The basic RNA-binding domain of HIV-2 Tat contributes to preferential trans-activation of a TAR2-containing LTR

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ABSTRACT

Human immunodeficiency viruses HIV-1 and HIV-2 encode a Tat protein that trans-activates the respective viral genome through RNA targets (TAR1 and TAR2). Tat-1 and Tat-2 have considerable homology. However, an interesting biological observation has been that Tat-1 activates the HIV-1 and HIV-2 LTRs equally while Tat-2 activates the former, in comparison to the latter, poorly. Here, we present evidence that it is the TAR2 RNA target together with the basic RNA-binding protein domain of Tat-2 that dictate this non-reciprocity in trans-activation.

INTRODUCTION

Human immunodeficiency virus (HIV) encodes for two viral trans-activators, Tat and Rev. Tat functions primarily at the level of viral transcription while Rev participates in the post-transcriptional processing of viral mRNAs (see review 1). An important concept that has emerged from mechanistic studies on both Tat and Rev is that of trans-regulation through the use of RNA sequence elements. Specifically, Tat is known to recognize a small nascent viral leader RNA (TAR; 2, 3, 4) while Rev recognizes a larger structured RNA (RRE; 5) which is found in all maturely transcribed unspliced messages.

tat is an essential gene for HIV (6). Closely related, though non-identical, Tat proteins are found for the two HIV serotypes (HIV-1 and HIV-2; 7, 8, 9, 10). Although the linear size of HIV-2 Tat (Tat-2; e.g. 130 aa for ROD isolate) is larger than HIV-1 Tat (Tat-1; 86 aa for HXB2 isolate), an alignment of the amino acids sequences between the two proteins shows conserved domains (e.g. cysteine rich region, basic region; 11). Interestingly, at the RNA target level, the HIV-2 trans-activation responsive (TAR2) element is also larger than its TAR1 counterpart (approximately 50 nucleotides for TAR1 and 120 nucleotides for TAR2; 7, 12, 13). An examination of the respective secondary structures reveals that TAR1 configures into a single stem-bulge-loop hairpin while TAR2 adopts a complex bifurcated RNA structure that consists of two separable hairpins (7, 12, 13).

Previous functional studies using HIV-1 or HIV-2 Tat and the respective LTRs have revealed a non-reciprocity in trans-activation. Specifically, while Tat-1 trans-activates LTR-1 and

LTR-2 with equivalent efficiencies, Tat-2 activates its homologous LTR roughly five to ten times better than its heterologous counterpart (7–9, 11–13). A mechanistic explanation for the latter observation is not apparent, although we previously had suggested that the second hairpin in TAR2 may confer a better 'fit' to Tat-2 (12).

To better understand potential interactions between domains in Tat proteins and TAR RNAs, we made chimeric forms of Tat that exchanged discrete regions between Tat-1 and Tat-2. When we assayed 9 such permutations in Tat, we correlated the presence of the Tat-2 basic domain (amino acids 66 to 88) with functional preference for TAR2. Parallel biochemical studies confirmed that a peptide encompassing the Tat-1 basic domain (amino acids 37 to 61) bound TAR1 ($K_d = 16\text{nM}$) and TAR2 ($K_d = 20\text{nM}$) RNAs *in vitro* equally while the corresponding Tat-2 peptide bound TAR-2 ($K_d = 16\text{nM}$) three fold better than TAR1 ($K_d = 48\text{nM}$). These findings indicate that the non-reciprocity in Tat-2 trans-activation is, in part, determined by its basic RNA-binding domain and by the manner in which this domain interacts with RNA targets.

MATERIALS AND METHODS

Plasmid Constructions

Plasmid pLTR-CAT is as previously described (3). It contains the complete HIV-1 U3 region, the TAR1 sequence, and a CAT reporter gene. pLTR1/TAR2-CAT was derived from pLTR-CAT using PCR splicing (14). The HIV-1 TAR sequence (TAR1, nucleotides +1 to +58; +1 being the mRNA cap site) in pLTR-CAT was replaced with the exact HIV-2 TAR sequence (TAR2, nucleotides +1 to +156). pLTR2-CAT contains the HIV-2 LTR placed upstream of the CAT reporter (7).

pYC4 and pYC5 are plasmids driven by the SV40 early promoter that express the first coding exon of the *tat* gene from HIV-1 (SF2) or HIV-2 (Rod), respectively. The protein sequences expressed are shown in figure 1A. To construct plasmids that express Tat-1/Tat-2 chimeric proteins, the first exon of Tat was divided into A, B, C, and D domains (Fig. 1A). Domain A encompasses codon 1 to 21 of Tat-1 (A1), or codon 1 to 49 of Tat2 (A2). Domain B is from codon 22 to 36 for Tat-1 (B1), and from codon 50 to 65 for Tat-2 (B2). Domain C is from codon 37 to 61 for Tat-1 (C1), and codon 66 to 88 for Tat-2 (C2).

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Domain D consists of the rest of the first exon of either Tat-1 (D1 codon 62 to 72) or Tat-2 (D2 codon 89 to 97). Amino acid sequences corresponding to each domain are shown in figure 1A. Exchanges between *tat-1* and *tat-2* were accomplished using PCR splicing (14). The various final combinations are shown in figure 2. We also replaced codon 49 to 57 of pYC4 (Tat-1) and codon 78 to 84 of pYC5 (Tat-2) with a sequence that encode for nine consecutive arginine residues (R9). These plasmids are designated as pTat1/R9 (pA1B1R9D1) and pTat2/R9 (pA2B2R9D2).

To make radiolabeled TAR1 or TAR2 probes, synthetic oligonucleotides that span HIV-1 TAR (TAR1, +1 to +58) or HIV-2 TAR (TAR2, +1 to +156) were ligated into the *HindIII* and *BamHI* sites of pGEM4Z. These plasmids are designated as pYC48 and pYC1. All constructions were sequenced directly to verify correct identity.

Cell culture, transfection, and RNA and protein analysis

HeLa cells were maintained in Dulbecco's minimal medium containing 10% fetal bovine serum (Gibco). Transfections were carried out using the calcium phosphate method (15). HeLa cells (0.5×10^6 cells) were transfected with 1 μ g of a plasmid that expresses either wild type or chimeric Tat protein driven by the SV40 early promoter and 1 μ g of either pLTR-CAT, or pLTR1/TAR2-CAT, or pLTR2-CAT. CAT assays were done as described previously (16). Each transfection was performed a minimum of three times. For RNA analysis, HeLa cells (0.5×10^7 cells) were co-transfected with 5 μ g of plasmid that expresses either wild type or chimeric Tat protein and 5 μ g of either pLTR-CAT or pLTR1/TAR2-CAT and 5 μ g of a human β -globin expressing plasmid (used as an internal transfection control). Cellular RNA was isolated by the hot phenol method, and S1 nuclease analysis was performed as previously described (3).

RNA-peptide filter binding assays

Synthetic peptides that contain amino acid residues 37 to 62 of HIV-1 Tat (strain HXB2), or amino acid residues 66 to 91 of HIV-2 Tat (strain Rod), or the Tat2 basic domain containing insertion of 9 arginines, or a mutated Tat1 basic domain peptide

were used (Fig. 5E). Uniformly labeled TAR1 and TAR2 RNA probes were made by *in vitro* transcription using T7 polymerase in the presence of [32 P]-CTP (3000 Ci/mmol, Amersham) according to manufacturer's protocol (Promega). Probes were gel purified prior to use.

Binding reactions were performed by incubation on ice for 20 minutes. 50 μ l of binding reaction contains $1 \times$ binding buffer (10 mM Tris-HCl, pH7.5, 70 mM NaCl, 0.2 mM EDTA, and 0.01% NP-40) (17), 1 nM of labeled TAR1 or TAR2 probes (5,000 to 10,000 cpm), and varying amounts of Tat-1 or Tat-2 peptide. The reactions were then filtered onto nitrocellulose paper using a microfiltration apparatus (Bio-Rad). The filter paper was washed with $1 \times$ binding buffer five times. Bound probes were quantitated using a phosphorimager (Fuji).

RESULTS

The basic RNA binding domains of Tat1 and Tat2 contribute to specificity of trans-activation

It has been shown that the Tat protein of HIV-1 (Tat-1) can trans-activate with equal efficiencies either the HIV-1 LTR or the HIV-2 LTR (7–9, 11–13). However, the Tat protein of HIV-2 (Tat-2) preferentially trans-activates the HIV-2 LTR (7–9, 11–13). To determine which domain(s), if any, within Tat-1 or Tat-2 confers specificity to trans-activation, we exchanged corresponding regions between Tat1 and Tat2 (Fig. 1A and 2) and made 7 chimeric Tat proteins. To test these chimeric proteins appropriately, we also constructed a new reporter plasmid pLTR1/TAR2-CAT to be used in comparisons with pLTR-CAT (HIV-1 LTR) and pLTR2-CAT (HIV-2 LTR). pLTR1/TAR2-CAT differs from pLTR-CAT in that a TAR2 sequence exactly replaces the TAR1 sequence (Fig. 1C). Thus except for this singular change, the two reporters are otherwise isogenic and are both driven by the same U3. We, therefore, expect that differences between these two reporters in response to the same trans-activator must be due to the respective TARs and not to non-identities in the U3 sequences of HIV-1 versus HIV-2 (11).

Figure 1. Schematic representations of protein, peptide, and LTR-CAT constructs. (A) A comparative alignment of the amino acid sequences of the first exons of Tat-1 and Tat-2. For purposes of domain exchanges the first exon was divided into four portions: A (N-terminus), B (cysteine rich), C (basic), and D (auxiliary) generally in the manner suggested by Kuppuswamy et al. (32). (B) Amino acid sequences of Tat-1 and Tat-2 basic domains. Tat-1 or Tat-2 domain contains amino acids 37 to 62 or amino acids 66 to 91 from the respective protein. (C) Representations of pLTR-CAT and pLTR1/TAR2-CAT. pLTR1/TAR2-CAT is identical to pLTR-CAT (3) except that the TAR1 (cross-hatched) region (80 nucleotides) of pLTR-CAT was replaced by 157 nucleotides that contain the TAR2 (solid) sequence.

We assayed 11 different forms of Tat using the three LTR-CAT (pLTR-CAT, pLTR2-CAT, and pLTR1/TAR2-CAT) reporters. A typical trans-activation experiment is shown in figure 3A. The basal activities of pLTR-CAT, pLTR2-CAT and pLTR1/TAR2-CAT (Fig. 3A, Basal) were comparable, although in our assays the basal expression of pLTR1/TAR2-CAT was routinely 2 fold higher than either pLTR-CAT or pLTR2-CAT. When pLTR-CAT, pLTR2-CAT, or pLTR1/TAR2-CAT was trans-activated by Tat-1 (Fig. 3A, Tat1) or by Tat-2 (Fig. 3A, Tat2) we saw results compatible with previous findings (7–9, 11–13). Specifically, Tat-1 trans-activated pLTR-CAT, pLTR2-CAT and pLTR1/TAR2-CAT with similar folds over their respective basal activities (Fig. 3A, Tat1), while Tat-2 trans-activated either pLTR2-CAT or pLTR1/TAR2-CAT about 3 to 10 fold better than pLTR-CAT (Fig. 3A, Tat2). Thus, the mere presence of TAR2 in an otherwise HIV-1 LTR background reconstituted the preferential Tat-2 trans-activation of the entire HIV-2 LTR (compare lanes 1 to 1.2 to 2 for Tat2, Fig. 3A; see also ref. 7).

Because binding by Tat to TAR RNA is an essential prerequisite for trans-activation (17–21), we explored whether the RNA-binding domain of Tat-2 might be a determinant for this non-reciprocity. This was tested initially by comparing the activities of chimeric proteins A1B1C2D1 (Fig. 3A, A1B1C2D1) and A2B2C1D2 (Fig. 3A, A2B2C1D2). One should note that the former differs from wildtype Tat-1 only in its C2 domain while the latter deviates from Tat-2 in having a C1 domain. In co-transfections, we found that A1B1C2D1 trans-activated preferentially pLTR1/TAR2-CAT and pLTR2-CAT over pLTR-CAT (10 to 15 fold difference; Fig. 3A, see A1B1C2D1) while A2B2C1D2 trans-activated all three reporter plasmids comparably

(Fig. 3A, A2B2C1D2). This result suggests that the particular presence of the C2 (RNA-binding) domain markedly specified the preference for TAR2 RNA. The serotypic origin of the other domains (A, B, D) apparently had little influence on this specificity (see Fig. 3B). This was further verified using the Tat1/R9 and the Tat2/R9 expression vectors (Fig. 3A). These two proteins are serotypically different in the A, B, and D domains (Fig. 2) but share a common C domain that consists of 9 reiterated arginines which is capable of binding to TAR RNA (22). We found that their relative activation of pLTR-CAT, pLTR1/TAR2-CAT and pLTR2-CAT to be virtually identical (Fig. 3A; Tat1/R9, Tat2/R9). This finding is compatible with a pre-eminence of the C domain in determining specificity of LTR trans-activation.

We confirmed these results using other chimeric Tat constructions (Fig. 3B). In parallel with Tat-1, Tat-2, A1B1C2D1, A2B2C1D1, Tat1/R9, and Tat2/R9, 5 additional permuted forms of Tat were compared for trans-activation of pLTR-CAT versus pLTR1/TAR2-CAT. The results yielded a surprisingly strict correlation (Fig. 3B). We found that all proteins containing the Tat-1 basic domain (C1; e.g. Tat-1, A2B1C1D1, A1B2C1D1, A2B2C1D1, A2B1C1D2, A2B2C1D2) or R9 trans-activated pLTR-CAT slightly better than pLTR1/TAR2-CAT (Fig. 3B). Conversely, those containing the Tat-2 basic domain (C2; e.g. A1B1C2D1, A1B2C2D1) behaved like wild type Tat-2 (Fig. 3B). They uniformly activated pLTR1/TAR2-CAT more efficiently than pLTR-CAT (Fig. 3B). Thus there appears to be a slight relative preference for TAR1 conveyed by C1 and a greater relative preference for TAR2 conveyed by C2.

Substituting the RNA binding domain of Tat2 with nine arginine residues or with the Tat1 basic domain changes specificity of trans-activation

One interpretation of the above results is that the RNA-binding domain of Tat-2 is crucially responsible for the non-reciprocity in trans-activation. A particularly informative finding was the functional comparison between the Tat1/R9 and Tat2/R9 (Fig. 3A, 3B) expression vectors. Trans-activation results from these two proteins confirmed that, in distinction to the C2 domain, the 'neutral' 9 arginine domain does not significantly discriminate between TAR1 versus TAR2 (Fig. 3A, B).

We verified our CAT-assay results using S1 nuclease analyses of steady state RNAs produced from pLTR-CAT or pLTR1/TAR2-CAT (Fig. 4). Both plasmids were separately co-transfected into HeLa cells with Tat1 (Fig. 4A, lanes 4, 5), Tat2 (Fig. 4A, lanes 6, 7), pA2B2C1D2 (Fig. 4A, lanes 8, 9), pA1B1C2D1 (Fig. 4A; lanes 10, 11), pTat1/R9 (Fig. 4B, lanes 12, 13), or pTat2/R9 (Fig. 4B, lanes 14, 15). We analyzed the relative magnitudes of trans-activation by comparing the TAR1-containing versus the TAR2-containing reporter. TAR1 or TAR2 specific expression was measured using single stranded gene-specific probes that discriminate between the two RNAs. In these experiments, TAR1-containing RNAs were identified by an 80 nucleotide band protected from S1 digestion (see –I; Fig. 4 lanes 4, 6, 8, 10, 12, 14). Similarly, TAR2-specific signal was represented in a 163 nucleotide band (see –II; Fig. 4 lanes 5, 7, 9, 11, 13, 15). All assays also included a co-transfected human β -globin plasmid whose signal (– β , Fig. 4 lanes 4–15) was used to internally control for transfection efficiency. We quantitated the relative trans-activation in RNA using a Fuji phosphorimager (Fig. 4C). The S1 results agree with the findings from the CAT assays (Fig. 3). We found that Tat1 (Fig. 4A,

Figure 2. Nine chimeric proteins that contain different combinations of the A, B, C, and D domains from either Tat-1 or Tat-2; and two Tat proteins that contain a 9 arginine substitution for the C domain. The Tat-1 and Tat-2 A, B, C, and D domains are as shown in Fig. 1A. Different chimeric proteins were made by exchanging domains between Tat-1 and Tat-2. Protein Tat1/R9 or Tat2/R9 was made by replacing amino acids 49 to 57 (Tat-1) or amino acids 78 to 84 (Tat-2) with 9 arginine residues.

Figure 3. TAR-specific trans-activation correlates with the alternate presence in Tat of either the C1 or C2 basic domain. (A) Typical CAT activities from pLTR-CAT (TAR1; labeled as 1), or pLTR1/TAR2-CAT (labeled as 1.2), or pLTR2-CAT (labeled as 2) without trans-activator (Basal; 0.01%, 0.03%, 0.01% acetylation respectively), or after co-transfection with Tat-1 (Tat1; 14.8%, 27.4%; 17.2% acetylation), co-transfection with A2B2C1D2 (A2B2C1D2; 17.4%, 23.7%, 19.6% acetylation), or co-transfection with A1B1C2D1 (A1B1C2D1; 0.66%, 8.8%, 9.4% acetylation), or co-transfection with Tat-2 (Tat2; 1.4%, 17.8%, 21.0%), or co-transfection with Tat1/R9 (Tat1/R9; 14.1%, 18.1%, 12.9% acetylation), or co-transfection with Tat2/R9 (Tat2/R9; 26.4%, 28.2%, 33.5%). Basal activity of pLTR1/TAR2-CAT is approximately 2 fold higher than either pLTR-CAT or pLTR2-CAT. This was verified based on longer exposures of the thin layer chromatogram and on repeated experiments using higher amounts of extract. Cm, 14 C-chloramphenicol; Ac, acetylated 14 C-chloramphenicol. (B) Normalized histogram of the preference by the 11 forms of Tat protein for TAR1 versus TAR2. Results are the average of 3 experiments in addition to that shown in panel A. For normalization the ratio of trans-activation of Tat-1 for TAR1- or TAR2-containing LTR was set as 1. Values shown are mean $\pm$ 1 SD (error bar).

lanes 4, 5), A2B2C1D2 (Fig. 4A, lanes 8, 9), Tat1/R9 (Fig. 4B, lanes 12, 13), and Tat2/R9 (Fig. 4B, lanes 14, 15) all activated the TAR1-containing reporter comparably or slightly better (see corrected ratios of 0.9 to 0.6; see TAR2/TAR1*, Fig. 4C) than the TAR2-containing reporter. However, A1B1C2D1, and Tat2 (the two proteins with a C2 domain) activated the TAR2 containing reporter considerably better than the TAR1 reporter (see TAR2/TAR1* ratios of 12 and 5.3; Fig. 4C). This is consistent with a role of C2 in determining a preference for TAR2 RNA.

Differences in the relative binding affinities of Tat-1 and Tat-2 basic peptides for TAR1 or TAR2 RNAs

The above findings suggest that the basic RNA binding domains of Tat-2 determines non-reciprocity in trans-activation. We investigated whether this functional observation could be correlated with the relative affinity of recognition by the C2 domain for either TAR1 or TAR2 RNA. We, therefore,

measured the binding affinities of the Tat-1 and the Tat-2 basic peptides (Fig. 5E) to either TAR1 or TAR2 RNA (Fig. 5). As additional comparisons, we also measured the affinity of the R9 domain for either RNA (Fig. 5C) and the affinity of a mutated Tat peptide for the TAR1 RNA (Fig. 5D).

The two wildtype Tat peptides used for our assays contain either the RNA binding domain from Tat-1 or Tat-2 (Fig. 1B). We assayed peptide-RNA complex formation using a filter binding approach (23). In our binding studies, we maintained radiolabeled TAR1 or TAR2 RNA at a constant level and increased input peptide over a 1 to 250 nM range (Fig. 5). Saturation binding curves were obtained for Tat-1 peptide binding to TAR1 or TAR2 RNAs (Fig. 5A), for Tat-2 peptide binding to the same two probes (Fig. 5B), for Tat2/R9 peptide binding to TAR1 or TAR2 (Fig. 5C), and for the control mutant peptide which was substituted in four out of six arginine residues in the Tat1 basic domain (mTat1; Fig. 5D). We found that the Tat-1 basic peptide bound TAR1 and TAR2 RNA with virtually

Figure 4. S1 nuclease analysis of RNAs expressed from pLTR-CAT or pLTR1/TAR2-CAT after trans-activation by Tat-1 (lanes 4, 5), Tat-2 (lanes 6, 7), A2B2C1D2 (lanes 8, 9), A1B1C2D1 (lanes 10, 11), Tat1/R9 (lanes 12, 13), or Tat2/R9 (lanes 14, 15). Panel A contains experiments that were performed simultaneously; panel B represents experiments done on a different day from panel A. Lanes 1, 2, and 3 show the sizes of intact input LTR/TAR1 probe (Probe 1, 261 nucleotides; lane 1), input LTR/TAR2 probe (Probe 2, 349 nucleotides; lane 2), and input β -globin probe (Probe G, 640 nucleotides; lane 3). Lanes 4, 6, 8, 10, 12, and 14 contain S1-protected signals from RNA species expressed from pLTR-CAT. pLTR-CAT was trans-activated by Tat-1 (lane 4), Tat-2 (lane 6), A2B2C1D2 (lane 8), or by A1B1C2D1 (lane 10), or by Tat1/R9 (lane 12), or by Tat2/R9 (lane 14). pLTR-CAT specific RNA is represented by an 80 nucleotide protected signal ($-I$). Lanes 5, 7, 9, 11, 13, and 15 show protected RNA expressed from pLTR1/TAR2-CAT after trans-activated by Tat-1 (lane 5), Tat-2 (lane 7), A2B2C1D2 (lane 9), by A1B1C2D1 (lane 11), by Tat1/R9 (lane 13), or by Tat2/R9 (lane 15). Twice as much trans-activator (10 μ g) and reporter (10 μ g) plasmids were used in the A1B1C2D1 experiments. pLTR1/TAR2-CAT specific RNA is represented by a protected 163 nucleotide band ($-II$). A human β -globin plasmid was also co-transfected as an internal control. β -globin specific transcript (β) is represented by a 210 nucleotide S1-protected band. All S1 protected bands were quantitated using a phosphorimager (Fuji). Panel C shows the quantitation of the TAR1 (*) and TAR2 (°) protected bands in arbitrary Fuji units from each experiment. TAR2/TAR1 represents uncorrected ratios; TAR2/TAR1* represents ratios corrected for differences in 32 P-dCTP incorporated into the TAR2 versus TAR1 protected bands. All S1 experiments were performed at least twice; results differ by less than 15% from experiment to experiment.

Figure 5. Comparisons of the binding affinities of Tat-1 peptide, or Tat-2 peptide, or Tat2/R9, or a mutant Tat1 (mTat1) peptide for TAR1 and TAR2 RNA. Binding assays were performed using 32 P-radiolabeled TAR1 or TAR2 RNA probes in 50 μ l of binding reactions containing increasing amounts of Tat-1 (panel A), or Tat-2 (panel B), or Tat2/R9 (panel C), or mTat1 peptide (panel D). Panel E shows the actual amino acid sequences of the four peptides. Saturation binding curves were plotted either using a linear (top) or a semi-log (bottom) abscissa scale. Calculated K_d of Tat-1 for TAR1 is 16 nM; Tat-1 for TAR2 is 20 nM; Tat-2 for TAR1 is 48 nM; Tat-2 for TAR2 is 16 nM; Tat2/R9 for TAR1 is 12 nM; Tat2/R9 for TAR2 is 14 nM. mTat1 showed no measurable binding to TAR1 (panel D). K_d was calculated based upon the concentration of peptide necessary to achieve 50% saturation binding of probe.

indistinguishable affinities (Fig. 5A, upper and lower panels), the corresponding Tat-2 peptide clearly bound TAR1 less well than it bound TAR2 (Fig. 5B, upper and lower panels). Based upon 50% saturation binding of RNA probe, the K_d s for Tat-1/TAR1 and Tat-1/TAR2 were 16 nM and 20 nM, respectively. The K_d s for Tat-2/TAR1 and Tat-2/TAR2 were 48 nM and 16 nM. Interestingly, the corresponding K_d s for Tat2/R9 (12 nM for TAR1; 14 nM for TAR2; Fig. 5C) is consistent with its functional inability to discriminate between

TAR1 versus TAR2 reporters (Fig. 3, 4). mTat1, as expected, failed to bind TAR1 RNA (Fig. 5D).

DISCUSSION

It has been shown previously that Tat-2 trans-activates its homologous HIV-2 LTR 5 to 10 times more efficiently than the heterologous HIV-1 LTR (7–9, 11–13). This observation stands in distinction to findings that the HIV-1 Tat protein trans-activated

both viral LTRs equally (7–9, 11–13). In earlier studies, we and others (12, 13) have suggested that differences in the TAR RNA structures between HIV-1 and HIV-2 might explain, in part, this non-reciprocity. We had observed that the addition of a second stem-loop structure to TAR1 modified it into a better trans-activation responsive element for Tat-2 (12). Our current study extends this observation and demonstrates that the Tat-2 basic domain (amino acids 66 to 88) defines an optimal interaction with TAR2 RNA. Our evidence indicate that it is the relative affinity of this interaction (compared to the corresponding interaction with TAR1) that is ultimately reflected in the preferential trans-activation of the HIV-2 LTR.

As yet, the exact mechanism by which Tat trans-activates the HIV LTR is not known (see review, 1). For HIV-1, it has been suggested that the trans-activator protein is brought to the LTR promoter by a nascent TAR RNA tether (24–28). Subsequently, Tat is envisioned to interact with promoter and/or upstream factors, thereby influencing transcriptional machinery (26–29). Thus, inefficiencies in Tat-2 trans-activation of the HIV-1 LTR could be due either to sub-optimal recognition of TAR1 RNA and/or to sub-optimal interaction(s) with HIV-1 promoter/upstream elements. Relevant to the latter point is the fact that the HIV-1 and HIV-2 U3s are neither identical in sequence (11) nor in function (30, 31).

We explored the two possible explanations for inefficient Tat-2 trans-activation of LTR1 by constructing chimeric reporters and chimeric Tat proteins (Figs. 1, 2). In making the pLTR1/TAR2-CAT plasmid, we generated a hybrid gene that contained the HIV-1 promoter/upstream elements with a HIV-2 TAR RNA leader. The fact that pLTR1/TAR2-CAT responded to Tat-2 in a manner identical to that previously described for the complete HIV-2 LTR (Fig. 3A; ref. 7) suggested that, at the target level, TAR2 (and not promoter/upstream elements) is the primary determinant for Tat-2 specificity.

The protein domain(s) within Tat-2 responsible for preferential trans-activation of the HIV-2 LTR was characterized using 11 different forms of Tat protein (Fig. 3B). We designed our chimeric exchanges based upon extensive mutagenesis results from other investigators (32–35). Although we did not make all possible combinatorial swaps between Tat-1 and Tat-2, our results were sufficient to implicate the C2 domain as dictating a preference for a TAR-2 containing-LTR (Fig. 3). This specificity persists even in a Tat protein that except for C2 is identical to Tat-1 (see A1B1C2D1, Fig. 3). The results derived from substituting a 9 arginine domain for C1 or C2 (Fig. 3B) were also informative. Our interpretation here is that a basic peptide domain is absolutely necessary to maintain trans-activation function; however, a homogeneously charged domain (9 arginines) cannot distinguish between TAR1 versus TAR2. It is the particular arrangement of the amino acids in the C2 domain that determines a preference for TAR2. Our findings also allowed us to conclude that the A, B, and D regions, of Tat-1 and Tat-2 appears to be functionally equivalent and can be qualitatively interchanged. In making the C domain exchanges, we have noticed that, in certain instances (e.g. A1B1C2D1; Fig. 3A), while qualitative trans-activation (i.e. preference for either TAR1 or TAR2) is preserved quantitative trans-activation (i.e. magnitude of trans-activation) is reduced (by 2 fold in Fig. 3A; compare A1B1C2D1 to Tat1 or Tat2). Thus although the C domain may largely determine target preference, the context in which it is found influences the overall efficiency of function of the whole protein.

When synthetic peptides containing the Tat-1 or Tat-2 basic domains were challenged with TAR1 or TAR2 RNA, we found complementing biochemical results. In these assays, Tat-1 peptide bound to TAR1 or TAR2 with indistinguishable affinities (Fig. 5A) which is consistent with its functional phenotype. In contrast, the Tat-2 peptide showed a small but clearly discriminatory difference between the two probes (Fig. 5B). The K_d of Tat-2 was calculated to be 48 nM for TAR1 and 16 nM for TAR2. Interestingly the R9 domain, which failed to distinguish between a TAR1 or a TAR2 reporter in functional assays (Fig. 3), also bound TAR1 and TAR2 RNAs equally well (Fig. 5C). Thus, these *in vitro* findings suggest sub-optimal binding of Tat to RNA as one explanation for the functionally inefficient trans-activation of TAR1 by Tat-2. We do not exclude the possibility that other factors might influence this interaction intracellularly.

It has been proposed that the efficiency of intracellular Tat function impacts on the replicative host range and cytopathic properties of HIV (36). In this regard, the kinetics of virus replication after entry into host cells are important for the relative viability of one virus strain compared to another. Because HIV undergoes multiple rounds of replication during productive infections, small differences that occur during each round accrue into large overall selective advantages/disadvantages. One can envision how increasing or decreasing Tat trans-activation efficiencies even by small amounts could have important evolutionary impact on a particular HIV. In this study we describe differences in trans-activation between Tat proteins in the range of 5 to 15 fold. While these magnitudes are numerically small, understanding the mechanistic reasons for these effects may help elucidate some of the selective pressures that occur during viral infections.

Since the completion of this study, work from Elangovan et al. (J. Virol., 1992) has also implicated the basic domain of Tat-2 in determining preferential trans-activation of the HIV-2 LTR.

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