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Delineating Minimal Protein Domains and Promoter Elements for Transcriptional Activation by Lentivirus Tat Proteins

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Lentivirus Tat proteins comprise a novel class of RNA-binding transcriptional activators that are essential for viral replication. In this study, we performed a series of protein fusion experiments to delineate the minimal protein domains and promoter elements required for Tat action. We show that a 15-amino-acid region of equine infectious anemia virus (EIAV) Tat protein, when fused to the GAL4 or LexA DNA binding domain, can activate transcription in appropriate promoter contexts. In the natural human immunodeficiency virus type 1 long terminal repeat, activation by Tat is dependent on multiple binding sites for the cellular transcription factor SP1. We delineate a 114-amino-acid region of the SP1 glutamine-rich activation domain that when fused to the GAL4 DNA binding domain can support transcription activation by Tat. Using these Tat and SP1 derivatives, we show that Tat activation can be reconstructed on a completely synthetic promoter lacking all *cis*-acting elements unique to the human immunodeficiency virus long terminal repeat. Our results indicate that lentivirus Tat proteins have essential properties of typical cellular transcriptional activators and define useful reagents for studying the detailed mechanism of Tat action.

Human immunodeficiency virus (HIV), simian and bovine immunodeficiency viruses (6, 26), and equine infectious anemia virus (EIAV) (9) are lentiviruses that encode a novel class of transcription activators termed Tat proteins. Tat proteins differ from any known cellular activator in that they are targeted to and activate transcription from nascent RNA.

Typical cellular transcriptional activators are composed of two separable functional domains: the transcription effector or activation domain, and a promoter-targeting domain that directs the transcriptional activation region to the promoter (27). The results of protein fusion experiments (31, 33) have shown that Tat also contains two separable functional domains: an arginine-rich RNA binding domain that targets Tat to nascent TAR RNA, and a transcriptional activating region. For example, HIV type 1 (HIV-1) Tat sequences have been fused to either RNA (Rev [21] or R17 [31]) or DNA (GAL4 [16, 33]) binding domains. These chimeric Tat proteins activate transcription efficiently when targeted artificially to their appropriate RNA or DNA binding sites within the vicinity of the promoter.

The HIV-1 long terminal repeat (LTR) is an extremely complex regulatory element that contains binding sites for a variety of cellular transcription factors, including: NF-κB/*rel* (24), SP1 (14), *ets* (32), *myb* (7), USF (10), GATA-3 (36), YY1 (22), LBP (37). We have taken a long-term strategy to delineate the minimal protein sequences and promoter elements essential for transcription activation by Tat. In this report, we describe minimal Tat derivatives, SP1 derivatives, and promoters that support Tat responsiveness.

Recent studies have shown that a Tat-R17 chimeric protein, containing a 15-amino-acid core domain of the distantly related EIAV Tat protein, can activate transcription from nas-

cent RNA (8). To determine whether the core region of EIAV Tat (8) can also function when bound to the promoter, the 15-amino-acid EIAV Tat core region was fused in frame to GAL4(1-95), which contains the DNA binding and dimerization domains of the yeast activator GAL4. The resulting GAL4-ETat expression plasmid was cotransfected into HeLa cells with the reporter G6 −80/+84 HIV LTR. Figure 1A (lanes 2 and 4) shows that GAL4(1-95)–ETat activates transcription as efficiently as GAL4-HTΔC, which contains the HIV-1 Tat activation domain (residues 1 to 49) fused to GAL4(1-95). Note that GAL4-HTΔC lacks the Tat RNA binding domain and thus cannot work through the TAR RNA element.
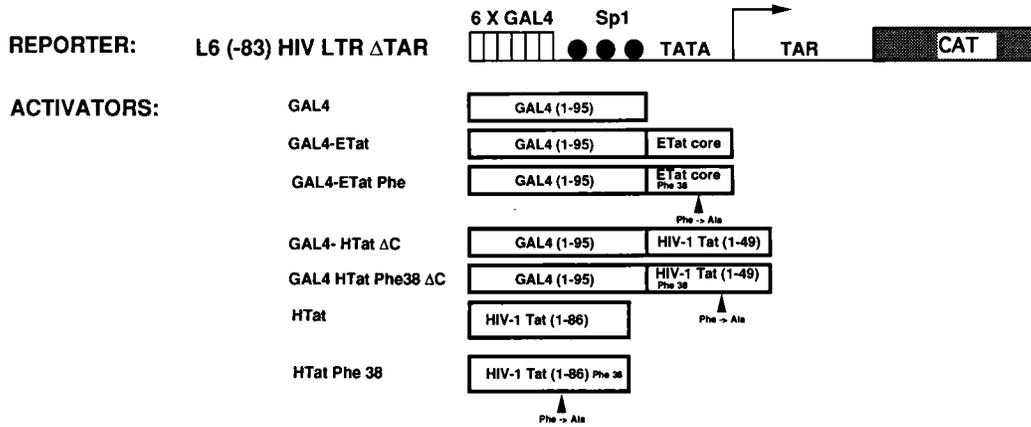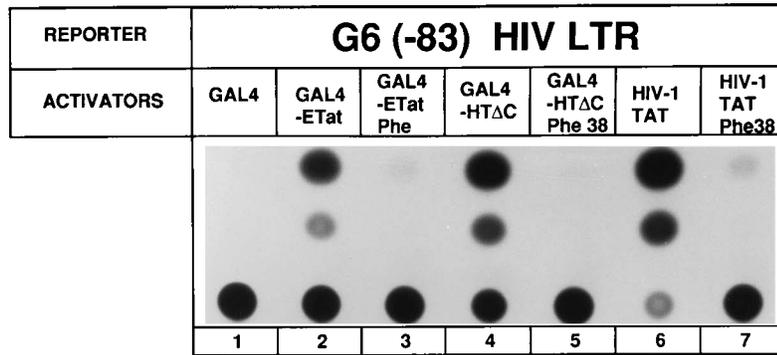
The central Phe residue within the 15-amino-acid core region of EIAV Tat is conserved among all known lentivirus Tat proteins (4). We therefore introduced a Phe→Ala mutation into each of these GAL4-Tat derivatives. Figure 1A shows that this single amino acid mutation abolished transcription activation by GAL4-ETat, GAL4-HTΔC, and HIV-1 Tat. We conclude that the 15-amino-acid core region of EIAV Tat can function efficiently when bound to HIV-1 LTR promoter DNA in human cells and that the highly conserved central Phe residue within this core region is critical for activity.

The activation domain of HIV-1 Tat resides within its amino-terminal 47 amino acids and consists of an acidic amino terminal domain, a Cys-rich domain, and the conserved core region (18, 28, 30). Substitutions within any of these domains can abolish function of wild-type Tat as well as Tat derivatives that act by targeting nascent RNA (31) or upstream promoter DNA (33). Figure 1B shows the ability of the minimal core region of HIV-1 Tat to activate transcription when targeted to upstream promoter DNA. The 15-amino-acid core regions of HIV-1 Tat and EIAV Tat were first fused in frame to the full-length bacterial repressor protein LexA(1-202) and subcloned into a mammalian expression vector. The LexA derivatives were cotransfected into HeLa cells together with an L6 (−83) HIV LTR ΔTAR reporter (Fig. 1B). Whereas the LexA-ETat derivative activated transcription, the Lex-HTat core derivative was inactive (compare lanes 2 and 4). Once
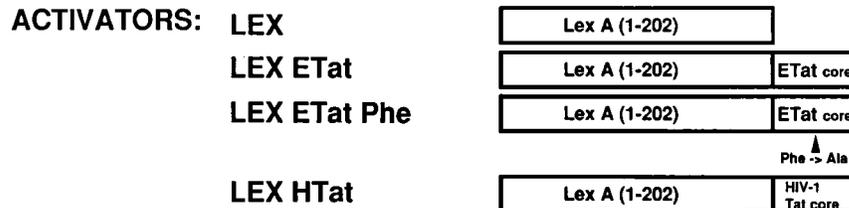
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A

REPORTER	G6 (-83) HIV LTR						
ACTIVATORS	GAL4	GAL4 -ETat	GAL4 -ETat Phe	GAL4 -HTΔC	GAL4 -HTΔC Phe 38	HIV-1 TAT	HIV-1 TAT Phe38

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |

REPORTER: L6 (-83) HIV LTR ΔTAR   [6 X GAL4] Sp1 ●●● TATA   TAR   CAT

ACTIVATORS:

GAL4 — GAL4 (1-95)

GAL4-ETat — GAL4 (1-95) | ETat core

GAL4-ETat Phe — GAL4 (1-95) | ETat core Phe 38 ↑ Phe -> Ala

GAL4- HTat ΔC — GAL4 (1-95) | HIV-1 Tat (1-49)

GAL4 HTat Phe38 ΔC — GAL4 (1-95) | HIV-1 Tat (1-49) Phe 38 ↑ Phe -> Ala

HTat — HIV-1 Tat (1-86)

HTat Phe 38 — HIV-1 Tat (1-86) Phe 38 ↑ Phe -> Ala

B

REPORTER	L6 (-83) HIV LTR ΔTAR			
ACTIVATORS	LEX	LEX -ETat	LEX -ETat Phe	LEX -HT

| | 1 | 2 | 3 | 4 |

REPORTER: L6 (-83) HIV LTR ΔTAR   [6 X LEXA] Sp1 ●●● TATA   ΔTAR   CAT

ACTIVATORS:

LEX — Lex A (1-202)

LEX ETat — Lex A (1-202) | ETat core

LEX ETat Phe — Lex A (1-202) | ETat core ↑ Phe -> Ala

LEX HTat — Lex A (1-202) | HIV-1 Tat core

A

REPORTER	L6 G1 (-31) HIVLTR			
GAL4-SP1 DERIVATIVES	GAL4 (1-95)	GAL4 (1-95) -SP1 Ser/Thr + GLN	GAL4 (1-95) - SP1 GLN	GAL4 (1-95) - SP1 GLN Δ
ACTIVATORS	LEX / LEX-ETAT / HIV-1 TAT	LEX / LEX-ETAT / HIV-1 TAT	LEX / LEX-ETAT / HIV-1 TAT	LEX / LEX-ETAT / HIV-1 TAT

B

REPORTER	L6 G1 (-31) HIVin			
GAL4-SP1 DERIVATIVES	GAL4 (1-95)	GAL4 (1-95) SP1 Ser/Thr + GLN	GAL4 (1-95) - SP1 GLN	GAL4 (1-95)- SP1 GLN Δ
ACTIVATORS	LEX / LEX-ETAT	LEX / LEX-ETAT	LEX / LEX-ETAT	LEX / LEX-ETAT

C

REPORTERS :

L6 G1 (-31) HIVLTR

6 X LEX A   1 X GAL4   HIV TATA   TAR   CAT
HIV LTR sequences -31 +84

L6 G1 (-31) HIVin

6 X LEX A   1 X GAL4   HIV TATA   polylinker   CAT
HIV LTR sequences -31 +1

ACTIVATORS :

GAL4 DERIVATIVES :

GAL4(1-95) — GAL4 (1-95)

GAL4(1-95) -SP1 Ser/Thr+GLNA — GAL4 (1-95) S/T GLN A

GAL4(1-95) -SP1 GLNA — GAL4 (1-95) GLN A

GAL4(1-95) - SP1 GLN Δ — GAL4 (1-95) GLN Δ

LEXA and TAT DERIVATIVES :

LEX(1-202) — LEX A (1 - 202)

LEX(1-202) - ETAT — LEX A (1 - 202) ETAT core
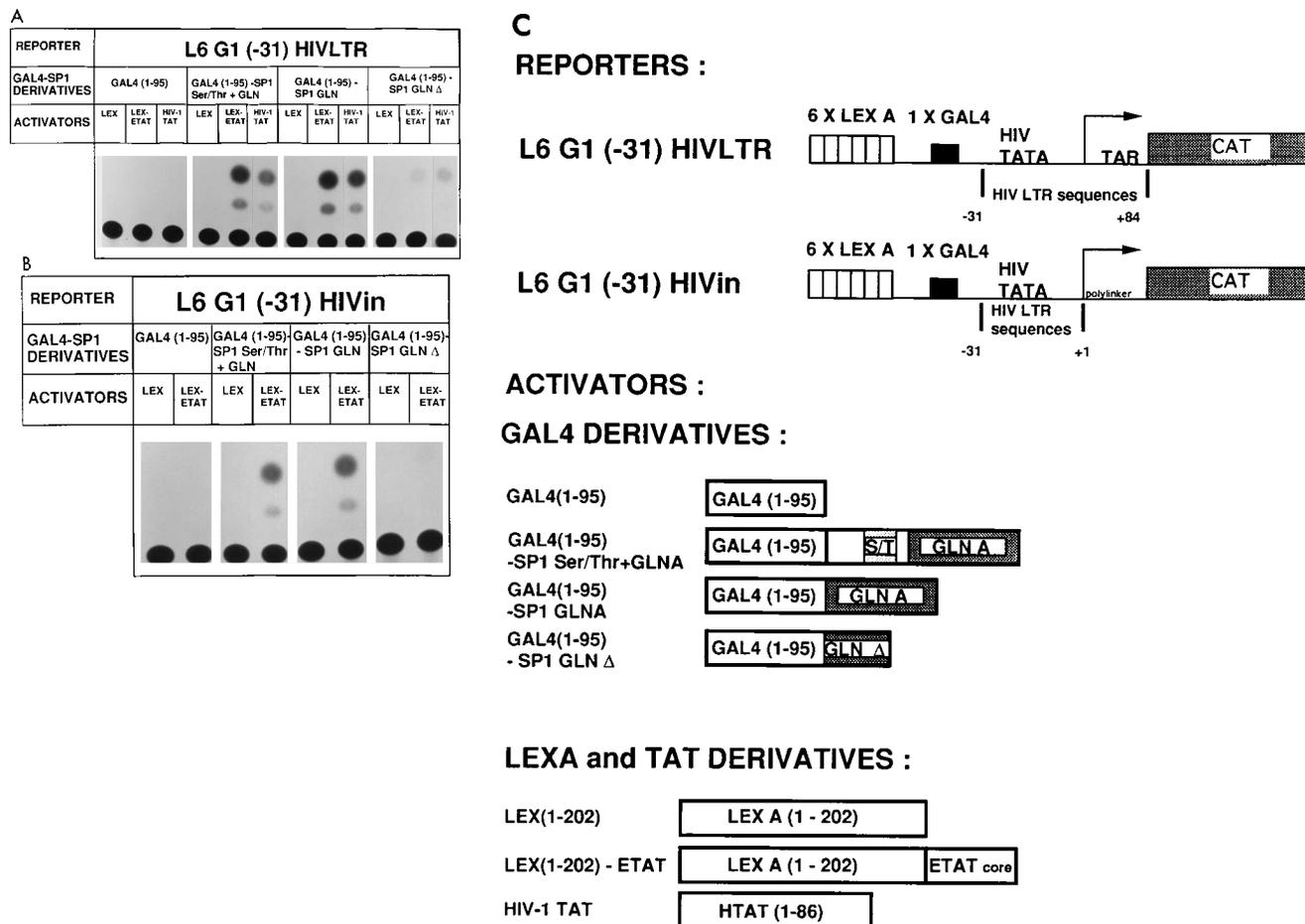
HIV-1 TAT — HTAT (1-86)

FIG. 2. Lex-Tat and GAL4-SP1 derivatives cooperate to activate transcription. (A) The 114-amino-acid glutamine-rich region A of SP1 can support Tat-mediated transcriptional activation. Deletion mutants of SP1 region A containing the Ser/Thr- and glutamine-rich regions (GLN) or only a 60-amino-acid subdomain of the GLN region were fused to GAL4 residues 1 to 95 and cloned into a cytomegalovirus-driven expression vector as indicated. L6G1 (−31) HIV-1 LTR was constructed by cloning six LexA sites upstream of the previously described G1 (−31) HIV-1 LTR reporter (31). The LexA, Lex-ET, and HIV-1 Tat expression plasmids and the L6G1 (−31) HIV LTR reporter were cotransfected into HeLa cells in the presence of expression plasmids indicated above the lanes by using the calcium phosphate coprecipitation technique. (B) Sequences downstream of the transcription start site are not required for activation by Lex-Tat. Six LexA sites and one GAL4 site were cloned upstream of HIV LTR positions −31 to +1 as shown. This reporter was then transfected into HeLa cells with various combinations of the LexA and GAL4 derivatives as described for panel A. CAT activity was determined 48 h posttransfection. (C) Schematic diagram of reporters and activators.

again, the Phe→Ala mutation within the core region of LexA-ETat (Lex-ETat Phe) abolished activity (lane 3). These combined data indicate that the core region of HIV-1 Tat is unable to activate transcription when tethered to upstream HIV-1 LTR promoter DNA under conditions in which the core element of EIAV Tat functions efficiently.

We have previously shown that HIV-1 Tat activates transcription from the HIV-1 LTR by cooperating with promoter-proximal cellular transcription factors, in particular SP1 (3, 33): a GAL4-SP1 derivative, containing the activation region A of SP1 fused to GAL4(1-147), cooperated with RNA-bound HIV-1 Tat to stimulate transcription greater than 80-fold (33). Activation region A of SP1 contains both a Ser/Thr-rich region

FIG. 1. Transcription activation by HIV-1 and EIAV Tat fusion proteins. (A) The CAT assay is shown above, and a schematic diagram of the reporter and activator proteins is shown below. GAL4-ETat contains the 15-amino-acid ETat core region (8) fused to GAL4(1-95), which contains the DNA binding and dimerization domains of the yeast activator protein GAL4. The G6 (−83) HIV-1 LTR reporter was constructed by cloning five GAL4 17-mer DNA binding sites upstream of the previously described G1 (−83) HIV LTR reporter (33). GAL4(1-95) sequences were first cloned into the PECMV NH vector, a mammalian expression plasmid identical to the previously described PECE except that the simian virus 40 early promoter was replaced with cytomegalovirus (CMV) immediate-early promoter and the plasmid backbone is pSP72 (Promega). By using PCR technology, all EIAV Tat and HIV-1 Tat sequences and mutants were cloned in frame into the PECMV-GAL4(1-95) expression plasmid and sequenced. This reporter was then cotransfected into HeLa cells in the presence of either GAL4(1-95), CMV-HTat, CMV GAL4-HTΔC, or GAL4-ET, using the calcium phosphate coprecipitation technique (33). At 48 to 72 h after transfection, the cells were harvested and CAT activity was measured. GAL4-ETat and GAL4-HTΔC Phe38 mutants contain the previously described Phe→Ala mutation within the HTat or ETat core domain as indicated in panel B. (B) The CAT assay is shown above, and a schematic diagram of the reporter and activator proteins is shown below. The DNA sequences encoding the core region of either EIAV Tat or HIV-1 Tat, depicted in the diagram, were fused in frame to the bacterial protein LexA(1-202), giving rise to Lex-ETat or Lex-HT, respectively. The Phe→Ala mutation was introduced into the EIAV Tat core region as shown. We constructed a L6 (−83) HIV LTR ΔTAR reporter in which the GAL4 sites in the previously described G6 (−83) HIV-1 LTR ΔTAR construct (31) were replaced by six LexA operator DNA binding sites obtained from the previously described L6E1bCAT reporter (23). LexA(1-202), Lex-ETat, Lex-ETat Phe, and Lex-HT expression plasmids were individually cotransfected with the L6 (−83) HIV LTR ΔTAR reporter into HeLa cells by the calcium phosphate coprecipitation technique. After 48 to 72 h, the cells were harvested and CAT activity was measured.
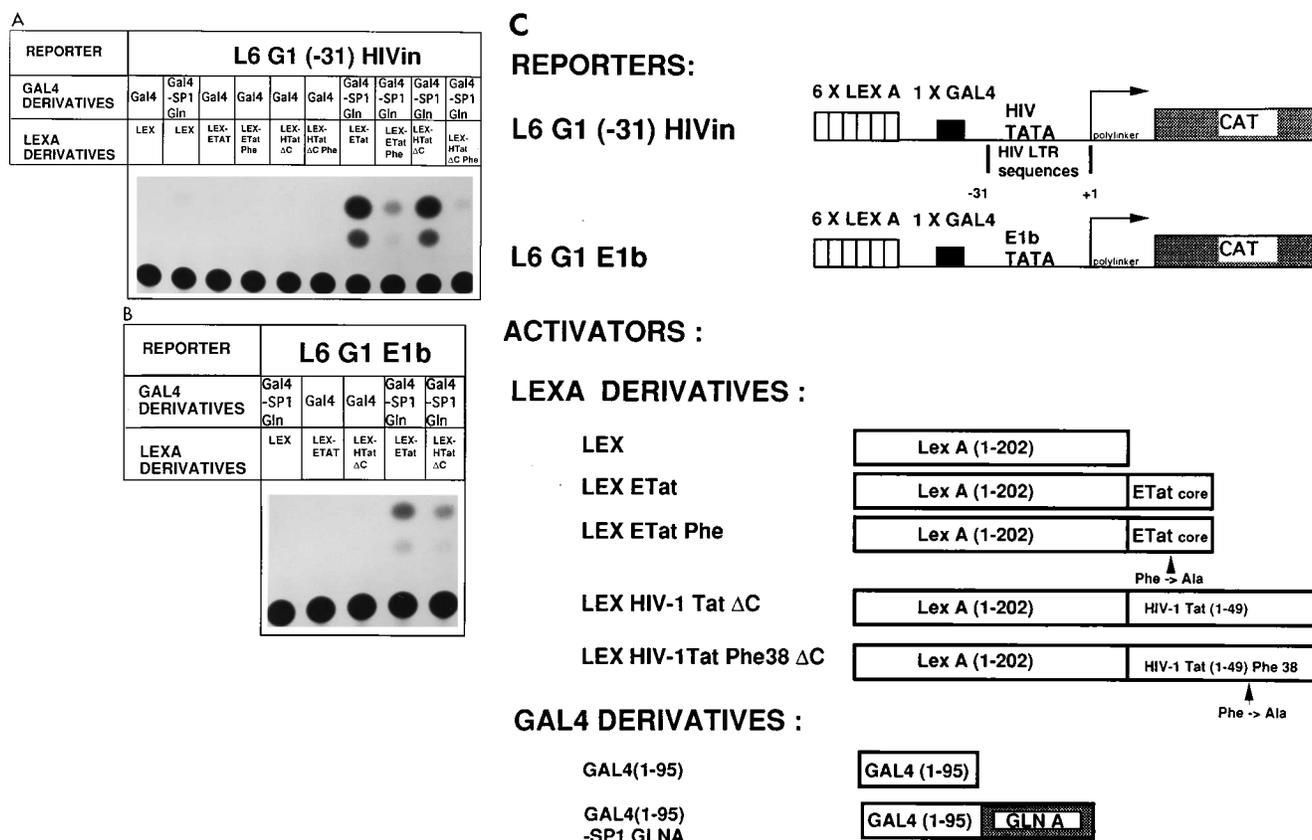
FIG. 3. Lentivirus Tat derivatives activate transcription from simplified promoters. (A) LexA-ETat and LexA-HTΔC derivatives cooperate with the minimal glutamine-rich activation domain A of SP1 to activate transcription. The indicated combinations of LexA, LexA-ETat, LexA-ETat Phe, LexA-HTΔC, and LexA-HTΔC Phe derivatives in the presence of either GAL4(1-96) or GAL4-SP1 GLN were cotransfected together with the L6 G1 (−31) HIVin reporter into HeLa cells. Cells were harvested 48 to 72 h after transfection and processed as previously described. (B) Transcription activation from a completely synthetic promoter. Six LexA sites were cloned upstream of the previously described G1E1bCAT reporter (20). This L6 G1 E1b CAT reporter was then cotransfected into HeLa cells together with the indicated combinations of LexA and GAL4 derivatives. Cells were harvested 48 to 72 h posttransfection, and CAT activity was assayed. (C) Schematic diagram of reporters and activators.

and a Gln-rich region (15). To determine the contribution of these two motifs in Tat activation, we constructed GAL4-SP1 derivatives containing the glutamine-rich domain A alone deleted of the Ser/Thr-rich domain (GAL4-SP1 GLN) or a portion of the Gln-rich subdomain (GAL4-SP1 GLNΔ) (Fig. 2A). These derivatives were then cotransfected with Lex, Lex-ETat, Lex-ETat Phe (containing the Phe→Ala mutation), or the HIV-1 Tat expression plasmids and either of two reporter plasmids, L6 G1 HIV LTR chloramphenicol acetyltransferase (CAT) (containing six Lex sites, one GAL4 site, and HIV LTR sequences between −31 and +84) or L6 G1 HIVin CAT (containing six Lex sites, one GAL4 site, and HIV LTR sequences between −31 and +1) (Fig. 2C). As shown in Fig. 2A, a 144-amino-acid glutamine-rich fragment lacking the Ser/Thr-rich domain efficiently supported activation by Lex-ETat or HIV-1 Tat. Further deletion into the glutamine-rich region (GAL4-SP1 GLNΔ) eliminated Tat responsiveness.

Figure 2B shows that similar results were obtained with the L6 G1 HIVin CAT reporter: once again, the 114-amino-acid fragment of the SP1 glutamine-rich region A supported activation by LexA-ETat. Furthermore, these results show that

sequences downstream of position +1 are dispensable for activation by LexA-ETat.

We next directly compared the abilities of EIAV and HIV-1 Tat activation domains to cooperate with the 114-amino-acid glutamine-rich activation domain of SP1. Figure 3A shows that GAL4-SP1 GLN, Lex-ETat core, and Lex-HTΔC by themselves failed to activate transcription significantly. However, Lex-ETat or Lex-HTΔC together with GAL4-SP1 GLN activated transcription in a cooperative fashion to comparable levels. Mutation of the Phe residue within the core region of these Lex-Tat derivatives severely impaired activity. We conclude that the 15-amino-acid core region of ETat and the 47-amino-acid activation region of HIV-1 Tat can cooperate with the 114-amino-acid glutamine-rich region of SP1 with similar efficiencies and that this activity requires the conserved Phe residue within the Tat core.

Several reports have suggested that sequences surrounding the HIV-1 LTR TATA element as well as the transcription initiation site and leader region may be involved in transcriptional regulation (29). To define further the role of HIV-1 LTR cis-acting sequences in Tat function, the GAL4-SP1 GLN, Lex-ETat, and Lex-HTΔC derivatives were cotransfected into HeLa cells together with a reporter, L6 G1 E1b CAT, that contains six LexA operator sites inserted upstream of the GAL4 site in the previously described synthetic G1E1bCAT reporter (20). Thus, this synthetic promoter lacks any natural HIV LTR sequences and contains no promoter element unique to the HIV LTR. Figure 3B shows that on L6 G1 E1b CAT, both Lex-ETat and Lex-HTΔC can cooperate with GAL4-SP1 GLN to activate transcription. We conclude that no cis-acting sequence unique to the HIV-1 LTR is essential for activation by Lex-ETat.
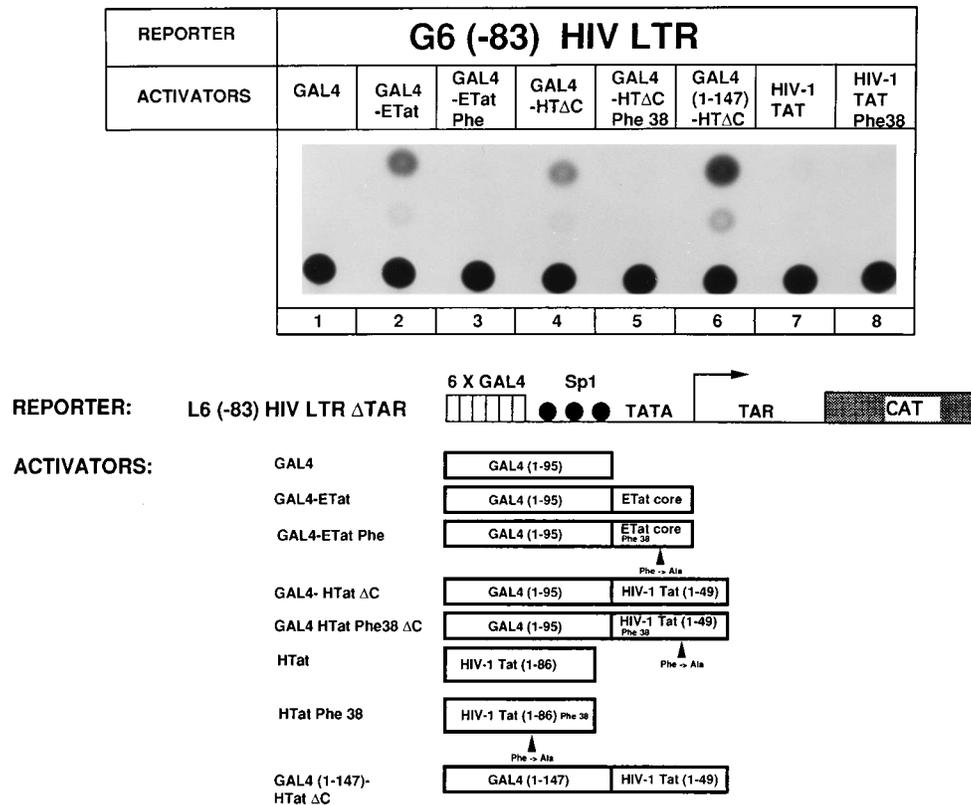
FIG. 4. Transcription activation by GAL4-Tat derivatives in CHO cells. The G6 (−83) HIV LTR reporter, depicted in Fig. 1A, was cotransfected into CHO cells together with either GAL4(1-95), GAL4-ETat, GAL4-ETat Phe, GAL4-HTΔC, GAL4-HTΔC Phe, or GAL4(1-147)–HTΔC derivatives by using the DEAE-dextran technique (20). Cells were harvested 48 to 72 h posttransfection, and CAT activity was measured.

Tat activation of the HIV-1 LTR is severely impaired in most rodent cell lines (2, 12, 25). This defect is rescued in human-rodent hybrid cells harboring human chromosome 12 (2, 25). To gain insight into the nature of the defect in rodent cells, the G6 (−83) HIV LTR reporter was transfected into CHO cells in the presence of either an HIV-1 Tat, GAL4-ETat, or GAL4-HTΔC expression plasmid. As expected from previous studies, Fig. 4 shows that in CHO cells, HIV-1 Tat activated transcription from the G6 (−83) HIV LTR very poorly (compare with Fig. 1A). In contrast, transcription was activated by GAL4(1-147)–HTΔC and to a lesser extent by GAL4(1-95)–ET and GAL4(1-95)–HTΔC (lanes 6, 2, and 4, respectively). In all instances, the Phe residue within the core region of these GAL4-Tat derivatives was critical for activity. We conclude that in CHO cells, Tat can activate transcription when bound to GAL4 DNA binding sites upstream of the transcription initiation site but not when targeted through nascent TAR RNA. The most likely interpretation of these results, and those of related studies (1, 21), is that in vivo the Tat-TAR interaction requires a human factor that is absent (or not functional) in CHO cells.

The results presented here and in previous protein fusion studies indicate that lentivirus Tat proteins have the hallmarks of typical transcription activators: they are modular (31, 33), they can act from upstream promoter DNA (33), and their activation regions cooperate with cellular transcription factors such as SP1 (3, 33, 35). Even the ability to activate transcription from nascent RNA is no longer unique to lentivirus Tat proteins: the acidic activator VP16 can activate HIV-1 LTR-directed transcription when tethered artificially to nascent RNA (34).

Two models have been proposed to explain how HIV-1 Tat activates transcription (5, 11, 13). First, the RNA enhancer model posits that RNA-bound HIV-1 Tat interacts with the upstream promoter to stimulate transcription initiation. Second, the processivity model postulates that HIV-1 Tat interacts with transcription complexes assembled at the HIV LTR promoter to increase the processivity of RNA polymerase II (5, 11, 17, 19). If the HIV-1 LTR directed an unusually poorly processive RNA polymerase II, one would expect to find a unique promoter element that is specifically required for Tat function. On the contrary, we show that Tat can activate transcription from a totally synthetic promoter lacking all cis-acting elements unique to the HIV-1 LTR.

Transcription activation by Tat appears to involve a complex series of protein-protein interactions between the activator domains of SP1 and HIV-1 Tat, putative adaptor molecules, and specific general transcription factors. The exact sequence of events leading to Tat activation of transcription awaits detailed analyses using Tat-responsive in vitro transcription systems. We anticipate that the minimal LexA-Tat and GAL4-SP1 derivatives described here will facilitate such in vitro mechanistic studies.

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