α−β Chimeric Oligonucleotides Form a New Stable "Snail-like" Structure

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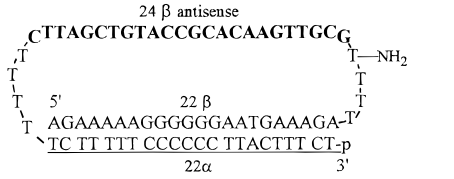
Abstract: The structure formed by a chimeric α−β-oligonucleotide containing the 3′-end α-sequence complementary to the 5′-end β-sequence is described. Chemical strand cross-linking was used to probe the structure of this oligonucleotide. The terminal sequences form a stable parallel duplex, and the internal sequence of the oligonucleotide forms a circle-like loop. This new molecule is resistant to nucleolytic degradation and also promotes efficient RNase H-mediated cleavage of a complementary ribooligonucleotide. Oligonucleotides with this structure therefore should prove useful in the control of gene expression.

Introduction

One of the most promising pharmaceutical applications of oligonucleotides (ODNs) is as modulators of gene expression. Generally speaking an oligonucleotide must possess several properties to be used as a therapeutic agent. Among the most important properties of antisense ODNs are the resistance to nucleolytic digestion and the ability to bind selectively to the target RNA molecule to form substrate efficiently hydrolyzed by RNase H.[1]

The development of antisense and antigene oligonucleotide technology has stimulated the search for new oligonucleotide structures such as hairpins,[2,3] dumbbells,[4] and looped[4,5] and circular[6−11] ODNs. These structures are more resistant to nucleases than natural oligodeoxyribonucleotides.[2,4,6] In addition these molecules display their own specific properties. Circular ODNs are able to form both stable duplexes[6] and triplexes.[7−10] Moreover, these ODNs bind more tightly and selectively to the complementary DNA or RNA single strand than the normal Watson−Crick complement.[7] However, circular oligonucleotides cannot be easily coupled to various groups needed to facilitate their transport through the cell membrane.



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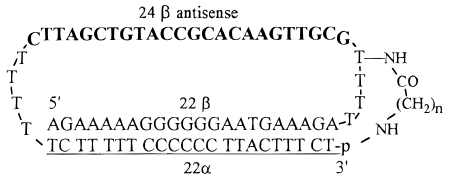

I'

24 β antisense	5′ CTTAGCTGTACCGCACAAGTTGCG	3′	II
27β	5′ AGAAAAAGGGGGGAATGAAAGATTTTT (NH2)	3′	III
22α	5′ TCTTTTTCCCCCCTTACTTTCTp	3′	IV

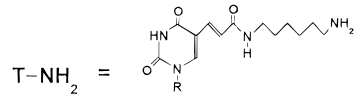

Figure 1. Oligonucleotides under study. R is a sugar residue; p is a phosphate group; $n = 1, 2, 7$. The 27-mer β-oligonucleotide contains the sequence of the 22-mer β-fragment with the addition of five thymidine residues.

In this paper we characterize a new structure formed by a chimeric α−β-oligonucleotide containing a 3′-end α-sequence complementary to its 5′-end β-sequence. Because α-ODNs specifically form parallel duplexes with complementary β-ODNs instead of antiparallel ones,[12] the 3′-end α-sequence of the chimeric α−β-oligonucleotide forms a stable parallel duplex with its 5′-end β-sequence. The inner part of the oligonucleotide forms a circle-like loop (Figure 1). This "snail-like" structure may be considered as a bifunctional molecule: the looped part

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has the features of an antisense, and the double helical part may either form a triplex with a single-stranded DNA or RNA or be used as a decoy for sequence specific DNA binding proteins. This molecule displays a high resistance to nucleases due to the inner duplex formed by the terminal fragments that usually are the most sensitive to nuclease attack.

Experimental Procedures

Oligonucleotide Synthesis. Oligonucleotides were synthesized in an automated DNA synthesizer, Applied Biosystems 391A. Small-scale dN-CPG (Applied Biosystems) was used as a polymer support. 5′-*O*-(4,4′-Dimethoxytrityl)-3-(*N*,*N*-diisopropylamino)-β-cyanoethyl phosphites of 2′-deoxyribonucleosides were from Applied Biosystems. The α-anomeric phosphoramidites were prepared as described earlier.[13] To introduce a deoxythymidine residue having an aliphatic amino group into oligonucleotides **I** and **III**, a modified phosphoramidite, Amino-Modifier C6 dT (Glen Research), was incorporated into oligonucleotides in accord with the routine protocol. 3′-Phosphorylated oligonucleotides **I** and **IV** were prepared using 5′-phosphate-ON (Clontech) as the first monomer in the oligonucleotide synthesis. Synthesized oligonucleotides were deprotected by routine phosphoramidite procedures and purified by reversed-phase HPLC.

Thermal Stability Determination. Absorbance vs temperature curves were recorded at 260 nm using a Hitachi 150-20 spectrophotometer with a thermostated cell. The buffer contained 50 mM 2-morpholinoethanesulfonate (MES) and 20 mM $MgCl_2$; pH was from 4.5 to 6.0. The ODN solutions were heated at 95 °C for 5 min to destroy any secondary structure and annealed to 10 °C. The temperature was scanned at a heating rate of 0.5 °C from 10 to 90 °C. Melting temperatures were obtained from the maximum value of the first-derivative plots of absorbance vs temperature.

Oligonucleotide Strand Cross-Linking. Oligonucleotide strand cross-linking induced by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) was performed in the buffer containing 50 mM MES and 20 mM $MgCl_2$; pH was from 4.5 to 6.0. The 76-member linear oligonucleotide **I** or two linear oligonucleotides **III** and **IV** were lyophilized and dissolved in 30 μL of the buffer to obtain an oligonucleotide concentration of 10^{-3} M per monomer. The reaction solutions were heated to 90 °C and then allowed to cool slowly to 0 °C. Then 3 mg of EDC was added, and the reaction mixtures were incubated for 48 h at 4 °C. ODNs were precipitated by adding 100 μL of 2 M $LiClO_4$ water solution and 1 mL of acetone.

Oligonucleotide strand cross-linking induced by BrCN was performed in the buffer containing 1 M MES, pH 7.6, and 20 mM $MgCl_2$. The 76-member linear oligonucleotide **I** or two linear oligonucleotides **III** and **IV** were lyophilized and dissolved in 30 μL of the buffer to obtain an oligonucleotide concentration of 10^{-4} M per monomer. The reaction solutions were heated to 90 °C and then allowed to cool slowly to 0 °C. Then 3 μL of BrCN solution (5 M solution in acetonitrile, Aldrich) was added, and the reaction mixtures were incubated for 1−5 min at 0 °C. ODNs were precipitated as described above.

Nucleolytic Degradation of Oligonucleotides. Oligonucleotide resistance to nucleolytic degradation was studied in murine DUNNI fibroblast lysate prepared as described earlier.[14] Oligonucleotide labeling was performed by using 10 units of T4 polynucleotide kinase (New England Biolabs) in 70 mM Tris−HCl buffer, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, and [γ-^{32}P]ATP (3000 Ci/mM, Amersham) for 1 h at 37 °C. The ^{32}P-labeled ODNs were incubated in 50 μL of the lysate at 37 °C. At various times (0.25, 0.5, 1, 2, 4, and 18 h), 5 μL aliquots were removed and the reaction was stopped by extraction with phenol−chloroform−isoamyl alcohol (25:24:1). Oligonucleotides were precipitated from aqueous samples by 10 volumes of acetone containing 2% $LiClO_4$, dried, and dissolved in 4 μL of formamide−water (4:1), 0.01% bromophenol blue, and 0.01% xylene cyanol. Samples were analyzed in a 15% denaturing polyacrylamide gel. Degradation of oligonucleotides was quantitated by liquid scintillation counting of radioactive bands corresponding to intact and degradated oligonucleotides.

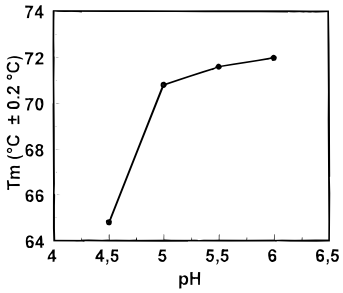

Figure 2. Variation of the thermal stability of the duplex **III**−**IV** in accordance with pH (duplex concentration 1.0 μM). The accuracy of T_m determination is ±0.2 °C.

RNase H-Mediated Cleavage. In order to study RNase H triggering activity of the oligonucleotides **I** and **II**, they were mixed (5 μM concentration) with a complementary 5′-^{32}P-labeled oligoribonucletide (see above) and 37.5 U of *Escherichia coli* RNase H in 200 μL of 20 mM Tris−HCl, pH 7.6, 10 mM $MgCl_2$, 100 mM KCl, and 1 mM dithiotreitol at 37 °C. At the time indicated in Figure 4, 2 μL of 0.5 M EDTA was added to a 20 μL aliquot, and then the hydrolysis products were precipitated by 1 mL of acetone containing 2% $LiClO_4$, dried, dissolved in 4 μL of formamide−water (4:1), 0.01% bromophenol blue, and 0.01% xylene cyanol, and analyzed by electrophoresis in a 20% denaturing polyacrylamide gel. A crude product of the oligoribonucleotide **V** synthesis was used as an RNA marker.

Results

Oligonucleotide Design and Synthesis. We chose a 24-mer β-nucleotide sequence complementary to the translation initiation region of *env* RNA of the Friend murine retrovirus for the chimeric 76-member ODN **I** loop part (Figure 1). The 5′-end 22 β-nucleotides and the complementary 3′-end 22-mer α-nucleotides were designed to form a parallel duplex. In order to decrease structural constraints related to the formation of the inner duplex, tetrathymidylate linkers were introduced between the loop sequence and the terminal duplex-forming sequences. Since the 76-mer **I** may form not only intra- but also intermolecular duplexes, we covalently cross-linked the strands. The structures obtained were then analyzed using electrophoresis in a denaturing polyacrylamide gel. In order to perform the cross-linking, we introduced a phosphate group at the 3′-end and an amino group inside the oligonucleotide chain at the 26th thymidine residue. The 3′-end phosphate was either used directly for the strand cross-linking or previously modified by carboxyl-containing linkers as described below. The β 24-mer **II** having the same sequence as the loop of **I**, the β 27-mer containing the amino group **III**, and the α 22-mer with the 3′-end phosphate **IV** were also synthesized as model compounds (Figure 1).

Thermal Denaturation Experiments. We studied the thermal stability of the duplex formed by the 5′- and 3′-end fragments of the ODN **I** under different concentrations (from 0.28 to 2.7 mM). The data obtained show that a 10-fold increase in ODN **I** concentration does not lead to an increase in the T_m. This is consistent with the denaturation of the intramolecular duplexes.[15,16] At the same time, a concentration increase (from 0.35 to 3.7 mM) for the intermolecular duplex formed by control oligonucleotides **III** and **IV** results in a corresponding increase of the T_m.

As will be explained below, it was necessary to study the influence of pH on the duplex thermal stability before cross-linking the oligonucleotide strands. The data presented in Figure 2 show that the T_m is slightly decreased at the lower pH values.

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Table 1. Efficiency of the Strand Cross-Linking

duplex	3′-end residue	cross-linked duplex yield, % EDC pH 4.5	EDC pH 5.0	EDC pH 5.5	EDC pH 6.0	BrCN pH 7.6
I	phosphate		0			0
	Gly		10			6
	β-Ala		12			7
	7-Ahp		20			8
III-IV	phosphate	0	3	0	3	0
	Gly	18	25	20	24	19
	β-Ala	40	50	45	48	25
	7-Ahp		30		25	23

Nevertheless, the ODNs **III** and **IV** can form stable duplexes even at pH 4.5. This result allows us to consider that the intramolecular duplex formed by the ODN **I** is also stable at pH 4.5−5.5.

Strand Cross-Linking. For cross-linking the snail-like structure, we had no information about the optimal distance between the two functional groups responsible for the cross-linking reaction. We introduced as nucleophile an amino group at the 26th thymidine which may react either directly with the 3′-end phosphate or with a carboxyl group introduced at the phosphate. In this view, we have modified the 3′-end phosphate by amino acids with different numbers of methylene groups [glycine (Gly), *β*-alanine (*β*-Ala) and 7-aminoheptanoic acid (7-Ahp)] using the method of *N*-hydroxybenzotriazole esters.[17,18] We used the duplex **III**−**IV** in order to optimize the coupling conditions. The water soluble 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC) under varied pH (from 4.5 to 6) and BrCN were used as coupling reagents. We did not detect the cross-linked product as the result of the direct interaction of the amino group with the 3′-end phosphate. The reaction between the carboxy and amino groups was most efficient when the EDC was used at pH 5.0 and the ODN **IV** was modified by the *β*-Ala residue (Table 1).

Optimal conditions established for the synthesis of the cross-linked duplex **III**−**IV** (0.5 M EDC concentration, 50 mM MES, pH 5.0, 20 mM $MgCl_2$, 4 °C, 48 h) were used to cross-link duplex **I**. Before cross-linking, the 3′-end phosphate of **I** was modified by the amino acid residues mentioned above. Purification of the cross-linked product **I**′ was carried out using a 15% denaturing gel. The yield of the cross-linked product was found to be about 10, 12, and 20% for the Gly, *β*-Ala, and 7-Ahp, respectively (Table 1).

To confirm that the cross-linked product had the structure corresponding to the 76-member snail-like molecule but not the 152-member dimeric duplex, we compared the migration rate in a 15% denaturing gel of the cross-linked product **I**′ and a circular 76-member oligonucleotide prepared using template-guided chemical ligation[6] (Figure 3). The product **I**′ migrates more slowly than its linear precursor **I** (lanes 1 and 2, Figure 3), but has the same mobility as the circular 76-mer (lane 4, Figure 3). At the same time the cross-linked product migrates like a linear 120-member oligonucleotide which was used as a control of the oligonucleotide length (lane 3, Figure 3). Therefore, the cross-linked product **I**′ is not a 152-member dimeric duplex that would migrate more slowly than the 120-mer (lane 3, Figure 3). These results confirm that the product of the oligonucleotide **I** cross-linking has the snail-like structure.

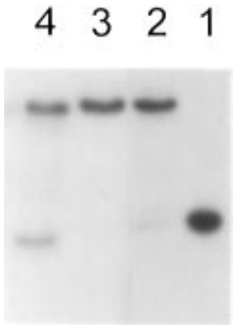

Figure 3. Radioautograph of a 15% denaturing polyacrylamide gel showing the relative mobilities of the linear oligonucleotide **I** (lane 1), the cross-linked product **I**′ (lane 2), a linear 120-mer (lane 3), and a circular 76-mer with traces of the linear precursor (lane 4).

In order to show that this new structure is covalently bound by the linker 3′-*p*-$NH(CH_2)_6CONH$−, we carried out a selective hydrolysis of the **I**′ product phosphoramide bond by acetic acid (15% CH_3COOH, 50 °C, 30 min).[17] The linear starting ODN **I** is formed as a result of the phosphoramide bond hydrolysis (data not shown). This result confirms that **I**′ is obtained from **I** by cross-linking the 3′-end and the thymidine 26.

Nucleolytic Degradation and RNase H Activity. We investigated the resistance of the non-cross-linked α−*β* chimeric 76-mer **I** to nucleolytic digestion in a lysate of murine DUNNI fibroblasts. The oligonucleotide samples were prepared in two different ways: with previous hybridization permitting the ODN **I** to form the inner duplex and without it. The latter sample was heated to 95 °C and then quickly frozen to prevent any secondary structure formation. We also used the 76-member linear *β*-ODN as a control. The half-life of oligonucleotides in this medium is 18 h for the ODN **I** having the snail-like structure, 2 h for the same ODN without structure, and 0.5 h for the control linear 76-member *β*-ODN. These results show that the snail-like structure of the ODN **I** increases significantly its stability in the biological medium compared to both *β* and α−*β*-oligonucleotides without secondary structure. In fact, the presence of the α-fragment at the 3′-end of the linear ODN **I** increases its stability against the nucleolytic degradation by a factor of 4. The stability of the same oligonucleotide with the snail-like structure however is increased by a factor of 9.

It was important to check the ability of the snail-like ODN to form a hybrid duplex with RNA that can be efficiently cleaved by *E.* coli RNase H. Indeed, RNase H plays a key role in the inhibition of gene expression by antisense oligonucleotides.[19] Figure 4 presents the results obtained after the RNase H hydrolysis of the 26-member ribooligonucleotide AGAAUC-GACAUGGCGUGUUCAACGCA (**V**) complementary both to the antisense loop part of the 76-mer **I** and to the linear *β*-ODN **II**. The sequence of this ribooligonucleotide is therefore identical to the targeted translation initiation region of the murine Friend retrovirus *env* RNA. The snail-like oligonucleotide **I** displays the higher activity for RNA digestion when compared to the linear control *β*-ODN **II**. Figure 4 shows that after 2 min of incubation the levels of the target RNA **V** degradation are about 100% and 70% in the presence of the oligonucleotides **I** and **II**, respectively. However, final products formed as a result of RNase H-produced hydrolysis are shorter when triggered by the oligonucleotide **I** (5−8-mers) than by the oligonucleotide **II** (9−11-mers) (Figure 4).

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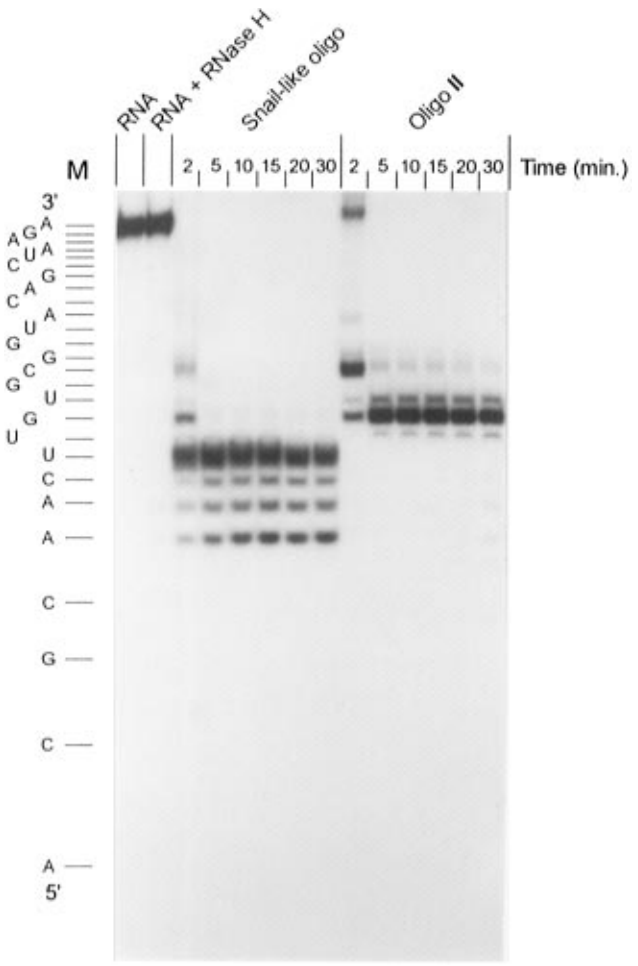

Figure 4. Time dependent *E. coli* RNase H-mediated degradation of the 26-member ribooligonucleotide **V** in the presence of ODNs **I** and **II**. The degradation time is noted at the top of the figure. The sequence of the ribooligonucleotide **V** is shown on the left. M corresponds to the migration of the nonpurified ribooligonucleotide **V** containing all intermediate products of the oligonucleotide synthesis.

Discussion

An oligonucleotide can form a snail-like secondary structure only if the duplex formed by its terminal fragments is parallel (Figure 1). The simplest approach to form such DNA duplexes is to use an α-oligonucleotide as one of the strands. Indeed, these unnatural ODNs have a parallel orientation when they are hybridized to complementary β-strands.[12] Besides, α-oligonucleotides are more resistant to nuclease degradation than their β-counterparts.[20] As 3′-exonucleolytic activities are important in biological *media*, we used the α-fragment for the making of the 3′-end of the ODN **I**.

The snail-like structure may be stabilized by the formation of an intramolecular link between the 3′-end of the oligonucleotide and an aliphatic amino group specifically introduced into the oligonucleotide. Condensation of the phosphate and aliphatic amino groups situated in the different strands of the oligonucleotide duplex was studied using initial conditions developed for chemical ligation in DNA duplexes[21] and employing EDC or BrCN as condensing agents. Two important modifications of the reaction conditions were made. An increased EDC concentration (0.5 M) was used similar to that proposed for the synthesis of oligonucleotide phosphoramidate derivatives in aqueous solutions.[22] The reaction pH was varied since EDC is known to modify thymidine and guanosine residues in single stranded DNA regions.[22] The rate of this reaction is maximal at neutral pH and decreases significantly at acidic pH. Since the snail-like oligonucleotide **I** contains a long single-stranded fragment that could be modified by EDC under pH 6, it was necessary to decrease the pH of the EDC-induced strand cross-linking. Besides, pH 4.5−5.0 is found to be optimal for the reaction of terminal phosphates with amines.[22] A decrease of the pH may disrupt the duplex stability, but in our case the melting experiments showed that the intermolecular duplex **III**−**IV** is stable even at acidic pH. We assumed that the intramolecular duplex **I** is also stable at low pH. These results allowed us to study EDC-induced strand cross-linking in the duplexes **I** and **III**−**IV** under acidic pH where T and G modification is improbable.

We also used BrCN as a cross-linking agent. In the case of chemical ligation using BrCN the reaction time is only 1−3 min (compared to 24−48 h for EDC) and BrCN does not make heterocyclic base modification. Despite these advantages, however, BrCN-induced cross-linking was less efficient compared to EDC (Table 1).

It should be noted that EDC-induced strand cross-linking was efficient only if the 3′-end phosphate was modified by amino acid residues. This may be due to higher reactivity of a carboxyl group compared to phosphate, but the stereochemical factor may be more significant. A flexible amino acid polymethylene chain could indeed provide a more advantageous conformation of the reacting groups. The snail-like structure is rigid and the cross-linking is the most efficient in the case of the longest 7-Ahp linker (Table 1). For the cross-linking of the intermolecular duplex **III**−**IV**, containing a flexible tetrathymidylate chain that does not participate in the duplex formation, the β-Ala linker is the most favorable (Table 1).

Therefore, the following results allow us to conclude that the oligonucleotide **I** has the snail-like structure: (i) The melting temperature study shows that the T_m of duplex **I** does not depend on its concentration while such a dependence is observed for the intermolecular duplex **III**−**IV**. This result is consistent with the intramolecular association of the ODN **I**. (ii) The cross-linking of the 3′-terminal phospate modified by the amino acid residues of the ODN **I** with the amino linker modified thymine 26 indicates that these two positions are close in the secondary structure of the ODN **I**. Covalent bond formation was confirmed by the selective hydrolysis of the phosphoramide bond by acetic acid. (iii) The cross-linked product **I**′ has the same migration rate in denaturing PAGE as a circular oligonucleotide of the equivalent size, which is in agreement with a circle-like structure of the ODN **I** in solution. Such biological properties of the snail-like ODN **I** as its resistance to nuclease degradation and its ability to trigger RNase H-mediated RNA hydrolysis were also investigated.

We observed that the ODN **I** previously hybridized to form a secondary structure was 9 times more stable in the DUNNI fibroblast cellular lysate than the nonstructured ODN **I** and 36 times more stable than the natural oligodeoxyribonucleotide. This result means that the snail-like structure provides an important increase of oligonucleotide resistance to nuclease degradation. On the other hand, the antisense part of the ODN **I** is found to be able to hybridize to the complementary RNA

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strand and thus to trigger the RNase H-mediated cleavage of the RNA (Figure 4). Moreover, the efficiency of this degradation is higher than the one triggered by the nonmodified oligonucleotide **II.** Besides, shorter final products result from the RNase H digestion of the template oligoribonucleotide **V** triggered by ODN **I**. These results allow us to suggest that the conformation of the duplex formed between the oligoribonucleotide **V** and the snail-like oligonucleotide **I** is more favorable for the RNase H activity if compared to the duplex formed by the oligonucleotide **II**. Some additional experiments are in progress in our laboratory in order to confirm this hypothesis.

These properties make snail-like ODNs promising molecules for antisense strategy. The possibility for the double-stranded portion of the molecule to form a triple helix with an appropriate RNA sequence and as a consequence to inhibit gene expression is now under investigation.

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