Exon2 of HIV-2 Tat contributes to transactivation of the HIV-2 LTR by increasing binding affinity to HIV-2 TAR RNA

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ABSTRACT

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) express related Tat proteins that are encoded in two exons. Tat proteins bind directly to the TAR RNA element contained in the 5' ends of viral transcripts and thereby stimulate transcription through an as yet unidentified mechanism. We have investigated the functional significance of exon2 of the HIV-2 Tat protein by examining properties of proteins consisting of exon1 alone or exon1+2. In transactivation assays *in vivo*, exon2 modestly increased HIV-2 Tat stimulation of transcription from the HIV-2 long terminal repeat (LTR) but had no effect on transcription from the HIV-1 LTR. In HeLa cells, exon2 increased transactivation of the HIV-2 LTR by approximately three-fold, while in COS and Jurkat cells this value was less than two-fold. In binding assays *in vitro*, exon2 increased the binding affinity of the HIV-2 Tat protein to HIV-2 TAR RNA. Results with GAL4 fusion proteins and a synthetic promoter containing GAL4 DNA binding sites indicated that exon2 does not contribute to the HIV-2 Tat activation domain. These observations suggest that exon2 of HIV-2 Tat contributes to transactivation of the HIV-2 LTR by increasing the binding affinity to HIV-2 TAR RNA.

INTRODUCTION

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) are closely related retroviruses that are the causative agents of acquired immunodeficiency syndromes (AIDS) (1). HIV-1 is the predominant cause of AIDS worldwide, while HIV-2 is largely restricted to western Africa (2,3). HIV-2 is likely to become increasingly more common worldwide, as HIV-2 infections are spreading rapidly in India (4). Both HIV-1 and HIV-2 encode a crucial gene, *tat*, whose protein product termed Tat-1 and Tat-2, respectively, regulates viral gene expression and is essential for efficient viral replication (reviewed in 5,6). Tat-1 and Tat-2 bind directly to the TAR RNA element at the 5' ends of nascent viral

transcripts. After binding to TAR RNA, Tat stimulates transcriptional initiation and elongation through a molecular mechanism that remains to be elucidated.

The HIV-1 TAR RNA core element (TAR-1), which encompasses nucleotides +18 to +44 (relative to the transcription start site), forms a stable stem-loop structure. The key determinant in TAR-1 RNA for specific binding *in vitro* of Tat-1 is a tri-nucleotide bulge at +23–25 and a few surrounding base pairs in the stem (7–10, reviewed in 11). Genetic experiments suggest that a cellular factor interacts with the TAR-1 RNA loop sequences to facilitate Tat-1 binding *in vivo* (12–14). Although studied much less extensively than TAR-1 RNA, the HIV-2 TAR RNA element (TAR-2) is known to encompass nucleotides +1 to approximately +90 (15). TAR-2 RNA has been predicted by computer analyses to form two stem-loop structures similar to the single TAR-1 stem-loop (16,17); a nuclease digestion analysis has largely verified this predicted structure (18). Deletion analyses have shown that each stem-loop in TAR-2 RNA can function independently, although the 5' proximal structure is significantly more active (16,19). The determinants within TAR-2 RNA that specify binding of Tat-2 include a di-nucleotide bulge in each of the stems that is similar to the TAR-1 bulge (18). Mutations in loop1 and loop2 reduce the TAR-2 element function (16), and by analogy to the TAR-1 element, these loop sequences are likely to interact with a cellular factor *in vivo* to facilitate Tat-2 binding to TAR-2 RNA.

Tat-1 (HXB2 isolate) and Tat-2 (ROD isolate) proteins are encoded in two exons and are 86 and 130 amino acid residues, respectively (see Fig. 1). The proteins are approximately 80% homologous over a stretch of 39 amino acids in their central regions, while the amino- and carboxyl-termini show no obvious similarities (20). Exon1 of both proteins was originally reported to possess full transactivation activity; exon1 of Tat-1 is 72 residues and that of Tat-2 is 99 residues (15,19,21,22). Recent evidence has shown, however, that under some circumstances exon2 may influence Tat-1 and Tat-2 activity (23–25).

Genetic and biochemical experiments have established that Tat-1 and Tat-2 contain two distinct functional domains: an RNA binding domain and an activation domain (9,10,12,26,27). The

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RNA binding domain consists of a stretch of basic amino acid residues that directs Tat to the TAR RNA element at the 5'-end of viral transcripts. The Tat-1 RNA binding domain consists of residues 49–57 and binds with high affinity to both TAR-1 and TAR-2 RNAs; consequently, Tat-1 has high transactivation activity for HIV-1 and HIV-2 LTRs (15,16,19,28). The Tat-2 basic domain consists of residues 78–90 and binds with high affinity only to TAR-2 RNA (29–31); consequently, Tat-2 has high activity for the HIV-2 LTR and relatively low activity for the HIV-1 LTR. The activation domain of Tat proteins interacts with the cellular transcription machinery to stimulate transcriptional elongation and in some cases, initiation (26,27,32–35). The activation domain of the Tat-1 protein has been shown through protein fusion experiments to encompass residues 1 through 48 (26,27). The Tat-1 activation domain includes the non-conserved amino terminal region and the conserved cysteine-rich and core regions (36–41). The activation domain of Tat-2 has not yet been defined.

In this study, we have examined the functional significance of exon2 of Tat-2 and have investigated whether it influences TAR RNA binding or contributes to the activation domain. We present evidence that under the conditions of our experiments, exon2 had no effect on the HIV-1 LTR but modestly increased Tat-2 transactivation of the HIV-2 LTR. In HeLa cells, exon2 increased transactivation of the HIV-2 LTR approximately three-fold, while in COS and Jurkat cells this value was less than two-fold. Using an *in vitro* gel shift assay, we show that exon2 increases the binding affinity of the Tat-2 protein for TAR-2 RNA. Using GAL4 fusions and a synthetic promoter containing GAL4 DNA binding sites, we show that exon2 does not contribute to the Tat-2 activation domain. Our results suggest that exon2 of Tat-2 contributes to transactivation of the HIV-2 LTR by increasing the protein's binding affinity *in vivo* to TAR-2 RNA.

MATERIALS AND METHODS

Plasmid constructs

The plasmids used to synthesize wild type TAR-1 RNA (+1 to +80) and wt_x TAR-2 RNA (+1 to +123) by *in vitro* transcription have been described previously (18). For expression in *E. coli*, the Tat-1 86R (86 residues of exons1+2, HXB2 isolate) and Tat-2 99R (99 residues of exon1, ROD isolate) genes were expressed from pGEX2T vector as fusions with glutathione-S-transferase (GST) as described previously (42). For construction of vectors expressing the full length 130R Tat-2 protein (130 residues of exons1+2, ROD isolate), exons 1 and 2 of Tat-2 were amplified by polymerase chain reactions (PCR); the junction between exon 1 and 2 was joined by a *Cla*I restriction enzyme site (ATCGAT, altering the serine codon at residue 100 from TCC to TCG). The resultant 130R Tat-2 gene was then inserted into the pGEX2T vector for expression in *E. coli*.

To express Tat proteins in mammalian cells, a modification was first performed on the pBC12/CMV vector that utilizes the cytomegalovirus immediate early promoter for expression (22). The IL-2 coding region from pBC12/CMV was replaced with 30 nucleotides from the polylinker [*Hind*III(5') and *Bam*HI(3')] of pGEM4Z (Promega). The Tat-1(86R), Tat-2(99R), and Tat-2(130R) genes were then inserted into this derivative of the BC12/CMV/ vector. The Tat-2(g) plasmid contains an HIV-2 proviral segment from coordinates +5783 to +8569; this plasmid is also based upon the BC12/CMV vector and is described in

(29). The β -galactosidase expression vector (pSV β -gal) used as a reference plasmid has been described previously (43).

To express GAL4/Tat fusion proteins, a derivative of the pSG424 (44) was first constructed that contained the polylinker [*Eco*RI(5') and *Xba*I(3')] from pGEM4Z (Promega) in place of the pSG424 polylinker. Into the resultant pSG424Z GAL4 expression vector were inserted PCR products containing: 48 Δ gene (termination codon after residue 48 in Tat-1 gene), the 130R Tat-2 gene (exon1+2), 77 Δ gene (termination codon after residue 77 in exon 1 of Tat-2 gene), Δ 8/33 gene (deletion of residues 8 through 33 in the 77 Δ gene), and Δ 8/47 gene (deletion of residues 8 through 47 in the 77 Δ gene). GAL4/Tat-1(86R) and GAL4/Tat-2(99R) have been described previously (31). HIV LTR-CAT reporter plasmids used in this study were pHIV-1CAT(SVori) (–454 to +80; 40) and HIV-2 LTR (–556 to +119; 15). CAT expression plasmids containing promoters with GAL4 DNA binding sites were G6(HIV-1)(–83)(–TAR) (27) and G5(SP1)(E1BTATA) (26). The plasmids constructed for use in this study were sequenced to verify identities.

Transfections and *in vivo* transactivation assays

Transfections were carried out in HeLa and COS cells using the calcium phosphate procedure as described (45). Culture dishes (6 cm) were transfected with 5 μ g of CAT reporter plasmids plus the indicated amounts of activator plasmids. In all transfections, appropriate amounts of CMV vector or GAL4 vector plasmid were included to bring the total amount of DNA of activator + vector to 1000 ng. Where indicated, 1 μ g of a β -galactosidase expression plasmid (43) was included as an internal reference for transfection efficiencies. Jurkat T cells were transfected by a lipofectamine procedure. Jurkat cells cultures were diluted to a density of 2×10^5 /ml at 24 h before transfection. The CAT reporter plasmids (5 μ g) and indicated amount of activator plasmids were mixed with lipofectamine reagent (10 μ l per culture dish; Bethesda Research Laboratories, Life Technologies) in 600 μ l of RPMI 1650 medium and incubated for 30 min at room temperature. Cells were washed once by centrifugation at $800 \times g$ for 5 min with serum-free growth medium (RPMI 1650) without antibacterial agents. Cells were seeded in 2.4 ml serum-free medium at a density of $\sim 2\text{--}4 \times 10^6$ per 6 cm plate. The plasmid–lipofectamine mix was added to the cell suspensions and incubated for 5 h at 37°C in a CO₂ incubator. Following incubation, 2 ml of complete growth medium (RPMI with 10% fetal bovine serum, PMA (1 μ g/ml), and PHA-M (50 ng/ml) was added to each culture dish. For transfections in all three cell lines, cell extracts were prepared at 48 h post-transfection and CAT enzyme assays were performed under conditions where conversions of chloramphenicol to its acetylated forms was always less than 50%. CAT enzyme assays were performed in a volume of 100 μ l containing 0.1 μ Ci of [¹⁴C]-chloramphenicol, 0.4 mM acetyl-coenzyme A, and 0.125 M Tris–HCl (pH 8.0) with the appropriate amount of cell extract. CAT assays were quantified with a Betagen Betascope 603 scanner. CAT units were arbitrarily defined as $50 \times (\% \text{ conversion of chloramphenicol to its acetylated forms}) / [\text{time (h) of reaction} \times \text{volume of cell extract} (\mu\text{l})]$. CAT expression was normalized to β -galactosidase expression (in HeLa and COS experiments) and the amounts of total cellular proteins (in all experiments).

TAR RNA binding assays

In order to purify Tat proteins for RNA binding assays, Tat-1(86R) and Tat-2(99R and 130R) proteins were expressed

from the pGEX2T vector as glutathione-S-transferase (GST) fusions with a thrombin proteolytic site engineered between Tat and GST. Tat proteins were purified and cleaved from GST as described (42). Gel shift assays were performed as described previously (31). Briefly, binding reaction mixtures (10 μ l) contained 0.4 pmol (40 nM) of uniformly labeled TAR-1 (+1 to +80) or wt_x TAR-2 (+1 to +123) RNA probes, 2 ng of poly(I.C), 0.5 μ g yeast tRNA, 50 mM Tris-HCl (pH 7.6), 20 mM KCl, and 0 to 80 pmol (0 to 800 nM) of Tat-1(86R) or Tat-2(99R and 130R) proteins. After incubations at room temperature for 10 min, the reaction mixtures were applied to a 6% nondenaturing polyacrylamide gel (acrylamide:bisacrylamide ratio of 75:1) in 50 mM Tris-glycine buffer (pH 8.8) which was run at room temperature.

Immunoprecipitations

COS cells (6 cm culture dishes) were transfected with 5 μ g of indicated Tat-2 plasmids by the calcium phosphate method. At 48 h post-transfection, cells were labeled with 100 μ Ci [³⁵S]-cysteine (NEN, 1140 Ci/mmol) for 3 h, and extracts were prepared and immunoprecipitations carried out as described (16). The antiserum against Tat-2 was obtained from the AIDS Research and Reference Reagent Program (provided by B.Cullen); this rabbit polyclonal antiserum against Tat-2 was raised against a synthetic peptide spanning amino acid residues 76 through 99 (16). Immunoprecipitates were analyzed on 15% SDS-polyacrylamide gels which were processed for fluorography.

RESULTS

The Tat-1 and Tat-2 proteins analyzed in this study are compared schematically in Figure 1. The indicated cysteine-rich (cys), core, and basic domains are >80% homologous (20), whereas the amino and carboxy termini show no obvious similarities. The

Figure 1. Comparison of Tat-1 and Tat-2 proteins. The two exon Tat-1 (HXB2 isolate) and two exon Tat-2 (ROD isolate) proteins are compared schematically. The junction between the exon1 and exon2 are indicated. The cysteine-rich regions apparently bind metal ions with specific coordinations that may be necessary for proper protein conformation (48,49). The core region has been identified by conservation of these sequences in the primate immunodeficiency viruses and equine infectious anemia virus (46). The basic region serves as a nuclear localization signal and also binds TAR RNA (11,38,50).

cys, core, and basic regions have been identified by work in several laboratories (especially refs. 37,46).

Immunoprecipitation analysis of Tat-2 proteins

To assess the functional significance of exon2 of the Tat-2 protein, we analyzed the transactivation properties of two Tat-2 expression vectors under control of the CMV immediate early promoter. The Tat-2(99R) vector contains exon1 and expresses the 99 residue protein, while the Tat-2(130R) vector contains a full length cDNA of exon1+2 and expresses the 130 residue protein. We carried out immunoprecipitation experiments in COS cells to compare the expression levels of Tat-2 proteins from these vectors. As a control in these experiments, we also analyzed a CMV-based expression vector, called Tat-2(g), that contains a genomic fragment from the HIV-2 provirus and was expected to express predominantly the 130 residue Tat-2 protein from a spliced mRNA. At 48 h post-transfection, cells were labeled with [³⁵S]-cysteine, extracts prepared, and immunoprecipitations performed with a Tat-2 antiserum. As demonstrated in Figure 2, the Tat-2(99R) and Tat-2(130R) vectors expressed the 99R and 130R proteins at similar levels. The Tat-2(g) vector expressed the full-length 130R protein at a level equivalent to that of the Tat-2(130R) vector; the Tat-2(g) vector also produced low levels of the 99R protein. In this study, we also used a CMV vector that expresses the 86R (exon1+2) HIV-1 Tat protein. We have shown previously that this 86R Tat-1 protein and the 99R Tat-2 protein from the Tat-2(99R) vector appear to be expressed at similar levels in COS cells (47).

Figure 2. Immunoprecipitation analysis of Tat-2 proteins. COS cells were transfected with vector (CMV) or the indicated Tat plasmids, labeled with [³⁵S]-cysteine, and immunoprecipitations performed with a Tat-2 antiserum. Lane labeled 'Cell alone' contains a control immunoprecipitation from non-transfected cells. Immunoprecipitations were analyzed on 15% SDS-polyacrylamide gels. Lane M contains molecular mass markers.

Figure 3. Tat transactivation of the HIV-1 and HIV-2 LTRs *in vivo*. The HIV-1 LTR-CAT reporter plasmid (A–C) or the HIV-2 LTR-CAT reporter plasmid (E–F) was transfected into the indicated cell line along with 0, 10, 100, or 1000 ng of Tat activator plasmids and a β -galactosidase expression plasmid for HeLa and COS cells. CAT expression measured at 48 h post-transfection was normalized to β -galactosidase expression and total protein amounts for HeLa and COS cells, and total protein amounts for Jurkat cells.

Effect of exon2 on Tat-2 transactivation of the HIV-1 and HIV-2 LTRs *in vivo*

To assess the effects of exon2 on Tat-2 function *in vivo*, we used plasmid co-transfection assays in HeLa, COS, and Jurkat T cell lines. HIV-1 and HIV-2 LTR-CAT reporter plasmids were co-transfected with increasing amounts of Tat expression plasmids and CAT expression was measured at 48 h post-transfection (Fig. 3). To adjust for transfection efficiencies in HeLa and COS cells,

a β -galactosidase reference plasmid was included in transfections and β -galactosidase expression was used to normalize CAT expression. The β -galactosidase plasmid could not be used in Jurkat T cells, however, as it was found to inhibit CAT expression from both the HIV-1 and HIV-2 LTRs. The explanation for this observation in Jurkat cells is unknown.

Results of transactivation assays were similar for the HIV-1 LTR-CAT reporter in all the cell lines tested. The HIV-1 LTR

Figure 4. Purified Tat proteins used in gel shift assay. Shown is a 15% SDS–polyacrylamide gel which was stained with Coomassie Blue. The indicated Tat proteins were expressed in *Escherichia coli* as fusions with GST. The Tat portions of the fusions were released from GST by cleavage with thrombin and purified as described in Materials and Methods. Each lane was loaded with 2 μ g of each preparation as determined by the Bradford protein assay; it was estimated that the Tat proteins (indicated by asterisks) composed 20% of each preparation.

responded most actively to the Tat-1 86R protein (Fig. 3A–C). The HIV-1 LTR-CAT responded significantly less actively to both the 99R and 130R Tat-2 vectors, showing maximal activities with 1000 ng activator plasmids that were 20–40% the level of the Tat-1 86R vector. Similar results as those shown in Figure 3 were obtained with the HIV-1 LTR in several independent experiments in all three cell lines. We conclude from these experiments that exon2 of the Tat-2 protein does not contribute to transactivation of the HIV-1 LTR under the conditions of our experiments.

Results of transactivation assays for the HIV-2 LTR-CAT reporter indicated that the 99R Tat-2 protein was less active than the 130R protein for activation of the cognate LTR (Fig. 3D–F). With 1000 ng of activator plasmid, the 99R vector was 28%, 61%, and 74% as active as the 130R vector in HeLa, COS, and Jurkat cells, respectively. Similar results as those shown in Figure 3 were obtained with the HIV-2 LTR in several independent experiments. We note, however, that in some experiments in Jurkat cells where transfection efficiencies were low, the 99R and 130R vectors displayed similar transactivation activities. We conclude from these experiments that exon2 of the Tat-2 protein can contribute modestly to transactivation of the HIV-2 LTR under the conditions of our experiments.

Effect of exon2 on Tat-2 binding *in vitro* to TAR-1 and TAR-2 RNAs

The *in vivo* transactivation results presented in Figure 3 suggested that exon2 may influence the binding affinity of Tat-2 for TAR-2

Figure 5. Gel shift assays with TAR-1 RNA. (A). The indicated molar ratios of 86R Tat-1, 99R Tat-2, and 130R Tat-2 proteins were incubated with 32 P-TAR-1 RNA (+1 to +80) and analyzed on 6% native polyacrylamide gel. (B). The % of free (unbound) 32 P-labeled TAR-1 RNA probe (40 nM input) in the gel at each Tat protein concentration was measured with a Betagen Betascope 603 scanner.

RNA but not TAR-1 RNA. To examine this possibility, we used a gel-shift assay to directly compare the *in vitro* binding affinities of purified 99R and 130R Tat-2 proteins to TAR-1 and TAR-2 RNAs. For comparative purposes, we also analyzed binding affinities of the 86R Tat-1 protein. Typical preparations of the 86R Tat-1, 99R Tat-2, and 130R Tat-2 proteins used in gel shift assays are shown in Figure 4. We first analyzed binding of the 86R Tat-1 protein and the 99R and 130R Tat-2 proteins to 32 P-labeled TAR-1 RNA (Fig. 5A). The 86R Tat-1 protein formed a weakly detectable complex at a 3:1 molar ratio of protein/RNA. We previously demonstrated that under these assay conditions the Tat-1 protein does not form a gel shift complex with a mutant TAR-1 RNA containing a deletion of the three nucleotide pyrimidine bulge at +23 to +25 (31). In contrast to the 86R Tat-1 protein, the 99R and 130R Tat-2 proteins did not form gel shift complexes with TAR-1 RNA at molar ratios up to 20:1. The amount of 32 P-labeled TAR-1 RNA remaining as free probe in the gel shown in Figure 5A was quantified (Fig. 5B). For the 86R Tat-1 protein, a molar ratio of approximately 20:1 was required to shift 50% of the TAR-1 RNA probe. For both the 99R and 130R Tat-2 proteins, molar ratios of >20:1 were required to shift 50% of the TAR-1 RNA probe. It is likely that this low affinity *in vitro* for TAR-1 RNA is involved in the relatively low transactivation activity of both the 99R and 130R

Figure 6. Gel shift assays with TAR-2 RNA. (A). The indicated molar ratios of Tat-1 (86R), Tat-2 (99R), and Tat-2 (130R) were incubated with 32 P-TAR-2 RNA (+1 to +123) and analyzed on 6% native polyacrylamide gel. (B). The % of free (unbound) 32 P-labeled TAR-2 RNA probe (40 nM input) in the gel at each Tat protein concentration was measured with a Betagen Betascope 603 scanner.

Tat-2 proteins for the HIV-1 LTR in HeLa, COS, and Jurkat cell lines (Fig. 3).

We next analyzed the binding of Tat proteins to a phenotypically wild type TAR-2 RNA called wt_x (Fig. 6). The wt_x TAR-2 element contains a substitution of three nucleotides at +10 to +12 that create an *Xba*I restriction enzyme site that facilitated the insertion of mutant TAR-2 RNA elements into HIV-2 LTR-CAT reporter plasmids (18). For the 86R Tat-1 protein, a single Tat-1/TAR-2 RNA complex was detected at a 1:1 molar ratio of protein to RNA (Fig. 6A). As the amount of Tat-1 protein was increased in the binding assay, a second distinct Tat-1/TAR-2 RNA complex was detected (molar ratios >3:1). An analysis of mutant RNAs containing deletion of bulge1, bulge2, or bulge1+2 in TAR-2 indicated that the slowly migrating complex formed between the 86R Tat-1 protein and wt_x TAR-2 RNA likely represents a single RNA with Tat-1 proteins bound simultaneously at each bulge binding site (Rhim and Rice, manuscript submitted).

When the 99R and 130R Tat-2 proteins were analyzed with TAR-2 RNA in the gel shift assay, complicated patterns of

complexes were observed as reported previously for the 99R protein (18,31,47). The complexes observed with TAR-2 RNA at molar ratios of Tat-2 proteins <10:1 are specific, relatively high-affinity protein/RNA complexes. They are not observed when 32 P-labeled anti-sense TAR-2 RNA is analyzed in the binding assay (31). They are not competed when an excess of unlabeled tRNA is included in the binding assay, but they are efficiently competed when an excess of wild type TAR-1 or TAR-2 RNA is included in the assay (31). Importantly, they are not observed when the di-nucleotide bulges at nucleotides +27,28 and +62,63 in stem-loop1 and stem-loop2, respectively, are deleted (18). Additionally, when two contiguous arginines in the 99R Tat-2 basic domain are mutated to alanine, these complexes are no longer observed in the gel shift assay (47). The complexes observed at a molar ratio of 20:1 in Figure 6A may represent non-specific binding of Tat-2 to the 32 P-labeled TAR-2 RNA, as at this high protein concentration, complexes can be observed with TAR-2 RNA containing deletions of both di-nucleotide bulges in stem-loop1 and stem-loop2 (18).

Protein/TAR-2 RNA complexes formed by the 130R protein appeared to migrate slightly more slowly than those formed by the 99R protein; this is likely the result of the larger molecular mass of the 130R protein. For the 99R and 130R Tat-2 proteins, protein/RNA complexes were first observed at molar ratio of 3:1 and 1:1, respectively (Fig. 6A). In contrast to the binding by the 86R Tat-1 protein, none of the protein/TAR-2 RNA complexes appeared preferentially at low protein concentrations for either the 99R or 130R Tat-2 protein.

For the Tat-1 protein, 50% of the TAR-2 RNA probe was bound at a molar ratio of approximately 10:1 (Fig. 6B). For the 99R Tat-2 protein, 50% of the TAR-2 RNA probe was also bound at a molar ratio of approximately 10:1. For the 130R Tat-2 protein, 50% of the TAR-2 RNA probe was bound at a molar ratio of approximately 5:1. These data indicate that the 130R Tat-2 protein possesses a higher binding affinity for TAR-2 RNA than does the 99R protein. This observation offers an explanation for the higher transactivation activity of the 130R Tat-2 protein than the 99R protein for the HIV-2 LTR (Fig. 3).

Relative transactivation activities of GAL4/Tat-1 and GAL4/Tat-2 fusion proteins

To characterize the activation domain of the Tat-2 protein and determine if exon2 contributes to the activation domain, we compared the transactivation activities of various fusions of Tat-2 to the yeast GAL4 DNA binding domain. GAL4/Tat fusion proteins are able to activate synthetic promoters containing GAL4 DNA binding sites (26,27). The affinity of GAL4/Tat fusions for GAL4 DNA binding sites in these artificial promoters should be equal and, therefore, the interactions of the Tat-2 moiety of these fusions with the cellular transcription machinery can be compared. We previously presented evidence that a GAL4 fusion to the 99R (exon1) Tat-2 protein was several-fold more active on such artificial promoters than a GAL4 fusion to the 86R Tat-1 protein (31).

To investigate whether exon2 of the Tat-2 protein can contribute to the activation domain, we analyzed fusions of GAL4 to the 130R Tat-2 protein and the 99R Tat-2 protein. For comparative purposes, we also analyzed transactivation properties of fusions of GAL4 to the 86R (exon1+2) Tat-1 protein, 72R (exon1) Tat-1 protein, and the activation domain alone of Tat-1 (called 48Δ). To characterize the Tat-2 activation domain, we focused on amino acid residues 1 through 77 in the Tat-2 protein

Figure 7. Transactivation of GAL4/Tat fusion proteins *in vivo*. (A). The synthetic promoter based upon HIV-1 LTR core sequences (−83 to +25 with TAR RNA element deleted; ref. 27) was co-transfected into HeLa cells along with 0, 10, 100, or 1000 ng of the indicated GAL4/Tat activator plasmids and a β -galactosidase expression plasmid. CAT expression was measured at 48 h post-transfection and was normalized to β -galactosidase expression and total protein amounts. (B). The indicated HIV LTR reporter plasmids (5 μ g) were transfected into HeLa cells with 1 μ g of GAL4/Tat activator plasmids and a β -galactosidase expression plasmid. CAT expression was measured at 48 h post-transfection and was normalized to β -galactosidase expression and total protein amounts.

(see Fig. 1), as the analogous region of the Tat-1 protein (residues 1 through 48) comprises that protein's activation domain (26,27). We therefore analyzed a GAL4 fusion to the first 77 residues of Tat-2 (called 77 Δ), as well as two GAL4 fusions in the 77 Δ protein background that contain amino terminal deletions encompassing residues 8 through 33 (called Δ 8/33) and 8 through 47 (called Δ 8/47).

We assayed transactivation activities of the GAL4/Tat fusions for a synthetic promoter containing GAL4 DNA binding sites located 5' to the core HIV-1 LTR sequences (deleted for the TAR element). The synthetic promoter [called G6(HIV-1)(-83)(-TAR); ref. 27] was co-transfected into HeLa cells with increasing amounts of GAL4/Tat expression plasmids (Fig. 7A). For the Tat-1 fusions, the GAL4/86R, GAL4/72R, and GAL4/48 Δ fusions activated the reporter plasmid and produced 3,230, 5,360, and 19,468 CAT units, respectively, with 1000 ng plasmid. This result suggests that truncation of Tat-1 before the basic domain increases the protein's ability as a GAL4 fusion to interact with the transcription machinery. This result agrees with a previous study that reported that a GAL4 fusion of the first 48 residues

of Tat-1 is a more potent activator of such artificial promoters than a fusion of the full-length (exon1+2) Tat-1 protein (26). We obtained similar transactivation results as those shown in Figure 7A using another synthetic promoter (26) containing GAL4 DNA binding sites located 5' to the adenovirus E1B core promoter (data not shown).

For Tat-2 fusions, the GAL4/130R and GAL4/99R proteins were equivalent in their ability to activate the CAT reporter and produced 9,335 and 9,217 CAT units, respectively, with 1000 ng plasmid. This result suggests that exon2 does not contribute to the Tat-2 activation domain. The GAL4/77 Δ protein was similar in transactivation activity to the GAL4/130R and GAL4/99R proteins with 10 and 100 ng input plasmid, but was only approximately 50% as active with 1000 ng input plasmid. This result indicates that unlike the Tat-1 protein, truncation of the Tat-2 protein just preceding the basic domain does not increase the transactivation function of a GAL4 fusion.

The GAL4/ Δ 8/33 fusion was reduced in the ability to transactivate the CAT reporter at all input plasmid amounts; it was 17% as active as the GAL4/130R and GAL4/99R proteins

at 1000 ng input plasmid. The GAL4/ Δ 8/47 fusion was inactive in the ability to transactivate the CAT reporter at all input plasmid amounts; it was 0.6% as active as the 130R and 99R fusions with 1000 ng input plasmid. These results imply that residues 8 through 33, although not absolutely required, do contribute to the Tat-2 activation domain. Deletion of residues 8 through 47 abolished function of the GAL4 fusion, indicating that the region between residues 34 to 47 are required for the activation domain function. It is not clear, however, whether these segments of the Tat-2 amino terminus are required for efficient interaction with the transcription machinery, or are simply required for the accumulation of stable protein. Our previous immunoprecipitation analysis of the expression levels of the Δ 8/33 and Δ 8/47 proteins (not as GAL4 fusions) suggested that these mutations result in unstable proteins (52). In this study, the Δ 8/47 protein expressed from the CMV vector was not detected in immunoprecipitation experiments (data not shown). The Δ 8/33 and Δ 8/47 proteins can be expressed, however, as stable proteins in *Escherichia coli* (51). It is possible that GAL4 fusions containing the Δ 8/33 and Δ 8/47 proteins are unstable, and this is the explanation for their deficiencies in transactivation of the synthetic promoter.

The GAL4/Tat proteins were also assayed for their ability to activate the natural HIV-1 and HIV-2 LTRs (Fig. 7B). In these transactivation experiments, the GAL4/Tat proteins function by binding through the Tat basic domain to TAR RNAs transcribed from the viral LTRs. 1 μ g of the GAL4 parent vector or GAL4/Tat fusion plasmid were co-transfected with an HIV-1 or HIV-2 LTR-CAT reporter plasmid. For the HIV-1 LTR, the GAL4/86R and GAL4/72R proteins displayed the highest transactivation functions. The GAL4/48 Δ protein was unable to stimulate expression of the HIV-1 LTR, as it does not contain the Tat-1 basic domain required for binding to TAR-1 RNA. The GAL4/Tat-2 99R and 130R fusions displayed several-fold lower transactivation than the GAL4/86R and GAL4/72R fusions for the HIV-1 LTR. This result was expected, as the natural Tat-2 proteins stimulate the HIV-1 LTR relatively weakly (see Fig. 2; refs. 15,16,19,28). The GAL4/Tat-2 fusions in the 77 Δ background were greatly reduced for transactivation of the HIV-1 LTR (<6% activity of the 130R fusion), as these fusions do not contain the Tat-2 basic domain required for binding to TAR-2 RNA. For the HIV-2 LTR-CAT reporter, the GAL4/86R and GAL4/72R proteins displayed high transactivation functions (Fig. 7B). The GAL4/Tat fusion deleted for the basic domain (48 Δ , 77 Δ , Δ 8/33, and Δ 8/47) were unable to activate the HIV-2 LTR. GAL4/99R and GAL4/130R fusions resulted in 5,812 and 15,848 CAT units, respectively; this result was expected, as the natural 130R protein is more active than the natural 99R protein for the HIV-2 LTR in HeLa cells (Fig. 3), probably because the 130R protein has higher binding affinity to TAR-2 RNA (Fig. 6).

DISCUSSION

We have presented evidence here that exon2 of the Tat-2 protein can contribute modestly to transactivation of the HIV-2 LTR but not HIV-1 LTR. In HeLa cells, exon2 increased Tat-2 transactivation of the HIV-2 LTR by three-fold, while in COS and Jurkat cells this value was less than two-fold. Our biochemical evidence supports these *in vivo* transactivation results; exon2 increases the binding affinity *in vitro* of Tat-2 to TAR-2 RNA. Interestingly, exon2 (ROD isolate) contains the sequence KKQKK

(residues 112–116) that is well conserved in different HIV-2 isolates (20). This patch of basic residues surrounding a glutamine is similar to a crucial sequence found in the Tat-1 RNA binding domain. The Tat-1 protein (HXB2 isolate) contains the sequence RRQRR at residues 52–56, and this sequence is involved in the direct binding of Tat-1 to TAR-1 RNA (11). It is possible, therefore, that this stretch of basic residues in exon2 of Tat-2 contributes to binding to TAR-2 RNA. A detailed mutagenesis study of exon2 will be required, however, to identify the amino acid residues in exon2 that contribute to binding to TAR-2 RNA. We note that data has been presented indicating that exon2 of Tat-2 can contribute significantly to transactivation of both HIV-1 and HIV-2 LTRs in Jurkat cells (24). Our results have shown no evidence that exon2 contributes to transactivation of the HIV-1 LTR (Fig. 2). The reasons for the different transactivation results obtained here and the previous study are not known, but may be the consequence of variations in experimental conditions.

Using GAL4 fusions and a synthetic promoter containing GAL4 DNA binding sites, we have examined and compared the activation domains of the Tat-1 and Tat-2 proteins (Fig. 7). For Tat-1, a GAL4 fusion containing the first 48 amino terminal residues appears to interact more efficiently with the cellular transcription apparatus than does a fusion with the full length 86R (exon1+2) or 72R (exon1) proteins. This finding implies that the Tat-1 activation domain may be partially masked in the natural 86R or 72R proteins, and an interaction with TAR-1 RNA or a cellular co-factor may be required *in vivo* to fully expose the activation domain. In this regard, we have observed that a cellular protein kinase specifically binds *in vitro* more efficiently to a Tat-1 protein truncated after residue 48 than to the 86R or 72R Tat-1 protein (51; Herrmann and Rice, manuscript submitted). Additionally, our recent results have indicated that this kinase activity is able to hyperphosphorylate the carboxyl terminal domain (CTD) of the largest subunit of RNA polymerase II.

For Tat-2, a GAL4 fusion containing the entire 99 residues of exon1 was as active in transactivation of the synthetic promoter as a fusion containing the 130 residues of exon1+2, suggesting that exon2 is not a component of the Tat-2 activation domain. In contrast to Tat-1, truncation prior to the Tat-2 basic domain at residue 77 does not increase the transactivation property of a GAL4 fusion for the synthetic promoter (Fig. 7A). Thus in the context of the natural Tat proteins, the activation domains of the Tat-1 and Tat-2 proteins are likely to exist as significantly different structures. Consistent with this proposal is our observation that the Tat-1 protein possesses a compact and perhaps globular structure, while the Tat-2 protein possesses an extended and non-globular structure (42). Future insights into the activation domains of Tat-1 and Tat-2 will require the identification of the cellular co-factor(s) that interacts with the activation domains and mediates Tat transactivation.

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