We investigated the binding of an antisense oligodeoxynucleotide to a stem-loop structure corresponding to the mini-exon sequence of the protozoan parasite *Leishmania amazonensis*. This oligomer was designed to anneal to the single-stranded region adjacent to the bottom of the hairpin and to fold back on itself, giving rise to a "double-hairpin" complex that involved a local triple. This imposed the recognition, by the third strand, of a "purine" strand containing 6 interspersed pyrimidines out of 15 nucleic acid bases. The sequence of the complementary oligonucleotide was derived from the so-called pyrimidine motif; the third strand of the anti-mini-exon oligomer was parallel to the purine strand of the target. Electrophoretic mobility shift assays and footprinting studies demonstrated that such an antisense oligomer was able to bind to both the DNA and RNA versions of the *Leishmania* hairpin. These double hairpin complexes allowed the formation at pH 6.0 of a triple-stranded structure, despite the presence of 4 A:T*G and 2 G:C*T triplets out of 15.

The common feature of messenger RNAs from trypanosomatids is the presence at their 5’ end of a short sequence named “mini-exon” acquired during maturation of premessenger RNAs through a trans-splicing mechanism (1). This mini-exon motif, which is absolutely required for translation, is therefore a very attractive target for the design of antisense oligonucleotides as anti-parasitic agents (2). Potentially, a single complementary sequence will prevent the synthesis of all parasitic proteins. Indeed, oligonucleotides targeting the mini-exon sequence of *Trypanosoma brucei* (3, 4) or of *Leishmania amazonensis* (5) were shown to inhibit *in vitro* translation in cell-free extracts. Moreover, an acidine-linked 9-mer, complementary to the 5’ end of *T. brucei* mRNAs specifically killed cultured procyclic forms of this parasite *in vitro* (6). More recently, it was reported that an anti-mini-exon phosphorothioate 16-mer, either free or associated to low density lipoproteins, displayed leishmanicidal properties against amastigotes of *L. amazonensis* grown in murine macrophages (7, 8).

It was previously shown that the mini-exon sequence of *L. amazonensis* could fold into a hairpin secondary structure (9), which weakened the binding of antisense oligonucleotides (10). Rather than competing with the intramolecular hairpin, we considered the possibility to bind an oligomer to the folded structure. This can be achieved in different ways: (i) binding to the stem via a triple-stranded structure (11) or to the loop, leading to half pseudoknot structure (12), (ii) using an oligonucleotide that bridges the single-stranded parts, upstream and downstream of the stem (13, 14), or (iii) selecting oligomers from a random population that recognize the folded target (15–17). The resulting complexes might actually stabilize the hairpin and interfere with mRNA translation.

Alternatively, we previously described a strategy that allows accommodation of a stem-loop structure into a so-called "double hairpin" complex (18, 19). The antisense oligonucleotide forms a short Watson-Crick duplex with a single-stranded sequence at the bottom of the hairpin and then folds back to give rise to a triple-stranded structure with both this short duplex and the stem of the hairpin. This approach was demonstrated using a model stem-loop purposefully designed to promote a triple helical structure; an antisense oligopurine was targeted to a hairpin made exclusively of purines on the 5’ side and consequently of pyrimidines on the 3’ side, thus leading to the formation of canonical T:A*T and C:G*C triplets (where the colon denotes Watson-Crick base pairing and the asterisk denotes Hoogsteen hydrogen bonding with the third strand). The resulting complex involved 16-base triplets in which the two pyrimidine strands were connected by a (T) loop.

The formation of triple helices is restricted to homopurine homopyrimidine sequences (2). Unfortunately, nucleic acid bases are not appropriately distributed for triple formation in the hairpin derived from the *L. amazonensis* mini-exon. Therefore we had to design an oligonucleotide that aimed at forming a double hairpin complex involving a triple-stranded structure with a target sequence comprising all four bases. In particular two Cs of two G-C pairs should be read by the third strand. We demonstrated that an antisense oligomer composed of the four bases was able to bind to either a DNA or a RNA folded hairpin corresponding to the *L. amazonensis* mini-exon sequence through the formation of G:C*T and A:U*T triplets, in addition to the canonical ones.

**MATERIALS AND METHODS**

**Oligonucleotide Synthesis**—The oligonucleotides used throughout this study (see sequences in Fig. 1) were prepared “trityl on” using conventional phosphoramidite chemistry. They were purified in one step by reverse phase high pressure liquid chromatography; an acetonitrile gradient in a 100 mM triethylammonium acetate buffer (pH 7.0) was used for elution. Purity was evaluated by electrophoresis of radio-labeled oligonucleotides on a 20% polyacrylamide gel containing 7 M urea, using [γ-32P]ATP (37.5 MBq/mm) from DuPont NEN. The oligoribonucleotide 35rLa was prepared by *in vitro* transcription with T7 RNA polymerase as described previously (20).

**Electrophoretic Mobility Shift Assay**—Radio-labeled mini-exon oligonucleotide 35rLa or 35rLa (0.1 μM) was incubated with a complementary oligonucleotide (2 μM) for 24 h at 4 °C in a 50 mM sodium acetate (pH 6.0), containing 10 mM magnesium acetate. The samples were then mixed (v/v) with the dye solution (10% glycerol, 0.05% bromophenol blue,**

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Fig. 1. Sequences of the mini-exon from *L. amazonensis* (top) and of complementary oligonucleotides. The target was prepared as DNA (35La) or RNA (35rLa). The anti-mini-exon 29DE was folded to show the anchor region complementary to the single-stranded sequence of the target and the putative third strand. Controls oligomers 10Cont and 29Cont are given below. Nucleotides in the target are numbered with roman numerals. The anchor region (antisense sequences) and the anchor binding site (target) are underlined.

Fig. 2. Electrophoretic mobility shift assays of hairpin-antisense oligonucleotide complexes with 35La (a) or 35rLa (b). The targets were run in the absence or in the presence of the oligonucleotide indicated at the top of the lanes. The position of linear (L), folded (F), and bound targets (C1, C2, and C3) are marked. Electrophoresis was performed at pH 6.0 on a 15% polyacrylamide gel.
labeled either on the target (35La) or on the anti-mini-exon strand (29DE). Footprinting performed with potassium permanganate showed a high reactivity of thymines 9–15 of 29DE in the complex, compared with 29DE alone (Fig. 3a). In contrast, T residues from positions 18–26 were significantly less susceptible to modification. This indicates that the 3' part of the oligonucleotide 29DE is protected through interaction with the target, whereas the T stretch is highly accessible. This might correspond to a loop structure, as confirmed by S1 mapping. Whereas this nuclease specific for single-stranded nucleic acids gave an all or nothing pattern of cleavage for 29DE alone, this oligonucleotide was specifically cleaved in the region corresponding to nucleotides 12–15 in the presence of 35La, i.e. in the T stretch that was highly reactive to KMnO₄ (Fig. 3b). A similar experiment performed with the oligomer 29Cont did not show such a pattern; however, a slightly increased sensitivity of residues located 3' to the anchor region was observed, indicative of a weak 35La-29Cont complex, in agreement with melting experiments (Fig. 3c).

Drastic changes of the KMnO₄ reactivity pattern were also observed for 35La upon addition of 29DE (Fig. 4). A reduced sensitivity of the T residues was generally observed in the anchor region; whereas Tᵥ and TᵥⅧ were fully protected, T₇ was still available for the reaction. This latter residue faced the 5’-terminal nucleotide of the anti-mini-exon oligomer 29DE. This means that the 10-base pair duplex was actually formed in the 35La-29DE complex, but despite potential cooperative interaction between the stem structure of 35La and the double-stranded anchor site, the junction was prone to transient opening. The reactivity pattern of T residues located in the stem of 35La was also informative; TₓⅦⅧ was protected, whereas the sensitivity of TₓⅦⅥ was exacerbated (Fig. 4). Assuming that the 3’ part of 29DE constitutes the third strand of a triple-stranded complex, TₓⅦⅧ could be next to the triple strand-double strand junction. A previous study (18) performed with a model sequence, designed to generate a double hairpin complex, has shown hyper-reactivity of the G residue located at the duplex-triplex junction, indicative of a distorted structure. A similar effect was also described for a linear triplex made from three independent strands (25, 26). In the present case the junction coinciding with a bulge might potentiate the conformation change. An additional effect could also contribute as discussed below for 10Cont.

The oligomer 10Cont, able to form a perfect duplex in the anchor region of 35La, yielded a different pattern of cleavage. As expected, this oligonucleotide protected Tᵥ and TᵥⅧ, (slightly less than 29DE), whereas Tₓ pairing with the 5’-terminal A of 10Cont was still reactive (Fig. 4). But TₓⅦⅥ, located in the stem remained accessible, in contrast to what was observed in the presence of 29DE. Surprisingly, TₓⅦⅥ was more reactive in the presence than in the absence of 10Cont, although it is located 6nt away from the 5’ end of the antimiexon oligomer (Fig. 4). This might suggest a conformation change of the target hairpin upon hybridization of the。

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**Fig. 3. Footprinting of 35La anti-mini-exon oligonucleotide complexes.**

α and b or 29Cont (c) were incubated at pH 6.0 with KMnO₄ (α) or S1 nuclease (b and c) under the conditions indicated under “Materials and Methods,” either in the absence (−) or in the presence of 35La (+). The sequence of the oligomers is indicated to the side of the panels. The left lane of a corresponds to the untreated labeled oligomer 29DE. Samples were analyzed on a 20% polyacrylamide gel containing 7 M urea.

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|   | a | b | c |
|---|---|---|---|
| 29DE | + | + | + |
| 29Cont | - | - | - |
| 35La | - | - | + |
10-mer, as observed for other complexes involving stem-loop structures (53).

S1 mapping of the target also revealed conformational changes upon binding of 29DE. In the absence of any added oligomer, 35La was cleaved by S1 nuclease at TXX, i.e. in the loop, and at TXXVI, which faces the bulge (Fig. 5). Both sites were protected in the presence of 29DE. Interestingly, the addition of 10Cont, which enhanced the reactivity of the bulged T to KMnO4, did not change the S1 sensitivity of the opposite strand at TXXVI. Neither was any change seen at TXX (Fig. 5). Therefore, 35La-29DE complex involves definitely more than the formation of 10 base pairs in the anchor domain. From the footprinting assays whose results are summarized in Scheme 1, it can be described as a double hairpin structure similar to the one reported previously for a model sequence (18); the 5’ end of 29DE is paired with the single-stranded region of 35La located at the bottom of the stem, whereas the 3’ moiety of the anti-mini-exon sequence forms the third strand of a triple-stranded structure, the T residues 11–14 being a connecting loop.

**Binding of 29DE to an RNA Target**—Because in the antisense strategy RNA is the physiological target of complementary oligonucleotides, it was of interest to monitor the binding of 29DE to 35rLa, an RNA hairpin homologous to the 35La DNA. We first studied the formation of 35rLa-29DE complex by band shift assay on a nondenaturing polyacrylamide gel. The RNA hairpin migrates as two bands, the most prominent one corresponding to the folded form of 35rLa (Fig. 2b). A weak band also appeared above the one corresponding to the linear 35rLa, suggesting the formation of a second type of complex that was not detected with the DNA target. No retarded bands were observed with either 10Cont or 29Cont (not shown). Therefore, as for the DNA target, the interaction between 35rLa and 29DE extends beyond a 10-base
The 10-mer, which forms a regular double-stranded heteroduplex, induced the cleavage of the RNA hairpin from Cv to Tx, the complexes formed by 35rLa with either 10Cont or 29DE, indicating that the 3' part of 29DE protected partially 35rLa and was not possible to recognize every double-stranded sequence even though the use of modified nucleic acid bases (32, 33), intercalating agent-oligonucleotide conjugates (34, 35), triplex dimerization (36) or “strand switching” (37, 38) extends the number of sequences that can give rise to stable triple-stranded structures.

We recently developed a new approach that aimed at recognizing hairpin loop structures via the formation of double-hairpin complexes (18, 19). Such complexes involve a local triple-stranded structure between the folded target and the antisense sequence, as demonstrated with a model sequence corresponding to the pyrimidine motif. As a contribution to the extension of the triplet alphabet, we investigated the formation of a double hairpin complex in which the purine strand of a target duplex contained 6 pyrimidines out of 15 bases. Systematic investigation of all possible triplet combinations had shown that for the DNA pyrimidine motif, inverted TA and GC pairs can be read by G and T or C, respectively, in the third strand (21–24). The optimal residual to recognize an inverted pair may vary with the surrounding bases; however, our antisense sequence was designed according to these previous reports to minimize the destabilization induced by a mismatched triplet, leading to the potential formation of T:A*T, G:C*T, and A:T*G triplets. Although these triplets are not isomorphous, our results summarized in Scheme 1 unambiguously showed that a double hairpin complex involving a nonperfect triple-stranded complex was able to form at pH 6.0 with a DNA hairpin.

Triple helix formation by oligonucleotides containing T, C, and G has been previously described for an oligoguanine-oligopyrimidine target (39). But this is to our knowledge the first time that a triple helix-forming oligomer allows accommodation of all four bases pairs in a complex with a significant stability. This should be related in part to the entropic contribution resulting from the linkage between the Hoogsteen and one Watson-Crick strand, as previously demonstrated with circular or clamp oligonucleotides (40, 41). The stability of such complexes could be further increased by the use of either mod-

**Fig. 6. Cleavage of 35rLa anti-mini-exon oligonucleotide complexes by E. coli RNase H.** Incubation was performed either at pH 6.0 (a) or at pH 7.3 (b) in the absence or in the presence of oligonucleotides 29DE or 10Cont, as indicated at the top of the lanes. The 35rLa sequence is given to the right.

1 R. Le Tinévez and J.-J. Toulmé, unpublished observations.
Antisense Oligonucleotides Targeted to DNA or RNA Hairpins

... selective targeting of RNA hairpins. This offers the possibility of blocking biological processes by promoting the formation of unstable polydT*polyrA:polydT, 6-diamidino-2-phenylindole, which have been shown to be native to the use of intercalating agents like berenil, ethidium, and...
Double Hairpin Complexes Allow Accommodation of All Four Base Pairs in Triple Helices Containing Both DNA and RNA Strands
Emanuelle Pascolo and Jean-Jacques Toumlé

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