Alternate strand recognition of double-helical DNA by (T,G)-containing oligonucleotides in the presence of a triple helix-specific ligand

Thérèse de Bizemont, Guy Duval-Valentin, Jian-Sheng Sun, Emile Bisagni1, Thérèse Garestier1 and Claude Hélène*

Laboratoire de Biophysique, CNRS URA 481, INSERM U 201, Muséum National d’Histoire Naturelle, 43 rue Cuvier, 75231 Paris Cedex 05, France and 1 Laboratoire de Synthèse Organique, CNRS UA 1387, Institut Curie-Biologie, Bâtiment 110, 91405 Orsay, France

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ABSTRACT

Triple helix formation requires a poly purine-polypyr imid ine sequence in the target DNA. Recent works have shown that this constraint can be circumvented by using alternate strand triplex-forming oligonucleotides. We have previously demonstrated that (T,G)-containing triplex-forming oligonucleotides may adopt a parallel or an antiparallel orientation with respect to an oligopurine target, depending upon the sequence and, in particular, upon the number of 5'-GpT-3' and 5'-TpG-3' steps [Sun et al. (1991) C. R. Acad. Sci. Paris Ser III, 313, 585–590]. A single (T,G)-containing oligonucleotide can therefore interact with two oligopurine stretches which alternate on the two strands of the target DNA. The (T,G) switch oligonucleotide contains a 5'-part targeted to one of the oligopurine sequences in a parallel orientation followed by a 3'-part that adopts an antiparallel orientation with respect to the second oligopurine sequence. We show that a limitation to the stability of such a triplex may arise from the instability of the antiparallel part, composed of reverse-Hoogsteen C.GxG and T.AxT base triplets. Using DNase I footprinting and ultraviolet absorption experiments, we report that a benzo[e]pyridoindole derivative [(3-methoxy-7H-8-methyl-11-[3-amino-propyl]-amino benzole[pyrido [4,3-b]indole (BePI)], a drug interacting more tightly with a triplex than with a duplex DNA, strongly stabilizes triplexes with reverse-Hoogsteen C.GxG and T.AxT triplets thus allowing a stabilization of the triplex-forming switch (T,G) oligonucleotide on alternating oligopurine–oligop yrimidine 5'- (Pu)14(Py)14-3' duplex sequences. These results lead to an extension of the range of oligonucleotide sequences for alternate strand recognition of duplex DNA.

INTRODUCTION

Oligonucleotides represent a class of ligands recognizing the major groove of double-stranded oligopurine–oligopyrimidine tracts in DNA by triplex formation (1,2). Triple helix-forming oligonucleotides can artificially control gene expression by competing with regulatory proteins, thereby inhibiting gene transcription both in vitro and in cell cultures. In addition they can be used as tools in molecular biology, e.g. for gene mapping or site-directed mutagenesis (for review see 3,4).

Four classes of base triplets have been described with unmodified oligonucleotides as third strands (C.GxC+, C.GxG, T.AxT, T.AxA) (5). (X.YZ base triplets: X, Y and Z are the nucleotides in the first, second and third strand of the triplex, respectively. ‘x’ indicates Watson-Crick interactions.) Base triplets involving T, C or G in the third strand may adopt two configurations; one of them corresponds to the hydrogen bonding scheme in co-crystals of A and T derivatives referred to as the Hoogsteen configuration (6). The other one involves a 180° rotation of the base in the third strand and will be referred to as reverse-Hoogsteen configuration. Adenine residues in the third strand adopt a single configuration for the formation of two hydrogen bonds: the resulting T.AxA base triplet belongs to the reverse-Hoogsteen category (7). Whereas Hoogsteen T.AxT and C.G.xC+ base triplets are isomorphic, the other triplets are not (Fig. 1).

In order to avoid distortions of the backbone of the third strand, triple helix formation occurs preferentially at oligopurine–oligopyrimidine target sequences. Polypurine sequences interrupted by one pyrimidine can form stable triple helices by inserting a non-natural nucleotide in the third strand (8). Recent work has demonstrated the ability of oligonucleotides to bind oligopurine sequences which alternate on the two strands of duplex DNA. An alternate-strand triple helix can be formed in different ways: (i) two pyrimidine oligonucleotides can be linked by their 3'- or 5'-ends to allow for recognition of alternating polypurine sequences (9-12); (ii) one can take advantage of the observation that (T,C)-containing oligonucleotides bind in a parallel orientation with respect to a polypurine target, whereas (G,A)-containing oligonucleotides bind in the opposite orientation. Therefore, oligonucleotides can be synthesized in such a way as to switch from one strand to the other one (13-16). For example, a 5'-(Pu)6(Py)6-3' double strand can be targeted by an
oligonucleotide containing a 5'-pyrimidine part and a 3'-purine part. A target containing a 5'-Pyb(Pu)3-GpT3'-sequence can be selectively bound by a third strand with additional nucleotides to connect the two binding domains at the site of crossover in the major groove (13). The length and the composition of this nucleotide link depends on the sequence located at the junction between the two domains. Recently, it has been reported that alternate strand binding can make use of a (T,G) sequence for the antiparallel sequence in the third strand instead of a (G,A) sequence and in particular on the number of 5'-TpG-3' steps and on the respective lengths of G and T tracts. Therefore an oligonucleotide containing only guanine and thymine residues may recognize polypurine tracts alternating on the two DNA strands (18). However the low stability of triplex-forming (T,G) oligonucleotide limits the use of the 'switch' strategy. To avoid this limitation one strategy consists in linking the (T,G) switched third strand to an intercalating agent such as acridine (19), as previously reported for triplex-forming (T,G) oligonucleotide limits the use of the C1′ atoms of the nucleotide in the third strand are shown in either the Hoogsteen mode of hydrogen bonding or in the reverse-Hoogsteen mode.

![Figure 1. Isomorphism of base triplets. The C1′ atoms of the three nucleotides belonging to each triplet are shown, with the C1′ atoms of the Watson–Crick base pair T-A or C-G located at fixed positions. The C1′ atoms of the nucleotide](image)

MATERIALS AND METHODS

DNA and chemical ligand

The benzo[e]pyridoindole derivative [3-methoxy-7H-8-methyl-11-[(3'-amino-propyl) amino] benzo[e]pyrido [4,3-b]indole (BePI), see Fig. 2] was synthetized as described by Nguyen et al. (22).

Oligodeoxyribonucleotides were synthetized on an automatic synthesizer by the Institut Pasteur (Paris) using the phosphoramidite method. Full length oligonucleotides were separated from shorter incomplete chains by electrophoresis on denaturing 20% acrylamide gels and recovered from mashed gel by diffusion in water. Oligonucleotide concentrations were determined by UV-absorption spectroscopy at 260 nm using a Kontron Uvikon 860 spectrophotometer at 20°C. One of the strands of the 44 base pair (bp) DNA fragment used in this study (44B1, see Fig. 3) was labelled at the 5'-end with [γ32-P]ATP (Amersham) and poly-nucleotide kinase (BioLabs).

Ultra-violet absorption

Thermal denaturation profiles were recorded with a Kontron Uvikon 820 spectrophotometer with 1 μM of each strand in 10 mM sodium cacodylate pH 7.2, 100 mM NaCl, 5 mM MgCl2 and 0.5 mM spermine. The BePI derivative was added at concentrations indicated in the figure legends. Thermostatic control of the sample cell holder was by circulating liquid (70% water/30% glycerol). The temperature of the bath was increased or decreased at a rate of 0.1°C/min, thus allowing complete thermal equilibrium of the cells between two measurements (separated by ~10 min). All melting curves were reversible, giving the same profile independently of whether the temperature was raised from 0 to 60°C (or 85°C) or decreased from 60 (or

![Figure 2. Structure of 3-methoxy-7H-8-methyl-11-[(3'-amino-propyl) amino] benzo[e]pyrido[4,3-b]indole used to stabilize triple helices](image)

![Figure 3. Sequence of the 44 bp DNA fragment (44B1-44B2) and the oligonucleotides (14A1, 14P1, 14A2, 14P2 and 28SW) used for footprinting and spectrophotometric experiments. Oligopurine–oligopyrimidine sequences are indicated by boxes. The oligopurine strand of Box I and the polypyrinidine strand of Box II are adjacent. Box I contains three 5'-GpT-3' and two 5'-ApG-3' steps, whereas Box II contains only one 5'-ApG-3' step (5'-ApG-3' or 5'-GpA-3' steps are indicated by black dots).](image)
85°C) to 0°C. The half-dissociation temperature of a third strand (temperature at which 50% of the triplex are dissociated into duplex target and free oligonucleotide) was estimated directly from melting curves or from derivative curves.

**DNase I reactions**

DNase I footprinting studies were carried out as described by Duval-Valentin et al. (23), with the following modifications. The 44 bp 44B1.44B2 duplex DNA fragment (20 nM) labelled on one strand (44B2) at the 5′-end was incubated overnight with the third strand (20 µM) at various temperatures in a 10 ml solution containing 20 mM Tris–HCl pH 7.2, 50 mM NaCl, 5 mM MgCl₂, 0.5 mM spermine and 5 mM CaCl₂. In some experiments, the BePI derivative was added at concentrations described in the figure legends. DNase I (0.2 µg/ml final) was added and digestion was stopped after different periods of time (depending on the temperature) by adding 1.5 µl of a mixture containing 100 mM EDTA and 10% SDS. After two ethanol precipitations, the digestion products were analyzed on a 20% polyacrylamide/7 M urea gel and autoradiographed with Fuji X-ray films at –80°C.

**Gel retardation assays**

Double-stranded target sequences radioactively labelled at one 5′-end (20 nM) were put in the presence of unlabelled oligonucleotides (20 µM) in a 10 µl solution containing 50 mM HEPES pH 7.2, 10 mM MgCl₂, 0.5 µM BePI. After overnight incubation at a precise temperature, 2 µl non-denaturing loading buffer (50% sucrose, 5 µg/µl tRNA) was added and samples were loaded on a 10% native polyacrylamide gel running under the same conditions (buffer, temperature) as the incubation gels (but without BePI). The gel was exposed to a phosphorimagery screen.

**RESULTS**

Figure 3 shows the sequences of oligonucleotides used in the present study. We call Box I, the 14 bp Watson–Crick duplex with three 5′-GpA-3′ steps and two 5′-ApG-3′ steps and Box II, the sequence d(A₁₀G₄·d(T₁₀C₄) with one 5′-ApG-3′ step only. These two contiguous boxes are flanked by two sequences rich in G·C base pairs which stabilize the ends of the duplex.

The (TG) oligonucleotides are designed to bind Boxes I and II in a parallel orientation (oligonucleotides 14P1 and 14P2) or in an antiparallel orientation (oligonucleotides 14A1 and 14A2) with respect of the oligopurine target strand. The switch (TG) oligonucleotide called 28SW is synthesized to bind both Boxes I and II by hybridization with 5′-G₂A₃G₄G₃₄-3′ on one strand in an antiparallel orientation and with 5′-A₁₀G₄-3′ on the other strand in a parallel orientation. The 28SW oligonucleotide is composed of the 14A1 and the 14P2 sequences (14A1-SW and 14P2-SW, respectively) linked together by a phosphodiester linkage.

**Triple helix recognition of Boxes I and II**

DNase I footprinting experiments were carried out to determine whether triplex formation occurred. We have previously shown that Box II was protected by 14P2 against DNase I digestion at 4°C (18). No footprint was detected with 14A1, 14P1 or 14A2 at the same temperature. The ‘switch’ oligonucleotide 28SW protected both boxes against DNase I attack at 4°C. It should be noted that runs of (dA)·(dT) present in Box II are cut poorly by DNase I which can be accounted for by their narrow minor groove (24).

**Table 1.** DNase I footprinting experiments performed on the double-stranded 44 bp DNA fragment with different oligonucleotides as third strands: (A) without BePI; (B) with 0.5 µM BePI

<table>
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<tr>
<td>14A1, 14P1, 14A2</td>
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<tr>
<td>14P2</td>
<td>+++</td>
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<tr>
<td>14P2-SW</td>
<td>+++</td>
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<tr>
<td>14A1-SW</td>
<td>+</td>
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<table>
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<tr>
<th>Third strand</th>
<th>BePI 0.5 µM</th>
<th>Incubation temperature (°C)</th>
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<td>14A2</td>
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<td>14A1-SW</td>
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The tables summarize DNase I protection experiments at various temperatures. The following symbols were used to evaluate the percentage of duplex protection by the third strand against DNase I attack: –, 0–25%; ±, 26–45%; +, 46–55%; ++, 56–85%; ++++, 86–100%. ‘ND’, non-determined. 14P2-SW and 14A1-SW indicate the two portions of the switch 28mer (28SW) (see Fig. 3).
The oligonucleotide 14P2 (5′-T(C/A)3'-7) adopts a parallel orientation relative to the polyurine sequence 5′-A10G3′. The switch oligonucleotide 28SW interacts with Box II in a parallel orientation and with Box I in an antiparallel manner. The temperature of incubation of the double helix in the presence of 20 μM 14P2 or 28SW was increased in order to determine the relative stability of the triplexes which was assayed by DNase I footprinting experiments (Table 1A). The decreasing order of relative stability as a function of temperature was: 14P2 > 14P2-SW >> 14A1-SW > 14A1, 14P1, 14A2 (no binding was detected with the last three oligonucleotides). The results show that there is no full protection of both boxes against DNase I detected with the last three oligonucleotides). The results show that there is no full protection of both boxes against DNase I cleavage with the 28SW oligonucleotide at temperatures >4°C. The 14P2 portion of the switch oligonucleotide (14P2-SW) was destabilized as compared to isolated 14P2. The lack of stability of the switch triplex formed with 28SW appears to be due—at least in part—to a poor affinity of 14A1-SW for its duplex target. 14A1-SW binding to Box I does not give a clear footprint at temperatures >4°C, in contrast to 14P2-SW which protects Box II from DNase I digestion up to 30°C. Therefore the binding of the 14A1-SW sequence should be increased if one wants to stabilize the switch triplex. We tested BePI derivatives as triplex helix-specific ligands. They were described previously as specific ligands for stretches of T.AxT base triplets in the Hoogsteen configuration (21).

### BePI effect on triple helix formation

We have previously shown that BePI derivatives (Fig. 2) intercalate preferentially between contiguous T.AxT triplets allowing triplex stabilization with a third strand containing thymine and cytosine residues (21). The effect of BePI was studied to determine whether it could stabilize triple helices formed by oligonucleotides containing thymine and guanine residues.

In order to study the effect of BePI on the triplexes formed between Box I and 14A1 or 14P1, DNase I footprinting experiments were performed on the duplex target incubated at 4°C with the oligonucleotides in the presence of increasing BePI concentrations. Up to 0.1 μM BePI, no triple helix was detected on Box I. In the presence of 0.3 μM BePI, 14A1 was bound to its target but not 14P1, whereas both 14A1 and 14P1 formed complexes at 1 μM BePI. The ligand effect on triplex stability with Box I could also be observed by UV absorption experiments (Table 2). Temperatures of half dissociation \( T_m \) of the third strand 14A1 or 14P1 were obtained by absorption spectrophotometry after subtraction of the duplex dissociation curve from the triplex curves recorded under the same conditions. Without BePI, no transition corresponding to dissociation of the (T,G) third strand was detected. In the presence of 3 or 10 μM BePI, an hyperchromism was associated with the dissociation of the third strand from the duplex. \( T_m \) values were calculated from these curves or from the corresponding derivatives: \( T_m(14A1) = 34°C \) and \( T_m(14P1) = 26°C \) with 3 μM BePI, and \( T_m(14A1) = 44°C \) and \( T_m(14P1) = 36°C \) with 10 μM BePI (Table 2). The triplex formed between Box I and the antiparallel 14mer (14A1) was therefore more stable (Δ\( T_m \) = 8°C) than the triple helix of Box I with 14P1. Additional experiments were carried out to check whether BePI could stabilize the triplexes with 14A2 or 14P2 on Box II. DNase I footprinting experiments showed that with 0.5 μM BePI, no stabilizing effect of the triple-helix specific ligand on the formation of triplexes involving 14A2 or 14P2 could be detected (Table 1). We used thermal denaturation experiments to monitor triplex formation between the 44 bp target 44B1,44B2 and 14A2 or 14P2 in the absence or in the presence of BePI. Absorbance at 260 nm was recorded versus temperature. No transition could be detected with 14A2 or 14P2 without BePI. In the presence of BePI derivatives (3 or 10 μM) an hyperchromism associated with the dissociation of the third strand from the duplex was detected on melting curves. \( T_m \) values of 14A2 and 14P2 were calculated from these curves or from the corresponding derivatives (Table 2): with 3 μM BePI, \( T_m(14A2) = 35°C \) and \( T_m(14P2) = 45°C \) with 10 μM BePI. \( T_m(14A2) = 43°C \) and \( T_m(14P2) = 53°C \). These results show that the stability corresponding to the parallel orientation (14P2) is favoured over that of the antiparallel orientation (14A2) even in the presence of BePI for a triple helix containing a single 5′-T$p$G-3′ step in the third strand.

### Sequence effect

The high number of (G/T) steps (5′-GpT-3′ + 5′-TpG-3′ steps) in 14A1 and 14P1 could have a destabilizing effect on triplex formation as suggested from molecular modeling and energy minimization (18). Box I was modified by decreasing the number of 5′-ApG-3′ and 5′-GpA-3′ steps from five to three while keeping the same base composition (four guanine and 10 adenine residues). Triplex formation was studied on a 26 bp duplex containing this modified Box I, called Box Ib (Fig. 4A). The oligonucleotides 14A1b and 14P1b could recognize the 26 bp target containing this modified Box Ib (Fig. 4A). The oligonucleotides 14A1b and 14P1b could recognize the 26 bp duplex in an antiparallel and parallel orientation, respectively (Fig. 4A). No transition corresponding to a triplex-to-duplex conversion was observed in the thermal denaturation curves in the absence of BePI. Experiments performed in the presence of 3 μM BePI showed superimposable thermal dissociation curves (Fig. 4B) for 14A1b and 14P1b with a \( T_m \) estimated to be ~46°C, i.e. 12 and 20°C higher than that of 14A1 and 14P1, respectively. In the presence of 3 μM BePI, the stability of the triple helices was the same for the parallel and antiparallel orientations with three (G/T) steps whereas the antiparallel orientation was favoured over the parallel one with a higher number of such steps (five) for a total number of 10 T.AxT and five C.GxG base triplets. In addition the lower number of steps increased the stability of both triple helices with a larger effect on the parallel orientation. These results are consistent with previous calculations and experiments which suggested that the energy penalty associated with the

<table>
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<th>Third strand</th>
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<td>14A1</td>
<td>34</td>
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<tr>
<td>14P1</td>
<td>26</td>
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<tr>
<td>14A1b</td>
<td>46</td>
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<tr>
<td>14P1b</td>
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<tr>
<td>14A2</td>
<td>35</td>
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<td>14P2</td>
<td>45</td>
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ND, non-determined.
each oligonucleotide described in Figure 3 and cleaved with stabilizes the triplex formed by 14A1 on Box I. At 30°C 14A1-SW motif. This is in agreement with the fact that BePI with the switch (T,G) oligonucleotide 28SW containing the detected without BePI. BePI stabilized the triple helix formed oligonucleotides. No clear footprint with the 14mers on Box I was attack of the targeted regions by 14A1, 14P2 or 28SW

0.5 µM BePI, Figure 5 A shows a total protection against DNase I. However the length of T and G stretches also plays an important role in determining both triplex stability and BePI binding. The switch oligonucleotide 28SW contains the same sequence as 14A1 called 14A1-SW. BePI was therefore expected to favour the antiparallel hybridization of the 14A1-SW sequence of 28SW. BePI was therefore expected to stabilise hybridization of the 14A1-SW sequence of 28SW. The duplex absorbance (Ad) was subtracted from the triplex absorbance (At) and d(At – Ad)/dT was plotted versus temperature.

Figure 4. (A) Sequence of the 26 bp DNA fragment and oligonucleotides used for melting experiments. Oligopurine–oligopyrimidine sequence is boxed. The Box Ib contains two 5′-GpA-3′ and one 5′-ApG-3′ steps (indicated by black dots). (B) Derivative of transition curves obtained by measuring the temperature dependence of absorbance at 260 nm of a solution containing 1 µM 26 bp DNA fragment with 1 µM 14A1b (- - - -) or 14P1b (- - - -) in the absence of BePI. The duplex absorbance (Ad) was subtracted from the triplex absorbance (At) and d(At – Ad)/dT was plotted versus temperature.

non-isomorphism of T.AxT and G.GxG base triplets was larger in the Hoogsteen than in the reverse-Hoogsteen configuration (Fig. 1). However the length of T and G stretches also plays an important role in determining both triplex stability and BePI binding.

The switch oligonucleotide 28SW contains the same sequence as 14A1 called 14A1-SW. BePI was therefore expected to favour the antiparallel hybridization of the 14A1-SW sequence of 28SW. In order to evaluate the relative stability of triplexes in the presence of 0.5 µM BePI, the target duplex was incubated with each oligonucleotide described in Figure 3 and cleaved with DNase I. The results are summarized in Table 1B. At 20°C with 0.5 µM BePI, Figure 5A shows a total protection against DNase I attack of the targeted regions by 14A1, 14P2 or 28SW oligonucleotides. No clear footprint with the 14mers on Box I was detected without BePI. BePI stabilized the triple helix formed with the switch (T,G) oligonucleotide 28SW containing the 14A1-SW motif. This is in agreement with the fact that BePI stabilizes the triplex formed by 14A1 on Box I. At 30°C with 0.5 µM BePI (Fig. 5B), 28SW protected both boxes whereas no footprint was detected with 14A1, indicating that the fixation of 14P2-SW induced binding of 14A1-SW. The triple helix with 14P2 on Box II was still detected at 30°C but Box II was less protected by 14P2 than by the switch 28mer containing the same sequence 14P2-SW. In the presence of BePI, the 14A1-SW sequence did not exhibit the destabilizing effect on 28SW binding observed in the absence of BePI. It should be noted that an enhanced cleavage occurred at the junction between duplex DNA and the triplex formed on Box I when 28SW was bound in the presence of BePI.

Sequence specificity

In order to test the sequence specificity of the switch oligonucleotide 28SW with the specific target (44B1.44B2) containing Boxes I and II and with a target containing one mismatch either in Box I (Box Im) or in Box II (Box IIm) (Fig. 6A), gel retardation assays were performed. At 10°C, pH 7.2 and with 0.5 µM BePI, the comparison of duplex and triplex populations shows that 28SW binds more efficiently to the DNA target containing alternating oligopurine sequences in Boxes I and II, than to DNA targets containing one central A·T base pair inversion either in Box I (Box Im) or in Box II (Box IIm) (Fig. 6B).

DISCUSSION

Pyrimidine-rich oligonucleotides bind to double-helical DNA in a parallel orientation with respect to the oligopurine strand of a double-stranded oligopurine–oligopyrimidine sequence. Cytosine residues need to be protonated, and the affinity of a pyrimidine third strand decreases at physiological pH. The use of G instead of C allows binding of the third strand in a pH-independent manner. Triplex formation with a (T,G) oligonucleotide requires relatively high Mg 2+ concentrations (5–20 mM).

A recent study concluded that at low guanine content (from 22 to 50%) triplex formation could not be detected with (T,G)-containing oligonucleotides whatever the orientation of the third strand (25). The 14P2 oligonucleotide used in our study contains 10 thymines and four guanines (%G = 28) and is able to bind its target at 30°C without BePI. Therefore a high G content is not a prerequisite for triplex formation with a (T,G)-containing third strand. In addition, an oligonucleotide containing many adjacent guanines is able to self-associate to form quadruplex structures, thus decreasing the concentration of free (T,G) oligonucleotides available for triplex formation (26).

One of the important parameters that must be taken into account in designing oligonucleotides directed to the major groove of double-stranded DNA is the isomorphism of base triplets (Fig. 1). When the C1′ atoms of Watson–Crick base pairs are fixed, the position of the C1′ atom in the third strand depends on the base triplet. Both T.AxT and C.GxG+ triplets are isomorphous in the Hoogsteen configuration. Despite the backbone distortion that results from the non-isomorphism of other base triplets, oligonucleotides differing from those containing only pyrimidines are able to form triple helices with oligopurine-oligopyrimidine sequences of DNA. For example reports have shown that oligonucleotides containing either G and T (25–30), G and A (28,31,32), or G, T and C (33) form triple helices.

With all nucleotides in the anti conformation, Hoogsteen hydrogen bond formation leads to a parallel orientation of the third strand with respect to the oligopurine target strand. Reverse-Hoogsteen hydrogen bonding corresponds to an anti-parallel orientation. Oligopyrimidines bind in a parallel orientation with respect to the polypurine strand of target sequences. Oligopurines bind in an antiparallel orientation. For oligonucleotides containing G and T, the orientation depends on the base sequence. Theoretical calculations suggested that the intrinsic stability of T.AxT and C.GG base triplets favours the Hoogsteen mode of hydrogen bonding. However the energy penalty associated with the distortion of the backbone imposed by the...
non-isomorphism of these two triplets is less important for the reverse-Hoogsteen configuration (see Fig. 1). Therefore it is expected that the orientation of a (T,G) oligonucleotide depends on the number of 5'-GpT-3' and 5'-TpG-3' steps in the third strand [called (G/T) steps in the following discussion] and on the length of the G and T tracts. Here we have shown that the orientation of a third strand containing 10 T and four G shifts from a parallel to an antiparallel orientation when the number of (G/T) steps increases. Footprinting experiments showed that a 14mer containing five (G/T) steps (14A1) was bound to the purine target in an antiparallel orientation. In the presence of BePI the antiparallel orientation was still more stable than the parallel one. When the number of (G/T) steps decreased from five to one with the same base composition, the orientation became parallel (oligonucleotide 14P2) as previously observed with another oligonucleotide (5'-T4CT4G6-3') targeted to the polypurine tract of HIV (33).

A comparison of the stability of triplexes with (T,G) third strands using UV spectroscopy is difficult in the absence of BePI because no transition could be detected in melting curves with a (T,G)-containing third strand without triplex-specific ligand. For example, even though a DNase I footprint on 44B1.44B2 was detected with 14P2, showing that a triple helix was formed under these conditions without BePI, no hyperchromism was detected on the melting curves. Oligonucleotides containing stretches of guanosine residues are known to form quadruplexes. Therefore self-association could compete with triplex formation (26). However this competition should be detected both by footprinting and UV melting. The hyperchromism associated with thermal dissociation is determined to a great extent by stacking interactions. A strong stacking interaction in single-stranded oligonucleotides containing G and T (or G and A) might not give rise to an additional hypochromism when the triplex is formed. This could explain the absence of transition in UV melting curves even when footprinting experiments reveal triplex formation. The absence of hyperchrom-
of a (T,G)-containing third strand and the number of (G/T) steps stability should be evaluated for each (T,G) oligonucleotide tested.

nucleotides which give no hyperchromism but the BePI effect on (T,G)-containing third strands. This could be useful for the (G/T) steps. Similarly T10 G 4  and T 5 G 2 T 5 G 2  have similar stability in both parallel orientation; the oligonucleotide T 5 G 2 T 5 G 2  with three in the parallel orientation (Table 2). Clearly the intrinsic triplex dissociation curves, allowing us to determine the m values of 8-oxoadenine (34).

We previously proposed a correlation between the orientation of a (T,G)-containing third strand and the number of (G/T) steps (18). Here we have shown that a 14mer 5′-T10G4-3′ with one 5′-TpG-3′ step binds in a parallel orientation. A systematic study concerning the orientation of (T,G)-containing third strands of various lengths, sequences and base compositions will be useful to fully understand the hybridization properties of (T,G)-containing oligonucleotides. Nevertheless, the results presented in Table 2 clearly show that both the number of (G/T) steps and the distribution of T and G tracts are important to determine triplex stability in the presence of BePI. For a given sequence the more stable complex (parallel or antiparallel) is predicted on the basis of the number of (G/T) steps. For example T3GT4GT3G2 with five (G/T) steps is more stable with an antiparallel orientation; in contrast T10G4 with a single (G/T) step is more stable in the parallel orientation; the oligonucleotide T3GT2T3G2 with three (G/T) steps has an intermediate behavior and binds equally well in both orientations. However it is difficult to predict the respective intrinsic stability of two oligonucleotides in the same orientation. Not only the number of (G/T) steps but also the lengths of G and T tracts play an important role. For example in the presence of 3 μM BePI, T3GT4GT3G2 and T10G4 have the same stability in the antiparallel orientation despite the great difference in number of (G/T) steps. Similarly T10G4 and T3GT2T3G2 have similar stability in the parallel orientation (Table 2). Clearly the intrinsic triplex stability is the resultant of several factors: the cooperativity of triplet stacking within T,AxT or C,GxG sequences, the energy penalty associated with backbone distortion at each (G/T) step (in both the third strand and the double helix) and the stacking interaction between a T,AxT and a neighbouring C,GxG triplet (in both 5′→3′ and 3′→5′ orientations).

In order to recognize oligopurine sequences alternating on the two strands of DNA, one can take advantage of the different orientations adopted by (T,G) oligonucleotides according to their sequence. Footprinting experiments showed that a switch oligonucleotide of 28 nucleotides (28SW) was able to bind an alternating target duplex 5′-(Pu)14(Py)14-3′ in the absence of any ligand (18). The switch 28mer oligonucleotide recognized Box II with a parallel orientation via Hoogsteen C,GxG and T,AxT base triplets and Box I with an antiparallel orientation via reverse Hoogsteen C,GxG and T,AxT base triplets. Although 28SW protected Boxes I and II against DNase I digestion, the N7 of guanine residues in Box I were not protected against DMS attack in contrast to guanine residues G19 to G22 in Box II which were partially protected (results not shown). In Box I, most of the guanine residues are located between two thymine residues. As shown by NMR experiments a distortion occurs at 5′-GpT-3′ and 5′-TpG-3′ steps due to the non-isomorphism of base-triplets (see Fig. 1) (35). This could explain the reactivity of these guanine residues to DMS reaction.

In the presence of BePI, DNase I footprinting experiments performed at 30°C showed that the switch oligonucleotide 28SW formed a triplex that was more stable than that obtained with separated 14A1 or 14P2 oligonucleotides. Moreover, whereas no footprint was detected on Box I with the sequence 14A1 alone, it was possible to detect a footprint on Box I when this sequence was linked to 14P2 (28SW oligonucleotide). Parallel binding of the 5′-T10G4-3′ portion of 28SW induced the antiparallel fixation of the 14A1 portion of 28SW. In contrast, in the absence of BePI the triplex helix formed by 28SW was less stable than that formed by 14P2 alone. Therefore, when the 3′-half of the 28SW oligonucleotide (14A1-SW) was not bound, this negatively-charged ‘tail’ attached to 14P2-SW destabilized the triplex formed with Box II.

BePI derivatives strongly stabilize triplexes with a (T,G) third strand in a concentration-dependent manner. Previous results obtained with (TC) oligonucleotides have shown that BePI stabilization was observed with triplexes rich in adjacent Hoogsteen T,AxT base triplets suggesting that BePI is preferentially intercalated between T,AxT base triplets (21). According to these previous results, the triplex with T10G4 oligonucleotide in the Hoogsteen configuration was expected to be stabilized by BePI derivatives as experimentally observed (Table 2). Here we have shown that BePI can also stabilize triple helices involving T,AxT and C,GxG base triplets in the reverse Hoogsteen configuration. Triplexes involving five (14A1), three (14A1b) or one (14A2) (G/T) steps in the third strand were stabilized.

As shown by DNase I footprinting experiments at increasing temperatures, the binding of a triplex-forming switch (T,G) oligonucleotide to an alternating oligopurine–oligopyrimidine sequence is stabilized by 25°C upon binding of BePI derivatives.

Gel retardation assay showed that 28SW can discriminate, even in the presence of BePI, between duplex DNA sequences that differ by one A,T base pair inversion. This is in agreement with previous studies showing that replacement of a single base pair in

**Figure 6.** (A) Sequence of the 44 bp DNA fragments used for specificity experiments. Boxes IIm and Im contain a central A,T base pair inversion in comparison with Boxes II and I, respectively. (B) Gel retardation assay of 44 bp fragments (containing Boxes II and I, or IIm and I, or II and Im) at 10°C in the presence of a non-specific oligonucleotide (C) or 28SW at 20 μM concentration in the presence of 0.5 μM BePI. Arrow D, target double helix; arrow T, triple helix (see Material and Methods for experimental conditions).
the center of an oligopurine-oligopyrimidine sequence is highly detrimental to triplex formation (36,37).

CONCLUSION

We have shown that natural oligonucleotides containing thymine and guanine residues can recognize, in a pH-independent manner, oligopurine sequences that alternate on the two strands of the duplex. This 'switch' triplex can be stabilized by BePI derivatives. The experimental results presented here also show that alternate-strand triple-helix formation with (T,G)-containing oligonucleotides requires appropriate target sequences with defined numbers of 5′-ApG-3′/5′-GpA-3′ steps in each of the oligopurine target sequence and an adequate length of G and T stretches in the third strand. A systematic study concerning the orientation of (T,G)-containing third strands of various lengths, sequences and base composition will be useful to understand the hybridization properties of (T,G)-containing oligonucleotides and therefore to extend the number of oligonucleotide sequences that can be used to target duplex DNA at specific sites where oligopurine sequences alternate on the two strands of the double helix.

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REFERENCES