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Site-specific cleavage of the transactivation response site of human immunodeficiency virus RNA with a tat-based chemical nuclease

(copper/phenanthroline/AIDS/human immunodeficiency virus types 1 and 2)

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ABSTRACT **tat, an essential transactivator of gene transcription in the human immunodeficiency virus (HIV), is believed to activate viral gene expression by binding to the transactivation response (TAR) site located at the 5′ end of all viral mRNAs. The TAR element forms a stem–loop structure containing a 3-nucleotide bulge that is the site for tat binding and is required for transactivation. Here we report the synthesis of a site-specific chemical ribonuclease based on the TAR binding domain of the HIV type 1 (HIV-1) tat. A peptide consisting of this 24-amino acid domain plus an additional C-terminal cysteine residue was chemically synthesized and covalently linked to 1,10-phenanthroline at the cysteine residue. The modified peptide binds to TAR sequences of both HIV-1 and HIV-2 and, in the presence of cupric ions and a reducing agent, cleaves these RNAs at specific sites. Cleavage sites on TAR sequences are consistent with peptide binding to the 3-nucleotide bulge, and the relative displacement of cleavage sites on the two strands suggests peptide binding to the major groove of the RNA. These results and existing evidence of the rapid cellular uptake of tat-derived peptides suggest that chemical nucleases based on tat may be useful for inactivating HIV mRNA *in vivo*.**

The development of agents that bind and cleave specific nucleic acid sequences is an attractive approach to inactivating viruses. Site-specific cleavage of DNA by using nucleic acid cleaving groups attached to DNA-binding molecules has been reported (1–13). Cleaving groups used in these studies include an enzymatic nuclease (9, 10) as well as chemical nucleases such as the Fe(II)–EDTA complex (1–4), the Cu(II)–phenanthroline complex [1,10-phenanthrolinecopper(II)] (5–8), and a derivative of ellipticine (11). Cu(II)–phenanthroline exerts its nucleolytic activity via oxidative attack on the C-1 hydrogen of the deoxyribose ring by a copper-oxo species generated in the presence of a reducing agent (13).

In contrast to the efforts devoted to the site-specific cleavage of DNA, much less attention has been given to site-specific cleavage of RNA (14), except by means of ribozymes (15). RNA-cleaving agents could be useful in inactivating viruses, including human immunodeficiency virus (HIV), the causative agent of AIDS. To achieve site-specific cleavage of HIV RNA, we utilized the viral tat protein, a potent transactivator of long terminal repeat-directed gene expression that is essential for viral replication (16, 17). tat stimulates the synthesis of viral mRNA and proteins >100-fold (18). The mechanism of tat-induced transactivation is yet to be understood; however, this process requires a sequence called the transactivation response (TAR) element, located at the untranslated 5′ end of the viral mRNA. The TAR element of HIV type 1 (HIV-1) is composed of the first 57 nucleotides and forms a stable stem–loop structure having a 3-nucleotide bulge on the stem (Fig. 1) (19). Purified tat and peptide fragments containing the nuclear targeting domain bind to TAR *in vitro* (20–24). The tat–TAR interaction is specific and of high affinity, with a dissociation constant, K_d, of 10^{-10} M (21). Mutational analysis of TAR reveals that mutations in the loop (nucleotides 30–33) and the base-paired part of the stem (nucleotides 8–11 and 48–51) have no effect on tat binding, although tat-mediated transactivation is reduced (21, 22). In contrast, the bulge (nucleotides 22–24) is critical for both tat binding and transactivation (21–24); base substitutions at nucleotide 22 significantly impair these functions (22, 24). Within the 86 amino acids of the tat protein, several domains have been identified: a cysteine-rich domain involved in dimerization (25), a putative activation domain (residues 38–48) (26, 27), and a nuclear targeting domain rich in basic amino acids (residues 49–57) (28).

Variants of tat having mutations in the nuclear targeting domain are inactive in transactivation and appear to reside primarily in the cytoplasm (29). Certain mutations in tat generate a transdominant phenotype (27, 30). Some of these may inhibit action of the wild-type tat by competing for binding to TAR (22). These observations suggest that reagents based on tat may also compete with tat for TAR binding and thus may have significant potential as antiviral agents.

A 24-amino acid proteolytic fragment of the Tat protein (amino acid residues 49–72) that specifically binds the TAR sequence has been identified (23). On the basis of this observation, we have chemically synthesized a 25-residue peptide designated Tat24C (Fig. 1*A*) and converted it to a nuclease by covalently attaching a 1,10-phenanthroline moiety. The resultant modified peptide (Tat24C-phen) binds to RNA sequences containing TAR sites and causes site-specific cleavage.

MATERIALS AND METHODS

5-Iodoacetamido-1,10-phenanthroline. 5-Iodoacetamido-1,10-phenanthroline was synthesized from commercially available 5-nitro-1,10-phenanthroline as described by Chen and Sigman (12).

Tat24C. Tat24C, a peptide consisting of the 24-amino acid basic domain of HIV-1 tat plus an additional cysteine residue at the C terminus, was synthesized on a Beckman system 990 automated peptide synthesizer by using *tert*-butoxycarbonyl-conjugated amino acids. After the cleavage from the resin, the peptide was purified by HPLC liquid chromatography.

Peptide Modification. Tat24C (20 nmol) in dimethylformamide (DMF) was mixed with 200 nmol of 5-iodoacetamido-1,10-phenanthroline in DMF (100 μl) at 4°C in the dark, and the mixture was incubated at the same temperature for ≈12 hr. The covalently modified peptide, Tat24C-phen (Fig. 1 *A* and *B*) was then purified from unreacted 5-iodoacetamido-1,10-phenanthroline by passing the reaction mixture through

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Abbreviations: TAR, transactivation response; HIV, human immunodeficiency virus; HIV-1 and HIV-2, HIV types 1 and 2; MPA, mercaptopropionic acid.

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A

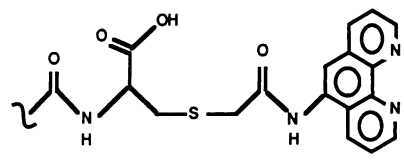
Tat

Met Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro Lys Thr Ala
Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His Cys Gln Val Cys Phe Ile Thr Lys Ala
Leu Gly Ile Ser Tyr Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly
Ser Gln Thr His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro Thr
Gly Pro Lys Glu

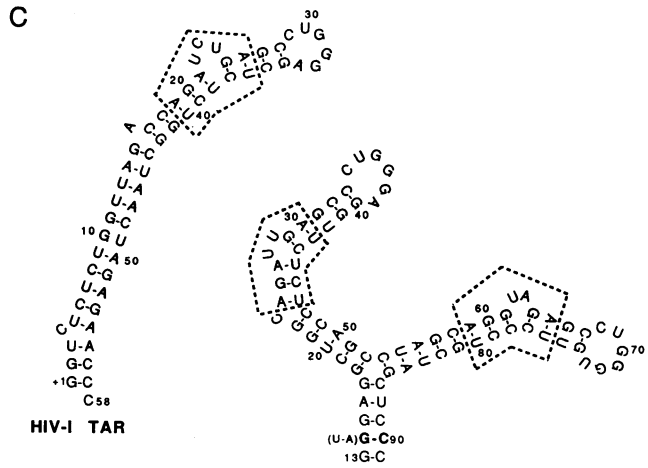
Tat24C-phen

Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His Gln Val Ser Leu
Ser Lys Gln Cys-phen

B



C



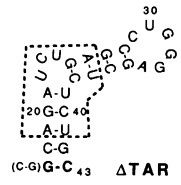


FIG. 1. (*A*) Primary sequence of HIV-1 tat protein (BRU isolate). Arginine and lysine residues in the basic nuclear targeting domain are shown in boldface. Tat24C-phen, based on the underlined 24-residue sequence of tat, is shown below with the addition to that sequence in boldface. (*B*) Chemical structure of the C terminus of Tat24C-phen, showing the 1,10-phenanthroline–cysteine linkage. (*C*) Sequences and predicted secondary structures of TAR RNAs. The base pairs at the base of ΔTAR and HIV-2 TAR were altered from the wild type (shown in parentheses) to facilitate *in vitro* transcription. Boxed regions in each RNA sequence show the probable sites for tat binding.

two successive Sephadex G-50 spin columns equilibrated in 70 mM NaCl/10 mM Tris·HCl, pH 7.5.

TAR RNAs. Several RNAs containing TAR sequences were synthesized by *in vitro* transcription of synthetic DNA templates by using phage T7 RNA polymerase (Promega) as described (31). Their sequences and predicted secondary structures are shown in Fig. 1*C*. HIV-1 TAR is the 57-nucleotide RNA stem–loop structure found in HIV-1 mRNA (nucleotides 1–57). ΔTAR is a truncated HIV-1 RNA containing the minimum tat binding site (nucleotides 17–43) (23). HIV-2 TAR includes the region of HIV-2 RNA essential for transactivation by HIV-2 tat (nucleotides 13–91) (32).

For uniformly labeled RNA, [α-^{32}P]CTP (DuPont/NEN) was included during the transcription, whereas 5′-end-labeled RNA was obtained by treating the transcribed RNAs with phage T4 polynucleotide kinase (United States Biochemical) and [γ-^{32}P]ATP. RNAs were finally purified by denaturing gel electrophoresis and were heated to 70°C in 70 mM NaCl/10 mM Tris·HCl, pH 7.5 buffer and slowly cooled to room temperature to facilitate formation of the native secondary structures.

Peptide Binding Assay. Uniformly labeled RNA (≈3 nM) was incubated with the peptide to be tested in 70 mM NaCl/0.2 mM EDTA/10 mM Tris·HCl, pH 7.5/5% (vol/vol) glycerol/0.1% (vol/vol) Nonidet P-40 for 20 min at 25°C. Samples were then electrophoresed on native 10% polyacrylamide gels and autoradiographed.

RNA Cleavage Assay. 5′-End-labeled RNA (≈3 nM) was incubated in 10 μl of buffer A (70 mM NaCl/10 mM Tris·HCl, pH 7.5/10 mg of tRNA per ml) in the presence of Tat24C-phen, and cleavage was initiated by adding $CuSO_4$ (to 10 μM) and mercaptopropionic acid (MPA) (to 2.5 mM) after 10 min at 22°C. After incubation for 17 hr, the reaction was stopped by adding 2,9-dimethyl-1,10-phenanthroline to 3 mM, tRNA to 0.2 mg/ml, and NaOAc to 0.3 M. RNA was then ethanol-precipitated, analyzed on 15% polyacrylamide sequencing gels, and autoradiographed.

RNA Sequencing. Sequencing of RNA was accomplished by using base-specific ribonucleases and uranyl nitrate-induced nonspecific photochemical cleavage of the RNA backbone at every nucleotide (33). For the G-specific reaction, end-labeled RNA (≈3 nM) was incubated with 1 unit of

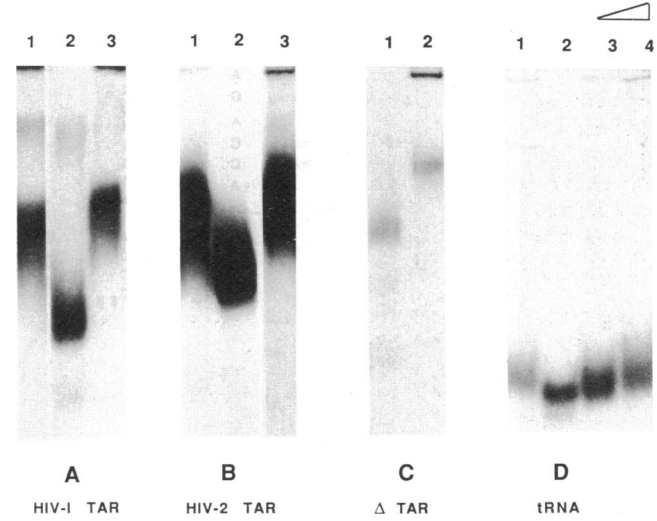


FIG. 2. Gel retardation assay showing specific binding between peptide constructs and TAR RNAs. (*A* and *B*) Binding of labeled 3 nM HIV-1 TAR (*A*) and labeled 3 nM HIV type 2 (HIV-2) TAR (*B*) to 7.5 nM Tat24C (lanes 1), control (no peptide added) (lanes 2), and 7.5 nM Tat24C-phen (lanes 3). (*C*) Binding of labeled ΔTAR to control (no peptide added) (lane 1), and 7.5 nM Tat24C-phen (lane 2). (*D*) Binding of end-labeled tRNA (control) to 7.5 nM Tat24C-phen (lane 1), control (no peptide added) (lane 2), 7.5 nM Tat24C (lane 3), and 150 nM Tat24C (lane 4).

ribonuclease T1 (BRL) for 10 min at 37°C in 10 μl of buffer A without tRNA. For the C-specific reaction, end-labeled RNA (≈3 nM) was treated with 0.2 unit of ribonuclease CL3 (Boehringer Mannheim) for 20 min at 37°C in 10 μl of buffer A without tRNA. For the uranyl nitrate ladder, end-labeled RNA (≈3 nM) in 10 μl of 70 mM NaCl/10 mM Tris·HCl, pH 7.5/20 mM uranyl nitrate was irradiated with 350-nm light [1.2 J in a Stratalinker (Stratagene)] at 25°C. RNAs were ethanol-precipitated and analyzed on sequencing gels.

S1 Nuclease Digestion. RNA (≈3 nM) was digested with 1 unit of S1 nuclease (BRL) in 10 μl of 30 mM sodium acetate, pH 5.0/50 mM NaCl/1 mM $ZnCl_2$ at room temperature for 5 min, and the reaction was stopped by adding EDTA to 10 mM. The digested RNA was ethanol-precipitated and analyzed on sequencing gels.

RESULTS AND DISCUSSION

Fig. 2 shows the results of the gel mobility-shift assay used to detect peptide binding. Discrete bands having retarded mobility were observed for samples containing both phenanthroline-modified and unmodified peptides, demonstrating the binding of both peptides to each of the three RNAs containing TAR elements (Fig. 2 *A–C*). Thus, the attachment of the extra cysteine and the phenanthroline moiety at the C terminus of the peptide does not substantially affect binding to the TAR site. Only slight retardation of tRNA was observed when it was incubated with high levels of peptide (Fig. 2*D*), indicating a low level of nonspecific binding.

To study the ability of Tat24C-phen to induce TAR-specific cleavage, we preincubated end-labeled RNAs with Tat24C-phen for 10 min at 22°C and then initiated cleavage by adding cupric ions and MPA. After the reaction, cleavage products were resolved on sequencing gels. The results with HIV-1 TAR are shown in Fig. 3*A*. Cleavage occurred primarily in the loop, especially at the uridine (nucleotide 30) to the 5′ side of the loop. A secondary cleavage site was also seen on the stem at nucleotides 12–14 and 18 (short arrows in Fig. 3*A*) and at nucleotides 43–45 on the complementary region (Fig. 3*B*). The cleavage pattern on opposite sides of the stem was shifted to the 5′ side, an indication that the cleaving moiety occupied the major groove (1, 3). A possible alternative binding mode, with the basic region of Tat24C-phen binding

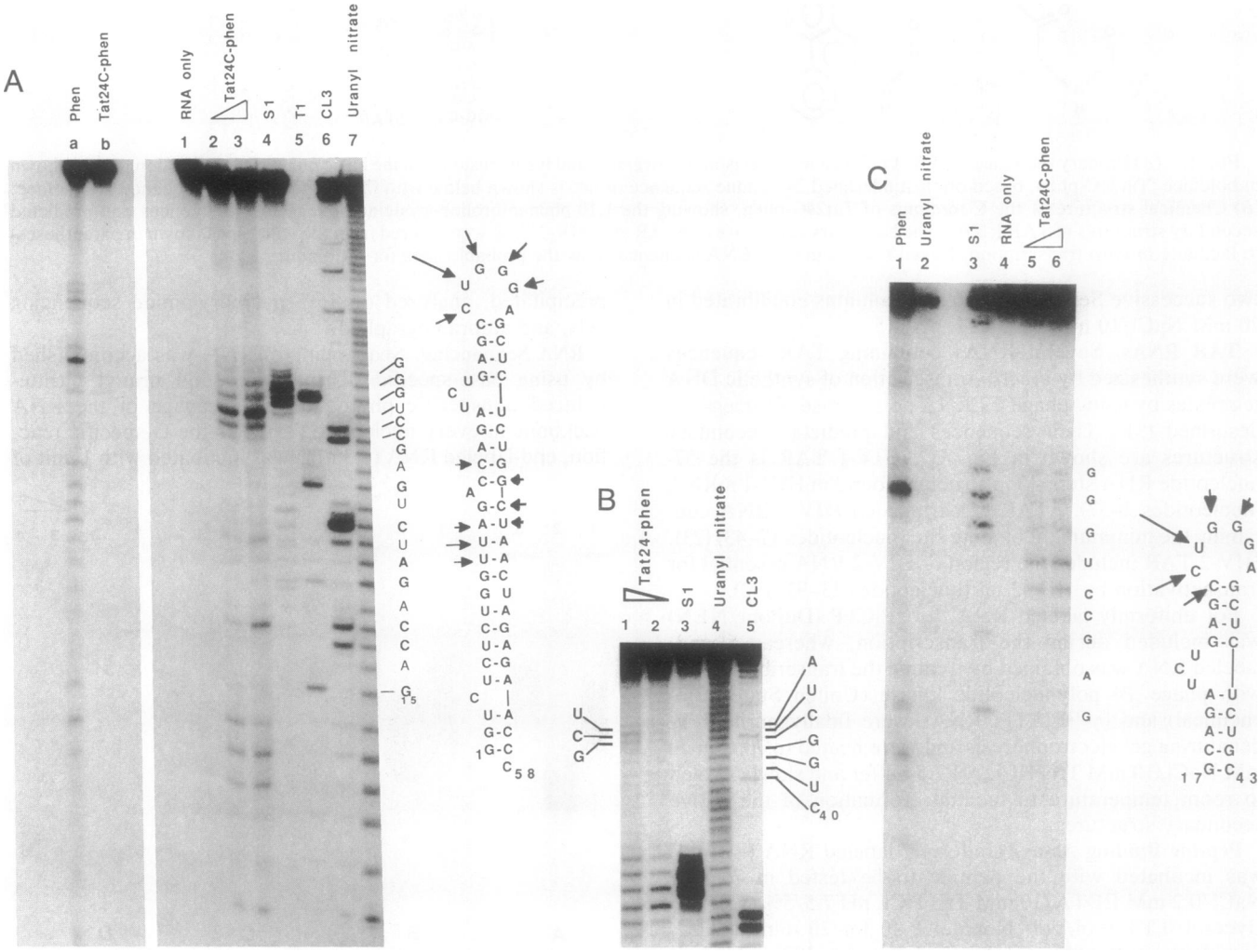


FIG. 3. Cleavage of RNA containing HIV-1 TAR. The cleavage sites on each substrate are indicated by arrows on the folded structure shown to the right of the autoradiogram. (*A*) Cleavage of full-length HIV-1 TAR RNA induced by 2 nM Tat24C-phen (lane 2) and 3 nM Tat24C-phen (lane 3), with addition of cupric ion and MPA to each; S1 nuclease (lane 4); G-specific reaction (RNase T1) (lane 5); C-specific reaction (RNase CL3) (lane 6); and uranyl nitrate ladder (lane 7). Control lanes: 1, RNA only; a, 40 μM Cu(II)–1,10-phenanthroline complex plus MPA; b, 3 nM Tat24C-phen plus MPA in the absence of Cu(II)—same as lane 3 except that no $CuSO_4$ was added. (*B*) Fine mapping of the cleavage sites in the 3′ half of HIV-1 TAR. All reactions were conducted as described for *A*. Lanes: 1 and 2, 3 nM Tat24C-phen (lane 1) and 2 nM Tat24C-phen (lane 2) with addition of cupric ion and MPA; 3, S1 nuclease; 4, uranyl nitrate ladder; 5, RNase CL3. (*C*) Cleavage of ΔTAR RNA under the same conditions as in *A*. Lanes: 1, Cu(II)–1,10-phenanthroline complex plus MPA; 2, uranyl nitrate ladder; 3, S1 nuclease; 4, buffer only; 5 and 6, 2 nM Tat24C-phen (lane 5) and 3 nM Tat24C-phen (lane 6) with addition of cupric ion and MPA to each. The direction of concentration change of Tat24C-phen is shown by the right-angled triangle above the appropriate lanes.

to the minor groove and the phenanthroline moiety reaching over to the major groove, is unlikely based on recent evidence suggesting that tat-derived peptides bind in the major groove (24) and the fact that, at least in the case of B-DNA, Cu(II)–1,10-phenanthroline prefers to bind to the minor groove (13).

Overall, the cleavage sites lie on either side of the bulge where tat is known to bind. Because Cu(II)–phenanthroline is known to preferentially cleave unpaired bases of RNA (34), HIV-1 TAR was incubated with Cu(II)–1,10-phenanthroline as a control. As shown by the cleavage pattern in lane a of Fig. 3*B*, free phenanthroline cleaves the RNA everywhere, but the unpaired bases at the loop and the bulge are most reactive. Thus, tethering phenanthroline to the peptide suppresses nonspecific cleavage on TAR RNA. In the absence of cupric ions, Tat24C-phen produces no cleavage (Fig. 3*B*, lane b). On the basis of scintillation counting of the radioactivity of excised gel bands, the overall efficiency of the peptide-induced cleavage is ≈20–30%. The cleavage pattern on ΔTAR, which lacks the base of the stem, is consistent with that of full-length TAR (Fig. 3*C*), suggesting that the presence of the minimum Tat binding site is enough for recognition by Tat24C-phen. On ΔTAR, the cleavage is restricted to the loop, but the pattern is different from that for cleavage by free phenanthroline (Fig. 3*C*, compare lane 1 with lanes 5 and 6).

In contrast to cleavage of HIV-1 TAR, for which the primary site is at the loop adjacent to the tat binding site, cleavage of HIV-2 TAR takes place mainly at the stem, roughly midway between the two loops (Fig. 4*A*, lanes 1 and 2). HIV-2 TAR comprises two stem–loops, each having a 2-nucleotide bulge, both of which have the consensus tat binding motif (23, 24). HIV-1 tat can transactivate HIV-2

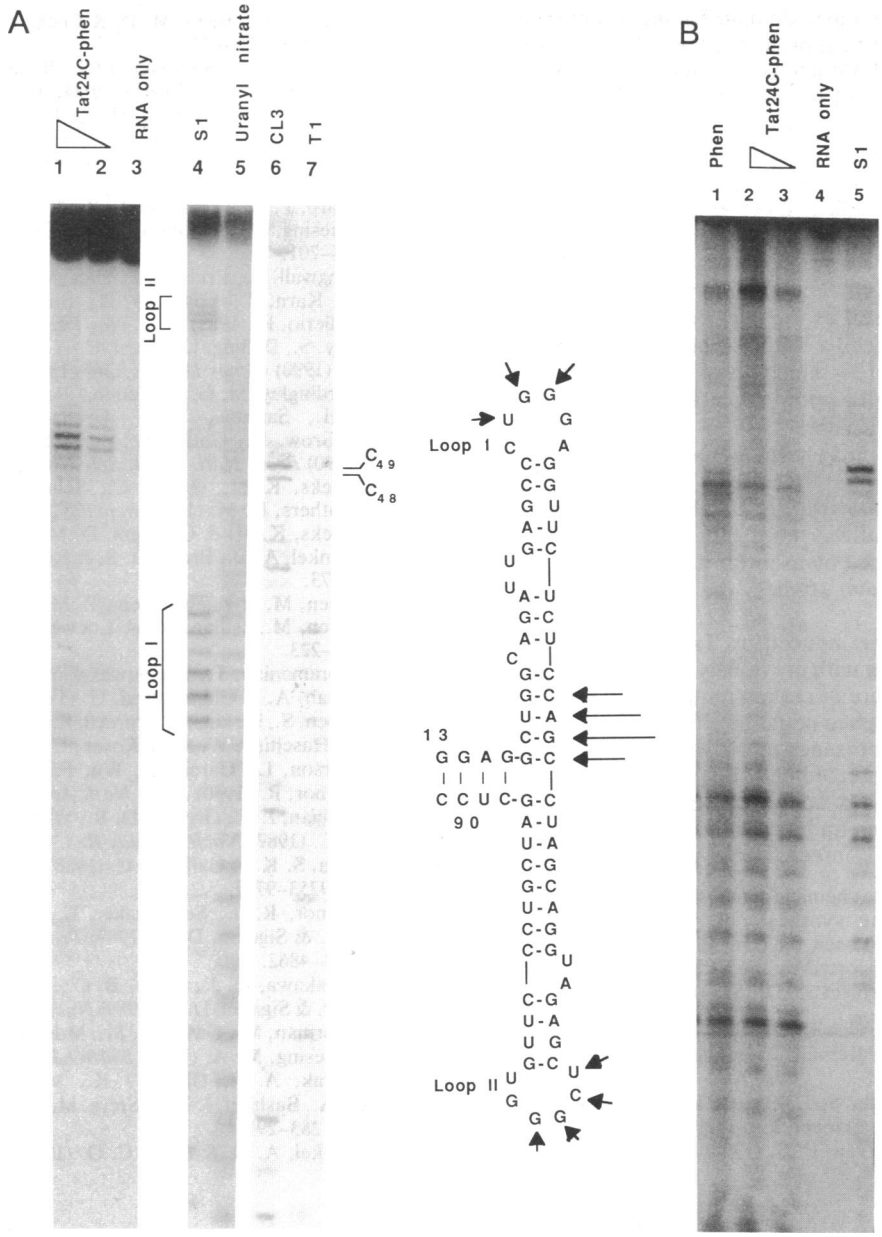


FIG. 4. Cleavage of HIV-2 TAR RNA and tRNA. (*A*) Cleavage of 3 nM HIV-2 TAR under the same conditions as in Fig. 3*A*. Lanes: 1 and 2, 3 nM Tat24C-phen (lane 1) and 2 nM Tat24C-phen (lane 2) with addition of cupric ion and MPA; 3, RNA only; 4, S1 nuclease; 5, uranyl nitrate ladder; 6, C-specific reaction (RNase CL3); 7, G-specific reaction (RNase T1) (*B*). Cleavage of tRNA under the same conditions as in Fig. 3*A*. Lanes: 1, 40 μM Cu(II)–1,10-phenanthroline complex plus cupric ion and MPA; 2 and 3, 6 nM Tat24C-phen (lane 2) and 3 nM Tat24C-phen (lane 3) with addition of cupric ion and MPA; 4, RNA only; 5, S1 nuclease. The cleavage sites on the HIV-2 TAR RNA are indicated by arrows. The direction of concentration change of Tat24C-phen is shown by the right-angled triangle above the appropriate lanes.

LTR-directed gene expression when either loop I or loop II is present [although the tat product of HIV-2 requires both loops for efficient transactivation (32, 35)]; hence, HIV-1 tat appears to bind to either stem–loop. Because the Tat24C peptide is based on the sequence of HIV-1 tat, the cleavage of both loops probably results from binding of the peptide to both elements. The major cleavage site for HIV-2 thus corresponds to the minor cleavage site for HIV-1 TAR [i.e., ≈3–8 base pairs from the bulge in the direction away from the loop(s)]. The higher level of cleavage at this site could be partly due to a superimposition of the cleavage resulting from tat binding on both stem–loops I and II. Alternatively, Tat24C-phen might bind only to stem–loop I, with the RNA folding so as to bring the two loops close together.

The cleavage observed on either side of the presumed Tat24C-phen binding sites (for both HIV-1 and HIV-2 TARs) may be due to flexibility in the C-terminal part of the peptide chain (exclusive of the basic domain having direct contacts with RNA), allowing it to approach either side of the binding site, especially if the TAR RNAs are bent so as to bring the opposite sides of the binding site closer than is indicated in Fig. 1*C*. An alternative explanation, that the peptide binds the symmetrical bulge sequence (UCU or UU) in either orientation, is unlikely because of the accurate discrimination between U-26-to-A-37 and A-26-to-U-37 TAR substrates in the binding of a similar but longer peptide (24), which indicates that sequence information beyond the symmetrical bulge is involved in peptide recognition.

When tRNA was used as the substrate for cleavage, the cleavage pattern induced by Tat24C-phen was identical with that caused by free Cu(II)–phenanthroline (Fig. 4*B*), indicating that Tat24C-phen does not induce site-specific cleavage on RNA lacking a TAR site.

In the absence of the reducing agent MPA, Cu(II)-complexed Tat24C-phen still cleaves TAR-containing RNA site specifically (not shown), presumably via a cupric ion-induced hydrolytic pathway (36), but with less efficiency. When MPA was replaced by ascorbic acid, a reducing agent more suitable for *in vivo* studies, the rate of cleavage increased (not shown).

The specific cleavage induced by Tat24C-phen on TAR-containing RNAs, along with previously reported evidence of the transdominant nature of certain mutations in tat (27, 30), indicates that Tat-24C-phen might be effective in inactivating HIV RNA even in the presence of native tat, which would be found inside an HIV-infected cell. Because chemically synthesized tat and peptides containing the nuclear targeting domain are rapidly taken up by cells (26, 37), Tat24C-phen is likely to also enter HIV-infected cells without difficulty.

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