RNA Conformation in the Tat−TAR 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 transactivation responsive region (TAR) RNA, a 59-base stem−loop structure located at the 5′-end of all mRNAs. We have used a site-specific cross-linking method based on 4-thiouracil (4-thioU) photochemistry to determine the conformation of TAR RNA and its interaction with Tat protein under physiological conditions. Three different TAR RNA constructs with a single 4-thioU residue at position 23, 38, or 40 were synthesized. Upon UV irradiation, 4-thioU at all three positions formed interstrand covalent cross-links in TAR RNA. Determination of cross-link sites by RNA sequencing revealed that 4-thioU at position 23 makes a direct contact with U40, while a 4-thioU at position 40 cross-links to C24 and C25, and at position 38, 4-thioU contacts G26 in TAR RNA. The addition of arginine did not alter the yield or the site of RNA−RNA cross-links. However, in the presence of Tat(38−72), UV irradiation of RNA modified with 4-thioU at position 23 or 38 resulted in RNA−protein cross-links, but no RNA−RNA cross-links were observed. 4-thioU at position 40 formed both RNA−RNA and RNA−protein cross-links in the presence of Tat(38−72). An intriguing finding of our studies was that a cross-linked TAR RNA with 4-thioU at position 40 retained specific Tat-binding activity. Our results establish four important conclusions about Tat−TAR structure. (1) U23 of free TAR RNA is in close contact with U40. (2) U40 is in close proximity to C24 and C25 both in free TAR RNA and in a complex with Tat. (3) Tat protein directly contacts U23, U38, and U40 in the major groove of TAR RNA. (4) Tat protein can recognize a TAR RNA structure containing an interrupted bulge which is formed by a covalent link between U40 and two bulge residues, C24 and C25. These structural studies provide new insights into tertiary folding of TAR RNA and its interaction with Tat protein.

RNA molecules can fold into extensive structures containing regions of double-stranded duplex, hairpins, internal loops, bulged bases, and pseudoknotted structures (Tinoco *et al*., 1990; Wyatt *et al*., 1989). Due to the complexity of RNA structure, the rules governing sequence-specific RNA−protein recognition are not well-understood. RNA−protein interactions are vital for many regulatory processes, especially in gene regulation where proteins specifically interact with binding sites found within RNA transcripts. One example of such interactions is the mechanism of transactivation in the human immunodeficiency virus type 1 (HIV-1). The promoter of the HIV-1, located in the U3 region of the viral long terminal repeat (LTR), is an inducible promoter which can be stimulated by the transactivator protein, Tat (Jones & Peterlin, 1994). As in other lentiviruses, Tat protein is essential for transactivation of viral gene expression (Cullen, 1992; Dayton *et al*., 1986; Fisher *et al*., 1986; Gaynor, 1992; Jeang *et al*., 1993). 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 (transactivation responsive) RNA element, a 59-base stem−loop structure located at the 5′-ends of all nascent HIV-1 transcripts (Berkhout *et al*., 1989). Upon binding to the TAR RNA sequence, Tat causes a substantial increase in transcript levels (Cullen, 1986; Laspia *et al*., 1989; Muesing *et al*., 1987; Peterlin *et al*., 1986; Rice & Mathews, 1988). The increased efficiency in transcription may result from preventing premature termination of the transcriptional elongation complex (Churcher *et al*., 1995; Graeble *et al*., 1993; Kao *et al*., 1987; Marciniak & Sharp, 1991; Zhou & Sharp, 1995). TAR RNA was originally localized to nucleotides +1 to +80 within the viral long terminal repeat (LTR) (Rosen *et al*., 1985). 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* (Garcia *et al*., 1989; Jakobovits *et al*., 1988; Selby *et al*., 1989). TAR RNA contains a six-nucleotide loop and a three-nucleotide pyrimidine bulge which separates two helical stem regions (Berkhout *et al*, 1989; Jakobovits *et al*, 1988; Muesing *et al*, 1987; Rosen *et al*, 1985). The trinucleotide bulge is essential for high affinity and specific binding of the Tat protein (Dingwall *et al*., 1989, 1990). The loop region is required for *in vivo*

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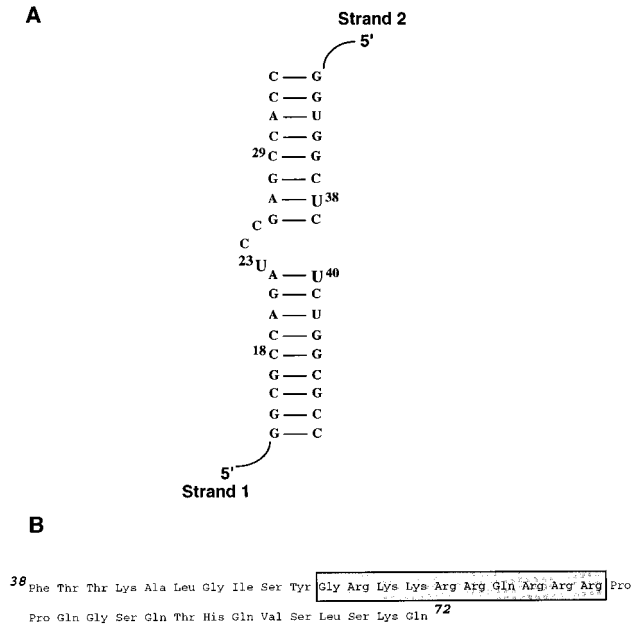

FIGURE 1: (A) The secondary structure of the designed duplex TAR RNA used in this study contains the minimal sequence required for *in vitro* Tat binding (Hamy *et al*, 1993). Duplex RNA contains the nucleotides C18−C29 and G36−G44 from the wild-type TAR RNA sequence and extra flanking base pairs to enhance hybridization of two RNA strands. Sequences containing the trinucleotide bulge and the complementary sequence are labeled as strand 1 and strand 2, respectively. Uridine 23 was substituted with 4-thiouridine during T7 polymerase transcription reactions. Incorporation of 4-thioU at position 38 or 40 was accomplished by chemical synthesis of RNA (Shah *et al*, 1994). Numbering of nucleotides in the duplex TAR RNA corresponds to their positions in wild-type TAR RNA. (B) The 36-residue peptide, amino acids 38−72, contains the RNA-binding domain of Tat. The arginine rich region of Tat, corresponding to the Tat 48−57 peptide, is highlighted.

transactivation but is not involved in Tat binding (Cordingley *et al*., 1990; Dingwall *et al*, 1990; Feng & Holland, 1988; Sumner-Smith *et al*., 1991). 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 (Hamy *et al*., 1993; Pritchard *et al*., 1994; Sumner-Smith *et al*, 1991).

We synthesized three different duplex TAR RNA constructs (Figure 1) with a single 4-thiouridine (4-thioU) residue at position 23, 38, or 40. Upon UV irradiation, these 4-thioU-labeled RNAs formed interstrand covalent cross-links in TAR RNA. Cross-link sites were determined by RNA sequencing, which revealed that 4-thioU at position 23 makes direct contact with U40 whereas at position 40 4-thioU cross-linked to both C24 and C25 residues in the bulge region. 4-thioU at position 38 photo-cross-linked to G26 in TAR RNA. To study the effect of free Arg and a Tat peptide on TAR RNA conformation, photo-cross-linking reactions were carried out in the presence of L-Arg and ADP-1, a Tat fragment which binds TAR RNA with high specificity and includes the basic region and part of the core region of Tat (Churcher *et al*., 1993). To avoid oxidation problems, we deleted the N-terminal cysteine residue from the ADP-1 sequence. Our results showed that the sites of cross-linking were unchanged in the presence of L-Arg. However, in the presence of Tat(38−72), UV irradiation of RNA modified with 4-thioU at position 23 or 38 resulted in RNA−protein cross-links, but no RNA−RNA cross-links were observed. 4-thioU at position 40 formed both RNA−RNA and RNA−protein cross-links in the presence of Tat-(38−72); however, the yields of RNA−RNA cross-links were decreased. Our results provide the first physical evidence for a Tat−TAR structure where U40 is located in the vicinity of the trinucleotide bulge 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 1X TBE buffer; binding buffer, 25 mM Tris-HCI (pH 7.5), 100 mM NaCI, 1 mM $MgCI_2$, and 0.1% Triton X-100; hydrolysis buffer, 50 mM Na_2CO_3−$NaHCO_3$ (pH 9.2); elution buffer, 1X TBE and 20% sodium acetate (3 M) (pH 5.5); and digestion buffer, 100 mM Tris-HCl (pH 7.8) and 10 mM $CaCl_2$.

Oligonucleotide Synthesis

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′TAATACGACTCACTATAG3′. DNA was deprotected in NH_4OH at 55 °C for 8 h and then dried in a Savant speedvac. 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 0.6 M sodium acetate. DNAs were ethanol precipitated and resuspended in DEPC (diethyl pyrocarbonate)-treated water. Concentrations of DNAs were determined by measuring absorbance at 260 nm in a Shimadzu UV spectrophotometer.

RNAs. All RNAs were prepared by *in vitro* transcription (Milligan *et al*., 1987). 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 reaction mixtures (20 μL) containing 8.0 pmol of template DNA, 40−60 units of T7 polymerase (Promega) was used. For the synthesis of 4-thioU-labeled RNA, UTP was replaced with 4-thioUTP (4 mM, final concentration) in the transcription buffer. 4-thio-UTP was synthesized according to the method of Stade *et al.* (1989). 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 30 min 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 (Kuchino & Nishimura, 1989). RNAs were gel purified on a denaturing gel, visualized by autoradiography, and recovered from gels as described above.

Site-specific incorporation of 4-thioU at position 38 or 40 in strand 2 was carried out by chemical synthesis of RNA using triazolouridine phosphoramidite as a precursor of 4-thioU (Shah *et al*., 1994). Synthesis of RNA, deprotection, characterization, and purification were performed as described earlier (Shah *et al*, 1994).

Site-Specific Photo-Cross-Linking Reactions

Before the photochemical reactions, RNA duplex was prepared by hybridizing two strands. Typically, 5 pmol of strand 1 or 2 RNA (5′ end labeled with ^{32}P) was mixed with 7.5 pmol of the complementary RNA strand in 10 μL of TK buffer, and 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 was UV irradiated (360 nm) for 5 min on ice in a Rayonet Photochemical Reactor (RPR-100). To study the effect of 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 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 mixture was electrophoresed on 8 M urea−20% acrylamide gels.

For kinetic experiments, 10 μL of preformed duplex RNA (0.5 μM) was irradiated for different time intervals as indicated in Figure 7. After irradiation, samples were analyzed on 8 M urea gels and quantified as described above.

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 chymotrypsin (sequencing grade, Boehringer Mannheim Biochemica) was added to the mixture and incubated for 4 h at 25 °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 and 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 picomole 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 phosphor image analysis.

Competition Assay

RNA−RNA cross-link formed by 4-thioU at position 40 was purified by denaturing gels. Competition experiments were performed in 10 μL volumes and contained 5 pmol of the end-labeled cross-linked RNA, 5 pmol of Tat(38−72), and increasing amounts of wild-type TAR or a mutant TAR duplex. Binding experiments were carried out in TK buffer. After addition of 10 μL of 30% glycerol, RNA−protein complexes were resolved on a nondenaturing 8% acrylamide gel.

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 HMP-linked polystyrene resin were obtained from Applied Biosystems Division, Perkin-Elmer. Trifluoroacetic acid, 1,2-ethanedithiol, phenol, and thioanisol were from Sigma. Tat-derived peptide (from amino acid 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 (King *et al*., 1990). After cleavage from the resin, peptide was purified by HPLC on a Zorbax 300 SB-C_8 column. The masses of fully deprotected and purified peptides were confirmed by FAB mass spectrometry; the calculated mass for Tat(38−72) ($C_{175}H_{298}N_{64}O_{43}$) = 4082.7, and the found mass 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 analog (4-thioU) at three critical residues, U23, U38, and U40. Photoactive analogs have been previously incorporated into DNA and RNA sequences to study nucleic acid structure and their interactions with proteins (Blatter *et al*., 1992; Gott *et al*., 1991; Hanna, 1989; Hanna & Meares, 1983; Harris *et al*., 1994; Liu *et al*., 1994; Musier-Forsyth & Schimmel, 1994; Willis *et al*., 1993; Wyatt *et al*., 1992; Yang & Nash, 1994). 4-thioU is a very useful photoactivatable probe which has been widely used to study RNA

structure (Döring *et al*., 1994; Dos Santos *et al*., 1993; Sontheimer & Steitz, 1993; Wollenzien *et al*., 1991). 4-thioU can be photoactivated at wavelengths >300 nm to form covalent cross-links to both RNA and proteins by a free radical mechanism (Brimacombe *et al*., 1988; Dos Santos *et al*, 1993). 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 (Hamy *et al*, 1993; Pritchard *et al*, 1994; Sumner-Smith *et al*, 1991). The duplex TAR sequence contained wild-type residues from C17 to C31. Flanking residues were added to achieve efficient annealing (Figure 1). Because U25 can be replaced with C or a propyl linker without the Tat binding being affected (Churcher *et al*, 1993; Delling *et al*., 1992), 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. Three different duplex TAR RNA constructs with a single 4-thioU residue at positions 23, 38, or 40 were synthesized. Labeling of strand 1 with 4-thioU at position 23 was accomplished during the transcription reaction in the presence of 4-thioUTP. Site-specific incorporation of 4-thioU at position 38 or 40 in strand 2 was carried out by chemical synthesis of RNA using triazolouridine phosphoramidite as a precursor of 4-thioU (Shah *et al*, 1994). Chemical syntheses of RNA, deprotection, characterization, and purification were performed as described earlier (Shah *et al*, 1994). After formation of a duplex TAR structure, UV 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.

Duplex TAR RNA Labeled with 4-thioU at U23 Forms an Interstrand RNA−RNA Cross-Link upon UV Irradiation

The 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 2). Irradiation of duplex TAR RNA yields an interstrand RNA cross-link with electrophoretic mobility less than that of strand 1 RNA (Figure 2, lane 2). The efficiencies of the photo-cross-linking reactions were very high, ≈45%.

It has been suggested by competition, chemical interference, and NMR experiments that free arginine interacts with TAR RNA in a manner similar to that of arginine in the context of short Tat peptides (Aboul-ela *et al*., 1995; Puglisi *et al*., 1992, 1993; Tan & Frankel, 1992; Tao & Frankel, 1992). 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-arginine (Figure 2, lanes 3 and 4). The electrophoretic mobilities and the yields of the cross-link products were unchanged in the presence or absence of arginine. These results show that the addition of L-arginine did not significantly change the RNA conformation.

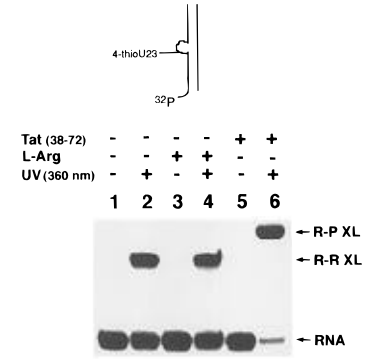

FIGURE 2: Cross-linking reactions of TAR RNA duplex labeled with 4-thioU at position 23. Strand 1 was labeled with ^{32}P at the 5′-end, annealed to its complement strand 2, and UV irradiated at 360 nm. Cross-linked products were resolved on a 20% acrylamide−8 M urea gel and visualized by autoradiography: lane 1, duplex RNA without irradiation; lane 2, duplex RNA with irradiation; lane 3, duplex RNA with 1 mM L-Arg and no irradiation; lane 4: duplex RNA irradiated with 1mM L-Arg; lane 5, duplex RNA with Tat(38−72) and no irradiation; and lane 6, duplex irradiated in the presence of Tat(38−72). The RNA−RNA cross-link and RNA−Peptide cross-link are indicated by R−R XL and R−P XL, respectively.

Duplex TAR RNA Labeled with 4-thioU at U23 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 (data not shown) only one slow-migrating RNA−peptide complex, indicating the absence of other nonspecific RNA−peptide complex formation (Churcher *et al*, 1993; Wang & Rana, 1995). 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 Experimental Procedures). Products of the photoreaction were analyzed by denaturing 8 M urea PAGE (Figure 2). 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 (Figure 2, lane 6). Both the peptide and UV irradiation are required for the formation of this cross-linked RNA−protein complex. This is shown by the fact that this cross-linked product is observed only when RNA is irradiated in the presence of Tat peptide (lane 6), and no cross-link is formed with peptide in dark without UV irradiation (lane 5). The formal possibility that the new mobility is due to an alternative RNA−RNA cross-link is ruled out by experiments presented below (Figure 3). The products of irradiation were also analyzed on a denaturing sodium dodecyl sulfate (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 yield is ≈90% as determined by a phosphor image 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.

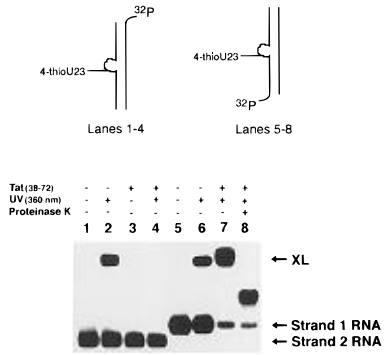

FIGURE 3: Determining the effect of Tat peptide on the RNA−RNA cross-linking reaction of TAR RNA duplex containing 4-thioU at position 23. Photoreactions of the duplex containing 5′-^{32}P-end-labeled strand 2 (lanes 1−4): lane 1, duplex RNA without irradiation; lane 2, duplex RNA with irradiation; lane 3, duplex RNA with Tat(38−72) and no irradiation; and lane 4, duplex RNA irradiated in the presence of Tat(38−72). Photoreactions of the duplex containing 5′-^{32}P-end-labeled strand 1 (lanes 5−8): lane 5, duplex RNA without irradiation; lane 6, duplex RNA with irradiation; lane 7, duplex RNA irradiated in the presence of Tat-(38−72); and lane 8, duplex RNA irradiated in the presence of Tat(38−72) and the sample treated with proteinase K for 15 min at 55 °C. RNA−RNA and RNA−protein cross-links are indicated as XL.

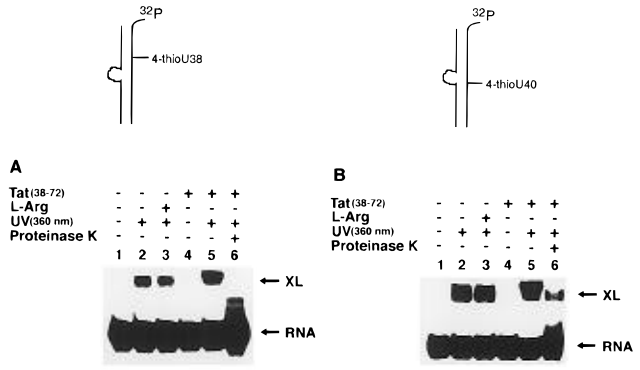

FIGURE 4: Cross-linking reactions of TAR RNA duplex modified with 4-thioU at position 38 (A) or position 40 (B). 4-thioU containing strand 2 was labeled with ^{32}P at the 5′-end, annealed to its complement strand 1, and UV irradiated at 360 nm. Cross-linked products were resolved on a 20% acrylamide−8 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-Arg; lane 4: duplex RNA with Tat-(38−72) and no irradiation; lane 5: duplex irradiated in the presence of Tat(38−72); lane 6, duplex RNA irradiated in the presence of Tat(38−72) and the sample treated with proteinase K for 15 min at 55 °C. RNA−RNA and RNA−protein cross-links are indicated by XL.

Formation of an RNA−RNA Cross-Link by 4-thioU at U23 Occurs in the Absence of Tat

To confirm that TAR RNA changes its conformation in the presence of Tat fragment and that there are no RNA−RNA cross-link products formed, we prepared two duplex TAR RNAs containing labeled strand 1 or strand 2 of the duplex at the 5′-end with ^{32}P and UV irradiated in the presence and absence of Tat(38−72). Results of these experiments are shown in Figure 3. 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). When strand 1 of the duplex was labeled, both the RNA−RNA and RNA−protein cross-links were observed, as expected (lanes 6 and 7). Proteinase K digestion of the RNA−protein cross-link resulted in a new band on the gel migrating slower than the free RNA and faster than the RNA−RNA and RNA−protein cross-links (lane 8). From these results, we conclude that RNA−RNA cross-link formation occurs in the absence of Tat protein, the protein forms a cross-link with strand 1 (4-thioU containing RNA), and an RNA−protein cross-link is the only photoproduct in the presence of Tat protein.

TAR RNA Labeled with a Single 4-thioU at either U38 or U40 Forms RNA−RNA and RNA−protein Cross-Links

4-thioU at position U38 or U40 was incorporated into strand 2 of the duplex during chemical syntheses of RNA as described earlier (Shah *et al*, 1994). The strand 2 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 strand 1 and strand 2 were 0.75 and 0.5 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 4). Interstrand RNA−RNA cross-link species with electrophoretic mobility less than that of strand 2 RNA were observed when these duplex RNAs were irradiated (Figure 4). The efficiencies of the photo-cross-linking reactions were very high (≈50%) when 4-thioU was placed at U40 as compared to when it was placed at U38 which cross-linked with lower efficiencies.

The effect of L-Arg on TAR RNA folding was determined by performing UV cross-linking reactions in the presence of 1 mM L-Arg or L-argininamide. Apparent electrophoretic mobilities and the yields of the cross-link products were unaffected by the presence of arginine (lane 3, Figure 4A,B). These results show that the addition of L-arginine did not significantly change the RNA conformation.

RNA−protein cross-links were observed when these two duplex RNAs were irradiated in the presence of a Tat peptide (38−72). Strand 2 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 UV irradiated with 360 nm light (see Experimental Procedures). Products of the photoreaction were analyzed by denaturing 8M urea PAGE (Figure 4). Irradiation of the RNA−peptide complex yields a new band with electrophoretic mobility less than that of strand 2 and the RNA−RNA cross-link (Figure 4 A,B, lane 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 4, Figure 4 A,B). Proteinase K digestion of the RNA−protein cross-link resulted in a loss of RNA−protein cross-link and a gain in free RNA as observed by band intensities on the gel (lane 6, Figure 4 A,B). As shown in Figure 4A (lane 6), proteinase K digestion of the RNA−protein cross-link with 4-thioU at U38 was complete and no remaining RNA−protein complex was observed on the gel. On the other hand, proteinase K digestion of RNA−protein cross-link with 4-thioU at U40 left a remaining band corresponding to an RNA−RNA cross-linked complex (Figure 4B, lane 6). Further confirmation that this remaining band is indeed an RNA−RNA cross-link in the presence of Tat is demonstrated by experiments presented below (Figure 5B).

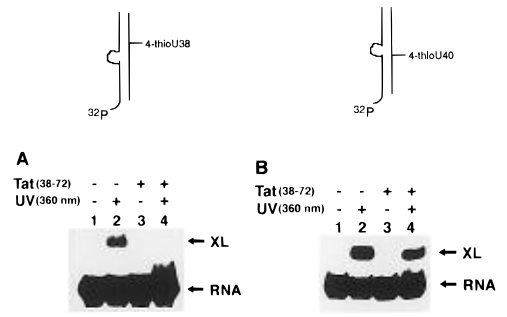

FIGURE 5: Determining the effect of a Tat fragment on the RNA−RNA cross-linking reaction of TAR RNA duplex labeled with 4-thioU at position 38 (A) or position 40 (B). Strand 1 of the duplex was 5′-end-labeled with ^{32}P in these experiments: lane 1, duplex RNA without irradiation; lane 2, duplex RNA with irradiation; lane 3, duplex RNA with Tat(38−72) and no irradiation; and lane 4, duplex RNA irradiated in the presence of Tat(38−72). RNA−RNA cross-links are indicated by XL.

Effect of Tat on RNA−RNA Cross-Link Formation

To further elucidate the effect of a Tat fragment on TAR RNA folding, we prepared two duplex TAR RNAs containing a single 4-thioU at either position 38 or 40, labeled strand 1 of the duplex at the 5′-end with ^{32}P, and UV irradiated the sample in the presence and absence of Tat(38−72). Since strand 1 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 5. When strand 1 of the duplex containing 4-thioU at position 38 in TAR RNA was labeled, an RNA−RNA cross-link product was formed in the absence of Tat peptide (Figure 5A, lane 2). There was no RNA−RNA cross-link product observed after the addition of the Tat peptide to the duplex (Figure 5A, lane 4). When strand 1 of the duplex containing 4-thioU at position 40 in TAR RNA was labeled, RNA−RNA cross-links were observed in the presence and absence of Tat peptide (Figure 5B, lanes 2 and 4). However, the yields of the RNA−RNA cross-link were reduced by the addition of Tat, as expected (Figure 5B, lane 4). From these results, we conclude that (i) for a duplex with 4-thioU at U38, RNA−RNA cross-link formation occurs only in the absence of Tat protein and (ii) for a duplex with 4-thioU at U40 RNA−RNA cross-linking takes place in the presence and absence of Tat protein.

Mapping the RNA−RNA Cross-Links

(i) 4-thioU at U23 Cross-Links to U40. To locate the cross-link site in this RNA−RNA cross-link, we labeled the strand 2 (complementary to the 4-thioU-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 6A, base hydrolysis of the cross-link produces an RNA ladder in which all fragments up to C39 are resolved. There is an obvious gap in the hydrolysis ladder after C39, indicating that U40 is the cross-linked base on strand 2 of the TAR RNA duplex (Figure 6A, lane 3). Base hydrolysis of non-irradiated RNA duplex showed no gaps in these positions, including U40 in the sequence (Figure 6A, lane 2).

To determine the effect of L-arginine on TAR RNA folding, we carried out the cross-linking reaction in the presence of 1 mM L-arginine. The RNA−RNA cross-link

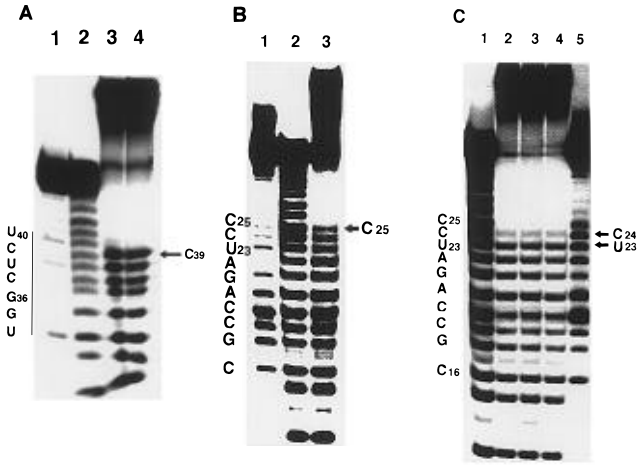

FIGURE 6: Mapping of the cross-linked base in the RNA−RNA cross-linked complexes by alkaline hydrolysis. (A) Analysis of the RNA−RNA cross-link containing 5′-end-labeled strand 2 in the duplex modified with 4-thioU at position 23: *B. cereus* ladder of strand 2 RNA (lane 1), hydrolysis ladder of strand 2 RNA (lane 2), hydrolysis ladder of the RNA−RNA cross-link (lane 3), and hydrolysis ladder of the RNA−RNA cross-link in the presence of L-Arg (lane 4). The sequence of the strand 2 of the duplex TAR from U34 to U40 is labeled, and a gap in the sequence is obvious after the C39 residue, indicating that U40 is the cross-linked base. (B) Sequencing of the RNA−RNA cross-link containing 5′-end-labeled strand 1 in the duplex modified with 4-thioU at position 38: *B. cereus* ladder of strand 1 RNA (lane 1), hydrolysis ladder of strand 1 RNA (lane 2), and hydrolysis ladder of the RNA−RNA cross-link (lane 3). The sequence of the strand 1 of the duplex TAR from C16 to C25 is labeled, and a gap in the sequence is obvious after the C25 residue, indicating that G26 is the cross-linked base. (C) Sequencing of the RNA−RNA cross-link containing 5′-end-labeled strand 1 in the duplex modified with 4-thioU at position 40: hydrolysis ladder of strand 1 RNA (lane 1), hydrolysis ladder of the RNA−RNA cross-link (lane 2), hydrolysis ladder of the RNA−RNA cross-link in the presence of L-Arg (lane 3), hydrolysis ladder of the RNA−RNA cross-link in the presence of the Tat fragment (lane 4), and *B. cereus* ladder of strand 1 RNA (lane 5). The sequence of the strand 1 of the duplex TAR from C16 to C25 is labeled. A decrease in the band intensity is obvious after U23, and a gap in the sequence is obvious after the C24 residue, indicating that C24 and C25 were the cross-link sites. Quantitation of band intensities in the hydrolysis ladder revealed that ≈70% of the cross-linking reaction occurs at C24.

in the presence of arginine was purified and sequenced as described above. This cross-link also showed a specific gap in the hydrolysis ladder after C39, indicating that the cross-link site was U40 (Figure 6A, lane 4). Thus, we conclude that 4-thio-U23 forms an interstrand cross-link with U40 in the TAR RNA duplex in the presence and absence of L-arginine.

(ii) 4-thioU at U38 Cross-Links to G26. To determine the cross-link site in this RNA−RNA cross-link, we labeled the strand 1 (complementary to the 4-thioU-containing strand) of the duplex with ^{32}P at the 5′-end, UV irradiated, purified, and sequenced as described above. As shown in Figure 6B, base hydrolysis of the cross-link produces an RNA ladder in which all fragments up to C25 are resolved. There is an obvious gap in the hydrolysis ladder after C25, indicating that G26 is the cross-linked base on strand 1 of the TAR RNA duplex (Figure 6B, lane 3). Base hydrolysis of nonirradiated RNA duplex showed no gaps in these positions, including G26 in the sequence (Figure 6B, lane 2).

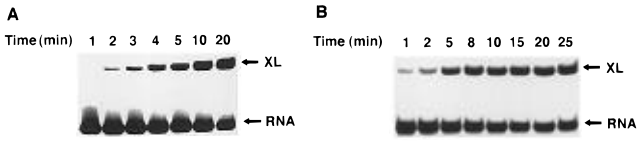

FIGURE 7: RNA−RNA cross-linking of the TAR duplex modified with 4-thioU at U23 (A) or U40 (B). RNA duplexes were irradiated for the indicated times and separated on a denaturing gel. Single-stranded and the cross-linked RNA are shown as RNA and XL, respectively.

(iii) 4-thioU at U40 Cross-Links to C24 and C25. To map the cross-link site in this RNA−RNA cross-link, we labeled the strand 1 (complementary to the 4-thioU-containing strand) of the duplex with ^{32}P at the 5′-end, UV irradiated, purified, and sequenced as described above. These experiments showed that there was one major and one minor cross-link site. As shown in Figure 6C, the major stop in the hydrolysis ladder was after U23, indicating that C24 is the cross-linked base. A complete gap in the hydrolysis ladder occurs after C24, indicating that C25 is also a cross-linked base on strand 1 of the TAR RNA duplex (Figure 6C, lane 2). Quantitation of band intensities in the hydrolysis ladder revealed that ≈70% of the cross-linking reactions invlove C24.

To determine the effect of Tat and L-Arg on RNA−RNA cross-linking, we carried out the cross-linking reaction in the presence of 1 mM L-arginine or L-argininamide. RNA-sequencing results showed that the cross-linked sites were unchanged by the addition of Tat fragment or L-arg (Figure 6C, lanes 3 and 4). Thus, we conclude that C24 is the major cross-link site which is unchanged by the addition of Tat or L-Arg.

Kinetics of RNA−RNA Cross-Link Formation

To further characterize the folding of TAR RNA in the trinucleotide bulge region, we determined the time dependence of two cross-linking reactions of RNA containing a single 4-thioU at position 23 or 40. Results of these experiments are shown in Figure 7. Photo-cross-linking reactions between two strands of TAR RNA were dependent on time of irradiation. The yields of cross-linked RNA species were increased with an increase in time of irradiation (Figure 7). After 20 min, there was no further increase in the cross-link yields. The rate of cross-linking reaction for a duplex containing 4-thioU at position 40 was approximately 2 times faster than for a duplex with 4-thioU at position 23.

Cross-Linked TAR RNA with 4-thioU at Position 40 Retains Specific Tat-Binding Activity

As shown in Figure 5B, a duplex with 4-thioU at U40 forms an RNA−RNA cross-link in the presence of Tat fragment. To determine whether this cross-linked RNA is representative of the native conformation, we measured the specific binding affinities of the cross-linked TAR RNA for Tat(38−72). The RNA−RNA cross-link was gel purified, renatured, and incubated with Tat(38−72) fragment. The specificity of the protein binding to the cross-linked RNA was established by competition experiments. Competition by wild-type and a mutant TAR RNA with the cross-linked RNA-peptide complex is illustrated in Figure 8. The wild-type TAR RNA competes efficiently for binding with the radiolabeled probe, while a mutant TAR RNA (lacking the trinucleotide bulge) does not compete as efficiently. Therefore, we conclude that the cross-linked TAR RNA with 4-thioU at U40 forms a specific RNA−protein complex with Tat(38−72). Remarkably, Tat protein can recognize a TAR RNA structure containing an interrupted bulge which is formed by a covalent link between U40 and two bulge residues, C24 and C25.

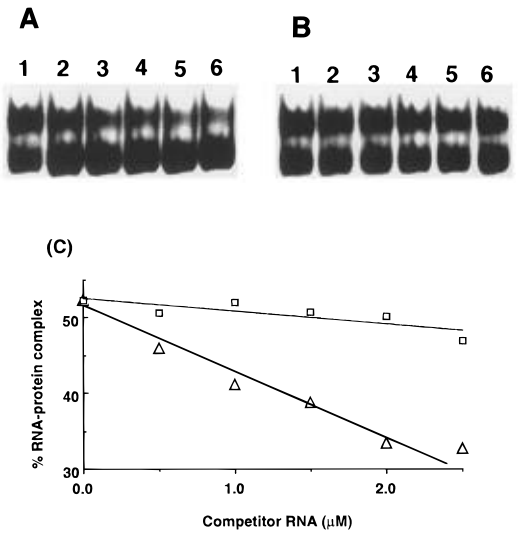

FIGURE 8: Specificity of Tat binding to an RNA−RNA cross-link formed by 4-thioU at position 40 determined by competition assays. Competition experiments were performed in 10 μL volumes and contained 0.5 μM end-labeled cross-link RNA, 0.5 μM Tat(38−72), and increasing amounts of wild-type TAR (A) or a mutant TAR duplex (B). Concentrations of the competitor RNA in lanes 1−6 were 0, 0.5, 1.0, 1.5, 2.0, and 2.5 μM, respectively. Binding experiments were carried out in TK buffer. After addition of 10 μL of 30% glycerol, RNA−protein complexes were resolved on a nondenaturing 8% acrylamide gel. (C) Quantitative analysis of competition experiments. The fraction of RNA in the RNA−peptide complex was determined by phosphor image analysis as described in Experimental Procedures: (△) wild-type TAR RNA competitor and (□) bulgeless mutant TAR RNA.

Cross-Linking Occurs at Amino Acid(s) in the Basic RNA-Binding Region of Tat

To identify the amino acid(s) of Tat which is involved in specific cross-linking with TAR RNA, a cross-linked RNA−peptide complex with RNA labeled at U23 with 4-thioU was prepared in preparative scale (see Experimental Procedure) purified from non-cross-linked TAR RNA by denaturing gels, and digested with trypsin and chymotrypsin. The digestion products were purified by 8 M urea−20% acrylamide gels and visualized by autoradiography. We carried out chymotryptic digestion on the RNA−protein cross-link which gave a new band on the gel with electrophoretic mobility slightly faster than that of the cross-link but slower than that of the free RNA (data not shown). We recovered ≈200 pmol quantities of a chymotryptic fragment of the cross-link, and subjected it to N-terminal sequencing. The amino acid-sequencing data showed that it had a sequence of Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln. This sequence matches residues 48−60 of Tat protein. Protein sequence analyses did not identify a nonstandard amino acid cycle which 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 (Musier-Forsyth & Schimmel, 1994).

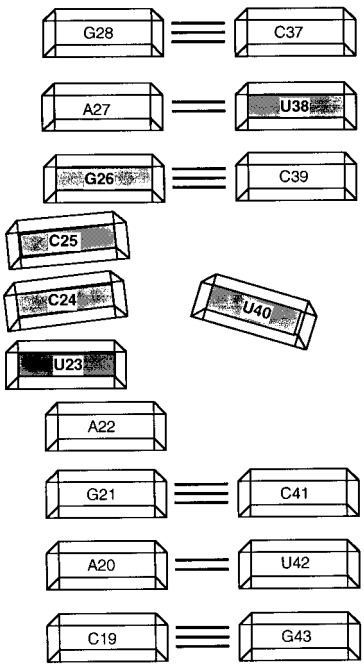

FIGURE 9: Proposed model for TAR RNA folding in the absence and presence of Tat. U23 of free TAR RNA is in close contact with U40. U40 is in close proximity to C24 and C25 both in free TAR RNA and in a complex with Tat. This does not imply that bases are hydrogen-bonded. In the protein−RNA complex, U23 is closer to G26 than in free RNA (Aboul-ela *et al*, 1995; Puglisi *et al*, 1992). Relative placement of C24 and C25 is derived from our cross-linking data. Residues shown in black are the cross-linked bases. Hydrogen bonds between base pairs are drawn as solid lines, two for A·U and three for G·C.

Trypsin digestion was used to infer the site of the cross-link in the Tat protein. Trypsin cleaves after Arg and Lys residues in a polypeptide chain. One or two amino acids covalently attached to RNA would have a negligible effect on the mobility of RNA. Treatment of the RNA−protein cross-link with trypsin resulted in a digestion product with electrophoretic mobility similar to that of free RNA (data not shown). If cross-linking reaction involves one of these amino acids in the arginine rich RNA-binding domain (49−57), trypsin cleavage would yield an RNA linked to one or two amino acids. This product would migrate with electrophoretic mobility similar to that of free RNA. This provides direct evidence that O^4 of U23 in the major groove of TAR RNA contacts the basic RNA-binding region of Tat protein.

DISCUSSION

We have used a site-specific cross-linking strategy to determine free TAR RNA conformation in solution and during its interactions with Tat protein. Our results establish four important conclusions about Tat−TAR structure. (1) U23 of free TAR RNA is in close contact with U40. (2) U40 is in close proximity to C24 and C25 both in free TAR RNA and in a complex with Tat. (3) Tat protein directly contacts U23, U38, and U40 in the major groove of TAR RNA. (4) Tat protein can recognize a TAR RNA structure containing an interrupted bulge which is formed by a covalent link between U40 and two bulge residues, C24 and C25. In light of our results, we suggest a model for TAR RNA (Figure 9). In free TAR RNA, U23 clearly interacts with U40. This precludes interaction of U40 with its complementary base, A22. Chemical and NMR studies support the notion that the A22·U40 base pair does not exist in free TAR RNA (Aboul-ela *et al*, 1995; Colvin & Garcia-Blanco, 1992). However, it has been suggested that the A22·U40 base pair is stabilized in the presence of Tat (Aboul-ela *et al*, 1995; Colvin & Garcia-Blanco, 1992). Our cross-linking experiments using 4-thioU modification at U40 showed that, in free TAR−RNA and the Tat−TAR complex, U40 interacts with C24 and C25 in the bulge region. Our data further show that the creation of an interrupted bulge by a cross-link formation between U40 and C24/C25 does not perturb the RNA structure recognized by Tat. These results strongly suggest that a stable A22·U40 base pair is not necessary in a Tat−TAR complex. We propose that, in free TAR−RNA and Tat−TAR complex, U40 is positioned in the trinucleotide bulge region and not base-paired with A22.

We do not see any RNA−RNA cross-link in the presence of Tat when TAR RNA was labeled with 4-thioU at position 23. The most logical conclusion is that an amino acid from the Tat sequence contacts U23 in the major groove of RNA and photoreacts with very high efficiencies. Another possible explanation is that the guanidinium group of an arginine from the Tat peptide interacts with G26 and draws U23 upward (Aboul-ela *et al*, 1995; Puglisi *et al*, 1992). Therefore, the inhibition of the RNA−RNA cross-link in the presence of Tat could be the result of both U23 displacement and efficient photoreaction between U23 and the interacting amino acid.

Between the trinucleotide bulge and the loop region (residues G26−C29), TAR RNA has an A-form double helix (Aboul-ela *et al*, 1995; Puglisi *et al*, 1992, 1993). In free TAR RNA, 4-thioU at U38 photoreacts with G26, although U38 is base-paired with A27. These data show that the cross-linking reaction depends upon the orientation and the efficiency of photoreaction between two bases in close proximity. In this case, the photoreaction between 4-thioU at U38 and functional groups of G26 is more efficient than those of A27. This finding could be explained by the inherently lower photoreactivity of adenosine. The addition of Tat completely abolished this RNA−RNA cross-link formation, and only RNA−protein cross-link was observed. The latter is likely due to efficient photoreaction between U38 and the interacting amino acid, as observed in U23-modified RNA.

The sites of the RNA−RNA cross-link and yields were not significantly affected by addition of L-Arg or L-argininamide. The finding that a single Arg at very high concentrations was unable to abolish RNA−RNA cross-link formation is probably due to weak binding of Arg to TAR RNA ($K_d \approx 10^{-3}$ M) and a fast exchange rate in the TAR−Arg complex.

Our findings presented here 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 drugs to inhibit Tat−TAR complex formation.

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