Hexitol Nucleic Acid-Containing Aptamers Are Efficient Ligands of HIV-1 TAR RNA†

Gaëlle Kolb,‡ Sandrine Reigadas,‡,⊥ Claudine Boiziau,‡,# Arthur van Aerschot,§ Andrey Arzumanov,|| Michael J. Gait,|| Piet Herdewijn,§ and Jean-Jacques Toulmé*,‡,⊥

INSERM U 386; Université Victor Segalen Bordeaux 2, 146 rue Léo-Saignat, F-33076 Bordeaux Cedex, France, Laboratory for Medicinal Chemistry, Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium, Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, United Kingdom, and Institut Européen de Chimie et Biologie, 2 rue Robert Escarpit, F-33607 Pessac, France

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ABSTRACT: The transactivation responsive element (TAR) plays a crucial role in the transcription of the HIV-1 genome upon specific binding of the viral protein Tat and cellular proteins. We have previously identified a RNA hairpin aptamer forming a stable and specific kissing complex with TAR RNA (Ducongé, F., and Toulmé, J. J. (1999) *RNA 5*, 1605−1614). We chemically modified this aptamer with hexitol nucleic acid (HNA) residues. We demonstrate that a fully HNA-modified aptamer is a poor ligand but, in contrast, mixmers containing both HNA and unmodified RNA nucleotides display interesting properties. Two HNA−RNA mixmers bind to TAR with an equilibrium dissociation constant in the low-nanomolar range and show a reduced nuclease sensitivity. In addition, they show a moderate dependence on magnesium ions for binding to TAR. These HNA−RNA mixmers are able to inhibit transactivation of transcription in an in vitro assay.

For more than twenty years, oligonucleotides have been a powerful tool for inhibiting gene expression. Indeed, different strategies were used for the identification of ligands able to bind to an mRNA target with high affinity and selectivity. In the antisense approach, chemically modified oligonucleotides were designed, essentially (i) to increase the affinity of the antisense sequences for this RNA target, (ii) to improve their nuclease resistance, and (iii) to promote their uptake in live cells (*1*). Whereas antisense oligonucleotides hybridize much better with single-stranded RNA regions, in vitro selection (or SELEX) permits the identification of high-affinity oligonucleotides targeting a folded structure (*2*). The selected molecules are called aptamers; both their sequence and their structure are responsible for the target recognition (*3*).

We previously conducted in vitro selections against the HIV-1 TAR[1] RNA stem-loop (*4*−*6*). This 59-nucleotide-long regulatory element is present at the 5′ end of all viral RNAs and plays a crucial role in viral transcription: TAR RNA is recognized by a ternary complex formed by the viral protein Tat (transactivator protein) and two cellular proteins, namely cyclin T1 and CDK9 (Figure 1A). This Tat-associated kinase hyperphosphorylates the C-terminal domain of RNA polymerase II, and consequently *trans*-activates the transcription machinery leading to the efficient synthesis of full-length viral RNAs (*7*). We are interested in strategies aiming at inhibiting the transcription transactivation step, thus abolishing viral replication. We previously identified against the TAR RNA of HIV-1, an aptamer called R06, which was proven to bind its target with high affinity and selectivity (*5*). The aptamer R06 folds into a stem-loop; the loop is eight-nucleotides long with six of them complementary to the six-nucleotide loop of TAR RNA (Figure 1B). This generates a loop−loop interaction also known as kissing complex. To investigate the biological properties of the selected oligomer, it was important to improve its nuclease stability. Post-selection modification is one way to circumvent nuclease digestion (*8*). This led us to use chemically modified R06 oligomers, taking care of keeping the characteristic A-type conformation of the selected aptamer. We previously investigated N3′→P5′ phosphoramidate (NP), 2′-*O*-methyl (2′O) or locked nucleic acid (LNA) derivatives of R06 (*9*−*11*). More recently, a novel modification of the sugar backbone was proposed: the furanose sugar moiety of an oligonucleotide is replaced by a hexitol, giving rise to so-called hexitol nucleic acid (HNA, Figure 1C). The six-membered hexitol ring mimics the furanose sugar in the 2′-exo, 3′-endo conformation (*12*). High-resolution NMR

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* Corresponding author: e-mail, jean-jacques.toulme@bordeaux.inserm.fr; telephone, 33(0)5 5757 1014; fax, 33(0)5 5757 1015.

‡ Université Victor Segalen Bordeaux 2.

⊥ Institut Européen de Chimie et Biologie.

Present address: Laboratoire de Neurobiologie des Affections de la Myéline, EA 2966, 146 rue Léo-Saignat, F-33076 Bordeaux Cedex, France.

§ Rega Institute for Medical Research.

|| Medical Research Council.

[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; TAR, transactivation responsive element; Tat, transactivator; HNA, hexitol nucleic acid; 2′O, 2′-*O*-methyl; NP, N3′→P5′ phosphoramidate; LNA, locked nucleic acid; EMSA, electrophoretic mobility shift assay; SPR, surface plasmon resonance; RU, resonance unit.

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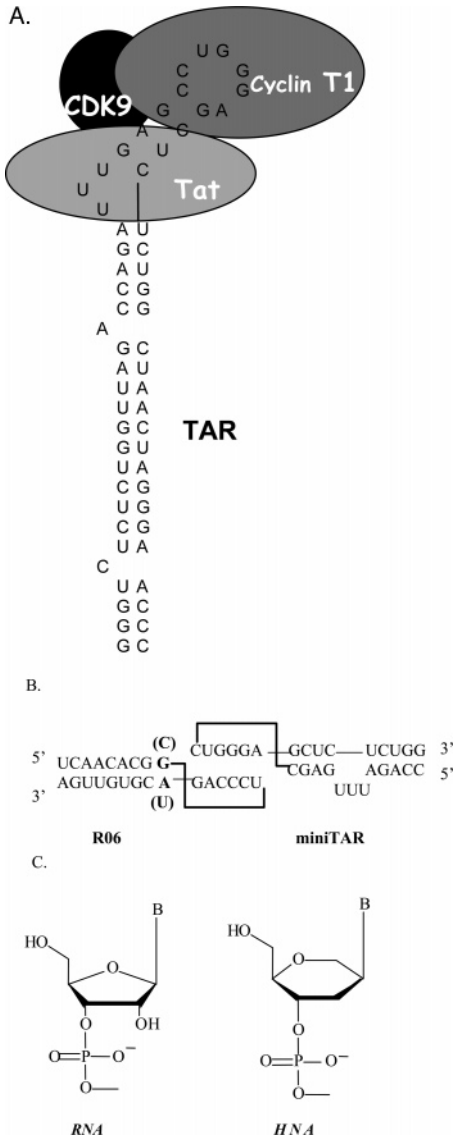

FIGURE 1: (A) Target and aptamer sequences. TAR RNA secondary structure is shown together with proteins involved in *trans*-activation of transcription. (B) Sequences and secondary structures of R06 aptamer and miniTAR RNA. The eight-nucleotide loop of R06 is partially complementary to the six-nucleotide loop of miniTAR RNA. The variant residues of the aptamer loop are indicated (C,U). (C) Schematic representation of HNA and RNA nucleotides.

studies of a HNA−RNA hybrid have shown a particular rigidity of the hexitol sugar moiety. This constrains HNA to adopt preferentially an A-type conformation (*13*). This might account in part for the high HNA−RNA duplex stability. Moreover, HNA-modified antisense oligonucleotides were shown to be translation inhibitors in a cell-free assay and in cultured cells, the high stability of the HNA−RNA duplex leading to a steric block of translation (*14*).

Several derivatives of the R06 aptamer, fully or partially HNA-modified, were synthesized. In the latter case, HNA and RNA nucleotides were interspersed in different ways in order to optimize loop−loop interaction. Interestingly, two HNA−RNA mixmers displayed an affinity similar to that of the RNA parent aptamer for TAR RNA. We investigated the binding properties of these derivatives. We also carried out in vitro transcription assay and demonstrated that HNA mixmers are potent and specific inhibitors of TAR-mediated transcription.

Table 1: R06- and HNA-Modified Aptamers[a]

		Tm (°C)
R06 RNA	C A C G G U C C C A G A C G U G	29±1.8
HNA GA	**C A C G G** U C C C A G **A C G U G**	20±0.3
HNA CU	**C A C G C** U C C C A G **U C G U G**	10±0.4
HNA11	C A C G G U C **C** C A **G** A **C G T G**	31±0.7
HNA12	C A C G **G** U **C** C C A **G** **A C G T G**	< 10
HNA13	C A C G **G** U C **C** C A **G** **A C G T G**	< 10
HNA14	C A C G **G** U C C **C** A **G** **A C G T G**	< 10
HNA15	C A C G G U C C **C** A **G** A **C G T G**	23±1.8
HNA17	C A C G **G** U **C** C C A **G** A **C G T G**	17±0.8
HNA18	C A C G G U **C C** C A G A **C G T G**	22±0.6
HNA19	C A C G G **T** C C **C** A G A **C G T G**	28±2.1

[a] HNA GA and HNA CU are fully HNA modified whereas HNA11−19 have both HNA-modified (bold) and RNA nucleotides (plain). HNA CU has a combination of mutated loop closing residues. The six central nucleotides of the loop are underlined. Melting temperatures of the aptamer−miniTAR complex (see Experimental Procedures) are given.

EXPERIMENTAL PROCEDURES

Oligonucleotides and Peptides. Synthesis of HNA oligomer and HNA−RNA mixmers was carried out as described previously (*15*, *16*). Both U and T HNA residues were used. Their sequences are given in Table 1. The 27-nucleotide-long RNA target, miniTAR, was prepared on an Expedite 8908 synthesizer (Applied Biosystems); LNA oligonucleotides were prepared as previously described (*11*). All the oligonucleotides were purified by electrophoresis on denaturating 20% polyacrylamide, 7 M urea gels, and desalted by extensive dialysis. The peptide Tat_{36} was purchased from Neosystem, Strasbourg, France.

Thermal Denaturation. The oligonucleotides (1 μM each) were preincubated for 30 min at 25 °C in 200 μL of a 20 mM sodium cacodylate buffer, pH 7.3, containing 140 mM potassium chloride, 20 mM sodium chloride, and 0.3 mM magnesium chloride. The thermal denaturation was generated by increasing the temperature from 2.5 to 95 °C, at 0.3 °C/min, and was followed by UV absorption at 260 nm on an Uvikon XL spectrophotometer. The melting temperature (T_m) was determined as the maximum of the first derivative of the melting curve.

Electrophoretic Mobility Shift Assay. For the miniTAR−oligonucleotide complex analysis, [^{32}P]-5′-end-labeled miniTAR (1 nM) was incubated with an increasing concentration of HNA oligonucleotide, in 10 μL of SE buffer (20 mM HEPES, pH 7.3, at 20 °C, 140 mM potassium acetate, 20 mM sodium acetate, 3 mM magnesium acetate) for 1 h at 23 °C. The samples were then loaded on a nondenaturing 15% polyacrylamide (75:1) gel in 50 mM Tris-acetate, pH 7.4 (at 20 °C), 3 mM magnesium acetate, equilibrated at 4 °C. The electrophoresis was run for 16 h at 10 V/cm at 4 °C. Complexes were then quantified on an Instant Imager apparatus (Hewlett-Packard). The dissociation constant (K_D) is deduced from data-point fitting with KALEIDAGRAPH 3.0 (Abelbeck Software, Reading, PA) according to eq 1

$$B = \frac{B_{max}[R06]_0}{[R06]_0 + K_D} \quad (1)$$

where B is the proportion of complex at a given aptamer

concentration, B_{max} is the maximum of complex formation, and $[R06]_0$ the total R06 concentration.

For the miniTAR−Tat competition experiments, [^{32}P]-5′-end-labeled miniTAR (1 nM) was incubated for 1 h at 4 °C, in SE buffer containing 1 mM dithiothreitol (DTT) and 0.01% Triton X100, in the absence or in the presence of 125 nM Tat_{36} peptide and in the absence or in the presence of an increasing concentration of HNA oligonucleotide. The complexes were loaded on a native 10% polyacrylamide (75:1) gel containing 44.5 mM Tris borate, pH 8.3, at 23 °C, 20 μM magnesium acetate, 0.01% Triton X100, and run for 2 h at 4 °C at 12 W. The same conditions were used for the HNA−Tat competition experiments with [^{32}P]-5′-end-labeled HNA11 or HNA14 (1 nM). For the HNA11−LNA5 competition experiment, [^{32}P]-5′-end-labeled miniTAR (1 nM) was incubated for 1 h at 4 °C, in SE buffer containing 1 mM dithiothreitol (DTT) and 0.01% Triton X100, in the absence or in the presence of 125 nM Tat_{36} peptide and 5 nM of HNA11 oligonucleotide and in the absence or in the presence of an increasing concentration of the LNA5 aptamer.

Surface Plasmon Resonance. A total of 200−300 resonance units (RU) of 3′-end-biotinylated miniTAR were immobilized on a carboxymethylated dextran sensorchip (CM5, BIAcore, Sweden) coated with streptavidin as previously described (*17*). The HNA oligonucleotide was prepared in the SE buffer and injected at a 20 μL/min flow rate, at 23 °C. The kinetic parameters were determined as previously described (*17*).

In Vitro Transcription Assays. In vitro transcription was performed at 30 °C for 20 min with 15 μL of HeLa cell nuclear extract in a 40 μL final volume containing 80 mM potassium chloride, 3.5 mM magnesium chloride, 20 mM HEPES (pH 7.9), 2 mM DTT, 10 μM zinc sulfate, 10 mM creatine phosphate, 100 μg/mL creatine kinase, 1 mg poly-[d(I−C)], 50 μM of ATP, GTP, CTP and 5 μM of UTP a-^{32}P (10 μCi) 10 nM of DNA template and 200 ng of Tat recombinant protein (*18*−*20*) were added as well as an increasing concentration of HNA-modified aptamers. The reaction was stopped by adding 50 μL of a solution containing 0.3 M sodium acetate pH 5.2, 20 mM EDTA, 1% SDS and 100 μg/mL of tRNA. Then, the transcripts were extracted by phenol/chloroform and precipitated with ethanol. The products were analyzed by electrophoresis on a denaturating 6% polyacrylamide gel, 7 M urea and revealed by autoradiography. The full-length transcripts were quantified by IMAGE 1.62 Software of the National Institutes of Health (Bethesda, USA).

Nuclease-Resistance Assay. [^{32}P]-5′-End-labeled HNA-modified aptamers (10000 cpm/sample, ≈340 Bq at 10 nM) were incubated at 37 °C in DMEM (Dubelcco's medium eagle modified) containing 5% fetal calf serum. Forty microliter aliquots were withdrawn at different incubation times. These samples were immediatly diluted with 50 μL of 0.3 M sodium acetate buffer pH 5.2, containing 20 mM EDTA, and 100 μg/mL tRNA. They were then extracted with phenol/chloroform and precipitated with ethanol. Samples were loaded on a denaturating 20% polyacrylamide, 7 M urea gel. The band corresponding to the full-length aptamer was quantified by Instant Imager.

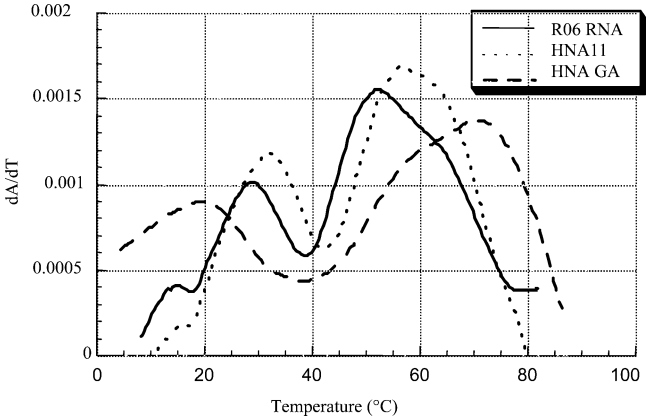

FIGURE 2: Melting transitions of loop−loop complexes. The denaturation of complexes between miniTAR, R06, HNA11, or HNA GA was carried out as described in Experimental Procedures.

RESULTS

The HNA Fully Modified Aptamer Is a Weak Ligand of HIV-1 TAR. We derived HNA-aptamers from the RNA hairpin R06 previously identified as a TAR RNA ligand through a SELEX procedure (*5*). The association between the aptamer and TAR hairpins was investigated by UV-monitored thermal denaturation. To make the synthesis and the analysis easier the stems were shortened. TAR was reduced to its 27-nucleotide-long apical region termed "miniTAR" and the aptamer R06 to a 16-mer with a 4 base pair stem (Figure 1B). Such a shortened TAR element has been previously shown to retain functional properties (*21*, *22*). The binding properties of these RNA truncated hairpins are identical to that of the full-length TAR and R06 molecules, indicating that the formation of the complex is driven by loop−loop interactions. Two fully modified HNA hairpins were synthesized (Table 1) corresponding to the selected sequence HNA GA and to a mutant HNA CU. The two hairpins differ only by the residues closing the aptamer loop. Even though these bases do not engage direct interactions with TAR RNA we previously demonstrated that the selected G,A combination is crucial for RNA-RNA loop−loop interaction. Substitutions at these positions are detrimental to complex formation; the C,U combination abolishes the association between TAR and the mutated R06 RNA.

The melting profile of miniTAR-aptamer mixtures shows two transitions (Figure 2), the broad one at high temperature corresponds to the overlapping signals of both miniTAR and hairpin stem denaturation whereas that at low temperature characterizes the dissociation of the loop−loop helix. As expected the stem of the HNA aptamers is much more stable than that of the parent R06 ($\Delta T_m \approx 18$ to 22 °C). But the introduction of hexitol sugars at every position in the loop was deleterious for the kissing complex formation. The HNA GA−miniTAR complex shows a ΔT_m of about −9 °C compared with the parent R06(RNA)−miniTAR complex (Figure 2 and Table 1). The aptamer mutant HNA CU is an even poorer ligand: a slight transition could be detected at about 10 °C (Table 1). As previously observed with the RNA aptamer, this means that the loop closing residues play a key role in the HNA−TAR kissing complex formation, suggesting that the overall organization of the chemically modified loop−loop complex share similar properties with the RNA−TAR complex. The sugar rings of HNA in HNA−

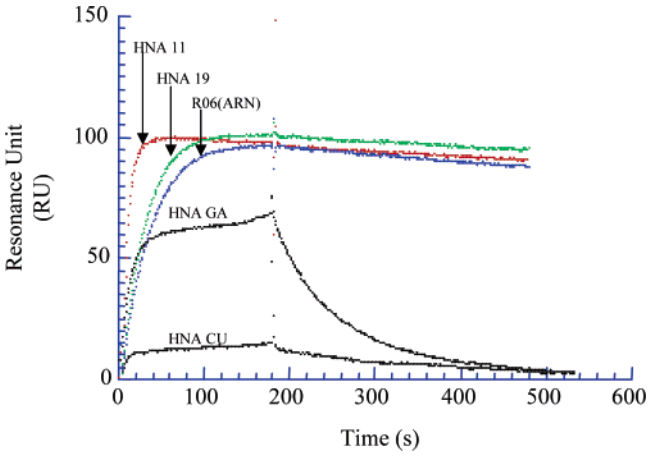

FIGURE 3: Sensorgrams of the R06- and HNA-modified aptamer−miniTAR complexes. Experiments were carried out in the SE buffer at 23 °C. 500 nM HNA11, HNA19, R06, HNA GA, or HNA CU (as indicated on figure) was injected on a miniTAR-functionalized sensor chip (see Experimental Procedures).

RNA hybrids were shown to have a rigid chair conformation (*13*). The rigidity induced by the HNA strand might not be favorable to a kissing interaction. It might prevent achievement of an optimal conformation, particularly at the junction between the stems and the loop−loop helix. We therefore synthesized a series of R06 derivatives with mixed RNA−HNA backbones.

A HNA−RNA "Mixmer" Is a Good TAR RNA Ligand. Eight mixmers were synthesized with empirically interspersed HNA and RNA residues (Table 1). Each aptamer derivative stem has the same fully modified HNA 3′ strand and unmodified RNA 5′ strand. This results in a stabilization of the stem compared to the parent aptamer R06 ($\Delta T_m \approx 6$ °C; not shown) as expected for RNA−HNA hybrids (*23*). This will also likely protect the oligomer against 3′ exonucleases. Four aptamers bore an RNA G,A combination (HNA11, 15, 18, and 19), three an HNA G,A combination (HNA12, 13, 14), and one a mixed HNA G, RNA A combination (HNA17). In addition, different HNA and RNA distributions were introduced into the aptamer loop (Table 1). The stability of the different mixmer−TAR complexes was first followed by thermal denaturation.

The HNA modification of the G,A closing residues largely destabilizes the kissing complex. No transition was observed for complexes between TAR and HNA12, 13 or 14 (Table 1). In contrast the loop of the strongest TAR ligands is closed by RNA G,A residues (23 °C < T_m < 31 °C; Table 1). The derivative HNA17 with a mixed HNA G, RNA A combination binds with an intermediate stability (T_m = 17 °C, Table 1). It must be noted that the HNA11−TAR complex has a slightly increased thermal stability compared to that of the parent R06 (Figure 2). The two best mixmers (HNA11: T_m = 31 °C and HNA19: T_m = 28 °C, Figure 2) were used for further investigations in comparison with R06(RNA), HNA GA, and HNA CU aptamers.

Surface plasmon resonance experiments were carried out in order to determine the association and dissociation rate constants. The different aptamers were successively introduced on a sensor chip on which miniTAR RNA was immobilized. In Figure 3 is represented the sensorgram obtained with different aptamers injected at a single concentration. The kinetic parameters listed in Table 2 were deduced from direct fitting of the curves. R06, HNA11, HNA19, and HNA GA are characterized by association rate constants in the same range (6.2 10^4 M^{-1} s^{-1} < k_{on} < 19.8 10^4 M^{-1} s^{-1}, Table 2). The dissociation rate constants for R06(RNA), HNA11 and HNA19 are also similar (1.5 10^{-4} s^{-1} < k_{off} < 3.6 10^{-4} s^{-1}). The fully modified HNA GA shows an increased dissociation rate (k_{off} = 115 10^{-4} s^{-1}, Table 2). The equilibrium constants deduced from these sensorgrams confirm that HNA11 and HNA19 form high affinity complexes with TAR (K_D = 1.5 nM and 1.7 nM, respectively, Table 2). In contrast, the fully modified aptamer HNA GA displays a moderate affinity due to a higher dissociation rate constant. The mutant HNA CU shows a slow association with TAR (Figure 3), but the curve could not be fitted to extract kinetic parameters. Electrophoretic mobility shift assays confirmed the results obtained from surface plasmon resonance experiments: HNA11, HNA19 and R06 display affinities for TAR in the low nanomolar range, whereas the fully modified aptamer HNA GA gives rise to a K_D of about 50 nM (Table 2).

Table 2: Equilibrium and Rate Constants of Aptamer−miniTAR Complexes[a]

	EMSA	surface plasmon resonance		
	K_D (nM)	k_{on} ($\times 10^4$ M^{-1} s^{-1})	k_{off} ($\times 10^{-4}$ s^{-1})	K_D (nM)
R06 RNA	2 ± 0.5	6.2 ± 0.2	3.6 ± 0.2	5.9 ± 0.5
HNA11	6 ± 0.4	19.8 ± 8.2	2.9 ± 0.1	1.5 ± 0.1
HNA19	6 ± 1.5	9.0 ± 2.1	1.6 ± 0.1	1.7 ± 0.3
HNA GA	46 ± 6.0	13.9 ± 6.4	115.0 ± 7.0	82.6 ± 5.5

[a] Dissociation constants (K_D) were determined by electrophoretic mobility shift assay (EMSA) and surface plasmon resonance (see text for details).

HNA Oligonucleotides Do Not Compete with Tat for the Association with TAR. The Tat protein and the aptamer bind to different parts of the TAR element: the kissing complex formation involves the loop, whereas it has long been shown that Tat associates in the region of the three-nucleotide bulge (Figure 1A). However, it was recently suggested that Tat also recognizes the TAR upper stem and loop (*24*). Indeed the N3′−P5′ phosphoramidate derivative of the R06 aptamer (*9*) and a LNA−DNA chimeric aptamer, LNA5 (unpublished), inhibit TAR−Tat association. It was therefore of interest to investigate the effect of HNA-modified aptamers on Tat−TAR complexes. We used a 36 amino acid long peptide that retains both the affinity and the specificity of the intact Tat protein (*25*) for electrophoretic mobility shift assays. As the migration of the TAR−Tat complex in a 3 mM magnesium containing buffer promotes the dissociation during migration (*9*) the assay was carried out in a buffer containing only 20 μM magnesium to allow both the aptamers and Tat to associate with TAR. RNA−RNA kissing complex formation is highly dependent on magnesium concentration. Consequently the R06 aptamer binds much more weakly to TAR at 20 μM magnesium than under selection conditions (3 mM magnesium) (*17*). Interestingly, HNA-modified aptamers efficiently bind to TAR at low magnesium concentration. The formation of the HNA11−miniTAR complex can be monitored by electrophoresis (Figure 4A; lane 2). The Tat_{36}−miniTAR complex is detected on the same nondenaturing polyacrylamide gel as a slow migrating band (Figure 4A; lane 3). The simultaneous addition of the Tat_{36} peptide and of the HNA11 aptamer

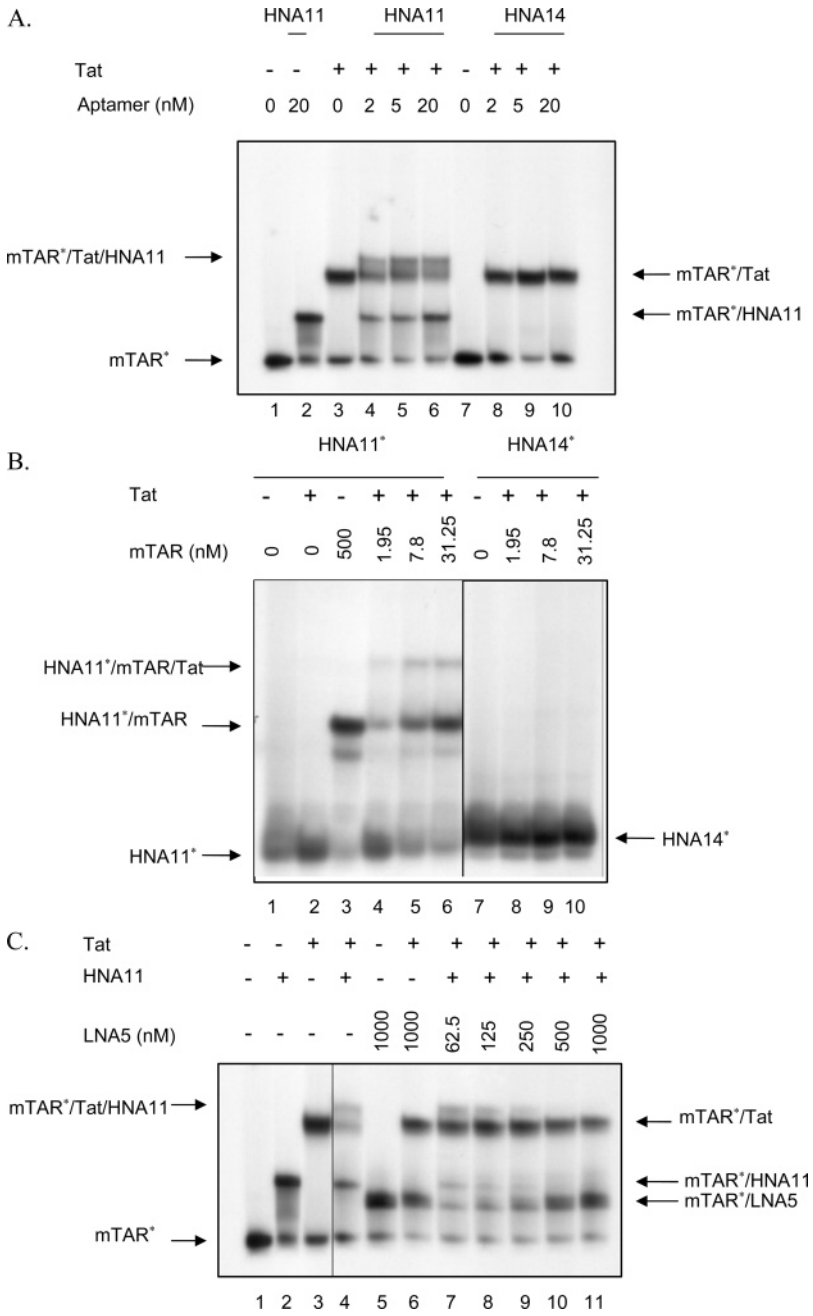

FIGURE 4: Competition between Tat peptide and aptamers for binding to TAR RNA. (A) Competition assay between HNA mixmers and Tat_{36} peptide with radiolabeled miniTAR RNA. 1 nM of radiolabeled miniTAR was incubated with or without 125 nM of Tat peptide, as indicated at the top of the lanes. Increasing amounts of HNA11 (left) or HNA14 (right) were added as indicated. (B) Competition assay between miniTAR RNA and Tat_{36} peptide with radiolabeled HNA mixmers. 1 nM radiolabeled HNA was incubated with or without 125 nM of Tat peptide, as indicated at the top of the lanes. Increasing amounts of miniTAR RNA was added as indicated. (C) Competition assay between HNA mixmers, LNA5 aptamer, and Tat_{36} peptide with radiolabeled miniTAR RNA. 1 nM radiolabeled miniTAR was incubated with or without 125 nM of Tat peptide and 5 nM of the HNA11 mixmer as indicated at the top of the lanes. Increasing amounts of LNA5 aptamer were added as indicated. Arrows indicate the different binary and ternary complexes between the aptamers, the peptide (Tat), and the TAR element (mTAR).

induces the appearance of a species migrating slower than the binary complexes Tat−miniTAR and HNA11−miniTAR (Figure 4A; lanes 4−6). Such a pattern was not observed when HNA14 which does not bind to TAR is substituted to HNA11 (Figure 4A; lanes 8−10). This suggests that the upper band seen with HNA11 corresponds to a ternary complex between the aptamer, TAR and Tat. This is confirmed by the experience shown in Figure 4B in which [^{32}P]-end-labeled HNA aptamer derivatives were used for monitoring the complexes. In agreement with the previous experiment a retarded band was shown upon addition of miniTAR to HNA11 (left panel) but not to HNA14 (right panel), corresponding to the kissing complex. A faint band moving ahead of the TAR−HNA11 major band was also seen which might correspond to an impurity or to a different conformation of the kissing complex. In the presence of both Tat_{36} and miniTAR a slowly migrating species was seen with HNA11, but not with HNA14 as expected for a ternary complex. A similar behavior was seen with HNA15 or HNA19 that do not bind to TAR either (not shown). Therefore the supershifted species requires the formation of a kissing complex. But the addition of the Tat peptide to the

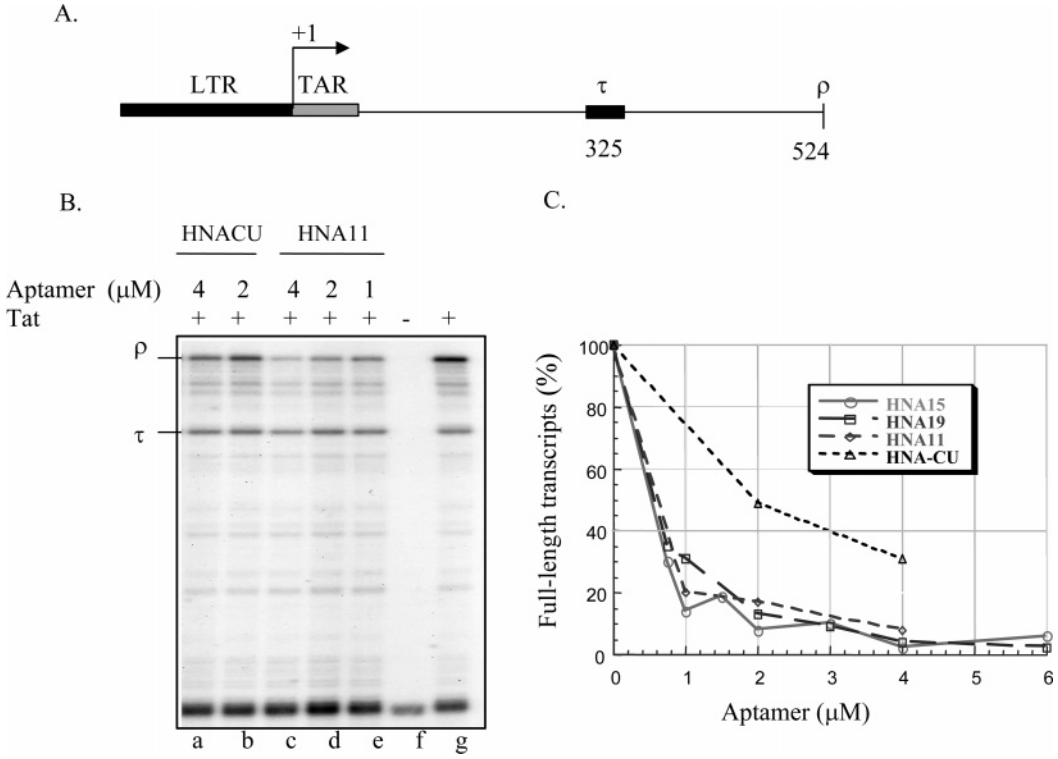

FIGURE 5: In vitro Tat-mediated transcription assay. (A) Schematic representation of the linearized DNA template containing the HIV-1 promoter (LTR), an artificial terminator sequence (τ), and the runoff terminator (ρ), giving rise to two RNA products, 325-nucleotides (τ) and 524-nucleotides (ρ) long. (B) Autoradiograph of the transcription products analyzed on a 6% polyacrylamide, 7 M urea gel. The DNA template (10 nM) was incubated in the presence of HeLa cell nuclear extract in the presence (lanes a−e, g) or in the absence (lane f) of 200 ng of recombinant Tat. HNA11 aptamer (lanes c−e) or HNA CU (lanes a−b) was added at concentrations of up to 4 mM. (C) Relative amount of full-length transcripts (ρ) as a function of aptamer concentration.

HNA hairpins does not result in a detectable complex. These results mean that in contrast to other modified anti-TAR aptamers the HNA derivative does not displace the Tat peptide from its TAR binding site. Chimeric LNA−DNA derivatives of the aptamer R06 were recently synthesized and investigated (*11*). A hairpin termed LNA5 with three LNA residues interspersed in a DNA loop generates a kissing complex with TAR and displaces the Tat_{36} peptide at high concentration (Darfeuille et al., unpublished). We used LNA5 as a competitor of HNA11 (Figure 4C). The two kissing complexes HNA11−mTAR and LNA5−mTAR show a different mobility. The addition of increasing amounts of LNA5 to a mixture containing miniTAR, Tat_{36}, and HNA11 results in the decrease of the band corresponding to the miniTAR−HNA11 binary complex and of the slowly moving one ascribed to the miniTAR−HNA11−Tat_{36} ternary complex to the expense of the miniTAR−LNA5 kissing complex. Therefore these results confirm that the aptamer HNA11 and the Tat_{36} peptide bind simultaneously to the TAR RNA element and do not compete with each other.

Inhibition of Tat-Mediated Transcription. We next investigated the ability of HNA-modified aptamers to inhibit the *trans*-activation of transcription mediated by the viral protein Tat. We used a DNA template containing the HIV-1 long terminal repeat. Two transcripts 325- and 524-nucleotides long are obtained, corresponding to the runoff transcript and internal artificial terminator (ρ and τ) (Figure 5A). The presence of HeLa cell nuclear extracts and of the protein Tat enhanced the transcription yield. The addition of HNA aptamers to the in vitro transcription mixture reduces the RNA synthesis in a dose-dependent manner (Figure 5B and C).

The mixmers HNA11, HNA15, and HNA19 inhibit the transcription with an IC50 < 750 nM whereas HNA CU which is a poor TAR ligand shows a weaker inhibition with an IC50 around 2 μM. The fully modified HNA GA is also a weak inhibitor of TAR dependent transcription (data not shown), indicating that the efficiency of inhibition is related to the binding affinity of the aptamer. Since the HNA mixmers do not compete with Tat binding, these results suggest that these modified aptamers interfere with the association of proteins to the TAR loop.

HNA-Modified Aptamers Are Resistant to Nucleases. The nuclease resistance of the different aptamers was tested in a cell culture medium (DMEM supplemented with 5% fetal calf serum). The fully modified HNA aptamers HNA GA and HNA CU are strongly resistant to nuclease digestion and no degradation was observed up to 48 h (Figure 6). As far as the mixmers HNA19 and HNA11 are concerned, degradation was observed and the half-lifetime is estimated around 90 min (Figure 6). This is significantly higher than that of the parent R06(RNA) which has a half-life of 10 min under the same conditions (not shown). Therefore, the introduction of HNA modifications increases the half-lifetime of the mixmers and renders the fully modified HNA aptamers strongly resistant to nucleases. Nuclease resistance of mixmers might be further improved by substituting 2′-O-methyl nucleosides for ribonucleosides. This would likely generate A-type molecules able to recognize the TAR hairpin.

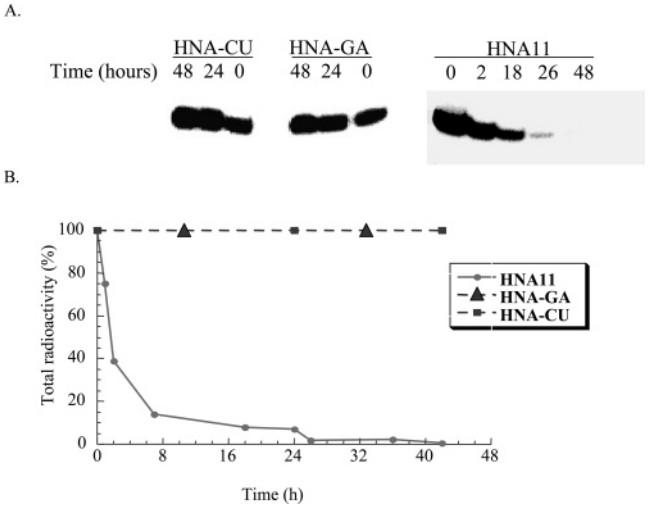

FIGURE 6: Nuclease resistance assay. (A) HNA GA, HNA CU, and HNA19 were incubated in DMEM medium containing 5% fetal calf serum. Aliquots were withdrawn at different times and electrophoresed on a denaturating 20% polyacrylamide, 7 M urea gel. (B) Amount of intact aptamers as a function of time (see Experimental Procedures for details).

DISCUSSION

Conserved regions of the HIV-1 RNA are valid targets for the development of inhibitors of retroviral replication. The TAR hairpin present at the 5′ extremity of the RNA genome is of key interest due to its role in the *trans*-activation of transcription (*26*). Different artificial ligands have been tested to this end (*27*). Small molecules were designed, either rationally (*28*−*30*) or after a computational approach (*31*). Peptides and peptidomimetics have been designed on the basis of the Tat minimal TAR binding region (*32*), and a peptoid−peptide hybrid was identified thanks to a combinatorial approach (*28*). Even though the TAR hairpin is not favorable for antisense hybridization, some interesting ligands have been designed, with K_Ds in the nanomolar range. Such molecules were able to inhibit Tat-dependent transcription in vitro and in cell culture (*20, 33, 34*).

In vitro selection allowed the identification of an RNA aptamer, R06, forming a kissing complex with the TAR target (*17*). This aptamer shows both a strong affinity and a high specificity for TAR. It is of interest to identify nuclease-resistant derivatives of R06 retaining the binding characteristics of the parent R06. Hexitol nucleic acid-modified oligonucleotides, which bind RNA with an increased affinity compared to RNA (*23*) and which adopt the C2′exo−C3′endo conformation, are candidates of interest (*13*). The fully modified HNA derived from R06 shows a reduced affinity for the TAR RNA element compared to the parent RNA aptamer. This is at variance from the N3′→P5′ phosphoramidate and 2′-O-methyl R06 derivatives which displayed binding properties analogous to that of the RNA aptamer. The behavior of the HNA analogue is reminiscent to what we previously observed for the fully modified LNA aptamer (*11*). The chemistry of the G,A residues closing the aptamer loop are of key importance: for the HNA series the presence of riboA and riboG nucleosides at these positions led to strong TAR ligands. These residues have been demonstrated to be important for kissing complex formation (*5*). The G,A combination leads to optimal stacking interactions at the loop−loop helix and stem junction. It also allows an increased inter-strand distance, thus reducing electrostatic repulsion between the phosphate groups (*35*). The rigidity induced in the sugar moiety in LNA and HNA might prevent optimal positioning of the residues at the junction. It is also of interest to note that an inter-strand hydrogen bond involving the sugar of the G residue has been suggested by a molecular dynamics study (*35*). The presence of HNA guanine is more detrimental to kissing interaction than that of a HNA adenine.

HNA mixmers containing HNA and RNA residues in the loop show strong binding properties (as far as G and A closing residues are provided as ribonucleosides). This is reminiscent to the results obtained with LNA. As few as two to three modified residues provide the aptamer with an affinity equivalent to that of the parent aptamer. The rules for the location of these modified residues are presently unknown but likely depend on the interaction with the adjacent residues, that is, on the loop sequence.

The effect of the association of the aptamer with TAR on the binding of the protein Tat indicates different structural properties of the kissing complex. Whereas the N3′→P5′ phosphoramidate aptamer (*9*) or the LNA−DNA mixmer (unpublished) displaces the Tat peptide from TAR the HNA−RNA mixmer gives rise to a ternary complex. Even if the recognition of TAR by both the N3′→P5′ phosphoramidate aptamer and the HNA mixmer relies on loop−loop interactions, the conformation of the TAR element is likely to be different in the two types of complexes. Consequently, the recognition of the same site might lead to different properties, making aptamers very subtle ligands allowing one to decipher structure−function relationships.

The magnesium dependence of kissing interactions also relates to different complex conformation. Whereas HNA and LNA mixmers bind to TAR with a similar affinity ($T_m \approx 31$ °C) in the presence of 3 mM magnesium a 10-fold excess of the LNA mixmer is required to compete with the HNA mixmer for binding to TAR at lower magnesium concentration (20 μM) as shown by electrophoretic mobility shift assays (Figure 4C). This might be related to the inter-strand phosphate-phosphate distance at the loop−loop helix aptamer stem junction. The HNA mixmer which is less dependent on magnesium ions might be a candidate of interest for biological assays. Indeed the inhibition of in vitro transcription make them promising tools.

In summary, from this work and previous reports we clearly showed that the recognition of the same target, the TAR RNA element of HIV-1, by different chemically modified derivatives of the parent RNA aptamer involves very subtle differences. Even though the primary recognition mode remains the same for all the aptamer derivatives, that is the formation of a loop−loop complex, the conformation of the complex is nicely tuned by the oligonucleotide chemistry. Among the nuclease resistant RNA mimics investigated so far only N3′→P5′ phosphoramidate and 2′-O-methyl residues are tolerated at the positions closing the aptamer loop (*10, 36*). Interestingly these derivatives as well as LNA−DNA (*11*) and HNA−RNA mixmers bind to the TAR hairpin with similar affinity. But the latter analogue does not compete with Tat in contrast to the other ones. Therefore these aptamer derivatives constitute a class of tools that can nicely modulate biological interactions.

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