RNA−Protein Interactions in the Tat−*trans*-Activation Response Element Complex Determined by Site-Specific Photo-Cross-Linking[†]

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ABSTRACT: Transcriptional regulation in human immunodeficiency virus type 1 (HIV-1) requires specific interactions of Tat protein with the *trans*-activation responsive region (TAR) RNA, a 59-base stem−loop structure located at the 5′-end of all HIV transcripts. We have used a site-specific cross-linking method based on 6-thioguanosine (6-thioG) photochemistry to determine the conformation of TAR RNA and its interaction with Tat protein under physiological conditions. Two different TAR RNA constructs with a single photoactive nucleoside (6-thioG) at position 21 or 26 were synthesized. Upon UV irradiation, 6-thioG at both positions formed interstrand covalent cross-links in TAR RNA. Determination of cross-link sites by RNA sequencing revealed that 6-thioG at position 21 contacts U42, while a 6-thioG at position 26 cross-links to C39. The addition of arginine did not alter the site of RNA−RNA cross-links; however, the yields of 6-thioG26-C39 cross-link were decreased. In the presence of a Tat fragment, Tat(38−72), UV irradiation of RNA modified with 6-thioG at position 21 resulted in RNA−protein cross-links but no RNA−RNA cross-links were observed. 6-thioG at position 26 formed both RNA−RNA and RNA−protein cross-links in the presence of Tat(38−72). Our results provide direct evidence that, during RNA−protein recognition, Tat is in close proximity to O^6 of G21 and G26 in the major groove of TAR RNA.

The promoter of the HIV-1,[1] located in the U3 region of the viral long terminal repeat (LTR), is an inducible promoter that can be stimulated by the *trans*-activator protein Tat (*1*). As in other lentiviruses, Tat protein is essential for *trans*-activation of viral gene expression (*2*−*6*). In the absence of Tat, most of the viral transcripts terminate prematurely producing short RNA molecules ranging in size from 60 to 80 nucleotides. The Tat protein is a small, cysteine-rich nuclear protein containing 86 amino acids and comprised of three important functional regions: core (32−48), basic (49−60), and Gln(61−72). HIV-1 Tat protein acts by binding to the TAR (*trans*-activation responsive) RNA element, a 59-base stem−loop structure located at the 5′ ends of all nascent HIV-1 transcripts (*7*). Upon binding to the TAR RNA sequence, Tat causes a substantial increase in transcript levels (*8*−*12*). TAR RNA was originally localized to nucleotides +1 to +80 within the viral long terminal repeat (LTR) (*13*). Subsequent deletion studies have established that the region from +19 to +42 incorporates the minimal domain that is both necessary and sufficient for Tat responsiveness in vivo (*14*−*16*). TAR RNA contains a six-nucleotide loop and a three-nucleotide pyrimidine bulge that separates two helical stem regions (*7, 10, 13, 14*). The trinucleotide bulge is essential for high affinity and specific binding of the Tat protein (*17, 18*).

Mutational analyses have shown that sequences in the loop of TAR RNA are required for *trans*-activation (*19, 20*) and not for Tat binding (*21*−*23*). The loop may provide the binding site for cellular factor(s) involved in *trans*-activation (*24*−*27*). Recently, it has been shown that the cellular factor TRP-185 regulates RNA polymerase II binding to HIV-1 TAR RNA (*28*). A group of cellular cofactors has also been identified that stimulate the binding of RNA polymerase II and TRP-185 to TAR RNA (*29*). The apical loop is not required for Tat binding and can be dispensed with in TAR models (*30*). Since Tat can bind duplex TAR RNA (without the loop residues) with approximately half the affinity for wild-type TAR RNA, chemically synthesized duplex TAR RNAs without loop residues have been used to study the role of various functional groups in Tat recognition (*22, 23, 31*). Recently, we and others have performed RNA−protein cross-linking studies on synthetic TAR RNA duplex structures containing Tat-binding bulge sequences (*32, 33*).

Understanding the principles of Tat−TAR interactions is a crucial step for drug design. Although high-resolution NMR information is limited to the TAR RNA component (*34, 35*), a structure of the RNA−protein complex is still missing. Therefore, new methods are needed to determine the topology of RNA−protein complexes under physiological conditions. Here we have used a site-specific photo-cross-linking method to probe Tat−TAR interactions.

We synthesized two duplex TAR RNA constructs (Figure 1) with a single 6-thioguanosine (6-thioG) residue at position 21 or 26. Upon UV irradiation, these 6-thioG labeled RNA formed interstrand covalent cross-links in TAR RNA. Cross-link sites were determined by RNA sequencing, which revealed that 6-thioG at position 21 contacts U42, whereas

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[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; TAR, *trans*-activation response element; EDTA, ethylenediaminetetraacetic acid; 6-thioG, 6-thioguanosine.

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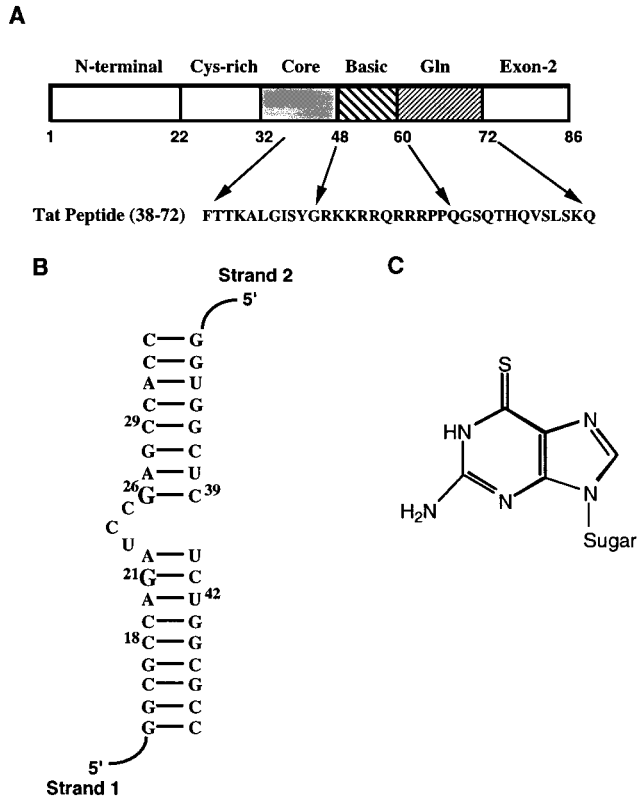

FIGURE 1: (A) Regions of the HIV-1 Tat protein and sequence of the Tat(38−72) peptide. (B) The secondary structure of the designed duplex TAR RNA used in this study contains the minimal sequence required for in vitro Tat binding (*23*). Duplex RNA contains the nucleotides C18−C29 and G36−G44 from the wild-type TAR RNA sequence, and extra flanking base pairs to enhance hybridization of two RNA strands. Sequences containing the trinucleotide bulge and the complementary sequence are labeled as strand 1 and strand 2, respectively. Incorporation of 6-thioG at position 21 or 26 was accomplished by chemical synthesis of RNA (*38*, *39*). Numbering of nucleotides in the duplex TAR RNA corresponds to their positions in wild-type TAR RNA. Sites of 6-thioG incorporation, G21 and G26, are highlighted. (C) Structure of 6-thioguanosine.

at position 26, 6-thioG cross-linked to C39 residue. To study the effect of L-argininamide (a TAR RNA ligand) and a Tat peptide on TAR RNA conformation, photo-cross-linking reactions were carried out in the presence of L-argininamide and ADP-1, a Tat fragment that binds TAR RNA with high specificity and includes the basic and part of the core regions of Tat (*30*). To avoid oxidation problems, we deleted the N-terminal cysteine residue from the ADP-1 sequence. Our results showed that the sites of cross-link were unchanged in the presence of L-argininamide. However, in the presence of Tat(38−72), UV irradiation of RNA modified with 6-thioG at position 21 resulted in RNA−protein cross-links but no RNA−RNA cross-links were observed. 6-ThioG at position 26 formed both RNA−RNA and RNA−protein cross-links in the presence of Tat(38−72); however, the yields of RNA−RNA cross-link were decreased. Our results provide direct evidence that, during RNA−protein recognition, Tat is in close proximity to O^6 of G21 and G26 in the major groove of TAR RNA.

EXPERIMENTAL PROCEDURES

Buffers. All buffer pH values refer to measurements at room temperature. TK buffer: 50 mM Tris-HCI (pH 7.4), 20 mM KCI, and 0.1% Triton X-100. Transcription buffer: 40 mM Tris-HCI (pH 8.1), 1 mM spermidine, 0.01% Triton X-100, and 5 mM DTT. TBE buffer: 45 mM Tris−borate, pH, 8.0, and 1 mM EDTA. Sample loading buffer: 9 M urea, 1 mM EDTA, and 0.1% bromophenol blue in 1× TBE buffer. Binding buffer: 25 mM Tris-HCI (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, and 0.1% Triton X-100. Hydrolysis buffer: 50 mM Na_2CO_3/$NaHCO_3$, pH 9.2. Elution buffer: 1× TBE and 10% sodium acetate (3 M), pH 5.5. Digestion buffer: 100 mM Tris-HCl (pH 8.5).

Oligonucleotide Synthesis: (A) DNAs. All DNAs were synthesized on an Applied Biosystems ABI 392 DNA/RNA synthesizer. The template strand encodes the sequence for the duplex TAR RNA (Figure 1). The top strand is a short piece of DNA complementary to the 3′ end of all template DNAs having the sequence 5′TAATACGACTCACTAT-AG3′. DNA was deprotected in NH_4OH at 55 °C for 8 h and then dried in a Savant Speed-Vac. The samples were resuspended in sample loading buffer and were purified on 20% acrylamide−8 M urea denaturing gels, 50 cm × 0.8 mm. Gels were run for 3 h at 30 W until xylene cyanol tracking dye was 5 cm from the bottom of the gel. DNAs were visualized by UV shadowing, excised from the gel, and eluted in 50 mM Tris, 16 mM boric acid, 1 mM EDTA, and 0.5 M sodium acetate. DNAs were ethanol-precipitated and resuspended in DEPC (diethyl pyrocarbonate) treated water. Concentration of DNAs were determined by measuring absorbance at 260 nm in a Shimadzu UV spectrophotometer.

(B) RNAs. RNAs were synthesized by chemical and enzymatic methods. Chemical syntheses were performed on ABI synthesizer model 392 using standard protocols. All the monomers of (2-cyanoethyl)phosphoramidites were obtained from Glen Research (Sterling, VA). To introduce 6-thioG at position 21 or 26 in TAR RNA, we synthesized a cyanoethyl-protected 6-thioG phosphoramidite, S^6-cyanoethyl-5′-*O*-(dimethoxytrityl)-N^2-benzoyl-2′-*O*-(*t*-butyldimethylsilyl)-6-thioguanosine 3′-*O*-[2-cyanoethyl(*N*,*N*-diisopropylamino)phosphoramidite], as described by Christopherson and Broom (*36*) and Adams et al. (*37*). Incorporation of 6-thioG into the RNA sequences and characterization of modified RNAs were carried out as described earlier (*38*, *39*).

All other RNAs were prepared by in vitro transcription (*40*). The template strand of DNA was annealed to an equimolar amount of top strand DNA and transcriptions were carried out in transcription buffer and 4.0 mM NTPs at 37 °C for 2−4 h. For reactions (20 μL) containing 8.0 pmol of template DNA, 40−60 units of T7 polymerase (Promega) was used. Transcription reactions were stopped by adding an equal volume of sample loading buffer. RNA was purified on 20% acrylamide−8 M urea denaturing gels as described above. RNAs were stored in DEPC water at −20 °C.

RNAs were 5′-dephosphorylated by incubation with calf intestinal alkaline phosphatase (Promega) for 1 h at 37 °C in 50 mM Tris-HCl, pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine. The RNAs were purified by multiple extractions with Tris-saturated phenol and one extraction with 24:1 chloroform:isoamyl alcohol followed by ethanol precipitation. The RNAs were 5′-end-labeled with 0.5 μM [γ-^{32}P] ATP (6000 Ci/mmol) (ICN) per 100 pmol of RNA by incubating with 16 units of T4 polynucleotide kinase (New England Biolabs) in 70 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 5 mM DTT. RNAs were gel-purified on a

denaturing gel, visualized by autoradiography, and recovered from gels as described above.

Gel Retardation Assays. RNA−protein binding reactions (20 μL) contained 0.5 μM 5′-^{32}P-end-labeled RNA and increasing concentrations of Tat (0−0.5 μM). Complex formation was performed in TK buffer with incubation at room temperature for 1 h. Complexes were separated from unbound RNA by electrophoresis in nondenaturing 8% polyacrylamide gels containing 0.1% Triton X-100. Gels were run in a cold room at 300 V for 2 h. The relative amounts of free and bound RNA were determined by phosphorimaging.

Site-Specific Photo-Cross-Linking Reactions. Before the photochemical reactions were performed, RNA duplex was prepared by hybridizing two strands. Typically, 5 pmol of strand 1 or 2 RNA (5′-end-labeled with ^{32}P) were mixed with 7.5 pmol of the complementary RNA strand in 10 μL of TK buffer; the mixture was heated at 70 °C for 1 min and allowed to cool slowly to room temperature. Under these conditions, more than 95% duplex was formed as confirmed by nondenaturing polyacrylamide gels (data not shown). For RNA−RNA cross-linking reactions, 10 μL of 0.5 μM preformed duplexes were UV-irradiated (360 nm) for 5 min on ice in a Rayonet photochemical reactor (RPR-100). To study the effect of L-Arg on RNA structure, RNA−RNA cross-linking reactions were performed in the presence of 1 mM L-Arg or L-argininamide. For RNA−peptide cross-linking reactions, 5 pmol of preformed duplex was mixed with 6 pmol of Tat(38−72), and the mixture was incubated at room temperature for 10 min, and irradiated for 5 min as described above. After irradiation, 2 μL of yeast tRNA (20 μg/μL) and 12 μL of sample loading buffer were added to each sample, and the samplers were electrophoresed on 8 M urea−20% acrylamide gels. Efficiencies of cross-linking were determined by a PhosphorImage analysis (Molecular Dynamics).

Large-Scale Preparation and Enzymatic Digestion of the RNA−Peptide Cross-Link. Preparative-scale duplex RNAs were formed by mixing 2 nmol of strand 1 RNA and 3 nmol of strand 2 RNA in 1 mL of TK buffer, and annealed as described above. RNA−protein complex formation was accomplished by the addition of 2.4 nmol of Tat(38−72). The RNA−protein complex was incubated at room temperature for 10 min and irradiated as described for analytical scale. The photoproducts of the reaction were ethanol-precipitated and separated from free RNA on a 20% denaturing polyacrylamide gel. The band corresponding to the cross-linked product was excised from the gel, eluted into 2 mL of elution buffer, and desalted on a C_{18} column. The purified cross-link was dissolved in 20 μL of digestion buffer and 1 μg of trypsin (sequencing grade, Boehringer Mannheim Biochemica) was added to the mixture, which was incubated for 3 h at 37 °C. The reaction was stopped by adding 20 μL of sample loading buffer and analyzed on a denaturing gel. The digestion product was visualized by UV illuminationand purified from the gel as described above. The sample was desalted on a C_{18} column, dried, and submitted for peptide sequencing.

RNA Sequencing. Alkaline hydrolysis of RNAs was carried out in hydrolysis buffer for 8−12 min at 85 °C. RNAs were incubated with 0.1 unit of RNAse from *Bacillus cereus* (Pharmacia) per pmol of RNA for 4 min at 55 °C in 16 mM sodium citrate, pH 5.0, 0.8 mM EDTA, and 0.5 mg/mL yeast tRNA (Gibco-BRL). This enzyme yields U- and C-specific cleavage of RNA. Sequencing products were resolved on 20% denaturing gels and visualized by phosphorimage analysis.

Competition Assay. Competition experiments were performed in 10 μL volumes and contained 5 pmol of the end-labeled RNA, 6 pmoles of Tat(38−72), and increasing amounts of wild-type TAR or a mutant TAR duplex. Photo-cross-linking reactions were performed as desribed above. RNA−RNA and RNA−protein cross-link products were resolved on 20% denaturing gels and visualized by phosphorimage analysis.

Peptide Synthesis. All Fmoc-amino acids, piperidine, 4-(dimethylamino)pyridine, dichloromethane, *N*,*N*-dimethylformamide, 1-hydroxybenzotriazole (HOBT), 2-(1*H*-benzotriazo-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), diisopropylethylamine, and 4-hydroxymethylphenoxy-linked polystyrene resin were obtained from Applied Biosystems Division, Perkin-Elmer. Trifluoroacetic acid, 1,2-ethanedithiol, phenol, and thioanisole were from Sigma. Tat-derived peptide (from amino acids 38 to 72) was synthesized on an Applied Biosystems 431A peptide synthesizer using standard FastMoc protocols. Cleavage and deprotection of the peptide was carried out in 2 mL of reagent K for 6 h at room temperature. Reagent K contained 1.75 mL of TFA, 100 μL of thioanisole, 100 μL of water, and 50 μL of ethanedithiol (*41*). After cleavage from the resin, peptide was purified by HPLC on a Zorbax 300 SB-C_8 column. The mass of fully deprotected and purified peptides were confirmed by FAB mass spectrometry; calculated mass for Tat(38−72) C_{175} H_{298} $N_{64}O_{43}$, 4082.7; found, 4083.7 (M + H).

RESULTS

Experimental Design. The goal of our study was to investigate the folding of TAR RNA and its interactions with Tat at the protein binding site. We planned to site-specifically label TAR RNA with a photoactive analogue (6-thioG) at two critical residues in the upper and lower stem regions, G21 and G26. Chemical synthesis of long oligoribonucleotides, such as 29-base TAR RNA, is still not very efficient. To circumvent this problem, two short oligoribonucleotides were synthesized and annealed together to form a duplex RNA that contained the trinucleotide bulge to create a structure similar to the Tat binding site in TAR RNA (*22*, *23*, *31*). The duplex TAR sequence contained wild-type residues from C18 to C30. Flanking residues were added to achieve efficient annealing (Figure 1). Because U25 can be replaced with C or a propyl linker without affecting the Tat binding (*30*, *42*), we substituted U25 with C in our duplex TAR RNA constructs. Therefore, strand 1 in our model duplex contained only one uridine, U23, in the RNA sequence. Two different duplex TAR RNA constructs with a single 6-thioG residue at positions 21 or 26 were synthesized. Site-specific incorporation of 6-thioG at position 21 or 26 was carried out by chemical synthesis of RNA using a cyanoethyl-protected 6-thioG phosphoramidite, S^6-cyanoethyl-5′-*O*-(dimethoxytrityl)-N^2-benzoyl-2′-*O*-(*t*-butyldimethylsilyl)-6-thioguanosine 3′-O-[2-cyanoethyl(*N*,*N*-diisopropylamino)phosphoramidite] (*36*, *37*). Incorporation of 6-thio-G into the RNA sequences and characterization of

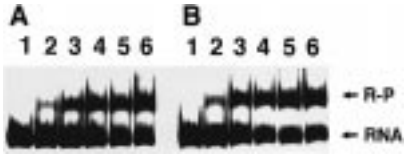

FIGURE 2: Binding of Tat(38−72) peptide to TAR RNA (A) or 6-thioG21-modified TAR RNA (B). Binding reactions contained 0.5 μM of 5′-^{32}P-end-labeled RNA and increasing concentrations of Tat peptide. Lane 1 was a control lane without the peptide. RNA−peptide complexes are shown as RP.

modified RNAs were carried out as described earlier (*38, 39*). After formation of a duplex TAR structure, ultraviolet irradiation (360 nm) was used for short periods of time to create interstrand RNA−RNA, and RNA−protein cross-links. RNA−RNA cross-links were purified by 8 M urea−20% acrylamide gel electrophoresis, and cross-link sites were mapped by RNA sequencing.

Tat Binding to Modified TAR RNA. To characterize and evaluate the Tat binding capabilities of 6-thioG containing TAR RNA, we determined the dissociation constants of modified Tat−TAR complexes. Equilibrium dissociation constants of the Tat−TAR complexes were measured using direct electrophoretic mobility assays (*30*, *43*, *44*). A Tat fragment, Tat(38−72), was used in these measurements. A typical gel of these experiments is shown in Figure 2. These results showed that Tat(38−72) binds the 6-thioG21-modified TAR duplex RNA with a K_D of 0.30 μM. To compare the Tat-binding affinities of the modified RNA to wild-type RNA, we synthesized an unmodified RNA duplex (Figure 1). Dissociation constants of the unmodified Tat−TAR complexes were determined under the same conditions used for 6-thioG21−Tat(38−72) complexes. These experiments showed that the unmodified TAR duplex binds Tat with a K_D of 0.15 μM. A relative dissociation constant (K_{rel}) can be determined by measuring the ratios of the modified TAR to wild-type TAR dissociation constants (K_D) for Tat(38−72). Our results demonstrate that the calculated value for K_{rel} was 2.0. Similar experiments were performed using 6-thioG26-modified TAR RNA duplex and the calculated value for K_{rel} was 1.46. These results indicate that incorporation of 6-thioG in the TAR duplex did not significantly alter the structure of the RNA, thus preserving the Tat-binding affinities of the RNA.

Duplex TAR RNA labeled with 6-ThioG at G21 Forms an Interstrand RNA−RNA Cross-Link upon UV Irradiation. Strand 1 of duplex TAR RNA was labeled at its 5′-end with ^{32}P and annealed to its complementary strand as described in Experimental Procedures. The concentrations of strand 1 and strand 2 were 0.5 and 0.75 μM, respectively. Duplex formation was confirmed by nondenaturing gel electrophoresis showing a slow-migrating band, indicating that a complete duplex was formed under these conditions (data not shown). Duplex RNA was UV-irradiated with 360 nm light, and electrophoresed on denaturing 8 M urea gels (Figure 3). Irradiation of duplex TAR RNA yields an interstrand RNA cross-link with electrophoretic mobility less than that of strand 1 RNA (Figure 3, lane 2). The efficiencies of the photo-cross-linking reactions were high, ∼26%.

It has been suggested by competition, chemical interference, and NMR experiments that free arginine or argininamide interacts with TAR RNA in a manner similar to that

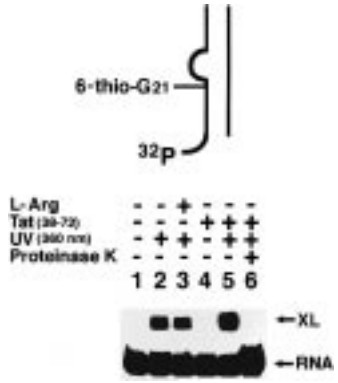

FIGURE 3: Cross-linking reactions of TAR RNA duplex labeled with 6-thioG at position 21. Strand 1 was labeled with ^{32}P at the 5′-end, annealed to its complement strand 2, and UV-irradiated at 360 nm. Cross-linked products were resolved on a 20% acrylamide−8 M urea gel and visualized by autoradiography. Lane 1, duplex RNA without irradiation; lane 2, duplex RNA with irradiation; lane 3, duplex RNA irradiated with 1 mM L-argininamide; lane 4, duplex RNA with Tat(38−72) and no irradiation; lane 5, Duplex RNA irradiated in the presence of Tat(38−72); lane 6, duplex RNA irradiated in the presence of Tat(38−72) and the sample was treated with proteinase K for 15 min at 55 °C. RNA−protein cross-links are indicated as XL.

of arginine in the context of short Tat peptides (*34*, *45*−*48*). To determine the effect of L-arginine on the conformation of RNA, we carried out the photo-cross-linking reaction in the presence of 1 mM L-argininamide (Figure 3, lane 3). The electrophoretic mobilities and the yields of the cross-link products were unchanged in the presence of arginine, ∼24%. These results suggest that arginine does not interact with G21 and its addition did not change the RNA conformation significantly around G21 position in the lower stem of TAR RNA.

Duplex TAR RNA Labeled with 6-ThioG at G21 Forms a Cross-Link with a Tat Fragment. The concentrations of TAR RNA and the Tat peptide employed for cross-linking reactions were 0.5 and 0.6 μM, respectively. Under these conditions, electrophoretic mobility shift assays revealed only one slow-migrating RNA-peptide complex, indicating the absence of other nonspecific RNA-peptide complex formation (*30*, *43*). Strand 1 of the duplex TAR RNA labeled at its 5′-end with ^{32}P was incubated with the Tat peptide for 15 min in 25 mM Tris (pH 7.4) and 100 mM NaCl and UV-irradiated with 360 nm light (see Materials and Methods). Products of the photoreaction were analyzed by denaturing 8 M urea PAGE (Figure 3). Irradiation of the RNA−peptide complex yields band with electrophoretic mobility less than that of strand 1 and similar to that of the RNA−RNA cross-link (Figure 3, lane 5). Both the peptide and UV irradiation are required for the formation of this cross-linked RNA−protein complex because this cross-linked product is observed only when RNA is irradiated in the presence of Tat peptide (lane 5), and no cross-link is formed with peptide in the dark without UV irradiation (lane 4). The formal possibility that the new mobility is due to an alternative RNA−RNA cross-link was ruled out by perfroming proteinase K digestion of the RNA−protein cross-link. RNA−protein cross-link products are degraded by proteinase K enzyme while RNA−RNA cross-link species are stable under these conditions (*32*). Proteinase K digestion of the RNA−protein cross-link resulted in a loss of RNA−protein cross-link and a gain

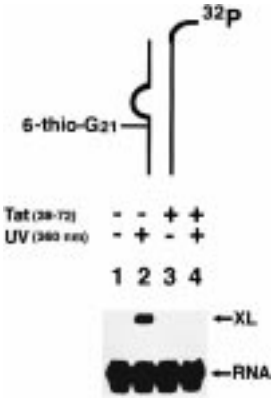

FIGURE 4: Determining the effect of a Tat fragment on the RNA−RNA cross-linking reaction of TAR RNA duplex labeled with 6-thioG at position 21. Strand 2 of the duplex was 5′-end-labeled with ^{32}P in these experiments. Lane 1, duplex RNA without irradiation; lane 2, duplex RNA with irradiation; lane 3, duplex RNA with Tat(38−72) and no irradiation; lane 4, duplex RNA irradiated in the presence of Tat(38−72). RNA−RNA cross-links are indicated by XL.

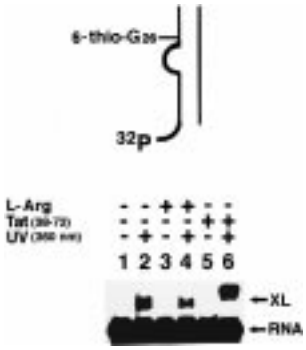

FIGURE 5: Cross-linking reactions of TAR RNA duplex labeled with 6-thioG at position 26. Strand 1 was labeled with ^{32}P at the 5′-end, annealed to its complement strand 2, and UV-irradiated at 360 nm. Cross-linked products were resolved on a 20% acrylamide−8 M urea gel and visualized by autoradiography. Lane 1, duplex RNA without irradiation; lane 2, duplex RNA with irradiation; lane 3, duplex RNA with 1 mM L-argininamide and no irradiation; lane 4, Duplex RNA irradiated with 1 mM L-argininamide; lane 5, duplex RNA with Tat(38−72) and no irradiation; lane 6, duplex irradiated in the presence of Tat(38−72). RNA−RNA and RNA−protein cross-links are indicated as XL.

in free RNA as observed by band intensities on the gel (Figure 3, lane 6). The products of irradiation were also analyzed on a denaturing SDS−15% polyacrylamide gel. Again, a photoproduct with electrophoretic mobility less than that of TAR RNA was observed, which was dependent on the presence of RNA and peptide (data not shown). The photoproduct yields were ∼45% as determined by a PhosphorImage analysis. Since the cross-linked RNA-peptide complex is stable to alkaline pH (9.5), high temperature (85 °C), and denaturing conditions (8 M urea, 2% SDS), we conclude that a covalent bond is formed between TAR RNA and the peptide during the cross-linking reaction.

Formation of RNA−RNA Cross-Link by 6-ThioG at G21 Does Not Occur in the Presence of Tat. To determine the effect of Tat on TAR RNA conformation and RNA−RNA cross-link formation, we performed the photo-cross-linking reactions in the presence of Tat(38−72) fragment. We prepared duplex TAR RNAs containing labeled strand 2 of the duplex at the 5′-end with ^{32}P and UV-irradiated them in the presence and absence of Tat(38−72). Results of these experiments are shown in Figure 4. When strand 2 of the duplex was labeled, an RNA−RNA cross-linked product was formed in the absence of Tat peptide (lane 2). There was no RNA−RNA cross-link product observed after the addition of the Tat peptide to the duplex (lane 4). From these results, we conclude that RNA−RNA cross-link formation does not occur in the presence of Tat protein, the protein forms a cross-link with strand 1 (6-thioG21 containing RNA), and an RNA−protein cross-link is the only photoproduct in the presence of Tat protein.

TAR RNA Labeled with 6-ThioG at G26 Forms RNA−RNA and RNA−Protein Cross-Links. 6-ThioG at position G26 was incorporated into strand 1 of the duplex during chemical syntheses of RNA as described above (*38*). Strand 1 of duplex TAR RNA was labeled at the 5′-end with ^{32}P and annealed to its complementary strand as described in Experimental Procedures. The concentrations of strands 1 and 2 were 0.5 and 0.75 μM, respectively. Duplex formation was confirmed as described above. Duplex RNAs were UV-irradiated with 360 nm light and electrophoresed on denaturing 8 M urea gels (Figure 5). Interstrand RNA−RNA cross-link species with electrophoretic mobility less than that of strand 1 RNA were observed when these duplex RNAs were irradiated (Figure 5). The yields of RNA−RNA cross-link formation were ∼18%.

The effect of arginine on TAR RNA folding was determined by performing UV cross-linking reactions in the presence of 1 mM L-argininamide. Apparent electrophoretic mobilities of the cross-link products were unaffected by the presence of argininamide (lane 4, Figure 5). However, the yields of RNA−RNA cross-link were lowered, ∼10%. These results show that arginine interacts with G26 and its presence may change the RNA conformation in the G26 region to affect the RNA−RNA cross-link formation.

RNA−protein cross-links were observed when the duplex RNA was irradiated in the presence of a Tat peptide (38−72). Strand 1 of the duplex TAR RNA labeled at the 5′-end with ^{32}P was incubated with the Tat peptide for 15 min in 25 mM Tris (pH 7.4) and 100 mM NaCl and then UV-irradiated with 360 nm light (see Experimental Procedures). Products of the photoreaction were analyzed by denaturing 8 M urea PAGE (Figure 5). Irradiation of the RNA−peptide complex yields a new band with electrophoretic mobility less than that of strand 1 and the RNA−RNA cross-link (lane 6, Figure 5). The yields of photo-cross-linking reactions were ∼22%. The band representing the cross-link products contains both the RNA−protein and RNA−RNA cross-link species (see below). Since RNA−protein cross-link was the major product in this band because only 7% RNA−RNA cross-link formation occurred in the presence of Tat peptide (Figure 6), it was difficult to separate these two species under our electrophoretic conditions (lane 6, Figure 5). However, it is also possible that RNA−RNA cross-link products adopt a different conformation in the presence of protein or RNA−protein cross-link and migrate with electrophoretic mobility less than that of RNA−RNA cross-link (lane 2, Figure 5). Both the peptide and UV irradiation are required for the formation of this cross-linked RNA−protein complex since no cross-link is formed with peptide in the dark without UV irradiation (lane 5, Figure 5).

Effect of Tat on RNA−RNA Cross-Link Formation. To further elucidate the effect of a Tat fragment on TAR RNA

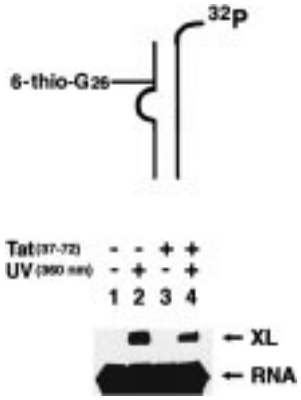

FIGURE 6: Determining the effect of a Tat fragment on the RNA−RNA cross-linking reaction of TAR RNA duplex labeled with 6-thioG at position 26. Strand 2 of the duplex was 5′-end-labeled with ^{32}P in these experiments. Lane 1, duplex RNA without irradiation; lane 2, duplex RNA with irradiation; lane 3, duplex RNA with Tat(38−72) and no irradiation; lane 4, duplex RNA irradiated in the presence of Tat(38−72). RNA−RNA cross-links are indicated by XL.

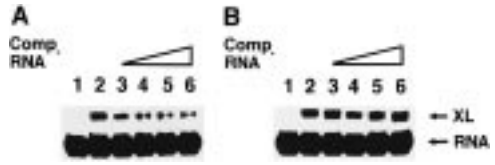

FIGURE 7: Specificity of the Tat−TAR cross-linking reaction determined by competition assays. RNA−protein complexes were formed between 0.25 μM ^{32}P-5′-end-labeled TAR RNA duplex modified with 6-thioG at position 21 and 0.5 μM Tat(38−72) in the presence of unlabeled wild-type TAR RNA (A) or bulge mutant TAR RNA (B). Concentrations of the competitor RNA in lanes 2−6 were 0, 0.5, 0.75, 1.0, and 1.25 μM, respectively. Lane 1 was a control RNA−peptide complex without UV irradiation. RNA−protein cross-links are indicated as XL.

folding, we prepared a duplex TAR RNA containing a single 6-thioG at position 26, labeled strand 2 of the duplex at the 5′-end with ^{32}P, and UV-irradiated in the presence and absence of Tat(38−72). Since strand 2 is end-labeled in these experiments, only RNA−RNA cross-link products can be seen on the gel. Results of these experiments are shown in Figure 6. When strand 2 of the duplex containing 6-thioG at position 26 in TAR RNA was labeled, RNA−RNA cross-links were observed in the presence and absence of Tat peptide (lanes 2 and 4, Figure 6). However, the yields of RNA−RNA cross-link were reduced by the addition of Tat, ∼7%. From these results, we conclude that for a duplex with 6-thioG at position 26, RNA−RNA cross-linking takes place in the presence and absence of Tat protein.

Specificity of the RNA−Protein Cross-Link Formation. Specificity of the cross-linking reaction was established by competition experiments (*49*). Cross-linking reactions were performed in a 15 μL volume containing 0.25 μM 5′-^{32}P-labeled 6-thioG21-modified TAR RNA duplex, 0.5 μM Tat peptide, 25 mM Tris-HCl (pH 7.4), 100 mM NaCl, and up to 1.25 μM unlabeled competitor RNA. Cross-linked products were separated by 8 M urea−20% polyacrylamide gels and visualized by phosphorimage analysis. Figure 7 shows that cross-linking was inhibited by the addition of unlabeled wild-type TAR RNA and not by a mutant TAR RNA lacking the trinucleotide bulge. Similar results were obtained when 6-thioG26-modified TAR RNA

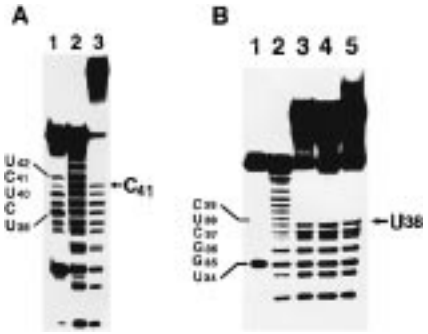

FIGURE 8: Mapping of the cross-linked base in the RNA−RNA cross-linked complexes by alkaline hydrolysis. (A) Analysis of RNA−RNA cross-link containing 5′-end-labeled strand 2 in the duplex modified with 6-thioG at position 21: lane 1, *B. cereus* ladder of strand 2 RNA; lane 2, hydrolysis ladder of strand 2 RNA; lane 3, hydrolysis ladder of the RNA−RNA cross-link. The sequence of the strand 2 of the duplex TAR from U38 to U42 is labeled, and a gap in the sequence is obvious after residue C41, indicating that U42 is the cross-linked base. (B) Sequencing of the RNA−RNA cross-link containing 5′-end-labeled strand 2 in the duplex modified with 6-thioG at position 26: lane 1, *B. cereus* ladder of strand 2 RNA; lane 2, hydrolysis ladder of strand 2 RNA; lane 3, hydrolysis ladder of the RNA−RNA cross-link; lane 4, hydrolysis ladder of the RNA−RNA cross-link in the presence of L-argininamide; lane 5, hydrolysis ladder of the RNA−RNA cross-link in the presence of Tat(38−72). The sequence of the strand 2 of the duplex TAR from U34 to C38 is labeled, and a gap in the sequence is obvious after residue U38, indicating that C39 is the cross-linked base.

duplex was used for RNA−protein cross-linking reactions (data not shown). Therefore, we conclude that formation of a specific RNA−protein complex between 6-thioG-modified TAR RNA and Tat is necessary for photo-cross-linking.

Mapping the RNA−RNA Cross-Links. High-resolution mapping of the RNA−RNA cross-links was accomplished by using 5′-end-labeled strand 2 and subjecting the cross-linked RNA to partial alkaline hydrolysis in parallel with an un-cross-linked control sample (*50*, *51*). Hydrolysis products of RNA were analyzed on denaturing polyacrylamide gels. The hydrolysis ladder of the cross-linked sample is identical to that of un-cross-linked control RNA up to the point of cross-linking because in both RNAs the released 5′-end-labeled hydrolysis fragments are linear. Beyond the cross-linking site, the hydrolysis fragments from the cross-linked RNA are nonlinear and migrate anomalously slowly, introducing a gap in the ladder relative to the un-cross-linked control RNA. The position of this gap defines the site of cross-linking reaction. The precise cross-linked nucleotide is determined by base-specific enzymatic cleavage of un-cross-linked RNA. Results of these experiments are presented below.

(i) 6-ThioG at G21 Cross-Links to U42. To locate the cross-link site in this RNA−RNA cross-link, we labeled the strand 2 (complementary to the 6-thioG-containing strand) of the duplex with ^{32}P at the 5′-end, UV-irradiated, and purified the cross-link by denaturing gels. The cross-link site was mapped by partial RNase digestion and alkaline hydrolysis of the gel-purified cross-link. As shown in Figure 8A, base hydrolysis of the cross-link produces an RNA ladder in which all fragments up to C41 are resolved. There is an obvious gap in the hydrolysis ladder after C41, indicating that U42 is the cross-linked base on strand 2 of the TAR

RNA duplex (Figure 8A, lane 3). Base hydrolysis of nonirradiated RNA duplex showed no gaps in these positions, including U40 in the sequence (Figure 8A, lane 2). Thus, we conclude that 6-thioG21 forms an interstrand cross-link with U42 in the TAR RNA duplex.

(ii) 6-ThioG at G26 Cross-Links to C39. To map the cross-link site in this RNA−RNA cross-link, we labeled strand 2 (complementary to the 6-thioG-containing strand) of the duplex with ^{32}P at the 5′-end, UV-irradiated, purified, and sequenced as described above. As shown in Figure 8B, base hydrolysis of the cross-link produces an RNA ladder in which all fragments up to U38 are resolved. There is an obvious gap in the hydrolysis ladder after U38, indicating that C39 is the cross-linked base on strand 2 of the TAR RNA duplex (Figure 8B, lane 3). Base hydrolysis of nonirradiated RNA duplex showed no gaps in these positions, including C39 in the sequence (Figure 8B, lane 2).

To determine the effect of Tat and arginine on RNA−RNA cross-linking, we carried out the cross-linking reaction in the presence of Tat(38−72) or 1 mM L-argininamide. The RNA−RNA cross-links in the presence of argininamide or Tat were purified and sequenced as described above. RNA sequencing results showed that the cross-linked sites were unchanged by the addition of Tat fragment or L-argininamide (Figure 8B, lanes 4 and 5). Thus, we conclude that 6-thioG26 forms an interstrand cross-link with C39 and this cross-link site is unchanged by the addition of Tat or L-argininamide.

Cross-Linking Occurs at Amino Acid(s) in the Core/Basic Region (Ala42−Arg57) of Tat. To identify the amino acid(s) of Tat that are involved in specific cross-linking with TAR RNA, a cross-linked RNA−peptide complex with RNA labeled at G21 with 6-thioG was prepared in preparative scale (see Experimental Procedures), purified from non-cross-linked TAR RNA by denaturing gels, and digested with trypsin. RNA modified with 6-thioG at position 21 was chosen for these experiments because this RNA produced high yields of RNA−protein cross-links and no RNA−RNA cross-links in the presence of Tat (Figures 3 and 4). The digestion products were purified by 8 M urea−20% acrylamide gels and visualized by autoradiography. We carried out a tryptic digest on the RNA−protein cross-link, which gave a new band on the gel with electrophoretic mobility slightly faster than the cross-link but slower than the free RNA (data not shown). We recovered ∼150 pmol quantities of the tryptic fragment of the cross-link and subjected it to N-terminal sequencing. The amino acid sequencing data showed that it had a sequence of Ala-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg. This sequence matches residues 42−57 of Tat protein. Protein sequence analyses did not identify a nonstandard amino acid cycle that might be obtained from a covalently modified residue. This is not an unexpected result due to the length of the fragment and relatively harsh conditions of peptide sequencing, which could result in the destruction of the cross-link between the peptide and RNA (*52*). In a previous study from our laboratory, we used 4-thioU-modified TAR RNA to form Tat−TAR cross-links and we were unable to locate the cross-linked amino acid by Edman protein sequencing (*32*). These results indicate that an amino acid (or acids) from the Ala42−Arg57 region of Tat contacts O^6 of G21 in the major groove of TAR RNA.

DISCUSSION

We have used a site-specific photo-cross-linking strategy to determine TAR RNA conformation in solution and its interactions with Tat protein. Photoactive analogues have been previously incorporated into DNA and RNA sequences to study nucleic acid structure and their interactions with proteins (*52*−*61*). Thio-modified nucleoside analogues are excellent molecules to probe RNA structure. For example, 4-thioU is a very useful photoactivatable probe that has been widely used to study RNA structure (for an excellent review, see ref *62*). In addition to 4-thioU, DNA sequences containing 6-thiodeoxyguanosine have been previously synthesized and used to probe DNA interactions with *Eco*RV restriction endonuclease and modification methylase (*63*). In another study, DNA containing 6-thio-dG at specific sites has been successfully used to analyze Max−DNA interactions [Q. Dong, A. Broom, Y. Ebright, and R. Ebright, personal communication]. Recently, 6-thioG was used to study dynamics of RNA−protein interactions in the HIV-1 Rev−RRE complex (*39*). In this study, we incorporated 6-thioG at position 21 or 26 in TAR RNA and used this modified RNA to probe RNA dynamics and RNA−protein interactions in the Tat−TAR complex. Our results show that in free TAR RNA, 6-thioG at position 21 formed a cross-link with U42 while 6-thioG at position 26 is in close contact with C39. Tat(38−72) formed cross-links with both TAR RNA constructs containing 6-thioG at position 21 or 26. Our results therefore provide direct evidence that, during RNA−protein recognition, Tat is in close proximity to O^6 of G21 and G26 in the major groove of TAR RNA.

Tat protein recognizes the trinucleotide bulge in TAR RNA. Key elements required for TAR recognition by Tat have been defined by extensive mutagenesis, chemical probing, and peptide binding studies (*21*, *23*, *30*, *31*, *42*, *46*, *64*−*68*). Tat interacts with U23 and two other bulge residues, C24 and U25, act as spacers because they can be replaced by other nucleotides or linkers (*22*, *30*). In addition to the trinucleotide bulge region, two base pairs above and below the bulge also contribute significantly to Tat binding (*30*, *65*). Phosphate contacts below the bulge at positions 22, 23, and 40 are critical for Tat interactions (*23*, *30*, *67*). In a chemical cross-linking study, Gait and co-workers showed that a TAR duplex containing a trisubstituted pyrophosphate replacing the phosphate at 38−39 reacted specifically with Lys51 in the basic region of Tat(37−72) peptide (*33*). Recently, we carried out 4-thiouracil-mediated photo-cross-linking studies on Tat−TAR complex and showed that Tat interacts with U23, U38, and U40 in the major groove of TAR RNA (*32*). Our cross-linking results presented here demonstrate that Tat interaction with G21 and G26 also takes place in the major groove of RNA. Taken together, these studies establish that Tat binds TAR RNA at the trinucleotide bulge region and interacts with two base pairs above and below the bulge in the major groove of RNA.

When G26 was substituted with 6-thioG, it cross-linked to C39 while 6-thioG at position 21 formed a cross-link with U42. Between the trinucleotide bulge and the loop region (residues G26−C29), TAR RNA has an A-form double helix (*34*, *35*, *47*, *48*). Therefore, it is not surprising that 6-thioG26 cross-linked to C39 because they are base-paired. However, the 6-thioG21 did not cross-link to its complementary

nucleotide; rather, it formed a cross-link with U42. We suggest two explanations to interpret this result. (1) Recent NMR and photo-cross-linking studies suggest that the A22·-U40 base pair is not stable in TAR RNA (*32*, *34*). Due to unstacking of U40, the G21·C41 base pair is destabilized and dynamics of RNA structure results in a favorable photoreaction between 6-thioG21 and U42. Protein binding stabilizes base-pairing in the lower stem helical region and inhibits this interaction. (2) The cross-linking reaction depends on the orientation and the efficiency of photoreaction between two bases in close proximity. In this case, the photoreaction between 6-thioG at position 21 and functional groups of U42 is more efficient than those of C41. The observation of a good photoreaction between 6-thioG26 and C39 supports our first explanation.

The addition of Tat completely abolished the G21−U42 RNA−RNA cross-link formation and only RNA−protein cross-link was observed. This is likely due to the change in RNA structure by stabilization of the lower stem region upon protein binding and efficient photoreaction between G21 and the interacting amino acid. The G26−C39 RNA−RNA cross-link formation occurred in the presence and absence of Tat; however, the cross-link yields were decreased in the presence of Tat because protein side chains can react with 6-thioG and compete with the RNA−RNA cross-linking reaction. In agreement with NMR studies (*34*, *35*), this result suggests that G26 is base-paired with C39 in free TAR and there is not a drastic structural change in RNA upon Tat binding. Similar results were obtained in the presence of L-arginine for G26−C39 RNA−RNA cross-linking reaction. It is interesting to note that arginine inhibited G26−C39 cross-link formation and had no significant effect on the G21−U42 cross-linking reaction. NMR studies showed that arginine binding to TAR RNA induces a specific pattern of chemical shift changes (*34*). The N7 of G26 and the N1 of U23 undergo upfield shifts of over 3 ppm that are much greater than any observed elsewhere in the molecule (*34*). These interactions place the guanidinium and ϵNH groups within hydrogen-bonding distance of G26−N7. There is no evidence of arginine interaction with G21 by NMR experiments. Our results are in agreement with NMR data and we observe arginine interaction with G26 and not with G21. Given the weak binding of arginine to TAR RNA ($K_d \sim 10^{-3}$ M) and a fast exchange rate in the TAR−Arg complex, our photo-cross-linking results are remarkable and demonstrate that these methods can be applied to trap fast and dynamic interactions.

Enzymatic digest and protein sequencing studies showed that amino acid(s) from the Ala42−Arg57 region of Tat are involved in cross-linking with 6-thioG21. It is an interesting result that trypsin cleaved after Lys41 and Arg57 in the Tat peptide present in the RNA−protein cross-link while other Lys and Arg residues in the basic region were not cleaved. It is quite possible that only Lys41 and Arg57 were exposed and accessible by the enzyme in a Tat−TAR cross-linked complex. This protection of the basic sequence of Tat suggests that the arginine region is covered by TAR RNA in a Tat−TAR complex. Recent chemical cross-linking studies also showed that Lys51 of Tat(37−72) peptide reacts specifically with TAR RNA duplex containing a trisubstituted pyrophosphate replacing a phosphate at 38−39 (*33*). These findings are consistent with a recent model proposed for Tat−TAR recognition suggesting that Tat makes initial contacts with TAR RNA in the widened major groove and causes a structural change in RNA where TAR RNA appears to be folded around the Tat peptide (*69*).

Our results provide new insights into tertiary folding of TAR RNA and its interaction with Tat under physiological conditions. These studies are crucial for understanding the mechanism of gene regulation in HIV-1 and designing new unnatural peptides to inhibit Tat−TAR complex formation (*70*).

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