Oligonucleotides Containing 2-Aminoadenine and 2-Thiothymine Act as Selectively Binding Complementary Agents

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ABSTRACT: A pair of complementary oligodeoxynucleotides (ODNs) uniformly substituted with 2-aminoadenine (A′) in place of adenine and 2-thiothymine (T′) in place of thymine did not hybridize to each other but did form very stable hybrids with unmodified complementary ODNs. These unusual properties were a consequence of the hydrogen-bonding properties of the two base analogs. Thermal denaturation studies of short duplexes which contained these bases demonstrated that the A′-T and A-T′ doublets formed stable base pairs whereas the A′-T′ doublet acted like a mismatch. Complementary ODNs substituted with these base analogs are referred to as SBC or selectively binding complementary ODNs. When used as a pair, these single-stranded ODNs invaded the ends of homologous duplexes and formed stable three-arm junctions under conditions where unmodified ODNs failed to give a product. SBC ODNs have a fundamental thermodynamic advantage in hybridizing to short segments of double-stranded nucleic acid and represent a new approach for the design of oligomeric probes and antisense agents. Many secondary structure features present in long single-stranded nucleic acids should be accessible to these reagents.

The use of oligodeoxynucleotides (ODNs)[1] as diagnostic probes and antisense agents is based upon the Watson−Crick base pairing of complementary nucleic acid sequences. These same hydrogen-bonding interactions also occur in long single-stranded DNA or RNA molecules, where they result in the formation of short, usually imperfect duplexes which can interfere with the hybridization of ODNs (Gamper et al., 1987; Chastain & Tinoco, 1993). Although numerous approaches have been described for the design of ODNs which overcome or exploit the natural tendency of DNA or RNA to base pair with itself, none provides a general solution to the problem.

Modified ODNs which form very stable hybrids have frequently been used to improve the efficiency of hybridization to single-stranded nucleic acid. Examples include ODNs with 2′-modified (Monia et al., 1993; Sproat & Lamond, 1993), N3′ → P5′ phosphoramidate (Gryaznov & Chen, 1994), and peptide (Nielsen et al., 1994) backbones, ODNs containing base analogs such as 2-aminoadenine (Lamm et al., 1991) or C5 propynylpyrimidines (Wagner et al., 1993), or ODNs conjugated to an intercalating agent (Asseline et al., 1984) or a minor groove binding agent (Afonina et al., 1996). However, none of these modifications guarantees efficient hybridization if the targeted sequence is already substantially base paired to another sequence in the same molecule.

Hybridization strategies which rationally exploit specific sequence or structural elements within a single-stranded target have also been described. Circular ODNs capable of triple-strand formation with a homopurine or homopyrimidine run exhibit enhanced binding affinities relative to control ODNs which only form a duplex (Prakash & Kool, 1991; Wang & Kool, 1994). Tethered ODNs complementary to two single-stranded sequences in close proximity to one another (Richardson & Schepartz, 1991) or separate ODNs which bind to contiguous sequences (Kutyavin et al., 1988) can exhibit cooperative binding. Localized hairpins are frequently found in long single-stranded nucleic acids and can be rationally targeted. Formation of a pseudoknot by hybridization of an ODN to a hairpin loop can significantly enhance hybrid stability (Ecker et al., 1992; Lima et al., 1992). Alternatively, an ODN can hybridize to the two single-stranded arms of a hairpin (Francois et al., 1994). In the special case where the hairpin stem contains a homopurine run, a single ODN can bind to both the stem and one of the flanking single-stranded arms (Brossalina & Toulme, 1993; Francois & Helene, 1995).

We describe the hybridization and strand invasion properties of a new class of modified ODNs designed to be used as paired complements. Substituted with 2-thiothymine and 2-aminoadenine, these selectively binding complementary (SBC) ODNs form very stable hybrids with complementary, unmodified sequences, yet they do not interact with each other. Hybridization of a complementary pair of single-stranded SBC ODNs to both strands of a short homologous DNA or RNA duplex should be kinetically favored since the pathway does not involve the formation of an intermediate Holliday junction and thermodynamically favored due to an increase in the number of base pairs (see Figure 1). As a consequence, we show that two SBC ODNs (but not the corresponding unmodified ODNs) strand invade both blunt-ended and recessed duplexes to form stable three-arm junctions. These model experiments suggest that the hybridization of paired SBC ODNs to long single-stranded

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[1] Abbreviations: A′, 2-aminoadenine; CPG, controlled pore glass; HPLC, high-performance liquid chromatography; ODN, oligodeoxynucleotide; SBC, selectively binding complementary; TAJ, three-arm junction; T_m, melting temperature; T′, 2-thiothymine.

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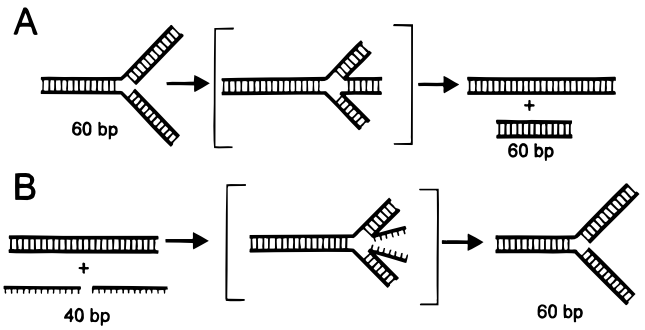

FIGURE 1: (A) Unmodified three-arm junction undergoes branch migration to yield two duplexes. During branch migration, crossover of strands occurs within a Holliday junction. In the presence of $MgCl_2$, the step time for branch migration is relatively long [hundreds of milliseconds (Panyutin et al., 1995)] because the four double-stranded arms of the junction stack to form two colinear helices (Lilley & Clegg, 1993). Branch migration in either direction preserves total base pairing. (B) Two SBC ODNs strand invade a longer homologous duplex to form a stable three-arm junction. Each SBC ODN is complementary to one strand of the duplex. Since the crossover junction contains three double-stranded and two single-stranded arms, its movement should be more facile than that of the highly structured Holliday junction. Strand invasion is driven by the formation of new base pairs. The total number of base pairs is indicated for the starting and ending states of the DNA, assuming each junction has three 20 bp long arms.

DNA or RNA should be less inhibited by the presence of secondary structure than a regular ODN.

MATERIALS AND METHODS

5′-*O*-(Dimethoxytrityl)-2-thiothymidine−3′-*O*-(2-cyanoethyl)-*N*,*N*′-diisopropylphosphoramidite was prepared using the procedure of Connolly and Newman (1989). 2,6-Diaminopurine 2′-deoxyriboside was synthesized as described by Fathi et al. (1990). 5′-*O*-(Dimethoxytrityl)-N^2,N^6-bis(phenoxyacetyl)-2,6-diaminopurine deoxyriboside−3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite was prepared essentially as described for the 2′-*O*-allyl analog (Sproat et al., 1991). A polystyrene support for DNA synthesis (Primer Support, Pharmacia) was modified with hexanol according to the procedure described for hexanol CPG (Gamper et al., 1993).

Oligonucleotide Synthesis. SBC ODNs were synthesized on a Pharmacia OligoPilot DNA synthesizer in 10 μmol scale using hexanol Primer Support. Deprotection and detachment from the solid support was accomplished with concentrated ammonia at 40 °C for 15 h. The remaining second phenoxyacetyl group on 2,6-diaminopurine residues was removed with a mixture of hydrazine/ethanolamine/methanol (1:5:5, v/v/v) (Polushin & Cohen, 1994). Purification of trityl-on ODNs was performed on a Hamilton PRP-1 (7.0 × 305 mm) reverse phase column employing a gradient of 5 to 40% acetonitrile in 0.1 M $NaClO_4$ (pH 7). After detritylation with 80% acetic acid for 15 min at room temperature, the ODNs were precipitated by addition of a 2% solution of $NaClO_4$ in acetone.

Enzymatic Digestion. To determine the stability of the modified bases to the deprotection conditions and evaluate the purity of synthesized ODNs, about 20 μg of each ODN was digested to nucleosides by a mixture of phosphodiesterase I, DNase I, and alkaline phosphatase. The hydrolysates were analyzed by reverse phase HPLC using a C18 (2 × 150 mm) column and a Waters 994 photodiode array detector. We detected less than 5% of the impurities derived from the modification of 2-thiothymine and 2,6-diaminopurine bases.

Thermal Denaturation Studies. Hybrids formed between complementary ODNs were melted at a rate of 0.5 °C/min in 200 mM NaCl, 0.1 mM EDTA, and 10 mM Na_2HPO_4 (pH 7.0) in a Lambda 2 (Perkin-Elmer) spectrophotometer equipped with a PTP-6 automatic multicell temperature programmer. Each ODN was mixed with an equimolar amount of complement to give a total strand concentration of 8×10^{-7} M. Prior to melting, samples were denatured at 100 °C and then cooled to the starting temperature over a 10 min period. The melting temperatures (T_m values) of the hybrids were determined from the derivative maxima. Free energies were calculated according to a "two-state" model by minimization of mean square errors between the calculated and experimental melting curves (Petershiem & Turner, 1983).

Gel Mobility Shift Assays. Sequential hybridization and strand invasion experiments were conducted at room temperature (25−27 °C) in 200 mM NaCl, 0.1 mM EDTA, and 10 mM Na_2HPO_4 (pH 7.0) as described in the figure legends. The concentrations of invading 20-mers and target duplex strands were 2.5×10^{-6} and 5×10^{-7} M, respectively. Prior to use, ODN **3** was 5′ end-labeled with ^{32}P using polynucleotide kinase and [γ-^{32}P]ATP. The final reaction volumes were 20−200 μL. Aliquots (5 μL) were removed at specific times into 5 μL volumes of cold dyes, ficoll and 2 mM $MgCl_2$, quickly frozen in a dry ice bath, and stored at −20 °C until they were analyzed. Immediately prior to loading onto a gel, each aliquot was thawed in an ice bath. Electrophoresis was conducted in a precooled 8% nondenaturing polyacrylamide gel (0.04 × 20 × 40 cm) containing 89 mM Tris-borate (pH 8.3), 2 mM EDTA, and 3 mM $MgCl_2$ for 4 h at 10 W. The dried gel was visualized by autoradiography, and bands were quantified using a BioRad GS-250 phosphorimager. Control studies showed that storage of aliquots prior to electrophoretic analysis did not alter the distribution of products.

RESULTS

Design of SBC ODNs. Selectively binding complementary ODNs are defined as self-complementary ODNs or pairs of complementary ODNs which do not interact with each other under physiological conditions and so exist as single-stranded molecules. While unable to base pair to each other, every SBC ODN can by design hybridize to a complementary DNA or RNA sequence with no mismatching. The hybrids so formed exhibit stabilities comparable to or greater than those of regular hybrids. These unique properties should promote strand invasion of paired SBC ODNs into a segment of double-stranded DNA or RNA. The kinetics of the reaction should be accelerated due to the reduced likelihood of forming transitory Holliday junctions, while the free energy of the complex should be reduced by the creation of additional base pairs. Figure 1 shows the stable three-arm junction formed when using SBC ODNs to strand invade a duplex. Also shown is the spontaneous decay of the corresponding unmodifed three-arm junction. These reactions will be described more fully once the experimental results have been presented.

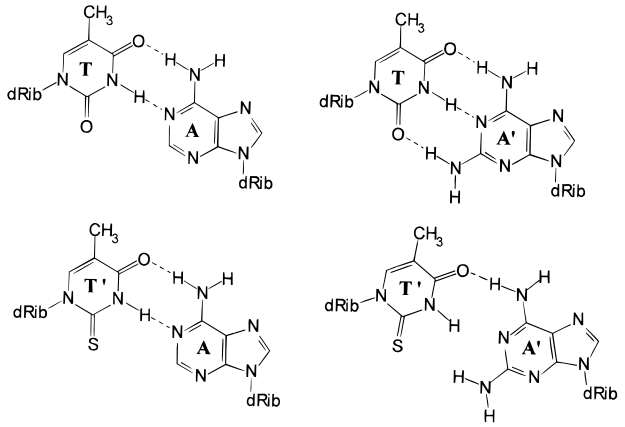

FIGURE 2: Base-pairing schemes for Watson−Crick doublets between adenine or 2-aminoadenine and thymine or 2-thiothymine.

Complementary 20-mers

ODN	Bases	Sequence
1a	A and T	5′-GTAAGAGAATTATGCAGTGC
1b	A′and T′	5′-GT′A′A′GA′GA′A′T′T′A′T′GCA′GT′GC
1c	A′and T	5′-GTA′A′GA′GA′A′TTA′TGCA′GTGC
1d	A and T′	5′-GT′**AAGAGAAT**′T′AT′GCAGT′GC
2a	A and T	5′-GCACTGCATAATTCTCTTAC
2b	A′and T′	5′-GCA′CT′GCA′T′A′A′T′T′CT′CT′T′A′C
2c	A′and T	5′-GCA′CTGCA′TA′A′TTCTCTTA′C
2d	A and T′	5′-GCACT′GCAT′**AAT**′T′CT′CT′T′AC

Double-Stranded 40-mers

ODN	Sequence
3	3′-ATAGTGAGTACCAATACCGT**CGTGACGTATTAAGAGAATG**
4	5′-TATCACTCATGGTTATGGC**AGCACTGCATAATTCTCTTAC**
3	3′-ATAGTGAGTACCAATACCGT**CGTGACGTATTAAGAGAATG**
5	5′-TATCACTCATGGTTATGGC**AGCACTGCATAATTCT**

FIGURE 3: Oligonucleotides used in this study. **1a** and **2a** are normal (unmodified) ODNs, while **1b** and **2b** are SBC ODNs. The bold portions of hybrids **3**−**4** and **3**−**5** are homologous to ODNs **1a** and **2a**. All of the 20-mer ODNs were synthesized on a hexanol Primer Support, leading to the introduction of a 3′ hexanol phosphate cap. Use of a modified polystyrene support permitted the synthesis of SBC ODNs with 3′ terminal A′ or T′ bases.

To accomplish the design goals, SBC ODNs were synthesized by substituting 2-aminoadenine (A′) for adenine (A) and 2-thiothymine (T′) for thymine (T). Figure 2 shows the hydrogen-bonded pairs formed by these bases. The A′-T base pair possesses an extra hydrogen bond relative to A-T (Howard & Miles, 1984) and is frequently used to stabilize nucleic acid hybrids (Azhikina et al., 1993; Sproat & Lamond, 1993). The A-T′ base pair exhibits the same hydrogen-bonding pattern as A-T, and its introduction into a hybrid usually increases the T_m by a small amount (Connolly & Newman, 1989; Newman et al., 1990; Kuimelis & Nambiar, 1994). By contrast, the A′-T′ doublet should act like a mismatch. Model building indicates that steric clash between the 2-thio group of thymine and the 2-amino group of adenine tilts the bases relative to each other, thereby allowing only one hydrogen bond to form.

Hybridization Properties of SBC ODNs. On the basis of the hydrogen-bonding properties of 2-aminoadenine and 2-thiothymine, we expected complementary ODNs which contained these bases to exhibit SBC properties. For the studies described here, two complementary 20-mer sequences (60% A/T) were synthesized with regular or modified adenine and thymine bases to generate the eight ODNs listed in Figure 3. Each of the 16 possible hybrids was formed in

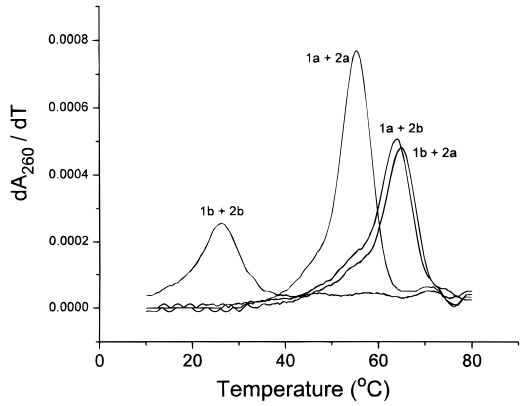

FIGURE 4: First-derivative melting curves of representative hybrids.

10 mM Na_2HPO_4 (pH 7.0) buffer containing 200 mM NaCl and 0.1 mM EDTA and assayed by ultraviolet monitored thermal denaturation.

Representative first-derivative melting curves are shown in Figure 4. The DNA−DNA hybrid (**1a**−**2a**) had a T_m of 55 °C, whereas the two alternative SBC−DNA hybrids (**1a**−**2b** and **1b**−**2a**) had T_m values approximately 10 °C higher. The relatively sharp, symmetric peaks obtained for the SBC−DNA hybrids indicated that A′-T and A-T′ base pairs did not alter the cooperativity of hybridization. The SBC−SBC hybrid (**1b**−**2b**) had a T_m of 26 °C, nearly 30 °C lower than that of the DNA−DNA hybrid and 40 °C lower than those of the two SBC−DNA hybrids. Above approximately 35 °C, the two complementary SBC 20-mers coexisted as single-stranded molecules capable of forming stable fully base-paired hybrids with normal complements. By these criteria, they met our definition of selectively binding complementary ODNs. The destabilizing effect of a A′-T′ doublet relative to an authentic mismatch was estimated in separate experiments which showed that the T_m of the control duplex (**1a**−**2a**) was on average depressed 2.4 °C for each introduction of a A′-T′ doublet versus 6.3 °C for each introduction of a T-T mismatch.

The free energy ($\Delta G°$) of each hybrid was determined using the two-state approximation for melting (Petershiem & Turner, 1983). These values are summarized together with the T_m results in Table 1. At 37 °C, the two SBC−DNA hybrids (**1a**−**2b** and **1b**−**2a**) were 1 kcal/mol more stable than the DNA−DNA hybrid (**1a**−**2a**) and 11 kcal/mol more stable than the SBC−SBC hybrid (**1b**−**2b**). On the basis of these differences, the equilibrium binding constant for the SBC−DNA duplexes was nearly 8 orders of magnitude greater than that for the SBC−SBC duplex. The free energy values for the other hybrids in Table 1 were as anticipated and further confirmed that the A′-T, A-T′, and A′-T′ base juxtapositions, respectively, stabilized, had little effect on, or destabilized the duplex under study.

Formation and Stability of Mobile Junctions. The hybridization properties of SBC ODNs **1b** and **2b** should favor the formation of stable, mobile three-way junctions between these ODNs and longer unmodified duplexes which contain the same sequences at one end. Two 40-mer hybrids (see Figure 3) were prepared to test this assumption, one with a blunt end (hybrid **3**−**4**) and the other with a five-base long recessed end (hybrid **3**−**5**). Three-arm junctions were formed two different ways. In the first protocol, each member strand of the longer duplex was separately hybrid-

Table 1: Melting Transition Temperatures and Free Energies for Hybrids Substituted with 2-Aminoadenine and/or 2-Thiothymine Base Analogs[a]

hybrid	A-T doublets[b]	T_m[c] (°C)	$-\Delta G°_{37}$[c] (kcal/mol)	$-\Delta G°_{60}$[c] (kcal/mol)
1a−**2a**	A-T	55	16.2	8.1
1b−**2a**	A-T′, A′-T	65	17.3	11.2
1a−**2b**	A-T′, A′-T	63	17.2	10.7
1b−**2b**	A′-T′	26	6.2	0.3
1a−**2c**	A-T, A′-T	59	17.4	9.3
1a−**2d**	A-T, A-T′	58	16.6	9.4
1b−**2c**	A′-T, A′-T′	51	13.2	7.0
1b−**2d**	A-T′, A′-T′	41	10.7	4.0
1c−**2a**	A-T, A′-T	60	18.8	10.0
1c−**2b**	A′-T, A′-T′	42	10.8	4.2
1c−**2c**	A′-T	66	20.3	12.1
1c−**2d**	A-T, A′-T′	35	8.6	2.3
1d−**2a**	A-T, A-T′	57	13.6	9.5
1d−**2b**	A-T′, A′-T′	47	11.3	6.9
1d−**2c**	A-T, A′-T′	41	10.1	5.4
1d−**2d**	A-T′	61	14.1	10.0

[a] Determined in 200 mM NaCl, 0.1 mM EDTA, and 10 mM Na_2HPO_4 (pH 7.0) with a 16 μM total nucleotide concentration. [b] The presence of A-T, A′-T, A-T′, and A′-T′ doublets in each hybrid is indicated. [c] Each reported value for T_m and free energy is an average of at least three separate experiments; uncertainties in T_m values and in free energies are estimated at ±1.0 °C and ±15%, respectively.

ized to the complementary "invading" 20-mer, after which both partial hybrids were combined to form a three-arm junction. Sequential hybridization permitted the formation of otherwise unstable junctions involving unmodified ODNs. In the second protocol, the longer duplex was preformed and then incubated at room temperature with the two invading 20-mers. The high T_m values of hybrids **3**−**4** and **3**−**5** (respectively, 69 and 68 °C) were expected to ensure that any three-arm junction would have been generated by strand invasion. Both SBC (**1b** and **2b**) and unmodified (**1a** and **2a**) ODNs were tested for branched molecule formation.

A gel mobility shift analysis was used to detect three-arm junction formation with the blunt-ended hybrid (Figure 5). Sequential hybridization using the SBC 20-mers generated a good yield of the three-arm junction (lane 5), whereas the same protocol using normal 20-mers generated much less of the desired junction (lane 3). Substitution of 2-aminoadenine for adenine in the unmodified ODNs to promote the formation of more stable branched molecules did not improve the yield (lane 4). Sequential hybridization using only one SBC or normal 20-mer failed to generate any branched product (lanes 7 and 8). The joint molecule formed in these reactions appears to have undergone spontaneous branch migration to yield a single-stranded 20-mer and a double-stranded 40-mer (Radding et al., 1977).

The stability of the three-arm junction depended upon whether normal or SBC 20-mers were used in the sequential hybridization protocol. When the junction contained unmodified ODNs, branch migration required many hours to resolve the four component strands into separate 40-mer and 20-mer duplexes (data not shown). The smear in lanes 3 and 4 of Figure 5 is attributable to this reaction occurring during the course of electrophoresis. By contrast, the three-arm junction formed using SBC ODNs was very stable and did not undergo branch migration (lanes 5 and 6 in Figure 5). This was expected since resolution of the junction would have been accompanied by a reduction in the total number of base pairs.

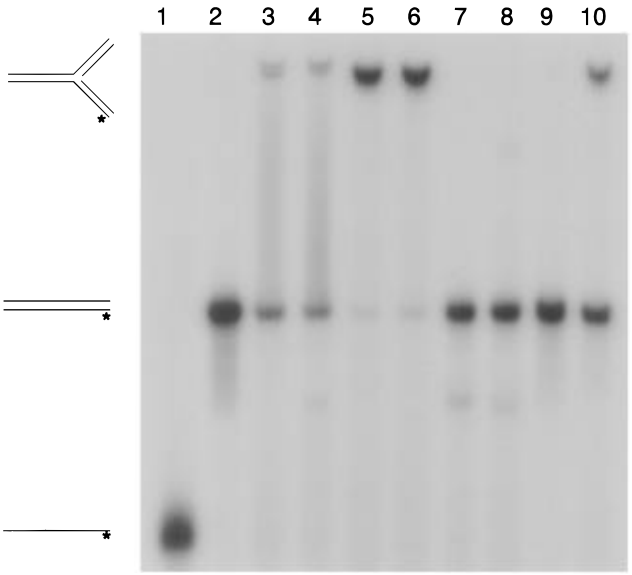

FIGURE 5: Gel shift analysis of branched molecules formed by the interaction of normal or SBC 20-mers with the blunt-ended duplex **3**−**4**. Reactions were carried out at room temperature (25−27 °C) in the standard buffer. ODN **3** was end-labeled and present at a final concentration of 5×10^{-7} M. When used in forming a branched molecule in this or other experiments, the molar ratio of ODNs **3**, **4**, **1a**−**c**, and **2a**−**c** was 1:1:5:5. Intermediate hybrids used in the sequential hybridization protocols were formed by incubating the respective strands for at least 10 min. Controls: lane 1, ODN **3**; lane 2, duplex **3**−**4**. Sequential hybridization of ODNs to form a three-arm junction followed by a 15 min incubation: lane 3, duplex **3**−**2a** added to duplex **4**−**1a**; lane 4, duplex **3**−**2c** added to duplex **4**−**1c**; lane 5, duplex **3**−**2b** added to duplex **4**−**1b**. Sequential hybridization of ODNs to form a three-arm junction followed by a 12 h incubation: lane 6, duplex **3**−**2b** added to duplex **4**−**1b**. Sequential hybridization to form a D-loop-like molecule: lane 7, ODN **4** added to duplex **3**−**2a**; lane 8, ODN **4** added to duplex **3**−**2b**. Strand invasion of duplex **3**−**4** to form a three-arm junction (12 h incubation): lane 9, ODNs **1a** and **2a**; lane 10, ODNs **1b** and **2b**. At the completion of each reaction, an aliquot was quickly frozen in dry ice and stored at −20 °C until it was analyzed by electrophoresis in a precooled (10 °C) 8% nondenaturing polyacrylamide gel run at room temperature. The closely spaced doublets in lanes 3 and 4 probably represent different conformers of the same three-arm junction; such doublets have been reported previously (Zhong et al., 1994).

A thermostability experiment showed that 50% of the three-arm junction formed between the SBC 20-mers and the blunt-ended 40-mer hybrid had resolved into the component duplex and single strands at 62 °C (Figure 6). This apparent T_m was only somewhat lower than the T_m of the blunt-ended 40-mer duplex in the same buffer and explained why the amount of three-arm junction increased above 65 °C. At those temperatures, both the three-arm junction and the underlying 40-mer duplex underwent denaturation. When aliquots of these solutions were rapidly cooled for storage, the three-arm junctions reformed.

Strand invasion of preformed hybrids was only observed with the SBC ODNs. These complementary ODNs formed stable three-arm junctions with both the blunt-ended hybrid (Figure 5, lane 10) and the recessed hybrid (Figure 7, left lanes 1−5). By providing an annealing site for one of the SBC ODNs, the recessed hybrid was more rapidly invaded. When incubated with the same hybrids, normal ODNs were devoid of strand invasion activity (Figure 5, lane 9; Figure

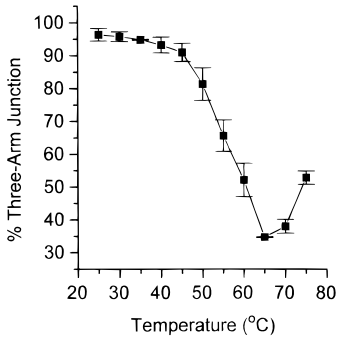

FIGURE 6: Thermostability of the three-arm junction formed between the blunt-ended duplex **3**−**4** and paired SBC 20-mers. The three-arm junction was formed by sequential hybridization of SBC ODNs **1b** and **2b** with duplex strands **3** and **4** as described in Figure 5. The temperature of the solution containing the junction was then raised in 5 °C increments starting from 25 °C. After 10 min of incubation at each temperature, an aliquot was quickly frozen in dry ice and stored at −20 °C while awaiting gel analysis. Bands corresponding to the three-arm junction and 40-mer duplex were detectable. The percent of three-arm junction was determined using a phorphorimager. Identical results were obtained when reaction aliquots were immediately run on a gel without prior storage.

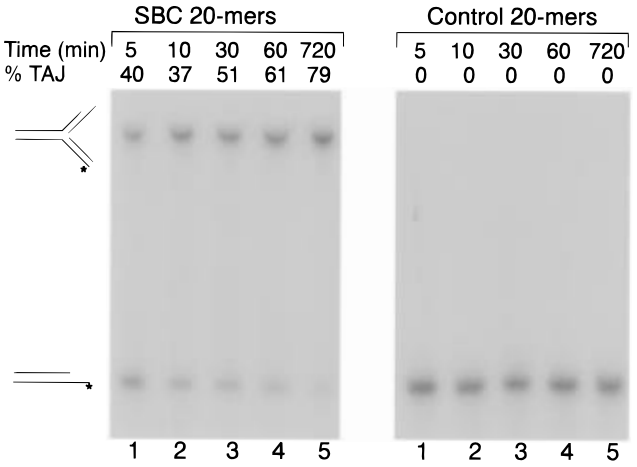

FIGURE 7: Strand invasion of the recessed duplex **3**−**5** by paired SBC or normal 20-mers. Reaction conditions and electrophoretic analysis were as described in Figure 5. The **3**−**5** duplex (4×10^{-7} M) was incubated with a 5-fold molar excess of normal ODNs **1a** and **2a** or SBC ODNs **1b** and **2b**. Aliquots were removed at the indicated times to determine the extent of strand invasion. The percent yield of three-arm junction was determined by phosphorimage analysis of the dried gel: left lanes 1−5, SBC ODNs; right lanes 1−5, normal ODNs. TAJ = three-arm junction.

7, right lanes 1−5). This was not surprising since these complementary ODNs hybridized to each other.

DISCUSSION

The single-stranded character of paired SBC ODNs is key to their remarkable strand invasion properties. At the concentrations used here, paired SBC ODNs can readily anneal to a homologous duplex which contains a single-stranded overhang or a transiently frayed blunt end. Invasion of the duplex by both ODNs follows. Use of a single self-complementary SBC ODN would reduce the molecularity of the annealing step and further promote strand invasion. In either case, strand invasion is energetically favored by the creation of one additional base pair for each step of invasion and may be kinetically favored by the unorthodox structure of the branch migration intermediate. Normally, strand exchange between two duplexes occurs within the context of a Holliday junction. In the presence of $MgCl_2$, the four double-stranded arms of a Holliday junction form two quasicontinuous helices (Lilley & Clegg, 1993) which retard branch migration (Panyutin & Hsieh, 1994; Panyutin et al., 1995). By contrast, the unusual junction formed when using SBC ODNs contains three double-stranded and two single-stranded arms (see the intermediate in Figure 1B). We hypothesize that the absence of a fourth duplex arm will increase conformational freedom at the crossover point and promote strand invasion both in the presence and in the absence of $MgCl_2$. The rate and extent of strand invasion observed here would have been improved by conducting the experiments at 37 °C instead of at room temperature. At the higher temperature, the paired SBC ODNs would have been totally single-stranded and the ends of the duplex more frayed.

Using two different protocols, the paired SBC ODNs formed stable, mobile three-arm junctions whereas the paired unmodified ODNs did not. While the SBC-containing junctions were slightly more stable than the unmodified junctions due to the presence of A′-T base pairs, we believe that the susceptibility of three-arm junctions to resolution is primarily determined by the relative base pairing count of branched versus linear associations of the strands. Extrusion of two SBC ODNs from a three-arm junction would be accompanied by a reduction in the total number of base pairs, making the branched molecule the more stable species despite the presence of a junction. On the other hand, extrusion of two normal ODNs from a three-arm junction should occur with no loss of base pairing. In this more typical situation, the three-arm junction would be less stable than the two component linear duplexes (Lu et al., 1991; Leontis et al., 1994) and susceptible to resolution (Panyutin & Hsieh, 1993).

For optimal strand invasion of a short duplex, each of the two SBC ODNs should form a hybrid with the respective complementary strand which is fully base-paired and has equal or greater stability than the starting duplex. Clearly, SBC ODNs with A′ and T′ substitutions meet these criteria. By contrast, two complementary ODNs with a limited number of mismatched bases at mutually exclusive positions might not interact with each other and yet form weak hybrids with complements possessing the wild-type sequences. Such ODNs would not likely strand invade and do not meet the definition of SBC ODNs. The robustness of a pair of SBC ODNs can be quantified by comparing the difference in stability between the normal hybrid and the SBC−SBC hybrid to the sum of the stability changes observed when only the Watson or the Crick strand of the hybrid is substituted with an SBC ODN. The greater this difference, the more powerful the SBC properties.

Rules for the design of SBC ODNs using A′ and T′ bases have yet to be defined. On the basis of the free energy values in Table 1, one can assume that each A′-T′ doublet in an SBC−SBC duplex contributes 0.8 kcal/mol of destabilization. This assumption provides a framework for predicting whether a given pair of ODNs can exhibit SBC properties. Both length and sequence will be important factors in determining the T_m of paired oligomeric complements. It is uncertain whether 2-aminoadenine will compromise the specificity of SBC ODNs. Although capable of degenerate

base pairing (Cheong et al., 1988), this analog is used in place of adenine by the S-2L cyanophage (Kirnos et al., 1977; Khudyakov et al., 1978), and the triphosphate of 2-amino-adenine acts as a true analog of ATP in transcription (Rackwitz & Scheit, 1977).

The design of the A′-T′ base pair has taken advantage of the absence of a functional group at the 2 position of adenine. Introduction of an amino group at this position together with substitution of sulfur for oxygen at the same position of thymine has provided two base analogs which permit substituted ODNs to discriminate between modified and natural complements. The design of G/C analogs for use in SBC ODNs must of necessity use different strategies. The synthesis and properties of ODNs substituted with such analogs will be described elsewhere (Woo et al., 1996).

The model strand invasion substrates employed in this study can be likened to the stem of a hairpin which might be found in a long single-stranded nucleic acid. While a traditional ODN is usually ill equipped to deal with such a structure, we have shown that paired SBC ODNs can readily invade a short duplex. In theory, paired SBC ODNs should not pay a penalty for binding to structured regions in single-stranded DNA or RNA due to a net increase in base pairing. By contrast, the binding of a traditional ODN to a long target is inhibited by the presence of secondary structure. From this analysis, it follows that the relative advantage of using paired SBC ODNs over a traditional ODN in hybridizing to a long single-stranded nucleic acid will be determined by the degree of secondary structure at the hybridization site, with localized hairpins providing the greatest advantage.

The inherent stability of RNA duplexes and the ability of RNA to form G-U base pairs favor intramolecular base pairing of these molecules within the cell. As a result, transcripts exist as highly folded, globular structures in which a large fraction of the bases are hydrogen-bonded (Laptev et al., 1993; Uhlenbeck, 1995). The preponderance of double-stranded segments in mRNA may explain why certain sense ODNs can act as antisense agents (Colige et al., 1993; Thierry et al., 1993). Generally, RNA folding programs cannot accurately predict the native structure of such long molecules because each can adopt many alternative conformations with nearly equivalent stabilities. This has hindered the development of antisense technology by making the selection of single-stranded regions in mRNA a largely empirical process.

The results presented here suggest that paired complementary SBC ODNs could be used as effective antisense agents. In this regard, paired SBC ODNs should be more tolerant of secondary structure features than a regular antisense ODN. Furthermore, SBC−RNA hybrids should be stabilized by the known propensity of A′- or T′-containing ODNs to form A-type duplexes (Connolly & Newman, 1989; Newman et al., 1990; Garriga et al., 1993). We have initiated studies using SBC ODNs to strand invade naturally occurring hairpin structures in RNA. Synthesis of SBC ODNs with modified RNA backbones should be chemically straightforward and may potentiate their use as antisense agents.

Besides hybridizing to single-stranded nucleic acids, paired SBC ODNs might also be used to target any sequence in a long double-stranded DNA. In this respect, strand invasion of SBC ODNs into the end of a duplex bears some similarity to the branch capture reaction (Quartin et al., 1989; Weinstock & Wetmur, 1990). The more general case of targeting an internal sequence distant from an end would be feasible if paired SBC ODNs can be used by the recA protein. Others have shown that this recombinase can catalyze the hybridization of both strands of a denatured restriction fragment to the complementary sequences in a duplex substrate (Jayasena & Johnston, 1993; Sena & Zarling, 1993). The resultant double D-loop is a stable structure which survives the removal of recA. While this technique does not work with short oligomeric complements, it might utilize paired SBC ODNs.

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