Functional Comparison of Transactivation by Simian Immunodeficiency Virus from Rhesus Macaques and Human Immunodeficiency Virus Type 1

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Simian immunodeficiency virus from rhesus macaques (SIV_{mac}), like human immunodeficiency virus type 1 (HIV-1), encodes a transactivator (*tat*) which stimulates long terminal repeat (LTR)-directed gene expression. We performed cotransfection assays of SIV_{mac} and HIV-1 *tat* constructs with LTR-CAT reporter plasmids. The primary effect of transactivation for both SIV_{mac} and HIV-1 is an increase in LTR-directed mRNA accumulation. The SIV_{mac} *tat* gene product partially transactivates an HIV-1 LTR, whereas the HIV-1 *tat* gene product fully transactivates an SIV_{mac} LTR. Significant transactivation is achieved by the product of coding exon 1 of the HIV-1 *tat* gene; however, inclusion of coding exon 2 results in a further increase in mRNA accumulation. In contrast, coding exon 2 of the SIV_{mac} *tat* gene is required for significant transactivation. These results imply that the *tat* proteins of SIV_{mac} and HIV-1 are functionally similar but not interchangeable. In addition, an in vitro-generated mutation in SIV_{mac} *tat* disrupts splicing at the normal splice acceptor site at the beginning of coding exon 2 and activates a site approximately 15 nucleotides downstream. The product of this splice variant stimulates LTR-directed gene expression. This alternative splice acceptor site is also used by a biologically active provirus with an efficiency of approximately 5% compared with the upstream site. These data suggest that a novel *tat* protein is encoded during the course of viral infection.

Molecularly cloned simian immunodeficiency virus from rhesus macaques (SIV_{mac}) (20, 23, 31, 32) is distantly related both serologically and genetically to human immunodeficiency virus type 1 (HIV-1), the etiologic agent of acquired immunodeficiency syndrome (20, 23, 24, 26-28, 31, 32). SIV_{mac} and HIV-1 also share biological features; both specifically infect cells that express the CD4 cell surface receptor and induce some cytopathic effects in these cells in vitro (9, 29, 32, 34, 37). Furthermore, SIV_{mac} has been reported to induce an immunodeficiency disease in rhesus macaques that is similar to human acquired immunodeficiency syndrome (9, 33).

DNA hybridization and sequence analysis demonstrated that SIV_{mac} has a genome organization similar to that of HIV-1 (6, 16, 23, 24). In addition to the *gag*, *pol*, and *env* genes found in all replication-competent retroviruses, SIV_{mac} contains a number of open reading frames with deduced amino acid homology to genes previously identified in HIV-1, including the transactivator gene referred to as *tat* or *tat3* (24). Comparison of the predicted protein sequences of SIV_{mac} *tat* with *tat3* indicates that these proteins contain only short regions of similarity (24). The protein encoded by *tat3* stimulates long terminal repeat (LTR) directed gene expression. Similarly, SIV_{mac}-infected cells contain a factor, presumably *tat*, which stimulates LTR-directed gene expression (2, 13). The mechanism of transactivation is unknown, and there is disagreement concerning the relative contributions made by transcriptional and posttranscriptional events to this process (3, 8, 14, 36, 41, 42, 44, 45, 50, 51).

The LTRs of SIV_{mac} and HIV-1 are also similar in their regulatory regions. Three sequences closely matching the consensus binding site for transcription factor Sp1 (25, 32) are present in both LTRs, as are sequences homologous to a core enhancer (32, 39). In HIV-1 a transactivator responder

(TAR) element sequence is located at the 3' end of U3 and extends into the R region of its LTR (45). This TAR element is required for response to *tat*-induced transactivation and functions in a position- and orientation-dependent manner. There is substantial but incomplete homology between the HIV-1 TAR region and the corresponding region of SIV_{mac} (24, 32).

Here we demonstrate that a fragment of SIV_{mac} containing the predicted *tat* gene encodes a product which stimulates LTR-directed gene expression. Transactivation of SIV_{mac} and HIV-1 LTRs fused to the bacterial chloramphenicol acetyltransferase (CAT) gene by both the SIV_{mac} and HIV-1 *tat* gene products results in comparable increases in both mRNA and CAT enzyme activities. In agreement with other workers, we found that the SIV_{mac} *tat* gene product only partially transactivates an HIV-1 LTR, whereas the HIV-1 *tat* gene product fully transactivates an SIV_{mac} LTR (2, 13). Moreover, we analyzed SIV_{mac} and HIV-1 *tat* genes mutated so as to encode proteins prematurely truncated at or near the beginning of coding exon 2. These constructs were less active than full-length products, but this effect was far less pronounced in HIV-1 than in SIV_{mac}. These results indicate that the SIV_{mac} and HIV-1 *tat* proteins are functionally homologous but not interchangeable. We also identified a splice acceptor site within coding exon 2 of *tat* which is used by a biologically active SIV_{mac} provirus. This result suggests that a novel *tat* protein is expressed during the course of viral infection.

MATERIALS AND METHODS

Plasmid constructions. SIV_{mac} fragments were isolated from the biologically active proviral clone BK28 (previously referred to as human T-lymphotropic virus type IV) (31, 32; M. Essex and P. Kanki, Letter, *Nature* (London) **332**:621-622). All LTR-CAT fusion genes were constructed by inserting restriction fragments (described below) into the *Bgl*II site

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of pSV0CAT (18) upstream of the CAT-coding region and simian virus 40 (SV40) polyadenylation sequences. pSHCAT contains the 997-base-pair (bp) *Bam*HI-*Hinc*II fragment from pBK28 and extends through the entire U3 region of the LTR to position +200 within U5. pSBCAT contains the 883-bp *Bam*HI-*Bal*I fragment from pBK28 and extends through the entire U3 region to position +100 within R. pMALCAT contains the 849-bp *Asu*II-*Hind*III fragment from pLAV-MAL (1) and extends through the entire U3 region to position +76 within R. pMALNCAT contains the 955-bp *Asu*II-*Nar*I fragment from pLAV-MAL and extends through the entire LTR to a position 3 bp downstream of the LTR.

All *tat*-expressing plasmids were constructed by inserting appropriate restriction fragments between the SV40 early promoter and large-T polyadenylation sequences of pSV2CAT (18). All of the fragments were inserted between the *Hind*III and *Hpa*I sites of pSV2CAT. pST contains the 2,686-bp *Hind*III-*Xmn*I fragment from pBK28 and thus includes both coding exons of SIV_{mac} *tat*. pSTAP contains the 386-bp *Hind*III-*Pvu*II fragment from pBK28 and thus includes coding exon 1 of SIV_{mac} *tat* and the first 28 bp of the intron. pSTS1 was derived from pST by inserting the synthetic oligonucleotide 5'-GGGTAAACCC-3' into a blunt-ended *Eco*O109 site located 18 bp downstream of the coding exon 2 splice acceptor consensus site. pSTS2 was derived from pST by inserting the same oligonucleotide into a blunt-ended *Acc*I site located 3 bp downstream of the coding exon 2 splice acceptor site. The oligonucleotide insertions in pSTS1 and pSTS2 were checked by dideoxy-chain termination DNA sequencing. pSTS2Δ was derived from pSTS2 by deleting the SIV_{mac} sequences downstream of the *Hpa*I site within the oligonucleotide insertion. pHT contains the 2,689-bp *Bam*HI fragment from pZIPNeotat_{III} (43) and thus includes both coding exons of HIV-1 *tat*. pHTΔA contains the 565-bp *Bam*HI-*Asp*-718 fragment from pZIPNeotat_{III} and thus includes coding exon 1 of HIV-1 *tat* and the first 307 bp of the intron.

pSV2ΔCAT was constructed by deleting the 515-bp *Hind*III-*Bal*I fragment from pSV2CAT. pCATSS was constructed by inserting the 982-bp *Sph*I-*Sau*3AI fragment of pSV2CAT between the *Sph*I and *Bam*HI sites of pUC18 and thus contains the entire coding region of the CAT gene. pGEMST1 was constructed by inserting the 386-bp *Hind*III-*Pvu*II fragment of pBK28 between the *Hind*III and *Hinc*II sites of pGEM2 and thus contains coding exon 1 of SIV_{mac} *tat* and the first 28 bp of the intron. pSP73ST2 was constructed by inserting the 164-bp *Nhe*I-*Xmn*I fragment of pBK28 between the *Xba*I and *Pvu*II sites of pSP73 and thus contains the last 63 bp of the SIV_{mac} *tat* intron and coding exon 2.

Transfections and CAT assays. HeLa cells (5×10^6 /ml to 8×10^6 /ml) were transfected by the DEAE-dextran procedure of Dorsett et al. (12). All transfections contained a total of 2.05 to 2.55 μg of DNA per ml, with equimolar amounts of all plasmids, except for pSV2ΔCAT, which was included at a 0.6 M ratio. Plasmid pSVK₁H (11), which contains the SV40 early promoter inserted into the vector pML, was included in transfections not containing transactivator plasmids. The inclusion of pSVK₁H maintained a constant concentration of the SV40 early promoter in all transfections. Cells were harvested after 44 to 48 h and divided into portions of 1/4 and 3/4 for the analyses described below.

CAT extracts were prepared from 1/4 of the transfected cells, and CAT assays were performed as previously described (18). Spots on thin-layer chromatography plates

corresponding to different forms of [¹⁴C]chloramphenicol were cut out and quantitated in a scintillation counter. Protein concentrations of the extracts were quantitated by using a Bio-Rad protein assay kit. Only values in the linear range of enzyme activity were used to determine the percent conversion of chloramphenicol. All values were normalized against the amount of protein contained in the extracts.

RNA isolation and analysis. RNA was prepared from 3/4 of the transfected cells by the procedure of Cohen and Meselson (7). All RNA samples were digested with RNase-free RQ-1 DNase (Promega Biotec).

The 5'-end-labeled probe used for S1 nuclease analysis was prepared as follows. Plasmid pCATSS was digested with *Bam*HI, and the 5' ends were dephosphorylated with calf intestine alkaline phosphatase. The linear DNA was end labeled with T4 DNA kinase and [³²P]ATP. The labeled fragment was then digested with *Asp*-718 and *Sph*I. The restriction enzyme *Asp*-718 digested the polylinker of pUC18 adjacent to the *Bam*HI site. This digestion released a 9-bp end-labeled fragment. The *Sph*I digestion released the 982-bp end-labeled probe. The unincorporated [³²P]ATP and the 9-bp *Bam*HI-*Asp*-718 fragment were removed by Sephadex G-50 (Pharmacia) chromatography. The probe was extracted twice with phenol-chloroform and once with chloroform, ethanol precipitated, and redissolved in 1 mM EDTA-10 mM Tris (pH 8.0). Probe (25 ng; about 3×10^5 cpm) and RNA samples (30 μg) were coprecipitated. The pellets were dissolved in 30 μl of hybridization buffer (4), heated to 85°C for 5 min, and then immediately brought to 50°C and incubated for 4 h. After the hybridization, the samples were digested with 600 U of S1 nuclease per ml at 30°C for 1 h under standard conditions (4). The protected fragments were purified by phenol-chloroform extraction and ethanol precipitated.

Antisense RNA probes for RNase protection experiments were synthesized with [³²P]UTP and either T7 or SP6 RNA polymerase as previously described (4). Probes for analysis of *tat* mRNAs were synthesized from either *Hind*III-linearized pGEMST1 (resultant probe, 428 nucleotides) or *Bgl*II linearized pSP73ST2 (resultant probe, 228 nucleotides). RNase protections were performed with 15 μg of RNA and 1.5×10^5 cpm of probe as previously described (4). Hybridizations were at 45°C for 17 h. Hybrids were digested with 40 μg of RNase A per ml and 1,000 U of RNase T1 per ml at 30°C for 45 min.

S1 nuclease and RNase protection products were electrophoresed on 6% polyacrylamide-7 M urea gels. Autoradiographs were quantitated by densitometry of exposures in the linear range of the film.

DNA sequence analysis. The *tat* gene from HXBc2 (15) contained within pZIPNeotat_{III} was sequenced by subcloning appropriate restriction fragments into M13mp19 and using the dideoxy-chain termination method (46).

RESULTS

SIV_{mac} encodes a functional *tat* gene product. To determine whether the putative *tat* gene of SIV_{mac} encodes a product that transactivates LTR-directed gene expression, we constructed a plasmid, pST, which contains both coding exons of *tat* under the transcriptional control of the SV40 early promoter (Fig. 1A). In addition, we constructed a plasmid, pSHCAT, which contains SIV_{mac} LTR sequences encompassing the entire U3 and R regions and extending to position +200 within U5 fused to the protein-coding region of the CAT gene (+1 defines the beginning of R and the start

FIG. 1. Plasmid constructions. (A) Structures of *tat*-expressing plasmids. Restriction fragments from either SIV_{mac} or HIV-1 were inserted between the SV40 early promoter (SV) and polyadenylation regions of pSV2CAT (18). The structure of pST, containing both coding exons of SIV_{mac}, is shown as an example. The solid boxes represent *tat* gene exons. The arrows represent antisense RNA probes used for RNase protection analysis of SIV_{mac} *tat* mRNAs. (B) Structures of LTR-CAT fusion genes. Various portions of the LTRs from SIV_{mac} (pSHCAT and pSBCAT) or HIV-1 (pMALNCAT and pMALCAT) were inserted upstream of the bacterial CAT gene (CAT) contained within pSV0CAT (18). Splice donor (SD) and splice acceptor (SA) sites are indicated. (C) Structures of pSV2CAT and pSV2ΔCAT. The gap in pSV2ΔCAT represents the portion of the CAT gene deleted from pSV2CAT. The rightward-facing arrow indicates the start site of transcription from the SV40 early promoter (SV). Also shown is the fragment used as a probe for S1 nuclease protection analysis of CAT-containing mRNAs. The dot represents the position of the ³²P end label. Abbreviations: H, *Hind*III; X, *Xmn*I; B, *Bam*HI; H2, *Hinc*II; Ba, *Bal*I; A, *Asu*II; N, *Nar*I.

site of transcription) (Fig. 1B). These plasmids were cotransfected into HeLa cells, and RNA and protein extracts were prepared 48 h later. Since we were specifically interested in examining the effects of the *tat* protein on LTR-directed gene expression, we performed these experiments in HeLa cells rather than in chronically infected or uninfected T cells. Uninfected T cells have relatively high basal levels of LTR-directed gene expression (39; unpublished data). This result suggests that chronically infected and uninfected T cells contain factors which affect LTR-directed gene expression independent of *tat*. Furthermore, HeLa cells support viral replication and can be transfected with greater efficiency than T cells (unpublished data).

All transfections contained the plasmid pSV2ΔCAT, which was derived from pSV2CAT (18) by deleting the 524-bp *Hind*III-*Bal*I fragment from the CAT protein-coding region, thus eliminating the ability of this plasmid to encode a functional enzyme (Fig. 1C). Because transcription from the SV40 early promoter is not affected by *tat* gene products (data not shown), the amount of mRNA transcribed from pSV2ΔCAT was used as an internal standard to normalize the amount of RNA between transfections.

We used an S1 nuclease protection assay to determine whether the *tat* gene product increases the steady-state levels of mRNA transcribed from the SIV_{mac} LTR. Total

RNA was hybridized to a ³²P-end-labeled DNA probe corresponding to the 982-bp *Sph*I-*Sau*3AI fragment of the CAT gene (Fig. 1C) and digested with S1 nuclease. When the SIV_{mac} LTR-CAT fusion gene was transfected in the absence of any *tat* gene products, no LTR-directed mRNA was detected (Fig. 2). However, cotransfection with pST, expressing both *tat*-coding exons of the SIV_{mac} gene, resulted in about a 120-fold increase in the level of mRNA (Fig. 2; Table 1). This value represents a minimal estimate of the level of transactivation, since no LTR-directed mRNA was detected above the background in the absence of *tat*. In these instances, levels of transactivation are reported as the ratio of the level of LTR-directed mRNA in the presence of *tat* compared with the lowest detectable signal from densitometric scans. This result indicates that SIV_{mac}, like HIV-1, encodes a transactivator protein which increases LTR-directed gene expression. We also used antisense RNA probes and RNase protections to measure the stimulation of LTR-directed mRNA accumulation. Similar levels of transactivation were detected regardless of the probe and the type of analysis used (data not shown).

The TAR region of the HIV-1 LTR is located between nucleotide positions -17 and +44 (21). Therefore, sequences extending into the U5 region of the HIV-1 LTR are not required for transactivation. To begin to delimit the TAR

FIG. 2. S1 nuclease protection analysis of *SIV_{mac}* LTR-directed mRNA expression in the presence of various *tat* gene products. HeLa cells were cotransfected with *SIV_{mac}* (pSHCAT) LTR-CAT fusion genes and various *tat*-expressing plasmids. All transfections also contained the internal control plasmid pSV2ΔCAT. Total RNA was hybridized with a 982-bp ³²P-end-labeled probe from the CAT-coding region. LTR-CAT-encoded messages protect a 784-nucleotide (nt) fragment. pSV2ΔCAT-encoded messages protect a 271-nucleotide fragment. The first seven and the last three lanes are from separate experiments. Each lane contained 15 μg of RNA. The levels of mRNA were quantitated by densitometry of autoradiographic exposures in the linear range of the film. Lane MW contained *Hae*III-digested pBR322.

FIG. 3. S1 nuclease protection analysis of HIV-1 LTR-directed mRNA expression in the presence of various *tat*-expressing plasmids. HeLa cells were cotransfected with HIV-1 (pMALCAT) LTR-CAT fusion genes and various *tat*-expressing plasmids. All transfections also contained the internal control plasmid pSV2ΔCAT. Total RNA was hybridized with a 982-bp ³²P-end-labeled probe from the CAT-coding region. LTR-CAT-encoded messages protect a 782-nucleotide (nt) fragment. pSV2ΔCAT-encoded messages protect a 271-nucleotide fragment. The first and last five lanes are from separate experiments. Each lane contained 15 μg of RNA. The levels of mRNA were quantitated by densitometry of autoradiographic exposures in the linear range of the film. Lane MW contained *Hae*III-digested pBR322.

region of *SIV_{mac}*, we constructed plasmid pSBCAT, in which the *SIV_{mac}* LTR is fused to the CAT gene at position +100 within R (Fig. 1A). When this plasmid was cotransfected with pST into HeLa cells, the levels of transactivation were similar to those observed with pSHCAT, indicating that nucleotides downstream of +100 of the *SIV_{mac}* LTR are not required for transactivation response (data not shown).

Cross-transactivation of *SIV_{mac}* and HIV-1. The predicted sequences of the *SIV_{mac}* and HIV-1 *tat* proteins are only 36% similar (24; see Fig. 6). To determine whether the different *tat* proteins transactivate one another's LTRs, we constructed the plasmid pMALCAT, containing the CAT gene fused to the HIV-1_{LAV-MAL} LTR (1) at position +76

within R (Fig. 1B). In addition, we constructed pHT, containing both coding exons of HIV-1_{HXB2} (15) *tat* under the transcriptional control of the SV40 early promoter (Fig. 1A). When the *SIV_{mac}* LTR-CAT fusion gene pSHCAT was cotransfected with pHT, an average increase in the level of mRNA of 110-fold was observed (Fig. 2; Table 1). This level of transactivation is comparable to the level observed for the *SIV_{mac}* *tat* construct pST (Table 1). When the HIV-1 LTR-CAT fusion gene pMALCAT was cotransfected with pST expressing the complete *SIV_{mac}* *tat* gene, an average increase in the level of mRNA of 71-fold was observed (Table 1; Fig. 3). In contrast, cotransfection of pMALCAT with pHT, expressing the complete HIV-1 *tat* gene, resulted in a

TABLE 1. Levels of transactivation of *SIV_{mac}* and HIV-1 LTRs^a

Transactivator	Avg ± SE (%) <i>SIV_{mac}</i> LTR transactivation expressed as:		Avg ± SE (%) HIV-1 LTR transactivation expressed as:	
	mRNA expression	CAT activity	mRNA expression	CAT activity
<i>SIV_{mac}</i>	122 ± 53 (100)	138 ± 44 (100)	71 ± 13 (100)	53 ± 28 (100)
HIV-1	110 ± 40 (97 ± 16)	74 ± 22 (54 ± 10)	367 ± 47 (515 ± 17)	234 ± 113 (450 ± 11)

^a HeLa cells were cotransfected with *SIV_{mac}* (pSHCAT) or HIV-1 (pMALCAT) LTR-CAT fusion genes and plasmids which express the complete *tat* genes from either *SIV_{mac}* (pST) or HIV-1 (pHT). The data represent the ratios of mRNA expression or CAT activity of LTR-CAT fusion genes in the presence of *tat*-expressing plasmids compared with those in the absence of *tat*-expressing plasmids. Therefore, these numbers represent levels of transactivation. The numbers in parentheses represent percent transactivation of LTR-CAT fusion genes in the presence of *tat*-expressing plasmids relative to the level of transactivation obtained for pST expressing the complete *SIV_{mac}* *tat* gene. The activities of the different *tat*-expressing plasmids were compared in parallel transfection experiments. Each value represents the average of at least three experiments.

FIG. 4. Relative levels of transactivation of either SIV_{mac} or HIV-1 LTR-CAT fusion genes in the presence of various *tat*-expressing plasmids. A schematic representation of various *tat*-expressing plasmids is shown. The boxes represent the coding exons of either SIV_{mac} or HIV-1 *tat*. The lines represent introns. The triangles show the locations of either naturally occurring or introduced in-frame stop codons. HeLa cells were transfected with either SIV_{mac} or HIV-1 LTR-CAT fusion genes and the various *tat*-expressing plasmids, and the levels of stimulation of CAT enzyme activity and mRNA accumulation were determined as described in Materials and Methods. All values are shown as percentages of transactivation in the presence of plasmids that express mutant *tat* genes relative to the level of transactivation in the presence of plasmids that express either wild-type HIV-1 (pHT) or SIV_{mac} (pST) *tat* genes. The activities of the different *tat*-expressing plasmids were compared in parallel transfection experiments. Each value represents the average of at least three experiments. Standard errors are given. n.d., None detected.

367-fold average increase in the level of mRNA (Table 1; Fig. 3). These results indicate that both the complete SIV_{mac} and HIV-1 *tat* gene products can transactivate an SIV_{mac} LTR equally well. However, the complete SIV_{mac} *tat* gene product can transactivate an HIV-1 LTR only about 20% as well as the complete HIV-1 *tat* gene product.

As noted above, pMALCAT contains all of the DNA sequences necessary to fully respond to the HIV-1 transactivator. Nevertheless, it only partially responded to the SIV_{mac} transactivator. To determine whether the HIV-1 LTR contains additional sequences which would allow it to fully respond to SIV_{mac} *tat*, we constructed pMALNCAT, which contains the entire HIV-1_{LAV-MAL} LTR fused to CAT at a position 3 bp past the end of the LTR (Fig. 1B). When this LTR-CAT fusion gene was cotransfected with pST, we detected a 60-fold increase in CAT activity, compared with a 400-fold increase when it was cotransfected with pHT (data not shown). These results are consistent with the differences in the levels of transactivation determined for pMALCAT and suggest that the HIV-1_{LAV-MAL} LTR does not contain sequences outside of the previously defined TAR region which would allow it to respond more fully to the SIV_{mac} transactivator.

Transactivation primarily increases mRNA accumulation. Transactivation by *tat3* was reported to be caused by enhancement of the translational efficiency of TAR-containing mRNAs by as much as 1,000-fold (14, 44). However, subsequent studies in other laboratories have revealed a translational component of 3- to 30-fold above a substantial in-

crease in mRNA accumulation (8, 51). To address the relative roles of transcriptional and translational effects of transactivation, we measured CAT enzyme activity in cell extracts prepared from the same transfections used for RNA analysis. In all of our studies, the levels of increase in CAT enzyme activity were similar to the levels of increase of mRNA accumulation (Table 1; Fig. 4). The fact that there were apparently concomitant increases in both LTR-directed CAT enzyme activity and mRNA levels indicates that the predominant effect of transactivation is at the level of either transcriptional initiation or mRNA stabilization.

Activity of truncated *tat* gene products. Inspection of the published sequences of 13 isolates of HIV-1, 3 isolates of SIV_{mac}, and 1 isolate of HIV-2 revealed in-frame stop codons immediately following coding exon 1 of *tat* in all isolates (38). Splicing would remove these stop codons, but they would be present in mRNAs that retain the *tat* intron. This observation suggests that a truncated *tat* protein corresponding precisely to an exon 1 product would be encoded by unspliced mRNAs. To measure the activities of such truncated *tat* gene products, we constructed plasmids that express coding exon 1 of either SIV_{mac} (pSTΔP) or HIV-1 (pHTΔA) (Fig. 4). These plasmids were constructed such that all consensus splice acceptor sequences were removed.

Cotransfection of pSTΔP with either pSHCAT or pMALCAT into HeLa cells resulted in approximately 5% or less of the mRNA accumulation and CAT activity achieved with the wild-type plasmid pST (Fig. 2 to 4). These results indicate that the product of coding exon 1 of SIV_{mac} is far less active

than the complete *tat* product. In contrast, we saw small but consistent differences in the activity of the coding exon 1 product of the HIV-1 *tat* gene compared with that of the complete gene product. The product of just coding exon 1 stimulated CAT activity from an HIV-1 LTR-CAT fusion gene to the same extent as the complete product. However, in five of six experiments the level of mRNA transcribed from an HIV-1 LTR was 40% lower in the presence of only the exon 1 product (Fig. 3 and 4). This suggests that coding exon 2 affects the activity of the complete protein. This is further supported by experiments which measured stimulation of the SIV_{mac} LTR by either the exon 1 product or the complete product of the HIV-1 *tat* gene. The exon 1 product resulted in about 55% of the mRNA accumulation and about 165% of the CAT activity achieved with the complete product (Fig. 2 and 4). These results were averaged from six separate experiments, and in all cases the trend was the same. These results indicate that, as in SIV_{mac}, the portion of the HIV-1 *tat* protein encoded by coding exon 2 affects *tat* activity.

To confirm that coding exon 2 of SIV_{mac} *tat* is required for full activity, we constructed additional plasmids with stop codons inserted in frame within coding exon 2. The first plasmid, pSTS1, contains a stop codon positioned six codons after the predicted splice acceptor site. Cotransfection of this plasmid with pSHCAT resulted in about 60% of the CAT activity and 55% of the mRNA accumulation achieved with the wild-type plasmid pST (Fig. 2 and 4). Similarly, cotransfection of pSTS1 with pMALCAT resulted in about 35% of the CAT activity and 45% of the mRNA accumulation achieved with pST (Fig. 3 and 4). These results indicate that coding exon 2 is required for activity and suggest that the first six amino acids encoded by exon 2 can, at least partially, fulfill this requirement.

We also constructed pSTS2, which contains a translational stop codon positioned one codon after the splice acceptor site. Surprisingly, cotransfection of this plasmid with either pSHCAT or pMALCAT resulted in either about 90 or 70% of the CAT activity and either 134 or 250% of the mRNA accumulation achieved with pST (Fig. 2 to 4). The enhanced stimulation of mRNA accumulation was detected in three of five transfections with the SIV_{mac} LTR and three of three transfections with the HIV-1 LTR. These results were surprising, since pSTS2 is expected to encode a *tat* protein similar to plasmids that express just exon 1. To examine this result further, we deleted the portion of coding exon 2 of *tat* downstream from the stop codon insertion in pSTS2. This plasmid, pSTS2Δ, is expected to encode a *tat* protein corresponding to all of coding exon 1 and one amino acid of coding exon 2. Cotransfection of pSTS2Δ with pSHCAT resulted in about 20% of the CAT activity and about 15% of the mRNA accumulation achieved with pST (Fig. 2 and 4). In addition, cotransfection of pSTS2Δ with pMALCAT resulted in no detectable mRNA accumulation and about 5% of the CAT activity achieved with pST (Fig. 3 and 4). These observations posed an apparent contradiction between the requirement for SIV_{mac} *tat* coding exon 2 sequences and the full activity of a predicted truncation generated by a stop codon insertion in pSTS2.

Analysis of the levels and structures of *tat* mRNAs. To address the discrepancy between the results obtained with pSTS2 and those obtained with the other *tat*-expressing plasmids, we analyzed the levels and structures of *tat* mRNAs by RNase protection with a ³²P-labeled antisense RNA probe spanning the coding exon 1-intron junction (Fig. 1). A 386-nucleotide protected fragment is expected from

FIG. 5. RNase protection analysis of SIV_{mac} *tat* RNAs. Total RNA was isolated from HeLa cells transfected with various *tat*-expressing plasmids or from HuT78 cells infected with SIV_{mac}. The RNAs were analyzed with either a 428-nucleotide (nt) antisense RNA probe corresponding to the exon 1-intron junction or to a 228-nucleotide antisense RNA probe corresponding to the intron-exon 2 junction. (A) RNAs were isolated from transfections containing various *tat*-expressing plasmids and analyzed with the coding exon 1-intron junction probe. Unspliced messages protect a 386-nucleotide fragment, and spliced messages protect a 358-nucleotide fragment. Each protected fragment consists of a pair of bands, because the RNA probe used in this experiment contained two 3' ends because of premature termination during the *in vitro* synthesis reaction. Lane MW contained *Hinf*I-digested pBR322. (B) RNAs were isolated from transfections containing either pST or pSTS2 and were analyzed with the intron-coding exon 2 junction probe. Unspliced messages encoded by pST protect a 164-nucleotide fragment, and messages spliced at the predicted splice acceptor site protect a 101-nucleotide fragment. Unspliced messages encoded by pSTS2 protect a 103-nucleotide fragment. The smaller protected fragment of about 86 nucleotides is indicative of splicing at a position 15 nucleotides downstream of the site used by transcripts expressed by pST. Lane MW contained *Hpa*II-digested pBR322. (C) RNA was isolated from SIV_{mac}-infected HuT78 cells and analyzed with the intron-coding exon 2 junction probe. Unspliced messages protect a 164-nucleotide fragment. Messages spliced at the upstream site protect a 101-nucleotide fragment, and messages spliced at the downstream site protect an 86-nucleotide fragment. Lane MW contained *Hpa*II-digested pBR322.

unspliced mRNAs, and a 358-nucleotide protected fragment is expected from mRNAs which use the predicted splice donor site at the end of coding exon 1 of SIV_{mac} *tat*. This analysis revealed that all of the plasmids expressed comparable levels of *tat* mRNA (Fig. 5A). Furthermore, analysis of cytoplasmic RNA indicated that all of the *tat* mRNAs were transported equally well from the nucleus (data not shown). These experiments also indicated (i) that mRNAs expressed by pST are spliced with about 30% efficiency, (ii) that the splice donor site of *tat* mRNAs expressed by pSTΔP is not spliced to cryptic downstream splice acceptor sites, (iii) coding exon 1 of *tat* mRNA expressed by pSTS1 was spliced with about the same efficiency as the *tat* mRNA expressed by pST, (iv) there was about a twofold enhancement of splicing of *tat* mRNA expressed by pSTS2 compared with pST, and (v) there was no detectable splicing of *tat* mRNA expressed by pSTS2Δ. To compare the *tat* mRNAs expressed by pST and pSTS2 further, we analyzed them with an antisense RNA probe spanning the intron-coding exon 2

junction (Fig. 1A). This experiment (Fig. 5B) indicated that *tat* mRNAs expressed by pST protect a fragment of about 164 nucleotides expected for unspliced messages and a fragment of about 101 nucleotides expected for messages spliced at the predicted splice acceptor sequence. In addition, *tat* mRNAs expressed by pSTS2 protect a fragment of about 103 nucleotides expected for unspliced messages and a fragment of about 86 nucleotides indicative of splicing at a site about 15 nucleotides downstream of the predicted splice acceptor site. These results indicate that the stop codon inserted into *tat* mRNAs transcribed by pSTS2 and pSTS2Δ disrupts splicing at the predicted splice acceptor site and, in the case of pSTS2, activates a downstream splice acceptor site.

To determine whether the splice acceptor site activated by the insertion in pSTS2 is used in virus-infected cells, we prepared total RNA from SIV_{mac} (isolate BK28)-infected HuT78 cells and SIV_{mac}-transfected HeLa cells. These RNAs were analyzed by RNase protection with the exon 2 probe (Fig. 5C; data not shown). This experiment revealed a fragment of about 86 nucleotides predicted for mRNAs spliced at the downstream splice acceptor site. This fragment was present at about 5% of the level of the fragment predicted for splicing at the upstream site. These results indicate that splicing at this downstream site is a normal feature of SIV_{mac} during viral infection.

DISCUSSION

Previous results (2, 13) have demonstrated that transfection of SIV_{mac} and HIV-1 LTR-CAT constructs into SIV_{mac}-infected cells leads to increased CAT activity compared with uninfected cells. These results suggested the presence of a virus-induced activator of LTR-directed gene expression. Here we show that this effect is directly mediated by a product encoded by a fragment of SIV_{mac} containing the predicted *tat* gene. In cotransfection experiments with plasmids which express the SIV_{mac} *tat* gene under the transcriptional control of the SV40 early promoter and plasmids containing SIV_{mac} LTR-CAT fusion genes, LTR-mediated gene expression was about 120-fold above the level found without transactivators.

SIV_{mac} and HIV-1 *tat* most likely function through a common mechanism. The two viruses are biologically similar, and their *tat* proteins can transactivate one another's LTRs (2). Cross-transactivation is not completely reciprocal; i.e., although HIV-1 *tat* fully transactivates the SIV_{mac} LTR, SIV_{mac} *tat* only partially transactivates the HIV-1 LTR, suggesting that the proteins have somewhat different specificities. Comparison of their predicted amino acid sequences reveals two centrally located conserved regions. The first is cysteine rich and encompasses residues 50 to 76 in SIV_{mac} and 22 to 47 in HIV-1 (Fig. 6). Of these 27 amino acids, 19 are identical and 5 represent conservative substitutions. This region in HIV-1 has been shown to bind metal ions which appear to coordinate folding and facilitate dimer formation (17). The predicted structure of the *tat*-metal complex is distinct from the zinc finger structure predicted for a number of eucaryotic nucleic acid-binding proteins (5). Adjacent to the cysteine-rich domain is a conserved highly basic region of 9 to 11 amino acids that is similar in structure to known nuclear translocation signals (48) (Fig. 6). The conservation of these regions suggests that they correspond to functional domains. The remaining regions of the proteins display poor homology. In addition, the SIV_{mac} protein is 28 amino acids larger than the HIV-1 protein in the amino-

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SIVmac METPLREQENSLESSNERSSSCILEADATTPESANLGEEIL
HIV-2 METPLKAPESSLKSCNEPFSRTSEQDVATQELARQGEEL
HIV-1 ME-PVDPRL-----EPWK

SIVmac SOLYRPLEACYN1TCYCKKCCYHCQFCFLKKGLGICYEQSR
HIV-2 SOLYRPLETCNN1SCYCKRCCYHCQMCFLNKGLGICYERKG
HIV-1 HPGSQPKTAC1TN-CYCKKCCFHCQVCFITKALGISYGRKK

SIVmac KRRRTPKKAKANTSSASNKLI2PNRTRHCQPEKAKKETVEK
HIV-2 RRRRTPKKTKTH2PSPTPKSISTRTGDSOPTKKOKKTVEA
HIV-1 RRQRRFAHQNSQTHQASLSKQ2TSQPRGDP2TGPKE-----

SIVmac AVATAPGLGR
HIV-2 TVE3TDIGPGR
HIV-1 -----

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FIG. 6. Deduced amino acid sequences of *tat* proteins from SIV_{mac}, HIV-1, and HIV-2. Dashes indicate gaps introduced to maintain alignment. The large box indicates amino acid residues which make up a cysteine-rich domain conserved among all three proteins. The smaller box indicates a highly basic region found in all three proteins. The underlined amino acids are the first residues encoded by coding exon 2. The predicted amino acid sequence of the SIV_{mac} *tat* protein was based on the DNA sequence of Hirsch et al. (24) and H. Kornfeld and J. I. Mullins (unpublished data). The predicted amino acid sequence of the HIV-1 *tat* protein from HXBC2 was based on our DNA sequence (see Materials and Methods). The predicted amino acid sequence of HIV-2 was from Guyader et al. (19).

terminal region preceding the cysteine-rich domain and 15 amino acids larger in the region of the protein encoded by exon 2. The differences between SIV_{mac} and HIV-1 in these regions may account for the different specificities of their *tat* proteins.

We found concomitant increases in the amounts of SIV_{mac} and HIV-1 LTR-directed enzyme activity and mRNA. These results support the hypothesis proposed for HIV-1 that the predominant effect of transactivation is to increase either transcriptional initiation or mRNA stabilization rather than translational efficiency (36, 41, 42). At least part of transactivation by HIV-1 results from a direct effect on transcription. Nuclear runoff experiments have indicated that *tat* stimulates transcriptional initiation (22, 42). Furthermore, extracts from HIV-1-infected cells stimulate in vitro transcription from an HIV-1 LTR (40). Finally, it has been reported that in the absence of *tat*, HIV-1 LTR-directed transcription is prematurely terminated within the TAR sequence (30). *tat* presumably relieves this termination and allows transcription to proceed. However, other studies have indicated that translational events account for all (14, 44), or at least a significant component of (8, 51), transactivation. Likely reasons for these discrepancies are the RNA quantitation methods used and the inherent difficulties of establishing comparable base-line levels for RNA and protein expression. In addition, it has been suggested that cell type-specific factors differentially affect the translational efficiency or stability of mRNAs (36, 51). The two studies that reported the highest translational contribution to transactivation also detected the highest levels of transactivation based on CAT activity (44, 51). These results were obtained in experiments with either virus-infected cells or cells which constitutively express *tat3*. Although other researchers also used stably transformed cell lines, they did not detect the very high levels of transactivation described in the former reports (8, 36). Since *tat3* apparently does not specifically bind to TAR-containing DNA or RNA (17), it probably interacts with TAR through another molecule. Possibly the transactivator has a higher affinity for a target molecule that is part of a transcription complex than it does for a target

molecule that is part of a translation complex. Therefore, higher concentrations of *tat3* may be required for significant binding of the transactivator to the latter to result in a detectable translational component to transactivation.

Our findings reported here indicate that the exon 1 products of the *SIV_{mac}* and HIV-1 *tat* genes are less active than the full-length proteins, albeit to different extents. pSTΔP, expressing coding exon 1 of *SIV_{mac} tat*, transactivates LTR-directed gene expression about 20-fold less than pST, which expresses both coding exons. Another plasmid, pSTS2Δ, was about 5- to 16-fold less active than pST in stimulating LTR-directed gene expression. This plasmid has coding exon 2 deleted downstream of an oligonucleotide insertion located 3 nucleotides past the splice acceptor site. This mutation prevents splicing and should therefore encode a protein corresponding to an exon 1 product. Taken together, these results indicate that the portion of the *SIV_{mac} tat* protein encoded by coding exon 2 is required for full activity in HeLa cells. Whether coding exon 2 is required in other cell types has not been determined.

To analyze the contribution of coding exon 2 of *SIV_{mac} tat* further, we constructed plasmid pSTS1, containing an in-frame stop codon inserted 18 nucleotides past the splice acceptor sequence. This plasmid was about 50% as active as pST in stimulating LTR-directed gene expression. These results support the observation that amino acids encoded by coding exon 2 are required for full activity of *SIV_{mac} tat*. Furthermore, they suggest that the first six amino acids encoded by coding exon 2 can partially fulfill this requirement.

Our results for HIV-1 *tat* are in contrast to those of previous studies which have concluded that the exon 1 product of HIV-1 *tat* is functionally equivalent to the complete protein (8, 47, 49). However, these previous studies measured only protein expression and did not examine the effects of exon 2 on mRNA accumulation. Our studies also indicate that deletion of exon 2 from HIV-1 *tat* does not decrease CAT activity. However, it reduces the mRNA levels about twofold. Given the lack of internal controls for CAT activity inherent in these experiments, it is difficult to determine whether CAT expression always reflects a real effect.

In-frame stop codons are located just after the end of coding exon 1 of the *tat* gene from a number of isolates of HIV-1, HIV-2, and *SIV_{mac}* (38). The conservation of these stop codons suggests that truncated *tat* proteins can be encoded by unspliced mRNAs. A truncated HIV-1 *tat* protein, presumably encoded by coding exon 1, has been identified (B. Cullen, personal communication). Identification of similarly truncated proteins from *SIV_{mac}* will require generation of *SIV_{mac} tat*-specific antisera. The contribution made by truncated *tat* proteins to the biology of *SIV_{mac}* and HIV-1 remains to be established.

One of the in vitro-generated mutations in *SIV_{mac} tat* (pSTS2) used in these studies appeared to be more active in stimulating LTR-directed mRNA accumulation than was wild-type *tat*. This mutation contains an insertion in coding exon 2 which disrupts splicing at the predicted splice acceptor site and activates a splicing event about 15 nucleotides downstream. The activated splice acceptor sequence, ACC-CAACAG, is similar to the consensus sequence, PyNPyPy PyNCAG (35). This alternatively spliced mRNA should encode a *tat* protein with a five-amino-acid internal deletion. RNase protection experiments indicate that this alternative splice acceptor site is also used by a biologically active *SIV_{mac}* provirus with an efficiency of about 5% compared

with that of the upstream site. Furthermore, we have isolated cDNA clones derived from *SIV_{mac}*-infected Hut78 cells which use the *tat* splice donor and the alternative splice acceptor site (manuscript in preparation).

The predicted sequence of the HIV-2 *tat* protein (19) is considerably more homologous to the *SIV_{mac} tat* protein than to the HIV-1 *tat* protein (Fig. 6). Functional studies demonstrated that both HIV-2 and HIV-1 *tat* gene products significantly transactivate HIV-2 LTR-driven CAT enzyme activity (13, 19). In contrast, the HIV-2 *tat* gene product only partially transactivates an HIV-1 LTR. These results are consistent with our findings for *SIV_{mac}*, further indicating a close evolutionary relationship between *SIV_{mac}* and HIV-2. However, amino acids encoded by coding exon 2 are apparently less important for the activity of HIV-2 *tat* than for that of *SIV_{mac} tat* (13).

The *tat* gene product is required for the replication and cytopathicity of HIV-1 in culture (10, 15a). Assessing the contribution made by transactivation to the in vivo pathogenicity of HIV-1 requires an understanding of the mechanisms and components involved in this process. Further comparative studies between *SIV_{mac}* and HIV-1 should help clarify this aspect of HIV-1 pathogenicity.

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