Binding of a Host Cell Nuclear Protein to the Stem Region of Human Immunodeficiency Virus Type 1 *trans*-Activation-Responsive RNA

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Human immunodeficiency virus type 1 (HIV-1) transcription is regulated by both viral and host cell factors. Although the viral *trans*-activator protein, Tat, and its *cis*-responsive element, *trans*-activation-responsive (TAR) RNA, have been identified and characterized, the mechanism of HIV-1 transcriptional regulation has not been satisfactorily described. Whereas Tat is necessary to activate transcription, additional factors, derived from the host cell, are important in regulating HIV-1 transcription. To identify such host cell-specific factors, we used an RNase protection mobility shift assay and UV cross-linking to detect a 140-kDa HeLa cell nuclear protein that binds specifically to TAR RNA. By extensive mutational analysis, we determined that the binding of this protein is dependent on both the sequence and the structure of the TAR RNA stem region. Other groups have shown that the production of prematurely terminated transcripts from the HIV-1 promoter is also dependent on the sequence and structure of the TAR RNA stem. This correlation with our results suggests that the TAR RNA stem-binding protein is involved in the production of prematurely terminated transcripts from the HIV-1 promoter and in the regulation of HIV-1 gene expression.

Human immunodeficiency virus type 1 (HIV-1) transcription is regulated by viral and cellular factors, which act on viral *cis*-responsive elements to either induce or repress viral gene expression (for reviews, see references 4 and 18). The HIV-1 *trans*-activator protein, Tat, interacts with its *cis*-responsive element, *trans*-activation-responsive (TAR) RNA, to increase greatly the synthesis of full-length viral transcripts. TAR forms a stable stem-loop structure and is located at nucleotide positions +1 to +57 within the 5' untranslated region of all viral mRNAs (3, 14) (Fig. 1). Although Tat binds to the pyrimidine bulge and the immediate flanking base-paired sequences of TAR RNA *in vitro* (6, 19, 23), this interaction is not sufficient to activate transcription *in vivo* (6, 19). For example, Tat binds to TAR RNAs with mutations in the loop; however, these mutations do not support Tat-mediated *trans* activation *in vivo*. This suggests that host cell factors are involved in regulating the HIV promoter. Indeed, Barry et al. (1) have shown that levels of HIV-1 transcriptional activity vary up to 1,000-fold between cell types. Further, studies of rodent-human cell hybrids demonstrate that whereas certain rodent cells, such as CHO cells, either do not support Tat-mediated *trans* activation or do so poorly, Tat-mediated *trans* activation is increased markedly in rodent-human hybrid cells which retain human chromosome 12 (9, 16). Additionally, Marciniak et al. have identified a 68-kDa HeLa cell nuclear protein which binds to the loop of TAR and increases *trans* activation *in vitro* (12, 13). These observations demonstrate that host cell factors play an important role in regulating HIV transcription.

To identify such host cell-specific factors, we used an RNase protection mobility shift assay and UV cross-linking to detect a 140-kDa HeLa cell nuclear protein that binds specifically to TAR RNA. Through extensive mutational analysis, we determined that the binding of this protein is

dependent on both the sequence and the structure of the TAR RNA stem region. Interestingly, other groups have shown that the production of prematurely terminated transcripts from the HIV-1 promoter is dependent on the sequence and structure of the TAR RNA stem (11, 17, 20, 21). This correlation with our results suggests that the TAR RNA stem-binding protein (SBP) is involved in the production of prematurely terminated transcripts and in the regulation of HIV-1 gene expression.

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MATERIALS AND METHODS

Plasmid constructs and *in vitro* transcription. An *in vitro* transcription-cassette vector, pT7pUC19, was constructed by cloning the bacteriophage T7 RNA polymerase promoter (5'-TAATACGACTCACTATA-3') into the *EcoRI*-*KpnI* site of pUC19 (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Synthetic TAR and TAR mutant oligodeoxynucleotides (synthesized on an Applied Biosystems 380B DNA synthesizer) were then cloned into the *KpnI* and *HindIII* sites of pT7pUC19. The plasmids were linearized at *HindIII* and transcribed *in vitro* with [α -³²P]CTP or [α -³²P]UTP (>400 Ci/mmol) (Amersham, Arlington Heights, Ill.) essentially as described by the supplier of the transcription kit (Promega, Madison, Wis.). After treatment with RNase-free DNase (1 U/ μ g of DNA, 37°C, 15 min), the transcripts were purified on an 8% bisacrylamide-7 M urea gel, eluted in 0.5 M ammonium acetate-1 mM EDTA-0.1% sodium dodecyl sulfate (SDS), extracted once in phenol-chloroform and once in chloroform, ethanol precipitated, and resuspended to 10⁵ cpm/ μ l in water. The secondary structures and free energies of the mutant TAR RNAs were predicted with an RNA-folding computer program (26).

RNase protection mobility shift assay. The RNase protec-

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FIG. 1. Secondary structure of HIV-1 TAR RNA positions +1 through +57. TAR RNA has been divided into sections as follows: four stem regions (region I, bases 5 to 9 and 50 to 54; region II, bases 10 to 15 and 44 to 49; region III, bases 17 to 21 and 39 to 43; and region IV, bases 25 to 28 and 35 to 38), a 3-base pyrimidine bulge ($U_{22}C_{23}U_{24}$), a six-member loop (CUGGGA), and unpaired nucleotides (C_4 and A_{16}).

tion mobility shift assay is a modification of one used to demonstrate sequence-specific binding of the HIV-1 Rev protein to the Rev-responsive element RNA (25). The reaction mixture consisted of 50% (vol/vol) nuclear extract (5 to 10 mg/ml in buffer D, which consisted of 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.9], 100 mM KCl, 20% [vol/vol] glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol [5]), 1.5 mM MgCl₂, and 10⁵ cpm of radiolabelled transcript, in a total volume of 10 µl. The reaction mixture was incubated for 15 min at room temperature, and then 1 µl of a solution of RNases A (1 mg/ml) and T₁ (5,000 U/ml) was added and the incubation was continued for 15 min. One microliter of loading dye (97% glycerol, 0.1% xylene cyanol, 0.1% bromophenol blue) was added, and the reaction mixture was loaded on a 4% native polyacrylamide gel (acrylamide-bisacrylamide [80:1]–50 mM Tris–50 mM glycine [unadjusted pH ~ 8.8]) and run at 100 V for 5 h or until the xylene cyanol had migrated approximately 12 cm. The gel was then dried and exposed to X-ray film overnight at –70°C with an intensifying screen. Competition assays were performed by incubating the nuclear extract with nonradiolabelled competitor RNA for 10 min prior to adding the ³²P-labelled TAR RNA.

UV cross-linking. The binding reactions were performed as described above. After the RNase treatment, the samples were transferred to ice and irradiated for 30 min with shortwave UV light at 8 mW/cm² (measured at the source) from a distance of 5 cm. An equal volume of 2× SDS sample buffer was then added, and the samples were placed in a 100°C water bath for 5 min and separated on an SDS–6% polyacrylamide gel. The dye front was allowed to migrate off the gel to remove ³²P-labelled degradation products. The gel was then fixed in 40% methanol–10% acetic acid, dried, and exposed to X-ray film at –70°C with an intensifying screen.

FIG. 2. RNase protection mobility shift assay. An RNase-resistant RNA-protein complex was formed when ^{32}P -labelled TAR RNA was incubated with HeLa nuclear extract (NE) (arrow, lane 2) but was not formed in the absence of nuclear extract (lane 1). Complex formation was abolished when RNase was added prior to the addition of nuclear extract (lane 3), when 0.1% SDS was present (lane 4), and when the nuclear extract was treated with 2 μg of proteinase K (lane 5).

RESULTS

A HeLa cell nuclear protein binds to TAR RNA. To investigate whether a HeLa cell nuclear protein forms a specific complex with TAR RNA, radiolabelled TAR RNA was incubated with HeLa nuclear extract, and then nonspecifically bound RNA was digested with RNases A and T₁. The resulting complex was resolved on a high-ionic-strength, low-cross-linked, native polyacrylamide gel. A distinct band of altered electrophoretic mobility was observed when TAR RNA was incubated with HeLa nuclear extract (Fig. 2, lane 2). This complex was not formed when the RNase was added prior to the addition of nuclear extract (Fig. 2, lane 3). This control shows that the protected RNA (lane 2) is not an inherently RNase-resistant fragment bound nonspecifically to protein or hybridized to nucleic acid in the nuclear extract. The complex was also not formed in the presence of 0.1% SDS (Fig. 2, lane 4) or when the extract was preincubated with proteinase K (Fig. 2, lane 5). The absence of complex formation under these conditions demonstrates the RNA-protein nature of the complex. This TAR RNA protein binding activity was also detected in HUT-78 nuclear extract (data not shown).

TAR RNA deletion analysis. To determine the sequences of TAR RNA essential to the formation of this RNA-protein complex, several deletion mutants of TAR were constructed and tested in the gel mobility shift assay (Fig. 3 shows the sequences and Fig. 4 shows the predicted secondary structures and stabilities of all TAR RNA mutants). In TM12, the stem regions III (bases 17 to 21 and 39 to 43) and IV (bases 25 to 28 and 35 to 38) and the pyrimidine bulge ($U_{22}C_{23}U_{24}$)

	1	10	20	30	40	50
WT TAR	GGUCUCUCUGGUUAGACCAGAU	CUGAGCCUUGGAGCUCUCUGG	CUAACUAGGGAACC			
TM12	GGUCUCUCUGGUUAG-----	CCUGGGA-----	CUAACUAGGGAACC			
TM27	GG-----	CCAGAU	CUGAGCCUUGGAGCUCUCUGG	-----	CC	
TM30	GGUCUCUCUGGUUAGACCAGAU	CUGAGCCU-----	AGCUCUCUGGCUAACUAGGGAACC			
TM29	GGUCUCUCUGGUUAGACCAGAU	-----	GAGCCUUGGAGCUCUCUGGCUAACUAGGGAACC			
TM31	GGUCUCUCUGGUUAGACCAGAU	CU-----	CUGGCUAACUAGGGAACC			
TM40	-----	GUUAGACCAGAU	CU-----	CUGGCUAAC-----		
TM38	GGUCUCUCUGGUUAGACCAGAU	CUGAGCCUUGGAGCUCUCUGG	CUA-----			
TM18	<u>GCAAGGGAUCAAUGGUCUCUC</u>	<u>UCUGGAGCAGAGACCGAUUGGUCUCUG</u>				
TM22	GGUC <u>AGGGA</u> GGUAGACCAGAU	CUGAGCCUUGGAGCUCUCUGG	CUAACU <u>UCUCU</u> UACC			
TM23	GGUCUCUCU <u>UCAAUC</u> ACCA	GAUCUGAGCCUUGGAGCUCUCUGG	<u>GAUUGG</u> AGGGAACC			
TM24	GGUCUCUCUGGUUAG <u>AGUUCU</u>	CUGAGCCUUGGAGCUC <u>AGACC</u> CUAACUAGGGAACC				
TM25	GGUCUCUCUGGUUAGACCAGAU	CUC <u>GC</u> UGGAG <u>CGAG</u> CUCUCUGGCUAACUAGGGAACC				
TM26	GGUCUCUCUGGUUAGACCAG <u>AGAGAGACCCU</u>	CUCUCUGGCUAACUAGGGAACC				
TM37	GGUCUCUCUGGUUAG <u>CA</u> GAUCUGAGCCUUGGAGCUCUCUGGCUAACUAGGGAACC					
TM39	GGUCUCUCUGGUUAG--CCAGAU	CUGAGCCUUGGAGCUCUCUGG	CUAACUAGGGAACC			

FIG. 3. Sequence of wild-type (WT) TAR RNA and mutant TAR RNAs which have been cloned into the *in vitro* transcription vector pT7pUC19. Deletions are indicated by dashes; base substitutions are boldface and underlined.

have been deleted, whereas the lower stem regions I (bases 5 to 9 and 50 to 54) and II (bases 10 to 15 and 44 to 49) and the six-member loop (CUGGGA) have been retained. In a second deletion mutant, TM27, the lower stem regions I and II, the unpaired residues C₄ and A₁₆, and the base pair U₃ · A₅₅ have been deleted, whereas the upper portion of the molecule (stem regions III and IV, the pyrimidine bulge, and the loop) and the first two G · C base pairs of the stem have been left intact (Fig. 4). Nuclear protein binding to the TM12 transcript was abolished (Fig. 5, lane 3). Thus, the stem regions I and II and the loop sequences are not sufficient to support the RNA-protein complex formation. This result suggests that regions within the upper portion of TAR RNA (the unpaired adenine at position +16, stem region III, the UCU bulge, and stem region IV) are necessary for protein recognition. Protein binding to the transcript of TM27 was detectable yet substantially reduced (Fig. 5, lane 4). This demonstrates that while TM27 may contain the minimum sequences necessary for protein recognition, the lower stem regions (deleted in this mutant) play an important role in stabilizing the RNA-protein interaction.

The TAR RNA pyrimidine bulge, stem IV, and the loop are essential for Tat *trans* activation (19–21). To test whether these regions are essential to the binding of the host cell nuclear protein, we constructed three TAR mutants with deletions in these regions. The three G residues of the loop (G₃₁G₃₂G₃₃) were deleted in TM30, the pyrimidine bulge (U₂₂C₂₃U₂₄) was deleted in TM29, and both the loop and stem region IV (sequences 25 to 38) were deleted in TM31 (Fig. 4). Neither the 3-base deletion in the loop (TM30) nor the more extensive deletion (TM31) had an effect on protein binding (Fig. 5, lanes 5 and 7, respectively). Protein binding to the bulge deletion mutant (TM29), however, was markedly reduced (Fig. 5, lane 6; an additional, faster-migrating RNA-protein complex was formed with the TM29 mutant, presumably because of the extended 9-bp stem between A₁₆ and the loop). These deletion studies establish that stem region IV and the loop are not essential for the TAR RNA-protein complex formation and indicate that the pyrimidine bulge is important.

Two lower-stem deletion mutants were created to examine the contribution of this region to the TAR RNA-protein interaction. In the mutant TM40, the loop and stem regions I and IV have been deleted (compare TM31, in which just the loop and stem region IV have been deleted; Fig. 4), and in TM38 bases 47 to 57 have been deleted (Fig. 4). The TAR RNA-binding protein did not form a complex with either of these mutant TAR RNAs (Fig. 5, lanes 8 and 9). This experiment confirms that a structured base-paired stem in the lower region of TAR RNA is necessary to support TAR RNA protein binding activity.

TAR RNA primary sequence analysis. We next analyzed the effects of TAR RNA primary sequence mutations on the TAR RNA-protein interaction. Several compensatory mutations were designed to alter the primary sequence of TAR and yet maintain as far as possible the secondary structure of the wild-type molecule. In TM18, the sequences of stem regions I, II, III, and IV have been switched (for example, a G · C has become a C · G), whereas the non-base-paired nucleotides (the cytosine residue at position +4, the adenine residue at position +16, and the bulge and loop) have not been altered. The mutations in TM18 have the predicted effects of collapsing the pyrimidine bulge, of leaving only the U₂₂ unpaired, and of altering the structure of the loop and stem region IV (Fig. 4). The TM18 transcript was not bound by the nuclear protein (Fig. 5, lane 10). This result shows that the primary sequence of the stem is important for protein recognition and demonstrates that the presence of the singly bulged nucleotides (C₄ and A₁₆) is not sufficient to support protein binding.

We constructed several base pair-switching mutants (TM22, region I; TM23, region II; TM24, region III; and TM25, region IV) (Fig. 4) to determine specifically which region of the TM18 RNA molecule is responsible for dismissing protein binding activity. The primary sequence alterations in stem regions I (TM22) and II (TM23) greatly reduced protein binding activity (Fig. 5, lanes 11 and 12) and thereby demonstrate that the primary sequence of the lower two stem regions is important for the stable binding of the host cell factor. The primary sequence switch in stem region III (TM24) has the predicted effects of reducing the 5-bp stem III to a 4-bp stem and of altering the composition of the bulge from UCU to UUC (Fig. 5). Protein binding to TM24 RNA was less efficient than to the wild-type TAR RNA (Fig. 5, lane 13). The implication from this experiment is that the primary sequence of stem region III and the composition of the pyrimidine bulge contribute to stable TAR RNA-protein binding. The base pair switches in stem IV (TM25) alter substantially the predicted composition and structure of the bulge, stem region IV, and the loop (Fig. 4). For example, the bulge is reduced to a single residue (U₂₂), the stem region IV is extended into an interrupted 5-bp stem, and the loop is curtailed to five nucleotides instead of the usual six. Surprisingly, TM25 RNA bound to the nuclear protein with a level of activity somewhat higher than that of wild-type TAR RNA (Fig. 5, lane 14). The mutational analysis described above demonstrates that the primary sequence of the stem regions I, II, and III plays an important role in the binding of this host cell factor to TAR RNA.

To extend the primary sequence analysis, we changed the sequences of both the bulge and the loop to their complements in the mutant TM26 (the UCU bulge was changed to AGA, and the loop sequence CUGGGA was changed to GACCCU; Fig. 4). The level of protein binding to TM26 RNA was markedly lower than that for wild-type TAR RNA (Fig. 5, lane 15). Since the loop is not essential for the

FIG. 4. Computer-predicted secondary structures and free energies (in kilocalories per mole) of TAR RNA and TAR mutants. Base substitutions are boldface.

binding of this protein, the reduction in binding is most likely due to the changes in the primary sequence of the bulge, particularly the U₂₂-to-A₂₂ alteration. It appears then that the binding site of this nuclear protein overlaps with the binding site of Tat (23).

TAR RNA point mutations. Having established that the primary sequence of stem region III is important to TAR

RNA protein binding, we wished to determine whether the secondary structure of this region affects protein binding. We therefore constructed a mutant, TM37, containing the single base substitution of C₁₈ to A₁₈, which is predicted to selectively disrupt the base pairing in stem region III and to position a guanine residue opposite the single adenine residue at position +16 (Fig. 4). Protein binding to this

FIG. 5. RNase protection mobility shift assay of TAR RNA mutants. The appearance of the slightly faster-migrating band beneath the major band (arrow) varied between nuclear extract preparations and does not exhibit a different binding specificity. This minor complex is probably a partial proteolytic digestion product of the major complex. -NE, no nuclear extract.

mutant was undetectable (Fig. 5, lane 16). This indicates that the secondary structure of stem region III and the contexture of the unpaired adenine residue (A_{16}) play important roles in the RNA-protein complex formation. To focus on the role of the single bulged adenine residue at position +16, this nucleotide was deleted in TM39 (Fig. 4). Binding activity was reduced with this molecule (Fig. 5, lane 17). The RNA-protein band seen with TM39, however, was not a simple quantitative reduction in signal intensity. Rather, it appears that there has been a selective loss of binding, compared with the wild type. This suggests that the TAR RNA-binding protein is a protein complex rather than a single polypeptide and that one of the proteins of the complex requires contact with the bulged adenine residue at position +16. Notably, the unpaired adenine at this position is highly conserved among HIV isolates (15).

Competition assay. To further investigate the roles of the primary sequence and secondary structure of stem region III in host cell factor binding, we performed competition assays with wild-type TAR and the mutants TM24 and TM37. In these assays, 0.1, 0.5, and 1.0 μ g (approximately 100-, 500-, and 1,000-fold molar excesses, respectively) of nonradiolabelled competitor RNA were incubated with the nuclear extract for 10 min prior to the addition of the 32 P-labelled TAR RNA. Protein binding was efficiently inhibited by competition with the wild-type TAR RNA (Fig. 6, lanes 3, 4, and 5), whereas the TM24 and TM37 transcripts were relatively inefficient competitors (Fig. 6, lanes 6, 7, and 8 [TM24], and lanes 9, 10, and 11 [TM37]). These competition assays demonstrate that alterations of either the primary sequence or the secondary structure of TAR RNA stem region III result in a less suitable substrate for host cell protein binding.

UV cross-linking. The molecular mass of the TAR RNA-

FIG. 6. Competition assays with nonradiolabelled RNA competitors. Nuclear extracts were incubated with 0.1, 0.5, or 1.0 μ g (approximately 100-, 500-, and 1,000-fold molar excesses, respectively) of cold RNA competitor prior to the addition of 32 P-labelled TAR RNA, as indicated above the lanes. -NE, no nuclear extract.

FIG. 7. SDS-PAGE analysis of UV-cross-linked RNA-protein complexes. A 140-kDa protein (arrow) is cross-linked to TAR, TM31, and TM25 RNAs (lanes 2, 4, and 6) but not to TM12, TM18, or TM37 RNA (lanes 3, 5, and 7). -NE, no nuclear extract.

binding protein was estimated by UV cross-linking the ^{32}P -labelled RNA to the protein and analyzing the products by SDS-polyacrylamide gel electrophoresis (PAGE). A 140-kDa protein (which typically migrated as a doublet) was cross-linked to TAR RNA and the mutant RNAs TM31 and TM25 (Fig. 7, lanes 2, 4, and 6), which also bind protein in the gel shift assay, but not to the mutant RNAs TM12, TM18, and TM37 (Fig. 7, lanes 3, 5, and 7), which do not bind protein in the gel shift assay. Additionally, the 140-kDa band was abolished by competition with nonradiolabelled TAR RNA but not by competition with the mutant RNAs, which bind weakly in the gel shift assay (data not shown).

DISCUSSION

HIV-1 transcriptional activity is modulated by both viral and host cell factors. The viral *cis*-responsive element, TAR RNA, is an essential transcription regulatory element through which *trans*-acting factors exert their effect. We have used an RNase protection mobility shift assay and UV cross-linking to show that a 140-kDa HeLa nuclear protein binds specifically to TAR RNA. By mutational analysis of TAR RNA, we show that the sequence and structure of stem regions I, II, and III, the pyrimidine bulge, and the unpaired adenine (position +16) are important to this interaction. Neither the loop nor stem region IV, however, is essential for the binding of this host cell protein to TAR RNA. The TAR RNA SBP is distinct in both its site of binding to TAR RNA and its molecular mass, from previously reported specific TAR RNA-binding cellular proteins (8, 13, 24). Recently, Wu et al. (24) reported two HeLa cell nuclear proteins (TRP-185 and TRP-140) which bind to TAR RNA. TRP-185 binding is dependent on the loop sequences of TAR RNA and requires an additional host cell factor, whereas TRP-140 is apparently nonspecific.

Possible function of TAR RNA SBP. One of the primary effects of Tat is to facilitate the elongation of initiated transcripts which would otherwise terminate shortly beyond the 3' end of TAR (7, 10–12, 17, 21). These prematurely terminated transcripts, 55 to 70 bases long, accumulate in uninduced cells and reflect a high rate of nonproductive

initiation events from the HIV promoter (1, 10, 11, 17, 21, 22). Ratnasabapathy et al. (17) have shown that positioning the TAR region (or inducer of short transcripts) immediately downstream of heterologous promoters activates transcription, causes premature termination, and results in the production of short transcripts. Since mutations disrupting the sequence and structure of the stem inhibit the production of short transcripts (17, 20, 21), the generation of short transcripts appears dependent on the integrity of the TAR RNA stem. For example, an antisense mutant is not capable of inducing the synthesis of short transcripts (17). Interestingly, our mutant TM18 (which has an antisense stem) does not support SBP binding. Moreover, the production of short transcripts is independent of the TAR loop (17), as is the binding of SBP. Several other TAR stem mutants, reported in the literature, do not support the production of short transcripts (11, 20). These correlations suggest that the SBP is involved in the production of short transcripts from the HIV promoter.

The sequence and structure of the TAR RNA stem are remarkably well conserved among HIV-1 isolates (2). This evolutionary conservation demonstrates that there exists strong selective pressure to maintain the binding site of this cellular protein. We therefore believe that this protein will prove to be an important factor in the regulation of HIV-1 gene expression.

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