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Sequence-specific interaction of Tat protein and Tat peptides with the transactivation-responsive sequence element of human immunodeficiency virus type 1 *in vitro*

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ABSTRACT **Bacterially expressed Tat protein of human immunodeficiency virus type 1 binds selectively to short RNA transcripts containing the viral transactivation-responsive element (TAR). Sequences sufficient for Tat interaction map to the distal portion of the TAR stem–loop. We show that critical sequences for Tat binding are located in the single-stranded "bulge," but no requirement for specific "loop" sequences could be demonstrated. TAR RNA competed for complex formation, and TAR mutants exhibited up to 10-fold reduced affinity for Tat. Synthetic peptides containing the basic region of Tat bound selectively to TAR RNA and exhibited the same sequence requirements and similar relative affinities for mutant TAR RNA as the intact protein. These results suggest that Tat contains a small RNA-binding domain capable of recognizing TAR and implicate functional relevance for direct Tat–TAR interaction in transactivation.**

The human immunodeficiency virus type 1 (HIV-1) Tat protein is essential for transactivation of viral gene expression (see refs. 1 and 2 for recent reviews). Tat exerts its effect via a cis-acting target sequence, termed the transactivation-responsive element (TAR), located immediately downstream from the site of transcription initiation in the viral long terminal repeat (1, 2). Tat acts via novel mechanisms to stimulate HIV-1 gene expression. It has been shown to increase mRNA synthesis both at the level of initiation of transcription and at the level of stabilization of elongation by RNA polymerase II (1, 2). Additionally, posttranscriptional effects have been detected (1, 2). Accumulated evidence indicates that TAR is recognized as RNA and encompasses a 57-base-long stem–loop structure located at the 5′ end of all HIV-1 transcripts (1, 2). Mutational analysis of TAR has indicated that sequences in both the TAR "loop," which contains the conserved pentanucleotide sequence 5′-CUGGG-3′, and "bulge" sequence are critical for transactivation (3–6).

Transactivation by Tat may be mediated through specific recognition of the TAR structure by Tat itself or by a host cell protein(s) (7–9). *In vitro* experiments (10) demonstrating the recognition and specific binding of purified Tat to TAR RNA suggest that direct binding of Tat to TAR may be involved in transactivation by the viral protein. The current studies were undertaken to map the critical regions of TAR and Tat required for specific interaction *in vitro*.

MATERIALS AND METHODS

Expression of Tat in *Escherichia coli*. The coding sequence of the HIV-1 strain BRU Tat gene was cloned and expressed in the T7 vector pET8c (11). Tat was purified by a modification of previously described methods (12). A 0.5-liter culture was pelleted and resuspended in 50 mM Tris·HCl/200 mM NaCl/2 mM EDTA, pH 8.0, at 4°C. Phenylmethylsulfonyl fluoride and L-1-tosylamido-2-phenylethyl chloromethyl ketone were added to 2 mM and 1 μM, respectively, and the cells were disrupted in a Stansted press. The lysate was centrifuged at 10,000 × *g* for 45 min and the supernatant was subjected to precipitation with 35% saturated ammonium sulfate. The pellet was resuspended in 10 ml of 50 mM Tris·HCl/20 mM NaCl/2 mM EDTA/6 M guanidine hydrochloride, pH 8.0, and dialyzed overnight against the same buffer. Aliquots (2 ml) were flushed with N_2, adjusted to 20% (vol/vol) 2-mercaptoethanol, and incubated at 40°C for 90 min. The reduced material was injected onto an RPC-HR 10/10 semipreparative column. Tat was eluted with a 0–100% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Tat-containing fractions were lyophilized and stored under N_2 at −20°C. For binding experiments, Tat was dissolved in 20 mM Hepes, pH 8.0/10 mM 2-mercaptoethanol at ≈1 mg/ml.

Tat–RNA Binding. Binding reactions were carried out in 15 μl of 25 mM Tris·HCl, pH 8.0/50 mM NaCl/1 mM $MgCl_2$/5 mM spermidine/0.5 mM dithiothreitol, 5% (vol/vol) glycerol containing ≈40 pg of radiolabeled probe RNA, 0.4 μg of yeast tRNA, and 1 μg of sheared salmon sperm DNA (10). Incubation was for 20 min at 30°C, after which the binding reactions were loaded on a 4% polyacrylamide gel (50:1 acrylamide/*N,N′*-methylenebisacrylamide) in 0.5× TBE buffer (45 mM Tris base/45 mM boric acid/0.5 mM EDTA, pH 8.3) and electrophoresed at 100 V for 4 hr at 0–4°C. Binding reaction mixtures generally contained 300 ng of purified Tat. Tat–TAR complexes were stable in the presence of 10 μg of tRNA (a 25,000-fold molar excess relative to the radiolabeled TAR), although 0.4 μg of tRNA was generally sufficient to remove background binding seen with nonspecific probes.

Tat Peptides. Peptide synthesis was carried out as described (13). Peptide purity was confirmed by analytical HPLC and identity by a combination of amino acid analysis, mass spectrometry, and sequence analysis. Peptide–TAR binding reactions were carried out under identical conditions to Tat binding and, unless specified, reaction mixtures contained 100 ng of peptide, 1 μg of yeast tRNA, and 1 μg of salmon sperm DNA.

TAR Transcripts and Mutagenesis of TAR. A TAR template plasmid, pC3-6, that allowed the first 76 bases of HIV mRNA to be transcribed from the T7 polymerase promoter in

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Abbreviations: HIV, human immunodeficiency virus; TAR, transactivation-responsive element; TAR^{WT}, wild-type TAR; IRE, iron-responsive element.

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pGEM-3 (Promega) was constructed. A *Bgl* II–*Hin*dIII fragment (nucleotides 473–531 of HIV clone HXB2) was inserted between the *Eco*RI and *Hin*dIII sites of pGEM-3 by using a synthetic oligonucleotide to reconstruct the first 18 nucleotides (nos. 455–472) of authentic HIV mRNA and generate an *Eco*RI site adjacent to the cap site at nucleotide 455. T7 transcripts therefore contained the additional nucleotides 5′-GGG-AGA-CCG-GAA-TTC-3′ from pGEM-3 at their 5′ end upstream from the HIV cap site. TAR transcripts were synthesized by standard protocols using T7 polymerase and were radiolabeled by incorporation of [α-^{32}P]UTP (14). Template DNA was linearized with *Hin*dIII for run-off transcription to generate a 91-nucleotide-long RNA.

Construction of TAR Mutants. TAR sequences in pC3-6 were modified by using standard techniques (15) to substitute synthetic oligonucleotide duplexes for wild-type sequences between unique restriction enzyme sites. Plasmids from randomly chosen transformant colonies were sequenced to confirm the presence of the desired mutations.

RESULTS

Mutational Analysis of TAR. The HIV-1 *tat* gene product, Tat, was expressed in *E. coli* and purified (*Materials and Methods*; data not shown). The protein was >95% pure and was biologically active, being capable of transactivating the HIV long terminal repeat when added to the supernatant of tissue culture cells in the presence of chloroquine (ref. 16; data not shown). Conditions were established (*Materials and Methods*) for selective binding of purified Tat to a 91-nucleotide RNA transcript containing the (+)-sense TAR sequence. A computer-generated structure predicted that this RNA would form a stem–loop structure characteristic of functional TAR (Fig. 1*A*). To identify the structural features of TAR involved in Tat binding *in vitro*, a series of plasmids encoding mutant TAR templates were constructed (Fig. 1*B*), and the resulting TAR transcripts were used in gel shift analysis with purified Tat (Fig. 2). Binding of Tat to wild-type TAR (TAR^{WT}) was evident as a prominent retarded complex composed of a major and minor (slower migrating) species.

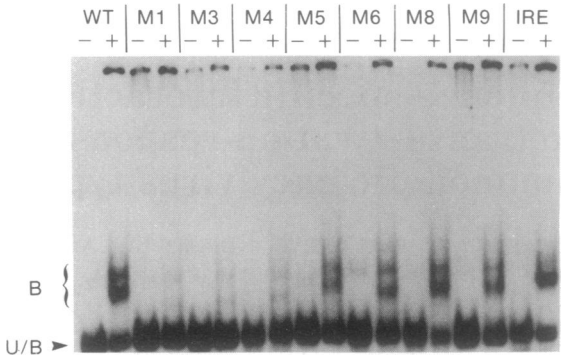

FIG. 2. Binding of Tat to TAR and mutant TAR transcripts. TAR transcripts were incubated with purified Tat (see *Materials and Methods*). Complexes were resolved in a nondenaturing 4% acrylamide gel. Binding reactions were with the indicated wild-type (WT) and mutant TAR transcripts (Fig. 1) in the absence (−) or presence (+) of *E. coli*-synthesized Tat. B, bound; U/B, unbound.

Binding of Tat to a mutant TAR transcript (M1) containing the inverse complement of TAR nucleotides 20–40 (the distal portion of the stem–loop) was barely detectable under these assay conditions. Mutant M1 differed from TAR^{WT} only in the position and sequence of the single-stranded loop and bulge. Similar to the other TAR variants tested, M1 contained identical vector and HIV sequences flanking the predicted stem–loop secondary structure and had a comparable Gibbs free energy value (−35.3 kcal/mol for TAR^{WT} versus −32.5 kcal/mol for M1 TAR) (17). The weak complexes formed with M1 TAR were disrupted by a 2- to 3-fold increase in nonspecific competitor RNA (data not shown). Nevertheless, Tat retained some preferential affinity for this molecule compared to the nonspecific competitor present in great excess. In contrast, specific Tat–TAR^{WT} complexes still formed in the presence of a 2.5 × 10^5-fold molar excess (a 30-fold increase) of nonspecific competitor RNA (data not shown). These results indicated that the RNA sequence or

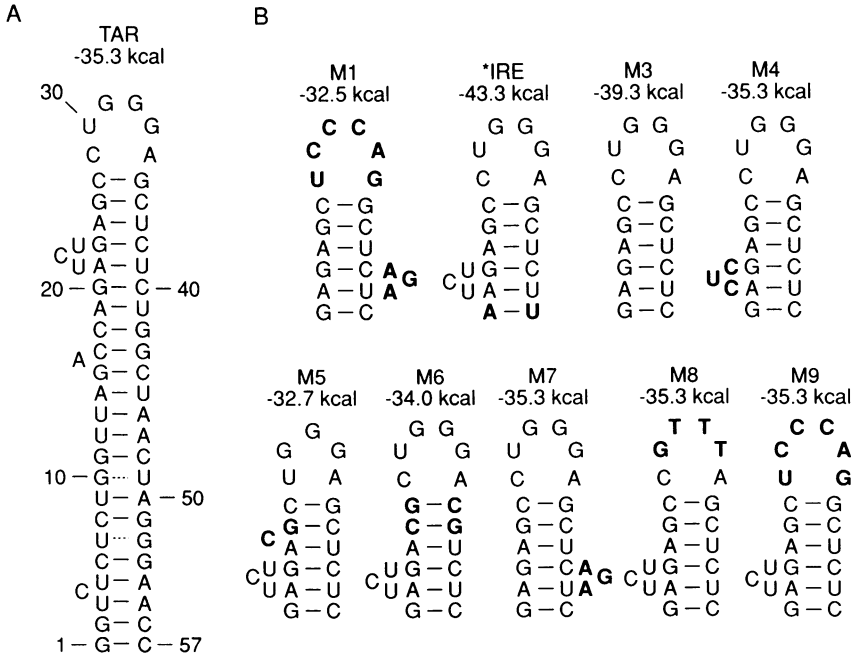

FIG. 1. HIV-1 TAR and TAR mutants. (*A*) HIV-1 TAR. The 57-base RNA stem–loop is defined as TAR. Nucleotide notation throughout designates nucleotide position 1 of TAR as residue 455 in the complete HXB2 sequence (GenBank). (*B*) Distal portion (nucleotides 20–40) of TAR mutants M1–M9. Nucleotides differing from wild type are indicated in bold; predicted secondary structures and free energy values (kcal/mol) were calculated by the program of Zuker and Stiegler (17). Stem sequences of IRE TAR flanking the sequence shown were replaced by those from the stem of the iron-responsive element (IRE) of ferritin mRNA (18).

secondary structure in the distal portion of the TAR stem–loop is critical for high-affinity Tat binding *in vitro*.

A second mutant, IRE TAR (Fig. 1*B*), has bases 21–39 of the TAR^{WT} stem–loop structure, including the single-stranded bulge and loop, flanked by heterologous sequences derived from the IRE of ferritin mRNA (18). The IRE sequences form a stable stem structure with no primary sequence homology to TAR. Tat was capable of binding strongly to the IRE TAR transcript (Fig. 2), indicating that the 19 bases composing the distal portion of the TAR stem–loop are sufficient for binding. This result is consistent with mutational analyses, which have shown that the primary nucleotide sequence of the stem plays no role in TAR-mediated transactivation by Tat as long as the potential secondary structure of the transcript is maintained (3, 4, 6, 19).

Mutations in the distal portion of TAR (Fig. 1*B*) including the single-stranded bulge were therefore analyzed for their effect on Tat binding (Fig. 2). M3 TAR, which had the unpaired bases of the bulge deleted, showed little Tat binding. In addition, a mutant (M4) that retained the bulge but had transition mutations at each position within it was also unable to complex effectively with Tat. Two additional mutants were constructed and assayed for Tat binding: M5, which had a 2-base mutation at the base of the loop to disrupt the secondary structure of the terminal loop sequence; and M6, which contained an additional compensatory mutation predicted to reestablish secondary structure similar to TAR^{WT}. Reduced binding to each of these mutant transcripts was observed compared with the TAR^{WT} transcript (Fig. 2 and below). These data demonstrate the major importance of the single-stranded bulge for Tat recognition *in vitro* and are consistent with the functional importance of these sequences *in vivo* (4–6).

Two TAR transcripts mutated in the loop (M8 and M9; Fig. 1*B*) were tested in Tat binding; both M8, which is functionally inactive *in vivo* (4), and M9 bound Tat efficiently, suggesting that loop sequences play no role in Tat–TAR interaction *in vitro*.

Tat Peptides Bind TAR *in Vitro*. By analogy with other eukaryotic transcriptional activators, we surmised that a separable functional domain of Tat might be responsible for TAR binding (20). Peptide $P_{37–72}$ was capable of binding TAR RNA *in vitro* (Fig. 3*A*, lanes 2 and 3) under conditions identical to those used for Tat (lanes 4 and 5). Binding was specific, since $P_{37–72}$ was also unable to bind to M1 TAR (lanes 7 and 8).

To determine whether the $P_{37–72}$–TAR interactions mimic those of Tat, the same TAR mutations were assayed for their effect on binding of peptide $P_{37–72}$ in the gel mobility-shift assay (Fig. 3*B*). The binding phenotype of all the TAR variants mirrored that observed in binding experiments with Tat protein. Notably, binding activity was retained by IRE TAR (lane 4) and loop mutants M8 and M9 (lanes 6 and 8), whereas the bulge mutants M3 and M4 (lanes 14 and 16) showed no binding. TAR mutants M5 and M6 (lanes 10 and 12) were each partially defective in binding activity, forming a weak complex with faster mobility than that formed with TAR^{WT}. The significance of this faster migrating complex is not understood, but it is clear that alteration of sequences at the base of the loop prevents formation of a complex with the mobility of that seen with TAR^{WT}.

Relative Affinity of Tat for TAR^{WT} and TAR Mutants. To determine the relative affinity of Tat or $P_{37–72}$ for different TAR structures, the ability of mutant TAR molecules to compete for Tat or $P_{37–72}$ binding to TAR^{WT} was measured. Binding reactions containing increasing quantities of unlabeled competitor were set up and the fraction of probe complexed in the presence of competitor was quantitated. The data from these experiments are summarized in Table 1. TAR^{WT}, IRE TAR, and M9 TAR competed equivalently for

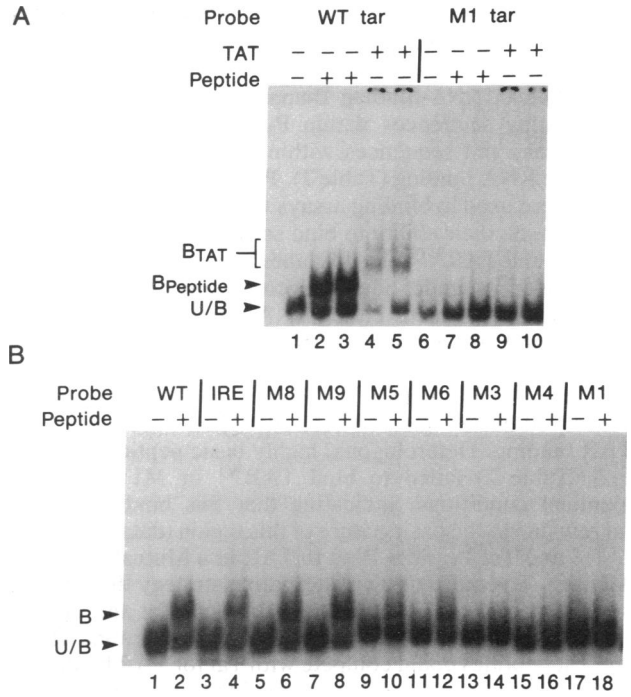

FIG. 3. Binding of Tat peptide to TAR or mutant TAR RNA. (*A*) Binding of Tat or peptide $P_{37–72}$ with TAR^{WT} (lanes 1–5) or M1 TAR RNA (lanes 6–10) analyzed by gel mobility shift. Samples contained RNA alone (lanes 1 and 6), RNA with 50 ng of $P_{37–72}$ in the presence of 0.4 μg (lanes 2 and 7) or 1 μg (lanes 3 and 8) of tRNA, and RNA with 300 ng of Tat in the presence of 0.4 μg (lanes 4 and 9) or 1 μg (lanes 5 and 10) of tRNA. (*B*) Binding of $P_{37–72}$ to mutant TAR transcripts. The indicated TAR transcripts were incubated in the absence (−) or presence (+) of 50 ng of $P_{37–72}$. B, bound RNA; U/B, unbound TAR RNA.

Tat or $P_{37–72}$, indicating that loop sequences and stem sequences do not substantially affect Tat or $P_{37–72}$ affinity *in vitro*. However, competition with mutants M3 and M4 indicated a reduced affinity for TAR molecules mutated in the bulge. These two mutants competed for Tat or $P_{37–72}$ with efficiencies only 10-fold lower than TAR^{WT}. A mutant TAR molecule deleted of nucleotides 23–32 (M10) also competed poorly for Tat and $P_{37–72}$, even though it did not contain the intact loop or bulge sequences of TAR^{WT}. Consistent with direct binding analysis, mutants M5 and M6 appeared to have intermediate phenotypes, exhibiting a small but reproducible reduction in affinity for both Tat (0.26 and 0.4) and $P_{37–72}$ (0.15

Table 1. Competition by TAR mutants

Competitor	Relative efficiency	
	Tat	$P_{37–72}$
TAR^{WT}	1.0	1.0
IRE TAR	1.1	1.0
M9	0.8	1.6
M3	0.09	0.1
M4	0.12	0.12
M5	0.26	0.15
M6	0.4	0.22
M10	0.045	0.125

Values indicate the relative affinity for competitor compared to TAR^{WT}, calculated from the concentration of competitor required for 50% competition compared to concentration of TAR^{WT} required for equivalent competition. Complexes were separated by electrophoresis and quantitated by direct Cerenkov counting of excised bands. Percent bound values were normalized by considering that fraction bound in the absence of added competitor (50–70% of input counts) as "100% bound."

and 0.22). These data support the direct binding data as described above and suggest that the single-stranded bulge plays a critical role in selective Tat/P_{37-72}–TAR binding.

Location of RNA-Binding Domain in Tat. Short peptides representing sequences within P_{37-72} were synthesized to further map the sequences within Tat that are involved in selective RNA binding (Table 2). Peptides P_{43-72}, P_{48-72}, and P_{58-72} were used in binding assays with TAR^{WT} and M1 TAR to determine their ability to bind selectively. Both P_{43-72} and P_{48-72} bound TAR^{WT} (Fig. 4*A*, lanes 3 and 4) but failed to bind M1 transcripts under identical conditions (lanes 8 and 9). P_{58-72} did not bind to either RNA transcript in this assay (lanes 5 and 10). Sequences between amino acids 48 and 72 are therefore sufficient for recognition and binding to TAR, and amino acids within the region 48–58, containing the conserved basic region GRKKKRRQRRR, are essential for TAR binding. Heterologous, highly basic peptides (P_{365} and P_{364}, Table 2) failed to bind TAR^{WT} or M1 TAR under identical conditions, indicating that this binding was not merely due to the basic nature of this region (data not shown).

Tat and Tat Peptides Bind to TAR in a Mutually Exclusive Manner. To confirm by an alternative strategy that the same sequences within TAR are bound by both Tat and the small peptides, an experiment was performed to determine whether the Tat peptides could compete with Tat for TAR binding. An initial control experiment confirmed that heterologous basic peptides fail to compete for TAR binding (data not shown). Competition assays using P_{37-72}, P_{43-72}, and P_{48-72} revealed that comparable concentrations of these peptides were able to disrupt the Tat–TAR complex and form complexes of different mobility, characteristic of peptide–TAR complexes (Fig. 4*B*, lanes 4–7, 9–12, and 14–17). No evidence for higher-order complexes was detected. indicating that both Tat and the short peptides containing the basic region of Tat bind in a mutually exclusive manner to TAR. This result is consistent with the peptides and Tat binding to the same or to overlapping sequences on TAR, a conclusion supported by the mutational analysis.

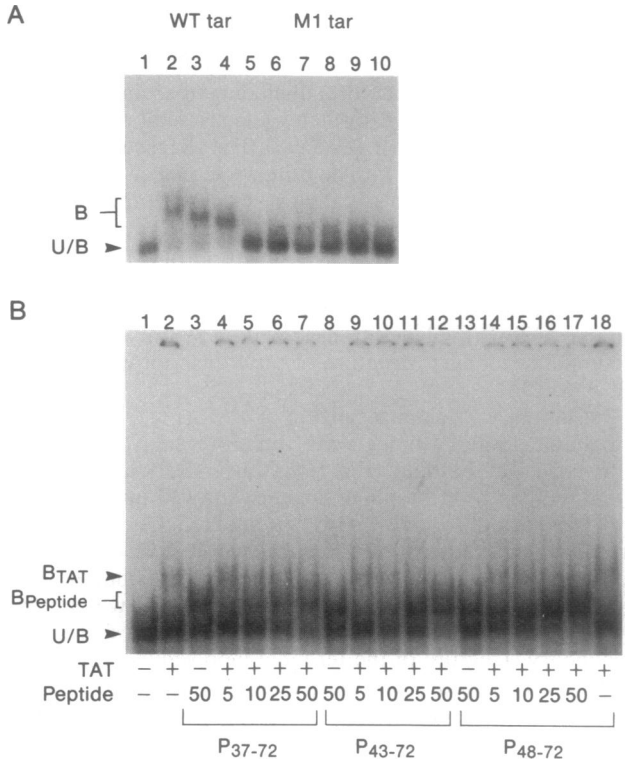

FIG. 4. Tat peptide–TAR interaction. (*A*) Binding of Tat peptides with TAR (lanes 1–5) or M1 TAR RNA (lanes 6–10) assayed by gel shift. Lanes 1 and 6, no peptide; lanes 2 and 7, 100 ng of P_{37-72}; lanes 3 and 8, 100 ng of P_{43-72}; lanes 4 and 9, 100 ng of P_{48-72}; lanes 5 and 10, 100 ng of P_{58-72}. (*B*) Competition for TAR by Tat peptides. Competition binding mixtures (see *Materials and Methods*) contained TAR RNA and 300 ng of Tat alone (lanes 2 and 18) or with the addition of competitor peptide P_{37-72} (lanes 4–7), P_{43-72} (lanes 9–12), or P_{48-72} (lanes 14–17). Mixtures contained 5 ng (lanes 4, 9, and 14), 10 ng (lanes 5, 10, and 15), 25 ng (lanes 6, 11, and 16), or 50 ng (lanes 7, 12, and 17) of the relevant competitor peptide. In some instances, peptide was incubated with TAR in the absence of Tat (lane 3, P_{37-72}; lane 8, P_{43-72}; lane 13, P_{48-72}). The positions of Tat–TAR (B_{TAT}) and peptide–TAR ($B_{Peptide}$) complexes are indicated; U/B, unbound TAR.

DISCUSSION

Our results confirm that recombinant Tat protein specifically binds TAR transcripts *in vitro*. In addition, a 36-amino acid peptide derived from the Tat sequence is capable of selective interaction with TAR^{WT}. Mutational analysis of TAR indicated that sequences important for both Tat–TAR and P_{37-72}–TAR interactions map to the distal 19 bases of the RNA stem–loop (nucleotides 21–39). Examination of TAR transcripts with mutations in this region, by direct binding and competition studies, indicated that the region of primary importance for Tat and P_{37-72} binding is the single-stranded bulge region of TAR (nucleotides 22–24). In contrast, mutations in the loop did not affect Tat–TAR interaction and a mutation at the base of the loop, similar to one previously shown to reduce Tat binding (10), had an intermediate effect on binding of Tat and P_{37-72}. Mutations within the bulge and at the base of the loop have been shown to have dramatic effects on Tat transactivation (3–6). This correlation of *in vitro* binding and *in vivo* functional data suggests that the defect in these TAR mutants may be caused by inefficient recognition and binding of Tat. It is interesting that although the affinity of Tat for TAR has been reported to be high (10), the difference in relative affinity of Tat for functional and nonfunctional TAR structures is relatively small. The affinity of Tat for a bulge-deleted TAR is only 10-fold lower than for TAR^{WT}.

Since TAR loop mutants are functionally defective (ref. 3 and unpublished data) yet retain wild-type ability to bind Tat

Table 2. Structures of synthetic peptides

Peptide	Sequence	Binding*
Tat-based		
P_{37-72}	CFTTKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQ	+
P_{43-72}	LGISYGRKKRRQRRRPPQGSQTHQVSLSKQ	+
P_{48-72}	GRKKRRQRRRPPQGSQTHQVSLSKQ	+
P_{58-72}	PPQGSQTHQVSLSKQ	−
Heterologous†		
P_{365}	RLRPGGKKKYKLKC	−
P_{364}	LGDPKPKKNKKPKNPC	−

*Binding assays were carried out with TAR and M1 TAR; +, specific binding to TAR.
†The P_{365} and P_{364} peptides contain sequences from HIV-1 Gag protein and herpes simplex virus glycoprotein B, respectively.

in vitro, Tat binding cannot be sufficient for TAR function *in vivo*. Loop sequences may be involved in additional molecular interactions *in vivo*. Indeed a HeLa cell TAR-binding factor, which exhibits a somewhat higher affinity for wild-type TAR compared to one mutated in the loop, has recently been identified (9). Such a factor might modulate the affinity of Tat for TAR *in vivo*; alternatively, TAR function might be dependent on the simultaneous binding of more than one factor, each with specificity for different elements in TAR. We note that recent observations by Southgate *et al.* (21) preclude an essential role for host sequence-specific TAR-binding proteins in Tat transactivation. Their experiments demonstrated that a chimeric Tat–Rev protein is capable of transactivating an HIV promoter in which TAR has been replaced by the HIV-1 Rev-responsive element (RRE), and support a model in which TAR is a direct binding site for the Tat protein. Nevertheless, a host factor responsible for modulating specific Tat–TAR interaction may be irrelevant when Tat is tethered to nascent RNA through Rev–RRE interactions.

The short peptides that bind TAR encompass the conserved basic motif (GRKKKRRQRRR, residues 48–58) that contains the nuclear localization signal of Tat. Tat molecules with mutations in this region are inactive in transactivation and appear to be located primarily in the cytoplasm of the cell (19, 22). Interestingly, Hauber *et al.* (23) described a mutation within this region that resulted in reduced transactivation yet did not prevent localization of the protein to the nucleus. This phenotype would be predicted for mutants capable of entering the nucleus but incapable of productively interacting with TAR and supports the potential importance of Tat–RNA interaction in Tat function *in vivo*.

Specific binding of short peptides to TAR indicates that the RNA-binding domain of Tat is functionally distinct and separable from the cysteine-rich and amino-terminal domains defined by mutational analysis (19, 22–25). An essential component of the binding domain is the basic region, which also appears to function in nuclear localization. A role for arginine-rich basic amino acid motifs in sequence-specific recognition of RNA has been described for the N proteins of λ and related phages (26). An arginine-rich domain of N proteins is responsible for recognition of *nut* sites in phage RNA transcripts (26). Remarkably, *nut* sites are comprised, in part, of a short RNA stem–loop structure reminiscent of the HIV TAR. The precise functional relevance of Tat–TAR interaction in TAR-mediated transactivation and the putative role of host TAR-binding factors await further study.

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