Aptamers Targeted to an RNA Hairpin Show Improved Specificity Compared to that of Complementary Oligonucleotides†

Fabien Darfeuille,‡ Sandrine Reigadas,‡ Jens Bo Hansen,§ Henrik Orum,§ Carmelo Di Primo, and Jean-Jacques Toulmé*

INSERM U386, Université Victor Segalen, 33076 Bordeaux, France, Institut Européen de Chimie et Biologie, 33607 Pessac, France, and Santaris Pharma, Fruebjervej 3, DK-2100 Copenhagen, Denmark

Received March 31, 2006; Revised Manuscript Received July 31, 2006

ABSTRACT: Aptamers interacting with RNA hairpins through loop−loop (so-called kissing) interactions have been described as an alternative to antisense oligomers for the recognition of RNA hairpins. R06, an RNA aptamer, was previously shown to form a kissing complex with the TAR (trans-activating responsive) hairpin of HIV-1 RNA (Ducongé and Toulmé (1999) *RNA* 5, 1605). We derived a chimeric locked nucleic acid (LNA)/DNA aptamer from R06 that retains the binding properties of the originally selected R06 aptamer. We demonstrated that this LNA/DNA aptamer competes with a peptide of the retroviral protein Tat for binding to TAR, even though the binding sites of the two ligands do not overlap each other. This suggests that upon binding, the aptamer TAR adopts a conformation that is no longer appropriate for Tat association. In contrast, a LNA/DNA antisense oligomer, which exhibits the same binding constant and displays the same base-pairing potential as the chimeric aptamer, does not compete with Tat. Moreover, we showed that the LNA/DNA aptamer is a more specific TAR binder than the LNA/DNA antisense sequence. These results demonstrate the benefit of reading the three-dimensional shape of an RNA target rather than its primary sequence for the design of highly specific oligonucleotides.

RNA folding is a strong limitation to the use of antisense oligonucleotides (*1*) and siRNA (*2*). Even though many RNA structures play an important role in gene expression (*3*−*8*), such regions are generally not considered as valid targets for complementary regulatory oligomers: intramolecular RNA−RNA interactions prevent or at least weaken the intermolecular RNA−oligonucleotide association. A limited number of studies have been devoted to the targeting of RNA motifs. Different types of strategies have been developed (*9*, *10*). First, the energy required for opening the RNA structure can be minimized. One generally takes advantage of peculiarities (loops, bulges) for choosing the target sequence, thus reducing the price paid for opening the structure (*11*). Second, one can make use of high affinity derivatives that promote the binding of the oligomer at the expense of intramolecular pairing (*12*). A number of oligonucleotide analogues with modified bases, sugar, or internucleoside linkers have been developped to this aim. Of course, high affinity derivatives can be introduced in sequences targeted to optimized sequences. However, one should be aware that oligomers displaying a high binding constant for a target being part of a secondary structure may also bind to an accessible (single-stranded) mismatched sequence. An equivalent amount of complexes will be formed with the intended folded target or with a noncomplementary linear sequence if the penalty energy to be paid for opening the structure in the first case is equivalent to the destabilization introduced by the mismatches in the second case. In other words, increased affinity might be a source of decreased specificity, a dilemma that has been recognized for a long time (*13*, *14*).

Ligands that interact with the folded structure, using, for instance, local triple helix formation (*15*−*17*) or aptamer recognition (*9*, *18*), may circumvent or at least limit this problem. We explored this latter strategy over the past few years and identified aptamers interacting with RNA hairpins through loop−loop interactions (*19*, *20*). The in vitro selection carried out against the trans-activating responsive (TAR[1]) element of HIV-1 led to the characterization of aptameric RNA hairpins that form kissing complexes with TAR, involving at most the formation of six base pairs (*21*). TAR is a 59 nucleotide long imperfect hairpin located at the very 5′ end of the retroviral RNA that *trans*-activates the formation of full length HIV transcripts upon binding to the viral protein Tat and host cellular factors (*3*, *22*, *23*).

We investigated the properties of nuclease resistant aptamers derived from the selected RNA candidates by the introduction of locked nucleic acid (LNA) residues. LNAs are nucleic acid analogues, with a 2′-O→4′-C-methylene linkage (Figure 1A) (*24*−*26*) showing unprecedented affinity for the complementary RNA sequence (*27*). We designed an LNA/DNA chimera hairpin with a four base pair stem containing two LNA pairs at the bottom and four interspersed

† S.R. is the recipient of a SIDACTION fellowship (grant 23041-00-02/AO15-1). This work is partly supported by the "Agence Nationale de la Recherche sur le SIDA".

* Corresponding author: Tel: 33 (0)5 57 57 10 14. Fax: 33 (0)5 57 57 10 15. E-mail: jean-jacques.toulme@bordeaux.inserm.fr.

‡ Equal first authors.

§ Santaris Pharma.

[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; TAR, *trans*-activation responsive element; Tat, *trans*-activator protein; LNA, locked nucleic acid; EMSA, electrophoretic mobility shift assay.

10.1021/bi0606344 CCC: $33.50
Published on Web 09/08/2006

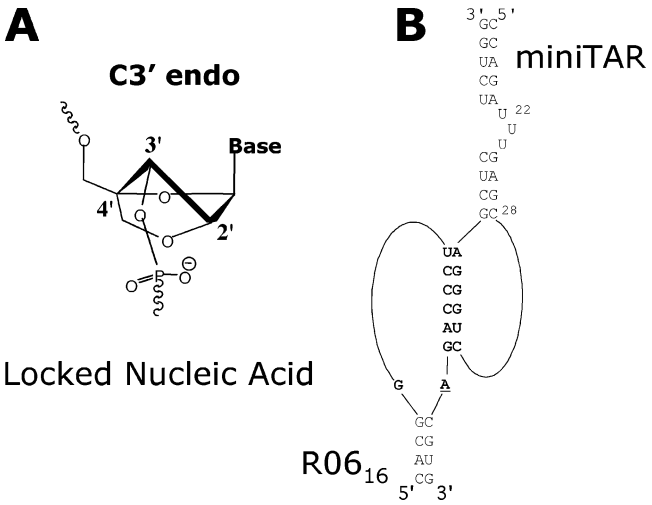

FIGURE 1: Scheme of the locked nucleic acid monomer and of the TAR−aptamer kissing complex. (A) Schematic representation of the locked nucleic acid monomer. (B) Sequence and secondary structure of the loop−loop complex formed between the $R06_{16}$ aptamer and miniTAR RNA used in this study. The consensus octamer obtained from in vitro selection and the loop bases of the TAR element susceptible to base pairing with the aptamer loop are shown in bold. The substitution of the closing A residue of the aptamer loop (underlined) by a C generates the $LD06_{16}$(gc) variant used in Figure 3A. The numbering of the TAR residues refer to the full length element.

LNA residues in the eight nucleotide loop (*28*). This chimeric aptamer binds to the TAR hairpin with an affinity similar to that of the parent RNA aptamer, very likely through the formation of a six base pair loop−loop helix. The chemically modified LNA/DNA octamer, homologous to the aptamer loop that can also give rise to the same six base pairs, binds as strongly to the TAR loop as the chimeric aptamer. We demonstrate in this article that the LNA/DNA aptamer highly discriminates between two TAR variants, differing only by a single mutation in the loop, whereas the LNA/DNA antisense sequence does not. Moreover, this modified aptamer competes with a peptide derived from the viral protein Tat for binding to TAR, whereas the antisense LNA/DNA octamer does not. Because the binding sites of the aptamer and the Tat peptide, the apical loop and a bulge, four base pairs below the loop, respectively, do not overlap, this suggests a teleo-specific effect. The aptamer, but not the antisense octamer, switches the conformation of the TAR bulge region to a structure that is no longer recognized by the Tat peptide.

MATERIAL AND METHODS

Oligonucleotides. DNA and RNA were synthesized on an Expedite 8908 synthesizer. Oligomers containing LNA units were synthesized as previously described on an automated DNA synthesizer (*29*). All oligonucleotides were purified by electrophoresis on denaturing 20% polyacrylamide/7 M urea gels and desalted on Sephadex G-10 spin columns. Oligomers were solubilized in sterile RNase- and DNase-free distilled water and stored at −20 °C. The concentration of all oligomer solutions was quantitated using spectrometric absorbance values at 260 nm. The molar absorptivity (ϵ_{260}) of LNA-containing oligonucleotides was calculated assuming identical absorptivities for LNA and DNA nucleotides.

Melting Curves. Melting curves were collected by measuring the change in absorbance at 260 nm using a Uvikon UV/Vis spectrophotometer (BIOTEK) equipped with a 10-position sample holder and a Peltier temperature control accessory. Samples (1 μM final concentration of each oligomer in the mixture, in 200 μL of 20 mM cacodylate buffer (pH 7.3 at 20 °C) containing 140 mM potassium chloride, 20 mM sodium chloride and 0.3 mM magnesium chloride) were heated at a rate of 0.4 °C/min from 5 °C to 90 °C. Samples were overlaid with 300 μL of mineral oil to prevent evaporation at high temperatures. An initial 30 min equilibration time was included prior to the temperature ramping. The midpoint of the melting transition, T_m, was given as the peak of the first derivative of the UV melting curve.

Electrophoretic Mobility Shift Assays. MiniTAR RNA−aptamer complexes were monitored by an electrophoretic mobility shift assay (EMSA) at room temperature as described previously (*30*). Briefly, a total of 0.5 nM [^{32}P] 5′-end-labeled aptamer $LD06_{16}$ or anti-loop $asLD_8$ were incubated with increasing concentrations of miniTAR BRU or MAL (Figure 2A) for 1 h at 23 °C in a 20 mM HEPES buffer at pH 7.3, at 20 °C, containing 20 mM sodium acetate, 140 mM potassium acetate, and 3 mM magnesium acetate (R buffer). Binding reactions were loaded onto running native gels equilibrated at 4 °C (15% (w/v) acrylamide and 75:1 acrylamide/bis(acrylamide) in 50 mM Tris-acetate (pH 7.3 at 20 °C) and 3 mM magnesium acetate). The electrophoresis was carried out at 300 V at 4 °C for 4 h. The radioactivity was quantified with an Instant Imager apparatus (Hewlett-Packard). The apparent dissociation equilibrium constant, *K*d, was deduced from data point fitting of the titration curves using Kaleidagraph 3.0 (Abelbeck software) according to eq 1.

$$B = \frac{B\text{max}\,[RNA]_0}{K\text{d} + [RNA]_0} \quad (1)$$

where *B* is the proportion of complex, *B*max is the maximum of complex formed, and $[RNA]_0$ is the miniTAR total concentration.

Footprinting of TAR/Oligonucleotide Complexes. Ten picomoles of in vitro transcribed TAR RNA were dephosphorylated with the Shrimp Alkaline Phosphatase (SAP, USB Corporation) and then radioactively labeled at the 5′ end with [γ-^{32}P] ATP and the T4 polynucleotide kinase (T4 PNK, USB Corporation) according to the manufacturer's instructions. The labeled fragments were purified by polyacrylamide gel electrophoresis. In total, 0.2 picomoles of 5′-end-labeled TAR RNA were incubated in 50 mM Tris-acetate buffer at pH 7.5 at 20 °C, containing 50 mM sodium acetate and 5 mM magnesium acetate in the presence or the absence of 10 pmol of LNA/DNA chimera oligonucleotides for 30 min at 37 °C. Subsequently, 2 μL of a fresh solution of 25 mM lead(II) acetate was added, and the incubation was continued for 2 or 5 min. Reactions were stopped by adding 5 μL of 0.1 M EDTA, and the labeled transcripts were precipitated, suspended in gel loading buffer and analyzed on an 8% polyacrylamide/8 M urea sequencing gel run in TBE.

Aptamer/Tat Competition Assays. 1 nM ^{32}P-labeled miniTAR was incubated with 100 nM Tat peptide, Tat_{36} (amino acids 37−72 of the Tat protein) (Neosystem) and with

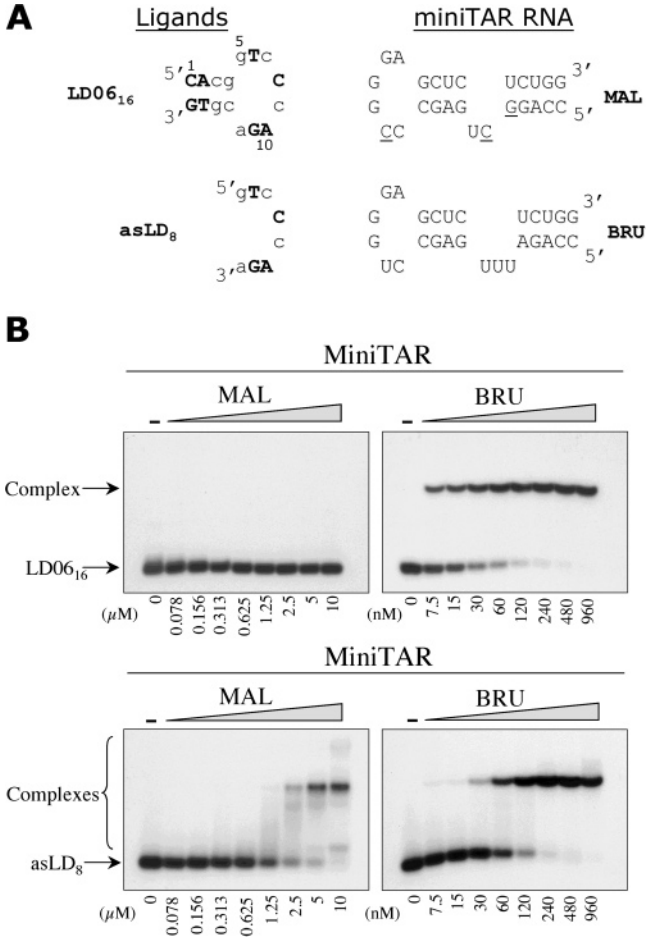

FIGURE 2: Analysis of oligonucleotide−miniTAR complexes by electrophoretic mobility shift assays. (A) Sequence of oligonucleotides used in this experiment. Capital letters represent RNA, capital letters in bold represent LNA, and lower case letters represent DNA. Underlined bases for miniTAR MAL are those that differ from miniTAR BRU. The A residue in the 12th position was substituted by a C for generating a G,C variant that no longer binds to mini TAR BRU or MAL (*28*). (B) Radiolabeled LD06$_{16}$ (top panels) or asLD$_8$ (bottom panels) derivatives were incubated in R buffer at 23 °C with increasing amounts of miniTAR MAL as indicated (left panels) or of miniTAR BRU (right panels).

increasing concentrations of aptamer LD06$_{16}$ or anti-loop asLD$_8$ in R buffer containing 0.01% Triton X-100 and 1 mM DTT at 37 °C for 2 h. Complexes were loaded onto running native gels (10% (w/v) acrylamide and 75:1 acrylamide/bis-(acrylamide) in 50 mM Tris acetate (pH 8.3 at 20 °C) and 20 μM magnesium acetate) and electrophoresed for 2 h at 12 W, at 4 °C. The gel was dried and visualized by autoradiography.

RESULTS

The RNA hairpin R06 previously selected against the TAR RNA element of HIV-1 (*21*) was truncated to generate the aptamer R06$_{16}$ (Figure 1B), which retains the originally selected 8 nucleotide loop and a 4 bp stem as well as the binding properties of the parent aptamer (*28*). A chemically modified analogue, LD06$_{16}$, was then prepared in which the two base pairs at the bottom of the stem and four loop nucleotides (T6, C8, A10, and G11) were introduced as LNA residues in a DNA context (Figure 2A). Such chimeric oligonucleotides were previously shown to adopt A-type helix geometry (*25*, *27*) and are, therefore, excellent mimics of the originally selected RNA aptamer. The chimera LD06$_{16}$ gives rise to a loop−loop complex with miniTAR (a RNA hairpin corresponding to the apical part of the TAR element) characterized by a T_m similar to that of the unmodified TAR RNA-aptamer RNA complex (*28*).

Table 1: Melting Temperature (T_m) of Aptamer and Antisense Oligonucleotides Complexed with BRU or MAL MiniTAR Variants

Ligands	Sequence 5'-3'	Tm (°C)	
		BRU	MAL
R06$_{16}$	GUC $_5$,CACG C $_3$,GUGC C AGA	30.5 ± 1.5[a]	< 10
asR$_8$	$_5$,GUCCCAGA$_3$,	20.3 ± 0.4[b]	ND[c]
LD06$_{16}$	gTc $_5$,**CA**cg **C** $_3$,**GT**gc c a**GA**	31.8 ± 0.4[a]	<10
asLD$_8$	$_5$,g**T**c**C**c**AG**a$_3$,	32.6 ± 0.5	20.9 ± 0.9
asL$_6$	$_5$,**TCCCAG**$_3$,	44.6 ± 0.9	> 35[d]
asL$_8$	$_5$,**GTCCCAGA**$_3$,	43.8 ± 0.5	> 35[d]

[a] Capital letters represent RNA, capital letters in bold represent LNA, and lower case letters represent DNA. T_m values are the average and standard deviation of at least three independent experiments. [a] and [b] T_m values are from refs (*28*) and (*34*), respectively. [c] ND: not determined. [d] No accurate determination of the T_m value was possible because the transition corresponding to the dissociation of the complex partly overlapped the melting of the TAR stem.

Stems are crucial structural determinants of stable loop−loop complexes because they provide continuous stacking from one end of the complex to the other through the loop−loop helix (*31*−*33*). This accounts for the increased stability of the mini-TAR−R06$_{16}$ complex (T_m = 30.5 °C) over that of the one formed between miniTAR and asR$_8$, the linear RNA octamer corresponding to the R06 loop (T_m = 20.3 °C), even though six base pairs can be formed in both complexes (Table 1; (*21*, *34*)). In contrast the antisense sequence asLD$_8$ corresponding to the apical loop of the chimera LD06$_{16}$ binds as strongly to TAR as the LNA/DNA aptamer (T_m = about 32 °C). The fully LNA-modified octamer asL$_8$ is an even stronger ligand than the antisense chimera (T_m = 43.8 °C; Table 1). As expected, the terminal bases of this octamer do not contribute to binding because the antisense hexamer asL$_6$ shows the same T_m as that obtained with asL$_8$ (Table 1).

LNA/DNA Aptamer Is a More Specific TAR Binder than the Antisense Sequence. Up to this point, experiments were carried out with the BRU variant of TAR, which was used as a target for the in vitro selection (*21*). The loop of the TAR MAL variant differs by one base from TAR BRU, the U residue being substituted by a C (Figure 2A). Consequently the binding of TAR MAL to the aptamer LD06$_{16}$ or to the antisense asLD$_8$ generates an A,C mismatch. The binding of LD06$_{16}$ and asLD$_8$ oligonucleotides to either mini-TAR BRU or MAL was compared by electrophoretic mobility shift assay. The addition of miniTAR BRU to radio-labeled

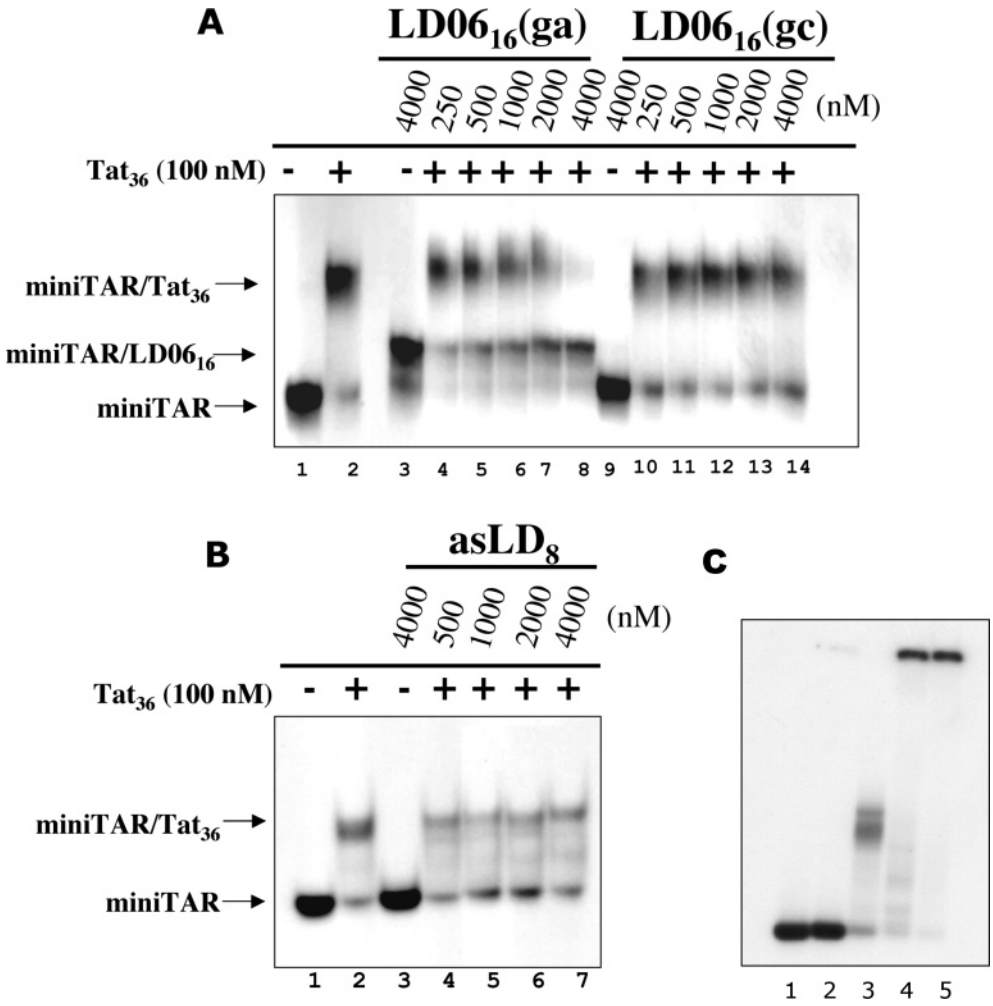

FIGURE 3: Inhibition of Tat−TAR interaction by chimeric LNA/DNA aptamers or an antisense oligomer. (A) The LD06$_{16}$ aptamer was used with either G,A (left) or G,C loop-closing deoxynucleotides (right) (see legend Figure 2). ^{32}P-labeled miniTAR BRU (2 nM) was incubated at 37 °C in the absence (lane 1) or in the presence of 100 nM Tat peptide (Tat$_{36}$; lanes 2, 4−8, and 10−14). Aptamer variants were added either to miniTAR alone (lanes 3 and 9) or to the miniTAR−Tat peptide complex at the concentrations (nM) indicated at the top of each lane. Experiments were analyzed on a 10% polyacrylamide gel in Tris-borate buffer at pH 8.3 at 20 °C containing 20 μM magnesium acetate, following incubation in R buffer as described in Materials and Methods. (B)^{32}P-labeled miniTAR (2 nM) was incubated at 37 °C in the absence (lane 1) or in the presence of 100 nM of the Tat peptide (lane 2 and 4−7). asLD$_8$ was added either to miniTAR alone (lane 3) or to the miniTAR−Tat peptide complex at the concentrations (nM) indicated at the top of each lane. Experiments were analyzed as in A. (C) ^{32}P-labeled asLD$_8$ (2 nM) was incubated at 37 °C in the absence (lane 1) or in the presence of the Tat peptide (1 μM) (lane 2). MiniTar (1 μM) was added to asLD$_8$ in the absence (lane 3) or in the presence of the Tat peptide (lane 4: 0.5 μM; lane 5: 1 μM).

LD06$_{16}$ induced the appearance of a low mobility species at a low concentration (<10 nM), whereas no retarded band was observed with TAR MAL up to 10 μM (Figure 2B, top). A quantitative analysis of the experimental results according to eq 1 led to an apparent *K*d of 22.2 ± 1.5 nM for the miniTAR BRU−LD06$_{16}$ complex. Therefore, this modified aptamer behaves as the parent RNA aptamer and discriminates between the two targets differing by a single base (*21*). In contrast with asLD$_8$, retarded bands were detected both with miniTAR BRU (apparent *K*d = 40.1 ± 3.8 nM) and with miniTAR MAL (apparent *K*d of about 2.5 μM) (Figure 2B, bottom). In addition, in this latter case, several slowly moving bands that likely correspond to different complexes are visualized (Figure 2B, bottom, left). Therefore, the antisense oligomer displays a reduced specificity of binding than the aptamer.

LNA/DNA Aptamer Inhibits the Binding of a Tat Peptide to TAR (the Antisense Oligomer Does Not). The Tat$_{36}$ peptide containing amino acids 37−72 of the Tat protein corresponds to the minimal recognition motif that binds to TAR and displays the same affinity, for both BRU and MAL viral targets, as the intact Tat protein (*35*). The effect of LNA/DNA oligomers on the Tat$_{36}$−TAR interaction was analyzed by electrophoretic mobility shift assay. To allow Tat$_{36}$ binding to TAR, the assay had to be carried out at low magnesium concentrations (20 μM) (*30*). Despite the reduced affinity of LD06$_{16}$ for TAR under these conditions compared to that under in vitro selection conditions (3 mM Mg^{2+}), a specific inhibition of Tat$_{36}$ binding to TAR was observed, and the addition of increasing amounts of LD06$_{16}$ reduced the intensity of the band corresponding to the TAR−Tat peptide complex (Figure 3A, lanes 4−7). A complete dissociation of the Tat peptide−miniTAR BRU complex was obtained with a 4 μM aptamer (lane 8), whereas a band corresponding to the miniTAR−aptamer complex was concomitantly observed. In contrast, no competition was detected with the aptamer LD06$_{16}$(gc) in which the G,A residues closing the aptamer loop were substituted by G,C (Figure 3A, lanes 10−14). This G,C variant does not bind to TAR, emphasizing the key role played by these residues

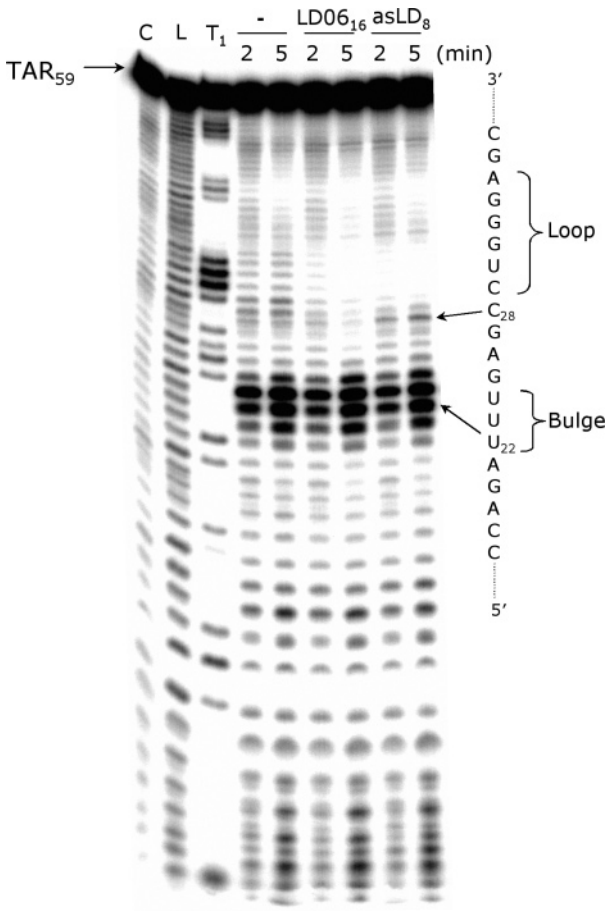

FIGURE 4: Lead(II) footprints of the complexes between TAR RNA and the chimeric LNA/DNA aptamer or antisense oligomer. [^{32}P] 5′-end-labeled TAR RNA in the absence of the ligand (−), in the presence of the aptamer $LD06_{16}$, or in the presence of the antisense oligonucleotide was incubated for 2 and 5 min with 25 mM lead-(II) acetate as described in Materials and Methods. The C and L lanes correspond to the untreated TAR RNA and the alkaline ladder, respectively. The T_1 lane corresponds to the TAR RNA digested with RNase T_1. (RNase T1 preferentially cleaves after G residues in a single-stranded context). Part of the apical sequence of TAR is given to the right (see Figure 1).

in kissing complex formation (*28*). The Tat_{36} peptide does bind to the MAL variant of TAR, but no displacement of this peptide from miniTAR MAL was induced by $LD06_{16}$ (data not shown). Because this oligomer is a poor TAR MAL ligand (see above), this indicates that the competition between the aptamer and Tat_{36} for binding to TAR BRU is directly related to the $LD06_{16}$−TAR complex formation.

We then carried out a similar experiment with $asLD_8$, which is as strong a TAR ligand as $LD06_{16}$ (Table 1 and Figure 2). However, the miniTAR−Tat_{36} complex was not out competed by this LNA/DNA antisense chimera, that is, no dissociation of the complex was detected even in the presence of 4 μM oligonucleotide (Figure 3B, lanes 4−7). It should be noted that no retarded band was detected upon addition of $asLD_8$ to miniTAR BRU (Figure 3B, lane 3). This is not related to the absence of interaction between the two partners under these experimental conditions. This should rather be ascribed to the low difference in molecular weight between the complex and miniTAR, which is not resolved on this gel. Indeed, under similar electrophoresis conditions, a miniTAR−$asLD_8$ complex was detected when the oligonucleotide $asLD_8$ was radiolabeled instead of miniTAR (Figure 3C, lane 3). Moreover, the addition of the Tat peptide to the miniTAR−$asLD_8$ complex resulted in the appearance of a species of reduced mobility, likely corresponding to the formation of a Tat−miniTAR−$asLD_8$ ternary complex (Figure 3C, lanes 4−5).

Footprinting of TAR/Oligonucleotide Complexes. The above results for the complexes with the aptamer $LD06_{16}$ on the one hand and with the antisense $asLD_8$ on the other hand suggest a different conformation of TAR BRU in the two cases. We performed footprints on the two complexes, using lead(II) as the reagent (*36*). In the absence of any added ligand, strong cleavage sites were observed in the TAR bulge (Figure 4). Weak non specific cleavage sites were observed at every other position of the TAR hairpin under the conditions used for the experiment. In the presence of either $LD06_{16}$ or $asLD_8$, we observed a generally reduced sensitivity to Pb^{2+} in the loop region. But on the 5′ side of the TAR loop, the C28 position was protected in the presence of the aptamer, whereas it was sensitized in the presence of the antisense octamer (Figure 4). No difference was detected in the bulge, indicating that the binding of the LNA/DNA oligomers does not alter its conformation.

DISCUSSION

Locked nucleic acid nucleotides were used to generate a modified version of the RNA kissing aptamer R06, previously obtained through in vitro selection against the TAR RNA element of HIV-1 (*21*). The 2′-*O* → 4′-*C*-methylene linkage that locks the ribose ring in a C3′-endo conformation (N-type) provides LNA antisense oligomers with a very high affinity for complementary RNA compared to that for antisense RNA (*26*). $LD06_{16}$, a LNA/DNA chimera derived from a truncated R06 aptamer, generates a complex with TAR as stable as the one obtained with the parent RNA aptamer (*28*). The six central bases of the R06 loop, 5′GUCCCAGA3′, are complementary to the TAR loop. This RNA octamer is a poor TAR ligand compared to the aptamer, demonstrating that the stem of the RNA aptamer contributes to the complex formation (*34*). In contrast, there is no difference in the thermostability of the complexes formed between TAR and either the aptamer $LD06_{16}$ or the antisense octamer $asLD_8$. Therefore, the association between this antisense 8-mer and the RNA target follows the rules previously reported for LNA−RNA duplexes (i.e., LNA residues are responsible for an increased affinity compared to the antisense RNA octamer), whereas no benefit is brought by the chemical modification in the aptamer context (*26*). This means that the loop−loop helix in the kissing complex cannot assimilate to a canonical A-type double helix. This is even more clearly demonstrated with the fully modified LNA hexamers and octamers that are characterized by a T_m of about 44 °C (Table 1), whereas the LNA homologue of R06 does not form a detectable complex under the same conditions (*28*). This conformational difference of the TAR complexes suggests that different properties might be expected for the two types of ligands: antisense on the one hand and aptamer on the other. Indeed, even though $LD06_{16}$ and $asLD_8$ bind to their target with a similar affinity, we demonstrated that they display fundamental differences with respect to specificity of target recognition and competition with Tat.

The specificity of binding is a key issue for strategies, making use of complementary oligonucleotides, which aimed at inhibiting the expression of a target gene. The length of the sequence is the first parameter driving the specificity of the effects induced by antisense oligonucleotides. The minimal length for ensuring the unicity of a target in the human genome ranges from 15 to 19 nucleotides, depending on the G,C content (*37*). On the one hand, long sequences increase the probability of generating mismatched hybrids with nontarget sequences stable enough under the experimental conditions, thus inducing nonintended effects. On the other hand, short sequences might have more than one matched target site and consequently inhibit several genes. This trend is exacerbated when using oligomer derivatives of high affinity as previously demonstrated for the 2′-*O*-methyl/DNA chimeric antisense oligomers. The increased affinity of such oligonucleotides compared to those of methylphosphonate/DNA chimeras resulted in both increased antisense effects and decreased specificities (*14*). The results obtained with the MAL variant of TAR that differs from BRU essentially by a U to C substitution in the loop and the octamer $asLD_8$ are relevant. $asLD_8$ forms a very stable complex with the matched viral RNA target, but it also binds with a significant affinity to the mismatched one. The presence of an internal A,C mismatch out of 6 base pairs does not prevent its association to a nontarget site. Moreover, multiple complexes are formed (Figure 2B). Although, we did not attempt to ascribe any of the multiple bands seen on the electrophoresis gel with the $asLD_8$/miniTAR mixture, some complex might involve an interaction of the antisense octamer with one strand of the TAR. Indeed, the oligomer is partly complementary to the 5′ end of the TAR RNA; the strong affinity of this LNA/DNA chimera likely allows both the invasion of the double-stranded stem and the formation of a stable mismatched duplex. Several octamers could bind simultaneously to a single target, leading to the complicated pattern seen on Figure 2B. This low specificity of antisense sequences is expected to be more important when the length of the oligonucleotide or the LNA content of the oligomer is increased. Indeed the T_m of the mismatched complex between TAR MAL and the antisense asL_8 is higher than that of the matched complex between TAR BRU and the aptamer $LD06_{16}$. Footprints with the LNA hexamer asL_6 actually revealed interactions with the stem of TAR (not shown). Although no data is available, it is likely that chemically modified antisense oligomers of increased affinity targeted to the top part of TAR used in previous studies will display a similar or even higher lack of specificity (*38*).

In contrast the aptamer $LD06_{16}$, which contains the $asLD_8$ sequence, binds to the BRU, not to the MAL variant of TAR. In addition, a single band is observed on the electrophoretic gel (Figure 2B). This means that the partially complementary regions in the stem, which are putative binding sites for the antisense $asLD_8$ are not recognized by the aptamer. Therefore, hairpin aptamers discriminate matched from mismatched targets not only on the basis of sequence complementarity but also on the basis of its structure. This definitively provides aptamers with a clear benefit over antisense oligomers.

A different structure of the duplex between the TAR loop and the complementary sequence of the aptamer and of the antisense oligomer was revealed by lead footprints. Generally two kinds of RNA-induced cleavage can be detected on RNA: weak unspecific cleavages due to free lead ions and strong specific ones due to ions coordinated to the RNA. Strong cleavage sites were observed at the level of the TAR bulge, in reasonable agreement with previous results; this bulge was suggested to be a binding site for calcium, magnesium, and lead ions (*39*). The only changes in the lead-induced cleavage pattern in the presence of oligonucleotides were located in the TAR loop. A difference was observed between aptamer and antisense complexes on the 5′ side of the TAR loop. This suggests a slightly different structural organization at the junction between the TAR upper stem and the duplex involving the loop. Pseudo-half knot formation between hairpin loop and antisense sequences have been described in which the heteroduplex is stacked on the 3′ strand of the hairpin stem (*11*). This is structurally different from the compact complex depicted for RNA/RNA kissing complexes (*31*−*33*). Surprisingly, no cleavage site was observed in the loop−loop helix, even though the kissing complex was reported to accommodate two magnesium ions (*31*−*33*).

The TAR RNA element contributes to the regulation of the HIV-1 genome transcription through the interaction with several proteins, including the retroviral trans-activator protein Tat. Our results demonstrate that $LD06_{16}$ specifically interferes with the binding of a Tat peptide to TAR, an effect reminiscent of what was previously obtained with the N3′-P5′ phosphoramidate R06 aptamer derivative (*30*). Because the aptamer recognizes a site (the apical loop) that does not overlap the Tat binding site (the bulge region), this suggests that the aptamer switches the TAR element to a conformation no longer appropriate for Tat binding. However, we did not detect any modification of the lead-induced cleavage pattern in the bulge upon aptamer binding. It is known that Tat binding leads to a major reorganization of the bulge region (*40*, *41*). Alternatively, the structure of the TAR element in the TAR/$LD06_{16}$ complex might prevent the transition from the free to the Tat-bound state.

The antisense $asLD_8$ does not displace the Tat/TAR complex. The different behavior of antisense $asLD_8$ and aptamer $LD06_{16}$ with respect to the competition with the Tat_{36} peptide for TAR binding is likely not related to the structural difference on the 5′ side of the TAR loop because the Tat recognition site is not known to extend to the top part of the TAR upper stem. The difference might originate in the kinetic properties of the complexes: the aptamer/TAR complex is much longer lived than the antisense/TAR complex ($k_{off} = 8.2$ and 45×10^{-3} s^{-1}, respectively; (*28*)). NMR investigations are under way for analyzing the structural differences between various oligonucleotide−TAR complexes.

ACKNOWLEDGMENT

We are grateful to Cedric Belair for helpful technical assistance.

REFERENCES

1. Vickers, T. A., Wyatt, J. R., and Freier, S. M. (2000) Effects of RNA secondary structure on cellular antisense activity, *Nucleic. Acids Res. 28*, 1340−1347.

2. Vickers, T. A., Koo, S., Bennett, C. F., Crooke, S. T., Dean, N. M., and Baker, B. F. (2003) Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis, *J. Biol. Chem. 278*, 7108−7118.
3. Berkhout, B. (2000) Multiple biological roles associated with the repeat (R) region of the HIV-1 RNA genome, *Adv. Pharmacol. 48*, 29−73.
4. Berkhout, B., Vastenhouw, N. L., Klasens, B. I., and Huthoff, H. (2001) Structural features in the HIV-1 repeat region facilitate strand transfer during reverse transcription, *RNA 7*, 1097−1114.
5. Blight, K. J., and Rice, C. M. (1997) Secondary structure determination of the conserved 98-base sequence at the 3′ terminus of hepatitis C virus genome RNA, *J. Virol. 71*, 7345−7352.
6. Kim, C. H., and Tinoco, I., Jr. (2000) A retroviral RNA kissing complex containing only two G.C base pairs, *Proc. Natl. Acad. Sci. U.S.A. 97*, 9396−9401.
7. Paillart, J. C., Skripkin, E., Ehresmann, B., Ehresmann, C., and Marquet, R. (1996) A loop-loop "kissing" complex is the essential part of the dimer linkage of genomic HIV-1 RNA, *Proc. Natl. Acad. Sci. U.S.A. 93*, 5572−5577.
8. Reynolds, J. E., Kaminski, A., Carroll, A. R., Clarke, B. E., Rowlands, D. J., and Jackson, R. J. (1996) Internal initiation of translation of hepatitis C virus RNA: the ribosome entry site is at the authentic initiation codon, *RNA 2*, 867−878.
9. Toulmé, J.-J., Di Primo, C., and Moreau, S. (2001) Modulation of RNA function by oligonucleotides recognizing RNA structure, *Prog. Nucleic Acid Res. Mol. Biol. 69*, 1−46.
10. Toulmé, J. J., Darfeuille, F., Kolb, G., Chabas, S., and Staedel, C. (2003) Modulating viral gene expression by aptamers to RNA structures, *Biol. Cell 95*, 229−238.
11. Ecker, D. J., Vickers, T. A., Bruice, T. W., Freier, S. M., Jenison, R. D., Manoharan, M., and Zounes, M. (1992) Pseudo-half-knot formation with RNA, *Science 257*, 958−961.
12. Compagno, D., Lampe, J. N., Kutyavin, I. V., Yurchenko, L., Lukhtanov, E. A., Bourget, C., Gorn, V., Gamper, H. B., and Toulmé, J. J. (1999) Antisense oligonucleotides containing modified bases inhibit translation of Leishmania amazonensis mRNAs by invading the mini-exon hairpin, *J. Biol. Chem. 274*, 8191−8198.
13. Herschlag, D. (1991) Implications of ribozyme kinetics for targeting the cleavage of specific RNA molecules in vivo: More isn't always better, *Proc. Natl. Acad. Sci. U.S.A. 88*, 6921−6925.
14. Larrouy, B., Boiziau, C., Sproat, B., and Toulmé, J. J. (1995) High affinity or selectivity: a dilemma to design antisense oligodeoxynucleotides acting in RNase H-containing media, *Nucleic Acids Res. 23*, 3434−3440.
15. Brossalina, E., and Toulmé, J. J. (1993) A DNA hairpin as a target for antisense oligonucleotides, *J. Am. Chem. Soc. 115*, 796−797.
16. François, J. C., and Hélène, C. (1995) Recognition and cleavage of single-stranded DNA containing hairpin structures by oligonucleotides forming both watson-crick and hoogsteen hydrogen bonds, *Biochemistry 34*, 65−72.
17. Le Tinévez, R., Mishra, R. K., and Toulmé, J. J. (1998) Selective inhibition of cell-free translation by oligonucleotides targeted to a mRNA structure, *Nucleic Acids Res. 26*, 2273−2278.
18. Mishra, R. K., Le Tinévez, R., and Toulmé, J. J. (1996) Targeting nucleic acid secondary structures by antisense oligonucleotides designed through in vitro selection, *Proc. Natl. Acad. Sci. U.S.A. 93*, 10679−10684.
19. Aldaz-Carroll, L., Tallet, B., Dausse, E., Yurchenko, L., and Toulmé, J. J. (2002) Apical loop-internal loop interactions: a new RNA-RNA recognition motif identified through in vitro selection against RNA hairpins of the hepatitis C virus mRNA, *Biochemistry 41*, 5883−5893.
20. Boiziau, C., Dausse, E., Yurchenko, L., and Toulmé, J. J. (1999) DNA aptamers selected against the HIV-1 TAR RNA element form RNA/DNA kissing complexes, *J. Biol. Chem. 274*, 12730−12737.
21. Ducongé, F., and Toulmé, J. J. (1999) In vitro selection identifies key determinants for loop-loop interactions: RNA aptamers selective for the TAR RNA element of HIV-1, *RNA 5*, 1605−1614.
22. Karn, J. (1999) Tackling Tat, *J. Mol. Biol. 293*, 235−254.
23. Rana, T. M., and Jeang, K. T. (1999) Biochemical and functional interactions between HIV-1 Tat protein and TAR RNA, *Arch. Biochem. Biophys. 365*, 175−185.
24. Elayadi, A. N., and Corey, D. R. (2001) Application of PNA and LNA oligomers to chemotherapy, *Curr. Opin. Invest. Drugs 2*, 558−561.
25. Petersen, M., Nielsen, C. B., Nielsen, K. E., Jensen, G. A., Bondensgaard, K., Singh, S. K., Rajwanshi, V. K., Koshkin, A. A., Dahl, B. M., Wengel, J., and Jacobsen, J. P. (2000) The conformations of locked nucleic acids (LNA), *J. Mol. Recognit. 13*, 44−53.
26. Orum, H., and Wengel, J. (2001) Locked nucleic acids: a promising molecular family for gene-function analysis and antisense drug development. *Curr. Opin. Mol. Ther. 3*, 239−243.
27. Bondensgaard, K., Petersen, M., Singh, S. K., Rajwanshi, V. K., Kumar, R., Wengel, J., and Jacobsen, J. P. (2000) Structural studies of LNA:RNA duplexes by NMR: Conformations and implications for RNase H activity, *Chemistry 6*, 2687−2695.
28. Darfeuille, F., Hansen, J. B., Orum, H., Di Primo, C., and Toulmé, J. J. (2004) LNA/DNA chimeric oligomers mimic RNA aptamers targeted to the TAR RNA element of HIV-1, *Nucleic Acids Res. 32*, 3101−3107.
29. Koshkin, A., Nielsen, P., Mledgaard, M., Rajwanshi, V. K., Singh, S., and Wengel, J. (2002) LNA (Locked Nucleic Acids): an RNA mimic forming exeedingly stable LNA:LNA duplexes, *J. Am. Chem. Soc. 120*, 13252−13253.
30. Darfeuille, F., Arzumanov, A., Gryaznov, S., Gait, M. J., Di Primo, C., and Toulmé, J. J. (2002) Loop-loop interaction of HIV-1 TAR RNA with N3′→P5′ deoxyphosphoramidate aptamers inhibits in vitro Tat-mediated transcription, *Proc. Natl. Acad. Sci. U.S.A. 99*, 9709−9714.
31. Beaurain, F., Di Primo, C., Toulmé, J. J., and Laguerre, M. (2003) Molecular dynamics reveals the stabilizing role of loop closing residues in kissing interactions: comparison between TAR-TAR* and TAR-aptamer, *Nucleic Acids Res. 31*, 4275−4284.
32. Chang, K. Y., and Tinoco, I. (1997) The structure of an RNA "kissing" hairpin complex of the HIV TAR hairpin loop and its complement, *J. Mol. Biol. 269*, 52−66.
33. Lee, A. J., and Crothers, D. M. (1998) The solution structure of an RNA loop-loop complex: the ColE1 inverted loop sequence, *Structure 6*, 993−1005.
34. Ducongé, F., Di Primo, C., and Toulmé, J. J. (2000) Is a closing "GA pair" a rule for stable loop-loop RNA complexes? *J. Biol. Chem. 275*, 21287−21294.
35. Churcher, M. J., Lamont, C., Hamy, F., Dingwall, C., Green, S. M., Lowe, A. D., Butler, J. G., Gait, M., and Karn, J. (1993) High affinity binding of TAR RNA by the human immunodeficiency virus type-1 tat protein requires base-pairs in the RNA stem and amino acid residues flanking the basic region, *J. Mol. Biol. 230*, 90−110.
36. Lindell, M., Romby, P., and Wagner, E. G. (2002) Lead(II) as a probe for investigating RNA structure in vivo, *RNA 8*, 534−541.
37. Hélène, C., and Toulmé, J. J. (1990) Specific regulation of gene expression by antisense, sense and antigene nucleic acids, *Biochim. Biophys. Acta 1049*, 99−125.
38. Arzumanov, A., Walsh, A. P., Rajwanshi, V. K., Kumar, R., Wengel, J., and Gait, M. J. (2001) Inhibition of HIV-1 Tat-dependent trans activation by steric block chimeric 2′−O-methyl/LNA oligoribonucleotides, *Biochemistry 40*, 14645−14654.
39. Olejniczak, M., Gdaniec, Z., Fischer, A., Grabarkiewicz, T., Bielecki, L., and Adamiak, R. W. (2002) The bulge region of HIV-1 TAR RNA binds metal ions in solution, *Nucleic Acids Res. 30*, 4241−4249.
40. Aboul-Ela, F., Karn, J., and Varani, G. (1996) Structure of HIV-1 TAR RNA in the absence of ligands reveals a novel conformation of the trinucleotide bulge, *Nucleic Acids Res. 24*, 3974−3981.
41. Du, Z., Lind, K. E., and James, T. L. (2002) Structure of TAR RNA complexed with a Tat-TAR interaction nanomolar inhibitor that was identified by computational screening, *Chem. Biol. 9*, 707−712.

BI0606344