Dissection of the Proposed Base Triple in Human Immunodeficiency Virus TAR RNA Indicates the Importance of the Hoogsteen Interaction†

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Received September 9, 1996; Revised Manuscript Received December 27, 1996⊗

ABSTRACT: A single arginine residue within the RNA-binding domain of the human immunodeficiency virus Tat protein makes a critical sequence-specific contact to TAR RNA. Arginine as the free amino acid also binds specifically to TAR and induces a change in RNA conformation similar to that induced by Tat peptides. NMR and biochemical studies have suggested that the arginine-binding site is stabilized by a base triple interaction between a bulged U and an A·U base pair in the adjacent stem. In this study, we have used chemical modification and mutagenesis experiments to examine the relative contributions of the Watson−Crick and Hoogsteen base-pairing partners of the proposed U-A·U base triple. We show that the Hoogsteen interaction is critical for arginine binding whereas the Watson−Crick interaction can be eliminated or replaced by other base−base interactions. The results are consistent with biochemical studies of the Tat−TAR interaction and support the base triple model for the structure of TAR.

The human immunodeficiency virus (HIV) Tat protein activates viral transcription by binding to an RNA site, TAR, located at the 5′ end of the viral mRNAs. Tat binding to TAR enhances the efficiency of RNA polymerase II elongation (Kao et al., 1987; Feinberg et al., 1991; Marciniak & Sharp, 1991; Kato et al., 1992; Laspia et al., 1993). Although the detailed mechansim of Tat activation is not well understood, it is clear that RNA binding is essential for Tat function and that Tat function is essential for viral replication. Thus, a detailed understanding of the Tat−TAR interaction may help in the design of inhibitors of a key step in the viral life cycle.

There have been numerous biochemical and genetic studies of the Tat−TAR interaction, and recent NMR studies have provided structural models for a key part of the interaction. It has been shown that peptides spanning the arginine-rich region of Tat bind specifically to TAR (Cordingley et al., 1990; Roy et al., 1990; Weeks et al., 1990; Calnan et al., 1991a) and that a single arginine residue is largely responsible for binding specificity (Calnan et al., 1991b). Other amino acids within this region, possibly others outside the region, and additional cellular proteins contribute substantially to the overall binding affinity and kinetic stability (Churcher et al., 1993; Tao & Frankel, 1993; Jones & Peterlin, 1994; Long & Crothers, 1995). Nonetheless, arginine, even as the free amino acid, binds specifically to TAR (Tao & Frankel, 1992) and induces a change in RNA conformation that largely, if not completely, mimics the conformation of Tat peptide−TAR complexes (Puglisi et al., 1992; Tan & Frankel, 1992; Aboul-ela et al., 1995). In one NMR-derived model of a TAR-arginine complex (Puglisi et al., 1992), it was proposed that a base triple is formed upon arginine binding in which a critical U in a three-nucleotide bulge forms a Hoogsteen interaction with an A·U base pair in the adjacent stem (Figure 1). This structure forms a binding pocket in which arginine can hydrogen-bond to a guanine base and to two nearby phosphates. The model is supported by a key experiment in which the putative U-A·U base triple was replaced by an isomorphous C^+-G·C base triple (Figure 1); the identical RNA structure was formed upon arginine binding (Puglisi et al., 1993). In neither case, however, was an additional imino proton resonance observed corresponding to the Hoogsteen part of the base triple, possibly because this proton is exposed to solvent and undergoes rapid exchange. A more recent and higher resolution NMR model (Aboul-ela et al., 1995) confirms many features of the earlier model, including the approximate location of the bulged U in the major groove, but the authors questioned whether the base triple actually

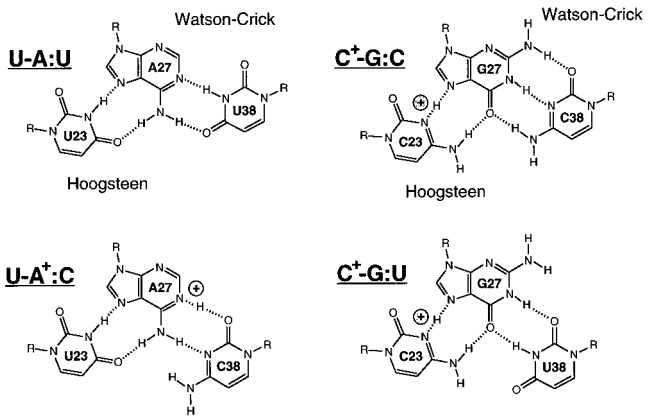

FIGURE 1: Schematic representation of potential base triple interactions in TAR and TAR mutants. Top left shows the proposed U-A·U base triple in wild-type TAR (Puglisi et al., 1992), top right shows the isomorphic C^+-G·C triple proposed to form at low pH (Puglisi et al., 1993), and bottom left and right show potential low-pH triples in mutants described in this paper.

† Supported by NIH Grant AI29135 and by the Gladstone Institute of Virology and Immunology.

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⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1997.

S0006-2960(96)02259-3 CCC: $14.00

forms. Further examination of the TAR structure by NMR supports formation of the base triple but suggests that the interaction might be dynamic in nature (Brodsky & Williamson, 1997).

In this paper, we have used additional biochemical and mutagenesis experiments to help evaluate the base triple model. The results suggest that the Hoogsteen part of the base triple, but not the Watson−Crick part, is essential for forming an arginine-binding site. The results can be reconciled with previous chemical substitution experiments seemingly inconsistent with the base triple model.

MATERIALS AND METHODS

Synthesis and Purification of RNAs. Wild-type and mutant TAR RNAs were synthesized using T7 RNA polymerase and synthetic DNA templates (Milligan & Uhlenbeck, 1989). RNAs were purified on 15% polyacrylamide/8 M urea gels as described (Calnan et al., 1991a).

Arginine Columns and Determination of Relative Arginine-Binding Dissociation Constants (K_ds). Ten micrograms of each RNA was folded by heating at 80 °C for 5 min and then slow cooling to room temperature in 0.5 mL of column buffer (10 mM Tris-HCl, pH 7.5, or 10 mM sodium phosphate, pH 5.5, and 0.2 mM EDTA) containing 100 mM NaCl. RNAs were loaded onto an equilibrated arginine−agarose column (8.4 μmol of L-arginine/mL of packed gel; Sigma) together with 5 μg of bulge-deleted TAR (BD TAR; a nonspecific control) and were eluted in 1-mL fractions using a 100-mL NaCl gradient from 100 to 500 mM, as described (Tao & Frankel, 1996). Elution positions of BD TAR and each TAR variant were determined by measuring A_{260} values of each fraction. Relative K_d values for some RNAs were determined by isocratic elution as described (Tao & Frankel, 1996), using the following equation: $K_d = [L](V_t - V_0)/(V_e - V_0)$, where V_t is the total column volume, V_0 is the void volumn, V_e is the elution volume, and [L] is the concentration of ligand bound to the column (Connell et al., 1993; Famulok, 1994). Relative K_ds for other RNAs were estimated from the salt concentration required to elute each RNA from an arginine column, using a standard curve relating K_d values measured by isocratic elution to salt-dependent elution (Tao & Frankel, 1996).

Hydrazine Modification Interference. Ten micrograms of 5′-^{32}P-labeled TAR RNA (2×10^6 cpm) was modified with hydrazine (HZ; Sigma) as previously described (Chen & Frankel, 1994). Modified RNAs were fractionated on an arginine−agarose column using standard NaCl gradients, and the fractionated RNAs were ethanol-precipitated and cleaved. Equal amounts of RNA, based on radioactivity, were dissolved in deionized formamide and analyzed on 20% polyacrylamide/8 M urea gels.

RNase Mapping. Ten nanograms of each 5′-^{32}P-labeled RNA (~50 000 cpm) was digested for 20 min on ice using 0.1 unit of RNase T1. Reactions contained 10 mM Tris-HCl (pH 7.5) or sodium phosphate (pH 5.5), 0.2 mM EDTA, and 70 mM NaCl. To probe arginine-bound conformations, RNAs were preincubated with 10 mM arginine (pH 7.0) for 10 min prior to RNase addition. Reactions were terminated by adding 10 μg of yeast tRNA (Sigma) and an equal volume of deionized formamide and immediately analyzed on 20% polyacrylamide/8 M urea gels.

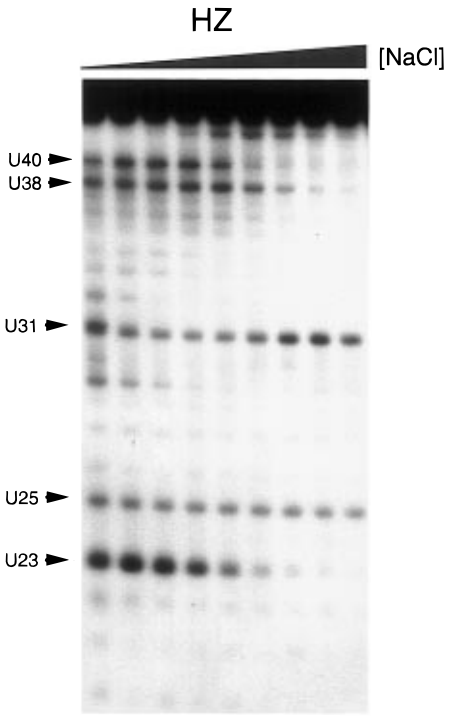


FIGURE 2: Hydrazine interference of arginine binding to TAR. TAR RNA was modified with hydrazine (on average at less than 1 site/molecule) and RNAs were eluted from an arginine−agarose column with an NaCl gradient. Fractions containing modified RNAs were cleaved and run on a gel, with salt concentration increasing from left to right. In the gel shown, every other fraction was loaded. Interference is indicated by the loss of bands in the late-eluting fractions, and the positions of modified uridines are indicated.

RESULTS

Hydrazine Modification Interference of Arginine Binding to TAR. As an initial test of the base triple model for the TAR−arginine complex, we chemically modified uridine residues in TAR using hydrazine (which cleaves the base) and asked whether both U23 and U38 of the proposed triple are required for arginine binding. Qualitatively, modification of either U23 or U38 interfered with binding; molecules containing modified uridines at either position eluted from an arginine affinity column at lower salt concentrations than unmodified TAR or TARs modified at U31 (in the loop) or U25 (the 3′ base of the bulge) (Figure 2). This result is consistent with the base triple model, although it is possible that modification of the uridines may disrupt the overall folding of TAR. Modification of U40, expected to disrupt the A·U base pair below the bulge, also interfered with binding. Similar results have been observed in chemical modification experiments with Tat-derived peptides (Weeks et al., 1990). Interestingly, modification of U23 caused TAR to elute 5−6 fractions earlier in the salt gradient than modification of U38, suggesting that U23 may be more important than U38 for arginine binding (see below).

Arginine Binding to Base Triple Mutant TARs. To further examine the relative importance of the U23-A27 and A27·U38 interactions of the proposed base triple, we measured arginine-binding affinities of a set of TAR mutants that retained the Hoogsteen interaction but disrupted the Watson−Crick interaction. Binding constants were determined (Tao & Frankel, 1996) both at neutral pH and at pH 5.5, since it was expected that some mutants would restore possible base−base interactions by base protonation at acidic pH.

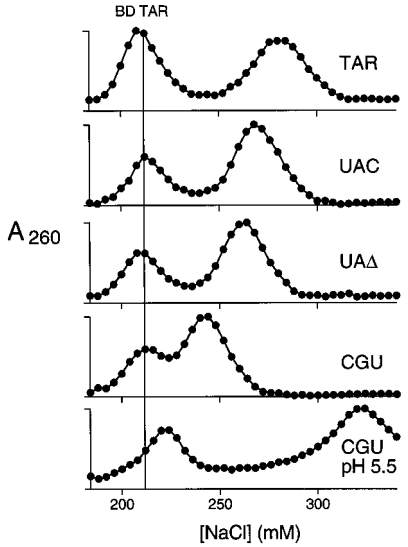
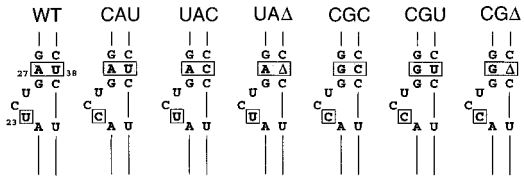


FIGURE 3: Arginine affinity column elution profiles of TAR mutants. Ten micrograms of each RNA was bound to a 1-mL arginine−agarose column and eluted with a 100-mL NaCl gradient. Fractions (1 mL) were collected and A_{260} was measured. Absorbance was normalized to the peak fraction for each pool. In all cases, 5 μg of bulge-deleted TAR RNA (BD TAR) was eluted in the same gradient to mark the position of nonspecific binding. BD TAR elutes from the gradient slightly later at pH 5.5 than at pH 7.5 (see profile for the CGU mutant).



pH 7.5

	WT	CAU	UAC	UAΔ	CGC	CGU	CGΔ
W-C pairing	yes	yes	no	no	yes	yes	no
Hoogsteen pairing	yes	no	yes	yes	no	no	no
Relative binding	1	0.15	0.6	0.55	0.18	0.23	0.27

pH 5.5

	WT	CAU	UAC	UAΔ	CGC	CGU	CGΔ
W-C pairing	yes	yes	yes	no	yes	yes	no
Hoogsteen pairing	yes	no	yes	yes	yes	yes	yes
Relative binding	1	0.15	0.92	0.31	1.3	2.0	2.1

FIGURE 4: Summary of arginine-binding affinities of TAR mutants. Schematic representations of the bulge region of TAR and TAR mutants are shown on top, and positions of the proposed base triple are boxed. The possibilities for Watson−Crick-like (including wobble) or Hoogsteen base pairing at neutral and low pH are indicated. Relative binding refers to the ratio of arginine-binding constants for each mutant compared to wild-type TAR, as determined by elution from an arginine−agarose column (see Materials and Methods; Tao & Frankel, 1996).

Two mutants, designated UAC and UAΔ (see Figure 4), disrupt the A27•U38 Watson−Crick interaction but showed only a marginal (∼2-fold) decrease in arginine-binding affinity at neutral pH compared to wild-type TAR (Figures 3 and 4). At acidic pH, the UAC mutant, but not the UAΔ mutant, bound arginine with the same specific binding affinity as wild-type TAR (Figures 3 and 4), consistent with formation of an A^+•C interaction having a wobble conformation similar to a Watson−Crick A•U base pair (Figure 1). The relatively modest (∼2-fold) decrease in arginine-binding affinity for these two mutants at neutral pH contrasts with the ∼7−10-fold decrease in Tat peptide or arginine binding affinity for a U23 → C mutant in which the Hoogsteen interaction has been disrupted, designated CAU in Figure 4 (Weeks & Crothers, 1991; Tao & Frankel, 1992). Unlike the UAC mutant, arginine-binding affinity of the CAU mutant did not increase at pH 5.5 (Figure 4); this mutant is not expected to form a Hoogsteen interaction at acidic pH. These results again suggest that U23 is relatively more important than U38, and indeed, U38 can be deleted entirely with only a modest effect on arginine-binding affinity.

It has been shown that an isomorphic base triple mutant, $C23^+$-G27•C38, binds arginine specifically only at acidic pH (Puglisi et al., 1993), supporting the importance of the U23-A27 Hoogsteen interaction. Using this triple mutant molecule as a framework, we constructed two additional mutants to further explore the relative importance of the Watson−Crick and Hoogsteen partners of the triple. In one mutant, designated CGU (see Figure 4), C38 was changed back to the wild-type U38, and it was expected that a G27•U38 wobble base pair would be formed (Figure 1). In a second mutant, designated CGΔ (see Figure 4), C38 was deleted. Interestingly, both CGU and CGΔ bound arginine with even higher affinity than wild-type TAR at acidic pH, further suggesting that the Hoogsteen interaction, but not the Watson−Crick interaction, of the base triple is critical for binding. The results may also suggest that structural flexibility at position 27, when not constrained by strict Watson−Crick pairing, favors formation of a tighter arginine-binding site.

RNase Probing of TAR Mutants. To ensure that the overall RNA structure was not significantly perturbed in the TAR mutants and to examine how the mutations affect the local secondary structure, we performed RNase T1 mapping experiments in the absence and presence of arginine at neutral and acidic pH to examine the accessibility of guanine residues within and surrounding the arginine-binding site. At pH 7.5, G26, the residue proposed to directly hydrogen-bond to arginine (Puglisi et al., 1992), was relatively resistant to T1 digestion in wild-type TAR in the absence or presence of arginine (Figure 5). In contrast, G26 became significantly more accessible in the UAC and UAΔ mutants, and arginine binding protected this residue from digestion. Residue G28 also showed increased accessibility in the unbound form and protection in the presence of arginine. Thus, it appears that the unbound structure of these mutants is somewhat more flexible than wild-type TAR but that the bound structures are similar. In contrast, G26 in the CGC, CGU, and CGΔ mutants was relatively inaccessible to digestion in the absence or presence of arginine, as observed for wild-type TAR.

At pH 5.5, as at pH 7.5, G26 and G28 in wild-type TAR were inaccessible to digestion (Figure 5). Interestingly, these bases became fully protected in the UAC mutant, even in the absence of arginine, suggesting that protonation of the adenine restored Watson−Crick-like base pairing and stabilized the unbound structure. In the UAΔ mutant, G26 was very sensitive to digestion and was only slightly protected by arginine binding, suggesting that the structure is unstable and correlating with the decrease in arginine-binding affinity (Figure 4). In the CGΔ mutant, G26 was partially sensitive to digestion and became protected in the presence of arginine, whereas in the CGU mutant, G26 was inaccessible even in the absence of arginine. It seems plausible that the increased arginine-binding affinity of the CGU mutant (Figure 4) results from the enhanced stability of the unbound structure whereas the increased affinity of the CGΔ mutant (Figure 4) results from the ability of G27 to adopt a more favorable geometry in the Hoogsteen pair. It appears that the CGΔ

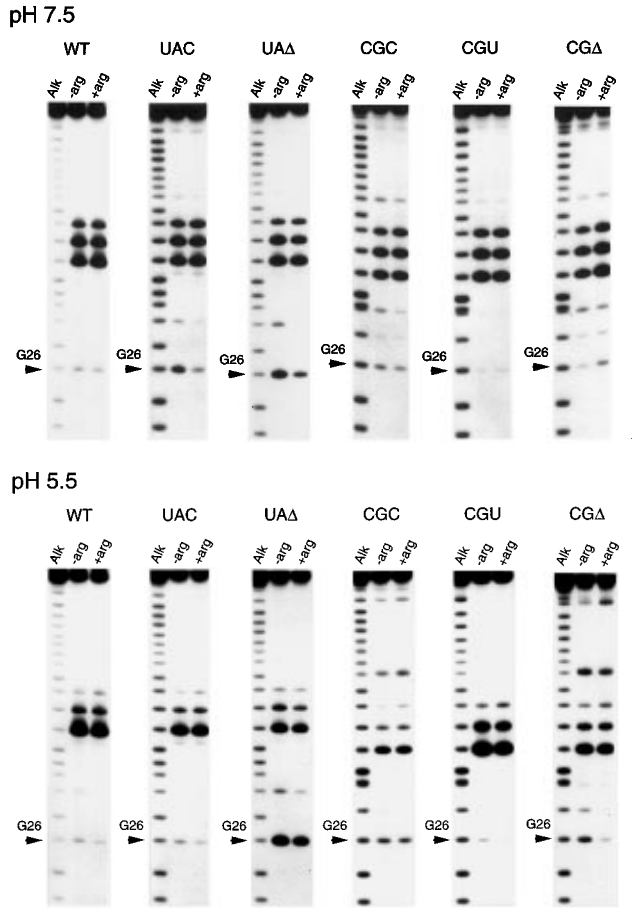
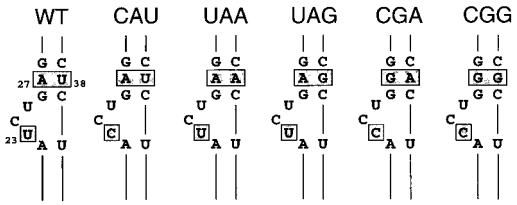


FIGURE 5: RNase mapping of TAR mutants. RNAs were cleaved with RNase T1, which cleaves at single-stranded G residues, in the absence or presence of 10 mM arginine at pH 7.5 or 5.5. RNase T1 activity is not generally inhibited at this concentration of arginine. An alkaline hydrolysis ladder is shown as a marker for each RNA, and the position of G26, which is proposed to directly hydrogen-bond to arginine, is indicated. Nucleotides G28−U31 in the CGC, CGU, and CGΔ mutants are compressed to form two strong bands that can be resolved using more highly denaturing gels containing 7 M urea and 40% formamide (data not shown; Tao & Frankel, 1996).

mutant may exist in equilibrium with an alternative structure in which C39 pairs with G27 (taking the place of the deleted C38) and U40 pairs with G26, as suggested by the enhanced digestion of G43 in the lower stem in the presence of arginine (Figure 5). Nevertheless, this mutant still binds arginine more tightly than does wild-type TAR or the CGC mutant (Figure 4), consistent with the idea that a flexible upper stem with imperfect Watson−Crick pairing may create a more favorable Hoogsteen geometry for arginine binding.

Arginine Binding to Mutants Containing Purine−Purine Substitutions. In the mutants described above, the Watson−Crick interaction of the base triple was disrupted either by deletion of a base or by replacement with a wobble base pair. To further assess the types of structural perturbations that support an arginine-binding conformation, we constructed an additional set of mutants in which the Hoogsteen interaction was maintained while the Watson−Crick partners were replaced by pairs of purines. In the context of the U-A Hoogsteen interaction, replacing the Watson−Crick base pair with an A·A pair (UAA mutant) decreased arginine-binding affinity to a level comparable to disruption of the Hoogsteen interaction (CAU mutant), and replacement with an A·G pair (UAG mutant) also resulted in weak binding (Figure 6).



	WT	CAU	UAA	UAG	CGA	CGG
pH 7.5						
Relative binding	1	0.15	0.15	0.21	0.41	0.47*
pH 5.5						
Relative binding	1	0.15	0.08	0.34	0.29	1.3*

FIGURE 6: Summary of arginine-binding affinities of mutants containing purine−purine substitutions. Schematic representations of the bulge region of TAR and TAR mutants are shown on top, and positions of the proposed base triple are boxed. Relative binding refers to the ratio of arginine-binding constants for each mutant compared to wild-type TAR, as determined by elution from an arginine−agarose column (see Materials and Methods; Tao & Frankel, 1996). Asterisks indicate that the CGG mutant RNA eluted from the arginine column as a broad peak, possibly suggesting conformational heterogeneity.

Although these results are inconsistent with the simple interpretation that a Hoogsteen interaction is sufficient to create an arginine-binding site, we do not know whether purine−purine pairs are being formed and whether particular types of pairings will disrupt the Hoogsteen interaction or the structure of the binding site. In the context of the C^+-G Hoogsteen interaction at pH 5.5, a G·G replacement of the Watson−Crick pair (CGG mutant) bound arginine with slightly higher affinity than wild-type TAR, suggesting that a G·G pair may create a better arginine-binding conformation than a Watson−Crick pair, whereas a G·A replacement (CGA mutant) bound relatively poorly (Figure 6). At pH 7.5, the CGA and CGG mutants bound with affinities only about 2-fold lower than wild-type TAR, perhaps suggesting that a single H-bond between the Hoogsteen partners can create a favorable binding geometry provided that the pairing does not compete with Watson−Crick or wobble pairing as would occur in the CGC and CGU mutants. The CGA mutant actually bound slightly better at pH 7.5 than at pH 5.5 (Figure 6), suggesting that G·A^+ pairing might compete with the C^+-G Hoogsteen interaction and result in a conformation unfavorable for arginine binding. It must be emphasized that while we have interpreted the effects of acidic pH based on protonation of adenines and cytosines, we cannot exclude the possibility that low pH has a more general effect on TAR structure or arginine binding, for example, by affecting the phosphate backbone. NMR experiments are required to define the precise pairing arrangements for all mutants described in this study.

DISCUSSION

The results presented here support a dynamic base triple model for the TAR-arginine complex and suggest (1) that the Hoogsteen interaction between the bulged base U23 and A27 in the upper helical stem is critical for forming an arginine-binding site, (2) that the Watson−Crick interaction between A27 and U38 is important for maintaining a stable conformation of unbound TAR but is dispensable when arginine is bound, and (3) that disruption of the Watson−Crick interaction may, in some cases, create more favorable arginine-binding geometries. The results help to explain a previous chemical substitution experiment in which substitution of A27 with *N*6-methyl-dA caused only a slight decrease

in Tat binding affinity (Hamy et al., 1993). This base analog removes one of the two hydrogen-bond donors from the N6 group of A27 and therefore is expected to disrupt the base triple. In light of the results presented here, it seems likely that the remaining single hydrogen-bond donor still allows formation of the Watson−Crick pair in the unbound state and formation of the Hoogsteen interaction when bound to arginine or Tat. In forming the Hoogsteen interaction, the bulged U is positioned to stack on the arginine guanidinium group (Puglisi et al., 1992), and it seems reasonable that the additional energy generated from π stacking or π-cation interactions helps stabilize the RNA structure even in the absence of the Watson−Crick interaction. The importance of the N7 group of A27, which is required to form the Hoogsteen interaction, has been demonstrated by diethyl pyrocarbonate modification interference (Weeks & Crothers, 1991) and by substitution experiments with 7-deaza-dA (Hamy et al., 1993). This base analog removes a hydrogen-bond acceptor and severely reduces Tat binding affinity.

NMR studies of a bovine immunodeficiency virus (BIV) Tat peptide−TAR complex suggest formation of a base triple similar to that proposed for HIV TAR. In this case, a single bulged U is positioned in the major groove near an A·U base pair, analogous to the bases in HIV TAR (Puglisi et al., 1995; Ye et al., 1995), and an additional imino proton corresponding to the Hoogsteen part of the triple has been observed (Ye et al., 1995). Interestingly, the A·U base pair in BIV TAR is not essential for BIV peptide binding (Chen & Frankel, 1994), suggesting that other hydrogen-bonding arrangements involving the bulged U, perhaps mimicking the Hoogsteen interaction, may be sufficient for creating an appropriate binding pocket.

Other arginine-binding RNAs have been identified by in vitro selection experiments (Connell et al., 1993; Connell & Yarus, 1994; Famulok, 1994; Tao & Frankel, 1996; Yang et al., 1996) and in the *Tetrahymena* group I intron (Yarus, 1988). In one in vitro selection study, a large set of TAR-like binding sites was found (Tao & Frankel, 1996). In these TAR-like RNAs, as in TAR (Figure 2), hydrazine modification of the Hoogsteen bulged U eliminated arginine binding whereas modification of the Watson−Crick U had little effect (Tao & Frankel, 1996). Thus, it appears that the arginine-binding motif of TAR may be represented by the sequence 5′ UX_nGA, where U forms a Hoogsteen interaction with the A, X_n is at least one unpaired nucleotide (Weeks & Crothers, 1991; Tao & Frankel, 1996), and G forms the arginine-binding site along with two adjacent phosphates. It is possible that such sites exist even in the absence of a Watson−Crick helix, provided that the surrounding RNA framework positions the bases in an appropriate orientation. This may be the case for some TAR-like RNAs in which small loops rearrange to form the arginine-binding site (Tao & Frankel, 1996). The HTLV-I Rex protein also appears to use a similar binding motif (Baskerville et al., 1995), and it may be anticipated that similar interactions will be found in other RNA−protein complexes.

ACKNOWLEDGMENT

We thank Cynthia Honchell and Joyce Heckman for technical assistance and Colin Smith, Ruoying Tan, and Jonathan Staley for comments on the manuscript.

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BI962259T