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Recognition and cleavage of hairpin structures in nucleic acids by oligodeoxynucleotides

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ABSTRACT

The possibility of designing antisense oligodeoxynucleotides complementary to non-adjacent single-stranded sequences containing hairpin structures was studied using a DNA model system. The structure and stability of complexes formed by a 17mer oligonucleotide with DNA fragments containing hairpin structures was investigated by spectroscopic measurements (melting curves) and chemical reactions (osmium tetroxide reaction, copper-phenanthroline cleavage). A three-way junction was formed when the oligonucleotide was bound to both sides of the hairpin structure. When the complementary sequences of the two parts of the oligonucleotide were separated by a sequence which could not form a hairpin, the oligonucleotide exhibited a slightly weaker binding than to the hairpin-containing target. An oligodeoxynucleotide-phenanthroline conjugate was designed to form Watson – Crick base pairs with two single-stranded regions flanking a hairpin structure in a DNA fragment. In the presence of Cu^{2+} ions and a reducing agent, two main cleavage sites were observed at the end of the duplex structure formed by the oligonucleotide – phenanthroline conjugate with its target sequence. Competition experiments showed that both parts of the oligonucleotide must be bound in order to observe sequence-specific cleavage. Cleavage was still observed with target sequences which could not form a hairpin, provided the reaction was carried out at lower temperatures. These results show that sequence-specific recognition and modification (cleavage) can be achieved with antisense oligonucleotides which bind to non-adjacent sequences in a single-stranded nucleic acid.

INTRODUCTION

Selective control of gene expression can be achieved by oligonucleotides complementary to selected sequences on messenger RNAs (1). This so-called 'antisense' strategy rests upon Watson–Crick base pair formation via hydrogen bonding interactions between two complementary nucleic acid sequences. Short-range and long-range interactions in single-stranded nucleic acids, such as mRNAs, make some sequences inaccessible to antisense oligonucleotides. Hairpin structures are often formed between neighboring complementary sequences. Most antisense oligonucleotides have been designed to form Watson–Crick base pairs with regions of mRNAs expected to be single-stranded on the basis of RNA folding programs to predict secondary structures (2–5). Vickers *et al.* have investigated the feasibility of targeting long antisense oligonucleotides (18–29 bases) to the stable RNA stem-loop structure of the TAR region of human immunodeficiency virus (6). They showed that these oligonucleotides were able to influence gene expression by disrupting the RNA secondary structure. Others have also targeted oligonucleotides against open regions in the loop sequence of hairpin structures (7, 8).

Oligodeoxynucleotides consisting of two oligomers linked by a tether have been shown to recognize secondary structures of RNA (9–11). These tethered oligonucleotides bind two single-stranded non-contiguous sites of a structured RNA. Here we describe an alternative way to recognize secondary structures in nucleic acids by designing oligonucleotides that are complementary to two non-adjacent sequences which are brought in close proximity through hairpin formation. Sequence-specific cleavage can be induced in the vicinity of the hairpin structure by a phenanthroline–oligonucleotide conjugate which is complementary to sequences on both sides of the hairpin. This study also shows that two separate sequences of a single-stranded fragment can bind a single oligonucleotide even in the absence of hairpin formation albeit with a lower affinity as compared to two non-adjacent sequences brought in close proximity by hairpin formation.

MATERIALS AND METHODS

Oligonucleotides

Unmodified oligodeoxynucleotides were purchased from the Pasteur Institute (France) and from Eurogentec (Belgium). They were purified by polyacrylamide gel electrophoresis. Their sequences described in Figure 1 were checked by Maxam–Gilbert sequencing (12). Molar extinction coefficients

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of these oligonucleotides were calculated according to reference 13. In order to synthesize the 1,10-phenanthroline-substituted 17mer (OP-17mer), a 5'-thiophosphate was attached to the 17mer oligonucleotide and reacted with (ω-bromopentylamido)-5-phenanthroline as described previously (14).

Melting experiments

The denaturation curves for oligonucleotides were obtained with an UVIKON 820 spectrophotometer. Target oligonucleotides (1 μM) and 17mer (1 μM) were mixed in 50 mM phosphate buffer (pH=7) containing 100 mM NaCl. The temperature was increased at a rate of 0.1°C/mn through a Haake PG 20 temperature programmer, connected with a Haake water circulating bath. The thermal dissociation curves were obtained by subtracting absorbance recorded at 258 nm from one recorded at 540 nm where none of the constituents absorb light. Before each experiment, all samples were heated to 90°C and then cooled slowly to room temperature. All melting transitions were reversible. Melting temperatures (T_m) were taken as the temperature corresponding to half-dissociation of the complexes.

Chemical and enzymatic assays

5'-end labelling was achieved by polynucleotide kinase with γ-^{32}P-radiolabelled ATP (Amersham). Target DNA was digested with P1 nuclease (GIBCO−BRL) using conditions recommended by the manufacturer. Osmium tetroxide was dissolved in distilled water as a 50 mM stock solution. Then, 5'-^{32}P-labelled DNA (10 nM) was incubated with 1 mM osmium tetroxide and 8 % pyridine in 50 mM phosphate buffer (pH=7) containing 100 mM NaCl and 0.5 μg/μl sonicated herring sperm DNA. Following quenching of osmium tetroxide reactions by ether extraction, the DNA was incubated in 1 M piperidine at 90°C for 30 min to cleave osmate adducts, followed by lyophilisation and analysis by denaturing polyacrylamide gel electrophoresis. OP-Cu cleavage of 5'-labelled 35mers and 36mers (10 nM) was obtained after incubation for 30 minutes at 20°C with 17mer or with non-specific 17mer (6 μM) in presence of 5 μM 1,10-phenanthroline, 2 mM β-mercaptopropionic acid, and 2.5 μM $CuSO_4$. In the OP-Cu cleavage reaction performed on 35mers and 36mers, it was important to add a non-specific 17mer as a potential copper chelator (via its phosphate groups). This non-specific 17mer ($^{5'}$ $dTG_3TG_3TG_3TGGTT$ $^{3'}$) was unable to bind to the target DNA fragments used in this report. OP-Cu cleavage reactions were quenched by adding neocuproine (20 μM). After electrophoresis, quantitative analysis of the gels was carried out with a Molecular Dynamics 400S phosphorimager.

Cleavage reactions by oligonucleotide covalently linked to 1,10-phenanthroline

Cleavage of 5'-^{32}P labelled target sequences (10 nM) with OP-17mer (1 μM) was carried out in a 50 mM sodium phosphate buffer (pH=7) containing 0.1 M NaCl. The cleavage reaction was initiated by the addition of 2 mM β-mercaptopropionic acid (MPA), and cupric sulfate (10 μM). After incubation, the reaction was quenched by adding 100 μM 2,9-dimethyl-phenanthroline. The samples were then analyzed by electrophoresis on 20% polyacrylamide/7 M urea, 29:1 cross-linked gels. Autoradiograms were obtained by exposing the gels to Fuji (X-ray) film at −20°C. The extent of cleavage was then analyzed on a LKB laser densitometer. Alternatively, a Molecular Dynamics 400S phosphorimager was used to quantitate the gels.

RESULTS

Recognition of two non-adjacent sequences

Two single-stranded nucleic acid fragments 35 and 36 nucleotides (nt) in length were used as targets for a 17mer oligonucleotide covalently linked to phenanthroline (Figure 1). Both the 35 and 36mer can form a hairpin structure with four G.C base pairs in the stem and four thymines in the loop and were called 35mer-$(CG)_2$ and 36mer-$(CG)_2$. These oligomers differ in sequence by only one nucleotide. Two other single-stranded DNAs (35mer-$(CA)_2$ and 36mer-$(CA)_2$) were also tested. They have the same sequence as the 35mer-$(CG)_2$ and 36 mer-$(CG)_2$ except that G residues in the stem were replaced by A residues (Figure 1). No hairpin structure was expected to be formed by these last two oligomers.

The 17mer oligonucleotide binds to both the 35 and 36mer-$(CG)_2$ with the same melting temperature (Tm) of 25 ± 1°C at 1 μM concentration in a pH 7 buffer containing 50 mM phosphate and 0.1 M NaCl (Figure 2A, 2C). All further experiments were carried out in this buffer. Note that the 17mer can form 17 base pairs with the 36mer-$(CG)_2$ but only 16 with the 35mer-$(CG)_2$. The fact that the double helical region of the 17mer/35mer-$(CG)_2$ complex is interrupted in its center and is missing one base pair in the complex does not influence its stability (Figure 2C).

For comparison with the 17mer/hairpin complexes, the 17mer bound to a 24 mer derived from the 36mer-$(CG)_2$ but lacking the hairpin sequence (12 bases) has a Tm of 45 ± 1°C, emphasizing the destabilizing role played by the intervening hairpin on complex stability (Figure 2B, 2C). A cytosine residue was inserted in the 24mer to introduce a bulged base in the complex. The 17mer binds to this 25mer with a melting temperature of 36.5 ± 1°C which is intermediate between the

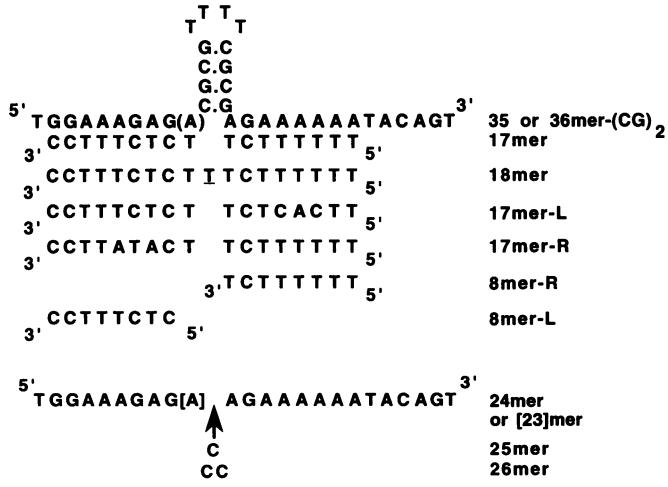

Figure 1. Sequences of oligonucleotides used in this report. The sequence of 36mer-$(CG)_2$, $^{5'}$ d $TG_2A_3GAG(A)CGCGT_4CGCGAGA_6TACAGT$ $^{3'}$ is identical to the 35mer-$(CG)_2$ sequence except for the addition of one adenine (in parentheses) located on the 5'- side of the hairpin structure $(CG)_2$-T_4-$(CG)_2$. In oligonucleotides 35mer-$(CA)_2$ and 36mer-$(CA)_2$, the stem nucleotides $(CG)_2$ are replaced by $(CA)_2$ in order to avoid hairpin formation. The 17mer-L, 17mer-R and 18mer sequences are indicated. The underlined thymine was inserted in the 17mer at the position of the hairpin site to obtain the 18mer. The arrow shows the insertion site of cytosine residues in the 24mer sequence leading to the 25mer and 26mer oligonucleotides. The adenine between brackets in the 24mer sequence was deleted in the 23mer oligomer.

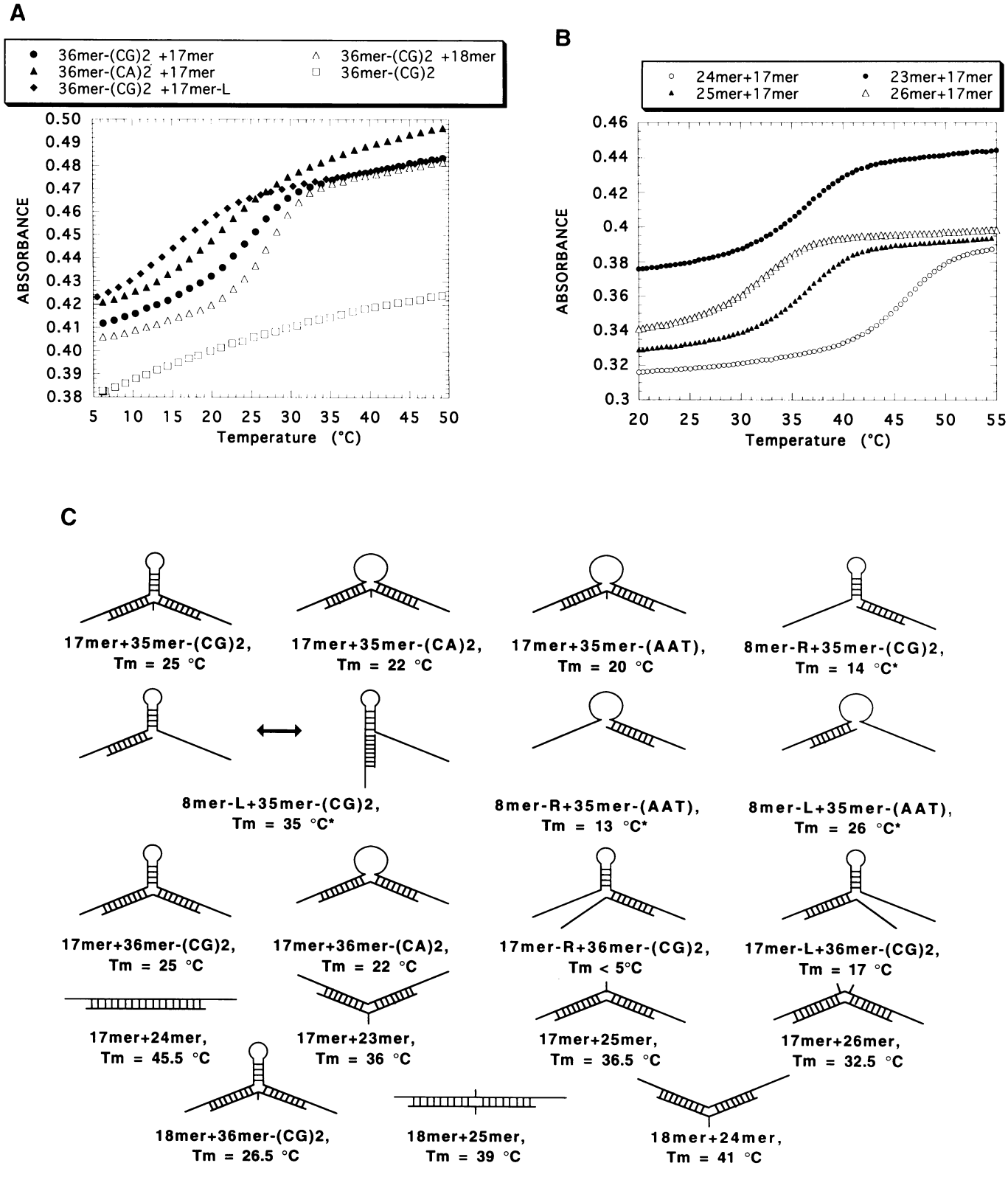

Figure 2. **A**— Melting curves measured at 258 nm for complexes of 17mer, 17mer-L and 17mer-R with hairpin-containing oligonucleotide 36mer-$(CG)_2$ and with oligonucleotide 36mer-$(CA)_2$ containing no hairpin, (36mer-$(CG)_2$, [□]; 36mer-$(CG)_2$ + 17mer, [●]; 36mer-$(CG)_2$ + 17mer-L, [◆] ; 36mer-$(CG)_2$ + 18mer, [△]; 36mer-$(CA)_2$ + 17mer, [▲]). **B**— Melting curves for complexes of 17mer with linear targets (24mer, [○] ; 25mer, [▲] ; 26mer, [△] and 23mer, [●]). **C**— Schematic representation of 17mer-, 18mer-, and 8mer-oligonucleotide complexes studied in this report. Melting temperatures are indicated below each complex at 1 μM concentration except for those indicated by an asterisk (*) where Tm was determined at 10 μM concentration of both oligonucleotides in the duplex.

Tm values of the 24mer/17mer and 35 or 36mer-$(CG)_2$/17mer complexes. It is noteworthy that a 18mer/24mer complex was more stable than the 17mer/25mer complex with a similar hyperchromism, suggesting that thymine insertion in the short oligonucleotide was less destabilizing than insertion of a cytosine in the longer oligomer (Figure 2C). The 17mer also binds to the 23mer which lacks a central adenine residue (compared to the 24mer) with a melting temperature of 36 ± 1°C (Figure 2C). When we used a 26mer which had two cytosine residues at the central position of the 24mer, the Tm value was 32.5 ± 1°C indicating that the hairpin structures in the 35 or 36mer-$(CG)_2$/17mer complexes were more destabilizing than the formation of a bulge with two extra-bases (Figure 2B, 2C). In addition, we found that the stability of the 18mer/25mer complex was intermediate between those of the 17mer/24mer and 17mer/25mer complexes, suggesting that the two mismatched nucleotides (T and C) were probably expelled outside the double helix (15, 16), thus allowing the two short double helices to stack on top of each other (Figure 2C).

The mutated oligonucleotides 17mer-L and 17mer-R are respectively targeted to the 5′ side (L) and the 3′ side (R) of the stem-loop structure and do not bind to the other side of the hairpin due to the presence of three mismatches (Figure 1). These two oligonucleotides bind to the 36mer-$(CG)_2$ with Tm of 17°C and < 5°C, respectively (Figure 1, 2A, 2C). The fact that the 17mer has a higher stability than the mutated oligomers strongly indicates that it binds to both sides of the stem-loop sequence. The difference in Tm values for 17mer-L and 17mer-R can be explained by the higher ratio of GC base pairs in the 17mer-L/36mer-$(CG)_2$ complex as compared to the 17mer-R/36mer-$(CG)_2$ complex (Figure 1).

As shown in the melting profiles in Figure 2A, the 17mer oligonucleotide could also bind to the 35 and 36mer-$(CA)_2$ substrates which were not expected to form a hairpin structure. Half dissociation of both 35 and 36mer-$(CA)_2$/17mer complexes occurred at 22 ± 1°C. When the 17mer was bound to 35 or 36mer-$(CA)_2$, 16 or 17 base pairs could form and the intervening sequence (12 nt) could loop out even though no hairpin was formed (Figure 2C). The small destabilization due to the absence of a hairpin structure in 35 or 36mer-$(CA)_2$/17mer complexes as compared to the 35 or 36mer-$(CG)_2$/17mer complexes suggested that the intervening sequence did not play a major role in stabilizing the binding of the 17mer.

Structure of complexes

Several methods were used to probe the structure of the complexes and to demonstrate the existence of the hairpin structure in the 17mer/35 or 36mer-$(CG)_2$ complex. Osmium tetroxide (OsO_4) is more reactive towards thymines in single-stranded than in double-stranded DNA and can be used as a specific probe for thymines in an altered environment (17). The 17mer was protected against osmium tetroxide cleavage reaction when bound to 35 and 36mer-$(CG)_2$ except for the two thymines in the center of the sequence, position 8 and 9 starting from the 5′ end (Figure 3). When the 17mer was bound to 35mer-$(CG)_2$ or 36mer-$(CG)_2$, these two thymines were also hyperreactive towards P1 nuclease digestion (which has a single-strand-specific endonuclease activity) (data not shown). The high reactivity of thymines at positions 8 and 9 reflects the structure of the three-way junction formed when the 17mer hybridizes to the hairpin-forming 35mer-$(CG)_2$ or 36mer-$(CG)_2$. On the basis of electrophoretic mobility of the species derived by shortening one arm of the junction and by chemical reactivity towards OsO_4, such a three-way junction was shown to adopt a Y-shaped extended conformation in the absence of Mg^{2+} ions (18–22). T-9, and to a lesser extent T-8, were as reactive when the 17mer was hybridized to 36mer-$(CG)_2$ (where an A–T(9) base pair could form) as when it was bound to 35mer-$(CG)_2$ where A–T(9) base pair could not form (Figure 3A). Therefore it is likely that the structure at the junction in the 17mer/36mer-$(CG)_2$ complex did not allow for complete base-pair formation of the central A.T base pair or that the altered environment at the three-way junction is favorable to OsO_4 reaction.

Copper-phenanthroline was used as a probe of the hairpin structure (Figure 3B, 3C). This chelate cleaves double-stranded DNA in the presence of a reducing agent (23–26). Hypersensitive cleavage sites were observed in the 35 and 36mer-$(CG)_2$ sequences within the 4 base pair stem of the hairpin structure. The cleavage sites exhibited an asymmetric distribution (Figure 3B and 3C). In the double-stranded region they were shifted towards the 3′-end of the oligonucleotide, suggesting that cleavage took place within the minor groove of the stem structure. Such a shift has already been observed in the case of double-stranded DNA (14, 25–26) and hairpin-containing single-stranded DNA (27). When the 17mer was bound the majority of the cleavage sites were shifted towards the adenine on the 3′-side of the hairpin and towards the first cytosine on the 5′-end of the hairpin stem, suggesting a conformational change in 35mer-$(CG)_2$ induced by 17mer binding (Figure 3B). It was noteworthy that the cleavage sites in the stem sequence existed even in presence of the 17mer, showing that the stem remained double-stranded when the 17mer was bound. When the 17mer-L was incubated with the 35mer-$(CG)_2$, no shift of cleavage sites was observed, indicating that the shift observed with 17mer was due to binding of both the 5′ and 3′ halves of the 17mer to the target sequence (data not shown). Similar cleavage shifts towards the 5′- and the 3′- sides of the hairpin were obtained with 36mer-$(CG)_2$ in presence of 17mer, suggesting that both complexes have similar conformations (Figure 3C).

No significant copper-phenanthroline cleavage was observed with the 35mer-$(CA)_2$ or 36mer-$(CA)_2$ in the absence or in the presence of the 17mer-L (for sequence see Figure 1), suggesting that no hairpin was formed in the $^{5'}(CA)_2\text{-}T_4\text{-}(CA)_2{}^{3'}$ sequence. The weak cleavage observed in Figure 3B was likely the result of an alternative structure of 35 or 36mer-$(CA)_2$ as shown in Figure 3B and 3C. In presence of 17mer, the 35mer-$(CA)_2$ was cleaved on both sides of the $^{5'}(CA)_2\text{-}T_4\text{-}(CA)_2{}^{3'}$ sequence (Figure 3B and 3C). Cleavage sites were observed at the adenines on the 3′ side of the $^{5'}(CA)_2\text{-}T_4\text{-}(CA)_2{}^{3'}$ sequence indicating that the junction at this position was reactive and had a similar structure to that obtained with 35mer-$(CG)_2$ when the hairpin was formed.

Two other oligomers 35mer-(CTCC) and 36mer-(CTCC) were designed as potential targets for the 17mer without hairpin structures and without the possibility to form C.A base pairs (see discussion). But alternative structures were observed with 35mer-(CTCC) where $^{5'}(CA)_2\text{-}T_4\text{-}(CA)_2{}^{3'}$ was replaced by $^{5'}(CTCC)\text{-}T_4\text{-}(CTCC)^{3'}$. With this target, OP-Cu cleavage occurred mainly within the T_4 sequence and the AGAG sequence on the 5′ side of the DNA fragment. These results are consistent with structures shown on Figure 3B, 3C. No cleavage shift or hypersensitive sites were observed when the mixture of 35mer-(CTCC) with 17mer was treated with $Cu(OP)_2$, as compared to the 35mer-(CTCC) alone (Figure 3B). No melting transition was observed

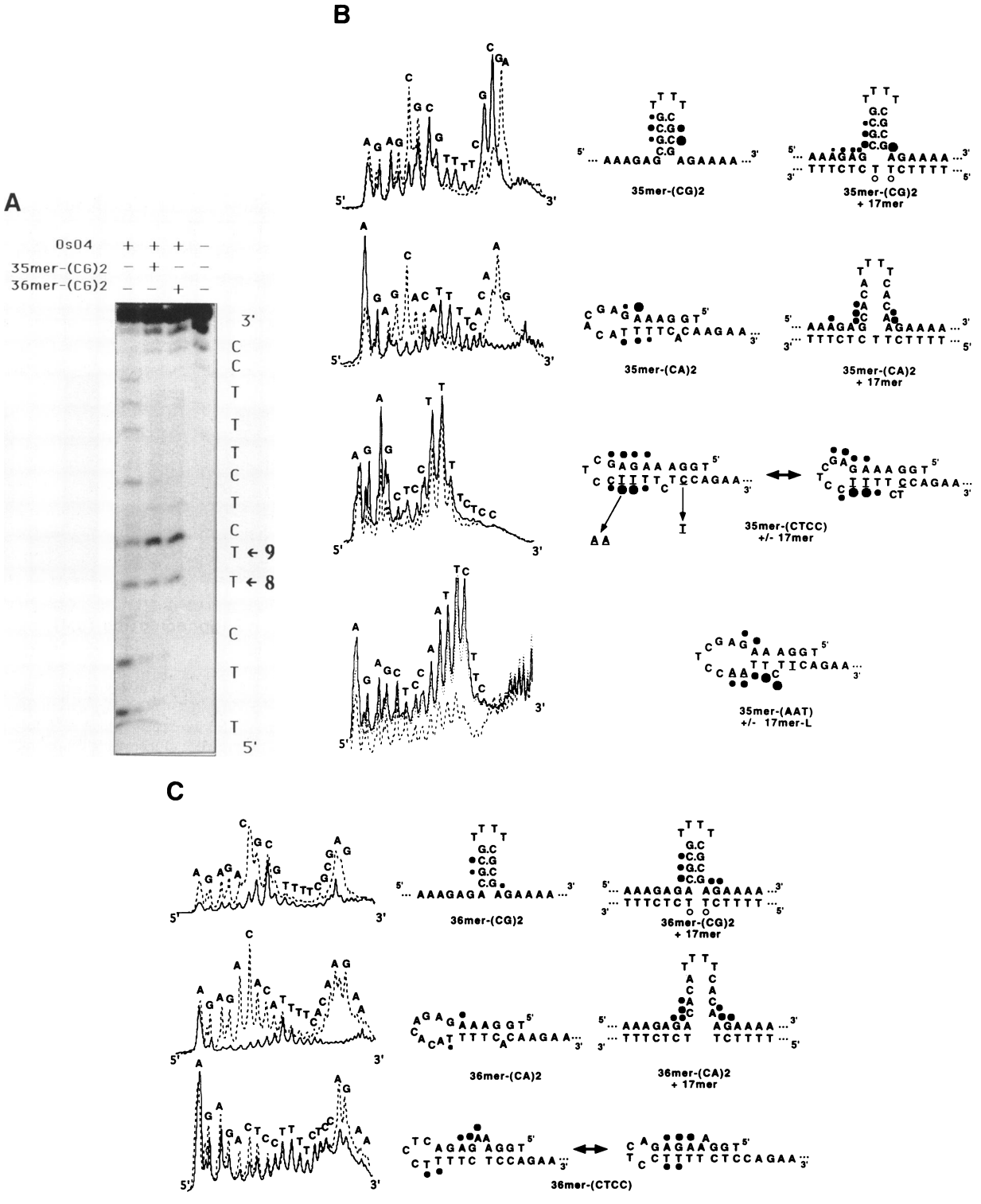

Figure 3. Scission of hairpin-containing 35mer-$(CG)_2$ and 36mer-$(CG)_2$/17mer complexes by chemical probes. **A**— Osmium tetroxide modification of thymine bases on the 17mer/35mer-$(CG)_2$ and 36mer-$(CG)_2$ complexes. OsO_4 reaction was performed for 10 seconds on the 5′-labelled 17mer (1 μM) incubated at 10°C with 35 or 36mer-$(CG)_2$ (10 μM). Small arrows show thymines that are hypersensitive to OsO_4 when the 5′-end labelled 17mer is bound to 35mer-$(CG)_2$ or 36mer-$(CG)_2$ (shown on Figure 3B, 3C as open circles). Other thymines are protected against OsO_4 reactivity indicating that they are base-paired with the 35 or 36mer-$(CG)_2$. **B**— Cleavage pattern obtained after densitometric analysis of OP-Cu reactions. OP-Cu cleavage of 5′-labelled 35mer-$(CG)_2$, 35mer-$(CA)_2$, 35mer-(CTCC) or 35mer-(AAT) (10 nM) was performed without (continuous line) and with 17mer (broken line) as described under Materials and Methods. Cleavage profiles obtained from densitometric analysis are presented with the size of black circles on the oligonucleotide sequences representing relative cleavage intensities. Alternative structures proposed for the 35mer-$(CA)_2$ and 35mer-(CTCC) are also shown. They were obtained from secondary structure calculations (2, 3, 35). Nucleotides underlined in the 35mer-(CTCC) sequence were changed to two adenines and one thymine in the 35mer-(AAT). The cleavage profile obtained with 35mer-(AAT)/17mer-L complex is presented as a dotted line. **C**— Cleavage pattern and schematic representation obtained after densitometric analysis of OP-Cu reactions performed on 36mer-$(CG)_2$, 36mer-$(CA)_2$ and 36mer-(CTCC) (10 nM) in the absence or in the presence of 17mer.

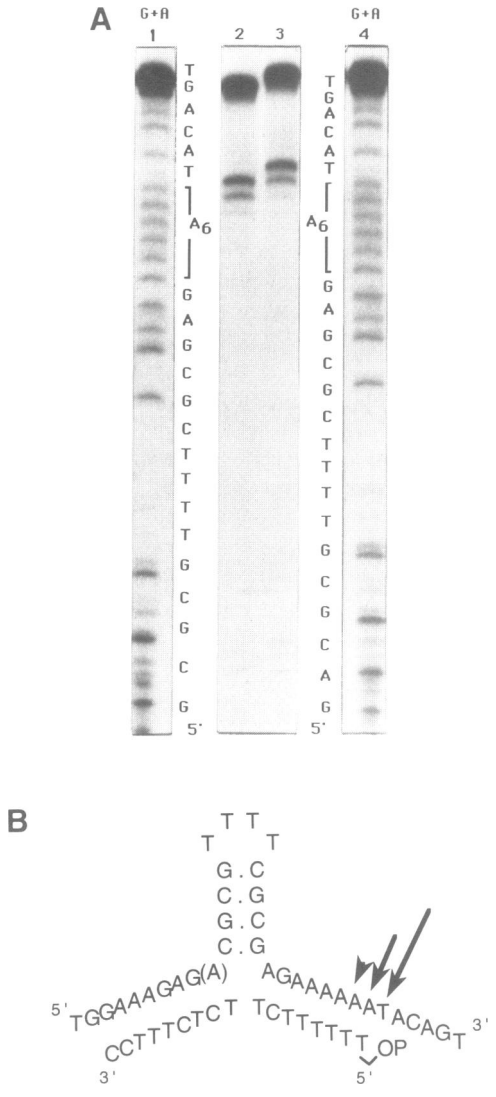

Figure 4. Scission of hairpin-containing 35 and 36mer-$(CG)_2$ by Cu-phenanthroline–oligomer conjugate. **A**— Cleavage of 5'- labelled target sequences 35mer-$(CG)_2$ (lane 2) and 36mer-$(CG)_2$ (lane 3) by OP-17mer at 20°C. Cleaved fragments resulting from G+A Maxam–Gilbert reactions are shown in lanes 1 and 4 (12). **B**— Cleavage pattern observed after incubation of OP-17mer with 35mer-$(CG)_2$. Arrow lengths represent cleavage intensities obtained from densitometric analysis of lane 2 on figure 4A.

for 1:1 mixtures of 35mer-(CTCC) and 17mer or 17mer-L, showing that the 17mer was unable to disrupt the alternative structure of 35mer-(CTCC) shown in Figure 3B.

The complex of 17mer with 35mer-(AAT) where the $^{5'}$(CTCC)-T_4-(CTCC)$^{3'}$ sequence was replaced by $^{5'}$(CTCC)-AATT-(CTTC)$^{3'}$ had a melting temperature of 20°C. Even though the 35mer-(AAT) could form a structure similar to that of 35mer-(CTCC) (Figure 3B), its stability was lower and was disrupted upon binding of the 17mer. Similar OP-Cu cleavage patterns were obtained with the 35mer-(AAT) alone and in presence of the 17mer-L (Figure 3B). With the 17mer cleavage intensities decreased. These results suggested that the 17mer-L oligomer could not bind to the 35mer-(AAT) but that the 17mer could bind and induced a pseudo-loop structure as in the 17mer/35mer-$(CA)_2$ complex (Figure 3B). It was noticeable that

Table 1. Cleavage efficiencies obtained after incubation at 20°C and 25°C of OP-17mer with 5'- labelled targets containing (35 and 36mer-$(CG)_2$) and not containing (35 and 36mer$(CA)_2$) a hairpin structure

Targets	Cleavage (%)	
Temperature	20°C	25°C
35mer-$(CG)_2$	44 ± 7	36 ± 5
35mer-$(CA)_2$	37 ± 7	17 ± 3
36mer-$(CG)_2$	42 ± 7	31 ± 5
36mer-$(CA)_2$	41 ± 3	18 ± 4

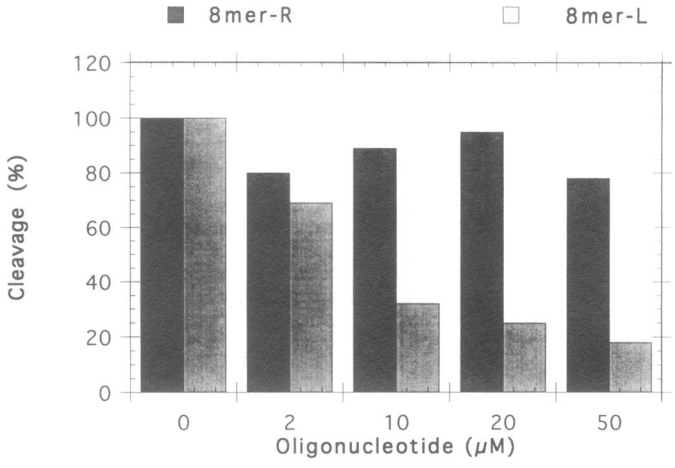

Figure 5. Competition between 8mer-L and 17mer-phenanthroline conjugate for the same target sequence on the 35mer-$(CG)_2$. Cleavage experiments with 17mer tethered to 1,10-phenanthroline (2 μM) were performed at 18°C for 2 hours under the conditions of Figure 4 in the presence of increasing concentrations of 8mer-L. The oligomer 8mer-R which does not bind at 18°C to 35mer-$(CG)_2$ was used as a control. The relative extent of cleavage was determined by densitometric analysis of 35mer-$(CG)_2$ cleavage products.

the OP-Cu-induced cleavage of 35mer-(AAT) decreased and that of 35mer-$(CA)_2$ increased in the presence of 17mer. These results suggested that OP-cleavage sites were created in the pseudo-loop structure of 35mer-$(CA)_2$/17mer, which might be due to the formation of C.A base pairs (see discussion) and that in the case of 35mer-(AAT), the pseudo-stem containing cytosine and thymine residues was not cleaved by the OP-Cu reagent.

Site-specific cleavage by the phenanthroline-17mer-conjugate

The complex formed by the 17mer-phenanthroline conjugate (OP-17mer) with the 35 and 36mer-$(CG)_2$ was incubated in the presence of copper ions and 3-mercaptopropionic acid. Strong cleavage of the 35 and 36mer-$(CG)_2$ was observed as shown in Figure 4A. The major sites of cleavage occurred in both cases at the same two bases (Figure 4B). These bases were also the most reactive when the 24mer was used as a substrate (without hairpin structure) for the 17mer-phenanthroline conjugate (data not shown). The same cleavage sites were observed when OP-17mer was incubated at 20°C with the 35mer-$(CA)_2$ and the 36mer-$(CA)_2$ (data not shown). The cleavage efficiency obtained with OP-17mer at 20°C and 25°C was determined for each complex (Table 1). At 20°C, the 35mer-$(CA)_2$ and the 36mer-$(CA)_2$ were cleaved by OP-17mer with nearly the same efficiency as the 35 or 36mer-$(CG)_2$. However at 25°C the cleavage efficiency dropped much more with the

$(CA)_2$-containing targets than with the $(CG)_2$-containing targets in agreement with the melting temperatures obtained for these complexes.

To demonstrate that cleavage involved binding of the 17mer to both sides of the hairpin structure of 35 or 36mer-$(CG)_2$, and not only to the 3′-side, two oligonucleotides, 8 nt in length, were used as competitors (Figure 1). 8mer-R is complementary to the sequence 3′ of the hairpin; it binds to the 35mer-$(CG)_2$ with a Tm of 14°C at 10 μM. 8mer-L binds to the sequence 5′ of the hairpin with a Tm of 35°C at 10 μM (28°C at 1 μM) (Figure 2C). As shown in Figure 5, 8mer-L competes with OP-17mer and inhibits cleavage of the 35mer-$(CG)_2$ target demonstrating that binding of the 17mer to the 35mer-$(CG)_2$ involves both the 3′-side of the hairpin structure where cleavage occurs and its 5′-side where 8mer-L competes. 8mer-R which did not bind at 18°C did not exhibit any inhibitory effect on the reactivity of the OP-17mer.

The large difference in Tm for 8mer-L and 8mer-R suggested that, in addition to its higher G.C content, 8mer-L could bind cooperatively with the four C.G base pairs of the stem in 35mer-$(CG)_2$. To test this hypothesis, the two 8mers were bound to 35mer-(AAT) which had no hairpin structure. The Tm was 13°C for 8mer-R and 26°C for 8mer-L at 10 μM, to be compared with 14 and 35°C, respectively, when 35mer-$(CG)_2$ was used as a substrate (Figure 2C) (a concentration of 10 μM was used for the 8mers, instead of 1 μM in all other experiments summarized in Figure 2C, in order to obtain a measurable Tm for 8mer-R complexes). The differences of 9°C between the Tm values of 8mer-L complexes with 35mer-$(CG)_2$ and 35mer-(AAT) suggested a cooperative binding of the 8 base pair double helix with four C.G base pairs of the stem in the 35mer-$(CG)_2$. When the 8mers were elongated to form 17mer-L and 17mer-R a destabilization of the complexes was observed as compared to 8mer-L and 8mer-R, respectively (Figure 2C). The non-hybridized part of the 17mer-L prevented any cooperative binding with the stem region. The higher Tm of the 17mer-L as compared to 17mer-R can be ascribed to the larger number of G.C base pairs formed by the former oligonucleotide with the 5′ side of the hairpin. When both sides of the 17mer could bind, the Tm of the complexes with 35mer- and 36mer-$(CG)_2$ was higher than with any of the 'mutants' 17mer-L and 17mer-R (Figure 2C), as expected if the 17mer was bound simultaneously to the 5′- and the 3′-side of the hairpin, forming a three-way structure.

DISCUSSION

Hairpin structures are present in regions of nucleic acids known to have a particular function such as recognition by proteins involved in controlling biological processes. Targeting an oligonucleotide to these hairpin structures could have a specific biological effect. This report demonstrates that an oligonucleotide (17mer) can bind to DNA containing a hairpin structure by forming a three-way junction. Spectroscopic studies indicate that this oligomer binds to the 35 or 36mer-$(CG)_2$ with a melting temperature of 25°C, a Tm lower than that obtained with the 24mer linear target sequence (45°C). The analysis of the results obtained with P1 nuclease assays and osmium tetroxide reactions suggests that the central thymine residues of the 17mer are not engaged in Watson–Crick base pairs with bases on the 35mer-$(CG)_2$ and 36mer-$(CG)_2$ targets. Under the conditions used in the study, a three-way DNA junction is assumed to adopt a Y-shaped structure rather than a T-shaped structure (18–22). In the Y-shaped structure, there is no stacking between any two arms in contrast to what is observed with a four-stranded junction (Holliday junction) (28, and for review 29).

As cleavage reactions by the phenanthroline-cuprous complex normally occur in the minor groove of a DNA duplex, the results obtained with OP-Cu cleavage of 35 or 36mer-$(CG)_2$ and 35 or 36mer-$(CA)_2$ (in the absence of the 17mer) strongly suggest that the 35 and 36mer-$(CG)_2$ exhibit a hairpin structure in solution and that the 35 and 36mer-$(CA)_2$ do not. The OP-Cu cleavage patterns of 35mer-$(CG)_2$ and 36mer-$(CG)_2$ were shifted (or enhanced) in the presence of 17mer. When the 17mer was bound to 35mer-$(CG)_2$ or 36mer-$(CG)_2$, the strongest cleavage sites were located on the 3′ side of the hairpin at the junction containing the 5′-half of the 17mer. These results show that this junction has an unusual conformation as described earlier (20).

The OP-17mer which has a 1,10-phenanthroline ring covalently linked to its 5′-end induced specific cleavage reactions on both 35mer-$(CG)_2$ and 36mer-$(CG)_2$ in the presence of Cu^{2+} and MPA. As expected the cleavage sites were localized near the 5′-end of the 17mer as indicated on Figure 4B. The results of the cleavage experiments performed in presence of 8mer-L showed that 35mer-$(CG)_2$ cleavage induced by OP-17mer did not occur when the 3′-part of the OP-17mer was not bound to the 5′-side of the 35mer-$(CG)_2$ hairpin sequence (Figure 5). Therefore, binding of the OP-17mer to both sides of the hairpin structure was necessary for specific induction of targeted cleavage. The 17mer sequence was such that binding of the 3′-part was much stronger than that of the 5′-part carrying the cleaving reagent. Therefore cleavage could be observed at temperatures where the 5′-part alone would not be bound, due to the cooperativity of binding of the two parts of the 17mer in a three-way structure.

In order to test whether the hairpin structure of the 35mer-$(CG)_2$ and 36mer-$(CG)_2$ was important for binding of the 17mer, two oligomers, 35mer-$(CA)_2$ and 36mer-$(CA)_2$, were synthesized with no stem-loop structure. In the 35mer-$(CA)_2$ and 36mer-$(CA)_2$, guanines were replaced by adenines in the stem sequence. Spectroscopic studies, OP-Cu cleavage and OP-17mer cleavage indicated that the 17 nucleotide-long oligomer could bind to the 35mer-$(CA)_2$ and 36mer-$(CA)_2$. Surprisingly, we found that 17mer could bind to these oligomers with a Tm value of 22°C which is only three degrees lower than the Tm for the 35mer-$(CG)_2$ and the 36mer-$(CG)_2$. OP-17mer could also induce site-specific cleavage in the 35 or 36mer-$(CA)_2$ oligonucleotides.

In the 35mer-$(CA)_2$/17mer and 36mer-$(CA)_2$/17mer complexes, 17mer binding induced the formation of a lariat structure by the $(CA)_2$-T_4-$(CA)_2$ sequence which brings the 5′ and 3′ sides of the target in close proximity. Binding of the 17mer could stabilize a hairpin with four C.A base pairs. Mismatched C.A base pairs have been observed in crystal structures (30–32) and in solution by NMR (30, 33–34). The fact that OP-Cu-mediated cleavage did not occur in the $(CA)_2$-T_4-$(CA)_2$ sequence of the 35mer-$(CA)_2$ or 36mer-$(CA)_2$ in the absence of 17mer suggested either that OP-Cu could not recognize a double-stranded DNA with C.A base pairs or that this sequence did not form any secondary structure. However when the 17mer was bound to the 35- or 36mer-$(CA)_2$ a structure was formed which was sensitive to $Cu(OP)_2$ cleavage with a distribution of cleavage sites close to that observed with 35 or 36mer-$(CG)_2$. This result suggests that a hairpin structure with C.A base pairs could form upon binding of the 17mer to both sides of the

$(CA)_2$-T_4-$(CA)_2$ sequence. It is noteworthy that the 35 and 36mer-$(CA)_2$ were likely to exhibit an alternative secondary structure (see Figure 3B, 3C). Similar structures were obtained with the 35mer-(CTCC) and the 35mer (AAT). In presence of the 17mer, only the structures of 35 and 36mer-$(CA)_2$ and 35mer-(AAT) were disrupted to form the pseudo-loop structure.

The use of oligonucleotides as gene control elements *in vivo* rest on a high selectivity of oligonucleotide binding to their cellular target. The oligonucleotide specificity requires a minimal length which ensures recognition of a single target in either a genome or a messenger RNA population. In human cells, the minimum length to have a unique target in the mRNA population should range from about 11 (with only Gs and Cs in the oligonucleotides) to 15 (only As and Ts) (1). Using short oligomers offers many advantages in the antisense strategy especially from the selectivity point of view (see reference 1 for a review).

In this report, we have tested the ability of an oligonucleotide tethered to 1,10-phenanthroline to recognize and cleave nucleic acids which possess a secondary structure. We have shown that oligonucleotides can hybridize to sequences located on both sides of a stem-loop structure. However, it was also shown that oligonucleotides could bind to two non-contiguous sequences which are not brought in close proximity by hairpin formation in the absence of oligonucleotide binding. These results confirm the fact that short antimessenger oligonucleotides must be used if specific and selective inhibition is contemplated. A long oligomer could recognize other target sequences than its completely complementary one by hybridizing to two non-adjacent sequences. Ribonuclease H which catalyzes the hydrolysis of the RNA moiety in oligodeoxynucleotide–messenger RNA hybrids, could cleave messenger RNA at non-specific sites if long oligomers were used.

Oligonucleotides can be designed to recognize two non-adjacent sequences in a single-stranded nucleic acid. Two oligonucleotides can be tethered to each other by a synthetic linker (see references 9–11). Here we have shown that a single oligonucleotide can also bind non-adjacent sequences. The intervening sequence needs not form a stable stem-loop structure for the oligonucleotide to bind. The sequence of the intervening segment in the target nucleic acid plays some role in determining complex stability as shown by comparing the results obtained with $(CG)_2$-T_4-$(CG)_2$, $(CA)_2$-T_4-$(CA)_2$ and (CTCC)AATT(CTTC). A stronger complex is obtained when a hairpin structure pre-exists before complex formation. Even though the complexes are less stable than when the two target sequences are adjacent this new strategy might find applications in the control of translation of mRNAs with known stable secondary structures.

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