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Biochemistry

Characterization of a "kissing" hairpin complex derived from the human immunodeficiency virus genome

(trans-activation response element hairpin/RNA–RNA interactions/antisense)

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ABSTRACT **Base-pair formation between two hairpin loops—a "kissing" complex—is an RNA-folding motif that links two elements of RNA secondary structure. It is also a unique protein recognition site involved in regulation of ColE1 plasmid DNA replication. The trans-activation response element (TAR), a hairpin and bulge at the 5′ end of the untranslated leader region of the human immunodeficiency virus 1 mRNA, enhances the transcription of the virus and is necessary for viral replication. Gel electrophoresis and absorbance melting curves indicate that a synthesized RNA hairpin (Tar*-16) with a loop sequence complementary to the TAR loop sequence (CUGGGA) associates specifically with a 16-nucleotide TAR hairpin (Tar-16) to form a stable complex. RNase T1 probing indicates that the three guanines in the Tar-16 loop become inaccessible in the complex. NMR imino proton spectra reveal that 5 base pairs are formed between the two hairpin loops (Tar-16 and Tar*-16); only the adenine at the 3′ terminus of the TAR loop does not form a base pair with the 5′-terminal uracil of the complementary loop. A 14-nucleotide hairpin [CCUA-(UCCCAG)UAGG] with a loop sequence complementary to the TAR loop is conserved within the *gag* gene of human immunodeficiency virus 1. A synthesized RNA hairpin corresponding to this conserved sequence also binds to the Tar-16 hairpin with high affinity. It is possible that the same RNA loop–loop interaction occurs during the viral life cycle.**

In *Escherichia coli*, the RNA primer required in ColE1 plasmid DNA replication and its natural antisense RNA can both adopt a stem–loop conformation and hybridize with each other to block the plasmid DNA replication (1). This hybridization process starts with the formation of base pairs between the complementary loop regions of two hairpins generating a "kissing" hairpin complex intermediate, which can be recognized specifically by the protein dimer ROM (RNA-one modulator) (2). A hairpin loop size of 6–8 nt and the stacking of purines at either end of the hairpin loops favor complex formation, as shown by mutation studies of the loop sequences (3, 4).

The trans-activation response element (TAR) at the 5′ end of the untranslated leader region of the human immunodeficiency virus 1 (HIV-1) mRNA increases the transcriptional efficiency of the provirus and is necessary for HIV replication (5, 6). The TAR element consists of a 59-nt hairpin ending in a loop of 6 nt with four consecutive purines in the sequence (CUGGGA) (7–10). The loop sequence can be recognized specifically by protein cellular factors and is essential for the transactivation of provirus transcription *in vivo* (11, 12). Decoy TAR hairpin loops have been used to sequester these cellular factors and, thus, inhibit the virus replication (13). The antisense sequence of the TAR region is not as effective as the sense TAR decoy in inhibiting HIV replication in cell culture (14). The reason for the inefficiency of the TAR antisense approach is not known.

The structure of the tip of the TAR hairpin loop has been extensively studied by NMR (15–18); the loop is dynamic, but there is considerable stacking of the loop purines on the 3′ side of the stem (16, 17). Here we show that an RNA hairpin with a loop sequence (UCCCAG) complementary to the TAR loop sequence (CUGGGA) associates specifically with the TAR hairpin loop to form a stable kissing complex. Native gel electrophoresis, absorbance melting curves, enzymatic probing, and imino proton NMR spectra indicate a stable complex with loop–loop base pairing. We note that a conserved 14-nt sequence (nt 1096–1109 in ref. 6) that can form a 4-bp stem with a UCCCAG loop sequence occurs within the *gag* gene of HIV. The presence of two conserved hairpins with complementary loop sequences suggests a biological role for their interaction.

METHODS

Oligonucleotide Synthesis and Enzymatic Probing of RNA Conformation. All RNA molecules were synthesized *in vitro* using T7 RNA polymerase with a synthetic DNA template (19) and were purified by denaturing polyacrylamide gel electrophoresis in 20% gels. RNA was 5′-end-labeled with ^{32}P by using alkaline phosphatase and T4 kinase sequentially as described (20). RNase T1 probing experiments were done in 10 mM Tris·HCl, pH 7.0/10 mM $MgCl_2$/100 mM NaCl at 4°C; each reaction mixture contained 3 μg of polycytidylic acid as a carrier. The cleavage reaction was terminated by adding 10 μl of buffer containing 9 M urea and 10% (vol/vol) glycerol and loaded directly onto a denaturing 20% polyacrylamide gel.

Electrophoretic Band-Shift Assays and Ultraviolet Melting Experiments. RNA was dissolved in 8 μl of incubation buffer (100 mM NaCl or 10 mM $MgCl_2$/0.1 mM EDTA/10 mM Tris·HCl, pH 7.0); after heating at 80°C for 30 sec, the sample was cooled immediately in an ice bath and equilibrated at 4°C for 30 min. The sample was then mixed with 2 μl of 40% (wt/vol) sucrose and loaded immediately into the gel electrophoresing at 5 W at 4°C. The electrophoresis buffer contained 100 mM NaCl or 10 mM $MgCl_2$ and 0.1 mM EDTA and 100 mM Tris·Hepes, pH 7.8; the nondenaturing 20% polyacrylamide gel was prepared using the same buffer with a 19:1 weight ratio of acrylamide to *N,N′*-methylenebisacrylamide. A_{280} was measured over a temperature range from 0 to 93°C by using a Gilford 250 spectrophotometer with a heating rate of 1°C/min controlled by a Gilford 2527 Thermoprogrammer. The melting buffer contained 0.1 mM EDTA, 10 mM sodium phosphate (pH 6.4), and 50 mM NaCl or 10 mM $MgCl_2$. Thermodynamic parameters were obtained by standard methods from van't Hoff plots (21).

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Abbreviations: HIV, human immunodeficiency virus; TAR, trans-activation response element; NOESY, nuclear Overhauser effect spectroscopy; 1D, one dimensional.

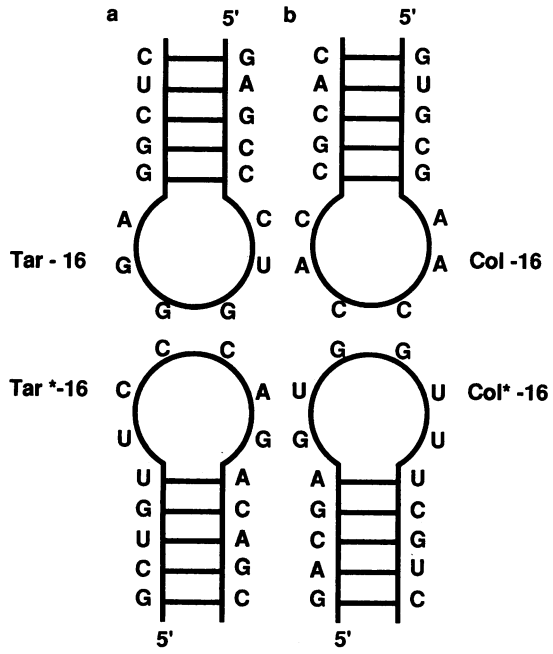

FIG. 1. Sequence and secondary structure of hairpins Tar-16 and Tar*-16 (*a*) and hairpins Col-16 and Col*-16 (*b*).

NMR Spectroscopy. RNA samples were dialyzed against 50 mM NaCl/0.1 mM EDTA/10 mM sodium phosphate, pH 6.4, for at least 48 hr. The final sample concentrations were 1–1.5 mM for the one-dimensional (1D) NMR studies. The 1D spectra in H_2O were taken on a GE GN-500 spectrometer at 5 or 10°C using a 1–1 water suppression pulse sequence (22) with the carrier frequency on the water resonance. The free induction decays were acquired with 4096 complex data points and 256 scans with a spectral width of 10,000 Hz. Data sets were processed by applying a 30° skewed sine bell and digital shift subtraction before Fourier transform using FELIX software.

The nuclear Overhauser effect spectroscopy (NOESY) spectra of 2.5–3 mM RNA were measured with a 200-msec mixing time using a 1–1 NOESY pulse sequence (23, 24) with the carrier frequency set on the water resonance at 3°C. The 2048 complex points in t_2 and 300–350 t_1 increments were acquired with 96 scans per t_1 increment and a spectral width of 12,000 Hz on a Bruker 600 spectrometer. Prior to the Fourier transformation, the data sets were processed by digital shift subtraction in both dimensions and apodized using a 30° skewed sine bell in the first dimension and 60° in the second dimension; the data were zero-filled in both dimensions to give a final 2048 × 2048 real data matrix.

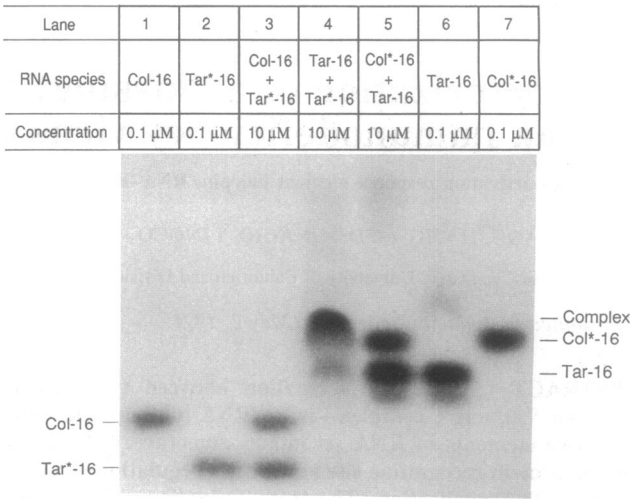

FIG. 2. Formation of a specific heterodimer between Tar-16 and Tar*-16 revealed by nondenaturing polyacrylamide gel electrophoresis. Lane 4 demonstrates that Tar-16 and Tar*-16 associate to form a species with a lower electrophoretic mobility than that of either hairpin. Lanes 3 and 5 are negative controls that show each component in the mixture of Tar*-16 plus Col-16 or of Tar-16 plus Col*-16 has the same electrophoretic mobility as the individual hairpins (lanes 1 and 2 and lanes 6 and 7, respectively). Tar-16 appears as a single band in a denaturing 20% polyacrylamide gel, although it shows a second band in lane 6 of the nondenaturing gel (presumably from a minor species). Each lane contains about 0.1 μM ^{32}P-labeled RNA and 3 μg of polycytidylic acid as a carrier. Lanes 3–5 also contain 10 μM unlabeled RNA making the final concentration for each species about 10 μM.

RESULTS

A Heterodimer Forms Specifically Between Tar-16 and Tar*-16. We synthesized two hairpins with complementary loops (Fig. 1*a*): the TAR sequence (Tar-16) and its complement (Tar*-16). The stem sequences were chosen to minimize duplex formation of the individual hairpins and to minimize heteroduplex formation involving the stems when the hairpins were mixed. The Tar-16 stem is the native sequence with an extra C·G closing base pair. We also synthesized two other hairpins with complementary loops, Col-16 and Col*-16 (Fig. 1*b*), derived from the wild-type kissing hairpins (RNA I and II) in Col E1 plasmid (4) to use as controls.

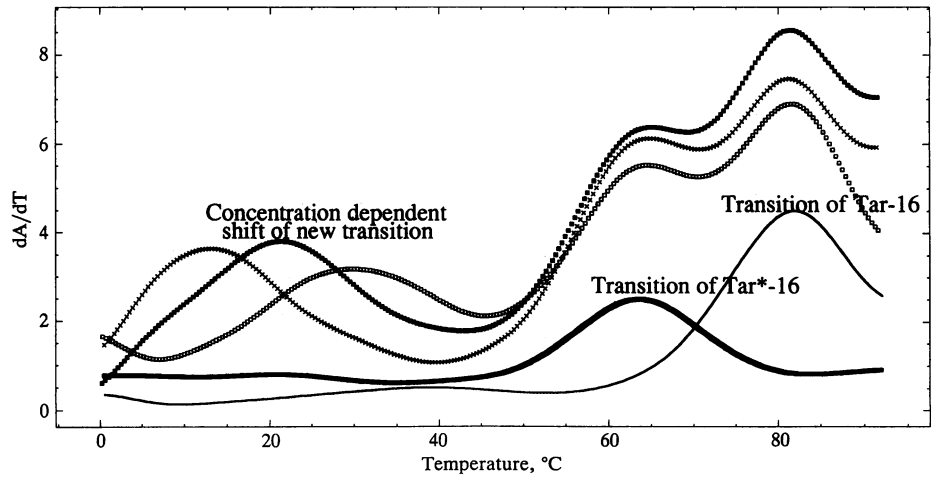

FIG. 3. Derivative plot, $[d(A_{280})/d(T)] \times 10^{+3}$, of ultraviolet melting profiles of both hairpins and the complex in 50 mM NaCl. Note that the transition temperature of each hairpin stem is concentration-independent and that of the loop–loop interaction is concentration-dependent. Bold line, 50 μM Tar*-16; solid line, 50 μM Tar-16; ×, 5 μM (Tar-16 + Tar*-16); solid squares, 50 μM (Tar-16 + Tar*-16); open squares, 500 μM (Tar-16 + Tar*-16).

Existence of a heterodimer between Tar-16 and Tar*-16 is shown by nondenaturing polyacrylamide gel electrophoresis (Fig. 2). At a 1:1 ratio, Tar-16 and Tar*-16 associate to form a stable complex (lane 4) with a lower electrophoretic mobility than either hairpin (lanes 2 and 6). No complex is formed for the noncomplementary mixtures of Tar*-16 and Col-16 or of Tar-16 and Col*-16 (lanes 3 or 5, respectively), ruling out the possibility of nonspecific aggregation. However, the complementary mixture of Col-16 and Col*-16 does form a complex as expected (data not shown). The thermodynamic stability of the Tar–Tar* complex was studied by absorbance melting experiments. A new melting transition occurs in the mixture of Tar-16 and Tar*-16 in addition to the higher-temperature transition for each hairpin stem. The melting temperature of this new transition is concentration-dependent and thus is consistent with the existence of a bimolecular complex (Fig. 3). The melting temperature for the kissing complex, each hairpin at 10^{-4} M, is 21 ± 2°C in 50 mM NaCl/10 mM sodium phosphate, pH 6.4. Values of ΔH° = −43 ± 3 kcal/mol and ΔG° (37°C) = −5.3 ± 0.5 kcal/mol (1 cal = 4.184 J) for the complex formation were obtained from experiments over a 100-fold range of concentrations (21). These thermodynamic data are consistent with the formation of 5 bp in an RNA duplex (25). In 10 mM $MgCl_2$, the melting temperature of the complex increased by 40°C (data not shown). The requirement of Mg^{2+} for stable complex formation was also reported for the ColE1 complex (4).

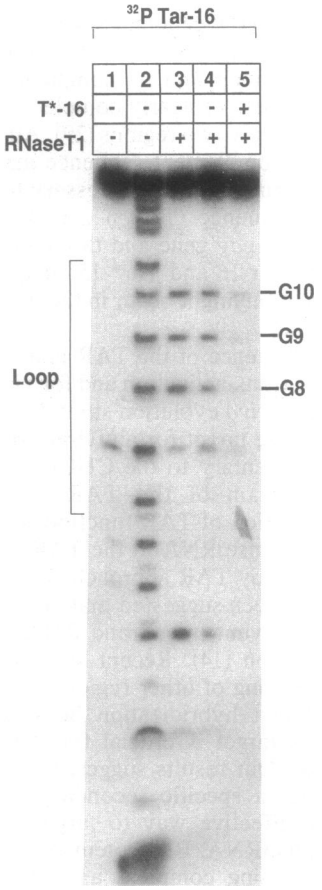

FIG. 4. G_{8-10} in the loop of Tar-16 becomes less accessible to RNase T1 cleavage in the presence of an equal amount of Tar*-16. Lanes: 1, untreated control; 2, hydrolysis ladder; 3, RNase T1 treatment of denatured Tar-16 (the reaction mixture contained 9 M urea); 4, RNase T1 treatment of native Tar-16 hairpin; 5, RNase T1 treatment of Tar-16 (1.0 μM unlabeled and 0.1 μM ^{32}P labeled) + Tar*-16 (1.1 μM unlabeled). Each lane contains 2.1 μM unlabeled Tar-16 and 0.1 μM ^{32}P-labeled Tar-16 unless otherwise specified.

The Complex Is Formed Through Base Pairing Between the Two Hairpin Loops. RNase T1 was used to probe the accessibility of the three guanines in the Tar-16 hairpin loop in the presence or absence of Tar*-16. These guanine residues became inaccessible to the RNase T1 cleavage (Fig. 4) upon the formation of the complex, indicating a loop–loop interaction between the two hairpins. One- and two-dimensional NMR spectra in H_2O were measured for each hairpin and for the complex to establish their secondary structures. The imino proton spectrum of Tar*-16 (Fig. 5*a*) shows characteristic imino resonances of a G·U base pair (G11* at 10.8 ppm and U6* at 12.3 ppm) (26) and indicates the formation of a hairpin with 4 nt in the loop. Tar-16 has loop imino resonances near 11 ppm (Fig. 5*b*) as was found (17); this indicates that the added C·G pair does not change the loop conformation of the wild-type TAR hairpin. Each of these five imino proton peaks is sharpened and shifted downfield when the two hairpins are mixed (Fig. 5*c*), thus indicating formation of new base pairs. The imino proton chemical shift of the closing base pair of each hairpin and the G·C base pair next to it in Tar*-16 also change in the complex. There is no major difference in the imino spectrum for the complex taken in 50 mM NaCl or 5 mM $MgCl_2$. There are 14 major imino proton resonances in the spectrum of the mixture: 9 intrastrand base-pairing imino peaks and 5 remaining imino peaks resulting from the interaction of the two hairpins (see Fig. 6 for the peak assignments). Analysis of a H_2O NOESY spectrum (27, 28) of the complex (Fig. 6) also verifies that three helical segments exist in this complex; two of them correspond to the stem of each hairpin (helix T and helix T*) and the third one is a new helix (helix K) containing 5 bp between the loops. The existence of helix K indicates that a complex is formed through the formation of 5 bp between the two hairpin loops (the corresponding five imino proton signals belong to G11*, U7, G8, G9, and G10).

A Conserved Hairpin with a Loop Sequence Complementary to the TAR Loop Exists in the HIV Genome. A conserved 14-nt sequence occurs within the *gag* gene of HIV-1 [5′-CCUA(UCCCAG)UAGG-3′] (29) that can form a stem of 4 bp with a loop sequence complementary to the TAR hairpin loop (Fig. 7*a*). This 14-nt sequence encodes a conserved peptide sequence Pro-Ile-Pro-Val-Gly of the gag protein. Deletion of a region of the viral RNA containing these 14 nt has been shown to impair the packing of the HIV genome (30). Secondary structure prediction (31) of an HIV-1 sequence of 200 nt indicates that this hairpin can form in the context of the surrounding viral sequence, although it is not calculated to be part of the lowest free energy secondary structure. To learn whether this TAR loop complement has the same ability as Tar*-16 to interact with Tar-16, we synthesized a 15-nt hairpin (Tar*-15) with sequence identical to the 14-nt HIV sequence except for an extra 5′ guanine. Electrophoretic band-shift assay shows that Tar*-15 also associates specifically with Tar-16 with an equilibrium dissociation constant, K_d, between 10^{-8} and 10^{-9} M in the presence of 10 mM $MgCl_2$ at 4°C (Fig. 7*b*).

DISCUSSION

A kissing complex between RNA I and RNA II of ColE1 plasmid is bound specifically by the helix–loop–helix protein dimer ROM (32, 33) to regulate the DNA replication of the plasmid in *E. coli* (2–4). It is the unusual structure created by the interaction of the two hairpins, not either hairpin, that is important for this specific RNA–protein recognition (3). Thus, the kissing complex is not only an important RNA folding motif but also may play a role as a protein recognition site. A similar long-range loop–loop interaction has been found by phylogenetic methods in the 23S rRNA of *E. coli* (34). Recently, two adjacent hairpins with complementary

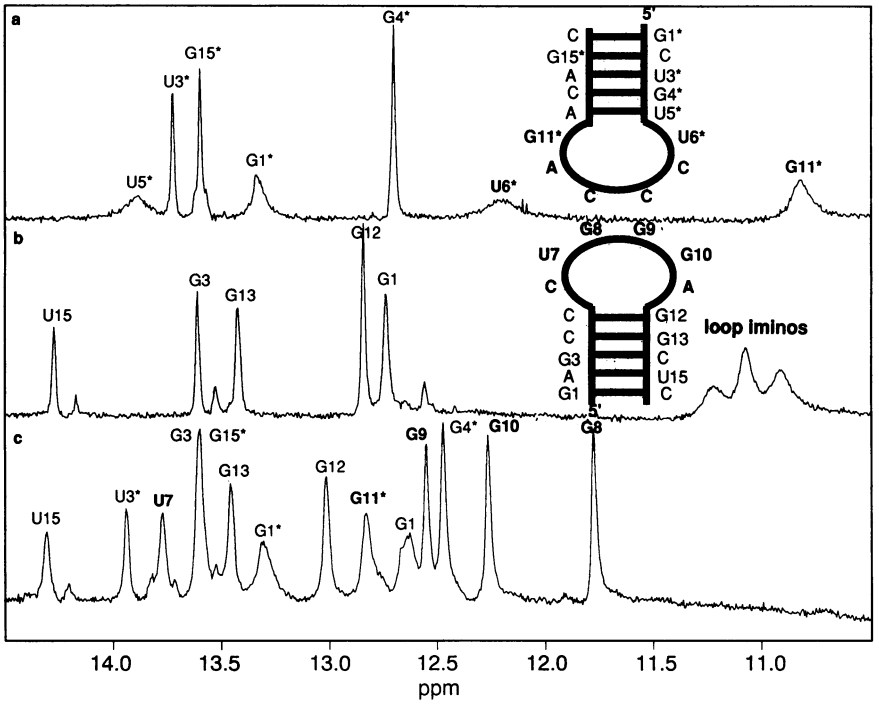

FIG. 5. Heterodimer between Tar-16 and Tar*-16 is a kissing complex formed through loop–loop base pairing of the two hairpins. (*a*) 1D imino proton spectrum assignment of Tar*-16 based on H_2O NOESY. (*b*) 1D imino proton spectrum assignment of Tar-16 based on analysis of H_2O NOESY spectra and previous work from this laboratory on a 14-mer TAR hairpin (5′-GAGCCUGGGAGCUC-3′) (17). The satellite peaks seen in the spectrum belong to a minor species. (*c*) 1D imino proton spectrum of the mixture of Tar-16 and Tar*-16 in 50 mM NaCl at 10°C (see Fig. 4 for spectrum assignment). The spectrum in *c* was shifted 100 points left to adjust for the temperature dependence of chemical shifts (spectra in *a* and *b* were measured at 5°C). Residues with boldface type are the nucleotides involved in new base pairing in the complex. Residues with * are the nucleotides of Tar*-16. Only the nucleotides having imino protons are numbered.

loop sequences were found in a consensus sequence required for RNA recombination in turnip crinkle virus (35).

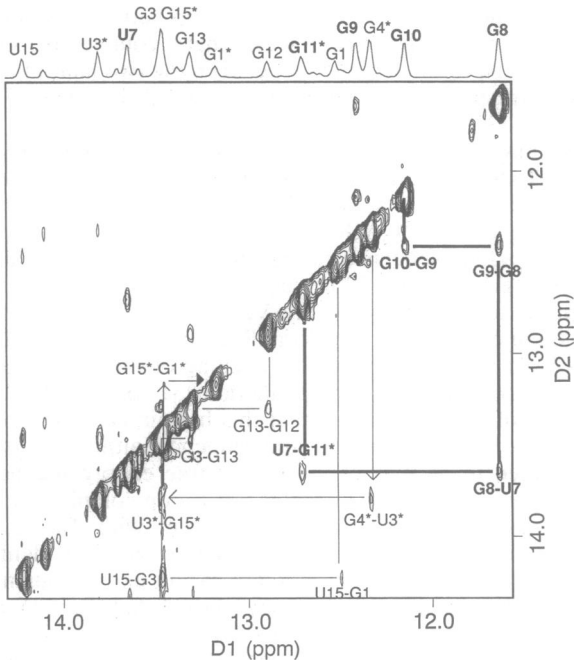

FIG. 6. H_2O NOESY spectra indicate the existence of three helical regions within the complex. Imino–imino portion of H_2O NOESY spectrum of kissing complex taken at 3°C is shown. There are three sequential NOE connectivities corresponding to three helical regions within the complex. The first walk (plain line) is assigned to the stem of Tar-16 (helix T) with NOE crosspeaks in the following order: G1–U15–G3–G13–G12 by using U15 as the reference. The Tar*-16 stem (helix T*) has a set of NOE crosspeaks (arrows) corresponding to the G1*–G15*–U3*–G4* walk with G1* as reference. A set of **G11*–U7–G8–G9–G10** interstrand NOE crosspeaks (bold line) defines the existence of 5 bp between the loops of the two hairpins. This assignment is consistent with the presence of imino-to-amino-to-H5 NOEs for the G·C base pairs and an imino-to-H2 NOE for the A·U base pair. No NOE is found between **U6*** and **G10**, G12, or U5* (the terminal base of helix K, T, or T*, respectively). All the nucleotides are labeled as in Fig. 5.

We have found a potential loop–loop interaction between two complementary hairpin loops with sequences that are highly conserved in HIV genomic RNA; one is the TAR loop and the other we designate as TAR*. Sequence covariation between the TAR loop and its complement could not be demonstrated because of nearly complete invariance of primary sequence in these two regions (29). An *in vivo* evolution study (36) of the complement sequence may show whether this primary sequence is really necessary for virus survival. The conserved sequence (TAR*) complementary to TAR present in the HIV *gag* gene and the high-affinity complex formed between Tar-16 and Tar*-15 suggest that the same loop–loop interaction might occur in the viral genome during the viral life cycle.

The primary sequence of the TAR hairpin loop is critical for Tat-mediated trans-activation and for viral replication, as suggested by an *in vivo* evolution study (36). Thus, the TAR loop is an appealing target for antiviral drugs. When a 6-nt sequence complementary to the CUGGGA TAR loop was inserted downstream of the TAR hairpin to form a pseudoknot, inhibition of TAR function was observed (37). However, an antisense RNA to the TAR region was not as effective as the decoy TAR approach (14) in inhibiting viral replication. It has been suggested that intrastrand secondary structure might prevent interstrand RNA–RNA interaction needed for inhibition (14). Recent studies of effective antisense RNA targeting of other regions of the HIV genome also show that fast hybridization between the antisense molecule and its target is crucial for the approach to be effective (38–40). Our results suggest that an antisense oligonucleotide with a specific secondary structure—a hairpin—can be an effective way to target a complementary hairpin loop in an mRNA. If a protein exists that specifically recognizes the kissing complex, as ROM does in ColE1 replication, the targeting efficiency and specificity might be further increased.

CONCLUSION

Two highly conserved stem–loop sequences occur in HIV-1 RNA. One contains the well-known TAR loop sequence (CUGGGA) in the 5′ untranslated leader region; the other

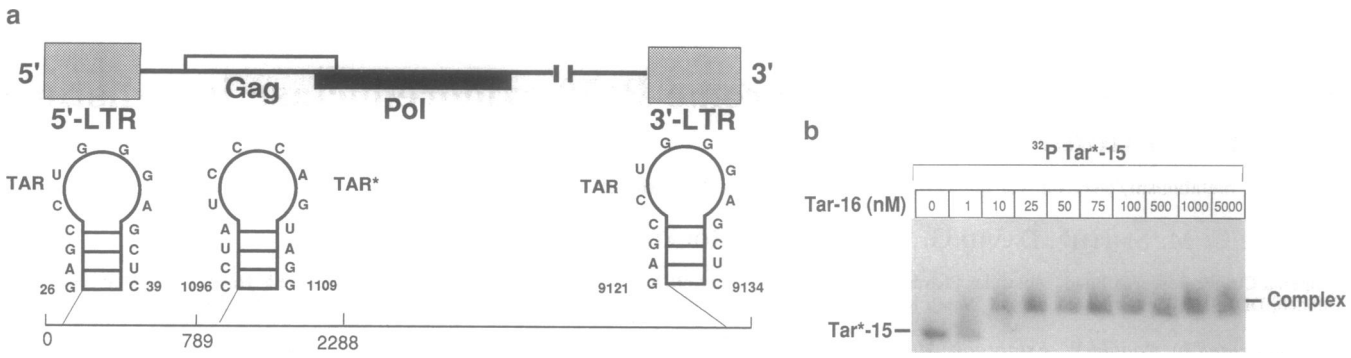

FIG. 7. (*a*) Locations of TAR and its complement TAR* within the HIV genome. There are two TAR sequences in each viral genome [in the 5′ and 3′ long terminal repeats (LTRs)]. The complement TAR* is located within the *gag* gene (nt 1096–1109 in ref. 6) about 1.1 kb away from the 5′ TAR loop. (*b*) Dissociation constant measurement of the complex between Tar*-15 and Tar-16 by an electrophoretic band-shift assay in 10 mM $MgCl_2$ at 4°C. Each lane contains about 0.5 nM ^{32}P-labeled Tar*-15 and unlabeled Tar-16 as indicated at the top of the gel. The dissociation constant is between 1 nM and 10 nM as shown by the concentration of Tar-16 required for the midpoint of the transition.

contains its complementary TAR* loop sequence (UCCCAG) in the gag region. The truncated forms of these two hairpin loops form a stable complex (K_d = 1–10 nM) in 10 mM Mg^{2+}. NMR studies on a model complex of these two hairpins with complementary loops show that five loop–loop base pairs are formed out of the six possible. The conserved primary sequences of these two hairpins plus the loop–loop interactions that have functional significance in other systems (2–4, 35) suggest that similar loop–loop interaction may have a role for the HIV *in vivo*.

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