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Transactivation of heterologous promoters by HIV-1 tat

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ABSTRACT

To determine whether HIV-1 tat can transactivate a heterologous promoter lacking HIV sequences other than the TAR element, TAR was placed downstream of the chicken β-actin promoter. Tat increased expression directed by the actin-TAR promoter to a degree equal to tat induction of the HIV-1 LTR. Optimal transactivation was observed when TAR was positioned downstream of the actin promoter such that the expected cap site of transcripts from this promoter would be the same as in transcripts directed by the HIV-1 LTR. Tat was able to transactivate, though to a lesser extent, a promoter consisting solely of a TATA element fused to TAR. Thus, tat induction does not require HIV-1 LTR promoter sequences other than TAR. Tat, when fused to the DNA binding domain of BPV-1 E2, was able to transactivate a truncated SV40 promoter containing upstream E2 binding sites, indicating that tat may be capable of transactivation when directed by a DNA binding protein to an upstream site in a heterologous promoter lacking all HIV sequences. Substitution of Ala for Lys at position 41 of tat in the tat-E2 fusion, a mutation which dramatically decreases tat transactivation of the HIV-1 LTR, eliminated this transactivation.

INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) is the predominant etiological agent of acquired immune deficiency syndrome (AIDS) (1, 2). The HIV-1 genome encodes a small nuclear localized protein, tat, which increases the expression of genes downstream of the HIV-1 long terminal repeat (LTR) by two to three orders of magnitude (3–5) and is essential for virus replication (6,7).

HIV-1 tat is an 86 amino acid protein encoded by two exons. The 72 amino acid segment present in exon 1 appears sufficient for full transactivation (8, 9). This protein has an essential cysteine rich region, which has been shown to play a role in the formation of metal-linked tat homodimers *in vitro* (10). It also contains an arginine rich region, which may be needed for nuclear localization, RNA binding and/or transactivation (9–15). The ability of tat to transactivate may be confered by two discreet regions: the amino-terminus, which is proposed to form an acidic amphipathic-helix activation domain (16), as well as a region centered at lysine 41 (17).

TAR, the target required for transactivation by tat, lies between −17 and +81 with respect to the HIV-1 LTR transcription start site (4). The minimal sequence required for activation may be as little as 25 to 30 nucleotides (18, 19). The RNA transcribed from the LTR has the potential to form a highly stable stem-loop structure from +1 to +59 with the mRNA cap site at the base of the stem (20, 21). Evidence indicates that the TAR element may act as an RNA, rather than DNA, target for tat (22–25). Tat protein binds to a small bulge in TAR RNA (26–28), while a six nucleotide loop in TAR RNA appears to bind a cellular protein which could act in concert with tat (29–31). Both the bulge and loop are critical for tat transactivation (32–34).

The mechanism of induction by tat is not entirely understood. Much evidence indicates that tat increases the rate of transcription from the HIV-1 LTR (20, 35–38). Tat has also been shown to increase transcript levels by relieving premature transcriptional termination at a discreet locus near the end of the TAR stem (39) and to increase the efficiency of transcriptional elongation (38,40). Still other evidence suggests that tat may increase the translational efficiency of TAR-containing RNA (23, 36, 41–45). However, in conflict with the model of translational enhancement by tat is the finding that tat does not transactivate mature TAR-containing RNA in nuclei or cytosol of primate cells (46). Demonstration of transactivation by tat *in vitro* (47) provided further evidence of a role for tat in the enhancement of elongation of transcripts initiated in the HIV-1 LTR. Evidence that tat transactivates by increasing the efficiency of transcriptional elongation of HIV transcripts may indicate that transcriptional complexes initiating in the HIV-1 LTR are defective for elongation.

A recent report showed that efficient tat transactivation only occurred when TAR was linked to the NF-κB and Sp1 promoter elements in the HIV-1 LTR (48). We wished to determine whether the HIV-1 LTR promoter environment is necessary for tat transactivation. We fused the HIV-1 TAR element to a heterologous promoter lacking all other HIV-1 LTR sequences and found that tat induced expression from this chimeric promoter as efficiently as from the HIV-1 LTR, indicating that HIV-1 sequences other than TAR are not required for full tat transactivation. We also found that tat, when fused to the DNA binding domain of bovine papillomavirus type 1 (BPV-1) E2, can transactivate a promoter having upstream E2 binding sites but lacking HIV-1 LTR sequences.

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MATERIALS AND METHODS

Cell lines

HeLa, cos7 and Balb/c 3T3 cells were all obtained from American Type Culture Collection. HeLa and 3T3 cells were propagated in Dulbecco's modified Eagle's medium (GIBCO) containing 10% donor calf serum (Hazleton) and 4 mM glutamine (Whittaker). Cos7 cells were grown in the same medium with substitution of 10% fetal bovine serum (Hazleton) for donor calf serum.

Plasmids

The promoter-lacking vector, pXB100, was derived from pBG312 (49) by replacement of the SV40 early promoter and adenovirus major late promoter with a polylinker. The chicken β-actin promoter (50,51) fragment from −270 to +1 was inserted into the XhoI-BamH1 sites of pXB100 to generate pXB101. The HIV-1 isolate HXB2CG was used for derivation of LTR, TAR and tat containing vectors. The HIV-1 LTR DNA fragment from −167 to +81 was introduced into pXB100 to create pXB301. Two actin-TAR vectors were constructed. The HIV-1 TAR element from −18 to +81 was inserted in the direct orientation at the β-actin transcription start site in pXB101, resulting in pXB302. In pXB315, synthetic deoxyribonucleotides were used to place the TAR sequence −19 to +59 just downstream of the β-actin TATA box such that the predicted transcription start site in TAR was precisely the same as in transcription directed by the HIV-1 LTR.

The chloramphenicol acetyltransferase (CAT) reporter gene derived from pSV2-CAT (52) was inserted between the promoter and the SV40 intron in pXB101, pXB301, pXB302 and pXB315. The 86 amino acid tat gene was expressed as a cDNA (constructed using synthetic oligonucleotides by Dr. M. Rosa) in pXB101. This vector, pXB101tat, was designed to express the tat protein but not rev. TATA-TAR vectors were constructed starting with pXB100 and inserting synthetic oligonucleotides which joined the β-actin TATA element −32 to −1 to HIV-1 TAR from +1 to +59. Wild type and deletions of +9/+10, +23/+24 and +44 were created.

In the E2-responsive reporter plasmid, pC515-9 (53), expression of CAT was directed by a truncated SV40 early promoter in which the enhancer was replaced with three E2 binding sites. Sequences encoding BPV-1 E2, E2C (the carboxy-terminal 249 amino acids of E2 contining DNA binding and dimerization activity but lacking transactivation activity), tat and tat-E2 fusion protein were all inserted into the expression vector pBG312 (49). E2 was inserted as a BamH1 fragment derived from pCO-E2 (53). E2C was taken as a BamH1 fragment from pYE2-R (54). pBG312tat was derived from pXB101tat. TatE2C was constructed by filling in the NcoI site at the initiation codon of E2C, ligating Bgl2 linkers and joining this end to a BamH1 site in tat. This insert was cloned into a BamH1 site of pBG312. This fusion construct encoded amino acids 1−62 of tat followed by a serine and then residues 162−410 of E2. The 41Lys→Ala mutation of tat was generated using polymerase chain reaction in which one of the oligonucleotide primers created the mutation.

Transient transfection assays

All cells were tranfected by an electroporation protocol similar to that described by Chu et al. (55). Typically, 20 μg of reporter and 20 μg of transactivator plasmids were introduced along with sonicated herring sperm DNA to a final of 400 μg total DNA per electroporation. Far lower amounts of pXB101tat could be transfected with similar results. CAT activity was measured 48 to 72 hours subsequent to electroporation by a thin layer chromatography assay (52) and by a differential extraction protocol (56). Equal amounts of total cell protein were used in each assay sample. Background counts of acetylated chloramphenicol, derived by assaying cells transfected with carrier DNA only, were subtracted from acetylated chloramphenicol levels of samples prior to determination of induction. Tat induction was computed as the fold-increase in acetylated cpm in the presence of tat over acetylated cpm in the absence of tat.

RESULTS AND DISCUSSION

Tat transactivation of a chimeric actin-TAR promoter

The chicken β-actin promoter was chosen for use as the basal promoter in TAR fusion vectors due to its low level of expression in most cell types tested. The HIV-1 TAR sequence was placed downstream of the β-actin TATA element in two constructs (Figure 1). In pXB302, the TAR sequence from −18 to +81 was inserted at the transcription initiation site of the actin promoter. In this vector, the TAR element was displaced downstream from the usual HIV-1 RNA cap site such that 18 additional nucleotides, which are not present normally in HIV-1 RNA, were expected to be transcribed. Therefore, in contrast to HIV-1 LTR transcripts, the cap site in pXB302 was not predicted to be involved in the stem-loop structure. Since TAR may be involved in the regulation of translation as well as transcription, it may be critical for the RNA cap site to be buried at the base of the stem. For this reason, as well as the possible

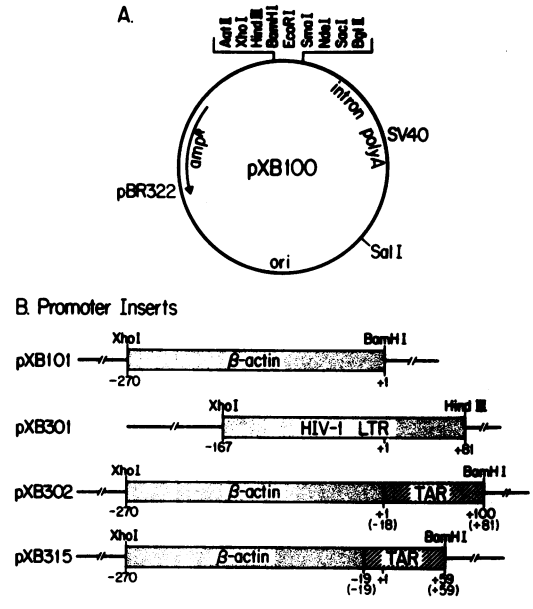

Figure 1. Map of vectors used. A, map of pXB100, which has a polylinker upstream of the SV40 splice and polyA signals. B, Four promoters were independently inserted into the polylinker of pXB100 such that transcription progressed in the direction toward the SV40 splice and polyA sequences. The restriction sites shown indicate the sites used to insert the promoters. The coordinates directly under the promoters indicate the expected positions relative to the transcription start sites, while the coordinates in parentheses indicate the positions of the TAR sequence when transcription is directed by the HIV-1 LTR. The CAT reporter gene was inserted into the BamHI site downstream of these promoters.

position-dependence of TAR as a transcriptional element, pXB315 was generated. In pXB315, the TAR sequence +1 to +59 was placed downstream of the actin promoter such that position +1 of TAR was in position +1 relative to the actin promoter. Thus, the resulting transcripts were predicted to have the cap site at the base of the TAR stem.

The tat responsiveness of the actin promoter and two actin-TAR fusion promoters was compared to that of pXB301 which consisted of the HIV-1 LTR from −167 to +81 (Figure 1). All vectors directed the expression of the CAT reporter. Reporter and tat expression vector constructs were introduced into HeLa cells in transient electroporations and CAT activity was determined. Results are shown in Figure 2. In the absence of tat, all four vectors displayed low CAT activities. Expression directed by the HIV-1 LTR was increased 142-fold by cotransfection of the tat vector. The uninduced expression levels from the two actin-TAR fusion promoters (pXB302CAT and pXB315CAT) were slightly greater than that from the actin promoter (pXB101CAT) indicating that the presence of TAR had no negative effect on the actin promoter in the absence of tat. This agrees with previous TAR deletion analyses (18, 20). Expression from the actin promoter alone was not altered significantly by the presence of tat.

The actin-TAR vector having the TAR element in the correct position relative to the HIV-1 LTR transcription start site (pXB315CAT) was induced 134-fold by pXB101tat cotransfection. The actin-TAR promoter consistently displayed a higher constitutive expression level than the HIV-1 LTR, so that while tat induction of HIV-1 LTR and actin-TAR were approximately equal, the final induced expression level of actin-TAR was two-fold greater.

The actin-TAR construct in which TAR was displaced slightly downstream of the RNA cap site (pXB302CAT) was induced only 34.6-fold by tat indicating that the TAR element displays position-dependence. This promoter gave an approximately four-fold lower transactivation than actin-TAR pXB315 in all experiments performed. The dependence on position could be due to a requirement for the RNA cap site to be involved in the stem structure or, more generally, to a requirement for TAR to be positioned proximal to the promoter.

Transient transfections into cos7 cells and the human hepatoma cell line Hep3B produced results similar to those above (data not shown). The increase in CAT protein levels in the presence of tat was mirrored by an increase in the steady-state level of RNA as determined by Northern blot analysis both in the case of the HIV-1 LTR and the actin-TAR promoter (data not shown). The length of transcripts detected in Northern blots was consistent with initiation at the start of the TAR element. However, high resolution analysis was not performed to determine whether the +1 position of TAR was actually the initiation site in actin-TAR transcripts.

These results indicate that tat can induce expression of a heterologous promoter containing only the HIV-1 TAR element from −19 to +59. It has been postulated that specific LTR promoter elements, in addition to TAR, may be required for tat activation (48). The chicken β-actin promoter has potential Sp1 binding sites but lacks sequences homologous to NF-κB binding sites which were found to be required for full tat activation of the HIV-1 LTR. We believe that enhancement by tat requires no other specific elements but TAR. However, we have not eliminated the possibility that a specific interaction of Sp1 with tat is required for full tat induction, though a requirement of NF-κB seems unlikely. If tat does transactivate primarily by increasing the rate of transcriptional elongation, it may be necessary to employ a promoter which is weak or has a specific defect in elongation in order to reconstitute full tat induction. Preliminary evidence indicates that the tat induction level of a strong promoter fused to TAR is significantly less than the induction of the HIV-1 LTR or the actin-TAR promoter (data not shown).

Tat transactivation of a minimal promoter

To determine whether tat can increase expression from a minimal promoter containing only a TATA element and TAR, a promoter was constructed such that the TATA element from the actin promoter was fused to the HIV-1 TAR +1 to +59 sequence (sequences upstream of the TATA element were from pBR322). The level of expression of the TATA-TAR vector in the absence of tat was 360-fold lower than that of the actin-TAR vector in the absence of tat (data not shown) indicating that this promoter is indeed severely defective. As shown in Table 1, CAT activity driven by the TATA-TAR promoter was increased 9.3-fold by

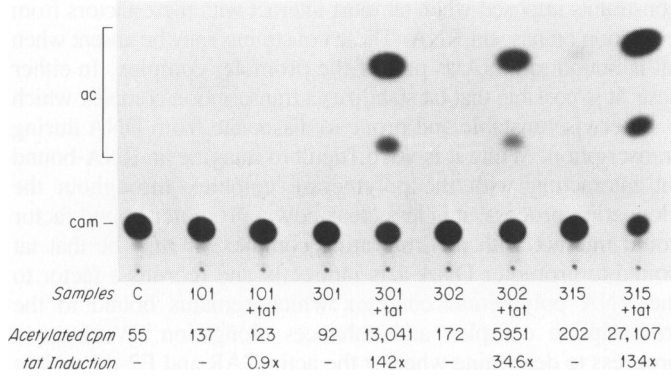

Figure 2. Expression of transiently introduced CAT vectors containing the HIV-1 LTR, actin or actin-TAR fusion promoters. pXB101CAT (actin promoter), pXB301CAT (HIV-1 LTR), pXB302CAT (actin-TAR fusion promoter with TAR displaced downstream in relation to its position in the LTR) and pXB315CAT (actin-TAR fusion promoter with TAR in the same position relative to the transcription start site as in the HIV-1 LTR) were introduced into HeLa cells by transient electroporation with or without the tat expression vector pXB101tat. A CAT assay was performed 3 days after electroporation. The reporter vectors are indicated by the three number designation of the plasmids. Sample C, cells were electroporated with carrier DNA only. Acetylated cpm were determined by a differential extraction protocol (56) using the same extracts as used in the chromatography.

Table 1. Tat transactivation of minimal promoter constructs expressing CAT following transient electroporation into HeLa cells[a]

Promoter construct	Induction by tat[b]
Actin-TAR	57.4
TATA	1.1
TATA-TAR	9.3
TATA-TAR(Δ+23,+24)[c]	1.4
TATA-TAR(Δ+9,+10)	2.6
TATA-TAR(Δ+44)	2.0

[a]Values represent the average of the results of three experiments.
[b]Values were computed as CAT activity (percentage conversion) in the presence of tat over CAT activity in the absence of tat for each promoter construct.
[c]Numbers in parentheses indicate base pairs deleted from TAR with +1 indicating the HIV-1 transcription cap site.

tat. This level of transactivation, while 6-fold lower than the tat induction of the actin-TAR promoter in this experiment, indicates that tat is capable of increasing expression from a promoter lacking all upstream elements. The low level of induction relative to actin-TAR and the HIV-1 LTR may indicate that a higher basal transcription rate may be needed for the full tat effect. It is also possible that tat needs to interact with specific transcription factors which are present in both the HIV-1 LTR and the chicken β-actin promoter complexes. Deletions in TAR which reduced the base-pairing in the stem (Δ+9,+10 and Δ+44) and a mutation which deleted two nucleotides in the bulge (Δ+23,+24) all decreased the transactivation by tat. The deletion of the TAR bulge produced the greatest decrease in transactivation. This result indicates that TAR secondary structure may be critical in this transactivation.

Transactivation by tat when tethered to promoter DNA

To determine whether tat could function as a transcription factor when bound to DNA at an upstream promoter site, a fusion protein was constructed. The fusion protein, tatE2C, consisted of the first 62 amino acids of tat placed at the amino-terminus followed by the last 249 amino acids of the bovine papillomavirus (BPV-1) E2 protein at the carboxy-terminus. BPV-1 E2 is a DNA binding protein which has an amino-terminal transactivation domain and a carboxy-terminal DNA binding and dimerization activity (57). As shown below, the DNA binding domain alone lacks detectable transactivation activity. The tatE2C chimeric protein was tested for transactivation of the reporter pC515-9 (53) in which three E2 binding sites were placed upstream of the enhancer-deleted SV40 promoter in pA10CAT. E2, E2C (the carboxy-terminal 249 amino acids having DNA binding and dimerization activity), tat and tatE2C were expressed using the adenovirus major late promoter augmented by the presence of the SV40 enhancer.

Table 2 shows the results of a CAT assay of transiently transfected Balb/c 3T3 cells. Neither tat nor E2C, introduced individually or together on separate plasmids, transactivated pC515-9. However, the tatE2C fusion protein significantly increased CAT expression. A direct comparison of the relative strengths of E2 and tatE2C cannot be done since E2 and tatE2C protein levels were not measured, and the half-life of these proteins could differ significantly. The transactivation activity of tatE2C appeared to be fairly weak since it was necessary to express this protein using a strong promoter to observe significant transactivation. TatE2C did not increase CAT expression of pA10CAT, which lacks E2 binding sites, indicating that DNA binding upstream of the promoter was required for transactivation (data not shown).

Table 2. Transactivation of pC515-9 by tatE2C fusion protein following transient electroporation into 3T3 cells[a]

Transactivator	Relative CAT activity[b]
none	1.0
pBG312E2	158
pBG312tat	0.8
pBG312E2C	0.9
pBG312tatE2C	22.9
pBG312tat(41A)E2C	1.6
pBG312tat + pBG312E2C	0.7

[a]Values represent the average of results from three experiments.
[b]CAT activity expressed relative to pC515-9 vector in the absence of transactivators.

This result suggests that tat has the capacity to act as a transcription factor when directed to an upstream site in a heterologous promoter lacking all HIV sequences. It should be noted that the HIV-1 LTR, the actin-TAR promoter and the truncated SV40 early promoter in pC515-9 all have Sp1 binding sites. The only promoter used in this work which lacked Sp1 sites was the TATA-TAR minimal fusion promoter. Thus, it is possible that Sp1 is required along with tat for full induction.

Berkhout et al. (48) found that a tat-jun fusion protein was incapable of significantly transactivating a promoter having upstream AP-1 sites. Our results may differ due to the strength of the promoter used to express the fusion protein and the efficiency of the electroporations, or due to a unique interaction between tat and the E2 DNA binding/dimerization domain which was lacking in the tat-jun fusion.

It is unclear whether the mechanism of transactivation by tatE2C is related to the normal activity of tat when bound to TAR RNA. Tat could transactivate simply due to its proposed acidic amphipathic-helical amino-terminus, since many acidic peptides will serve as transactivators when fused to a DNA binding domain. Tat appears to have a second domain required for transactivation as a change at position 41 from Lys to Ala has been shown to dramatically decrease tat transactivation of the HIV-1 LTR (12, 17). The tatE2C fusion protein having this mutation, tat(41A)E2C, did not significantly increase CAT expression directed by the E2-dependent reporter (Table 2) indicating that the acidic amino-terminus is not sufficient for transactivation.

It is possible that the mechanism of tat transactivation when bound to an upstream promoter DNA site through a fused heterologous DNA binding domain is similar to, though weaker than, transactivation when bound to TAR RNA. Tat may contact the same transcription factors, or RNA polymerase itself, whether bound to TAR RNA or, in the case of tatE2C fusion protein, to the E2 promoter site. This would be surprising considering the strict position-dependence of the TAR sequence in tat activation of the HIV-1 LTR and the actin-TAR promoter. However, the position-dependence of TAR could be due to constraints imposed when tat must interact with these factors from a position on nascent RNA. These constraints may be absent when tat is bound to DNA as part of the promoter complex. In either case, it is possible that tat stabilizes a transcription complex which is otherwise unstable and prone to dissociate from DNA during transcription. While it is not difficult to imagine an RNA-bound tat interacting with the polymerase complex throughout the elongation process, it is less clear how a promoter bound factor could interact with this migrating complex. It may be that tat bound to promoter DNA acts indirectly and recruits a factor to the RNA polymerase complex which remains bound to the transcription complex and enhances elongation. Work is in progress to determine whether the actin-TAR and E2-dependent promoters used in this work display the same transcript polarity as the HIV-1 LTR indicating a defect in elongation which might be overcome by the action of tat.

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