Triplex-forming oligonucleotides trigger conformation changes of a target hairpin sequence

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ABSTRACT

We used a DNA duplex formed between the 5′ end of a 69mer (69T) and an 11mer (OL7) as a substrate for BamHI. The former oligonucleotide folds into a hairpin structure, the stem of which contains a stretch of pyrimidines in one strand and consequently a stretch of purines in the other strand. The oligomer 69T was used as a target for complementary oligodeoxypyrimidines made of 10 nt (OL1), 16 nt (OL5) or 26 nt (OL2) which can engage the same 10 pyrimidine–purine–pyrimidine triplets with the 69T hairpin stem. Although the binding site of OL7 did not overlap that of OL1, OL2 or OL5, the BamHI activity on 69T–OL7 complexes was drastically modified in the presence of these triplex-forming oligomers: OL1 abolished the cleavage by BamHI whereas OL5 and OL2 strongly increased it. Using footprinting assays and point-mutated oligonucleotides we demonstrated that these variations were due to different conformations of the 69T–OL7 complex induced by the binding of oligomers OL1, OL2 or OL5. Therefore, oligonucleotides can act as structural switchers, offering one additional mode for modulating gene expression.

INTRODUCTION

Antisense oligonucleotides represent an interesting means to artificially regulate gene expression. Numerous examples are available of successful use of complementary oligonucleotides to selectively decrease the synthesis of the protein encoded by the target RNA (reviewed in 1–3). Although they constitute powerful tools in molecular genetics and potential interesting prototypes for therapeutic agents (4–6), these molecules are hampered with several limitations. Biochemical stability (regular oligonucleotides are degraded by nucleases), limited uptake by live cells and availability of the target sequence, presently constitute the major hurdles for the development of efficient antisense oligomers. Chemists have provided a number of oligonucleotide analogues that display nuclease resistance and fulfill the major requirement for antisense purposes: affinity and specificity for the target sequence (7–12). Uptake has been significantly improved using oligonucleotides conjugated either to polyacations or to hydrophobic ligands or carriers, like liposomes, lipoproteins or nanospheres (13,14). Target site selection is, however, still largely an empirical process.

Although RNA are single-stranded molecules, they are known to fold into ordered structures (15–18). Intramolecular folding will restrict the availability of the sequence for binding to the complementary one, thus leading to weak antisense effects (19–22). Several RNA structures have been demonstrated to play a key role in the modulation of gene expression. For instance, iron responsive element control the translability and the lifetime of messages coding for proteins involved in iron metabolism (23). Hairpin and pseudoknot structures are part of the signal that drives ribosomal frame-shifting or readthrough in retroviral genomes (24). TAR and RRE are folded motifs required for the synthesis and the maturation of the HIV mRNAs (25). The design of oligonucleotides able to bind to such regions might allow to modulate the function mediated by these structures. A few attempts have been made to design oligomers to disrupt RNA structures (26) or to bind to single-stranded regions in their vicinity (27–29).

We recently developed strategies to take into account the three-dimensional organisation of RNA targets. Antisense oligonucleotides were either selected from pools of oligodeoxynucleotide candidates (30,31) or designed to form local triple helical complexes with hairpin motifs. In the latter case, we have demonstrated that OL2, an oligopyrimidine 26 nt long, was able to bind to 69T, a 69mer DNA which formed a stem–loop structure derived from the mini-exon sequence of the protozoan parasite Leishmania amazonensis. This led to the formation of a 69T–OL2 ‘double-hairpin’ complex involving pyrimidine–purine–pyrimidine triplets (32,33). We report here the results of further studies performed with this 69mer DNA hairpin. We show that the binding of OL2 and other complementary oligonucleotides induced drastic conformation changes of the target, ultimately leading to either an increased or a decreased activity of a restriction enzyme on a remote site of 69T.

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Figure 1. Sequences of target (T) and antisense (OL) oligodeoxynucleotides (antisense sequences are italicized and underlined). Oligomers OL1, OL2, OL4, OL5, mOL5 and OL7 are shown bound to either 69T, 51T or m69T but all three targets contain binding sites for all oligomers. OL4, OL7 and the 5′ part of OL2, form Watson–Crick base pairs whereas OL1, OL5 and the 3′ part of OL2 are the third strand of triple-stranded complexes with the target. The <i>BamHI</i> site is indicated by dotted lines. The mutated bases in m69T and in mOL5 are boxed. The names for oligonucleotides were taken from a previous study with the 69T hairpin (33).

MATERIALS AND METHODS

Oligonucleotides

Oligodeoxynucleotides were obtained from Genset (Paris) and purified by electrophoresis on a 20% polyacrylamide–7 M urea gel. 5′-end-labelling was performed with T4 polynucleotide kinase (Ozyme) and [γ-32P]ATP (37.7 MBeq/mmol) from Dupont according to standard procedures (34). The sequences of oligonucleotides used in this study are shown in Figure 1.

<i>BamHI</i> digestion

The cleavage of 69T–OL7 complex by <i>BamHI</i> (Boehringer) was performed in a 50 mM sodium acetate buffer pH 6.0 containing 10 mM MgCl₂. The <i>32P</i> 5′-end-labelled 69T (5 × 10⁻⁸ M) was incubated at 20°C with 0.3 U/ml <i>BamHI</i> in the presence of 3 × 10⁻⁵ M OL7 and of the desired antisense oligonucleotide. Samples were then analyzed on a denaturing polyacrylamide gel. For quantitation of the cleavage yield, autoradiographs were scanned with a densitometer.

Footprinting analysis

Footprinting studies were performed essentially as described in (35). The <i>32P</i> 5′-end-labelled oligonucleotide was mixed with the desired complementary sequence(s) and incubated in a 50 mM sodium acetate buffer pH 6.0 containing 10 mM MgCl₂. Following a 30 min pre-incubation at 15°C, samples were reacted for 90 min at 25°C with diethyl pyrocarbonate (10% final concentration). After addition of 1 vol 5 mM EDTA, the mixture was extracted with ethyl ether and the DNA was precipitated with ethanol. The samples were then treated (30 min at 90°C) with 1 M piperidine, precipitated twice with ethanol and dissolved in 80% formamide containing bromophenol blue and xylene cyanol (0.05% each). The oligomers were then analyzed on a polyacrylamide gel containing 7 M urea in TBE (89 mM Tris, 89 mM borate, 2 mM EDTA) buffer pH 8.3.

RESULTS AND DISCUSSION

Antisense oligonucleotides modulate the cleavage of the 69T–OL7 complex by <i>BamHI</i>

The 69mer DNA 69T (Fig. 1) used in previous studies (32,33) displays a folded structure with a 13 bp stem. The 3′ strand of the stem contains 10 contiguous pyrimidines at the bottom. Consequently, the 5′ strand is made of 10 purines, adjacent to a single-stranded six purine motif (5′-AGGGAG), used as an anchor binding site for the 5′ end of the 26mer OL2. This oligopyrimidine was designed such that its 3′ part can fold back in a triple-stranded structure involving 16 base triplets upon binding to the 69mer (Fig. 1). We previously demonstrated that a 69T–OL2 ‘double-hairpin’ complex was actually formed at pH 6.0, in the presence of Mg²⁺ ions, adjacent to a single-stranded six purine motif (5′-AGGGAG), used as an anchor binding site for the 5′ end of the 26mer OL2. This oligopyrimidine was designed such that its 3′ part can fold back in a triple-stranded structure involving 16 base triplets upon binding to the 69mer (Fig. 1). We previously demonstrated that a 69T–OL2 ‘double-hairpin’ complex was actually formed at pH 6.0, in the presence of Mg²⁺ ions, adjacent to a single-stranded six purine motif (5′-AGGGAG), used as an anchor binding site for the 5′ end of the 26mer OL2. This oligopyrimidine was designed such that its 3′ part can fold back in a triple-stranded structure involving 16 base triplets upon binding to the 69mer (Fig. 1).
OL7 is complementary to nt 1–11 of 69T whereas the 6 nt long anchor of OL2 forms Watson–Crick base pairs with the purine stretch extending from nt 12 to 17. We might have expected a reduced restriction endonuclease activity in the presence of OL2 due to the steric hindrance induced by the triple-stranded structure and the loop of the double hairpin complex, next to the BamHI site. A similar trend was observed when OL4 + OL5 were added to the double hairpin except that the anchor (5′-end) remains unbound (Fig. 1). Indeed, no effect of OL7 was seen on the DEPC footprinting of 69T–OL5 complex (Fig. 1). This 69T–OL5 complex is therefore equivalent to the perfect non-perfect triplex between the 5′- and the 3′-end of OL2, respectively. They can bind simultaneously to the target 69mer and form a structure similar to the double hairpin except that the anchor (5′-CTCCCT) and the Hoogsteen strand are not connected to each other. Of note, OL5 can engage 10 triplets with the stem of 69T in the absence of OL4. This 69T–OL5 complex is therefore equivalent to the perfect triple helix formed by OL1 with the stem of the 69T hairpin, except that the 5′ terminal part of OL5 remains unbound (Fig. 1). Surprisingly, whereas OL5 stimulated the cleavage of the 69T–OL7 duplex by BamHI more efficiently than OL2, the triple helix-forming 10mer OL1 abolished the endonuclease activity. None of the oligomers OL1, OL2, OL4 or OL5 displayed any effect on the BamHI activity on a restriction site other than the one formed by 69T and OL7. These results suggest that the binding of the different antisense oligonucleotides OL1 to OL5 interfered with the association between 69T and the 69mer DNA and/or induced overall conformation changes of the 69T–OL7 complex.

In order to elucidate the reasons for altered cleavage by BamHI, we undertook a structural analysis of the various oligonucleotide–69mer complexes by diethylpyrocarbonate (DEPC) footprinting under the conditions used for restriction digestion of the 69T–OL7 duplex. This allowed the identification of purines not involved in Watson–Crick base-pairing. The formation of 69T–OL7 complex protected both A(3), G(5), G(6) and A(7) of 69T (Fig. 3, lane 1). Protection was still observed after addition of either OL1, OL2, OL5 or OL4 + OL5, indicating that the OL7–69mer complex was stable in the presence of antisense oligonucleotides (Fig. 3, lanes 2–6). Therefore, variations in the cleavage rate did not result from changes in the association between 69T and OL7, which would have been induced by the added oligomers. In particular, the inhibition of BamHI cleavage by OL1 did not reflect a disruption of the target–OL7 antisense duplex.

The 3′ end of 69T interacts with its 5′ end

DEPC footprinting showed that the reactivity of some purines of 69T was modified upon addition of OL7 outside its binding site; in particular we observed an increased reactivity of A and G residues from positions 12 to 17 (Fig. 3, lane 2). This suggested that this region of 69T is shielded from acylation by intramolecular interactions which are disrupted by the binding of OL7. The importance of the 3′ end of 69T in the above effects was demonstrated using 51T, a 3′ truncated version of the 69mer target in which the 18 nt downstream of the hairpin stem were deleted (Fig. 1). Indeed, no effect of OL7 was seen on the DEPC footprint of 51T (not shown) indicating that the 3′ end of 69T interacts with the 12–17 region. A hypothetical folded structure with a non-perfect intramolecular duplex between the 3′ and the 5′ ends of 69T is shown in Figure 4a. This gives rise to an extended double-stranded stem, which includes the six purines of the anchor binding site, taking into account two GT pairs. Two bulges, 5 and 3 nt long, are formed on the 3′ side. Whether the G(17)–C(58) and A(16)–T(59) pairs are formed is questionable as these purines were significantly more reactive than the three contiguous Gs at positions 13–15.

Non-perfect triplex is involved in the OL1–69T–OL7 complex

According to the model shown in Figure 4a, the binding of OL7 to 69T would only disrupt the terminal T(11)–A(68) pair. It is unlikely that the dramatic changes in the DEPC sensitivity of purines in the 12–17 region (Fig. 3, lane 2) result from the opening of a single base pair. It should rather be due to the formation of a new structure involving both OL7 and the 3′ end of 69T as no such change was seen with the shortened target 51T (not shown). Addition of OL1 to the 69T–OL7 complex resulted in a still higher modification of purines 12–17 of 69T (Fig. 3, lane 3) while no change was observed with OL1 in the absence of OL7 (not shown).
abolished the inhibitory effect of OL1 (Fig. 2b).

Second, the proposed structure for the OL1–69T–OL7 complex belongs to the pyrimidine–purine–pyrimidine motif, i.e. a pyrimidine third strand interacts with the purine strand of a double helix via the formation of Hoogsteen hydrogen bonds. Such a triple helix is not only dependent on the sequence, but also on pH as C–G.C+ triplets are more stable at acidic pH (39). Indeed, the above effects are no longer seen at neutral pH (not shown). Third, according to the model given in Figure 4b, the sequence of the S2–69 region in 69T is critical for the structure. In order to prove the role played by the nucleotides engaged in the non-perfect triplex we prepared m69T, a mutated 69T in which both C(58) and C(65) were substituted by A (Fig. 1). This oligomer behaved similarly to the truncated target 51T with respect to cleavage by BamHI and to DEPC footprinting (not shown). Therefore, residues C(58) and C(65) are crucial for the formation of a structure which prevents BamHI activity in the presence of OL1. This is a strong support to the model depicted in Scheme 1.

This structure also explains the effects observed in the presence of OL2. The formation of the 69T–OL2 double hairpin complex involves the 12–17 region in a triple strand which will move T(11) away from A(18), likely disrupting the non-perfect triplex formed by the 3′ end of 69T (Fig. 4b). As a consequence, the BamHI site of the 69T–OL7 duplex will show an increased availability. Indeed, the addition of OL2 to a 69T + OL7 mixture resulted in an increased cleavage efficiency (Fig. 2a). As expected, no such increase was seen with 51T (Fig. 2b).

### The 5′ part of OL5 interacts with 69T

The above model (Fig. 4b) did not predict the effect of OL5 on the cleavage of 69T by BamHI. As this oligomer is able to form 10 triplets with 69T we might have expected an inhibition similar to the one seen with OL1. In contrast, a considerable increase of the cleavage rate was observed (Fig. 2a). This may be explained either by electrostatic repulsion due to the 5′ non-bound part of OL5 or by an interaction of this region with the 13–17 nt sequence of the 69mer and unfolding of the non-perfect triplex.

DEPC footprinting of 69T–OL7 complexes showed a different pattern in the presence of either OL5 or OL1. In particular, the three Gs at positions 13–15 were partially protected with OL5 while A(16) and G(17) remained reactive (Fig. 3, compare lanes 3 and 5). This suggested an interaction between the 5′ part of OL5 and the G(13)–G(15) region. 32P end-labeled OL5 was probed with sodium permanganate which reacts with single-stranded thymidines. The binding of OL5 to 69T dramatically increased the reactivity of T(12) from OL5 (Fig. 5, arrow). The extent of modification of OL5 bound to 69T was further increased in the presence of OL7 but was decreased by the addition of OL4. Interestingly, A(23) of the target 69T, which faces T(12) of OL5 in the 69T–OL5 triple-stranded complex displayed a higher sensitivity to DEPC compared with 69T alone (Fig. 3, lane 5, arrow). No such modifications of the homologous base triplet were seen with either OL1 or OL2, suggesting a particular conformation of the triplex formed by OL5 which can be viewed as a kink opening the [T(46)–A(23)]69T – [T(12)]3′OL5 triplet. One might speculate that this structural change was related to a constraint imposed by the 5′ moiety of OL5 interacting with the 12–17 region of 69T. The role of this 5′ part of OL5 was demonstrated by the use of mOL5, an oligomer derived from OL5 in which four Ts were substituted for the four Cs not involved in

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**Figure 3.** DEPC-footprinting of 69T-antisense oligonucleotide complexes.32P 5′-end-labeled target (5 × 10–8 M) alone (lane 1) or mixed with OL7 (3 × 10–5 M) were reacted with diethylpyrocarbonate as described in Materials and Methods, either in the absence or in the presence of additional antisense oligonucleotides, as indicated at the top of the lanes, at the concentration indicated in the legend of Figure 2a. In lane 0 the sample was treated with piperidine without prior incubation with diethylpyrocarbonate. Samples were analyzed on a 20% polyacrylamide gel containing 7 M urea. The sequence of the 69T is indicated to the right.
triplex formation with the stem of 69T (Fig. 1). The reactivity changes described above for OL5 were no longer seen with mOL5 (not shown). Moreover, the addition of mOL5 to the 69T–OL7 complex inhibited the cleavage by BamHI (Fig. 2a). Therefore mOL5 behaved as OL1, underlining the role of the C residues from the 5′ part of OL5 to destabilize the 69T–OL7 non-perfect triplex shown in Figure 4b. Three G residues are present in the anchor binding site on 69T (positions 13–15). It is likely that an interaction takes place between this three G motif of the 69mer and the three Cs of OL5 (Scheme 1). Due to the polarity of the pyrimidine third strand in triple helices, OL5 runs parallel to the purine strand of 69T. Parallel-stranded DNA duplex has been previously described for AT sequences (40,41) involving reverse Watson–Crick interactions, and for homo base pairs at acidic pH (42). Recent work has shown a parallel duplex with Hoogsteen pairing in equilibrium with a regular Watson–Crick helix (43). Such an unusual parallel-stranded structure between the anchor binding motif and the 5′ end of OL5 could be promoted by the adjacent triple helix. Alternatively, one cannot exclude that the 3C motif of OL5 is flipped into the anti-parallel orientation giving rise to three Watson–Crick GC pairs, the T(5) and C(6) residues of OL5 connecting two parts of opposite polarities. This would likely induce a distortion which might explain both the reactivity of A(16) and G(17) to DEPC and the ‘kinked’ triplex in the middle of the stem.

CONCLUSION

Synthetic oligonucleotides are now routinely used to modulate gene expression. At the level of double-stranded DNA, triplex-forming oligomers have been shown to block transcription whereas targeting of RNA led to the inhibition of splicing, translation or reverse transcription (1). With single-stranded DNA molecules two major mechanisms accounted for the...
observed antisense effects: (i) direct competition between the sense RNA–antisense oligonucleotide complex and the machinery in charge of reading the genetic information encoded in the RNA (44) and (ii) induced degradation of the target RNA by RNases H (45,46). In the above study we showed that, upon binding, antisense oligonucleotides can induce conformation changes of the target, subsequently interfering with the activity of a restriction enzyme acting on a remote site. Such an effect has been suggested to explain the inhibition of translation of ICAM-1 mRNA by an antisense oligomer targeted to the 3′ untranslated region of the message (47). From our work it is clear that an antisense oligonucleotide can shift the equilibrium between unanticipated. Undoubtedly, such results are not restricted to the region of the message (47). From our work it is clear that an mRNA by an antisense oligomer targeted to the 3′ untranslated region of the message (47). This work also raised questions regarding non-intended effects of antisense oligonucleotides. The opposite properties displayed long range interactions are involved in secondary or tertiary RNA at a long distance from the actual binding site of the oligomer if alternative conformations. This might perturb biological processes like protein binding or RNA lifetime. This can take place at a long distance from the actual binding site of the oligomer if long range interactions are involved in secondary or tertiary RNA structures.

This work also raised questions regarding non-intended effects of antisense oligonucleotides. The opposite properties displayed by OL1 and OL5 on BamHI activity on the 69T–OL7 duplex were unanticipated. Undoubtedly, such results are not restricted to the particular hairpin we were working with. Similar effects can account for some of the numerous non-antisense properties reported with oligonucleotides. This also strengthen the requirement for several control oligomers in antisense studies (48).

Last, our results suggest that non-perfect triplexes can be stable under some conditions, without the help of chemically-modified bases, intercalating agents or reactive oligonucleotide conjugates. This situation can vary from one oligonucleotide analogue to another.

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REFERENCES


