Targeting nucleic acid secondary structures by antisense oligonucleotides designed through in vitro selection

(aptamer/hairpin structure/inhibition of translation)

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ABSTRACT Using an in vitro selection approach, we have isolated oligonucleotides that can bind to a DNA hairpin structure. Complex formation of these oligonucleotides with the target hairpin involves some type of triple-stranded structure with noncanonical interaction, as indicated by band-shift assays and footprinting studies. The selected oligomers can block restriction endonuclease cleavage of the target hairpin in a sequence-specific manner. We demonstrate that in vitro selection can extend the antisense approach to functional targeting of secondary structure motifs. This could provide a basis for interfering with regulatory processes mediated by a variety of nucleic acid structures.

Regulation of gene expression by antisense oligonucleotides has provided a powerful approach for studying the function of a gene and for inhibiting genes responsible for undesirable traits. The binding of a synthetic oligomer to a complementary site of a target RNA can prevent the maturation of the message, its transport from the nucleus to the cytoplasm, or its translation (for review, see ref. 1). Antisense oligonucleotides have also been shown to block cDNA synthesis by retroviral reverse transcriptases (2). However, single-stranded target RNA may adopt secondary structures that reduce accessibility to a complementary oligonucleotide, leading to a weaker antisense effect (if any) than expected (3–5).

One can avoid structured target regions by a systematic screening of the entire RNA length until an accessible target is reached, i.e., until inhibition of gene expression is observed (6). However, several RNA structures have been shown to play a role in regulatory processes, in particular in viruses (7–11). Binding an oligonucleotide to such regions will likely interfere with these processes. Complex formation can be achieved by increasing the affinity and/or the concentration of the antisense sequence to compete out the intramolecular folding of the target, but this might be a source of nonspecific effects (1, 12).

Alternatively, antisense oligomers could be designed to bind to intact RNA structures. This approach might be energetically favorable because the secondary or tertiary interactions within the targeted nucleic acid bases are preserved. Moreover, antisense sequences able to read the three-dimensional array of interacting sites in the structured RNA may provide a higher level of selectivity. It was previously demonstrated that a DNA hairpin can interact with a complementary oligonucleotide, forming a triple helix-mediated double hairpin structure (13, 14). However, as only homopurine/homopyrimidine double strands can form triplexes (15), this approach is restricted to stem-loop structures with appropriate sequences.

Combinatorial strategies have proved to be very powerful to identify RNA or DNA sequences exhibiting high affinity and selectivity for small ligands or macromolecules (refs. 16–20; for review, see ref. 21). In vitro selection has been used to generate oligonucleotides able to recognize a nucleic acid; numerous examples of RNA enzymes obtained from directed evolution are available (22–27). This strategy has also been applied against a double-stranded DNA, leading to the selection of oligoribopyrimidines interacting with the target through the formation of canonical T-A-T and C-G-C triplets (20). Single-stranded regions of folded targets have been identified by in vitro selection (19) or rational screening of modified oligonucleotide libraries (28). In these latter cases, conventional double-stranded structures resulted from the association between the target and the aptamers. We have applied an in vitro selection approach to isolate “aptastucts,” i.e., aptamer oligonucleotides able to interact with a structured nucleic acid.

MATERIALS AND METHODS

Materials. Oligodeoxynucleotides were obtained from Institut Pasteur (Paris, France). The library of random candidates was prepared by introducing simultaneously on the synthesizer, the four synths in equimolar amounts at each position from nucleotide 14 to nucleotide 29 (Fig. 1). Oligonucleotides were purified by reverse-phase HPLC, using an acetonitrile gradient (0–48%) in a 100 mM ammonium acetate (pH 7.0) buffer, and checked for purity by electrophoresis on a 20% denaturing polyacrylamide gel of 3P-5'-end-labeled products.

Chemical reagents were obtained from Aldrich, and [γ-32P]ATP was obtained from ICN. T4 polynucleotide kinase was from Boehringer Manheim, and Tag polymerase was from Stehelin (Basel, Switzerland). Restriction endonucleases and DNase I were purchased from Gibco. SI nuclease and T4 ligase were from Promega.

Selection. The selection procedure showed in Fig. 1 was performed from a starting oligodeoxynucleotide repertoire of 416 = 4.29 × 108 unique species, as described in a preliminary note (29). Briefly, target hairpin (33 nM), selector, and candidates (16.6 μM each) were mixed in a 50 mM Tris-acetate (pH 6.0) buffer containing 10 mM MgCl2. This resulted in more than a 400-fold excess of candidate sequences over the target, allowing strong competition for binding to the hairpin.

After addition of 20 units of SactI, the samples were incubated for 20 h at 21°C, precipitated with ethanol and redissolved in sterile water. After denaturation (10 min at 95°C), DNA was amplified with Tag polymerase (∼0.25 units), in the buffer supplied with the enzyme, in the presence of both primers for 30 cycles (10 s at 95°C, 30 s of reannealing at 40°C, and 30 s

Abbreviation: DMS, dimethyl sulfate.

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An analysis of Complexes. Before analysis, unless otherwise indicated, the oligonucleotide mixture (target plus candidate) was heated for 5 min at 75°C in 50 mM Tris-acetate buffer, containing 10 mM MgCl₂ (buffer A), and then allowed to come to room temperature in about 45 min, after which it was stored at 4°C for at least 3 h.

For electrophoretic mobility-shift assays, 32P-5'-end-labeled candidates (0.6 μM) were mixed with cold target (24 μM) in buffer A (pH 7.5) and loaded immediately onto a 15% non-denaturing polyacrylamide gel. For ligation experiments, the mix containing stoichiometric amounts of target and candidates in buffer A (pH 6.0) was incubated at 15°C for 16 h with 10 mM ATP and 10 units of T4 ligase. The products were then analyzed on a 20% denaturing polyacrylamide gel.

S1 mapping and DNase I footprinting experiments were performed on preformed complexes in buffer A at pH 6.0. S1 nuclease (15 units) was added to the complex (1.2 μM S'-end-labeled candidate and 24 μM target), and the digestion was carried out for 15 min at 20°C and stopped by the addition of 10 μg tRNA, 1 μl of 3 M sodium acetate (pH 5.6), and 100 μl of ethanol (95%). For DNase I footprinting, samples containing 1.2 μM S'-end-labeled target and 24 μM candidate were incubated for 5 min at 20°C with 0.6 units of DNase I. The reaction was stopped as indicated above.

Chemical [dimethyl sulfate (DMS), KMnO₄ and EDTA-Fe²⁺] footprinting was performed in buffer A at pH 6.0 as described (31) on a mixture containing 1.2 μM S'-end-labeled target and 24 μM candidate (DMS), 0.3 μM 32P-S'-end-labeled candidate, and 15 μM target (KMnO₄) or 0.3 μM S'-end-labeled target and 60 μM candidate. Samples were reacted with 5% DMS (final concentration) for 4 min at 85°C and precipitated with ethanol after addition of 15 μg of tRNA and sodium acetate up to a concentration of 0.5 M. For permanganate footprinting, complexes were incubated in the presence of 22.8 mg/ml KMnO₄ for 30 min at 4°C and precipitated with ethanol. After dissolution in water, samples were treated for 30 min at 90°C by 1 M piperidine and precipitated twice before electrophoresis. For OH footprinting, oligonucleotides were incubated for 3 min at 18°C with 13 mM EDTA and 6.7 mM ferrous ammonium sulfate in the presence of 1% H₂O₂ and 100 mM sodium ascorbate. The reaction was stopped as indicated above for DMS footprinting using a mix supplemented with 33 mM thiourea. Samples were then precipitated by ethanol and analyzed by electrophoresis.

Functional Studies of Complexes. Rsal activity was evaluated on complexes (1.2 μM target plus 12 μM candidates) preformed in buffer A (pH 7.5) and incubated for 3 h at 20°C with 2 units of RsaI. Products were analyzed by electrophoresis on a 20% denaturing polyacrylamide gel.

RESULTS AND DISCUSSION

Selection of Anti-Hairpin Candidates. A pool of 45-nt-long oligodeoxyribonucleotides, containing a random stretch of 16 nucleotides, constituted the population of candidates against a DNA hairpin used in previous work (13, 14). The random stretch was flanked on the 5' side by a multifunctional sequence: 3'TTTTTTCCCTGAGCAC that, in addition to being used for amplification and cloning, contained the binding site of the selector oligonucleotide and a six residue anchor 3'TCCCTC, complementary to the 5' single-stranded part of the target (Fig. 1a).

Our in vitro selection approach has the distinction of being a “single-pot” series of reactions that does not require a purification step before amplification and is applicable to DNA as well as RNA targets. This approach involves a “selector” oligonucleotide (5'GGGAGCTCGT) that, upon binding to the 5' end of a candidate, generates a SacI site, leading to the loss of one of the PCR primer binding sites after restriction digestion (Fig. 1b). The restriction site of synthesis at 72°C. After precipitation by ethanol and dissolution in water, a second step of amplification was performed under the same conditions except that only primer 1 was used, to obtain a second generation of single-stranded candidates. Four identical rounds of selection were performed, after which candidates digested by SacI and BamHI were cloned into pUC19 linearized by SacI and BamHI. Sequencing was performed using the dideoxyribonucleotide termination method (30).
(5’GAGGCTC) was derived from the 5’ end of the anchor region, leading to the choice of SacI, which is sensitive to DNA conformation (more active on linear DNA), has no start activity, and works well at low pH and under a broad range of salt conditions. The candidates interacting with the target structure are not accessible to selector binding and hence escape restriction digestion. After four rounds of selection/amplification, the candidate population was cloned, and we arbitrarily picked three of them for further studies.

**Characterization of Hairpin-Candidate Complexes.** We synthesized the three 26-mer candidates, C1, C2, and C3 (Fig. 1a), composed of the 16-base stretch (C116, C216, C316) linked to the 10-base common sequence (the four ‘T’ linker and the anchor). Based on sequence analysis, none of these candidates displayed any obvious way to interact with either the folded or the unfolded target DNA, except the expected formation of 6 bp with the anchor motif. Sequences of the three candidates were different from the triplex-forming oligopyrimidine CM used in previous work to accommodate the DNA hairpin (13, 14) (Fig. 1a). We analyzed the interactions of these three selected sequences with the target hairpin. As shown by electrophoretic mobility-shift assay, the oligomers C1, C2, and C3 form one major type of complex with the target DNA hairpin at pH values of both 7.5 (Fig. 1c) and 6.0 (not shown). Additional minor complexes of reduced mobility can be detected on overexposed gels under these experimental conditions (not shown). The mobility of complex also varied with the target/candidate ratio. Potential intermolecular multimeric complexes due to the self-complementarity in parts of the target sequence may explain these observations. This proposition is substantiated by the fact that the mobility of these complexes is sensitive to the target concentration (not shown).

The extent of shift and the ratio of different kinds of complexes with C1 or C3 are slightly different from that with C2 (Fig. 1c), indicating subtle variation in the shape of the complex as both the size and the charge are identical. However, the three types of complexes were indistinguishably by their circular dichroic spectra (not shown). An excess (about 100-fold) of the hexamer 5’CTCCCT (6CM), was needed to compete out the C2/target complex (not shown). This indicates that the 3’ end of C2, and not only its 6-nt anchor, contributes to its binding to the DNA hairpin. Similar results were obtained with C1 and C3.

When 32P-5’-end-labeled candidates were used, the resulting complexes with the target were enzymatically ligated (Fig. 2a), demonstrating that the 5’ ends of the candidates are adjacent to the 3’ end of the target, as expected from the design of the anchor region. S1 nuclease (Fig. 2b), KMnO4 (Fig. 2c), or OsO4 (not shown) footprinting assays showed that the accessibility of the thymine residues in the region between nucleotides 7 and 10 in candidates is increased in the presence of the target. This is consistent with a folded conformation of the candidates in the complex such that the “T-region” forms a loop. The hypersensitivity of C3 to S1 nuclease, compared with the other two candidates, denotes weaker interaction of the 3’ end, as cleavage extends beyond the “T-loop.” DMS and OsO4 footprinting of the target revealed only minor changes in the presence of either candidate; a slight protection of the Gs in the stem was seen (Fig. 3a). In contrast, the DNase I cleavage pattern of the target DNA was significantly changed; all three candidates yielded a similar pattern, consistent with the formation of a double-stranded structure with the 5’ part of the target, i.e., with the anchor region (Fig. 3b). Therefore, although the 3’ part of the candidate interacts with the target hairpin, that binding does not prevent DNase I activity. This result contrasts to that observed in the presence of CM, the 26-mer oligopyrimidine able to form a local triple helix; in this case, no DNase I cleavage was seen in the region, whereas a hypersensitive site appeared at the double helix/triple helix junction, in good agreement with DMS footprinting (13).

**Fig. 2.** (a) Electrophoretic analysis of ligation products of 32P-5’-end-labeled candidates with the target. Oligomers CM (lanes 1 and 2), C1 (lanes 3 and 4), C2 (lanes 5 and 6), or C3 (lanes 7 and 8) were incubated with the ligase mix, in the absence (odd lanes) or in the presence (even lanes) of the target. Lane M contains size markers: labeled target (44 nt) and CM oligomer (26 nt). The expected size of the ligation product (70 nt) is also marked to the left. S1 mapping (b) and KMnO4 footprinting analysis (c) of 32P-5’-end-labeled candidates C1, C2, or C3 in the absence (−) or in the presence (+) of the target. The sequence common to the three candidates is indicated to the right. Samples were analyzed on a 20% polyacrylamide/7 M urea gel.

These results strongly suggest that the complexes formed by the candidates do not correspond to canonical triple helices. This hypothesis was indeed confirmed by experiments performed at neutral pH; candidates did bind to the target hairpin, whereas CM did not, underlining the role played by C-G.C+ triplets in the latter, but not the former, complex. Indeed, C-G.C+-containing triplexex are much less stable at neutral pH than at acidic pH (32, 33). Hence, our selected candidates are viewed as aptastruc oligomers, i.e., aptamers able to interact with the target hairpin by some unconventional triple-stranded motif.

**Selected Oligonucleotides Prevent Restriction of the Target Hairpin.** We designed a restriction cleavage assay to monitor the interaction of the target with aptastruc sequences, taking advantage of an RsaI site located at the top of the stem. CM
protected the DNA hairpin from RsaI digestion at pH 6.0 (14), but not at pH 7.5 (Fig. 4, lane 1). Unrelated oligonucleotides (Fig. 4, lanes 17 and 18) also did not show a noticeable effect, but all three aptamers reduced the cleavage of the target hairpin by RsaI at pH 7.5, when present in 10-fold excess, with increasing efficiency in the order C1 < C2 < C3 (Fig. 4, lanes 4–6). The inhibition was nearly complete when a 20-fold excess of the aptamers was used (not shown). The connecting loop turned out to be important; the 16-mers C116 and C316, corresponding to the “third strand” of candidates C1 and C3, showed only a slight inhibitory effect when combined with the hexamer 6CM (Fig. 4, lanes 14 and 16).

It is likely that base pairing of the first six nucleotides of the candidates followed by folding through the loop sequence is critical for final complex formation. As bases in the loop are probably not engaged in the complex (the T residues remain accessible to structural probes), it is tempting to suggest that this region may contribute to kinetically promote the association, as demonstrated for clamps or circular oligonucleotides (34, 35). The molecular analysis presented here does not take into account possible multimeric complexes that correspond to low migrating species on nondenaturing gels (see above). In such supramolecular structures, the loop region of the candidates, along with the self-complementary regions of the target, also plays the anchoring role. This networking may contribute to the higher order organization of the resulting complex, whereas the interaction of the third strand alone may not be as stable as a canonical triple helix. This might have been an overriding factor during the selection cycles, hence resulting in the isolation of C1, C2, and C3 sequences.

In contrast, the homologous oligonucleotide C216 derived from C2 induces a very strong inhibition of RsaI cleavage, even in the absence of the hexamer 6CM (Fig. 4, lanes 12 and 15). This behavior might be due to the fact that G,T-containing
oligomers can bind with rather weak affinity to a target homopurine strand of a duplex to form triple-stranded helices (36); however, it is unlikely that a regular triple helix with a G,T-third strand is formed in this case because it would contain more than 50% of noncanonical triplets (15).

The inhibition of RsaI digestion of the DNA hairpin by aptastrucs C1, C2, or C3 was sequence-specific; none had any effect on the digestion of plasmid DNA by RsaI, demonstrating that the protection of the DNA hairpin was not due to a direct interaction of the oligomers with the enzyme. Also noteworthy, the selected candidates had no direct inhibitory effect on SacI, the enzyme used for in vitro selection.

Protection of the hairpin against cleavage by Rsai can be due either to a steric hindrance of the restriction site by the oligomer or/and a conformational change of the target DNA upon candidate binding, which makes the site less susceptible to Rsai activity. We favor the latter hypothesis as, first, the four nucleotide-shorter version of the aptastrucs (C122, C222, and C322; see Fig. 1a) blocked Rsai with an even greater efficiency than the parent aptastrucs (Fig. 4, lanes 7–9). Second, OH-footprinting showed that, while the sugar–phosphate backbone in the stem region of the hairpin becomes less accessible in the presence of C1, C2, or C3, a hyperactive site is formed at nucleotide 26 of the target (Fig. 3c). This hyperactive site moves 4 nucleotides closer to the 5′ end of the target when the shortened candidates, C122, C222, or C322, are used for complex formation. In all these cases, the hyperactive site is about 6 nucleotides away from the 3′ end of the candidate bound to the target, assuming an extended conformation of the oligonucleotide along the hairpin, with a 3′ to 4-base loop. This indicates that the influence of C1, C2, or C3 on the sugar–phosphate backbone conformation of the target is of similar kind, even though the sequence of the 3′ end of the candidate is varied.

CONCLUSION

Using an in vitro selection approach, we have extracted aptastrucs i.e. oligonucleotides interacting with a DNA structure, from a library of random oligonucleotides. The complexes formed by three selected candidates with their stem–loop target can be viewed as a double-hairpin wherein the 5′ end of the aptastruc oligomer is adjacent to the 3′ end of the target sequence. According to our anchor design, the first 6 nucleotides on the 5′ side of the aptastrucs are base paired with the target and extend the stem region of the hairpin (Fig. 5). The next few residues provide the loop of the hairpin turn as that the remaining sequence can be located in the major groove of the extended double helix, which bends at the double strand “triple strand” junction, toward the 3′ terminus of the candidate, thereby exposing the other side of the helix.

Despite differences in the accessibility of some bases to chemical and enzymatic probes, the three complexes adopt a similar overall structure. This may reflect a low selectivity of binding. As only four rounds of selection were performed, we likely selected a subclass of candidates that organize themselves on the folded hairpin with limited interactions with the target. We could likely obtain candidates that bind tighter and more specifically to the target after additional rounds of selection under more stringent conditions.

The initial base pairing of the anchor region is critical in the formation of the aptastruc/target complex. Indeed, oligonucleotides derived from the three aptastrucs by deleting the anchor did not display detectable affinity for the target with use of an electrophoretic mobility-shift assay. Conversely, the properties exhibited by the oligomers C1, C2, and C3 are not only accounted for by the 6-nt-long anchor, as indicated by the competition experiment between the hexamer and each of the three aptastrucs for binding to the DNA target. Moreover, no retarded band was seen when the anchor hexamer was used in native gel assays. Last, the oligopyrimidine CM that contains the same anchor as the three selected aptastrucs did not bind to the target at neutral pH, in contrast to what was observed with C1, C2, and C3. This indicates that the complex formed by the aptastrucs and the target involves different types of third-strand binding interaction than the one in the pyrimidine motif triple-stranded complex. It is worth mentioning that this anchor-driven selection undoubtedly restricted the repertoire of selectable candidates. Selection of candidates from an anchor-free library would likely provide different aptastruc sequences.

Therefore, we have demonstrated that in vitro selection extends antisense strategy to a new class of targets. But this study also points out the limitation of the antisense approach; oligonucleotides can bind to unintended sites that cannot be predicted on the basis of primary sequences, possibly giving rise to unexpected effects.

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