are selected for or against, depending on whether the virus is propagated in human or simian cells (21, 24). In some cases, this selection involves deletions that remove one or both 5′ splice acceptor sites but that retain the 3′ splice acceptor site (21). In all cases examined at least one of the three alternative splice acceptor sites is retained; therefore, presumably so is the ability to splice into *tat/rev* coding exon 2. Since the coding exon 2 portion of SIV_{mac} *tat* is required for full activity (40), retention of at least one splice acceptor site is most likely required for viral replication. Therefore, the presence of the three alternative splice acceptor sites may be a consequence of the selective pressures exerted on this region in different cell types. Whether they are used by viruses that do not contain the stop codon alternation is being examined.

One species of cDNA that we isolated (p5′.1) represents an mRNA with an unusual junction that joins a small untranslated 5′ exon to an exon beginning just before the *vpr* open reading frame. Comparison of the cDNA and provirus sequences indicated that imperfect direct repeats are located at the boundaries of the intron removed from this mRNA. Because of these repeats, the precise borders of the intron cannot be determined. If these cDNAs represent a spliced mRNA, then both the splice donor and splice acceptor sites must be contained within the repeated sequence 5′-ACU AGCA-3′ (donor, nucleotides 559 through 565; acceptor, nucleotides 6117 through 6123). However, this sequence does not contain a site closely matching a consensus splice donor (28). These cDNAs do not represent an artifact of PCR, since S1 nuclease protection analysis demonstrated that mRNAs containing this junction were present in the infected cells (data not shown). An alternative explanation for these cDNAs is that they represent the primary transcript from a provirus with an internal deletion. This possibility is being examined.

The 5′ untranslated ends of SIV_{mac} mRNAs fall into two classes. Both classes use a splice donor site at nucleotide 972; this site appears to be the major splice donor for all SIV_{mac} subgenomic-length mRNAs. These classes differ from each other by the splicing of an intron located between nucleotides 566 and 711 (+60 and +204 relative to the start of transcription). In chronically infected cells, approximately 10 to 20% of each size class of SIV_{mac} mRNA, including the ~9.6-, ~4.5-, and ~2.0-kb species, splices this intron. Although their use has not been demonstrated, the splice donor and splice acceptor sites for this intron are conserved in other isolates of SIV_{mac} as well as in SIV_{smm} and HIV-2; in contrast, these sites are not conserved in HIV-1 (31).

A feature of some cDNA clones that we isolated was the presence of noncoding exons, in addition to those found at the very 5′ end. Approximately 5% of the *rev* and *nef* cDNAs and 24% of the *tat* cDNAs analyzed contained these extra exons. Their significance is unclear; however, they may reflect inefficiency in viral splicing rather than the function of these particular mRNAs. The primary retroviral transcript cannot be constitutively spliced, since it serves not only as an mRNA but also as the genomic RNA contained within mature virions. Constitutive splicing of the primary transcript may be prevented by structural features of the viral splice sites or *cis*-acting sequences located within introns (22, 38). These sequences may provide targets with lower affinities for components of the cellular splicing machinery than similar sequences in constitutively spliced pre-mRNAs. The extra noncoding exons identified in some of the SIV_{mac} cDNAs may therefore represent promiscuous splicing events resulting from this inefficiency.

The position of the intron located at the very 5′ end of the SIV_{mac} mRNAs suggested that it overlapped the TAR element. TAR is a sequence element required for response to *tat*-mediated stimulation of LTR-directed gene expression and is located predominantly within the R region of the LTR (19, 29, 33). At least a part of the mechanism of *tat* action in HIV-1 requires an RNA hairpin structure adopted by the TAR element (4, 5, 12, 29, 32). Like HIV-1 TAR RNA, SIV_{mac} TAR RNA is predicted to adopt a stable secondary structure. However, instead of containing the single hairpin found in HIV-1, the SIV_{mac} TAR is predicted to contain at least two hairpin structures. The intron located at the 5′ end of SIV_{mac} mRNAs overlaps the predicted TAR structure and includes most of the second hairpin. We therefore tested by transient transfection analyses whether sequences contained within the intron are required for full response to *tat*-mediated *trans* activation. LTRs that are truncated at the splice donor site of this intron are about threefold less responsive to *tat*-mediated *trans* activation than LTRs that contain the entire intron. Thus, sequences contained within the 5′ intron are required for full responsiveness. However, since these results are based on transient transfection experiments, they do not address whether this requirement is at a transcriptional or posttranscriptional level. It will be of interest to determine whether splicing of the TAR intron effects a posttranscriptional component of *trans* activation and what role this splicing event may play in the life cycle of SIV_{mac}.

We previously demonstrated that the *tat* proteins of SIV_{mac} and HIV-1 are functionally homologous but not interchangeable (40). SIV_{mac} and HIV-1 *tat* are equally effective in *trans* activating the SIV_{mac} LTR, whereas SIV_{mac} *tat* is significantly less effective than HIV-1 *tat* in *trans* activating the HIV-1 LTR. The studies described in this report extend our comparison between SIV_{mac} and HIV-1 and point out that alternative splicing of the TAR element is another difference between these viruses. These and other differences may be important in the evaluation of SIV as a model for human acquired immunodeficiency syndrome.

In total, we identified at least 22 mRNA species derived from the 9.6-kb SIV_{mac} primary transcript. Regulation of alternative splicing and the resulting pattern of protein expression adds another level of complexity to the life cycle of the immunodeficiency viruses, already complicated by the presence of six nonstructural and presumed regulatory genes. Analysis of these subtleties of regulation in tissue culture-propagated virus should be extended to evaluate the pressures or consequences of alternative regulation that occur during replication and disease progression in vivo.

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ADDENDUM IN PROOF

After this work was submitted for publication, Schwartz et al. (S. Schwartz, B. K. Felber, D. M. Benko, E.-M. Fenyö, and G. N. Pavlakis, J. Virol. **64**:2519–2529, 1990) demonstrated complex patterns of RNA splicing in HIV-1.

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