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# Unusual Structure of the Human Immunodeficiency Virus Type 1 *trans*-Activation Response Element

RICHARD A. COLVIN[1,2] AND MARIANO A. GARCIA-BLANCO[1,2,3]*

*Section of Cell Growth, Regulation, and Oncogenesis,[1] Department of Microbiology and Immunology,[2] and Department of Medicine,[3] Duke University Medical Center, Durham, North Carolina 27710*

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**The *trans*-activation response element (TAR) of human immunodeficiency virus type 1 is a structured RNA consisting of the first 60 nucleotides of all human immunodeficiency virus type 1 RNAs. Computer analyses and limited structural analyses indicated that TAR consists of a stem-bulge-loop structure. Mutational analyses showed that sequences in the bulge are required for Tat binding, whereas sequences in both the bulge and the loop are required for *trans* activation. In this study, we probed the structures of TAR and various mutants of TAR with chemical probes and RNases and used these methods to footprint a Tat peptide on TAR. Our data show that the structure of wild-type TAR is different from previously published models. The bulge, a Tat-binding site, consists of four nucleotides. The loop is structured, rather than simply single stranded, in a fashion reminiscent of the structures of the tetraloop 5′-UUCG-3′ and the GNRA loop (C. Cheong, G. Varani, and I. Tinoco, Jr., Nature [London] 346:680–682, 1990; H. A. Heus and A. Pardi, Science 253:191–193, 1991). RNA footprint data indicate that three bases in the bulge are protected and suggest that a conformational change occurs upon Tat binding.**

Replication of the human immunodeficiency virus type 1 (HIV-1) requires two viral *trans* activators, Tat and Rev. Rev allows the expression of full-length unspliced viral transcripts, whereas Tat acts to increase viral transcription and translation in a manner not yet fully elucidated (6, 7, 27). Tat binds to an RNA target sequence termed the *trans*-activation response element (TAR) which is present in the first 60 nucleotides of all HIV-1 RNAs (8, 9, 29). TAR is a highly structured element that is postulated to form a hairpin and that contains a hexanucleotide loop and a trinucleotide bulge (15, 23). Most of the remaining RNA is probably in an A-form helical configuration (30).

Recent studies indicated that TAR does not have a direct role in transcription but serves to anchor Tat to a location near the start of transcription (1, 26, 28). By replacing the RNA-binding domain of Tat with an alternative RNA- or DNA-binding domain of a different nucleic acid-binding protein and replacing TAR with the appropriate target sequence, *trans* activation can be retained (1, 26, 28). This indicates that TAR does not directly interact with the transcriptional machinery.

Mutational analyses and modification interference studies indicated that Tat binds to TAR at the bulge, with U23 playing a particularly important role (24, 29). The bulge includes bases A22 and U23, which, when chemically modified, destroy the ability to bind Tat, and base U25, which, when chemically modified to an abasic site, shows enhanced Tat binding. The ability of a given TAR to facilitate *trans* activation depends on its ability to bind Tat (8, 9, 24, 25). Nevertheless, Tat binding is required but not sufficient for *trans* activation (8, 9, 24). Mutational analyses have shown that although sequences in the loop are not required for Tat binding, they are required for *trans* activation (2, 10). The loop may be the site of binding to a cellular factor involved in *trans* activation (12, 13, 20). The specificity of this interaction may be determined by the sequence and/or the structure of the loop (20).

Although several studies of the structure of TAR have been published, the complete structure of TAR and particularly that of the hexanucleotide loop have not been determined (3, 23, 30). We probed the structure of TAR with several chemical modifiers and RNases and found that the loop contains a secondary or tertiary structure, while the bulge consists of four nucleotides rather than of the previously proposed three. The same methods were used to probe the structure of TAR complexed with Tat. We show evidence of specific protection of part of the bulge and a conformational change of TAR in the helices flanking the bulge upon Tat binding.

## MATERIALS AND METHODS

**Plasmids.** TAR mutants 31/34 and Δ(+35/+38) TAR are described in reference 11. HIV5 [pT7TAR(U/+34)], HIV7 [pT7TAR(G/+29)], and HIV8 [pT7TAR(G/+29,C/+36)] are described in references 10 and 20. TAR+10s was constructed by replacing the *Bgl*II-*Sac*I fragment in the plasmid T7TAR (20) with the oligonucleotides 5′-GATCTGCTCT CAGCCTGGGAGCTGAGAGCT-3′ and 3′-ACGAGAGTC GGACCCTCGACTC-5′.

**Transcriptions and preparation of RNAs.** DNA templates were obtained by polymerase chain reaction of the inserts of plasmids described above. RNAs were transcribed by T7 RNA polymerase in such a way that the transcriptional start site corresponded to the authentic HIV-1 RNA +1 nucleotide (20, 21). The RNAs were denatured by heating at 65°C for 10 min and were allowed to renature by slow cooling to room temperature in 100 mM NaCl–10 mM $MgCl_2$ (5).

**Modifications, endonucleolytic cleavages, and primer extensions.** RNAs were treated with dimethyl sulfate (DMS), 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide methyl *p*-toluenesulfonate (CMCT), and kethoxal. DMS methylates the N1 position of adenines (A) and the N3 position of cytosines (C), CMCT reacts with position N1 of guanines (G)

* Corresponding author.

and position N3 of uracils (U), and kethoxal reacts with guanines at positions N1 and N2 (5, 14, 18, 22). Each reaction mixture contained $10^{-12}$ mol of RNA in 100 mM KCl, 10 mM $MgCl_2$, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 1 mM dithiothreitol, and 0.1% (vol/vol) Nonidet P-40. DMS (Mallinckrodt) was added to a final concentration of 0.5% (vol/vol) in a 100-μl total volume. The reactions proceeded for 20 min on ice. CMCT (Sigma) was added to a final concentration of 2.1 mg/ml in a 20-μl total volume. The reactions proceeded for 10 min at 30°C. Kethoxal (U.S. Biochemicals) was added to a final concentration of 0.75 mg/ml in a total volume of 100 μl. The reactions proceeded for 10 min at 30°C. For enzymatic reactions, $10^{-12}$ mol of each RNA was incubated with the indicated amount of RNase $T_1$ in a total volume of 20 μl for 30 min on ice or with the indicated amount of RNase A for 10 min on ice. Following incubation, the reaction mixtures were extracted once with phenol, once with phenol-chloroform (1:1), and once with chloroform, and they were precipitated with ethanol (5). Modifications were detected by primer extension with murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) as described elsewhere (22). An oligonucleotide complementary to the region from +60 to +80 of the HIV-1 transcript was labelled at the 5' end with T4 polynucleotide kinase (New England Biolabs) and 5' ATP to a specific activity of 500 μCi/mmol. The sequencing ladder was obtained with T4 DNA polymerase (Sequenase; U.S. Biochemicals) and with the 5'-labelled oligonucleotide described above on the plasmid T7TAR. The labelled cDNAs were separated in a 15% polyacrylamide (acrylamide-bisacrylamide [29:1]) sequencing gel.

**Gel retardation assays.** RNAs were synthesized and prepared as described above. A total of $10^{-12}$ mol of each RNA was added to $5 \times 10^{-12}$ mol of Tfr38 (29) and allowed to incubate at room temperature for 2 min. The samples were then separated by native polyacrylamide gel electrophoresis in a 5% polyacrylamide (acrylamide-bis [60:1]) gel containing 0.045 M Tris-borate, 0.045 M boric acid, and 0.002 M EDTA (pH 8.0) (29).

**Footprinting.** TAR RNA prepared as described above was incubated with $2.5 \times 10^{-12}$, $5 \times 10^{-12}$, or $1 \times 10^{-11}$ mol of Tfr38 at room temperature for 10 min. Following incubation, the complexes were treated with 0.5 μl of DMS for 20 min on ice. Otherwise, the modification reactions were as described above.

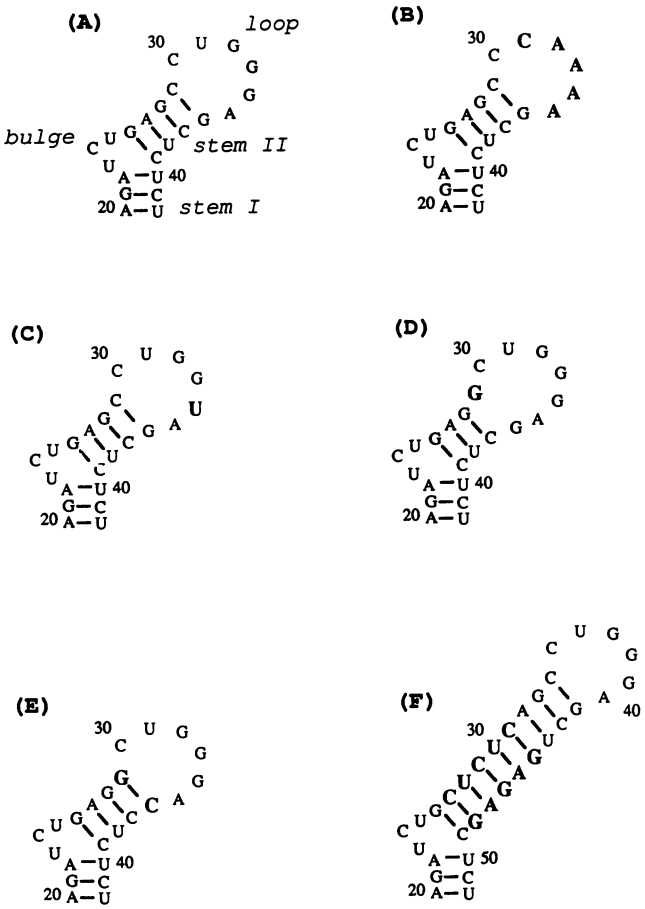


FIG. 1. Predicted partial structures of TAR RNA and various mutants. Model structures based on computer predictions made with the Zucker parameters are shown (31). Predicted structures of wild-type (A), 31/34 (B), HIV5 (C), HIV7 (D), HIV8 (E), and TAR+10s (F) TAR RNAs are shown.

## RESULTS

**Structural analysis of TAR and TAR mutants.** The bulge of wild-type TAR RNA has been predicted to contain three bases, U23, C24, and U25 (Fig. 1) (3, 23). In this study, chemical probes were used to detect bases that have their Watson-Crick base-pairing positions accessible to solvent (see Materials and Methods for details of chemical probes) (5, 22). Consistent with the bulge in the proposed structure, U23 and U25 were modified by CMCT (Fig. 2, lanes 3 and 4), and C24 was methylated by DMS (Fig. 2, lanes 1 and 2). Unexpectedly, DMS also methylated A22, indicating that it does not form a stable Watson-Crick base pair with U40 (Fig. 2, lanes 1 and 2). No evidence, however, that CMCT modified U40 was obtained. These data indicated that the wild-type TAR RNA contained a 4-base bulge from positions A22 through U25. Probing of TAR loop mutants 31/34, HIV5, HIV7, and HIV8 (Fig. 1) revealed the presence of the same 4-base bulge (Fig. 2B, lanes 1 and 2, and data not shown). This suggested that the structure of the bulge does not depend on a specific sequence in the loop.

The loop of TAR has been predicted to contain six unpaired bases, C30, U31, G32, G33, G34, and A35 (3, 22). DMS methylated A35 but not C30 (Fig. 2A, lanes 1 and 2). U31 and G32 were preferentially modified by CMCT in comparison with G33 and G34 (Fig. 2A, lanes 3 and 4). Finally, kethoxal strongly modified G32, but G33 and G34 were modified much less frequently (Fig. 2A, lanes 5 and 6). These results indicated that the loop contains either secondary or tertiary structures, as evidenced by the solvent inaccessibility of C30, G33, and G34.

Additional evidence that the loop of TAR is structured was obtained by partial digestion of TAR RNA with RNase $T_1$, a nuclease with specificity for single-stranded G residues (Gp ↓ N) in RNA molecules (Fig. 2A, lanes 10 and 11). In agreement with the CMCT and kethoxal results described above, G32, G33, and G34 showed decreasing susceptibilities to endonucleolytic cleavage by RNase $T_1$. Although it was not susceptible to modification by DMS, in partial digestions C30 was cleaved by RNase A. RNase A, a nuclease with specificity for pyrimidines (especially Up ↓ A), also cleaved 3' of U31 (Fig. 2A, lanes 7 through 9).

Probing of the TAR mutant RNAs yielded different results

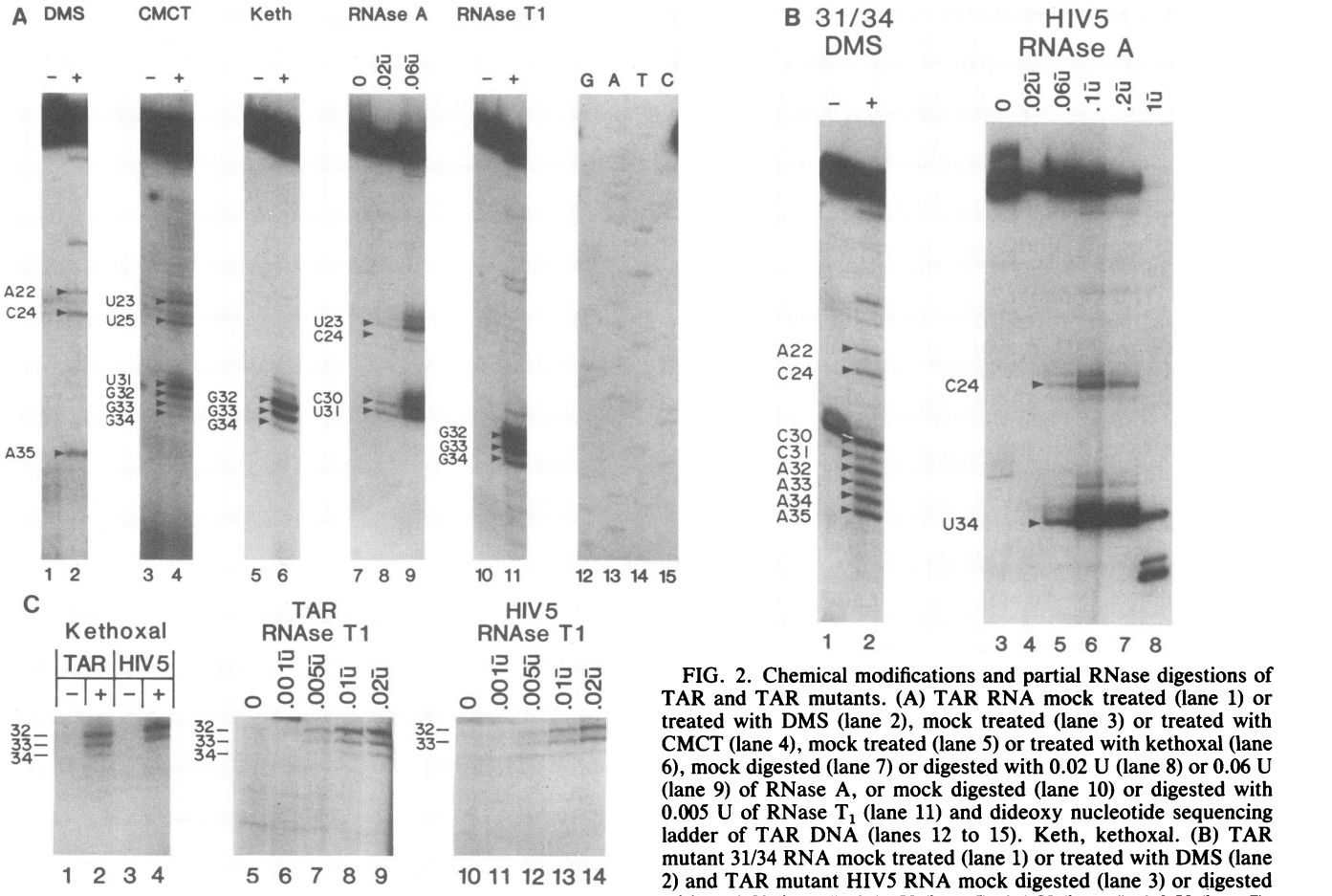


FIG. 2. Chemical modifications and partial RNase digestions of TAR and TAR mutants. (A) TAR RNA mock treated (lane 1) or treated with DMS (lane 2), mock treated (lane 3) or treated with CMCT (lane 4), mock treated (lane 5) or treated with kethoxal (lane 6), mock digested (lane 7) or digested with 0.02 U (lane 8) or 0.06 U (lane 9) of RNase A, or mock digested (lane 10) or digested with 0.005 U of RNase $T_1$ (lane 11) and dideoxy nucleotide sequencing ladder of TAR DNA (lanes 12 to 15). Keth, kethoxal. (B) TAR mutant 31/34 RNA mock treated (lane 1) or treated with DMS (lane 2) and TAR mutant HIV5 RNA mock digested (lane 3) or digested with 0.02 U (lane 4), 0.06 U (lane 5), 0.1 U (lane 6), 0.2 U (lane 7), or 1 U (lane 8) of RNase A. (C) TAR RNA mock treated (lane 1) or treated with kethoxal (lane 2); HIV5 RNA mock treated (lane 3) or treated with kethoxal (lane 4); TAR RNA mock digested (lane 5) or digested with 0.001 U (lane 6), 0.005 U (lane 7), 0.01 U (lane 8), or 0.02 U (lane 9) of RNase $T_1$; and HIV5 RNA mock digested (lane 10) or digested with 0.001 U (lane 11), 0.005 U (lane 12), 0.01 U (lane 13), or 0.02 U (lane 14) of RNase $T_1$. Positions of the chemical modifications or endonucleolytic cleavages were determined by primer extension with a 5′-labelled oligonucleotide spanning positions +60 to +80 on the RNA and reverse transcriptase. The same 5′-labelled oligonucleotide was used in the sequencing reactions.

(Fig. 2B). TAR 31/34 RNA has a mutant loop (CCAAAA), and all six nucleotides were equally modified by DMS (Fig. 2B, lanes 1 and 2). These results strongly suggested that the structure of the loop in TAR 31/34 is different from that of the loop present in wild-type TAR. Modification of C30 was difficult to interpret, since C30 appeared as a primer extension stop in unmodified control lanes for 31/34. Although TAR HIV5 contains only one base change from wild-type TAR (a G-to-U change at position 34), the structure of the HIV5 loop is significantly altered. U34 was modified by CMCT (data not shown), in contrast to G34 in TAR, which was only slightly modified by CMCT and kethoxal. The two remaining G residues in the loop of HIV5, G32 and G33, were modified by CMCT and kethoxal similarly to the corresponding residues of wild-type TAR. RNase $T_1$, on the other hand, cleaved after G32 and G33 equally, in contrast to the result with wild-type TAR (Fig. 2C, lanes 5 through 14). When TAR and HIV5 were probed with RNase A, a striking difference appeared in their cleavage patterns (Fig. 2A, lanes 7 through 9, and Fig. 2B, lanes 3 through 8). C30 and U31 of TAR were highly susceptible to cleavage by RNase A, whereas C30 and U31 of HIV5 were not cleaved by RNase A, even at concentrations of the enzyme that resulted in nonspecific cleavages. As with wild-type TAR, DMS did not methylate C30 of HIV5 (data not shown). These results suggested that the loop of HIV5, although differing in sequence by only one base, is structured differently from the loop of wild-type TAR. The structure of HIV5 TAR, with the exception of the loop, was indistinguishable from that of wild-type TAR in our assays (data not shown).

TAR mutant HIV7, a mutant expected to have an 8-base loop (Fig. 1), appeared to have an unstructured loop, with all the bases reacting as expected (data not shown). TAR mutant HIV8 complements the mutation in HIV7, recreating the possibility of a base pair between positions 29 and 36. This mutant had a 6-base loop with the same structure as the loop of wild-type TAR (data not shown). TAR mutants Δ(+35/+38) TAR and TAR+10s were not as tightly structured as the other TAR mutants under the conditions used in the assay, because in both cases, stem II (Fig. 1) demonstrated moderate accessibility to modification (data not shown). Unlike 31/34, HIV5, and HIV7, which are inactive, HIV8 *trans* activates as well as wild-type TAR, suggesting a correlation between wild-type structure and the ability to *trans* activate (10).

**Gel retardation assays.** We performed gel retardation assays with the various TAR mutants to determine which

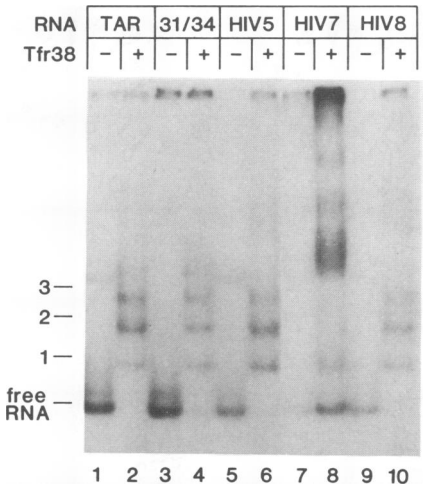


FIG. 3. Gel retardation assays of TAR and TAR mutants. TAR, 31/34, HIV5, HIV7, and HIV8 RNAs were incubated with the Tat peptide Tfr38 (28) (+) or mock incubated (−). Tfr38 was present in approximately a fivefold molar excess. The Tfr38-bound RNA and the free RNA were separated by electrophoresis in a native polyacrylamide gel.

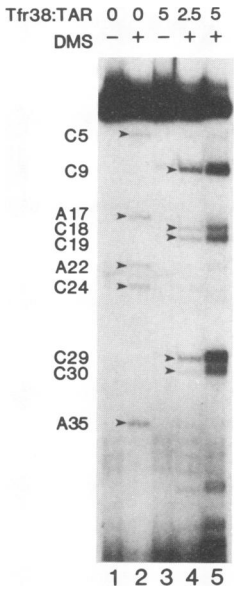


FIG. 4. Tfr38 footprint of TAR. Lanes 1 and 2, TAR mock treated and treated with DMS, respectively; lane 3, TAR incubated with 5 molar equivalents of Tfr38 and mock treated with DMS; lanes 4 and 5, TAR incubated with 2.5 or 5 molar equivalents of Tfr38, respectively, and treated with DMS. Lanes 1 and 2 appear in Fig. 2A.

aspects of the loop structure, if any, were important for binding Tat (Fig. 3). Tfr38, a 38-amino-acid fragment of Tat which retains the RNA-binding specificity of Tat, was used (29, 30). Unlike full-length Tat protein, Tfr38 is soluble in water and is not readily oxidized (29). RNAs with mutant loops were shifted by Tfr38 to the same extent as wild-type TAR in a manner reported previously (Fig. 3 and Table 1) (29). These mutants included 31/34, HIV5, and HIV8. On the other hand, those mutants [including Δ(+35/+38) TAR and TAR+10s] that showed aberrant stem II structures were not shifted to the same extent or to the same complexes as wild-type TAR (data not shown). It is possible that HIV7 forms dimers and is shifted by Tfr38 as such (Fig. 3). These results suggested that as expected, Tat binding does not correlate with loop structure but is sensitive to the structure of stem II.

**Footprinting of Tfr38 on TAR.** Chemical modifications of TAR were performed after the addition of Tfr38 (Fig. 4). Tfr38-TAR complexes were formed by incubation as described above for the gel retardation assays. Tfr38-TAR complexes were probed with DMS, CMCT, and kethoxal. Titration of Tfr38-TAR complex formation was used to determine the ratio of Tfr38 to TAR used (data not shown). DMS-modified positions C5, A17, A22, C24, and A35 showed equivalent protection from modification by DMS at a Tfr38/TAR molar ratio of approximately 2.5:1 (Fig. 4). Several bases exhibited enhanced reactivity to DMS in the presence of Tfr38; these were C9, C18, C19, C29, and C30. Modification of each of these bases was enhanced with an increase in the Tfr38/TAR ratio. This result suggests that upon Tfr38 binding, TAR undergoes a conformational change that makes the Watson-Crick base-pairing positions of these bases accessible to DMS. Tfr38 was also footprinted with CMCT and kethoxal (data not shown). U23 was protected from modification by CMCT, whereas U25 exhibited no evidence of protection. C24 was also specifically protected from RNase A cleavage by Tfr38 (data not shown). These data are consistent both with the modification interference data that indicate that U25 is not important in Tat binding and with the mutational analyses that indicate that TAR elements require only two pyrimidines in the bulge to bind Tat (29, 30).

TABLE 1. Ability of mutants to bind Tfr38[a] and to facilitate *trans* activation[b]

| RNA | Degree of [c]: | |
|---|---|---|
| | Tfr38 binding | *trans* activation |
| TAR | +++ | +++ |
| 31/34 | +++ | − |
| HIV5 | +++ | − |
| HIV7 | + | − |
| HIV8 | +++ | +++ |
| Δ(+35/+38) TAR | + | − |
| TAR+10s | + | NA |

[a] Results are based on gel retardation assays.
[b] See references 10 and 19.
[c] −, none; +, weak or moderate; +++, strong; NA, not applicable.

## DISCUSSION

Our current model for the structure of TAR is shown in Fig. 5. The salient features of this model are two helical stems, a 4-base bulge, and a 6-base loop that is structured in a manner consistent with a secondary interaction of C30 with G33, and G34 with the sugar-phosphate backbone in the vicinity of C30 and U31—an interaction similar to that seen in the tetraloop and the GNRA loop (4, 16). Although A22 was clearly seen to be modified in this assay, we did not detect modification at U40. There are at least two possible explanations consistent with this: the Watson-Crick base-pairing positions of U40 remained protected, although those of A22 did not, because of another aspect of the structure of

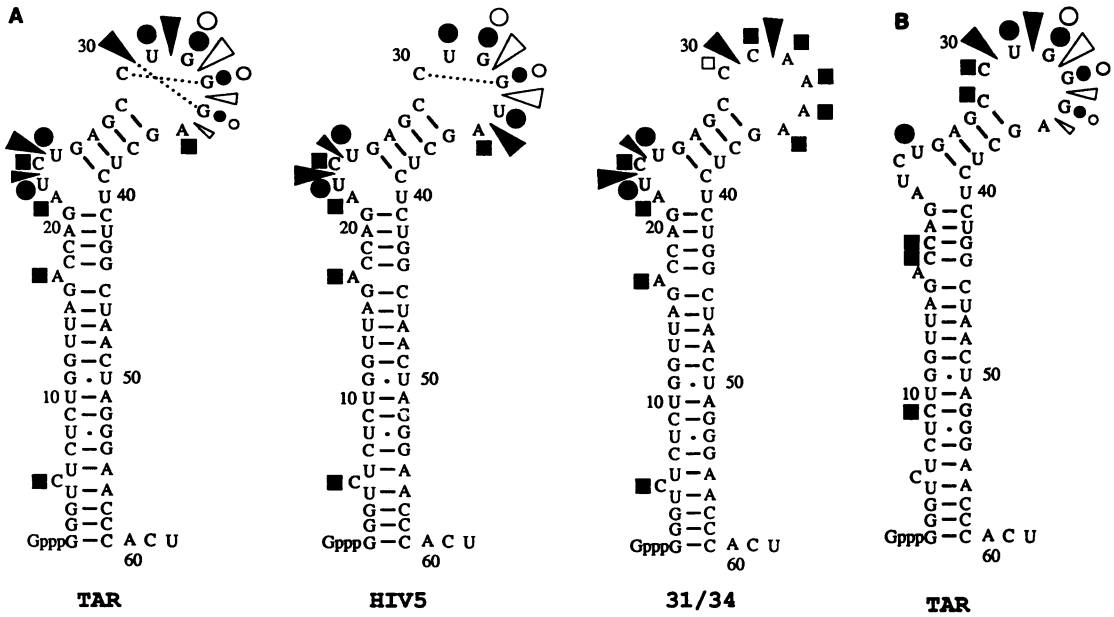


FIG. 5. (A) Summary of the structural data and proposed structures of the HIV-1 (TAR), HIV5, and 31/34 TAR RNAs. Models are based on data obtained by chemical modifications and sensitivities to nucleases. (B) Summary of modified or cleaved bases upon addition of Tfr38 to TAR RNA. Modifications by DMS (■), CMCT (●), and kethoxal (○) and cleavages by RNase $T_1$ (▷) and RNase A (►) are indicated. □, base whose reactivity with DMS could not be determined. The relative frequency of modifications or cleavages is indicated by the sizes of the symbols. Dotted lines indicate proposed interactions in the loop.

TAR or CMCT; alternatively, this assay did not detect modification at U40 because of the gel system.

Although not necessary for Tat binding, the wild-type loop is necessary for *trans* activation (2, 10). Our studies suggested that the requirement for sequences in the loop may reflect a structural requirement. Previously, RNA terminal loops have been shown by chemical modification studies and nuclear magnetic resonance spectroscopy to be structured (4, 16, 22). These loops are stabilized by base stacking and non-Watson-Crick hydrogen bonding between bases and also by interactions between the bases and groups in the sugar-phosphate backbone (4, 16). Some structured loops are important for protein binding, and thus, it may be the structure of the wild-type TAR loop that confers specific binding to cellular protein factors (12, 13, 20). An attractive possibility is that the specific *trans* activation mediated by TAR results from an RNA-protein complex containing TAR, Tat, and one or more cellular factors which bind to the loop. The role of this cellular factor(s) must be to stabilize the interaction between Tat and TAR. In this study, a single base change in the loop of HIV5 is sufficient to alter the overall structure of the loop of TAR. This may explain the drastic decrease in *trans* activation seen with this mutant.

Tat and Tfr38 have been previously shown to bind RNA bulges consisting of a single nucleotide with fairly high affinity and low specificity (9, 30). This is consistent with our data showing protection at C5, A17, A22, U23, C24, and A35. Given our model of the loop, A35 may be structured like a single bulged nucleotide. Alternatively, one Tfr38 molecule may bind A35 and the bulge simultaneously, since they may be topologically contiguous on the same side of the helix. Because *trans* activation has been shown to be very efficient and specific, this low-specificity Tat-TAR binding suggests that factors which enhance the interaction between Tat and TAR in vivo exist. It has been proposed that the bulge may be important in altering the secondary structure of the adjacent A-form helix, allowing accessibility to the major groove and thus the potential hydrogen-bonding donors and acceptors of adjacent bases (30). The helical structure (stem II) may be disrupted further upon the binding of Tat, as seen in Fig. 4. These results show that Tat binding results in DMS accessibility of the Watson-Crick positions of bases flanking the Tat-binding site, suggesting a conformational change in the stems. Although this result was not seen in every experiment (four of six), it was seen in every experiment in which protection was observed. It is likely that at these concentrations, DMS modifies the peptide to such an extent that the peptide is no longer active. An alternative explanation for the enhanced modification of certain positions by the addition of Tfr38 is that Tfr38 may sequester DMS into pockets around the RNA and enhance modification at these points. Upon probing with CMCT and kethoxal, an analogous change in the modification pattern was not seen. These chemicals may have modified the peptide to an inactive form, or positions may not have been accessible to these larger reactants. Similar conformational changes have been reported to occur in other RNAs, including the Rev-responsive element of HIV-1, upon binding of a specific binding protein (17).

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## ADDENDUM

The structures of several other TAR mutants were probed while the manuscript was under review. Most interestingly, mutation of C30 to A changed the patterns of modification of G32, G33, and G34 by kethoxal and CMCT, such that G33 and G34 were equally modified to levels much higher than those of their counterparts in wild-type TAR. G32, on the other hand, was only slightly modified by kethoxal and CMCT. These results lend support to the proposed interactions between nucleotides across the loop of wild-type TAR RNA.

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