

G/C-modified oligodeoxynucleotides with selective complementarity: synthesis and hybridization properties

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ABSTRACT

Modified oligodeoxyribonucleotides (ODNs) that have unique hybridization properties were designed and synthesized for the first time. These ODNs, called selective binding complementary ODNs (SBC ODNs), are unable to form stable hybrids with each other, yet are able to form stable, sequence specific hybrids with complementary unmodified strands of nucleic acid. To make SBC ODNs, deoxyguanosine (dG) and deoxycytidine (dC) were substituted with deoxyinosine (dI) and 3-(2'-deoxy- β -D-ribofuranosyl)pyrrolo-[2,3-d]-pyrimidine-2-(3H)-one (dP), respectively. The hybridization properties of several otherwise identical complementary ODNs containing one or both of these nucleoside analogs were studied by both UV monitored thermal denaturation and non-denaturing PAGE. The data showed that while dI and dP did form base pairs with dC and dG, respectively, dI did not form a stable base pair with dP. A self-complementary ODN uniformly substituted with dI and dP acquired single-stranded character and was able to strand invade the end of a duplex DNA better than an unsubstituted ODN. This observation implies that SBC ODNs should effectively hybridize to hairpins present in single-stranded DNA or RNA.

INTRODUCTION

Oligodeoxyribonucleotides (ODNs) do not effectively hybridize to complementary sequences which are already base paired. Without the assistance of recombinase enzymes such as recA (1), accessibility of ODNs to double-stranded DNA (dsDNA) is usually restricted to homopurine runs (2) or to extruded single-stranded sequences in supercoiled DNA (3). Although less of an issue with single-stranded DNA (ssDNA) or RNA, hybridization of ODNs to many sequences in these molecules can be compromised by intramolecular base pairing (4,5). While numerous hybridization strategies have been described to overcome or exploit secondary structure, none provides a general solution to the problem. Examples include modified ODNs which form unusually stable hybrids (6–12), ODNs which form

triple-stranded complexes (13), ODNs which hybridize to hairpins or contiguous flanking sequences (14–18), and the use of 'effector' ODNs (19) and 'tethered' ODNs (20) to improve binding affinity through cooperative interactions.

A pair of uniquely modified complementary ODNs (or a single self-complementary ODN) that do not hybridize to each other, yet do hybridize to unmodified complementary sequences might offer a general solution to the challenge of targeting any site in DNA or RNA. If such a pair of ODNs could be synapsed to a homologous region in dsDNA by recombination, a complement-stabilized or double D-loop (21–22) would be formed (Fig. 1a). Unlike a simple D-loop, the double D-loop is relatively stable and might inhibit gene expression. Alternatively, the same type of paired ODNs could be hybridized to a unique sequence in long, single-stranded nucleic acid. To the extent that sequence is involved in secondary structure (such as a localized hairpin; Fig. 1b), the paired ODNs should have an advantage over a standard ODN. Whether such ODNs are used as probes or antisense agents, their hybridization to a target should generate more new base pairs than an unmodified ODN. This is depicted in Figure 1.

We describe the synthesis of a cytosine (dC) analog. When incorporated into an ODN it rearranged to a nucleoside (dP) which formed 2-3 hydrogen bonds when opposite a guanosine (dG) and 1-2 hydrogen bonds when opposite an inosine (dI). When every dC and dG in a pair of complementary ODNs was substituted with dP and dI, respectively, the ODNs did not hybridize to each other yet did hybridize to unmodified, complementary ODNs. By this criterion, the ODNs demonstrated selective binding complementarity and are designated SBC ODNs. Although the SBC–DNA hybrids were less stable than the DNA–DNA hybrid, a self-complementary SBC ODN was more effective than the corresponding unmodified ODN in strand invading a homologous duplex DNA. Further development of the SBC concept will depend upon the synthesis of base analogs which form stronger pairs with the natural complement.

MATERIALS AND METHODS

Materials and their sources were as follows: DNA synthesis reagents, Glen Research; phosphodiesterase I (*Crotalus adamanteus* venom), alkaline phosphatase (calf intestinal) and DNase I, Amersham Life Science; T4 polynucleotide kinase (10 U/ μ l),

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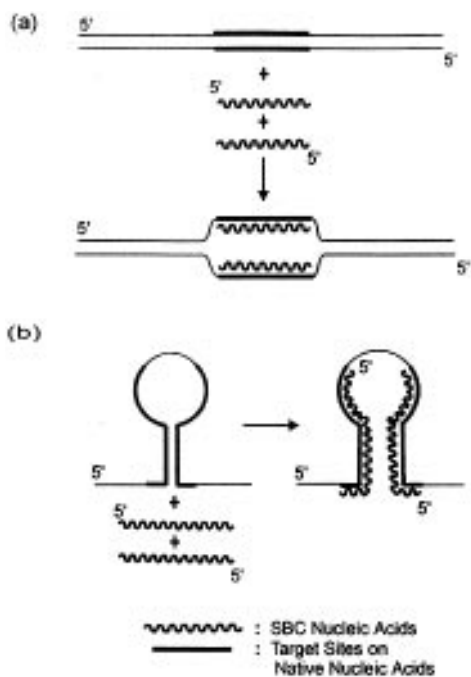


Figure 1. The possible applications of SBC ODNs. (a) The interaction of SBC ODNs with dsDNA to form a complement stabilized D-loop in the presence of a recombinase such as recA. (b) The strand invasion of a DNA or RNA hairpin by SBC ODNs. In each example hybridization leads to an increase in the total number of base pairs, thus providing a thermodynamic drive for the reaction.

Promega; [γ - ^{32}P]ATP, NEN Research. Commercial reagents were used as received. ^1H -NMR spectra were determined on a Varian Gemini-300. Elemental analysis was performed by Quantitative Technologies Inc. (Whitehouse, NJ). UV spectra were measured on a Beckman DU-40 spectrophotometer or a Perkin Elmer Lambda 2S UV/VIS spectrophotometer.

Preparation of 3-(2'-deoxy- β -D-ribofuranosyl)furano-[2,3-*d*]-pyrimidine-6(5*H*)-one (dF)

5-Ethynyl-2'-deoxyuridine (3 g, 11.9 mmol) (23) and copper (I) iodide (500 mg, 2.6 mmol) in a 250 ml two-necked round-bottomed flask were dried *in vacuo* for 3 h, placed under argon, and suspended in anhydrous DMF (35 ml) and triethylamine (15 ml). The solution was vigorously stirred at 120°C under argon and every 30 min fresh copper (I) iodide (250 mg, 1.3 mmol) was added until most of the starting material had reacted. After 2 h, the resulting mixture was filtered and the filtrate was concentrated *in vacuo* to dryness. The residue was suspended in acetone (100 ml) and stirred overnight. The desired product was filtered, washed with acetone (20 ml), and dried *in vacuo* to afford 2.2 g of dF as a slightly yellowish solid. The remaining product in mother liquor was further purified by silica gel column chromatography (elution solvent: 25% MeOH in EtOAc) to afford an additional 0.3 g of dF (total yield: 2.5 g, 83%); mp 167–168°C; UV (0.05 M $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7) λ_{max} 322 nm (ϵ 12 500). Anal. calcd for $\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_5$: C, 52.38; H, 4.80; N, 11.11. Found: C, 52.11; H, 4.81; N, 10.91. ^1H NMR (DMSO- d_6): the same as reported by Kumar *et al.* (24).

Preparation of 5'-O-(4,4'-dimethoxytrityl)-dF

dF (2.17 g, 8.6 mmol) was dried *in vacuo* at 60°C overnight and then added to 4,4'-dimethoxytrityl-chloride (3.51 g, 10.4 mmol) and anhydrous triethylamine (2.4 ml) in pyridine (30 ml). After 2 h at room temperature under argon, the resulting mixture was diluted with an equal volume of water and extracted with two 150 ml portions of ether. The ether layer was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in dichloromethane (20 ml) and the desired product (4.6 g) was precipitated by adding the solution to 400 ml of rapidly stirred hexanes. Filtration yielded 4.6 g (96%) of a white solid.

Preparation of 5'-O-(4,4'-dimethoxytrityl)-dF-3'-O-phosphoramidite

Chloro-[(β -cyanoethoxy)-*N,N*-diisopropylamino]-phosphine (2.9 g, 12.5 mmol) was added dropwise over 30 s to an anhydrous mixture of 5'-O-(4,4'-dimethoxytrityl)-dF (4.6 g, 8.3 mmol), diisopropylethyl amine (5.8 ml), and dichloromethane (27 ml) under argon (25). After 30 min at room temperature the reaction was stopped by adding anhydrous methanol (0.3 ml). The reaction mixture was extracted with 5% aqueous NaHCO_3 (2 \times 15 ml) and saturated aqueous NaCl (2 \times 15 ml). The organic layer was dried over anhydrous sodium sulfate, filtered and then evaporated under reduced pressure to afford a brown oil. This crude product was further purified by silica gel column chromatography using hexanes: CH_2Cl_2 :EtOAc: Et_3N (4:3:2:1 by vol) as the solvent system. Fractions containing the desired product were combined, evaporated to dryness, and redissolved in EtOAc (10 ml). Precipitation from rapidly stirred hexanes (400 ml) yielded 5.9 g (94%) of purified material. ^1H NMR (CDCl_3) δ 8.88 (d, J = 18.6 Hz, 1H), 7.5–7.2 (m, 10H), 6.81 (m, 4H), 6.32 (m, 1H), 5.62 (d of d, J = 9.5, 3.9 Hz, 1H), 4.70 (m, 1H), 4.19 (m, 1H), 3.8–3.4 (m, 12H), 2.77 (m, 1H), 2.59 (t, J = 9.4 Hz, 2H), 2.44 (m, 2H), 1.25–1.0 (m, 12H).

Conversion of dF to dP

dF (1 g, 3.96 mmol) was dissolved in 30% aqueous ammonium hydroxide (30 ml). After overnight at room temperature, the resulting solution was concentrated *in vacuo* to dryness. The residue was suspended in acetone (50 ml), stirred overnight, and the undissolved product filtered to afford 850 mg. The mother liquor was concentrated to dryness and the residue was suspended in acetone (10 ml) overnight with stirring to yield an additional 100 mg of insoluble product (total 950 mg, 95.4%). This compound was analyzed by HPLC, UV and NMR and shown to be identical to authentic 3-(2'-deoxy- β -D-ribofuranosyl)pyrrolo-[2,3-*d*]-pyrimidine-2(3*H*)-one (dP).

Synthesis and purification of ODNs

ODNs containing modified bases were synthesized on 1 μmol scale using standard procedures for an ABI-394 DNA synthesizer. ODNs with the dimethoxytrityl group were purified by HPLC using a Hamilton PRP-1 (7.0 \times 305 mm) reverse phase column employing a gradient of 5 to 45% CH_3CN in 0.1 M $\text{Et}_3\text{NH}^+\text{OAc}^-$, pH 7.5, over 20 min with a 2 ml/min flow rate. After detritylation with 80% acetic acid, the ODNs were precipitated by addition of 3 M sodium acetate and 1-butanol. The resulting ODNs were dried and further purified by using 20% denaturing PAGE as described by Hopkins *et al.* (26).

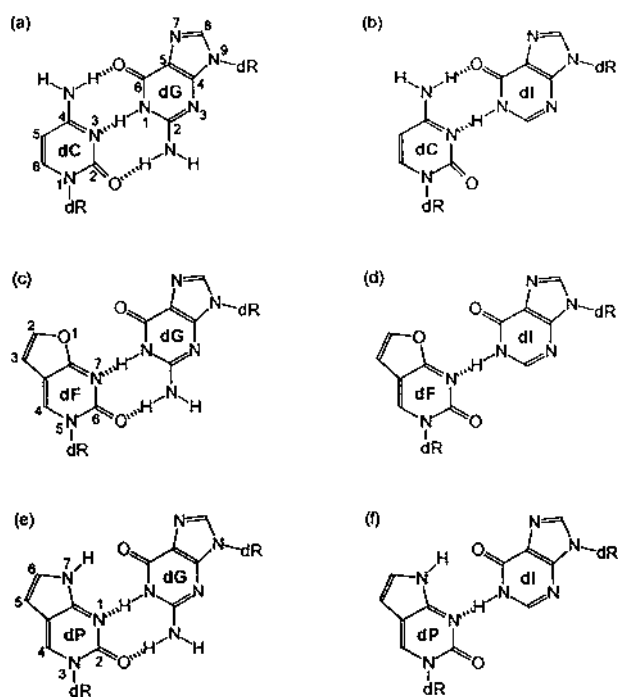


Figure 2. Base pairing schemes for dC and dG analogs.

Enzymatic digestion of ODNs

Enzymatic hydrolysis of ODNs was carried out as described by Woo *et al.* (27). The resulting hydrolysate was analyzed by HPLC with dual detection at 260 nm and 320 nm (Waters 994 Programmable Photodiode Array Detector) using a C-18 reverse phase column (Rainin, MicrosorbTMShort-One[®]). The solvent gradient was run at 1 ml/min as follows: solvent A, 0.1 M Et₃NH⁺OAc⁻, pH 7.5; solvent B, CH₃CN; a linear gradient 0 to 13% B over 10 min, a linear gradient to 100% B over 2 min, then isocratic 100% B for 3 min. Peaks were identified by comparison of retention times to those of authentic, commercial samples (dA, dG, dT and dC) and synthetic samples (dF and dP) prepared by known procedures (28).

Thermal denaturation data (T_m)

T_m values were recorded on a Perkin Elmer Lambda 2S UV/VIS spectrophotometer equipped with a temperature programmer (PTP-6) and interfaced to an IBM personal computer (PECSS software, Perkin Elmer). Scan rates were 0.5°C/min. Data were collected at 260 nm in the temperature range from 5 to 90°C. The T_m is defined as the temperature at half the maximal hyperchromicity using baseline correction at high and low temperature extremes (29). Samples were prepared by dissolving ODNs in TNM buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 50 mM NaCl, 10 mM MgCl₂]. To ensure complete hybridization of complementary strands (1:1 molar ratio) before collecting data, the samples were incubated at 90°C for 2 min and cooled to 3°C over 1 h. The concentration of hybridized ODNs was approximately 2 μM.

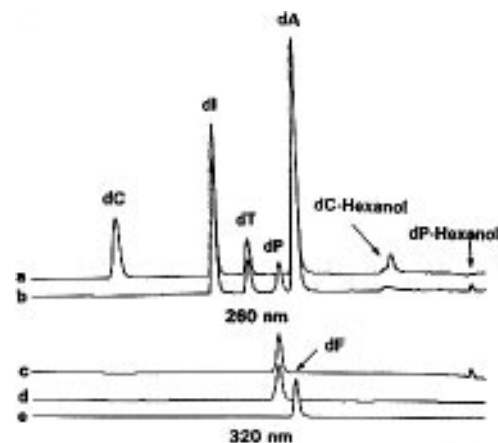


Figure 3. Reverse phase HPLC analysis: (a) enzymatic hydrolysate of Watson strand in **II** (see Table 1); (b and c) enzymatic hydrolysate of Watson strand in **VIII** (see Table 1); (d) authentic dP; (e) authentic dF. Detection was at 260 nm (a and b) or 320 nm (c, d and e). Retention times increase to the right.

Gel migration assay

ODNs with an asterisk (*) in Figures 5 and 6 were 5' ³²P-labeled using T4 kinase and [γ -³²P]ATP (30) and present at 0.5 μM unless otherwise indicated. Hybrids were formed by incubating the labeled ODN with a 2-fold molar excess of cold complementary ODN for 60 min at room temperature in 20 μl TNM buffer. These samples were then mixed with 20 μl loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 2.5% Ficoll type 400) and then kept on ice prior to gel electrophoresis. Aliquots (5 μl) were analyzed in a 12% non-denaturing polyacrylamide gel [19:1 acrylamide: bisacrylamide, 0.35 mm thick, 20 × 16 cm, polymerized and run in TBE buffer (89 mM Tris-borate/2 mM EDTA) containing 3 mM MgCl₂]. Pre-electrophoresis in a BioRad Protean[®]II xi apparatus was performed for 1 h at 200 V and 10°C. Samples were loaded, and the gel was run as before until the bromophenol blue dye had traveled ~15 cm (~5 h). The gel was dried and visualized with a phosphorimager (BioRad GS-250 Molecular Imager).

RESULTS AND DISCUSSION

Design and synthesis of SBC ODNs

The design paradigm for SBC ODNs is modification of complementary dA-dT or dG-dC bases such that the modified bases form only one hydrogen bond when paired to each other, yet can form two or even three hydrogen bonds when paired to the natural partner. We report the synthesis of a complementary pair of G/C-rich SBC 28mers substituted with deoxyinosine (dI) in place of dG and 3-(2'-deoxy-β-D-ribofuranosyl) furano-[2,3-d]-pyrimidine-6(5H)-one (dF) in place of dC. As shown in Figure 2, these modified bases should form two hydrogen bonds, respectively, with dC (2b) or dG (2c), yet only one hydrogen bond with each other (2d). Although the stabilities of the SBC-DNA hybrids might not be as good as DNA-DNA hybrids, the SBC-SBC hybrids would be much less stable, thus enabling the design goals.

The dG analog was simply prepared by removal of the N2 exocyclic amino group of dG to give deoxyinosine (dI). This

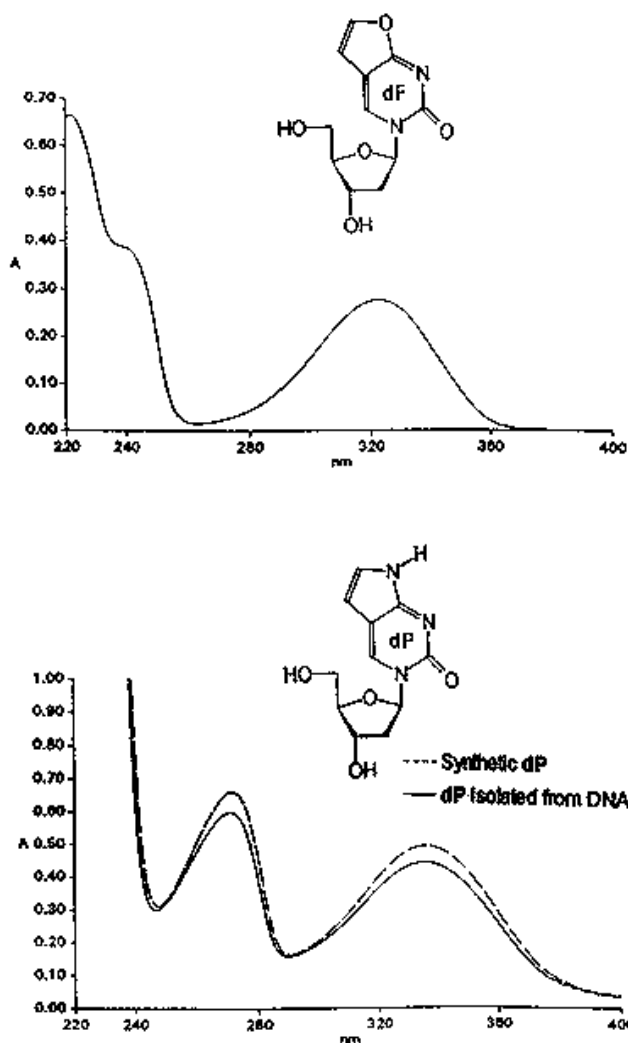


Figure 4. Ultraviolet spectra of **dF** (upper) and **dP** (lower).

nucleoside analog is known to preferentially pair with dC (31–32). The modified dC was designed to have no hydrogen bonding ability at the position equivalent to the N4 exocyclic amino group of dC. We chose the bicyclic nucleoside **dF** to fulfill this role. It was expected to be better than monocyclic dC analogs (33) because of its base stacking ability. The nucleoside **dF** was synthesized by copper (I)-catalyzed cyclization of the known antiviral nucleoside, 5-ethynyl-2'-deoxyuridine (23). Unlike **dF** analogs with a substituent at C2 (23, 34–35), preparation of the desired compound was very sensitive to solvent and reaction conditions; for example, if pyridine was used as a solvent the major product was a dimer of the starting material. **dF** was dimethoxy-tritylated and converted to its cyanoethoxy phosphoramidite by conventional methods (25).

SBC and unmodified ODNs were synthesized using standard procedures on an ABI-394 DNA synthesizer. Based upon recoveries of purified products, the average coupling yield was 91% for SBC ODNs and 94% for unmodified ODNs. The nucleoside composition of a representative SBC ODN was determined by reverse phase HPLC analysis of an enzyme hydrolysate. As shown in Figure 3, no peak corresponding to authentic **dF** was observed in the nucleoside hydrolysate from the modified ODN. Instead a peak

Table 1. T_m values for native and modified ODNs with **dI** and **dP**

Watson: 5' XTY AXA AXY ATX YYA YYA XXY AAY YAY X 3'							
Crick: 3' YAX TYT TYX TAY XXT XXT YYX TTX XTX Y 5'							
Hybrid	Watson		Crick		T_m (°C)	T_m Drop per Modified Base Pair	
	X	Y	X	Y			
I	C	G	C	G	75.6	0	
II	C	I	C	G	55.2	2.04	
III	C	G	C	I	62.8	1.83	
IV	C	I	C	I	43	1.92	
V	P	G	C	G	70.6	0.71	
VI	C	G	P	G	72	0.36	
VII	P	G	P	G	69.6	0.35	
VIII	P	I	C	G	48.2	1.61	
IX	C	G	P	I	57.2	1.08	
X	P	I	P	I	20.2	3.26	

corresponding to 3-(2'-deoxy- β -D-ribofuranosyl)-pyrrolo-[2,3-*d*]-pyrimidine-2(3*H*)-one (**dP**) was observed, suggesting that the **dF** had been converted to **dP** during the treatment with 30% aqueous ammonium hydroxide used in the final step in DNA synthesis.

The **dP** isolated from the modified ODN was identical to authentic **dP** by both UV analysis (Fig. 4) and HPLC coinjection in two gradient solvent systems. The **dF** nucleoside, when treated overnight with 30% aqueous NH_4OH at room temperature, rearranged to a compound (95.4% recovered yield) which had a ^1H NMR identical to that previously reported for **dP** (28). Based on these results we conclude that **dP** was the only dC analog detectable in the ODN hydrolysate and that >90% of the **dF** residues had been converted to **dP** by base treatment. Subsequent attempts to incorporate **dP** directly into ODNs using the phosphoramidite method were not successful due to instability during the iodine oxidation conditions employed in the standard ODN synthesis cycle.

Although **dP** still could hydrogen bond at N1 with the carbonyl group at C6 of dG or **dI**, this interaction should be relatively weak due to suboptimal orientation of the N1 hydrogen (Fig. 2e and f). Previous studies on the base pairing properties of **dP** have shown that it preferentially pairs with dG and that this base pair is slightly less stable than dC-dG (28).

Hybridization properties of SBC ODNs

Table 1 shows the hybridization properties of 28mer ODNs containing **dI** for dG, **dP** for dC, or both. The sequence, taken from pBR322 plasmid, had a G-C content of 60.7%. Introduction of either **dI** or **dP** into one or both strands of the duplex decreased its T_m by 1.8–2.0 or 0.4–0.7°C, respectively, per modified base pair. When only one strand of the hybrid was substituted with both **dI** and **dP**, the T_m dropped by 1.1–1.6°C per modified base pair. These values reflect a slight destabilization attributable to the dG-**dP** base pair and a larger destabilization due to the **dI**-dC base pair. When both strands of the hybrid were substituted with **dI** and **dP**, however, the T_m drop per modified base pair increased significantly to 3.3°C.

Some of the hybrids were analyzed by non-denaturing PAGE (Fig. 5). As shown in Table 1 and Figure 5, the SBC ODNs containing both **dI** for dG and **dP** for dC (Watson in VIII, Crick in IX and both Watson and Crick in X) did not form a stable hybrid with each other at room temperature (hybrid X; Fig. 5, lane 8), yet did form stable hybrids with their unmodified complementary

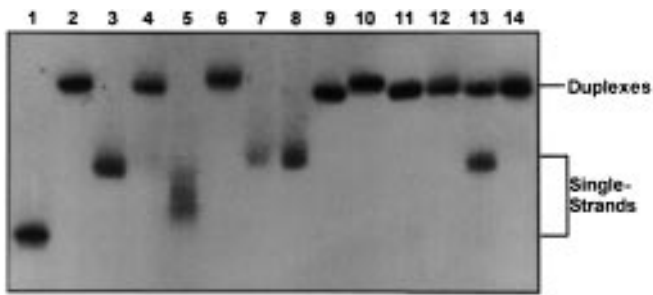


Figure 5. Gel mobility shift analysis of selected ODNs and hybrids listed in Table 1. ^{32}P end-labeled ODNs are denoted by an asterisk (*) and Watson/Crick strands by W/C (see experimental section for details). Single-stranded ODN was analyzed in lanes 1 (*W in I), 3 (*C in IV), 5 (*C in VII), and 7 (*C in X). Complementary ODNs were analyzed in lanes 2 (*W/C in I), 4 (W/*C in IV), 6 (W/*C in VII), 8 (W/*C in X), 9 (*W/C in III), 10 (*W/C in VI), and 11 (*W/C in IX). For lane 12 (W/*C in IX + C in I; molar ratio 2:1:3), the preformed hybrid IX was treated at room temperature for 60 min with unmodified Crick. For lane 13 (W in I + C in I + *C in IX; molar ratio 1:1:1), unmodified Watson (1 μM) was mixed simultaneously with unmodified Crick and SBC Crick and incubated at room temperature for 60 min. For lane 14 (W/C in VIII plus W/*C in IX; molar ratio 2:1:2:1), the hybrid VIII was mixed with the hybrid IX and incubated at room temperature overnight.

ODN strands (hybrid IX; Fig. 5, lane 11). As a result, these ODNs exhibited selective complementary binding. Despite the reduced stability of hybrids formed between SBC and normal ODNs, the normal Watson strand showed no preference for the normal Crick over the SBC Crick strand when equimolar of these three strands were mixed simultaneously at room temperature; about equal amount of duplexes I and IX were formed (Fig. 5, lane 13). Additionally, there was little, if any, strand displacement or strand exchange when the pre-formed DNA–SBC duplex IX was incubated with the normal homolog of the SBC strand or with SBC–DNA duplex VIII; not much single-stranded SBC was formed (Fig. 5, lanes 12 and 14). These data clearly demonstrate that the SBC ODNs described above behaved like natural ODNs when hybridized to unmodified complements, yet did not form stable hybrids with themselves.

Strand invasion of a DNA duplex

To determine whether an SBC ODN could strand invade dsDNA, a 17 base pair segment of hybrid X was synthesized as a single self-complementary ODN (XIII; Fig. 6A). Linking the complementary domains into one ODN was expected to improve the kinetics of strand invasion and the stability of the product. The chimeric SBC ODN had a T_m of 31 °C and hybridized to a partial DNA complement (Watson in XI) at room temperature (Fig. 6B, lane 4). The corresponding unmodified ODN (XII), derived from hybrid I, had a T_m of 80 °C and hybridized poorly to the same DNA complement (Fig. 6B, lane 3). A 48 bp duplex (XI) with one end homologous to the self-complementary ODNs was used as a substrate for strand invasion. Annealing was facilitated by the presence of two 5 base long single-stranded overhangs in XI which could hybridize to complementary four base long overhangs in the invading ODNs. The duplex can be likened to the stem of a hairpin that might exist in a long ssDNA. After 4 h at room temperature, a 10-fold excess of XIII converted 73% of XI to a three-way junction compared with 17% for XII (Fig. 6B, lanes

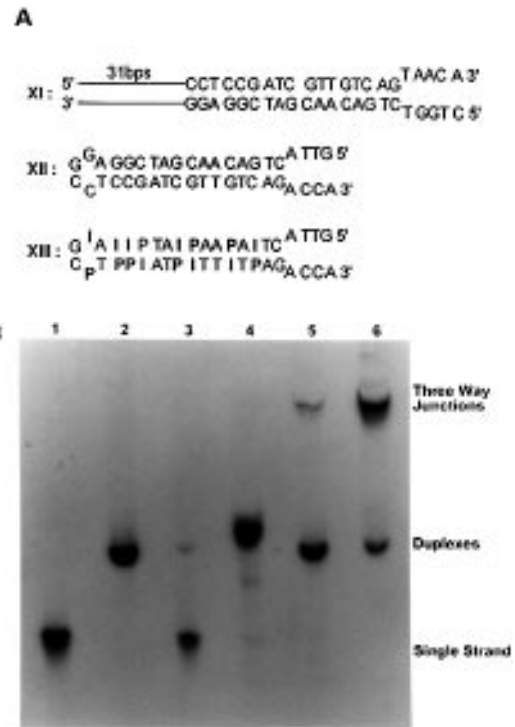


Figure 6. Gel mobility shift analysis of the strand invasion properties of normal and SBC self-complementary ODNs. (A) Sequences of the dsDNA duplex (XI) and the strand invading normal (XII) and SBC (XIII) self-complementary ODNs. Employing the convention of Table 1, the upper and lower strands of XI are Crick and Watson, respectively. (B) Gel mobility shift analysis of strand invasion. Reactions were incubated 4 h at room temperature in TNM buffer prior to electrophoresis. Unless otherwise indicated, hybrids were formed by mixing the labeled ODN (0.1 μM) with a 2-fold molar excess of cold complementary ODN. Single-strand (*W in XI) and double-strand (*W/C in XI) standards were run in lanes 1 and 2. Hybridization reactions between free *W strand of duplex XI and self-complementary ODNs XII or XIII (molar ratio 1:10) were analyzed in lanes 3 and 4. Strand invasion reactions between duplex XI (*W/C) and self-complementary ODNs XII or XIII (molar ratio 1:10) were analyzed in lanes 5 and 6.

6 and 5). Ongoing studies indicate that the self-complementary SBC ODN has a significant kinetic advantage over the unmodified ODN.

Conclusions

Based on thermal denaturation and non-denaturing gel mobility shift assays, we have designed and synthesized for the first time modified ODNs which exhibit selective complementary hybridization. A self-complementary version of one of these paired ODNs strand invaded a homologous double-stranded DNA better than the corresponding unmodified ODN. The possible diagnostic and therapeutic uses of these ODNs are being explored. Efforts to improve the hybridization properties of SBC ODNs including the modification of dA and dT are also underway.

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