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Circular dichroism and molecular modeling yield a structure for the complex of human immunodeficiency virus type 1 trans-activation response RNA and the binding region of Tat, the trans-acting transcriptional activator

(nucleic acid protein interaction/circular dichroism/energy minimization/AIDS)

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ABSTRACT Transcription in the human immunodeficiency virus type 1 (HIV-1) retrovirus is regulated by binding the viral Tat protein (trans-acting transcriptional activator) to the trans-activation response (TAR) RNA sequence. Here, vacuum UV circular dichroism (VUV-CD) is used to study the structure of TAR and its complex with two peptide fragments that are important for Tat binding to TAR. The VUV-CD spectrum of TAR is typical of A-form RNA and is minimally perturbed when bound to either the short or the long Tat peptide. The CD spectra of the complexes indicate an extended structure in the arginine-rich region of Tat from amino acid residue 47 through residue 58 and a short α -helix within the adjacent 59–72 region. Models of TAR and its peptide complexes are constructed to integrate these spectroscopic results with current biochemical data. The model suggests that (i) the arginine-rich 49–58 region is primarily responsible for electrostatic interactions with the phosphates of the RNA, (ii) the arginine side chains can additionally interact with substituent groups of the nucleotide bases to confer base recognition in the complex, (iii) the recognition of uracil-23 in TAR is facilitated by the peptide backbone, and (iv) the glutamine-rich face of an α -helix within the 59–72 region pairs to bases UGG at nucleotide positions 31–33 in the TAR loop and thus provides an additional motif in the Tat trans-activating protein to recognize TAR RNA.

Human immunodeficiency virus type 1 (HIV-1) possesses regulatory genes not found in other retroviral genomes (1). One of these genes encodes the trans-acting transcriptional activator (Tat protein), which upon binding to the nucleotide trans-activation response (TAR) sequence, activates all viral mRNA transcripts (see ref. 2 for a recent review). The TAR sequence is present at the 5' end of all HIV-1 mRNAs (3), suggesting that the binding of Tat to the RNA form of TAR is an important step in regulating transcription of HIV-1 genes (4–12), although the mechanism of this trans-activation remains unclear.

Tat is a small protein of 86 residues; however, only the 72 N-terminal amino acids are required for full activity (13). Mutations within the arginine-rich region from amino acid residue 47 through residue 58 yield a nonfunctional cytoplasmic Tat protein (14), and short peptides corresponding to this polybasic region show high affinity for the TAR sequence. A 9-residue peptide containing only arginine residues also binds TAR with high affinity, while an analogous peptide containing only lysine residues does not (12). Similarly, mutant forms of Tat in which residues 47–55 have been replaced by arginine

residues are fully active, while lysine substitutions yield a marginally active protein (12). Arginine-52 appears to be particularly important in binding TAR. One model proposes that the imino nitrogens of the guanidino side chain form specific networks of hydrogen bonds to bridge adjacent phosphodiester, linking nucleotides G21 to A22 and A22 to U23 in the TAR sequence (12). Recent molecular models of the arginine-rich region of Tat resulting from CD studies suggest that this basic region is an extended structure (15) or is unstructured in the absence of RNA (10). Upon binding to the TAR RNA, however, the peptide may become partially or fully structured and induce a conformational change in the RNA (10). Lengthening the peptides to include residues 47–72 enhances the stability but not the affinity of complexes with TAR (7).

The sequence in TAR that is recognized by the Tat protein spans nucleotides +19 to +42, and forms a stable hairpin that includes a six-nucleotide loop, a trinucleotide bulge, and an 8-base-pair (bp) stem (3, 16, 17). Mutations in the loop are deleterious for transactivation but have no effect on the binding of Tat protein or the polybasic peptides (5, 6, 9). Peptide binding studies show that the trinucleotide bulge and 5 bp adjacent to the bulge are important for Tat binding (8, 11). U23 in the bulge is invariant and is required for nucleoprotein complex formation (5, 6, 18). A recent model of TAR suggests that the major groove, where the bulge is located, is similar to that of a B-form double helix and that the Tat protein binds within this wider major groove (11).

MATERIALS AND METHODS

Synthesis. The RNA was the gift of Gabriele Varani, and Tat-(47–72)-hexacosapeptide was synthesized as described (15). Tat-(47–58)-dodecapeptide was synthesized with an Applied Biosystems 431A synthesizer using "Fast-moc" chemistry.

VUV-CD Measurements. TAR and TAR–Tat complexes for vacuum UV circular dichroism (VUV-CD) measurements were in 10 mM sodium phosphate buffer, pH 7.5/5% (vol/vol) glycerol/70 mM KF (10). Molar ratios for complex preparations were 1:1 as monitored by HPLC. Peptide samples were prepared in 10 mM sodium phosphate buffer (pH 7.5), 10 mM NaOH (pH 11), or 80% (vol/vol) CF₃CH₂OH. CD spectra were measured in 50- μ m path-length cells from

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Abbreviations: HIV-1, human immunodeficiency virus type 1; TAR, trans-activation response element; Tat protein, trans-acting transcriptional activator of HIV-1; C₁₈ HPLC, reverse-phase high-performance liquid chromatography on C₁₈ columns; VUV, vacuum ultraviolet; Tat-(47–58), peptide matching Tat sequence 47–58; Tat-(47–72), peptide matching Tat sequence 47–72.

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320 nm to 178 nm with a VUV-CD spectrophotometer as described (15). Measurements were performed at 20°C for the peptides alone and 5°C for TAR and its peptide complexes (5°C was required to stabilize the complexes). CD spectra are reported as the difference in extinction coefficient, $\Delta\epsilon$, per amide or per nucleotide. The VUV-CD data were analyzed for secondary structure by the method of Manavalan and Johnson (19). Purified peptides were analyzed for amino acid content and concentration on a Beckman 126AA system gold HPLC amino acid analyzer. The TAR extinction coefficient is 229,500 M⁻¹cm⁻¹ at 260 nm per TAR molecule.

Molecular Models of TAR and Its Tat Peptide Complexes. Initial models for the TAR RNA alone and TAR complexed with the Tat peptides were constructed by using the INSIGHTII program from Biosym Technologies, similar to previous work (15). Each model was subjected to energy minimization, followed by 1 ps of molecular dynamics at 500 K after equilibration, and then final energy minimization to a maximum derivative of 1.0 kcal per step by using the program DISCOVER from Biosym Technologies, with the AMBER (20) forcefields, running on a Silicon Graphics 4D30GTX Iris personal workstation. The structures were optimized in terms of the internal energies within each molecule and of the interaction energies between the molecules.

RESULTS AND DISCUSSION

CD Spectrum of TAR RNA. The VUV-CD spectra of double-stranded DNA and RNA are highly sensitive to the conformation of the polynucleotide helix. The spectrum of TAR RNA alone is typical for RNA and is usually associated with the A-conformation of a polynucleotide helix (Fig. 1).

CD Spectra of the Tat Peptides. The VUV-CD of Tat-(47–58) (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro) at pH 7, pH 11, and in 80% CF₃CH₂OH is characterized by a negative band about 198 nm, which is normally attributed to a random coil structure (Fig. 2). Analysis of these spectra by the variable selection method gives antiparallel β -strand, β -turn, and other structure (Table 1). Random coil is not "other," but is a dynamic system with a propensity for the ϕ - ψ angles associated with β -strand and β -turn. These analyses are typical for a random-coil CD.

The VUV-CD (Fig. 2) and subsequent secondary structure analyses (Table 1) of the longer Tat-(47–72) peptide (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Gln-

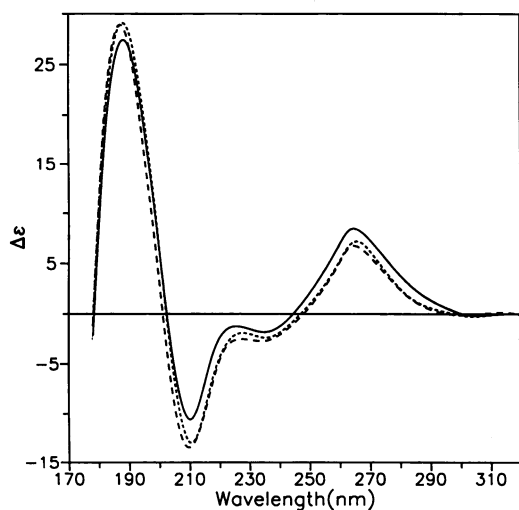


FIG. 1. VUV-CD spectra at 5°C of TAR (—), TAR-Tat-(47–58) complex (- - -), and TAR-Tat-(47–72) complex (- · -) in 10 mM phosphate buffer, pH 7.5/70 mM KF/5% glycerol, expressed as M⁻¹cm⁻¹ per base.

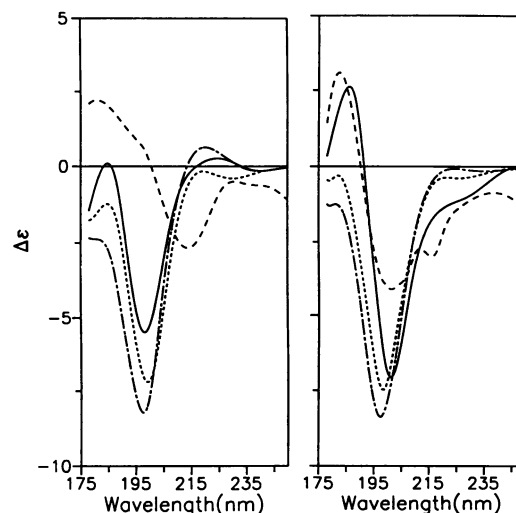


FIG. 2. VUV-CD spectra of the peptides Tat-(47–58) (Left) and Tat-(47–72) (Right) in 10 mM phosphate buffer (pH 7.5) (- · -), in 10 mM NaOH (pH 11) (- - -), and in 80% CF₃CH₂OH (—) as M⁻¹cm⁻¹ per amide basis. Difference spectra of the TAR-Tat peptide complexes minus the TAR RNA spectrum (- - -) are represented along with the corresponding peptide as M⁻¹cm⁻¹ per base.

Gly-Ser-Gln-Thr-His-Gln-Val-Ser-Leu-Ser-Lys-Gln) at pH 7 and pH 11 are similar to those of Tat-(47–58) (Fig. 2). In 80% CF₃CH₂OH, however, there is a significant increase in α -helix structure, which was not observed with the short peptide (Table 1). This difference is well illustrated in the emergence of a broad negative band at long wavelength, a positive band at 187 nm, and a red shift from 197.5 to 201 nm of the original negative band. This same CD, showing a higher α -helical content, was obtained in the presence of 2 mM SDS (results not shown). This increase in α -helix structure is most likely located at residues 59–72, since Tat-(47–58) in 80% CF₃CH₂OH shows no α -helical structure.

CD Spectrum of the TAR-Tat-(47–58) Complex. The CD of the complex between TAR and the Tat-(47–58) peptide is dominated by the RNA (Fig. 1). Moreover, the modest CD due to the protein contributes only at a wavelength below 240 nm (Fig. 2). The 265-nm band of the RNA is sensitive to base stacking. The decrease in $\Delta\epsilon$ upon forming the complex can be interpreted as a modification of the base stacking induced by the binding of Tat-(47–58). Overall, the spectrum, especially the 210-nm band, is still typical of the A-form; thus the TAR structure is only minimally perturbed in the complex.

A decrease in intensity for the 265-nm band of RNA is always accompanied by a decrease in intensity for the shorter wavelength bands, as shown in melting (21) and solvent perturbation studies (22). In these cases, the first crossover is virtually unchanged. Alternatively, when an A-form polynucleotide is perturbed toward B-form, the 265-nm band decreases and the 240-nm band increases in intensity with a

Table 1. Secondary structures predicted by variable selection for Tat-(47–58) and Tat-(47–72) in phosphate buffer (pH 7), sodium hydroxide (pH 11), or 80% CF₃CH₂OH

Peptide	Condition	H	A	P	T	O	Tot
Tat-(47–58)	pH 7	0.00	0.17	0.04	0.36	0.43	1.00
	pH 11	0.02	0.12	0.05	0.33	0.48	1.00
	80% TFE	-0.01	0.32	0.03	0.22	0.39	0.96
Tat-(47–72)	pH 7	0.04	0.11	0.01	0.37	0.48	1.00
	pH 11	0.06	0.16	0.00	0.31	0.46	1.00
	80% TFE	0.14	0.15	0.01	0.29	0.41	1.00

TFE, CF₃CH₂OH; H, helix; A, antiparallel β -sheet; P, parallel β -sheet; T, turn; O, other structure; Tot, total.

red shift of the crossover, while the shorter wavelength bands decrease in intensity (22). This observation is similar to the effect observed for the TAR complex (Fig. 1), including the increase in CD to the red-absorbing wavelengths of the protein bands. This suggests that upon binding, the TAR RNA assumes a somewhat more B-like character. In any event, the increases in the magnitude of the CD that we observe below 240 nm must be due to the protein. The difference CD shows a maximum at 185 nm and a minimum at 215 nm (Fig. 2). The negative band at 215 nm cannot be due to the RNA and matches very well with the CD of a β structure (21). If we scale the CD of the TAR RNA alone by the same percentage at all wavelengths to fit the 265-nm band of the complex (as suggested by solvent perturbations), we obtain a similar difference spectrum, but with intensities comparable to that of the uncomplexed peptides (results not shown). Together, these observations lead to the same interpretation—that Tat-(47–58) adopts an extended structure upon binding to TAR.

CD of the TAR–Tat-(47–72) Complex. The complex of TAR with the Tat-(47–72) peptide shows a CD that is very similar to that obtained for the TAR–Tat-(47–58) complex (Fig. 1). A clear difference occurs between 235 and 223 nm with a maximum at 225 nm. The intensity of the negative band near 210 nm is the same in both complexes but is slightly blue-shifted to 209 nm in the TAR–Tat-(47–72) complex. The crossover is blue-shifted from 203.5 to 201 nm. These results indicate that addition of the C-terminal sequences causes little additional modification of the TAR structure; therefore, the primary structural perturbations to TAR are associated with effects from the 47–58 region of the peptide.

The difference CD spectrum derived from the TAR–Tat-(47–72) complex and TAR alone shows two minima at 204 nm and 215 nm and a maximum at 185 nm (Fig. 2). Again, scaling the CD of TAR to account for solvent perturbation or B-form structure does not significantly change the extrema but does increase the intensity of the bands in the difference spectrum. The 215-nm band can be attributed to the β -structure of the polybasic region that is common between the short and the long peptide. However, the 204-nm band shows that there is an α -helix in the Tat peptide complexed to TAR.

The spectra in Fig. 2 are plotted on a per amide basis so that the bands associated with the β -structure would appear to be half the intensity in the longer oligomer as observed for the shorter peptide. The minimum at 204 nm is similar to the π - π^* transition at 205–208 nm of an α -helix (21). For short α -helices (seven to eight residues long), the characteristic CD bands are blue-shifted, with the positive band located at 185–187 nm and the negative band at 204–205 nm (23), as observed here. These results suggest the presence of a short α -helix, probably no longer than two turns, located at the C-terminal end of the Tat-(47–72) peptide when complexed to TAR. The n - π^* transition at 222 nm that is characteristic of an α -helix is reduced in intensity and broadened to a non-distinct band in the spectra of shorter peptides. In the present difference spectrum, the 215-nm band overlaps and obscures the n - π^* transition of the short α -helix, but this feature could account for the difference observed between 223 nm and 235 nm in the CD spectra of the TAR–Tat-(47–58) versus the TAR–Tat-(47–72) complexes (Fig. 1). The band at 204 nm is also observed for the Tat-(47–72) peptide alone in 80% $\text{CF}_3\text{CH}_2\text{OH}$ and is associated with an increase in α -helix structure (Table 1). Thus, it appears that the 47–58 region is an extended structure, while a short α -helix of seven or eight residues is located in the C-terminal 59–72 region.

Model of TAR RNA. We used the crystal structure of residues 49–65 from phenylalanine t-RNA, plus the intervening G18 residue, to set guidelines for the structural features that are important in defining the conformation of the TAR RNA. The model for the TAR RNA follows the struc-

tural guidelines set by the t-RNA template (Fig. 3). The continuity of the A-helix is maintained, and base stacking and base pairing are maximized as much as possible. The base-paired regions in the TAR model adopt an A-helical conformation, as suggested by the CD. Residues C24 and U25 of the bulge extend away from the A-helix, while U23 is stacked between residues A22 and G26. This places a kink in the A-helix towards the major groove but otherwise leaves the base stacking and pairing of the stem intact. Interestingly, U25 appears to form a hydrogen bond to the 2'-hydroxy group of the ribose and to O-4 of the base at C24. This hydrogen-bonding scheme, plus the stacking of U23, may render the two uracil bases less susceptible to electrophilic reagents such as diethyl pyrocarbonate (11).

In the model it was logical to pair C30 and G34. Thus, a C-G base pair separates the UGG (positions 31–33) loop and a single-base bulge (A35). Residues G32, G33, and G34 are stacked, while the adenine of A35 forms a triple base pair with C30-G34.

Model of the TAR–Tat-(47–58) Complex. Weeks and Crothers (11) suggested that arginine-rich peptides sit in the major groove of the A-helix of TAR. Earlier studies suggested that this region of the Tat protein is extended (15) and may be induced to form a specific structure upon binding to the TAR RNA (10). The current CD results further suggest that the RNA is only minimally perturbed by binding of this polybasic region. A model for the complex was constructed by systematically adding amino acids to the major groove of the TAR model until the full peptide was generated. In building this model, we oriented the peptide with the C terminus towards the loop of the RNA. Studies have shown

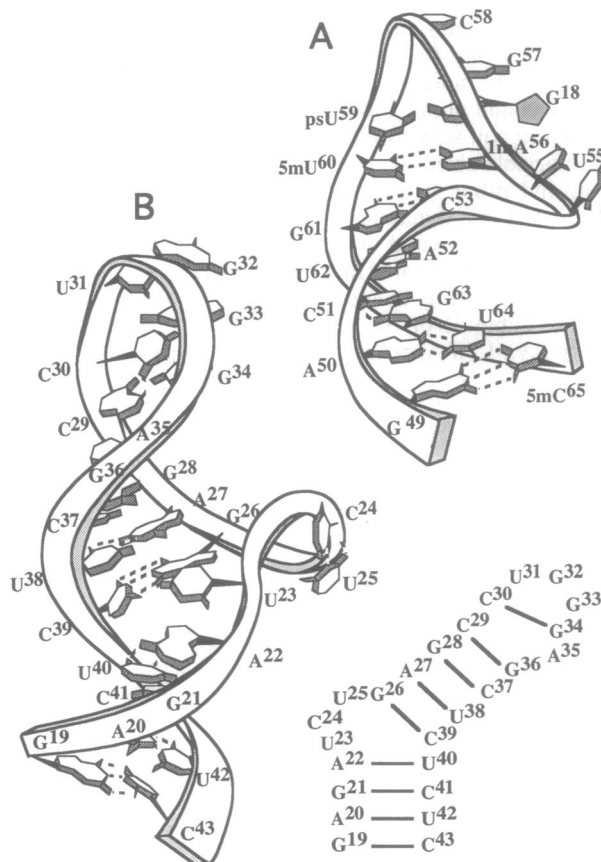


FIG. 3. Comparison of the phenylalanine t-RNA loop and bulge structure (A) with the TAR RNA model (B). Hydrogen bonds are drawn as broken lines. The base sequences are labeled along the ribbon backbone of each structure. The sequence of the TAR RNA is shown.

protein protects not only the bulge but also the loop region of TAR (27). Furthermore, mutations in the loop region of TAR have been shown to impede the binding of the longer Tat-(47–72) peptide but not the short Tat-(47–58) fragment (28). Thus, the loop region of the TAR RNA appears to be an additional site for recognition by the Tat protein.

The bulge and the loop of TAR therefore act in concert to confer sequence-specific binding by the Tat protein. The nucleotide sequences present in the loop and the bulge as well as the spatial relationship between the two are essential for trans-activation by the Tat protein in HIV-1 (16, 29). Insertion of extra base pairs in the stem that separates the bulge from the loop decreases the efficiency of trans-activation (30). Arginine-rich peptides have been shown to have very high affinities for the TAR sequence, while the C-terminal 59–72 residues do not appear to significantly enhance the affinity of Tat. However, mutations within this 59–72 region dramatically decrease Tat function (31). The intermolecular energy between TAR and residues 49–58 is calculated from our models to be -710 kcal/mol, with the electrostatic interactions accounting for fully 80% of the total energy. For the longer Tat peptide complex, the total intermolecular energy is -782 kcal/mol, with the four glutamine nucleotide pairs of our model contributing approximately -20 kcal/mol. Thus, it appears that residues 47–58 of the Tat protein, and indeed nearly any arginine-rich peptide, provide the majority of the binding energy, while the glutamine-rich-face confers additional specificity to the complex.

Previous studies have described cellular proteins that bind to the loop and the bulge of the TAR RNA (32–36). Therefore, a competition between these cellular proteins and Tat could be an important step in the activation of the virus. A host cell nuclear protein has been found that binds to the stem loop of TAR and promotes premature termination of transcripts from the HIV-1 promoter *in vivo* (37). Tat, with its higher affinity, could displace this protein from TAR and thus promote the elongation of the HIV-1 transcript, which is a Tat function (38). Another role of region 59–72 could be to facilitate the trans-activation by stabilizing the Tat–TAR complex, and to properly position the activating region of Tat.

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