

Functional Analysis of the Tat *trans* Activator of Human Immunodeficiency Virus Type 2

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The *trans*-activator (Tat) proteins of the related but distinct type 1 and type 2 human immunodeficiency viruses (HIV-1 and HIV-2) display incomplete functional reciprocity. One possible explanation for this observation, suggested by computer analysis of potential RNA secondary structures within the viral *trans*-activation response (TAR) elements, is that HIV-2 Tat requires the presentation of two viral RNA stem-loop sequences for full activity whereas HIV-1 Tat is maximally active upon presentation of a single stem-loop structure. Here, we demonstrate that the HIV-2 long terminal repeat indeed contains two functionally independent TAR elements. However, the second (3') TAR element of HIV-2 is significantly less active than the 5' TAR element and is functionally masked in the context of an intact HIV-2 long terminal repeat. Evidence is presented suggesting that the activities of these two HIV-2 TAR elements reflect, at least in part, their relative distances from the site of transcription initiation. Although the HIV-2 TAR element proximal to the viral mRNA cap site appears to be sufficient for effective *trans* activation by HIV-2 Tat *in vitro*, this functional redundancy may nevertheless serve to enhance HIV-2 replication in infected cells *in vivo*.

The pathogenic human retrovirus human immunodeficiency virus type 1 (HIV-1) is the predominant etiologic agent of acquired immune deficiency syndrome (9). Recently, a second retrovirus, HIV-2, has been isolated from patients with acquired immune deficiency syndrome in western Africa (5, 13). HIV-2 is more closely related to the simian immunodeficiency viruses (~75% homology to SIV_{MAC}) than to HIV-1 (~45% homology) (13, 17, 28) and appears to produce a significantly lower level of disease in infected individuals (19). However, HIV-2 displays a genetic complexity comparable to that of HIV-1 (13, 17). In particular, both HIV-1 and HIV-2 encode a regulatory protein, termed Tat, that functions to *trans* activate the expression of sequences linked to the viral long terminal repeat (LTR) promoter (1, 2, 8, 13) and whose expression (for HIV-1) has been shown to be required for viral replication (7, 12). Although the precise mechanism of action of Tat has yet to be clearly defined, it appears that Tat enhances both the level of viral mRNA synthesis and the translational utilization of LTR-directed transcripts (4, 6, 10, 16, 18, 24-26, 30). Evidence suggesting that the Tat proteins of HIV-1 and HIV-2 (hereinafter referred to as Tat1 and Tat2, respectively) each have the ability to effectively *trans* activate the expression of genes linked to the HIV-2 LTR has been presented elsewhere (1, 2, 8, 13). However, Tat1 was observed to be significantly more effective than Tat2 in enhancing HIV-1 LTR-specific gene expression (1, 2, 8, 13). These experiments have been interpreted to suggest that these Tat proteins may be directly involved in mediating the recognition of their respective viral LTR target sequences, the so called *trans*-activation response (TAR) elements (8).

The TAR element of HIV-1 is located within the LTR R region 3' to the transcription initiation site and has been

shown to be position and orientation dependent (11, 14, 18, 24, 26). Current evidence suggests that the TAR element is recognized as an RNA, rather than a DNA, sequence (4, 11, 24). The HIV-1 TAR element coincides with a highly stable predicted RNA secondary structure (Fig. 1), and the existence of this RNA secondary structure has been confirmed *in vitro* (24). Site-directed mutational analyses of the HIV-1 TAR region support the hypothesis that the integrity of this RNA structure is essential for *trans* activation by Tat1 (11, 14, 18, 27). In particular, these results suggest that the Tat1 response is mediated by the sequence-specific recognition of a specific RNA hexanucleotide loop sequence (5'-CUGGGX-3') presented in the context of a stable RNA secondary structure (11).

The sequence requirements for *trans* activation of HIV-2 LTR-specific gene expression by Tat2 remain less well defined. Recent evidence (2, 27) suggests that the HIV-2 TAR element coincides with a predicted RNA secondary structure that is somewhat more complex than that observed in HIV-1 (Fig. 1). This RNA secondary structure displays an additional loop within the predicted TAR recognition sequence, and it has been suggested that a requirement for recognition of two such structures by Tat2 might explain the incompletely reciprocal *trans* activation observed for the Tat1 and Tat2 proteins (2, 8). Here, we have attempted to address this question via an extensive mutational analysis of the HIV-2 TAR region. This study utilized constructions in which the HIV-2 LTR was linked *in cis* to reporter genes to measure the effects of TAR element mutations on Tat1 and Tat2 *trans* activation of HIV-2 LTR-specific gene expression. These results define the minimal sequences necessary for Tat1- and Tat2-mediated *trans* activation of the HIV-2 LTR and provide direct evidence for the functional importance of both of the predicted RNA stem-loop structures present in the HIV-2 TAR element.

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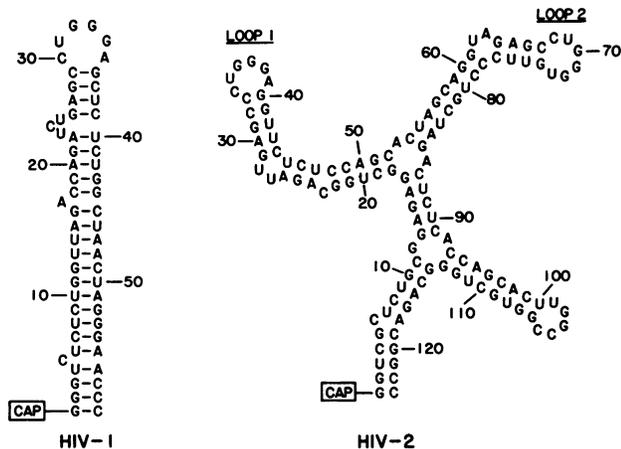


FIG. 1. Predicted RNA secondary structure of the TAR elements of HIV-1 and HIV-2. The HIV-1 TAR element is shown in its most stable predicted secondary structure ($\Delta G = -37.6$ kcal [1 cal = 4.184 J]/mol) (11, 24, 27). The distribution of significant secondary structures within the HIV-2 leader region (+1 to +545) was calculated for RNA segment sizes ranging from 30 to 300 bases, as previously described (20, 23). The segment scores which measure the statistical significance of the optimal secondary structure were computed by using the Cray operating system of a Cray X-MP/24 computer and the energy rules of Turner et al. (29). The indicated structure has a predicted stability (ΔG) of -62.0 kcal/mol.

MATERIALS AND METHODS

Construction of molecular clones. The expression vector pgTAT(HIV-1) (previously named pgTAT) we used contained a complete genomic copy of the HIV-1 *tat* gene under the control of the Tat nonresponsive cytomegalovirus immediate early promoter (22). Polyadenylation signals were provided by sequences derived from the genomic rat insulin II gene. pgTAT(HIV-2) was derived from pgTAT(HIV-1) by excision of the HIV-1-specific sequences by cleavage with *Sall* and *Bam*HI followed by substitution of a functionally equivalent *Hind*III to *Pvu*II fragment derived from a genomic clone of HIV-2 (13) (HIV-2_{ROD} sequence coordinates 5783 to 8430). Like pgTAT(HIV-1), pgTAT(HIV-2) therefore contained both *tat* coding exons separated by an intronic sequence primarily derived from the viral *env* gene. As neither pgTAT(HIV-1) nor pgTAT(HIV-2) encodes a functional copy of the viral Rev *trans* activator, both vectors predominantly express spliced mRNAs that encode full-length, two-exon forms of the viral Tat proteins (22).

We have previously (3) described the vectors pBC12/HIV-1/SEAP and pBC12/HIV-1/CAT, which contain the HIV-1 LTR linked in *cis* to the secreted alkaline phosphatase (SEAP) or chloramphenicol acetyltransferase (CAT) indicator gene, respectively. Substitution of a comparable HIV-2 LTR DNA fragment (HIV-2_{ROD} sequence coordinates 9270 to 9619) generated the equivalent HIV-2 LTR-based vectors pBC12/HIV-2/SEAP and pBC12/HIV-2/CAT. This HIV-2 LTR DNA fragment contains all the sequence elements known to be important for Tat *trans* activation of HIV-2 gene expression (2, 8). The 3' border of this HIV-2 LTR fragment, defined by an *Nla*IV site at position +123 relative to the start of transcription, was converted to a *Hind*III site by the addition of a synthetic DNA linker (5'-CAAGCTTG-3'). A unique *Bgl*II site was similarly introduced at HIV-2 LTR position -226. These introduced sites facilitated sub-

sequent molecular manipulation of the HIV-2 LTR fragment. The control vectors pBC12/CMV and pBC12 Δ I have been described elsewhere (6).

The HIV-2 LTR deletion mutants were derived by processive *Bal*31 exonuclease digestion (slow form; International Biotechnologies, Inc.) of pBC12/HIV-2/SEAP after initial cleavage with *Hind*III. *Bal*31-digested DNA was subsequently blunt ended by using Klenow DNA polymerase I and was ligated to a *Hind*III linker. HIV-2 LTR fragments of <350 base pairs were gel purified after cleavage with *Bgl*II and *Hind*III and recloned into the parental vector. The deletion endpoints of the resultant clones were determined by DNA sequencing, and selected examples were tested for the *trans*-activation phenotype. The deletion mutants, whose designations begin with pD, are named according to the 5' border of the deletion, e.g., pD+21/SEAP is identical to pBC12/HIV-2/SEAP except that it lacks HIV-2 LTR sequences between +21 and the *Hind*III site at +123.

Mutants p Δ L1/SEAP, p Δ L2/SEAP, and p Δ L1+2/SEAP were derived from pBC12/HIV-2/SEAP by oligonucleotide-directed mutagenesis. These mutations each resulted in 4-base sequence insertions in the predicted loop sequences. In the case of p Δ L1/SEAP, the predicted loop 1 sequence 5'-CUGGGA-3' (Fig. 1) was changed to 5'-CCGGCCGGGA-3'. This mutation also resulted in the introduction of a unique site for the restriction enzyme *Eag*I. In p Δ L2/SEAP, the predicted loop 2 sequence (Fig. 1) was changed from 5'-CUGGGU-3' to 5'-CAUGCAUGGU-3'. p Δ L1+2 contains both insertion mutations.

The deletion mutant p Δ S1/SEAP was constructed from p Δ L1/SEAP and from a *Bal*31 deletion mutant of the HIV-2 LTR whose endpoint was mapped to position +21 (pD+21/SEAP). The pD+21/SEAP vector was cleaved at *Hind*III, filled in by using Klenow DNA polymerase, and then cleaved at a unique *Xho*I site within the SEAP indicator gene. The insert was derived from p Δ L1 by cleavage at the introduced *Eag*I site (described above), removal of the resultant 5' overhang by S1 nuclease treatment, and further cleavage at the SEAP gene *Xho*I site. Ligation of these fragments resulted in a vector lacking nucleotides from positions +21 through +35, which essentially encompasses the 5' side of the first predicted stem in the HIV-2 TAR element (Fig. 1).

The CAT reporter gene analogs of these constructs were made by replacing the *Hind*III-*Bam*HI SEAP gene fragment with a 797-base-pair *Hind*III-*Bam*HI CAT gene fragment (3).

Cell culture and DNA transfection. Jurkat and COS cells were maintained as previously described (6, 21). COS cells were transfected with DEAE-dextran and chloroquine (6). Jurkat cells were electroporated with 10 μ g of plasmid DNA per 10⁷ cells by using the Bio-Rad gene pulser set at 960 μ F and 250 mV in a 250- μ l sample volume. SEAP and CAT assays were performed as previously described (3, 21) by using COS cell culture medium and Jurkat cell lysates harvested at 60 h posttransfection.

Immunoprecipitation analysis. A polyclonal rabbit antipeptide antiserum directed against a 25-amino-acid synthetic peptide consisting of residues 75 to 99 of the predicted HIV-2 *tat* open reading frame was prepared as previously described (16). Immunoprecipitation analysis of transfected cells was performed (22) by using a 1:150 dilution of the anti-Tat2 antiserum following a 2-h metabolic labeling with [³⁵S]cysteine.

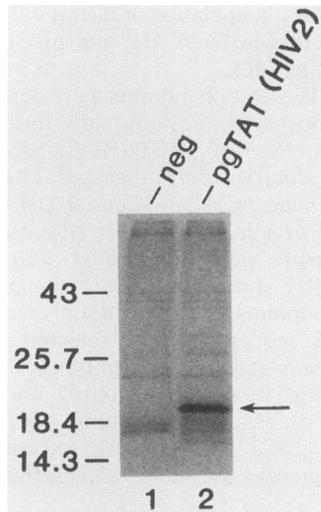


FIG. 2. Immunoprecipitation of the Tat2 protein. The Tat2 protein is visualized as an ~21-kilodalton protein present in cells transfected with the pgTAT(HIV-2) vector (lane 2) that is lacking in cells transfected with the parental pBC12/CMV vector (neg; lane 1). Molecular sizes (in kilodaltons) are shown at left.

RESULTS

We have previously described the genomic HIV-1 *tat* gene expression vector pgTAT(HIV-1) (22). In transfected COS cells, pgTAT(HIV-1) induces the efficient synthesis of the full-length 86-amino-acid form of the Tat1 protein (22). The Tat1 protein has been shown to migrate at an unexpectedly

slow relative molecular mass (M_r) of 15.5 kilodaltons on sodium dodecyl sulfate-polyacrylamide gels (22) and is predominantly localized to the nuclei, and particularly the nucleoli, of expressing cells (16).

The genomic HIV-2 *tat* gene expression vector utilized in this study, pgTAT(HIV-2), is structurally equivalent to the pgTAT(HIV-1) vector. To verify the utilization of this construct, we raised a polyclonal rabbit antiserum directed against a synthetic peptide derived from amino acids 75 to 99 of the predicted Tat2 open reading frame. This antibody specifically immunoprecipitated an ~21-kilodalton protein present in a COS cell culture transfected with pgTAT(HIV-2) that was absent from a control culture (Fig. 2). Therefore, the slow migration of the 130-amino-acid Tat2 protein recapitulates the anomalous migration of the Tat1 protein on sodium dodecyl sulfate-polyacrylamide gels. Immunofluorescence analysis using the rabbit polyclonal anti-Tat2 peptide antiserum also demonstrated that the Tat2 protein, like Tat1, is predominantly localized to the cell nucleus, where it is further concentrated in the nucleoli (data not shown).

Functional comparison of Tat1 and Tat2. We next compared the abilities of the Tat proteins of HIV-1 and HIV-2 to *trans* activate viral LTR-specific gene expression. For this purpose, we used vectors in which the SEAP indicator gene (3) was expressed under the control of either the HIV-1 LTR (pBC12/HIV-1/SEAP) or the HIV-2 LTR (pBC12/HIV-2/SEAP). A constant amount of these vectors (1 μ g) was then transfected into cultures of the HIV-1 replication-permissive cell line COS with a range of concentrations of pgTAT(HIV-1) or pgTAT(HIV-2). This experiment demonstrated that the HIV-2 Tat protein was two- to threefold less effective than the HIV-1 Tat protein in *trans* activating the HIV-1 LTR

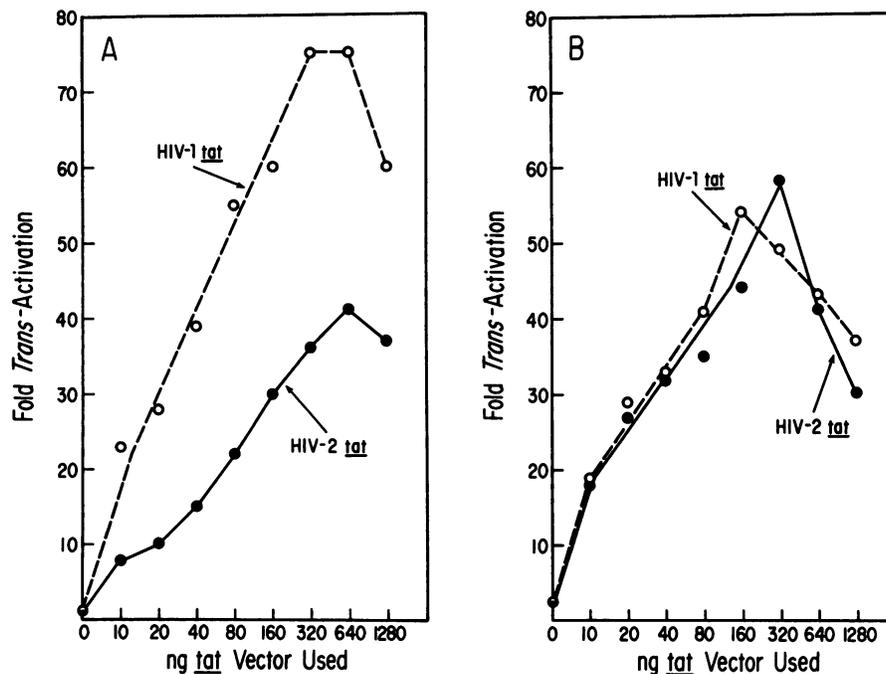


FIG. 3. Relative *trans* activation of the HIV-1 and HIV-2 LTRs mediated by various levels of Tat1 or Tat2. A constant level (1 μ g) of the indicator plasmid, pBC12/HIV-1/SEAP (A) or pBC12/HIV-2/SEAP (B), was cotransfected with increasing levels (10 to 1,280 ng) of pgTAT(HIV-1) or pgTAT(HIV-2). The total amount of DNA transfected per culture was maintained at 2.5 μ g by supplementation with the negative control vector pBC12/CMV (6). The levels of SEAP expression in the supernatant media were determined (3) at 60 h posttransfection. SEAP activity is expressed as a multiple of the basal level of LTR-specific gene expression.

over the entire range of Tat vector concentrations used (Fig. 3A). In contrast, both Tat1 and Tat2 yielded essentially the same dose-response curves when cotransfected with the HIV-2 LTR-based expression construct (Fig. 3B). This result suggests that the inability of Tat2 to fully *trans* activate the HIV-1 LTR is not a dose-dependent phenomenon. It is important to note that the equivalent response observed with the HIV-2 LTR did not appear to result simply from a more effective interaction of the Tat2 protein with the homologous HIV-2 LTR but instead seemed also to reflect a lower level of response mediated by the HIV-1 Tat protein. As previously reported (15), a decrease in *trans* activation was observed at high levels of Tat expression (Fig. 3). Subsequent transfection experiments therefore used the ratio of indicator plasmids to *tat* expression vector (4:1) predicted to yield maximal *trans* activation of the HIV-2 LTR.

Mutational analysis of the HIV-2 TAR element. In HIV-1, the sequence element required for TAR has been shown to coincide with a stable RNA stem-loop structure comprising the first 59 nucleotides of all HIV-1 transcripts (11, 18, 24, 26, 27) (Fig. 1). Evidence suggests that the loop RNA sequence 5'-CUGGGX-3' is the recognition sequence required for Tat *trans* activation, whereas the function of the stem is primarily the presentation of this loop sequence in the appropriate structural context (11, 18). Although the existence of the RNA stem-loop structure predicted for the HIV-1 TAR element has been confirmed by *in vitro* analysis (24), little is known about the HIV-2 TAR element. Therefore, we used previously described computer analysis techniques (20, 23) to predict the most stable secondary structure present within the HIV-2 LTR RNA leader. The structure obtained extends from positions +1 to +123 relative to the site of transcription initiation (Fig. 1) and is identical to the HIV-2 TAR structure previously proposed by Arya and Gallo (2) and by Selby et al. (27). Of interest is the fact that this structure is predicted to contain two RNA loops which match the TAR consensus sequence 5'-CUGGGX-3'.

In order to assess the importance of this predicted RNA secondary structure in mediating *trans* activation by Tat1 or Tat2, we constructed a nested set of HIV-2 LTR deletion mutants extending 5' from the introduced *Hind*III restriction enzyme site at position +123 (Fig. 4). The phenotypes of the mutants were determined by using two different reporter genes and two distinct cell lines to control for cell- or reporter gene-specific phenomena. Vectors based on the SEAP indicator gene were again used to quantitate the relative *trans* activation of each HIV-2 LTR deletion mutant in COS cells (Fig. 4A). With the exception of a small drop in *trans* activation by both Tat1 and Tat2 after deletion of HIV-2 LTR sequences between positions +106 and +123, this analysis revealed little significant phenotypic effect until TAR sequences between +45 and +50 were affected, when *trans* activation was essentially lost. Functional analysis in the CD4⁺ human T-cell line Jurkat of CAT reporter gene analogs of these same HIV-2 LTR mutants yielded essentially similar results (Fig. 4B). In this system, the loss of sequences 3' to +51 again resulted in a slight (<twofold) effect on HIV-2 LTR *trans* activation. However, deletion of nucleotide +51 led to a marked loss in *trans* activation, while loss of nucleotide +50 resulted in the ablation of HIV-2 LTR responsiveness to both Tat1 and Tat2. Both of these assays, therefore, focused attention on predicted HIV-2 TAR element stem-loop 1, and the results suggest that this TAR element is no longer functional when the length of this stem

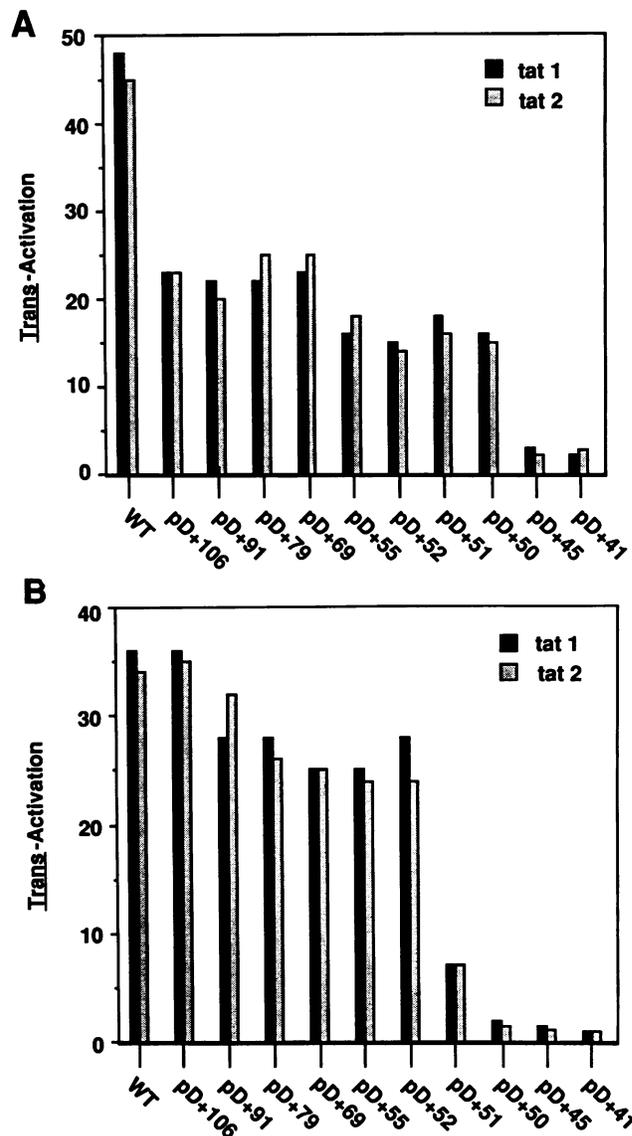


FIG. 4. *trans* activation of HIV-2 TAR deletion mutants by Tat1 or Tat2. (A) COS cell cultures were transfected with the indicated SEAP constructs based on the indicated HIV-2 LTR constructs in the presence of either pgTAT(HIV-1) or pgTAT(HIV-2). WT, Full-length HIV-2 TAR element, which is predicted to extend to position +123 relative to the cap site. Deletion mutants are named according to the 5' border of the deletion; i.e., pD+41 lacks HIV-2 TAR sequences between +41 and +123 inclusively. Levels of supernatant SEAP activity were determined (3) at 60 h posttransfection and are expressed as a multiple of the basal activity determined for each HIV-2 LTR construct when cotransfected with the negative control vector pBC12/CMV (6). No significant effects of the indicated mutations on basal LTR-specific gene expression were noted. (B) Materials and methods used were comparable with those described in the legend to panel A, except that the cell line was Jurkat and all constructs were based on the CAT indicator gene.

falls below ~10 base pairs (i.e., in pD+50). This result is very similar to data previously obtained for HIV-1, which demonstrated a minimal functional TAR element stem length of 9 base pairs (14, 18, 27). Most importantly, the results presented in Fig. 4 clearly show that Tat1 and Tat2 gave the same *trans*-activation phenotype in all the HIV-2 TAR

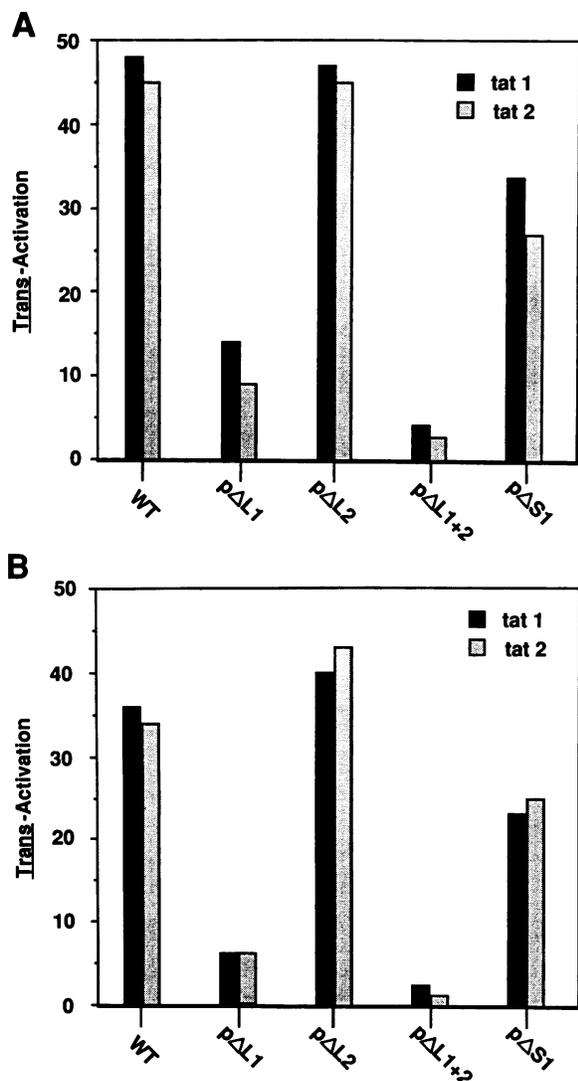


FIG. 5. HIV-2 LTR leader region, containing two TAR elements. (A) The indicated mutant HIV-2 LTRs were linked to the SEAP gene and transfected into COS cells. *trans* activation by Tat1 or Tat2 was calculated for each HIV-2 LTR construction by reference to a parallel cotransfection with the negative control vector pBC12/CMV (6). (B) Materials and methods used were comparable with those described in the legend to panel A, except that the cell line was Jurkat and all constructs were based on the CAT indicator gene.

mutants tested in both COS and Jurkat cells. The HIV-2 LTR sequence requirements for *trans* activation by Tat1 and Tat2 therefore appear to be identical.

HIV-2 contains two independently functional TAR elements. The evidence presented in Fig. 4 supports the hypothesis that the integrity of the 5' stem-loop predicted for the HIV-2 TAR element (Fig. 1, loop 1) is important for *trans* activation by both Tat1 and Tat2. However, these results do not indicate a functional role for the predicted stem-loop involving nucleotides +54 to +85 (Fig. 1). These data therefore raise the possibility that the predicted loop 2 does not play any role in the *trans* activation of the HIV-2 LTR. To more directly address this question, we introduced into either loop 1 (pΔL1) or loop 2 (pΔL2) mutations that are

predicted (11) to destroy the ability of the loop to mediate Tat *trans* activation but are not predicted (29) to affect the overall secondary structure of the HIV-2 TAR element. These constructs and another construct mutated in both predicted loop structures (pΔL1+2) were then introduced into COS cells (Fig. 5A) or Jurkat cells (Fig. 5B) in the presence or absence of pgTAT(HIV-1) or pgTAT(HIV-2). As predicted from the deletion analysis, mutation of loop 2 (pΔL2) resulted in no detectable phenotype in the context of an intact loop 1 structure. However, pΔL1 was observed to retain a significant capability for *trans* activation that was lost after the further mutation of loop 2 (pΔL1+2). This result suggests that the second loop structure predicted for the HIV-2 leader region is, in fact, a second independent TAR element. However, this second element is functionally masked in the presence of an intact loop 1 TAR element. Further, this 3' TAR element was found to be four- to eightfold less active than the 5' TAR element when found alone (Fig. 5).

It has been demonstrated that the effectiveness of the HIV-1 TAR element declines rapidly as it is moved away from the viral mRNA transcription start site (14, 26, 27). To test the hypothesis that the differential effectiveness of the two TAR elements present in the HIV-2 leader reflected their relative proximity to the cap site, we constructed a deletion mutant of pΔL1, called pΔS1, which lacked HIV-2 LTR nucleotides +21 to +35 inclusively. Phenotypic analysis of this mutant showed a significantly enhanced level of *trans* activation compared with that of the parental pΔL1 vector (Fig. 5). Therefore, we conclude that the ineffectiveness of the second, 3' TAR element of HIV-2 results at least in part from its suboptimal distance from the viral mRNA cap site.

DISCUSSION

In this study, we have used site-directed mutagenesis of the HIV-2 LTR, combined with transient expression assays in both nonlymphoid and lymphoid cells, to analyze *trans* activation by the HIV-2 *tat* gene product. In general, our results suggest that *trans* activation in the HIV-2 system occurs via a mechanism that is very similar to *trans* activation in HIV-1. However, while both Tat1 and Tat2 function via analogous sequence elements present at the 5' end of viral mRNAs, they display higher activities with their homologous viral TAR element (Fig. 3). This suggests that some differences in sequence specificity do exist.

Several investigators have previously reported that Tat2 is less effective than Tat1 in *trans* activating the HIV-1 LTR (1, 2, 8, 13). However, this phenomenon has previously been ascribed entirely to a more extensive TAR element sequence requirement for Tat2 function compared with that for Tat1 (2, 8). For example, Emerman et al. (8) have suggested that HIV-2 TAR element sequences between +1 and +53 are both necessary and sufficient for *trans* activation by Tat1, while full *trans* activation by Tat2 requires additional sequences between +53 and +99 that are unique to the HIV-2 TAR element. These observations led to the hypothesis that Tat1 requires only a single TAR element RNA stem-loop for full function (as is normally observed in HIV-1 transcripts; Fig. 1) while Tat2 requires the display of two functional TAR elements for maximal *trans* activation (2, 8). In contrast, our mutational analysis of the HIV-2 TAR element did not show any differential HIV-2 LTR sequence requirements for Tat1 or Tat2 function and, in particular, did not show any requirement for sequences between +53 and +99 for full

Tat2 *trans* activation of the HIV-2 LTR. These experiments were performed by using both lymphoid (Jurkat) and non-lymphoid (COS) cells and two distinct indicator genes (Fig. 4). Therefore, the results do not appear to represent a tissue- or construct-specific phenomenon. The reasons for the difference between these results and the data of Emerman et al. (8) are therefore unclear. One possible interpretation of the observations, i.e., that the HIV-2 LTR contains only a single functional TAR element, was directly tested by targeted mutagenesis (Fig. 5). This experimental approach showed that the second putative HIV-2 LTR TAR element was, in fact, functional when the more 5' TAR element was rendered inactive. However, the 3' TAR element was observed to be four- to eightfold less effective than the 5' TAR element when present alone (Fig. 5). It appears probable that the ineffectiveness of the 3' TAR element in HIV-2 is due at least in part to its distance from the viral RNA cap site (14, 27). Indeed, decreasing this distance by deletion mutagenesis was found to significantly enhance *trans* activation by both the Tat1 and Tat2 proteins (Fig. 5).

The results presented in this paper constitute the first demonstration that the HIV-2 LTR contains two distinct TAR elements. Our data also suggest that the second, 3' TAR element is functionally masked in the context of an intact HIV-2 LTR. We believe, however, that these observations, obtained by using transient assays of gene expression in tissue culture, do not preclude the possibility that this functional redundancy could bestow a significant replication advantage on HIV-2 *in vivo*. It remains unclear why the HIV-2 LTR contains two TAR elements when HIV-1 replicates very effectively with only one. However, the final resolution of this question appears likely to require a more complete understanding of the mechanism of action of both Tat1 and Tat2 than currently exists.

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