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Human immunodeficiency viral long terminal repeat is functional and can be trans-activated in *Escherichia coli*

(human immunodeficiency virus/promoter/trans-activation/expression)

FATAH KASHANCHI AND CHARLES WOOD*

Department of Microbiology, University of Kansas, Lawrence, KS 66045

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ABSTRACT **The long terminal repeat (LTR) of the human immunodeficiency virus (HIV) contains the viral promoter, which is responsible for viral gene expression in eukaryotic cells. We have demonstrated that HIV LTR can also function as a promoter in *Escherichia coli*. A recombinant plasmid containing the HIV LTR linked to the chloramphenicol acetyltransferase gene can express the enzyme efficiently upon transformation into bacteria. Mung bean nuclease analysis mapped the bacterial transcriptional start site of the promoter to the U3 region of the LTR, in contrast to transcription in eukaryotic cells, which initiates in the U3–R boundary of the LTR. The HIV LTR, besides being fully functional in *E. coli*, can also be specifically trans-activated by the HIV *tat* gene product. Trans-activation is demonstrated by an increase in chloramphenicol acetyltransferase activity as well as an increase in the mRNA level of the enzyme. This trans-activation of HIV LTR by tat protein in bacteria offers a useful system to investigate further the specific interaction between tat protein with HIV LTR and the mechanisms of trans-activation.**

Human immunodeficiency virus (HIV) has been implicated as the etiologic agent associated with AIDS (1–3). The viral genome consists of several characteristic retroviral genes—namely, *gag*, *pol*, and *env* (4–6). In addition, the HIV genome contains several regulatory genes—for example, the regulator of virion protein expression *rev* and the trans-activator gene *tat* (7–13).

The *tat*-encoded protein of HIV is a potent activator of viral gene expression (8, 9, 14). This protein is encoded by two separate exons of the *tat* gene, the first of which is sufficient for trans-activation (8, 14). The tat protein is required for high levels of viral gene expression, but its precise mode of action is unclear. Rosen *et al.* (15) suggested that tat is required for the active translation of viral mRNAs. Muesing *et al.* (16) have also shown that *tat* expression correlates with increased mRNA level. Thus, tat may function at both transcriptional and translational levels. Also tat has been demonstrated to act as an antiterminator, which allows high levels of full-length transcripts (17).

The sequence responsive to tat action (TAR) resides mainly in the R region of HIV long terminal repeat (LTR) and was mapped to −17 to +80 relative to the eukaryotic transcriptional start site (16). Deletions within this region inhibit the ability of tat to trans-activate, suggesting that tat binds directly to the DNA of the TAR region. Direct physical binding of tat to TAR sequences has not yet been demonstrated; *tat* may act indirectly via some cellular intermediates that then enhance viral expression.

Mitsialis *et al.* (18) showed that Rous sarcoma virus (RSV) LTR contains a promoter-like sequence that is functional in *Escherichia coli*. We show here that HIV LTR as well as several other retroviral LTRs are also functional in bacteria. The HIV LTR can further be specifically trans-activated by *tat*, and the TAR region on the LTR seems necessary for trans-activation. This system should provide a simple and useful means of studying the mechanism of trans-activation by *tat*.

MATERIALS AND METHODS

Cell Lines and Viral Clones. Human T-cell lymphotropic virus type III (HTLV-III)-infected HT-9 cells were obtained from R. C. Gallo and M. Popovic (National Institutes of Health). cDNA clones as well as an entire proviral clone were then cloned from a phage library constructed from infected HT-9 cellular DNA using standard procedures (19).

Chloramphenicol Acetyltransferase (CAT) Plasmid Constructions. HIV–CAT was constructed by removing the U3–R region of HIV LTR from a cDNA clone that was generated from HTLV-III-infected H-9 cells and cloned into vector $pCDV_1$ according to Okayama and Berg's procedures (20). This clone contains a 1.2-kilobase (kb) insert, which includes the 3′ end of the viral genome and the 3′ LTR that terminates at the poly(A) site in the R region (C.W., unpublished data). The U3–R region of the HIV LTR can be released by cutting with *Xho* I because an *Xho* I site occurs in the viral sequence 5′ of U3 and also in the vector immediately after the poly(A) tail (41 bases long) (Fig. 1). To construct HIV–CAT, the *Xho* I–*Xho* I fragment containing the U3–R region was then blunt-ended and ligated upstream of the CAT gene of a promoterless pSV_2-CAT plasmid (where SV_2 represents simian virus 40). The promoterless PSV_2-CAT plasmid was constructed by eliminating the simian virus 40 promoter (21) of pSV_2-CAT (*Nde* I and *Hin*dIII cut). ΔHIV–CAT, which lacks the TAR region, was constructed by inserting the *Pvu* II–*Pvu* II fragment of HIV LTR (Fig. 1) into the promoterless pSV_2-CAT plasmid.

HTLV-II–CAT was constructed similarly using an *Eco*RI fragment that contains the LTR from a plasmid containing the entire HTLV-II clone (22). Simian sarcoma virus (SSV)–CAT was constructed using the *Xba* I–*Bam*HI LTR fragment of λ-SSV-11 C11 DNA (23). All these promoter fragments were also inserted upstream of CAT gene using the same promoterless pSV_2-CAT plasmid.

The LTR–*tat* plasmid was constructed by inserting the *Sal* I–*Kpn* I fragment (exon I of *tat*) of an HTLV-III clone (3, 15) into the *Sal* I–*Kpn* I sites of pUC19, the ampicillin gene (*Amp*) of which had been replaced by the tetracycline gene (*Tet*) of pBR322. Then, the *Xho* I–*Xho* I U3–R fragment of HIV was inserted into the *Sal* I site, positioning the HIV promoter upstream of *tat*.

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Abbreviations: RSV, Rous sarcoma virus; SSV, simian sarcoma virus; tat, trans-activator protein encoded by *tat*; TAR, trans-activation responding sequence; LTR, long terminal repeat; HIV, human immunodeficiency virus; HTLV, human T-cell lymphotropic virus.

*To whom reprint requests should be addressed.

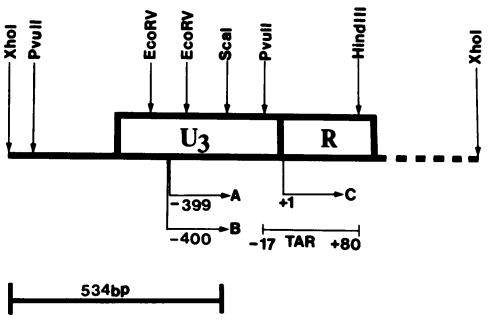

FIG. 1. Schematic representation of the restriction map of HTLV-III LTR from a cDNA clone used in this study. ▪ ▪ ▪, Poly(A) stretch (41 bases) of the cDNA and the *Xho* I linker of the vector to which it is connected (20). A and B, Putative transcriptional start sites at −399 and −400 in *E. coli*; C, normal eukaryotic start site at +1 as defined by the presence of a TATAAT box at the −30 region. The 534-bp probe was used for mapping the start sites.

Control Plasmids. A control *tat* plasmid, which made no functional tat protein, was also constructed using the same exon-I fragment of *tat* and cloning it into the *Sal I–Kpn* I site of pUC19 that expresses a truncated LacZ–tat protein. Promoterless CAT control plasmid was constructed by eliminating the simian virus 40 promoter of pSV_2-CAT (cut by *Nde* I and *Hin*dIII). Plasmid pDH5060, which expresses CAT using a bacterial promoter, was used as a control (24, 25).

CAT Assay. Cells containing plasmids were grown to A_{600} = 0.7 in 2 ml of LB medium (19)/0.2% glucose at 37°C. Cells were centrifuged (4,500 × *g* for 10 min), and the cell pellet was resuspended in 175 μl of hypotonic TE solution (25 mM Tris·HCl/10 mM EDTA, pH 8.0) with 250 μg of lysozyme. The resuspended cell pellet was further incubated at 37°C for 30 min for lysis. Cell lysates were centrifuged (12,000 × *g* for 5 min), and the supernatants were assayed for CAT activities by the spectrophotometric method of Shaw (26). Rate of increase in A_{420} was measured at 37°C to determine CAT enzymatic activity expressed as nmol of dithiobisnitrobenzoic acid reduced per min per mg of dry weight (27).

RNA Preparation. Total RNA was extracted from 15 ml of cells at A_{600} = 0.7 (19). Total RNA was measured at A_{260}, and appropriate amounts were filtered through nitrocellulose filter (0.45 μm, Schleicher & Schuell). Dot-blot hybridization was done as described (19), and a nick-translated CAT probe (19) was used for hybridization.

Mung Bean Nuclease Protection Assay. A modified procedure of Berk and Sharp (28) was used to map the start site of transcription. The probe used was from a subclone containing the *Xho* I–*Sca* I fragment of LTR (Fig. 1) inserted into the *Sal* I–*Sma* I site of pUC19. The probe can be released with an *Eco*RI–*Hin*dIII digest that cuts into the linker of the vector to yield a fragment of 534 base pairs (bp). Briefly, the *Eco*RI–*Hin*dIII 534-bp LTR fragment was labeled using [γ^{32}-P]ATP (150 μCi; 1 Ci = 37 GBq) and T4 polynucleotide kinase (29). Ten micrograms of total RNA and 200 μg of labeled DNA were resuspended in 20 μl of 80% formamide/0.4 M NaCl/0.04 M Pipes, pH 6.5/1 mM EDTA. The mixture was heated and incubated overnight at 45°C. Two hundred and seventy units of Mung bean nuclease (Pharmacia) and 280 μl of cold buffer (30 mM NaOAc, pH 4.6/50 mM NaCl/1 mM $ZnCl_2$/5% glycerol) was added. The mixture was incubated at 37°C for 1 hr. The nuclease-resistant hybrids were electrophoresed and visualized by autoradiography.

RESULTS

To test the expression of HIV LTR in bacteria we used the CAT gene as a marker. The DNA segment containing the U3–R region of the HIV LTR was inserted in front of the CAT gene as described. The resulting plasmid, HIV–CAT, was then transformed into *E. coli* HB101. The transformants were able to grow well on chloramphenicol plates, indicating that active CAT enzyme was being made from this plasmid. A spectrophotometric assay (26) was used to measure the amount of enzyme. An increase in enzymatic level was measured as an increase of A_{420}. The results (Fig. 2*a*) indicate that the HIV LTR was an active promoter and the level of CAT expression was comparable to that of a bacterial plasmid, pDH5060, which contains the bacterial CAT promoter. The specific enzyme levels for each plasmid construct were calculated and are summarized in Table 1, column 1. HIV–CAT and pDH5060 both showed similar levels of enzyme activity, about ten times greater than a control promoterless CAT plasmid. HIV–CAT and pDH5060 gave CAT activities of 3.4 and 2.94 nmol/min per mg of bacteria, respectively, whereas a promoterless CAT plasmid showed activity only slightly above background levels (0.38 nmol/min per mg of bacterial cells). Because other retroviral promoters, such as RSV LTR, have been reported to function in *E. coli*, we tested two other LTRs as well as two non-LTR eukaryotic promoters using our CAT assay system in bacteria. The two LTR–CAT plasmids, HTLV-II–CAT and SSV–CAT, were also quite active and gave comparable levels of CAT activity when compared with the bacterial plasmid pDH5060 (Fig. 2*a*). Essentially, only the LTR promoters

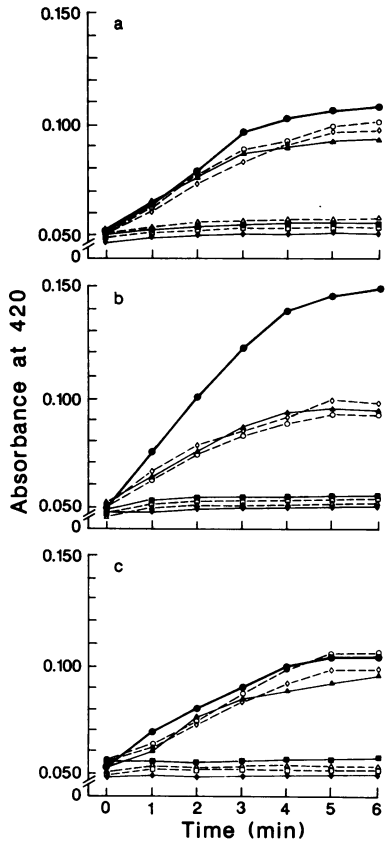

FIG. 2. CAT enzyme expression with different promoter constructs. (*a*) CAT activities of HB101 cells transformed with different CAT constructs. (*b*) CAT activities of HB101 cells cotransformed with different promoter elements and with LTR–*tat* plasmid. (*c*) CAT activities of HB101 cells cotransformed with different promoter elements and with an out-of-frame pUC19–*tat* construct. Cotransformants were selected and grown on ampicillin/tetracycline plates. Each CAT assay was repeated at least three times. The promoter element constructs used were as follows: ●, HIV–CAT; ○, HTLV II–CAT; ▲, SSV–CAT; ◇, pDH5060; ■, CAT; □, metallothionein–CAT; △, pSV_2–CAT (SV_2, simian virus 40); and ◆, HB101.

Table 1. CAT-specific activities directed by different promoters in *E. coli*

Construct	Cotransformation CAT, specific activity*		
	None	+LTR–*tat*	+*tat*†
HIV–CAT	3.40	6.61	3.18
HTLV-II–CAT	2.69	2.94	2.20
SSV–CAT	2.45	2.69	2.45
pSV_2–CAT	0.24	0.20	0.24
MT–CAT	0.24	0.00	0.38
pDH5060	2.94	2.69	2.94
CAT	0.38	0.30	0.38
HB101	0.00	0.00	0.00

MT, metallothionein; SV_2, simian virus 40.
*Expressed as nmol/min per mg of cells (dry weight).
†Out-of-frame pUC19–*tat*.

tested so far were functional, whereas the two other eukaryotic promoters, simian virus 40 and metallothionine, were not significantly functional and gave only background levels of CAT expression, similar to that of a promoterless CAT vector (Fig. 2*a*, Table 1).

The normal promoter of the HIV LTR, which functions in eukaryotic cells, was found to be in the U3 region of the LTR (4, 6), where the TATAAT signal sequence is found. To determine the position of the initiation site of the LTR promoter in bacteria, two sets of experiments were done: (*i*) deletion analysis to roughly locate the promoter region and (*ii*) Mung bean nuclease analysis to pinpoint the exact starting site of transcription. Deletion analysis was performed by removing small segments of the LTR in either the R or U3 regions (Fig. 1). A deletion clone that had part of the U3 region removed by deleting a 79-bp *Eco*RV segment was enough to abolish all promoter activity (data not shown). This result indicated that the promoter may be located within the *Eco*RV segment of U3, upstream from the normal HIV eukaryotic promoter.

To determine the exact transcriptional start site of HIV–CAT plasmid, a modified S1 analysis was done using Mung bean nuclease (30). The probe (Fig. 1) used was a 534-bp subcloned *Xho* I–*Sca* I LTR fragment. Total RNA was extracted from bacteria containing HIV–CAT and hybridized to the labeled probe. Mung bean nuclease was used to digest the unhybridized single-stranded regions. The Mung bean-digested reaction mixture was electrophoresed next to a sequencing reaction to determine the exact size of the protected fragment; the results are shown in Fig. 3. There were only two protected fragments, a strongly protected

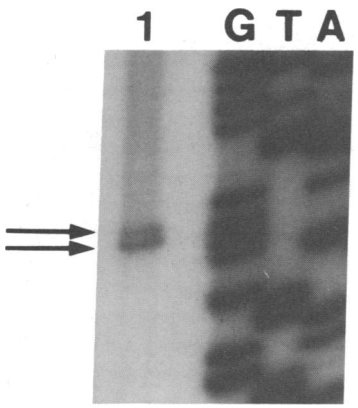

FIG. 3. Mung bean nuclease analysis of HIV-LTR-transformed HB101 cells. Lanes: 1, Two protected fragments of 261 and 262 bp (arrows) after Mung bean treatment using the 534-bp (*Xho* I–*Sca* I) LTR probe. G, T, and A lanes are from a single-stranded M13mp8 dideoxynucleotide sequencing reaction.

fragment of 261 bp and a weakly protected fragment of 262 bp. No other protected fragments were consistently detected, indicating that two sizes of mRNA were made and placing the initiation sites at −399 and −400 within the U3 region of the LTR (Fig. 1). Sequence of the U3 region of the HIV LTR around the initiation site was then analyzed and compared with other bacterial and retroviral promoters (Fig. 4). In comparison, the HIV LTR has a bacterial promoter-like sequence similar to RSV LTR, which also functions in bacteria. The sequence around −30 was nearly homologous to the model bacterial promoter, whereas the −10 region was less similar. The similarity appears to be sufficient for the viral LTR sequence to function as a genuine bacterial promoter.

The HIV promoter is trans-activated in eukaryotic cells by other viral proteins such as tat (8, 9, 14). Assessing whether the expression of CAT activity by HIV LTR can also be trans-activated in bacteria seemed important. To test this hypothesis we introduced a second plasmid containing *tat* into the bacteria, which already contained the HIV–CAT plasmid. The LTR-*tat* plasmid was constructed with a tetracycline-resistant marker, so when the HB101 cells were cotransformed by HIV–CAT and LTR–*tat* these cells could be selected for the presence of both ampicillin and tetracycline markers. The resulting CAT assays of the cotransformed cells are shown in Fig. 2*b* and Table 1. A 2-fold increase of CAT-specific activity from 3.4 to 6.61 nmol/min per mg of dry weight of bacteria occurred in the presence of LTR-*tat* plasmid. This trans-activation was HIV-LTR-specific in that none of the CAT vectors with other promoters, such as HTLV-II LTR or SSV LTR, could be trans-activated (Fig. 2*b*).

To further show that tat protein caused the increase in CAT expression, a control plasmid was constructed. This plasmid was constructed from pUC19, which uses the *lacZ* promoter to express cloned fragment as a LacZ fusion protein. The *tat* coding region was cloned into pUC19 after the *lacZ* ATG initiation site. The resulting plasmid uses the promoter and ATG initiation site of *lacZ*, but the *tat* fragment was inserted so that it would terminate shortly after initiation, and no functional LacZ–tat fusion protein could be made. When this control *tat* plasmid was cotransformed with the HIV–CAT plasmid into HB101 cells, no trans-activation was seen. Fig. 2*c* shows results of such an experiment, indicating no significant trans-activation, and the enzyme activity of HIV–CAT was comparable to the level of Fig. 2*a* when *tat* plasmid was absent. Thus, our results show specific trans-activation of the HIV LTR in *E. coli* by *tat*, and the tat protein may bind directly to the HIV promoter to specifically trans-activate CAT expression.

In eukaryotic cells the TAR region of the HIV LTR has been demonstrated (16) as responsible for tat trans-activation. To determine what part of the HIV LTR binds to tat, we constructed another plasmid without any TAR region. This plasmid, ΔHIV–CAT, was constructed by using the *Pvu* II–*Pvu* II fragment that contains the promoter region of HIV LTR and thus lacks TAR sequence (Fig. 1). This fragment was then inserted upstream of the CAT gene in the promoterless CAT plasmid. ΔHIV–CAT had similar CAT enzymatic activities when compared with the intact HIV–CAT construct, but upon cotransformation of LTR–*tat*, no trans-activation was detected (Fig. 5). Our results indicate that the TAR sequence (*Pvu* II–*Hin*dIII fragment) was directly responsible for trans-activation in our system. This result also implies that the tat protein may bind directly to the TAR region to trans-activate without involving any other cellular intermediates.

In HIV-infected cells tat protein was implicated to trans-activate at both transcriptional and translational levels (15, 16). Therefore, to find whether trans-activation of HIV–CAT

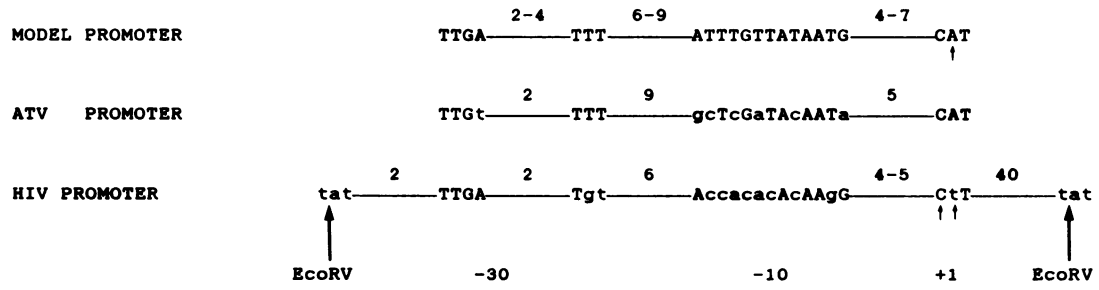

FIG. 4. Comparison of a model bacterial promoter with RSV and HIV promoters. The model promoter was based on sequences described by Hawley and McClure (31). The ATV promoter contains a sequence of RSV LTR that has promoter activities in *E. coli* (18). The proposed HIV transcriptional start site lies within the *Eco*RV–*Eco*RV region of the LTR in *E. coli*. Uppercase letters denote sequences homologous to the prokaryotic consensus sequence; small arrows represent the bases where mRNAs initiate.

expression by *tat* in *E. coli* could also be reflected at the transcriptional level, RNA dot-blot analysis was performed with the total RNA extracted from *E. coli* cells. Serial dilutions of different concentrations of different RNAs were dotted onto nitrocellulose filter and then hybridized with a ^{32}P-labeled CAT probe. The results (Fig. 6) indicate at least a 4-fold increase of CAT mRNA in the presence of the LTR–*tat* plasmid. This increase of CAT mRNA was not seen with any CAT plasmids containing other promoters, such as HTLV-II or SSV LTR when cotransformed with the same LTR–*tat* construct. This fact suggests that trans-activation of HIV occurs at the RNA level, which may account for the 2-fold increase of CAT-specific activity (Fig. 2*b*).

DISCUSSION

Our results strongly suggest that the HIV promoter is functional in *E. coli*. Previous reports (18, 32) along with these data indicate the presence of sequences within the retroviral promoters that can be recognized by *E. coli* RNA polymerase. At least for HIV, the region that functions as the prokaryotic promoter differs from that of eukaryotic cells. Mung bean analysis has shown the only transcriptional start site for *E. coli* RNA polymerase to be at −399 and −400 in the U3 region, whereas the binding site of eukaryotic RNA polymerase II is downstream at −30 in the LTR U3 region. The start site at −399 seems stronger, whereas the −400 site is weaker. Comparison of sequences within the *Eco*RV–*Eco*RV region of HIV LTR with that of 112 bacterial promoters by Hawley and McClure (31) as well as the sequence of RSV LTR, which also functions in *E. coli*, shows surprising identities—specifically at the −35 region, whereas the matches at the −10 region are less significant. However, given the variations even among bacterial promoters in these regions, similarities of the HIV sequence at −35 and −10 may be sufficient for it to function in bacteria.

Among all recombinant plasmids that we assayed LTR promoters were most active in *E. coli*. This list includes HIV, HTLV-II, SSV, and RSV promoters (18). Other eukaryotic and DNA viral promoters, such as metallothionein and simian virus 40 (SV_2) promoters did not show significant activity. Our experiments with bacterial cells that were transformed with pSV_2-CAT resulted in very small colonies on chloramphenicol plates after prolonged incubation (72 hr). These cells, however, did not show significant CAT activities with spectrophotometric analysis; however, this absence could be related to the sensitivity limitations of this assay.

Trans-activation of viral gene expression has been demonstrated for several retroviruses, including HTLV-I, HTLV-II (33), and RSV (34). Several groups (8, 14) have also shown that the HIV LTR is trans-activated in HIV-infected cells. This effect is mediated through the action of a virally encoded tat protein on a target sequence (TAR) in the HIV LTR (15). We have also shown the same trans-activating effect on HIV LTR upon cotransformation of LTR–*tat* and HIV–CAT plasmids into *E.coli*. Because both LTR–*tat* and HIV–CAT plasmids use the same HIV LTR promoter for expression of *tat* and CAT in bacteria, the HIV LTR itself, rather than the tat protein, could trans-activate the expression of LTR–CAT. However, we have seen similar trans-activation when a different *tat*-containing plasmid, RSV-*tat*, which uses RSV promoter, was tested in our bacterial system (unpublished results). Probably the increase of CAT-specific activity on the HIV-driven CAT plasmid is due to the direct

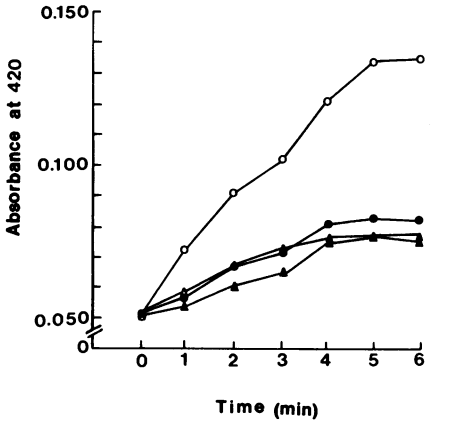

FIG. 5. CAT enzyme expression by HB101 cells transformed by either HIV–CAT or ΔHIV–CAT (deletion of TAR region) alone, as well as cotransformed with LTR–*tat*. ●, HIV–CAT; ▲, ΔHIV–CAT; ○, HIV–CAT cotransformed with LTR–*tat*; △, ΔHIV–CAT cotransformed with LTR–*tat*.

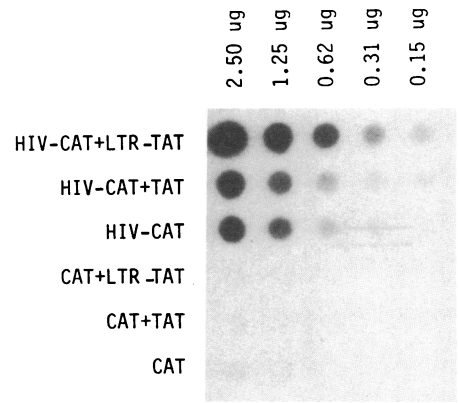

FIG. 6. RNA dot-blots of transformed and cotransformed HB101 cells with plasmids. HIV–CAT and LTR–TAT denote constructs with HIV promoters, CAT denotes a construct with no promoter, and TAT is the construct with *tat* gene inserted out-of-frame with which no functional tat is expressed.

trans-activation by tat protein. In parallel with CAT activity measurements, we have also shown increased levels of CAT mRNA transcribed from HIV LTR upon cotransformation of LTR-*tat* (Fig. 6). However, whether the increase in CAT mRNA is from the activation of transcription or increased stability of CAT mRNA or both is unclear.

That *tat* exerts its effect through TAR (16), possibly by direct interaction with the TAR region or via some other cellular intermediates, has been proposed (35). With our system it is unlikely that other bacterial intermediates than the expressed tat protein are involved in the trans-activation—unless similar factors are used in both prokaryotic and eukaryotic cells. Thus, tat may exert its effect by direct interaction with TAR sequences that activate the HIV LTR promoter. However, the possibility is still that tat may act by interacting directly with mRNA. The TAR sequence may efficiently fold into a stable stem-loop in the corresponding mRNA, with RNA secondary structure conceivably acting in trans-activation (16).

Haseltine and colleagues (15), using HIV-infected H9 cells, found unusually high levels of HIV LTR-directed protein synthesis, an increase in expression of >500- to 1000-fold relative to uninfected cells, that was due to the effect of tat protein. In our studies using bacterial host for LTR-driven CAT expression, we consistently observe only a 2- to 3-fold increase in CAT-specific activity upon bacterial expression of the tat protein. This noticeable difference in the protein-synthesis level can be explained in several ways. (*i*) Other eukaryotic cellular factors are missing in the prokaryotes, such as enhancer-binding proteins (36). (*ii*) Other transcriptional factors, such as SP1, may also bind to the G + C-rich areas of U3 enhancer region and increase transcription initiation along with tat, thus resulting in more protein synthesis (35). (*iii*) Another possibility is the coupling of transcription and translation in bacteria. If the secondary structure of the TAR in the mRNA is, indeed, important in interacting with tat protein to trans-activate, then this structure would be much less accessible for tat–TAR interaction in the prokaryotes as compared with the eukaryotes. Nevertheless, trans-activation in this prokaryotic system provides a useful system to further investigate the direct specific interaction between tat with its target sequences and the specific mechanisms of trans-activation.

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