Circular Dichroism Studies Suggest That TAR RNA Changes Conformation upon Specific Binding of Arginine or Guanidine†

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ABSTRACT: Short basic peptides from the HIV Tat protein bind specifically to a bulge region in TAR RNA, with a single arginine residue providing the only sequence-specific contact. The free amino acid arginine also binds specifically to TAR. Previous circular dichroism (CD) experiments suggested that peptide binding induces a conformational change in TAR. Here we confirm this observation using single arginine-containing peptides and show that arginine or guanidine binding also induces a conformational change in TAR. A peptide containing a single arginine within a stretch of histidines (CYHHHRHHHHHA) shows pH-dependent binding and a corresponding change in TAR conformation, as detected by a decrease in the CD signal at 265 nm. Arginine and guanidine, which bind to TAR with apparent K_d's of ~1.5 mM, induce similar CD changes. In contrast, lysine, which does not bind specifically to TAR, has no effect. Mutants of TAR that abolish specific binding (a U→C substitution in the three-nucleotide bulge, a deletion of the bulge, or an A-U to U-A base pair change above the bulge) show no change in the CD signal upon binding of peptides, arginine, or guanidine. The results suggest that binding of a single guandinium group to a specific site in TAR induces a change in RNA conformation.

The Tat protein from human immunodeficiency virus (HIV) is a potent activator of HIV transcription and is essential for viral replication. The precise mechanism by which Tat acts is still under investigation, but it appears that Tat works, at least in part, by increasing the efficiency of transcriptional elongation [see review by Frankel (1992) and references cited therein]. Transactivation is dependent on the specific interaction of Tat with the trans-acting responsive (TAR) element, an RNA hairpin located at the 5′ end of the viral mRNAs (Roy et al., 1990; Dingwall et al., 1990; Cordingley et al., 1990; Weeks et al., 1990; Calnan et al., 1991a).

Studies with peptides have shown that the TAR RNA-binding domain of Tat is contained within a short region of basic amino acids, having six arginines and two lysines within nine residues (Weeks et al., 1990; Cordingley et al., 1990; Calnan et al., 1991a). Further studies have shown that a single arginine residue provides the only sequence-specific contact with the RNA (Calnan et al., 1991b) and that the arginine must be surrounded by at least three basic acids on each side to provide nonspecific electrostatic contacts and increase the RNA-binding affinity (Calnan et al., 1991b; J. Tao and A. D. Frankel, submitted for pulication). The free amino acid arginine binds specifically to the same site in TAR as does the Tat peptide (Tao & Frankel, 1992). The observations that the Tat peptides are unstructured (Calnan et al., 1991a) and that free arginine can bind specifically to TAR have led to the conclusion that the precise three-dimensional structure of TAR must be a major determinant of Tat/TAR recognition. This is consistent with many studies of tRNA synthetases and bacteriophage R17 coat protein which show the essential role of RNA structure in RNA–protein recognition [see Rould et al. (1989, 1991), Ruff et al. (1991), and Witherall et al. (1991) for examples].

A considerable amount is known about TAR RNA structure and the requirements for Tat and arginine binding. Nuclease mapping experiments first showed that TAR forms a stable stem–loop structure (Muesing et al., 1987). Subsequent deletion analysis showed that the upper part of the stem–loop (nucleotides +19 to +42 relative to the start of HIV transcription) was sufficient for the Tat response in vivo (Jakobovits et al., 1988). The TAR hairpin contains a six-nucleotide loop and a three-nucleotide bulge (see Figure 1) that are both essential for Tat activity. Tat binding appears to be limited to the region around the bulge (Roy et al., 1990; Dingwall et al., 1990; Cordingley et al., 1990; Weeks et al., 1990; Calnan et al., 1991a,b) while cellular factors may bind to the loop (Marciniak et al., 1990; Wu et al., 1991; Sheline et al., 1991). Although the size of the bulge is not so critical for Tat binding (it must contain at least two nucleotides), there is an important requirement for uridine at the 5′-most position of the bulge (+23) (Weeks & Crothers, 1991; Sumner-Smith et al., 1991). The identity of several base pairs surrounding the bulge is also important; in particular, the two base pairs immediately above the bulge, G26–C39 and A27–U38, are essential (Weeks & Crothers, 1991; Berkhout & Jeang, 1991). In addition to these possible base contacts, it was found that ethylation of two phosphates, located at the junction of the double-stranded stem and bulge, strongly interfered with peptide or arginine binding (Calnan et al., 1991b; Tao & Frankel, 1992). This led to a model in which part or all of the specificity may result from a set of hydrogen bonds between the arginine guanidinium group and two highly oriented phosphates, an interaction termed the "arginine fork" (Calnan et al., 1991b). Another model, based on chemical modification studies of TAR (Weeks & Crothers, 1991), suggested that Tat peptides might interact with specific groups in the RNA major groove. Recent NMR studies suggest that arginine donates hydrogen bonds to the two phosphates and to G26 in the major groove and that the complex is stabilized by a base triple interaction between U23 and A27–U38 (Puglisi et al., 1992).

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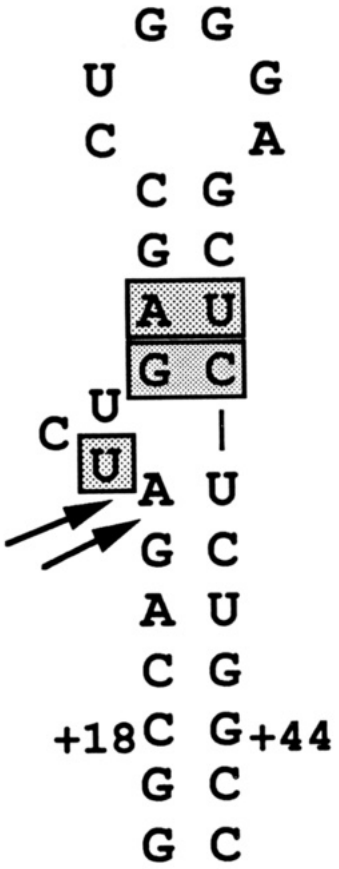

FIGURE 1: Sequence and secondary structure of TAR. The boxed uridine in the bulge and the boxed A-U and G-C base pairs are essential for Tat peptide binding (Sumner-Smith et al., 1991; Weeks & Crothers, 1991). The arrows mark the positions of phosphates whose modification interferes with binding (Calnan et al., 1991b; Tao & Frankel, 1992). The numbering shown is relative to the start of transcription from the HIV long-terminal repeat.

Although these studies clearly indicate that the TAR conformation is essential for the interaction with arginine, it also appears that there are differences in the free and bound RNA conformations. Difference circular dichroism (CD) spectra of TAR and peptide–TAR complexes initially suggested that the RNA may change conformation upon peptide binding, showing significant changes in the RNA CD spectrum near 265 nm (Calnan et al., 1991a). NMR studies of TAR and arginine–TAR complexes have confirmed that a conformational change occurs upon binding (Puglisi et al., 1992). To further examine the specificity and significance of these conformational changes, we have examined CD spectra of wild-type and mutant TAR RNAs and of peptide–RNA, arginine–RNA, and guanidine–RNA complexes. The results indicate that TAR RNA changes conformation upon specific binding of a guanidinium group.

MATERIALS AND METHODS

Peptides and Peptide Affinity Column. Peptides were synthesized using Fmoc chemistry and were purified and characterized as described (Calnan et al., 1991a). Concentrations were determined using the tyrosine absorbance (ϵ = 1420 M^{-1} cm^{-1} at 278 nm; Creighton, 1984). The histidine-containing peptide (CYHHHRHHHHHA; HR52) was coupled to an (aminohexyl)agarose resin via the cysteine thiol using a maleimidosuccinimide ester bifunctional cross-linker (J. Tao and A. D. Frankel, submitted for publication). One milligram of peptide was coupled to 1 mL of resin. To determine whether TAR RNA bound specifically to this peptide resin, a mixture of 10 μg each of in vitro-transcribed TAR, TAR_{UC} (TAR containing a U23→C substitution in the three-nucleotide bulge), and TAR_{BD} (TAR containing a deletion of the bulge) was bound to a peptide affinity column, and RNAs were eluted at 4 °C with a NaCl gradient and analyzed on 15% polyacrylamide gels as described (Tao & Frankel, 1992).

RNA Synthesis and Purification. TAR RNAs were transcribed in vitro using T7 RNA polymerase and synthetic oligonucleotide templates (Milligan & Uhlenbeck, 1989; Calnan et al., 1991a). All RNAs contained GG at their 5′ end, which increased the efficiency of transcription, and CC at the 3′ end to base-pair with GG. Wild-type TAR RNA contained sequences +18 to +44 of the HIV long-terminal repeat TAR site. RNAs were purified on 20% polyacrylamide/8 M urea gels and were eluted from the gels in 0.6 M sodium acetate, pH 5.5, 1 mM EDTA, and 0.1% SDS. RNAs were then loaded onto a DEAE column, eluted with NaCl, and ethanol-precipitated. Purified RNAs were resuspended in sterile deionized water and quantitated by spectrophotometry. RNA was internally labeled with ^{32}P during in vitro transcription as previously described (Calnan et al., 1991a).

Circular Dichroism (CD). CD spectra were measured using an Aviv model 62DS spectropolarimeter. Samples were prepared in 10 mM K_2HPO_4, pH 7.5, and 70 mM KF, and the temperature was maintained at 5 °C, unless described otherwise. RNA concentrations were 20–60 μg/mL. Spectra were recorded from 300 to 200 nm using a 1-cm path-length cuvette, and the signal was averaged for 5 s at each wavelength. Scans were repeated 5 times and averaged. The data presented are at dynode voltages below 600 V. The mean molar residue ellipticity of RNA and of peptide–, arginine–, and guanidine–RNA complexes was calculated per nucleotide of RNA. Peptide ellipticity was calculated per amino acid residue. Binding curves of L-arginine (Sigma Chemical Co.) or guanidine (hydrochloride salt; Pierce Chemical Co.) were determined by measuring the change in ellipticity at 265 nm, with 30-s averaging times at each concentration.

RESULTS

Specific Binding of a Single Arginine-Containing Peptide Induces a Change in the TAR CD Spectrum. Previous circular dichroism (CD) experiments suggested that TAR RNA may change conformation upon specific binding of Tat peptides (Calnan et al., 1991a). In these experiments, binding of a peptide containing the wild-type Tat sequence (residues 47–58; YGRKKRRQRRRP) showed a significant (approximately 15%) change in the CD spectrum near 265 nm. The change was initially assigned to the RNA because TAR alone shows a large maximum near this wavelength and because no known peptide conformation contributes at this wavelength. Tyrosine can sometimes give a CD signal in this region of the spectrum (Woody, 1985); however, the magnitude is generally smaller and the maximum is usually at a longer wavelength than that observed in the TAR difference spectrum. Experiments with arginine and guanidine, described below, will support the assignment of the CD change to TAR. We interpret the change as a change in the RNA conformation (see Discussion).

We first wished to determine whether specific binding of a single arginine-containing peptide (R52; YKKKRKKKK-KA; Calnan et al., 1991b) induced changes in the CD spectrum similar to those seen with the wild-type peptide. R52 was bound to TAR, and the spectrum of the peptide–RNA complex was compared to the spectrum of TAR alone (Figure 2A).

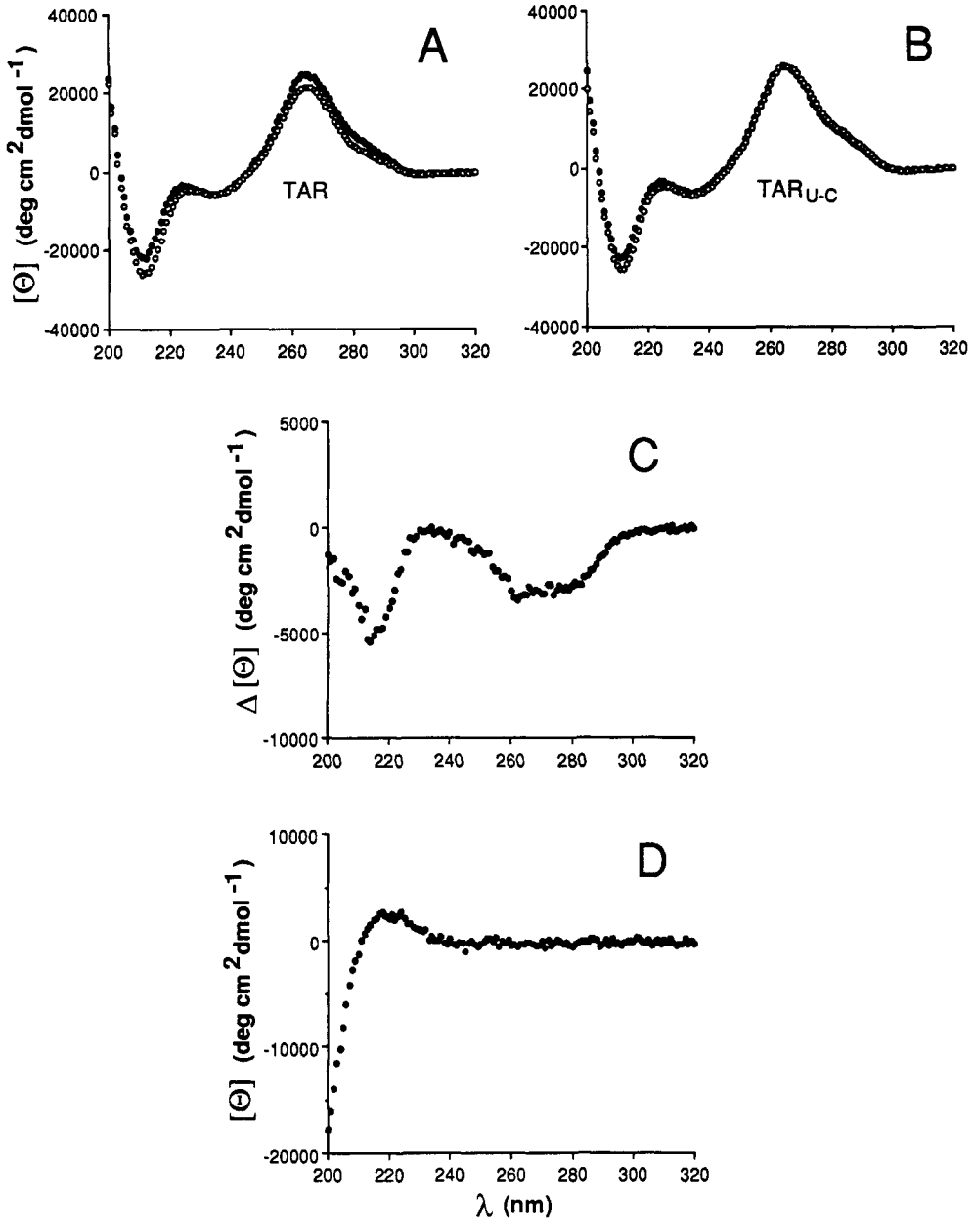

FIGURE 2: CD spectra of TAR RNA, TAR_{U-C} mutant, R52 peptide, and peptide–RNA complexes. (A) Spectra of TAR RNA alone at 40 μg/mL (●) and of the R52/TAR RNA complex at a 1:1 stoichiometry (○). (B) Spectra of TAR_{U-C} RNA alone at 40 μg/mL (●) and of the R52/TAR_{U-C} RNA complex at a 1:1 stoichiometry (○). (C) Difference spectrum of the spectra shown in (A). (D) Spectrum of R52 alone at 4 μM.

Just as with the wild-type peptide (Calnan et al., 1991a), there was an approximately 15% decrease in the maximum near 265 nm. In contrast, when R52 was bound to a mutant TAR (TAR_{U-C} contains a U22→C substitution in the bulge and binds Tat nonspecifically; Sumner-Smith et al., 1991; Calnan et al., 1991a; Weeks & Crothers, 1991), no change was observed in this region of the CD spectrum (Figure 2B). The difference spectrum between wild-type TAR and the R52–TAR complex (Figure 2C) is similar to that observed with the wild-type Tat peptide (Calnan et al., 1991a). There is a minimum near 216 nm that is likely to contain contributions both from the peptide and from conformational changes in TAR. Because the peptide and RNA signals overlap in this region of the spectrum, it is not possible to determine how much of the signal is due to peptide and how much is due to RNA. There is a second minimum near 265 nm which we interpret as a change in RNA conformation (see above and Discussion). The CD spectrum of R52 alone (Figure 2D) indicates a random-coil structure (Creighton, 1984) as seen with the wild-type peptide (Calnan et al., 1991a).

To further assess the specificity of the peptide-induced CD change near 265 nm, we compared the spectra of the R52–TAR complex to R52 complexes with two other mutant RNAs known to abolish specific binding (see Figure 1): TAR_{UA} contains an A27–U38 base pair to U27–A38 base pair substitution at the second base pair above the bulge (Weeks & Crothers, 1991), and TAR_{BD} contains a deletion of the bulge (Roy et al., 1990; Calnan et al., 1991a; Weeks & Crothers, 1991). As with TAR_{U-C} (Figure 2B), R52 binding did not change the TAR mutant CD spectra near 265 nm (Figure 3A,B; compare to wild-type TAR in Figure 2A). At these concentrations, R52 binds nonspecifically to all three mutant RNAs (Calnan et al., 1991b); data not shown). Thus, the change in the CD signal near 265 nm correlates with specific peptide binding to TAR and suggests either that the mutant RNAs cannot undergo the same conformational

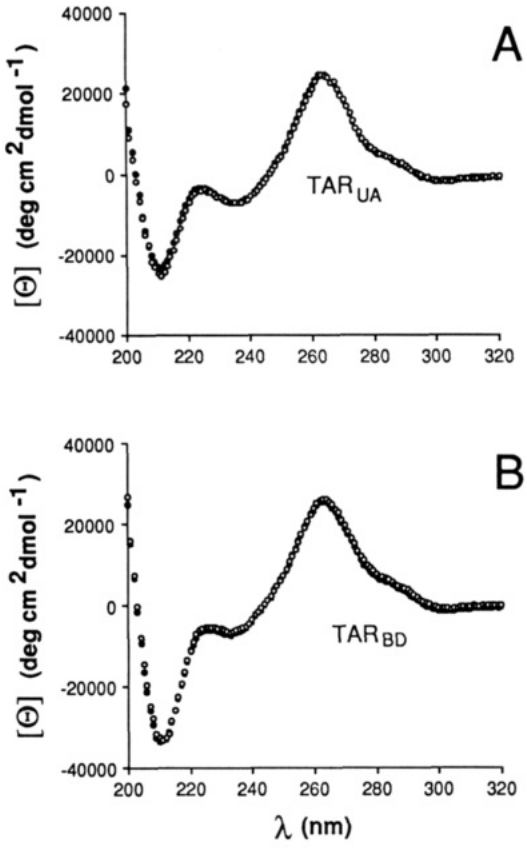

FIGURE 3: CD spectra of R52 peptide binding to mutant TAR RNAs. (A) TAR_{UA} (●) and R52/TAR_{UA} complexes (○). (B) TAR_{BD} (●) and R52/TAR_{BD} complexes (○). Peptide–RNA complexes were at a 1:1 stoichiometry.

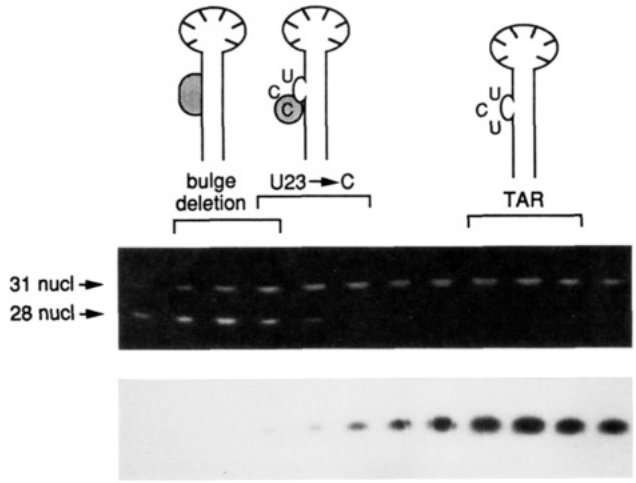

FIGURE 4: Binding of TAR, TAR_{U-C}, and TAR_{BD} to an HR52 peptide–agarose column. An equimolar mixture of TAR (31 nucleotides), TAR_{U-C} (31 nucleotides), and TAR_{BD} (28 nucleotides) was bound to the column, and RNAs were eluted with a NaCl gradient, as previously described using an L-arginine–agarose column (Tao & Frankel, 1992). Fractions were analyzed by gel electrophoresis (with salt increasing from left to right), and RNAs were visualized by ethidium bromide staining (top). Wild-type TAR was labeled with ^{32}P and autoradiographed (bottom) to determine its elution position compared to the same-sized TAR_{U-C} mutant. The three peak fractions for each RNA, determined by A_{260} and scintillation counting, are bracketed.

change as wild-type TAR or that specific binding is required to induce a conformational change.

pH-Dependent Binding of a Histidine-Containing Peptide. To more accurately assess the quality of the CD spectra, we devised a peptide in which the lysines surrounding the arginine

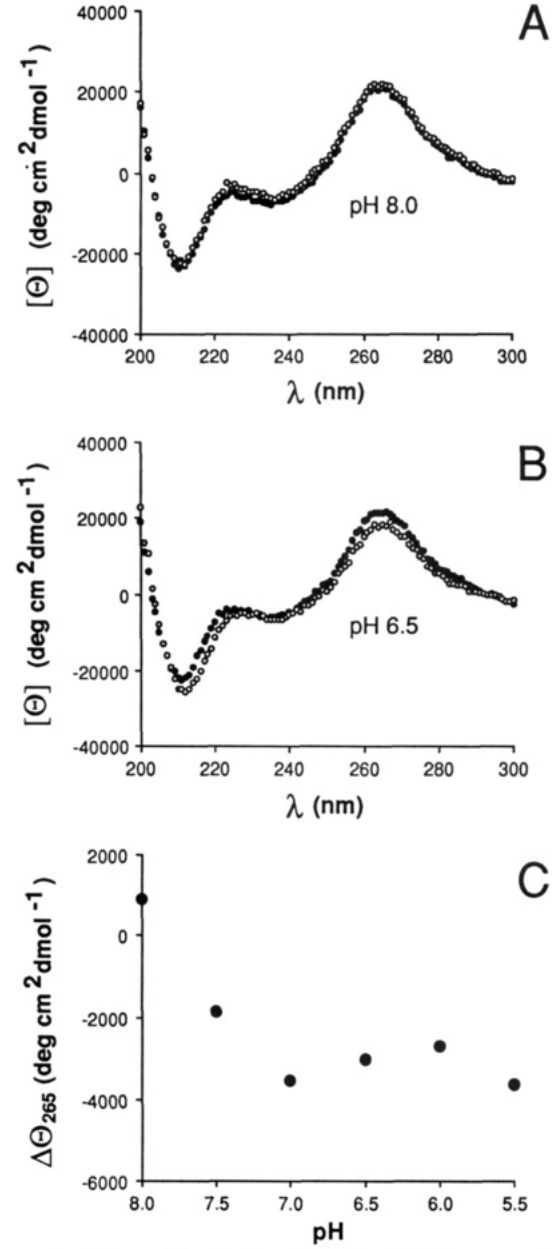

FIGURE 5: pH-dependent binding of HR52. (A) CD spectra of TAR (●) and HR52/TAR complexes (○) at pH 8.0. (B) Same spectra as in (A) at pH 6.5. (C) Change in ellipicity at 265 nm between free TAR and HR52/TAR complexes at different pHs. All complexes were at a 1:1 stoichiometry.

in R52 were replaced by histidines (HR52; CYHHHRHH-HHHA). We anticipated that peptide binding would be pH-dependent as the histidines became charged below neutral pH and reasoned that we could directly compare the spectrum of free peptide and free TAR in the same solution (above neutral pH) with the spectrum of the peptide–TAR complex (at lower pH). To first test whether the HR52 peptide bound specifically to TAR, we coupled HR52 to an agarose resin and asked whether a peptide affinity column could distinguish wild-type from mutant TAR RNAs, as previously demonstrated with an L-arginine column (Tao & Frankel, 1992) and with a wild-type Tat peptide column (J. Tao and A. D. Frankel, unpublished results). A mixture of 10 μg each of TAR RNA, the TAR_{U-C} mutant, and the TAR_{BD} mutant was loaded onto the HR52–agarose column, and RNAs were eluted with a salt gradient as described (Tao & Frankel, 1992). At pH 8.0, no specific binding was observed, with all three RNAs eluting at less than 50 mM NaCl (data not shown). In contrast, at

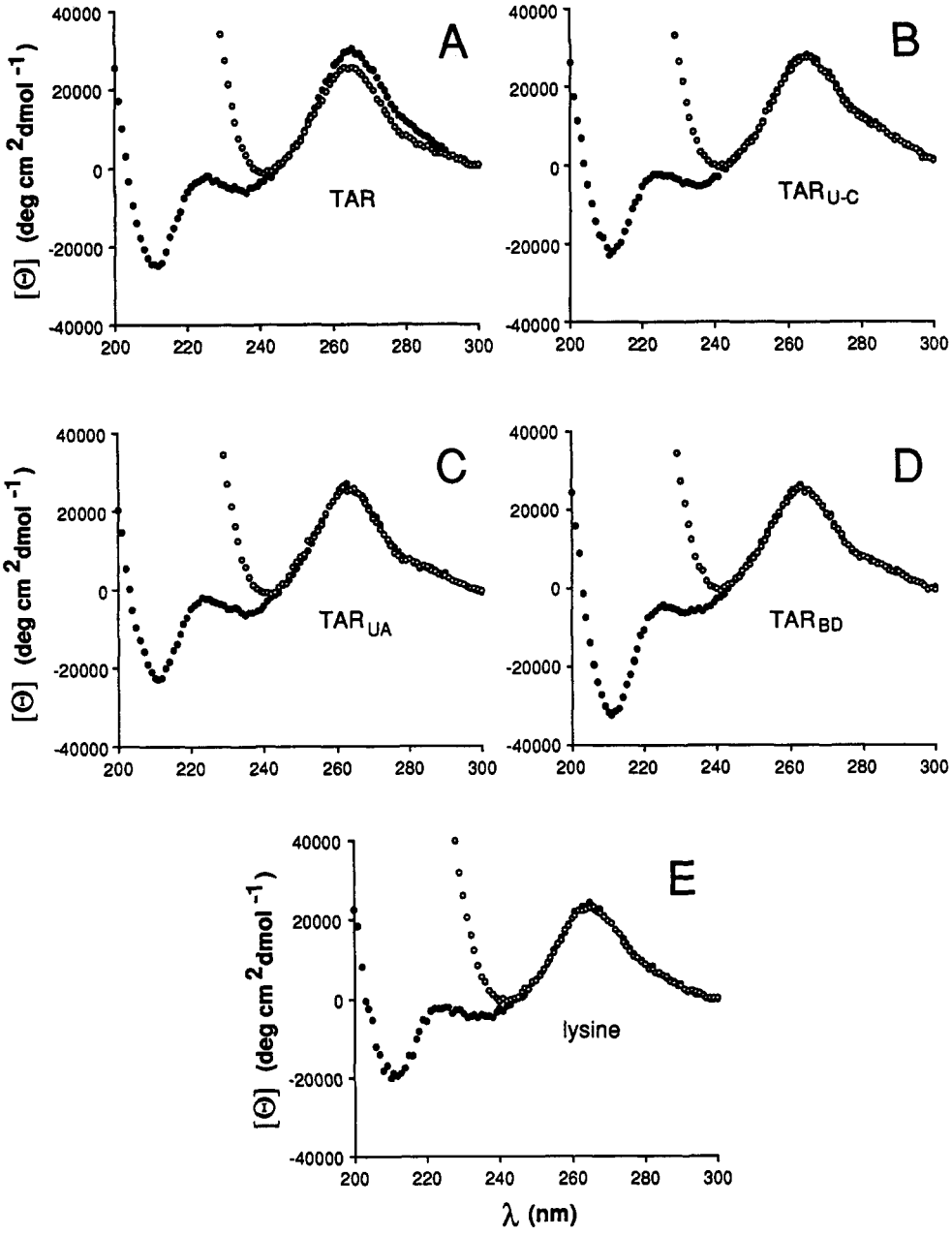

FIGURE 6: Effect of arginine on the CD spectra of TAR and mutant TARs, and effect of lysine on TAR. (A) Spectra of wild-type TAR in the absence (●) or presence (○) of 10 mM arginine. (B) TAR$_{U-C}$ in the absence (●) or presence (○) of 10 mM arginine. (C) TAR$_{UA}$ in the absence (●) or presence (○) of 10 mM arginine. (D) TAR$_{BD}$ in the absence (●) or presence (○) of 10 mM arginine. (E) Spectra of wild-type in the absence (●) or presence (○) of 10 mM lysine.

pH 6.0, wild-type TAR eluted from the column at 390 mM NaCl whereas TAR$_{U-C}$ and TAR$_{BD}$ eluted significantly earlier, at 300 and 270 mM NaCl, respectively (Figure 4). The specificity of HR52 binding is similar to that seen with wild-type Tat peptide binding (Calnan et al., 1991a; J. Tao and A. D. Frankel, unpublished results) and L-arginine binding (Tao & Frankel, 1992). At pH 6.5, TAR bound to the column specifically but eluted earlier in the salt gradient, consistent with partial deprotonation of the histidines (the pK of the imidazolium group is 6.00). These results support the important role of flanking charges in maintaining high-affinity RNA binding (Calnan et al., 1991b; J. Tao and A. D. Frankel, submitted for publication).

We next measured CD spectra of HR52 and TAR as a function of pH to determine whether changes in the spectrum correlated with pH-dependent binding. In the absence of binding (at pH 8.0), the spectrum near the RNA maximum (near 265 nm) was the same whether or not the peptide was present (Figure 5A). However, as the pH was lowered, allowing the peptide to bind, there was a decrease in the CD signal near 265 nm (see pH 6.5; Figure 5B). The pH-dependent change in ellipticity, monitored at 265 nm, is plotted in Figure 5C. The change is most simply interpreted as a change in TAR conformation and correlates with peptide binding. The CD spectrum of TAR showed no pH-dependent changes in the absence of peptide (data not shown).

Arginine and Guanidine Binding to TAR and TAR Mutants. Previous results from peptide competition experiments and with an L-arginine affinity column demonstrated that the free amino acid arginine binds specifically to TAR (with a K_i of ~4 mM) and that free arginine and arginine in the peptide bind to the same site in TAR (Tao & Frankel, 1992). Since the CD experiments with mutant RNAs (above) suggested that the conformational change in TAR requires specific peptide binding, we asked whether specific binding of L-arginine would also induce a change in the CD spectrum.

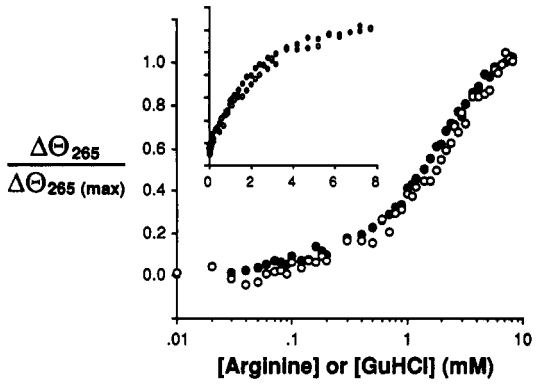

FIGURE 7: Arginine (●) and guanidine (○) titration of TAR RNA. The change in ellipicity was monitored at 265 nm, and the TAR concentration was 60 μg/mL. Values are plotted as the ratio of the change at 265 nm to the maximal change at 265 nm. Base lines were determined by averaging the first and last five points of each titration. Both linear (inset) and logarithmic concentration scales are plotted.

As seen with the peptides, arginine binding did indeed induce a similar CD change near 265 nm (Figure 6A). In contrast, arginine had no effect on any of the three mutant RNAs described above (Figure 6B–D), and lysine, which binds TAR nonspecifically, had no effect on the TAR spectrum (Figure 6E). Thus, specific binding of arginine to TAR is sufficient to induce a change in the CD spectrum and fully accounts for the changes observed near 265 nm. These results further demonstrate the specificity of arginine binding and of the CD changes, and strongly support the assumption that the change in ellipticity observed near 265 nm results from a change in TAR conformation.

It has been suggested that the guanidinium group of arginine forms a set of hydrogen bonds with specific acceptor groups on TAR RNA (Calnan et al., 1991b; Tao & Frankel, 1992; Puglisi et al., 1992). To ask whether the guanidinium group is sufficient for specific recognition, we recorded CD spectra of TAR and TAR mutants in the absence or presence of guanidine (hydrochloride). Guanidine induced a CD change in wild-type TAR similar to that seen with the peptide or with arginine but did not induce a change in any of the mutant TARs (data not shown). To compare arginine and guanidine binding, we determined binding curves by monitoring the change in ellipicity at 265 nm (Figure 7). Binding in both cases was saturable, with similar apparent K_d's of ~1.5 mM. The apparent K_d for L-arginine is in reasonable agreement with the K_i of ~4 mM previously determined using a peptide competition assay (Tao & Frankel, 1992). Although guanidine is generally used as a denaturant, specific binding to TAR occurs at a much lower concentration than that required to denature the RNA. The specific binding of guanidine suggests that the determinants of TAR recognition reside solely in the guanidinium group and that this single interaction is sufficient to induce a conformational change in TAR.

DISCUSSION

Previous studies have suggested that the precise conformation of TAR RNA plays an important role in recognition by Tat. The results presented here provide evidence for a conformational transition in TAR as the specific complex is formed. The binding of a single arginine guanidinium group to a specific site in TAR appears to be necessary and sufficient to induce the conformational change. Thus, the relationship between TAR RNA and the guanidinium group seems analogous to that of enzyme and substrate; TAR specifically recognizes a guanidinium group and undergoes a conformational change or "induced fit" upon binding. The significance of such a conformational change for the biological activity of Tat is unknown, but one might imagine that different conformations could influence the binding of additional cellular proteins to TAR (Marciniak et al., 1990; Wu et al., 1991; Sheline et al., 1991). Perhaps more likely, the conformational change may simply reflect a reorganization of specific RNA acceptor groups during binding.

What is the nature of the RNA conformational change? The CD experiments presented here cannot provide detailed information about specific conformational changes; however, the CD of nucleic acids is generally sensitive to changes in base stacking (Aboul-ela et al., 1988). Recent NMR studies of TAR and arginine–TAR complexes (Puglisi et al., 1992) are consistent with specific changes in base stacking upon arginine binding. In the unbound state, nucleotides in the bulge appear to be at least partially stacked between two A-form helices. When the complex with arginine is formed, the bases in the bulge become unstacked and the two double-stranded helices become coaxially stacked. The apparent net loss of stacking interactions is consistent with the observed decrease in the CD signal at 265 nm. The structure of bound TAR appears to be stabilized by a base triple interaction between U23 in the bulge and the A27–U38 base pair.

Changes in RNA conformation have been seen in other RNA–protein interactions. The most detailed insights are provided by crystallographic studies of tRNA synthetase–tRNA complexes (Rould et al., 1989, 1991; Ruff et al., 1991). Several types of conformational rearrangements are seen, including unstacking of bases and formation of non-Watson–Crick base pairs. Circular dichroism studies (Daly et al., 1990) and nuclease and chemical modification experiments (Kjems et al., 1991) with the HIV Rev protein suggest that an RNA conformational change may also occur upon its interaction with a specific RNA-binding site. Understanding the relationship between RNA conformational change and specific protein recognition is likely to provide interesting perspectives on molecular recognition and RNA function.

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Registry No. CYHHHRHHHHHA, 143493-40-7; L-arginine, 74-79-3; guanidine, 113-00-8.