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The Full-Length Tat Protein Is Required for TAR-Independent, Posttranscriptional *trans* Activation of Human Immunodeficiency Virus Type 1 *env* Gene Expression

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Tat is a protein that dramatically increases the expression of all genes expressed from the human immunodeficiency virus type 1 (HIV-1) long terminal repeat through interaction with a *cis*-acting target sequence referred to as TAR (for *trans*-acting responsive region). The *tat* gene is divided into two coding exons which, when translated, result in the synthesis of an 86-amino-acid protein. However, the 72-amino-acid segment encoded by the first coding exon of *tat* is sufficient to encode a fully active Tat protein in known assays. We examined expression of the *env* gene from an LTR that lacks TAR (designated dTAR-env). Surprisingly, only the full-length Tat peptide *trans* activated expression of the *env* gene from dTAR-env. Comparison of RNA and protein expression of the *env* gene in the presence of Tat indicated that the mechanism of *trans* activation is posttranscriptional rather than transcriptional. To test whether the TAR-independent Tat function is specific to the HIV-1 *env* gene, we analyzed expression of heterologous genes from the long terminal repeat lacking TAR. These heterologous genes were not *trans* activated by Tat in the absence of a TAR element, which suggests that the second-exon peptide of Tat has a sequence-specific role in TAR-independent *trans* activation of the HIV-1 *env* gene. Analysis of a mutant in the 5′ end of the *env* gene was used to identify a *cis*-acting sequence required for Tat responsiveness.

As with other members of the lentivirus family of retroviruses, the organization and expression of the human immunodeficiency virus type 1 (HIV-1) genome is complex. In addition to the usual retroviral genes that encode the proteins of the virus particle and the enzymes involved in nucleic acid replication, HIV-1 encodes at least six additional gene products. These novel retroviral proteins play critical roles both in regulation of HIV-1 gene expression and in morphogenesis and release of infectious HIV-1 virions. Tat is a *trans*-activator protein that dramatically increases the expression of all genes expressed from the HIV-1 long terminal repeat (LTR) (2, 31, 47). Genetic analysis of the *tat* gene indicates that expression of Tat is required for replication (8, 15). Tat function requires the presence of a *cis*-acting target sequence referred to as TAR (for *trans*-acting responsive region), located downstream of the site of transcription initiation (39), between nucleotides +14 and +44 in the LTR (16, 24, 42). TAR function is both position and orientation dependent and requires the presence of upstream promoter and enhancer elements (35, 39). The 5′ untranslated leader sequence transcribed from TAR is capable of forming a stable stem-loop structure (33), and mutations that change the sequence in the loop or disrupt the stem structure greatly reduce *trans* activation by Tat (14, 16, 43). These findings support the hypothesis that TAR functions as an RNA target (11, 22, 40). Furthermore, there is evidence that Tat can specifically bind to the three-nucleotide bulge of the TAR RNA stem-loop structure (10, 40, 52). However, binding of Tat to TAR RNA is not in itself sufficient for *trans* activation. Mutations in the loop of TAR RNA abolish *trans* activation but do not effect binding of Tat to the TAR RNA (40). Recent studies demonstrate that the loop is required for the binding of a 68-kDa cellular protein (32) and that additional cellular factors may also function in *trans* activation (9, 34). However, the precise role of the TAR RNA remains unknown. It has been known that introduction of an artificial target for Tat binding within the LTR has been used to restore Tat responsiveness of TAR-deleted promoters (3, 43, 48). Further, phorbol myristate acetate stimulation of lymphoid cells derived from the normal central nervous system resulting in TAR-independent *trans* activation of the LTR has been observed and is dependent on the HIV-1 enhancer (20, 50). These results suggest that TAR is not the actual sequence mediating *trans* activation of the HIV-1 LTR promoter, but that TAR may serve as an attachment site directing Tat to the LTR.

Many studies have shown that Tat increases the steady-state level of transcripts derived from genes linked to the HIV-1 LTR. However, alternative mechanisms have been invoked to explain the manner in which Tat enhances the rate of transcription. Tat interaction with the TAR region on RNA initiated by the HIV-1 LTR may increase the stability of the association between RNA polymerase and viral nucleic acid and permit the efficient synthesis of full-length transcripts (27, 28, 30). Conversely, in the absence of Tat, for RNAs initiated in the LTR, premature transcription termination is observed. Since such termination occurs with both viral and heterologous sequences linked to the HIV-1 LTR, it is likely that this poor processivity is linked to initiation at the HIV-1 LTR promoter. It was therefore proposed that Tat *trans* activates the HIV-1 LTR by relieving a specific block to transcription elongation by interaction with the TAR sequence. Although these results support the hypothesis that a primary role of Tat in transcriptional *trans*

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activation is to enhance transcription elongation, evidence have also been presented suggesting that Tat can increase the frequency of RNA initiation (21, 27) and the translational efficiency of TAR-containing mRNA (4, 5, 7, 38).

The *tat* gene is divided into two coding exons which, when translated, result in the synthesis of an 86-amino-acid (aa) protein (2, 47). However, the 72-aa segment encoded by the first exon of *tat* is sufficient to encode a fully active Tat protein in known assays (7, 45). Moreover, mutational analysis of Tat suggests the presence of at least three distinct functional domains affecting nuclear localization and *trans* activation (37, 41, 47), and all of these domains are localized in the peptide encoded by the first exon. Since the binding of Tat to TAR is not sufficient for *trans* activation, it is likely that one or more of these Tat domains or an unidentified domain interacts with specific cellular proteins that serve important functions in Tat *trans* activation.

The Tat protein is able to *trans* activate some heterologous viral promoters even though those promoter-enhancers lack an obvious TAR element (25, 49). Previous analysis of Tat *trans* activation of the murine cytomegalovirus major immediate-early promoter-enhancer used the HIV-1 *env* gene as an indicator gene (25). Here we describe experiments indicating that the *env* gene itself contains a *cis*-acting Tat-responsive region. However, in contrast to *trans* activation of the HIV-1 LTR TAR, *trans*-activated expression of the *env* gene requires the full-length 86-aa Tat protein.

MATERIALS AND METHODS

Plasmid construction. Plasmid TAR-env was constructed by deleting the *Eco*RI fragment containing the simian virus 40 poly(A) signal from pIIIenv3.1 (47). Thus, TAR-env still contains a Tat-responsive HIV-1 5′ LTR and open reading frames for *rev* and *env* genes as well as an intact HIV-1 3′ LTR. TAR-cat was made by replacing the HIV-1 *env* gene in plasmid TAR-env with the bacterial chloramphenicol acetyltransferase (CAT) gene. Plasmids dTAR-env and dTAR-cat are identical to TAR-env and TAR-cat, respectively, except that they contain a deletion of the entire TAR sequence from a *Bgl*II site (+20) in the R region of the HIV-1 5′ LTR to an artificial *Sal*I site (47) located upstream of the initiation codon of *rev*. Thus, dTAR-env still encodes *rev* and *env* genes. TAR-luc and dTAR-luc contain the *Xho*I-*Bam*HI and *Bgl*II-*Bam*HI luciferase gene fragments of the pGL2-Basic vector at the *Sal*I-*Bam*HI and *Bgl*II-*Bam*HI sites, respectively, in pTat120B.

HIV-1 vector YK130, which is a *tat*-deficient HIV-1 proviral DNA, was constructed by introducing the region from the *Sal*I site in the Tat coding region to the *Bam*HI site in the gp41 coding region of pIIIenv3.1 (47) into the infectious HIV-1 proviral clone pNL4-3 (1). Plasmid YK137 was made by deleting the region from the *Sph*I site to the *Apa*I site in the Gag coding region of the HIV-1 proviral vector YK130. YK138 contains a frameshift mutation obtained by *Sph*I digestion, treatment of T4 DNA polymerase, and then religation, in the *gag* gene of YK130. An *env*-deficient HIV-1 proviral clone, YK161, was made in two steps as follows. The infectious proviral clone pNL4-3 (1) was digested with *Stu*I and *Nhe*I, the digested DNA was treated with Klenow fragment, and the ends were religated (regenerating the *Nhe*I site) to produce clone pGB107. pGB107 thus contains a 426-bp in-frame deletion in the *env* gene and can produce truncated gp120 and intact gp41. pGB107 was digested with *Nhe*I (regenerated enzyme site), the digested DNA was treated with Klenow fragment, and the ends were religated, resulting in plasmid YK161. YK152-71 and YK152-10 were constructed by introducing a stop codon in 72nd and 11th amino acid codons, respectively, of the *tat* gene in the HIV-1 proviral clone YK161.

TAR-envΔ24 and dTAR-envΔ24 were made by deleting the 12-bp direct repeat sequences in the HIV *env* gene (nucleotides +32 through +55 from the ATG codon of the *env* gene) from TAR-env and dTAR-env, respectively, by oligonucleotide-directed site-specific mutagenesis.

Tat-expressing plasmids pTat120B and pTat120K encode only the first exon and the intact sequences of the HIV-1 *tat* gene (both exons), respectively (25). pUC19SXLTR was used as a negative effector plasmid (25).

Rev expression plasmid pRev was made in two steps as follows. The HIV-1 *env* expression vector pMcenvHc (25) was digested with *Xho*I and *Eco*RI (partial) to remove the HIV-1 3′ LTR, and the simian virus 40 poly(A) site was inserted into the *Xho*I-*Eco*RI site to produce pKIMenvSV. pKIMenvSV thus contains only two *Bgl*II sites in the *env* gene. The 579-bp *Bgl*II-*Bgl*II *env* fragment was deleted, resulting in plasmid pRev, which does not produce functional envelope glycoprotein.

Plasmid pSP6-env was constructed by inserting the 421-bp *Bam*HI-*Xho*I fragment of the HIV-1 *env* gene into *Sal*I-*Bam*HI sites of pGEM-3Z (Promega, Madison, Wis.) for in vitro transcription.

Cell culture and transfection. The $CD4^+$ HeLa cell line HeLaT4 (29) was provided by R. Axel (Columbia University). HeLa and HeLaT4 cells were grown in Dulbecco's modified Eagle's medium with 7% calf serum. In preparation for the syncytium assay, 0.6×10^6 or 0.8×10^6 HeLaT4 cells were plated in 5 ml of medium in a 60-mm-diameter tissue culture dish 1 day before transfection. A total of 8 μg of plasmid DNA (3 or 5 μg of test plasmid and 5 μg of effector plasmid) was prepared for each plate and then coprecipitated with 10 μg of calf thymus DNA and calcium phosphate in a final volume of 0.5 ml by the calcium phosphate transfection method described by Graham and van der Eb (19) and modified by Hopkins et al. (23). The plasmid precipitate was added to the culture medium for 4 h. At the end of the incubation, the culture medium was removed, and the cells were rinsed once with serum-free Dulbecco's modified Eagle medium and shocked with 15% glycerol in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline for 3 min at room temperature, after which the glycerol was removed and replaced with 5 ml of culture medium. One or two days after transfection, the plates were stained for counting syncytia.

For the CAT assay, luciferase assay, and immunoprecipitation, 0.6×10^6 HeLa cells were transfected by the calcium phosphate method described above. Two days after transfection, the cells were harvested for the CAT assay or labeled with [^{35}S]methionine for immunoprecipitation.

For total RNA preparation, 3×10^6 HeLa cells were plated in 150-mm-diameter tissue culture dish and transfected with 15 μg of test plasmid and 25 μg of effector plasmid. Two days after transfection, the plates were harvested for RNA isolation.

Fusion, CAT, and luciferase assays. For fusion assays, HeLaT4 cells were plated in a 60-mm-diameter grid dish (500 grids per dish). Cells were stained 1 or 2 days posttransfection with a solution of 5 g of methylene blue and 1.7 g of pararosamiline per liter in methanol, and syncytia (giant cells containing more than four nuclei) were scored by microscopic observation. CAT enzyme assays were done as described by Gorman et al. (19). All extracts were made 48

h posttransfection by freeze-thawing, and reactions were run in 250 mM Tris-HCl (pH 7.8) for 15 min. Acetylated and unacetylated forms were resolved by thin-layer chromatography, located by autoradiography, and excised for liquid scintillation counting. For the luciferase assay, cell extracts were prepared, and the luciferase activity was measured by using the Luciferase Assay System kit (Promega) and a luminometer monolight 2010 (Analytical Luminescence Laboratory, San Diego, Calif.) under the conditions supplied with the kit.

Immunoprecipitation analysis. Cell labeling, preparation of cell lysate, and immunoprecipitation were performed as described previously (25). Two days after transfection, cells were trypsinized, and 2×10^6 cells were incubated for 30 min in methionine-free medium, after which 250 μCi of [^{35}S]methionine was added for 12 h. Following the labeling, cells were lysed, precleared, and immunoprecipitated twice with AIDS patient serum. Immunoprecipitates were then subjected to electrophoresis on 10% polyacrylamide gels in the presence of 10% sodium dodecyl sulfate (SDS), followed by fluorography.

RNA analysis. Total cellular RNA was prepared by CsCl-isothiocyanate ultracentrifugation (6, 17). For Northern (RNA) analysis, RNA (25 μg) was electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose filter (Schleicher & Schuell, Keene, N.H.), and hybridized to a random-primer-labeled probe (12). Probe 1 (1.18-kb *Hin*dIII-*Hin*dIII *gag* gene fragment of pNL4-3), probe 2 (326-bp *Kpn*I-*Kpn*I *pol* gene fragment of pNL4-3), and probe 3 (421-bp *Bam*HI-*Xho*I *env* gene fragment of pNL4-3) were labeled with [α-^{32}P]dCTP (~3,000 Ci/mmol; Amersham) under conditions specified by the manufacturer (Boehringer Mannheim Biochemicals). Hybridizations were carried out at 68°C for 1 h in QuikHyb rapid hybridization solution (Stratagene), and the hybridized membranes were washed at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 15 min twice and at 60°C with 0.1× SSC–0.1% SDS for 30 min.

^{32}P-labeled SP6 transcripts were made from *Nde*I-linearized pSP6-env according to standard procedures supplied by the manufacturer of the in vitro transcription kit (Promega) and then purified by 5% acrylamide–8 M urea gel electrophoresis. For use in the RNase protection assay, the purified transcript (10^5 cpm) was mixed with 10 μg of total RNA, digested by RNase under conditions supplied by the RPA II kit instruction manual (Ambion, Austin, Tex.), and analyzed in a 5% acrylamide–8 M urea gel.

Virus production and infections. Magi cells (CD4-LTR/β-galactosidase indicator cells; provided by M. Emerman), which are derived from HeLaT4 cells by introduction of the *lacZ* gene promoted from the HIV-1 LTR (26), were maintained at 37°C in the same medium supplemented with 10% fetal bovine serum and the antibiotics hygromycin and G418. Five micrograms of each proviral DNA (YK161, YK152-71, or YK152-10) and *env* expression plasmid (TAR-env) was transfected into 6×10^5 HeLa cells on 60-mm-diameter tissue culture dishes by the calcium phosphate procedure. Jurkat cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Jurkat cells (5×10^6) were transfected by the DEAE-dextran method (2). On day 2 posttransfection, 5×10^4 Magi cells were plated onto a 24-well plate for infection the following day. For the cell-free infection, medium was collected from the transfected HeLa or Jurkat cells and subjected to low-speed centrifugation to remove cell debris. The top portion of the supernatant was removed, and DEAE-dextran was added to a final concentration of 8 μg/ml. One-quarter milliliter of diluted supernatant was added to the Magi cells. After 4 h of incubation, fresh medium was added. Two days postinfection, the virus titer of supernatant was quantified by scoring the number of cells that turned blue after treatment with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (26).

RESULTS

TAR-independent *trans* activation of the *env* gene by Tat. Previous analysis of HIV-1 *env* gene expression from a heterologous promoter-enhancer indicated that expression was augmented in the presence of Tat (25). To determine whether the *env* gene contains a *cis*-acting determinant that contributes to Tat-mediated *trans* activation of *env* gene expression, we examined *env* gene expression from an HIV-1 LTR that lacks a TAR element. Figure 1A shows the structures of the TAR-containing and TAR-deficient HIV-1 LTR expression constructs used to express the *env* gene. In dTAR-env, a crucial nucleic acid segment required for TAR function was deleted. The deletion includes the segment of the R region that contains TAR as well as the entire U5 region. Thus, the essential trinucleotide bulge and the six-nucleotide loop (10, 40, 52) (from the *Bgl*II site at +20 in the R region) were among the segments removed.

Initially, we used a highly sensitive and rapid syncytium assay to examine the expression level of the *env* gene in TAR-env and dTAR-env in the absence or presence of Tat. Expression of the HIV-1 *env* gene product induces syncytium formation in a CD4-expressing HeLa cell line, HeLaT4 (29). Therefore, by transiently transfecting HeLaT4 cells with *env* gene expression constructs and quantifying the numbers and relative sizes of the syncytia, it is possible to measure the relative efficiency of functional Env protein expression (25). An example of such an analysis is shown in Fig. 1A. Two alternative Tat expression constructs were used in an attempt to *trans* activate *env* gene expression; pTat120B encodes the 73-aa Tat peptide derived from the first *tat* coding exon, whereas pTat 120K encodes the full-length 86-aa Tat protein. As expected, Tat-mediated *trans* activation of the *env* gene, by both forms of Tat, from the TAR-containing LTR (TAR-env) was substantial (150- to 190-fold), as evidenced by the syncytium assay. In contrast, *trans* activation of the *env* gene from the LTR lacking an intact TAR element did not occur with the 73-aa Tat peptide. However, surprisingly, the intact 86-aa Tat protein *trans* activated *env* gene expression from dTAR-env by 2 orders of magnitude (Fig. 1A). These data are consistent with previous observations which indicate that the truncated version of Tat is sufficient to increase transcriptional expression through interaction with TAR. Interestingly, the data also point to a possible role for the full-length Tat protein in *trans* activation of the *env* gene through a TAR-independent mechanism.

We next wanted to determine whether the TAR-independent Tat-responsive sequence is in the residual LTR region of dTAR-env or in the *env* gene itself. Thus, we replaced the *env* gene with two other indicator genes and examined expression in the presence and absence of Tat. The bacterial CAT and firefly luciferase genes were placed under the control of the intact HIV-1 LTR or the LTR lacking the TAR region (Fig. 1B and C) and cotransfected into HeLa cells along with plasmids expressing either the 86- or 73-aa version of Tat. As expected, both forms of Tat dramatically increased the expression of either CAT or luciferase from the LTR containing TAR (Fig. 1B and C). However, for the

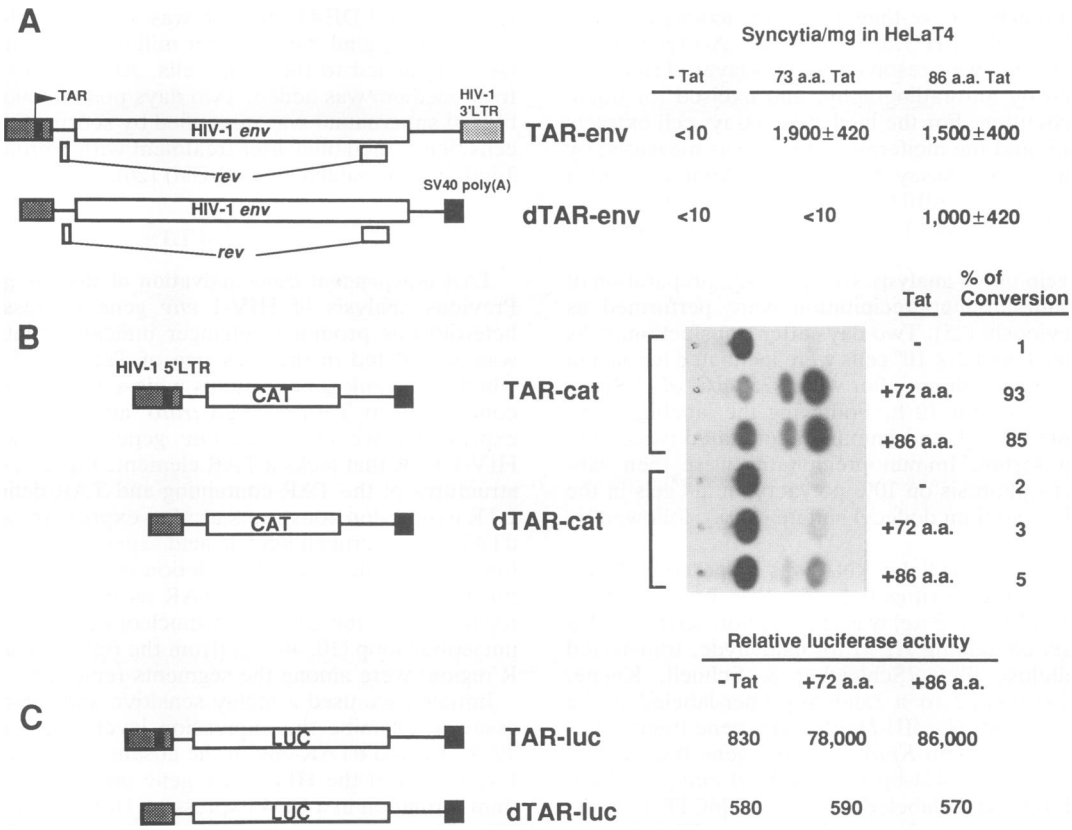

FIG. 1. Tat responsiveness of the TAR-deleted HIV-1 LTR. (A) For the fusion assay, HeLaT4 cells in 60-mm-diameter dishes were cotransfected with 3 μg of an *env* gene expression construct and 5 μg of an effector plasmid, pUC19SXLTR (−Tat), pTat120B (73 a.a. Tat), or pTat120K (86 a.a. Tat). The peak of syncytium formation was at 24 h posttransfection for TAR-env and at 48 h for dTAR-env. All data for the number of syncytia (multinucleate giant cells) represent the averages of at least seven assays. SV40, simian virus 40. (B and C) HeLa cells were transfected with 3 μg of the CAT (B) or luciferase (LUC; C) expression vector in the presence of 5 μg of pTat120B (+72 a.a. Tat), pTat120K (+86 a.a. Tat), or pUC19SXLTR (−Tat). All extracts were made 48 h posttransfection, and the activity of CAT or luciferase was measured as described in Materials and Methods.

constructs lacking the TAR element, there was little or no induction of the CAT or luciferase gene by either form of Tat; CAT gene expression from dTAR-cat exhibited only a small increase (two- to threefold *trans* activation), and luciferase gene expression exhibited no detectable increase, in the presence of Tat (Fig. 1B and C). These results contrast with those obtained with expression of the *env* gene from the LTR lacking the TAR element (Fig. 1A). Thus, these data are consistent with the presence of a *cis*-acting Tat-responsive element in the HIV-1 *env* gene rather than in the residual LTR of dTAR-env.

To ensure that the effector plasmids, pUC19SXLTR, pTat120B, and pTat120K, were not affecting DNA uptake during cotransfection, an assay was used in which plasmid pCH110 (Pharmacia Ltd.), which expresses the β-galactosidase gene, was cotransfected with pUC19SXLTR, pTat120B, or pTat120K. Uptake of pCH110 was quantified by scoring the number of blue cells after staining (26). Results of this analysis indicated that none of the effector plasmids inhibited DNA uptake during cotransfection (data not shown).

In these experiments, full-length Rev is expressed from dTAR-env, while pTat120B and pTat120K would not be expected to express functional Rev, since both Tat expression plasmids contain major deletions in the *rev* gene. Therefore, it seemed unlikely that the differential induction of *env* expression by the two Tat expression plasmids could be attributed to limiting amounts of Rev. However, we carried out a control experiment with a Rev expression plasmid (pRev) to examine the effect of additional Rev expression in a direct way. pTat120B or pTat120K, dTAR-env, and pRev were cotransfected into HeLaT4 cells, and *env* expression was again assessed by using the syncytium formation assay. The results of this experiment indicated that expression of Rev had no effect; the pattern of syncytium induction was identical to that elicited by the Tat expression constructs and dTAR-env alone (data not shown). These data indicate that the induction of *env* by pTat120K was not due to limitation of Rev expression.

***trans* activation of the *env* gene by Tat occurs through a posttranscriptional mechanism.** To examine Tat-induced Env expression more directly, and to characterize the mechanism by which Tat *trans* activates the *env* gene, we compared the steady-state levels of protein and RNA in the presence and absence of Tat. HeLa cells were transiently transfected with the same *env* gene expression constructs used in the syncytium assay in the presence or absence of the alternative forms of the Tat protein. To directly assess the relative level of envelope glycoprotein (Env-gp) in the presence and absence of Tat, we carried out immunoprecipitation analy-

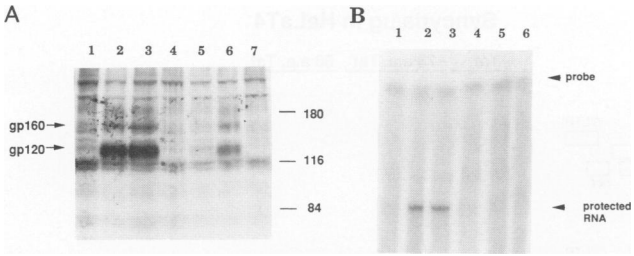

FIG. 2. Analysis of steady-state levels of protein and RNA. (A) HeLa cells were transfected with *env* gene expression constructs in the presence or absence of Tat, labeled with [^{35}S]methionine, and then immunoprecipitated with AIDS patient serum. Positions of the HIV-1 envelope glycoproteins and molecular size markers in kilodaltons are indicated. Lanes: 1, TAR-env plus pUC19SXLTR; 2, TAR-env plus pTat120B; 3, TAR-env plus pTat120K; 4, dTAR-env plus pUC19SXLTR; 5, dTAR-env plus pTat120B; 6, dTAR-env plus pTat120K; 7, pTat120B plus pTat120K. (B) Total RNA was harvested from HeLa cells after 48 h of transfection with the *env* expression vectors in the presence or absence of HIV-1 Tat. RNA samples (10 μg) were used in the quantitative RNase protection assay. A ^{32}P-labeled RNA probe was synthesized by in vitro transcription of pSP6-env that had been linearized with *Nde*I. Lanes: 1, TAR-env plus pUC19SXLTR; 2, TAR-env plus pTat120B; 3, TAR-env plus pTat120K; 4, dTAR-env plus pUC19SXLTR; 5, dTAR-env plus pTat120B; 6, dTAR-env plus pTat120K.

sis. Two days after transfection with the HIV-1 *env* expression vectors, protein was labeled with [^{35}S]methionine for 12 h, cells were lysed, Env-gp was immunoprecipitated with AIDS patient serum, and the precipitate was analyzed by autoradiography. As expected, the positive control plasmid (TAR-env) produced easily detectable amounts of HIV-1 Env-gp in the presence of either the 73-aa Tat peptide (Fig. 2A, lane 2) or the full-length 86-aa peptide (Fig. 2A, lane 3). In addition, this experiment demonstrated that, as expected from the results of the syncytium assay, the 86-aa HIV-1 Tat protein increases the amount of envelope glycoprotein expressed from dTAR-env (Fig. 2A, lane 6). Moreover, also consistent with results of the syncytium assay, there is relatively little *trans* activation of the *env* gene by the 73-aa Tat peptide (Fig. 2A, lane 5).

The results presented above demonstrate that the intact Tat enhances HIV-1 *env* gene expression directed by the HIV-1 LTR lacking TAR. To determine whether Tat functions to increase the pool of steady-state RNA, we carried out RNase protection analysis of RNA. An HIV-1 *env*-specific RNA probe transcribed from pSP6-env (see Materials and Methods) was hybridized to total cellular RNA extracted 48 h after transfection, and the RNA was subjected to RNase digestion. As a control for RNA integrity and uniform loading of the gel, total cellular RNAs were electrophoresed on agarose-formaldehyde gel, and rRNAs were examined. Two sharp, undegraded rRNA bands were detected in each sample (data not shown). Examination of the protected RNA by electrophoresis and autoradiography revealed a 421-base RNA molecule protected from RNase digestion in some of the samples (Fig. 2B). As expected, the protected 421-base RNA was observed after annealing with RNA extracted from cells transfected with TAR-env in the presence of either form of Tat (Fig. 2B, lanes 2 and 3). However, surprisingly, there was no detectable increase in the steady-state *env* RNA in cells transfected with dTAR-env in the presence of the 86-aa Tat protein (Fig. 2B, lane 6). No *env*-specific, protected RNA could be detected even after protracted exposure of the gel. This result indicates that the Tat-mediated *trans* activation of HIV-1 *env* gene expression probably is not likely to take place at the transcriptional level. Rather, the data are consistent with induction of *env* gene expression through a posttranscriptional mechanism. However, since the amount of RNA was below the limit of detection in the RNase protection assay, it cannot be conclusively stated that the induction is not at the level of transcription.

TAR obviates the requirement for the *cis*-acting Tat-responsive element in *env*. We next wanted to examine the relationship between the TAR element in the LTR and the *cis*-acting, Tat-responsive element of the *env* gene. It was clear from the results of the syncytium and immunoprecipitation assays described previously that the 73-aa Tat peptide can *trans* activate *env* gene expression; the presence of TAR apparently obviates the need for the *cis*-acting Tat-responsive element in *env* in the transient transfection assay (Fig. 1 and 2). Expression of the *env* gene normally occurs from a spliced mRNA. To examine *env* gene expression in a more natural context, we assessed Env-gp expression in full-length and nearly full-length virus. To determine whether the 73-aa Tat peptide is sufficient for *env* expression, or whether the 86-aa protein is necessary for efficient *env* gene expression, we constructed derivatives of full-length viral DNA that do not express functional Tat protein as a result of an in-frame deletion between the *vpr* and *tat* genes (YK130, YK137, and YK138) (Fig. 3). Env-gp expression from this construct could then be examined by genetic complementation with plasmids that express Tat. To examine the induction of *env* by both forms of Tat, YK130, YK137, or YK138 was cotransfected with either pTat120B or pTat 120K into HeLaT4 cells, and syncytium induction was measured. This assay revealed that expression of either the 73- or 86-aa version of Tat resulted in similar levels of *env* gene expression (Fig. 2). These results are similar to those obtained with TAR-env and indicate that even for full-length or nearly full-length viral DNA, the 73-aa peptide is sufficient for *env* gene expression provided that there is a functional TAR element in the LTR.

To determine whether the full-length Tat protein might play an obvious role in HIV-1 replication, we compared the ability of the first-exon peptide and the intact Tat protein to support replication of virus over a single cycle of infection. YK161 and YK152-71 are HIV-1 viral DNA clones containing the 86-aa intact Tat and the 71-aa first-exon peptide of Tat, respectively. YK152-10 is a *tat*-deficient HIV-1 viral clone that was used as a negative control. All of the clones contain a truncated *env* gene and so cannot produce functional envelope glycoprotein. Therefore, an *env* expression vector, TAR-env, was cotransfected with the viral DNA. Either HeLa or Jurkat cells were transfected, virus was harvested, and the virus titer was measured by infection of HeLaT4 cells containing an endogenous *lacZ* gene under the control of the HIV-1 LTR (26) (the experimental strategy is shown in Fig. 4A). The titer for YK152-71 was similar to that observed for YK161 (Fig. 4B). This finding indicates that in this assay, the 72-aa Tat peptide is sufficient for virus replication.

Identification of a *cis*-acting Tat-responsive element in the HIV-1 *env* gene. The TAR element in the LTR consists of a stable stem-loop structure (see the introduction). In addition, the 3′ border of the TAR core sequence (+30 to +44) contains a sequence almost identical to a 14-nucleotide sequence located between positions −2 and +12. Both repeated sequences and the stem-loop appear to be required for optimal TAR function (21). Because of these features of

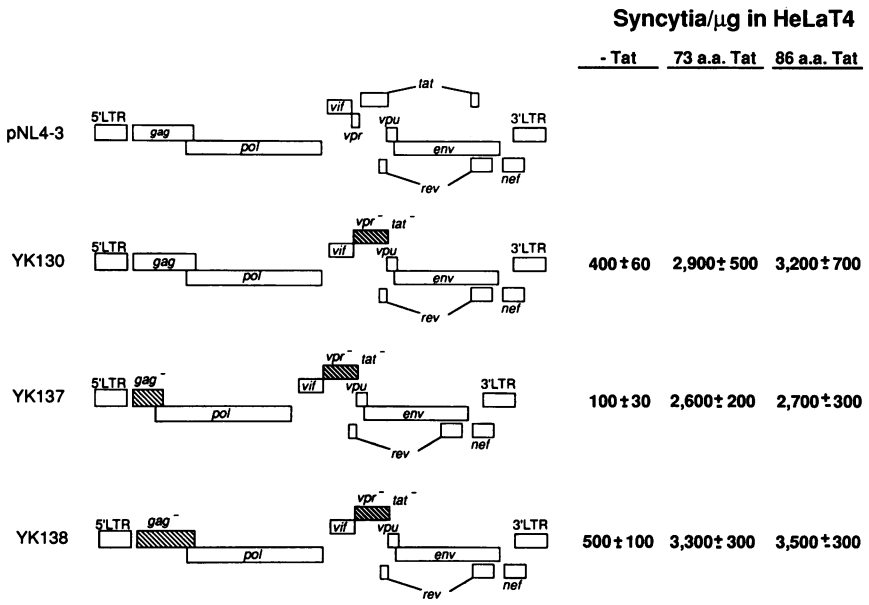

FIG. 3. Tat responsiveness of *env* gene expression of HIV-1 proviral DNA. For the fusion assay, HeLaT4 cells in 60-mm-diameter dishes were cotransfected with 5 μg of an HIV-1 proviral DNA and 5 μg of an HIV-1 *tat* gene expression construct, pTat120B (73 a.a. Tat), pTAt120K (86 a.a. Tat), or pUC19SXLTR (−Tat). One day posttransfection, cells were stained and scored for syncytia. The data are derived from at least four assays.

TAR, we searched the leader sequences and coding sequences of HIV-1 *env* for similar repeat sequences or regions that could form secondary structure similar to TAR. A segment of the *env* gene encoding part of the signal peptide (nucleotides 6279 through 6302 of HIV-1 strain HXB2) contains nucleotides that comprise a perfect 12-bp direct repeat (Fig. 5). Moreover, both repeats are situated within a larger segment (nucleotides 6274 to 6331) that could potentially form a stem-loop structure.

To determine whether this region of the *env* gene is important in *cis* for TAR-independent *trans* activation, we constructed an in-frame deletion mutation that removes a segment, the 12-bp direct repeat, of this region (dTAR-envΔ24). Interestingly, the deletion fully abrogated Tat responsiveness of *env* expression in the TAR-independent system (Fig. 6A). Env-gp expression from dTAR-envΔ24 was not increased by cotransfection with either pTat120K or pTat120B, as evidenced by both the syncytium assay and immunoprecipitation analysis (Fig. 6B). To determine whether Env-gp containing the 24-bp deletion was potentially capable of inducing syncytium formation, we juxtaposed *env* containing the 24-bp deletion to the wild-type HIV-1 LTR promoter (TAR-envΔ24). In the presence of Tat, the mutant form of Env was expressed and induced syncytium formation in HeLaT4 cells (data not shown). Taken together, these results indicate that there is a *cis*-acting element, which we designate E-TAR (Env TAR), required for the posttranscriptional Tat *trans* activation in the coding region of *env*, and that the second-exon peptide of Tat encodes a functional domain for the TAR-independent and posttranscriptional *trans* activation.

DISCUSSION

In this study, we have used the homologous promoter system (HIV-1 5′ LTR) and a transient expression assay in human cell lines permissive for HIV-1 infection (HeLa, HeLaT4, and Jurkat) to examine the role of the second-exon peptide of Tat protein in TAR-independent *trans* activation. Our results indicate that in the absence of the TAR element, the intact Tat protein containing the second-exon peptide is able to *trans* activate *env* gene expression. Further, the results indicate that induction is manifested, at least in part, through a posttranscriptional mechanism. Finally, the results indicate that this novel induction of gene expression by Tat requires a specific *cis*-acting sequence (E-TAR) in the HIV-1 *env* gene.

Posttranscriptional effects of Tat have been detected in several systems (7, 13, 38, 53). Thus, it has been suggested that *tat*-mediated enhancement of HIV-1-specific gene expression is due to both an increase in mRNA level and an increase in the utilization of that mRNA by the cellular translational machinery. A posttranscriptional role for Tat was convincingly demonstrated in a study in which LTR-containing plasmids of synthetic viral RNAs were introduced into *Xenopus* oocytes (4, 5). Those data suggest that an interaction between Tat and nuclear factors influences the ability of viral transcripts to be translated. The observations presented in Fig. 1 provide direct evidence that Tat *trans* activates gene expression via a bimodal mechanism, transcriptional and posttranscriptional activation. An implication of this observation is that the transcriptional effects seen with Tat are coupled to the posttranscriptional regulatory pathway.

The sequence in *env*, which we have designated E-TAR, is responsible for TAR-independent posttranscriptional Tat responsiveness. The nucleotides of E-TAR can potentially form a stem-loop structure containing a 12-bp direct repeat (Fig. 5). In addition, E-TAR possesses two potential loop sequences (AUGGG) similar to the critical region of the HIV-1 LTR TAR, CUGGG. We used deletion mutagenesis to examine the role of the 12-bp direct repeat within the stem-loop structure. Deletion of the 12-bp direct repeat in

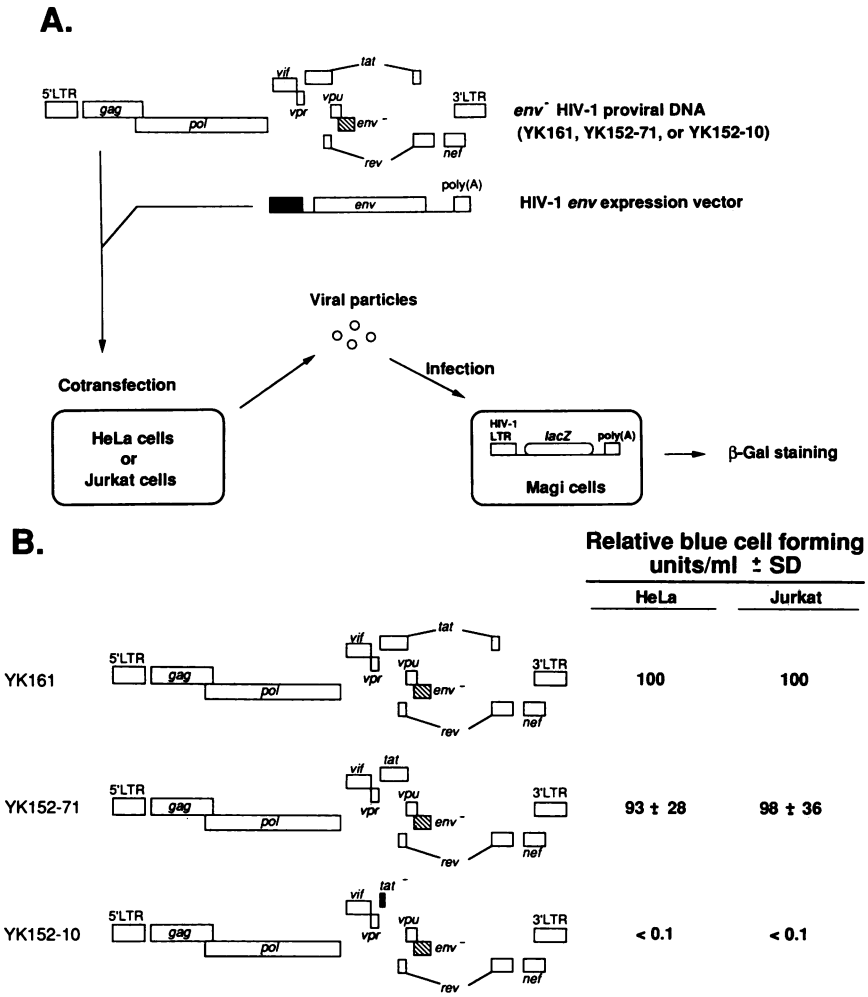

FIG. 4. Effect of Tat on the production of the HIV-1 replication. (A) Flowchart of the Magi assay. (B) HIV-1 proviral DNA, YK161 (intact Tat), YK152-71 (first-exon peptide of Tat), or YK152-10 (Tat⁻) was cotransfected with a HIV-1 *env* expression vector into HeLa or Jurkat cells, cell-free medium was collected, and dilutions of the virus were then used to infect the Magi cells (CD4-LTR/β-galactosidase [β-Gal] indicator cells). The infected Magi cells were fixed and stained as described in Materials and Methods. The numbers of blue cells in the infected Magi cells were counted 2 days after infection. SD, standard deviation.

E-TAR completely abrogated the *trans*-activation response. Deletion of E-TAR (dTAR-envΔ24) did not affect the basal activity of the HIV-1 LTR promoter significantly. We have analyzed the promoter function of the wild-type LTR by using an *env* gene lacking E-TAR (TAR-envΔ24). TAR-envΔ24 and TAR-env exhibited similar, low basal expression levels (data not shown). These observations demonstrate that the E-TAR element, like TAR, is not a *cis*-acting negative regulator of HIV-1-specific gene expression.

We did not detect a requirement for the full-length Tat protein in HIV-1 replication. The truncated version of Tat appeared to be sufficient for the production of viral progeny in HeLa and Jurkat cells (Fig. 4). In addition, in the presence of an HIV-1 LTR containing TAR, there was not a significant difference in *trans* activations by the intact and truncated versions of Tat. Thus, the general role of the full-length Tat protein is still enigmatic. However, we suggest that Tat positively modulates *env* gene expression, under some circumstances, to facilitate optimal virus replication.

What are other potential biological roles for the full-length Tat protein during HIV infection? It has been known that Tat

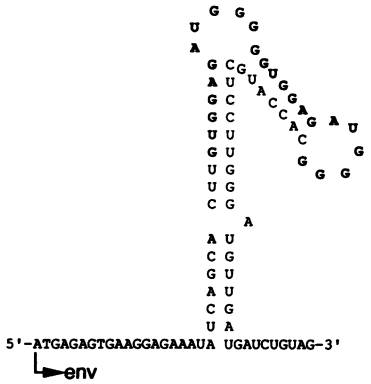

FIG. 5. Sequence of the E-TAR region in the HIV-1 *env* included in TAR-env and dTAR-env. A sequence around the 12-bp direct repeat in the *env* gene is shown in a possible secondary structure involving the 12-bp direct repeat. Also shown is the location of the start site of *env*. The location of a 12-bp perfect direct repeat is shown by boldtype letters. This structure has an estimated free energy of −13.5 kcal (ca. −56.5 kJ)/mol as determined by the procedure of Tinioco et al. (51). Deletion of the 24-bp region (the 12-bp direct repeat) would be expected to destabilize the putative secondary structure.

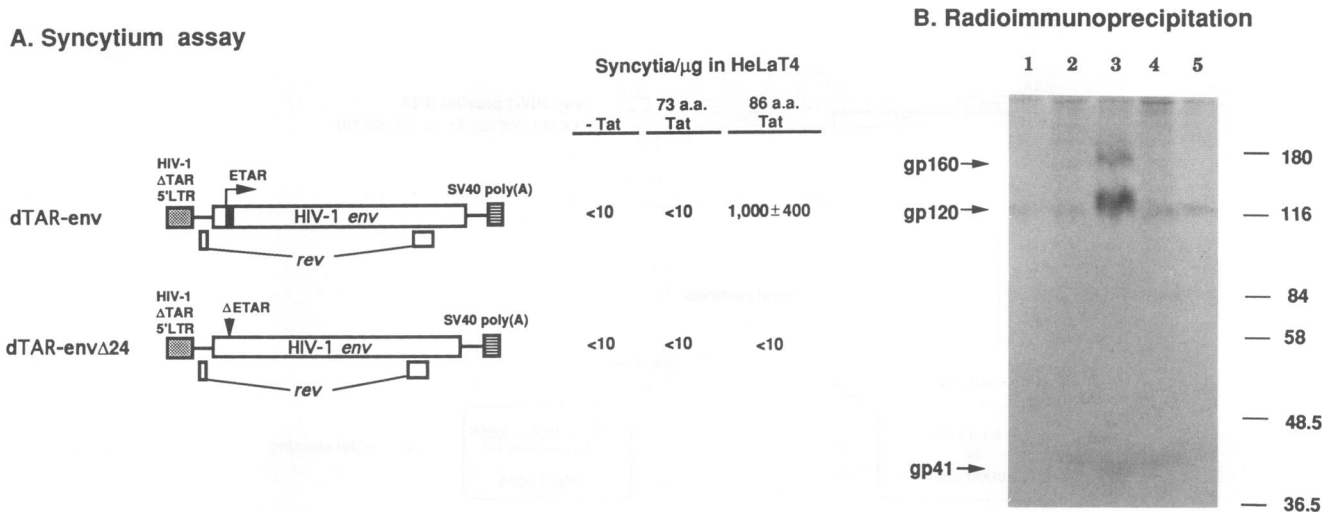

FIG. 6. Effect of deletion of the 12-bp direct repeat in *env* (E-TAR). (A) HeLaT4 cells were transfected with each of the HIV-1 *env* expression constructs, dTAR-env or dTAR-envΔ24, in the presence of pUC19SXLTR (−Tat), pTat120B (73 a.a. Tat), or pTat120K (86 a.a. Tat). Two days posttransfection, cells were stained and scored for syncytia. All data represent averages of at least five assays. (B) HeLa cells were transfected with the TAR-deleted HIV-1 LTR promoter-directed *env* gene expression constructs in the presence or absence of Tat, labeled with [^{35}S]methionine, and then immunoprecipitated with AIDS patient serum. Positions of the HIV-1 envelope glycoproteins and molecular size markers in kilodaltons are indicated. Lanes: 1, pTat120B plus pTat120K; 2, dTAR-env plus pTat120B; 3, dTAR-env plus pTat120K; 4, dTAR-envΔ24 plus pTat120B; 5, dTAR-envΔ24 plus pTat120K.

stimulates heterologous viral promoters in a TAR-independent manner (25, 49). Also, in HIV-1 infection, it has been observed that Tat induces tumor necrosis factor beta and interleukin-4 receptors in a human B-lymphoblastoid cell line (36, 44) and stimulates growth of cells derived from Kaposi sarcoma lesions (11). The results from those studies indicate that the HIV-1 Tat protein activates the expression of some viral and cellular genes during HIV-1 infection through a TAR-independent pathway. Further, a recent study demonstrates that Tat can interact with a region of the LTR other than the TAR element in cells derived from the central nervous system (50). It is possible that the activation of these cellular genes and the interaction of Tat with the enhancer region of the LTR require a full-length Tat protein.

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