Recognition and Cleavage of Single-Stranded DNA Containing Hairpin Structures by Oligonucleotides Forming Both Watson−Crick and Hoogsteen Hydrogen Bonds†

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Received June 6, 1994; Revised Manuscript Received October 7, 1994®

ABSTRACT: A new approach is described to design antisense oligonucleotides targeted against single-stranded nucleic acids containing hairpin structures by use of both Watson−Crick and Hoogsteen hydrogen bond interactions for recognition. The oligonucleotide has two different domains, one allowing double helix formation involving Watson−Crick base pairs and the other one forming a triple helix involving Hoogsteen-type base triplets in the major groove of a hairpin stem. Spectroscopic and gel retardation experiments provided evidence for such Watson−Crick/Hoogsteen (WC/H) recognition of hairpin structures in single-stranded DNA. An antisense oligonucleotide designed to form only Watson−Crick base pairs was unable to disrupt the stable stem structure of the target under conditions where the oligonucleotide designed with the Watson−Crick/Hoogsteen interactions could bind efficiently to the hairpin-containing target. The addition of one nucleotide to the oligonucleotide at the junction between the double helix and triple helix regions in WC/H complexes had an effect on stability which was dependent on the relative orientation of the Watson−Crick and Hoogsteen domains in the target. An oligodeoxynucleotide−phenanthroline conjugate targeted against such a hairpin-containing DNA fragment induced specific cleavage in the double-stranded stem. This WC/H approach may be useful in designing artificial regulators of gene expression.

Artificial control of gene expression can be achieved by using short oligonucleotides according to several strategies (Hélène & Toulmé, 1990). In the *antisense* strategy, the oligonucleotide is targeted to a specific messenger RNA and inhibits translation of the mRNA into the corresponding protein. In the *antigene* strategy, the oligonucleotide is targeted against a DNA double-stranded sequence; triple helix formation leads to transcription inhibition of a specific gene (Postel et al., 1991; Young et al., 1991; Duval-Valentin et al., 1992; Maher, 1992; Grigoriev et al., 1992). The recognition of a specific sequence in an mRNA by an oligonucleotide is based on Watson−Crick hydrogen-bonding interactions between complementary bases. In the antigene strategy sequence-specific recognition of the major groove of DNA at polypurine−polypyrimidine sequences is achieved via Hoogsteen or reverse Hoogsteen hydrogen bond formation between bases in the third strand and the purines of the Watson−Crick A·T and G·C base pairs (Thuong & Hélène, 1993). The third strand can be synthesized with pyrimidines only, in which case it adopts a parallel orientation with respect to the target polypurine sequence. Alternatively, it can be synthesized with purines in an antiparallel orientation. Third strands containing G and T can adopt a parallel or an antiparallel orientation depending on the sequence (Sun et al., 1991). An oligonucleotide containing T, C, and G has been described to bind in a parallel orientation with respect to the target polypurine sequence (Giovannangéli et al., 1992).

In the antisense strategy, the target sequence of the mRNA must be available for hybridization with the short oligomer. However, mRNAs are not single-stranded random coil nucleic acids but exhibit short-range and long-range internal structures such as hairpins. These hairpin structures may affect the affinity of oligonucleotides and their kinetics of binding and may even totally prevent hybridization (Lima et al., 1992). Recent alternative approaches have been proposed for targeting hairpin structures that are often too stable to be disrupted by complementary oligonucleotides. We have previously shown that oligonucleotides can recognize secondary structures in nucleic acids by designing them to bind two nonadjacent sequences brought into close proximity through hairpin formation (François et al., 1994). Oligonucleotides consisting of two oligomer sequences linked by a chemical tether can also bind two single-stranded noncontiguous sites in RNA with secondary structures (Richardson & Schepartz, 1991; Cload & Schepartz, 1991, 1994; Cload et al., 1993).

The formation of triple helices can also be of interest in the antisense strategy. For example, Giovannangéli et al. have shown that an oligopyrimidine can bind to a polypurine sequence of a single-stranded nucleic acid by forming both Watson−Crick and Hoogsteen hydrogen bonds (Giovannangéli et al., 1991, 1993). Duplex formation involving Watson−Crick pairing occurs with the 3′ part of the oligopyrimidine, and Hoogsteen hydrogen bonds are formed by the 5′ part of the oligonucleotide with this duplex. The oligopyrimidine recognizes its single-stranded target by forming a hairpin (Xodo et al., 1990; Giovannangéli et al., 1991, 1993). These oligonucleotide *clamps* have been shown to arrest DNA polymerase on a DNA template (Giovannangéli et al., 1993). Circular oligopyrimidines can also form triple

† This work was supported in part by the Ligue Nationale contre le Cancer and by Rhône Poulence Rorer.

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® Abstract published in *Advance ACS Abstracts,* December 1, 1994.

0006-2960/95/0434-65$09.00/0 © 1995 American Chemical Society

helices when bound to a single-stranded polypurine sequence (Kool, 1991; Dsouza & Kool, 1992; Prakash & Kool, 1992; Booher et al., 1994). More recently, it has been shown that an oligopyrimidine could be targeted against a DNA hairpin structure by forming both a triple helix on a single-stranded region and a second triple helix with the hairpin stem (Brossalina & Toulmé, 1993; Brossalina et al., 1993). The linker between the two oligonucleotide parts can be a non-nucleotidic linker (Giovannangéli et al., 1991; Letsinger et al., 1993) or a short oligonucleotide (Xodo et al., 1990; Kool, 1991; Dsouza & Kool, 1992; Prakash & Kool, 1992; Brossalina & Toulmé, 1993; Brossalina et al., 1993; Giovannangéli et al., 1993).

Here we describe an alternative way to recognize hairpin structures by designing oligonucleotides which interact with one of the two single-stranded sequences flanking the hairpin and with the double-stranded stem of the hairpin. The oligonucleotide has two different domains, one allowing double helix formation involving Watson–Crick base pairs and the other one allowing triple helix formation involving Hoogsteen-type base triplets in the major groove of the stem. The two domains of the oligonucleotide are contiguous, with no intervening sequences. Two orientations of the oligonucleotides can be defined depending on whether the Watson–Crick interactions occur on the 5′ or the 3′ side of the hairpin. These two possibilities will be abbreviated as WC→H and H→WC. Melting experiments and gel shift analysis were used to demonstrate that oligonucleotides could recognize a DNA hairpin in a sequence selective way. In addition we show that a WC/H oligonucleotide binds to a hairpin-containing target under conditions where the corresponding antisense oligonucleotide (with only Watson–Crick base pairs formed with both the single-stranded and hairpin regions) fails to bind. The influence of the WC/H oligonucleotide sequence at the junction between the single-stranded region and the double-stranded stem was analyzed in both Watson–Crick→Hoogsteen (WC→H) and Hoogsteen→Watson–Crick (H→WC) orientations. A phenanthroline–oligonucleotide conjugate was also used as a probe to study these Watson–Crick/Hoogsteen complexes. In the presence of Cu^{2+} ions and a reducing agent, this phenanthroline conjugate cleaved the stem of the hairpin of the single-stranded target by this new WC/H recognition.

MATERIALS AND METHODS

Oligonucleotides. Unmodified oligodeoxynucleotides were purchased from Genosys (England) and Eurogentec (Belgium). They were purified by polyacrylamide gel electrophoresis (PAGE). Their molar extinction coefficients were calculated according to Cantor and Warshaw (1970). Absorbances were measured at high temperature to determine concentrations. In order to synthesize the phenanthroline-substituted 17mer-A (OP-17mer-A), a 5′-thiophosphate was attached to the 17mer-A oligonucleotide and reacted with 5-(ω-bromohexanamido)-1,10-phenanthroline as previously described (François et al., 1989a,b).

Melting Experiments. Absorption spectra were recorded on an UVIKON 940 spectrophotometer. Melting curves were obtained by subtracting absorbance recorded at 260 nm from that recorded at 540 nm where none of the constituents absorbed light. Before each experiment, all samples were heated at 90 °C and then cooled slowly to 0 °C. Then the absorbance of samples was recorded upon raising the temperature at a rate ranging from 0.1 to 0.25 °C/min. The melting temperature (T_m) is defined as the temperature

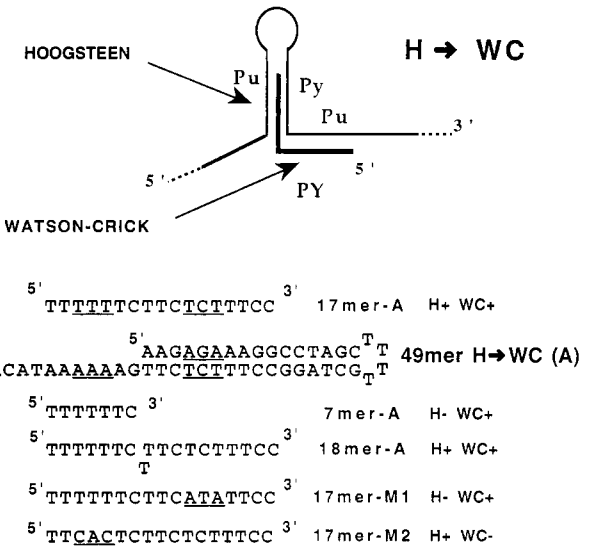


FIGURE 1: Sequences of the hairpin-containing oligonucleotide 49merH→WC(A) and its ligands, 17mer-A, 18mer-A, 7mer-A, 17mer-M1, and 17mer-M2. The sites of mismatches involving 17mer-M1 and 17mer-M2 are underlined. A hairpin structure (top panel, thin lines) can be recognized by oligonucleotides (top panel, thick lines) via both Watson–Crick and Hoogsteen hydrogen bond formation. Two orientations of the oligonucleotide target can be defined depending on whether the Watson–Crick interactions occur on the 5′ or the 3′ side of the hairpin (see text). These two possibilities, H→WC and WC→H, are shown in the top panel of this figure and in Figure 2, respectively. Similar models could be built with reverse Hoogteen instead of Hoogsteen hydrogen-bonding interactions by exchanging the Py and Pu strands of the hairpin stem.

corresponding to half-dissociation of the complex (Rougée et al., 1992). These T_m values were determined with an accuracy of ±1 °C from at least two independent experiments performed with the same constant heating or cooling rate. In order to maintain the hairpin structure and well-defined single- and double-stranded domains, the melting curves were recorded in a temperature range from 0 °C to the temperature just below the beginning of hairpin dissociation. The T_m values for disruption of the hairpin structure in 49merH→WC and 48merWC→H, in a pH 6 phosphate buffer (50 mM) containing 0.1 M NaCl, were 74 and 60 °C, respectively. Most of the thermal denaturation experiments were performed with target and oligonucleotide at 1 μM concentration in 50 mM phosphate buffer (pH 6 or 7) and 0.1 M sodium chloride with or without addition of spermine (0.2, 0.5, or 1 mM).

Gel Shift Assays. Binding of oligonucleotides to hairpin-containing targets was assayed by mobility shift experiments. The oligonucleotides were 5′-end-labeled by using polynucleotide kinase and γ-^{32}P-radiolabeled ATP (Amersham). Before incubation with the 5′-labeled oligonucleotides, target nucleic acids (50 nM) were heated at 90 °C and cooled quickly to 0 °C in order to prevent intermolecular dimerization. Both target and oligonucleotides were incubated at room temperature in a binding buffer containing 50 mM sodium phosphate, pH 6, sodium chloride (0.1 M), spermine (0.2 mM), $MgCl_2$ (5 mM), tRNA (0.5 μg/μL), and sucrose (10%). Samples were then analyzed on 10% acrylamide non-denaturing gels in a buffer containing 50 mM 4-morpholineethanesulfonic acid (MES) (Sigma) at pH 6 and in the presence of 5 mM $MgCl_2$. This pH 6 buffer (MESMg) was preferred in order to obtain a sufficiently stable pH of the gel during the migration [pK_a (MES) = 6.1]. Finally,

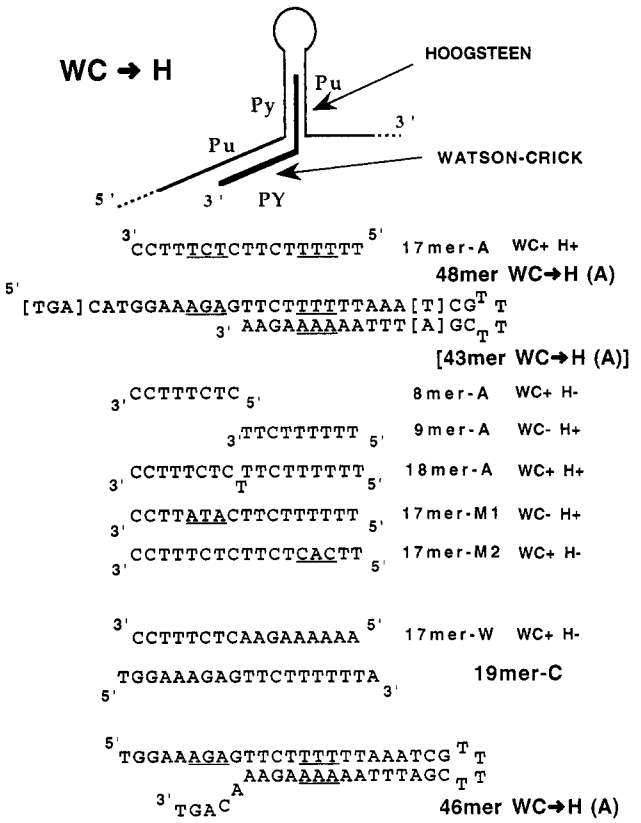


FIGURE 2: Sequences of the hairpin-containing oligonucleotide 48merWC→H(A) and specific oligopyrimidines 17mer-A and 18mer-A, which can form both Watson–Crick and Hoogsteen hydrogen bonds with the hairpin-containing substrate in the WC→H orientation. Mutated oligomer sequences 17mer-M1 and 17mer-M2 are also shown. The 43merWC→H(A) sequence is derived from the 48merWC→H(A) sequence. Three nucleotides at the 5′ end and one T·A base pair in the stem (shown in brackets) have been removed in 43merWC→H(A) as compared to 48merWC→H(A). Notice that both targets have the same binding site for 17mer. 17mer-W is complementary to the single-stranded polypurine sequence and to the polypyrimidine strand of the stem of 48merWC→H(A) and 43merWC→H(A). 19mer-C corresponds to the linear target of 17mer-W (in the Watson–Crick sense). 46merWC→H(A) is derived from 43merWC→H(A) by addition of a single-stranded sequence at the 3′ end.

gels were dried and autoradiographed with X-ray film. During migration, gels were thermostated at 20 °C in order to avoid any increase in temperature.

Cleavage Induced by Phenanthroline–Oligonucleotide Conjugate. The 5′-^{32}P-labeled hairpin-containing target (10 nM) was incubated with OP-17mer-A (1 μM) in a buffer containing 50 mM phosphate (pH 6 or 7), 100 mM NaCl, 2 mM mercaptopropionic acid, and 0.2 mM spermine where indicated. Cleavage was initiated by the addition of 5 μM cupric sulfate, and after incubation for 1 h at 20 °C the reaction was quenched by the addition of 2,9-dimethyl-1,10-phenanthroline (20 μM). After ethanol precipitation samples were analyzed by electrophoresis on 20% polyacrylamide/7M urea, 29:1, cross-linked gels containing standard Tris/borate/EDTA (90/90/2 mM). In order to determine the cleavage intensity at each nucleotide position in the target, gels were analyzed with a Molecular Dynamics 400 S Phosphorimager.

RESULTS

Design of Hairpin-Containing Targets. The targets used in this report have two different domains, a double-stranded stem and a single-stranded sequence, allowing both triple helix and double helix formation with an oligonucleotide ligand. The diagrams at the top of Figures 1 and 2 represent the two possibilities for designing targets. Depending on whether the purine-rich single-stranded region is on the 3′ side or the 5′ side of the hairpin, they will be referred to as Hoogsteen toward Watson–Crick (H→WC) (Figure 1) or Watson–Crick toward Hoogsteen (WC→H) (Figure 2). An oligonucleotide which is able to bind the single-stranded part of the target and not the double-stranded stem is $WC^+ H^-$; alternatively, a $WC^- H^+$ oligonucleotide can bind only the stem and not the single-stranded sequence (see Figures 1 and 2). The stem sequences were chosen to be long enough to have two distinct melting transitions corresponding to oligonucleotide dissociation and stem disruption. The oligonucleotides used to target hairpin structures in the present study were oligopyrimidines because we wanted to be able to exchange the Watson–Crick and Hoogsteen parts of the complexes, but of course, the Watson–Crick part does not need to be an oligopurine–oligopyrimidine sequence. We used a pyrimidine motif for triple helix formation with parallel binding to the polypurine sequence of the hairpin stem.

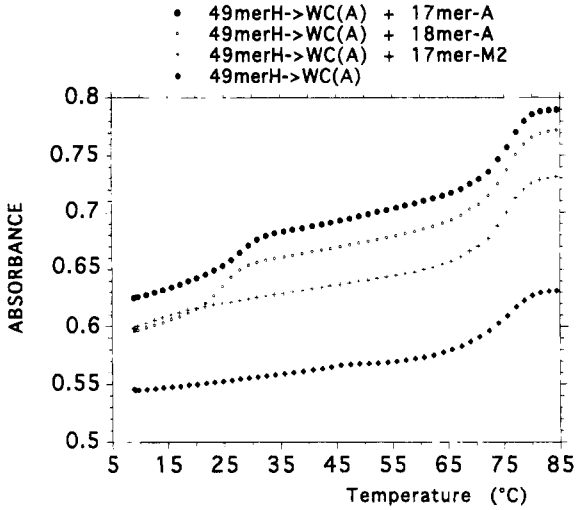


FIGURE 3: Melting curves measured at 260 nm for 1 μM 49merH→WC(A) (◆) and its complexes (at 1/1 μM) with 17mer-A ($H^+ WC^+$) (●), 18mer-A ($H^+ WC^+$) (○), and 17mer-M2 ($H^+ WC^-$) (+) in 0.1 M NaCl and 50 mM phosphate buffer, pH 6.

Watson–Crick/Hoogsteen Transitions. The same oligopyrimidine 17mer-A ($H^+ WC^+$ or $WC^+ H^+$) was targeted against two different nucleic acids, 49merH→WC(A) and 48merWC→H(A) (Figures 1 and 2, respectively). Mutant oligomers are also presented in both figures. 17mer-M1 could bind to the single-stranded sequence ($H^- WC^+$) of 49merH→WC(A), while 17mer-M2 could bind only via Hoogsteen interactions ($H^+ WC^-$). As shown on the melting profile presented in Figure 3, the melting transitions corresponding to oligopyrimidine dissociation and stem fusion were well separated. For the 17mer-A ($H^+ WC^+$)/49merH→WC(A) complex the corresponding T_m values at pH 6 were 27 and 74 °C, respectively. The absence of transitions at low temperature and at pH 6 showed that the mutant oligomers, 17mer-M1 ($H^- WC^+$) and 17mer-M2 ($H^+ WC^-$), did not bind to the 49merH→WC(A) target, suggesting that both parts of the oligopyrimidine 17mer-A ($H^+ WC^+$) were important for the binding to target 49merH→WC(A) (Table 1). As shown in Table 1 for 49merH→WC(A), the oligopyrimidine/target complex stability decreased when the pH was

Table 1: Melting Temperatures (T_m) for the Dissociation of Oligonucleotide Ligands (L) from a Hairpin-Containing Target (T), 49merH→WC(A)[a]

	49merH→WC(A) target (T)		
	pH 6	pH 7	pH 7, S^+ (0.5 mM)
concentration, T (μM)/L (μM)	1/1	1/1	1/1
oligonucleotides (L)			
17mer-A, H^+ WC^+	27	15	23
18mer-A, H^+ WC^+	24	10	22
17mer-M1, H^-, WC^+		<0	7
17mer-M2, H^+ WC^-	<0	<0	4
7mer-A, H^- WC^+			12

[a] See the sequences in Figure 1. T_m values were obtained with a concentration ratio of 1/1 μM (ligand/target) in a 50 mM phosphate buffer containing 0.1 M NaCl at pH 6 or 7 in the absence or in the presence of 0.5 mM spermine (S+). <0 indicates that no melting transition was observed between 0 and 90 °C except that of the hairpin duplex target.

raised from 6 to 7 as expected because cytosine protonation is required for triple helix formation. Spermine, which is known to stabilize triple helices, increased the stability of the complexes (Table 1).

7mer-A (H^- WC^+) targeted to the 3′ side of the single-stranded sequence of 49merH→WC(A) had a melting transition of 12 °C at pH 7 with 0.5 mM spermine (Table 1). This T_m value was lower than that obtained with 17mer-A (H^+ WC^+) (23 °C), but higher than those for the mutant oligomers (7 and 4 °C). The lower stability of the 17mer-M1 (H^- WC^+)/49merH→WC(A) complex as compared to the 7mer-A (H^- WC^+)/49merH→WC(A) complex demonstrates the destabilizing role of the unbound sequence in this WC/H structure.

The Watson−Crick and Hoogsteen parts were exchanged in 48merWC→H(A) [and 43merWC→H(A)] as compared to 49merH→WC(A) (compare Figures 1 and 2). The T_m values for the 17mer-A (WC^+ H^+)/48merWC→H(A) complex at pH 6 in the absence of spermine and for the 17mer-A (WC^+ H^+)/43merWC→H(A) complex at pH 7 in the presence of 1 mM spermine were 24 and 37 °C, respectively (Figure 4). 8mer-A (WC^+ H^-) targeted to the 5′ side of the single-stranded sequence of 43merWC→H(A) [and 48merWC→H(A)] that could form only the Watson−Crick complex was slightly more stable than the 17mer-A (WC^+ H^+) which could form both the Watson−Crick and Hoogsteen complexes (Figure 4). The stability of the 8mer-A (WC^+ H^-)/43merWC→H(A) complex is due to the high G·C content of the duplex and also to a cooperativity of double helix formation with the stem structure. This cooperativity between 8mer-A and the stem sequence of 43merWC→H(A) was suggested by measuring the T_m of the 8mer-A complex with a sequence (19mer-C; see Figure 2) where only the eight Watson−Crick base pairs could form without any further stacking with a preexisting double-helical stem. This T_m value was 3 °C below that of 8mer-A bound contiguously to the stem (Figure 4). The cooperativity disappeared when the hairpin was flanked by a single-stranded sequence on its 3′ side (see below and the 8mer-A (WC^+ H^-)/46merWC→H(A) complex in Figure 4). Also 17mer-M2 (WC^+ H^-), which could form the same Watson−Crick base pairs with 43merWC→H(A), was less stable than 8mer-A (WC^+ H^-) as a result of the destabilization provided by the additional non-hydrogen-bonded sequence on the 5′ side of 17mer-M2.

In order to study the effect of an additional base (a thymine) at the junction between the stem and the single-stranded structures, 18mer-A (WC^+ H^+ or H^+ WC^+) was compared to 17mer-A in the two WC/H complexes (Table 1 and Figure 4). The T_m value was slightly increased upon insertion of an additional thymine in the 17mer with 43merWC→H(A), but the 49merH→WC(A)/18mer-A complex was slightly less stable than the 49merH→WC(A)/17mer-A one (Table 1).

All melting transitions obtained with the stem-containing targets and oligonucleotides were reversible; i.e., the curves obtained upon heating and cooling were superimposed. 17mer-A was assayed under the same conditions (50 mM phosphate, pH 6, 0.1 M NaCl) on a complete double-stranded target to determine the stability of a triple-helical complex with 17 base triplets. As previously observed (Rougée et al., 1992), the heating and cooling curves were not superimposed in these conditions (the apparent T_m values are 28 °C for heating and 22 °C for cooling), showing that the kinetics of formation of 17 base triplets was slower than that of the mixed Watson−Crick/Hoogsteen complex. It should be noted that the formation of the Hoogsteen complex becomes intramolecular once the Watson−Crick complex is formed.

Gel Shift Experiments. Binding of 5′-labeled oligonucleotides 17mer-A, 17mer-AC, 18mer-A, 17mer-M1, and 17mer-M2 to hairpin-containing targets 49merH→WC(A) and 48merWC→H(A) was assayed by gel shift experiments (Figure 5). The gel retardation observed *at pH 6* indicated that 17mer-A and 18mer-A bound to the hairpin-containing targets at 20 °C but mutant oligomers did not. As observed in melting experiments, 18mer-A gave slightly less stable complexes than 17mer-A. When incubation and gel electrophoresis were carried out *at pH 7* and 20 °C, no complexes were seen with 17mer-A and 18mer-A. Only 17mer-AC formed a complex (data not shown). This oligomer had the same sequence as 17mer-A with cytosines replaced by 5-methylcytosines, which are known to stabilize C·G×C^+ base triplets at neutral pH. These results are fully consistent with triplex formation by the 5′ and 3′ halves of the 17mer oligonucleotide with 48merWC→H(A) and 49merH→WC-(A), respectively.

Comparison of Antisense and WC→H Oligonucleotides Targeted against Hairpin Structures. The 43merWC→H(A) and 48merWC→H(A) targets have the same binding site for 17mer and have only a few differences in sequence (Figure 2). 17mer-A (WC^+ H^+) could bind to 43merWC→H(A) by formation of both a double and a triple helix (T_m value = 37 °C). The corresponding antisense oligonucleotide 17mer-W (WC^+ H^-), which is complementary to the single-stranded polypurine sequence and to the polypyrimidine strand of the stem, did not bind to the hairpin target 43merWC→H(A) (Figures 2 and 4). The stem structure was too stable to be disrupted by the binding of 17mer-W (WC^+ H^-). On the contrary, 17mer-W (WC^+ H^-) and 17mer-A (WC^+ H^+) bound to the linear single-stranded target 19mer-C with T_m values of 52 and 34 °C, respectively (Figure 4). As expected, the stability of 17mer-A (WC^+ H^+)/19mer-C was less than that of 17mer-W (WC^+ H^-)/19mer-C since only half of 17mer-A was bound to 19mer-C. These results indicated that a hairpin can form a stronger complex with a WC→H oligonucleotide than with an "antisense" oligonucleotide.

Effect of Single-Stranded Regions Flanking the Hairpin on Complex Stability. All the targets mentioned above have

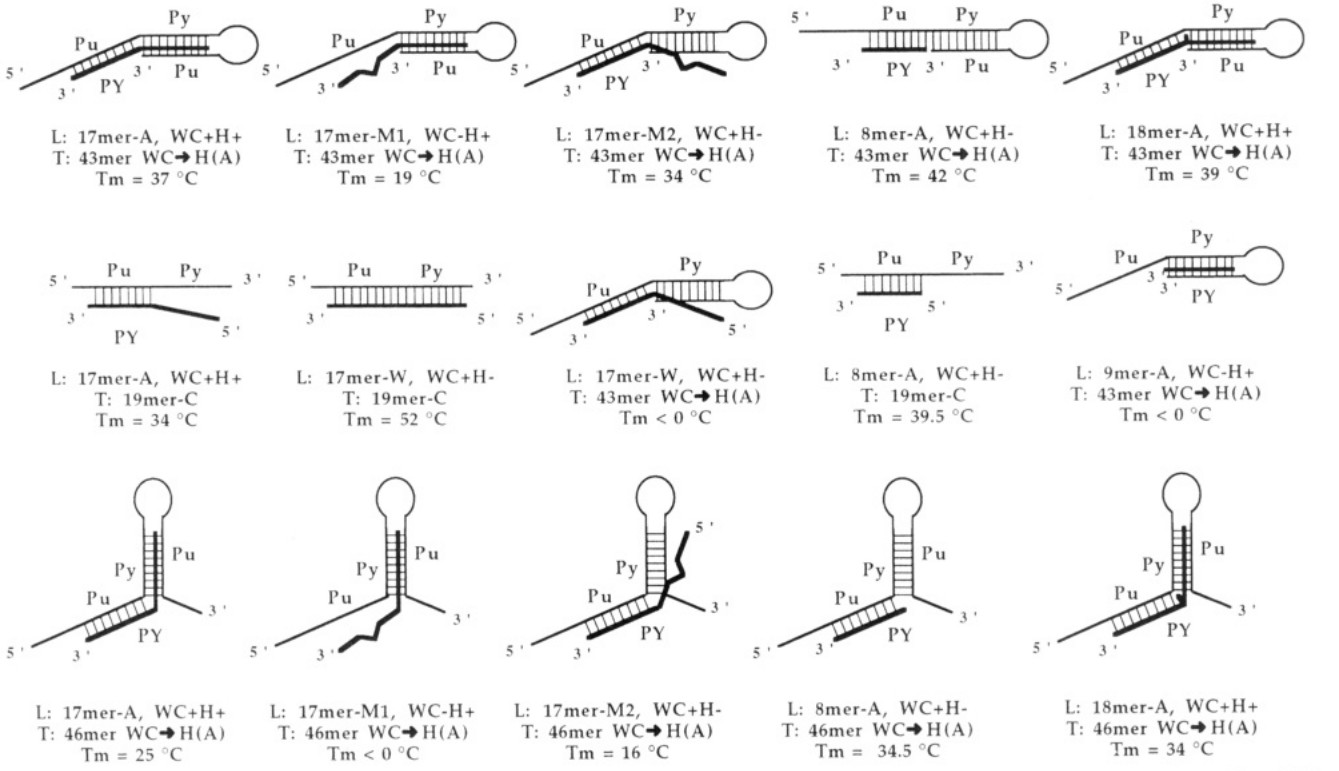


FIGURE 4: Schematic representations of the WC/H complexes formed between hairpin-containing targets (T) 43merWC→H(A), 46merWC→H(A), and 19mer-C and their ligands (L), 17mer-A, 18mer-A, 17mer-M1, 17mer-M2, 17mer-W, 8mer-A, and 9mer-A. The sequences of these oligonucleotides are presented in Figure 2. Melting temperatures are indicated below each complex at 2 μM in a pH 7 phosphate buffer (50 mM) containing 0.1 M NaCl and 1 mM spermine. The 43merWC→H(A) and 46merWC→H(A) targets have the same binding site for the 17mer as 48merWC→H(A), but an additional single-stranded sequence is present on the 3′ side of the hairpin in 46merWC→H(A) (see Figure 2). <0 indicates that no melting transition was observed between 0 and 90 °C except that of the hairpin duplex target.

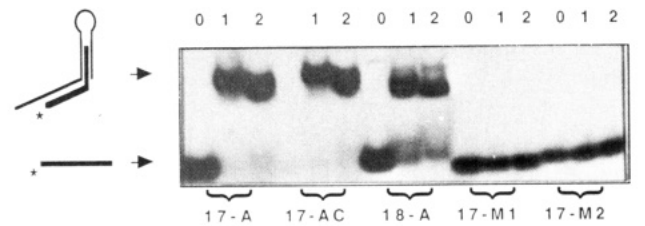
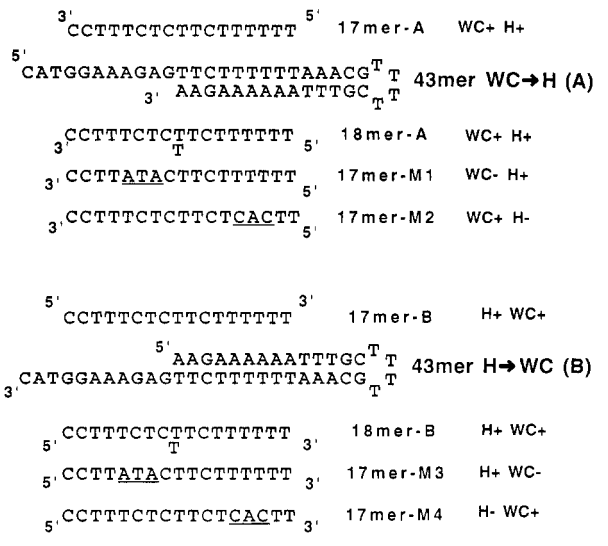


FIGURE 5: Gel shift experiment indicating that 17mer-A and 18mer-A bind to the hairpin-containing targets. The 5′-labeled oligomers were incubated at pH 6 as described in Materials and Methods in the absence (lanes 0) or in the presence of unlabeled target 49merH→WC(A) (lanes 1) or 48merWC→H(A) (lanes 2). 17mer-A, 18mer-A, 17mer-M1, and 17mer-M2 are abbreviated as 17-A, 18-A, 17-M1, and 17-M2, respectively. 17mer-A with cytosines replaced by 5-methylcytosines is abbreviated as 17-AC.

only one single-stranded sequence on one side of the double-stranded stem. In messenger RNA, secondary structures such as hairpins have typically two single-stranded sequences located on each side of the stem−loop sequence. In order to determine whether it was possible to target oligonucleotides against such hairpin structures, 46merWC→H(A) was synthesized (Figure 2). This oligomer has the same binding sequence as 43merWC→H(A) except that a single-stranded sequence is added to the 3′ end of the stem (Figures 2 and 4). As shown by the T_m values obtained, the binding of 17mer-A (WC^+ H^+) was weakened by the introduction of a single-stranded region at the 3′ end of the target (Figure 4). Nevertheless, the binding resulted from both Watson−Crick and Hoogsteen pairing as indicated by the behavior of mutated oligomers [compare T_m values for 17mer-M1 (WC^- H^+) and 17mer-M2 (WC^+ H^-) on 46merWC→H(A) and 43merWC→H(A) targets]. It is noteworthy that the 18mer-A (WC^+ H^+)/46merWC→H(A) complex was more stable than the 17mer-A (WC^+ H^+)/46merWC→H(A) complex. The same result was obtained with the 43merWC→H(A) target, but the difference in T_m values between 17mer-A and 18mer-A was lower than that obtained with the 46merWC→H(A) target (Figure 4). The octamer 8mer-A (WC^+ H^-), which corresponds to the Watson−Crick side of the oligonucleotide ligand, binds to 46merWC→H(A) and 43merWC→H(A) with T_m values of 34.5 and 42 °C, respectively, emphasizing the destabilizing effect of the additional single-stranded "tail" in 46merWC→H(A). The 8mer-A targeted to the 5′ side single-stranded sequence of 43merWC→H(A) had a higher melting transition due to a binding cooperativity between the short oligomer and the stem structure (see above) (Figure 4). This cooperative effect was lost when the 46merWC→H(A) with the 3′ "tail" was used (Figure 4).

Influence of WC→H or H→WC Orientation on Stabilities. In order to analyze the importance of the junction between the double-stranded stem and the single-stranded sequence, two different targets were assayed for binding of two oligonucleotides, 17mer-A (WC^+ H^+) and 17mer-B (H^+ WC^+), with reverse orientations (Figure 6). The orientation of the 43merWC→H(A) stem sequence is then of opposite polarity to that of 43merH→WC(B). Melting experiments were performed to compare both targets and both oligomers. As shown in Figure 6, the 17mer-A (WC^+ H^+)/43merWC→H(A) complex had a slightly higher stability as compared to the 17mer-B (H^+ WC^+)/43merH→WC(B) complex.

A thymine residue was incorporated in the 17mers at the junction between the Hoogsteen and Watson−Crick regions. No important differences in T_m values were observed when 17mer-A (WC^+ H^+) and 18mer-A (WC^+ H^+) were compared at pH 6 (Figure 6). A slight stabilization occurred at pH 7.



Oligo/target		pH 6, S+[a]	pH 7, S+[b]
17-A/43WC->H (A)	WC+ H+	30	37
17-B/43H->WC (B)	H+ WC+	28	30
18-A/43WC->H (A)	WC+ H+	30	39
18-B/43H->WC (B)	H+ WC+	23	24
17-M1/43WC->H (A)	WC- H+	-	19
17-M3/43H->WC (B)	H+ WC-	-	5
17-M2/43WC->H (A)	WC+ H-	-	34
17-M4/43H->WC (B)	H- WC+	-	20

FIGURE 6: Effect of the junction between the single-stranded and double-stranded portions of the target on binding stabilities. 18mer-A (WC^+ H^+) contains one additional thymine at the junction of 43merWC→H(A) as compared to 17mer-A (WC^+ H^+). Sequences of oligomers 17mer-B (H^+ WC^+) and 18mer-B (H^+ WC^+) and their target, 43merH→WC(B), are also indicated. 17mer-B and 18mer-B have a reverse orientation as compared to 17mer-A and 18mer-A. Both 43merWC→H(A) and 43merH→WC(B) have junctions with opposite orientations, 5′-AGTT-3′/3′-AA-5′ for 43merWC→H(A) and 3′-AGTT-5′/5′-AA-3′ for 43merH→WC(B). T_m values are obtained in a pH 6 or 7 phosphate buffer (50 mM) containing 0.1 M NaCl in the presence of spermine as mentioned. [a]1 μM/1 μM, 0.2 mM spermine. [b]2 μM/2 μM, 1 mM spermine.

In contrast, the addition of one base at the junction decreased the stability of 18mer-B as compared to 17mer-B when bound to 43merH→WC(B) (24 versus 30 °C at pH 7). Conformational energy minimization was performed with the JUMNA program (Lavery, 1988). This analysis suggested that the extra thymine was located within the major groove of the stem structure in the case of 43merWC→H(A), but was rotated outside of the double-helical structure in the case of 43merH→WC(B) (results not shown). Therefore it is possible that this thymine destabilizes the 18mer-(B) (H^+ WC^+)/43merH→WC(B) complex by changing the solvent structure in the vicinity of the WC/H junction.

As shown also in Figure 6, the mutated oligomers WC^+ H^-, 17mer-M2, and 17mer-M4 had different stabilities. At pH 7 in the presence of spermine, a difference of 10 °C was observed between 17mer-B (H^+ WC^+) and 17mer-M4 (H^- WC^+), but only a difference of 3 °C existed between 17mer-A (WC^+ H^+) and 17mer-M2 (WC^+ H^-). A similar difference in stability was observed with the mutated oligomers WC^- H^+ (compare 17mer-M1 and 17mer-M3 in Figure 6). It should be noted that hyperchromisms, which are related to base pair and base triplet stacking, are lower with mutated oligomers than with the specific 17mers, confirming that only the Watson–Crick double helices are formed in the WC^+ H^- and H^- WC^+ complexes. 17mer-M1 (WC^- H^+) was more stable than 17mer-M3 (H^+ WC^-) when bound to their respective inverted targets, even though they lead to the same mismatches. The difference was larger than when both Watson–Crick and Hoogsteen interactions took place (17mer-A and 17mer-B). Destabilization was always smaller when mismatches occurred in the Hoogsteen part. These results indicate that the orientation of the oligonucleotide designed in the Watson–Crick/Hoogsteen approach plays an important role in determining complex stability.

Site-Specific Cleavage of Hairpin Targets by the Phenanthroline–17mer Conjugate. The 1,10-phenanthroline–cuprous complex [$(OP)_2$–Cu^+] cleaves DNA in a reaction that requires oxygen and a reducing agent (Sigman, 1986, 1990; Sigman et al., 1979, 1993). The covalent linkage of 1,10-phenanthroline to an oligonucleotide brings the cleaving reagent into close proximity to the single-stranded or double-stranded complementary sequences and therefore confers upon this oligomer a targeted nucleolytic activity (Chen & Sigman, 1986, 1988; François et al., 1988a; François et al., 1989a,b; Sigman et al., 1993; Shimizu et al., 1994). A modified 17mer-A (WC^+ H^+) with a phenanthroline attached to its 5′ end was incubated with the 48merWC→H(A) target in the presence of copper ions and 3-mercaptopropionic acid. Cleavage of the target was observed as shown in Figure 7A. The cleavage sites were located within the hairpin stem in close proximity to the 5′ end of OP-17mer-A. Cleavage efficiencies are presented in Figure 7B. The cleavage sites on the upper and lower strands of the hairpin stem exhibited an asymmetric distribution. They were shifted toward the 3′ end, suggesting that cleavage took place from the minor groove as previously observed with phenanthroline cleavage (François et al., 1989a,b; Veal & Rill, 1989; Sigman et al., 1993).

The location of cleavage sites with respect to the target sequence indicated that OP-17-mer-A was bound in a parallel orientation with respect to the purine-rich strand of the stem sequence via Hoogsteen base triplet formation and in an antiparallel orientation with respect to the purine-rich single-stranded sequence via Watson–Crick base pairing. As shown in Figure 7A, cleavage efficiency was also increased upon addition of spermine and at acidic pH, in agreement with triple helix formation in the 17mer-A (WC^+ H^+)/48merWC→H(A) complex. Cleavage from the minor groove side of the double-helical region occurred as a result of phenanthroline intercalation within the stem structure, as previously described for a triple-helical complex (François et al., 1989a,b) (see Figure 7B for the position of intercalated phenanthroline). Cleavage was about twice as efficient on the oligopyrimidine strand of the stem as on the oligopurine strand.

A weak cleavage occurred at the thymine located at the junction between Hoogsteen and Watson–Crick domains. This weak site might result from cleavage of a peculiar conformation at the junction between the double- and triple-stranded helices by the phenanthroline moiety of a non-hydrogen-bonded OP-17-mer-A. This observation is in agreement with the reactivity of the 1,10-phenanthroline–cuprous complex, which has been extensively used as a chemical reagent to probe specific structures in hairpins (Drew, 1984; François et al., 1994), in bulge-containing duplexes (Williams et al., 1988), in three-way DNA junctions (Guo et al., 1990; Zhong et al., 1994; François et al., 1994), and in triple helices (François et al., 1988b).

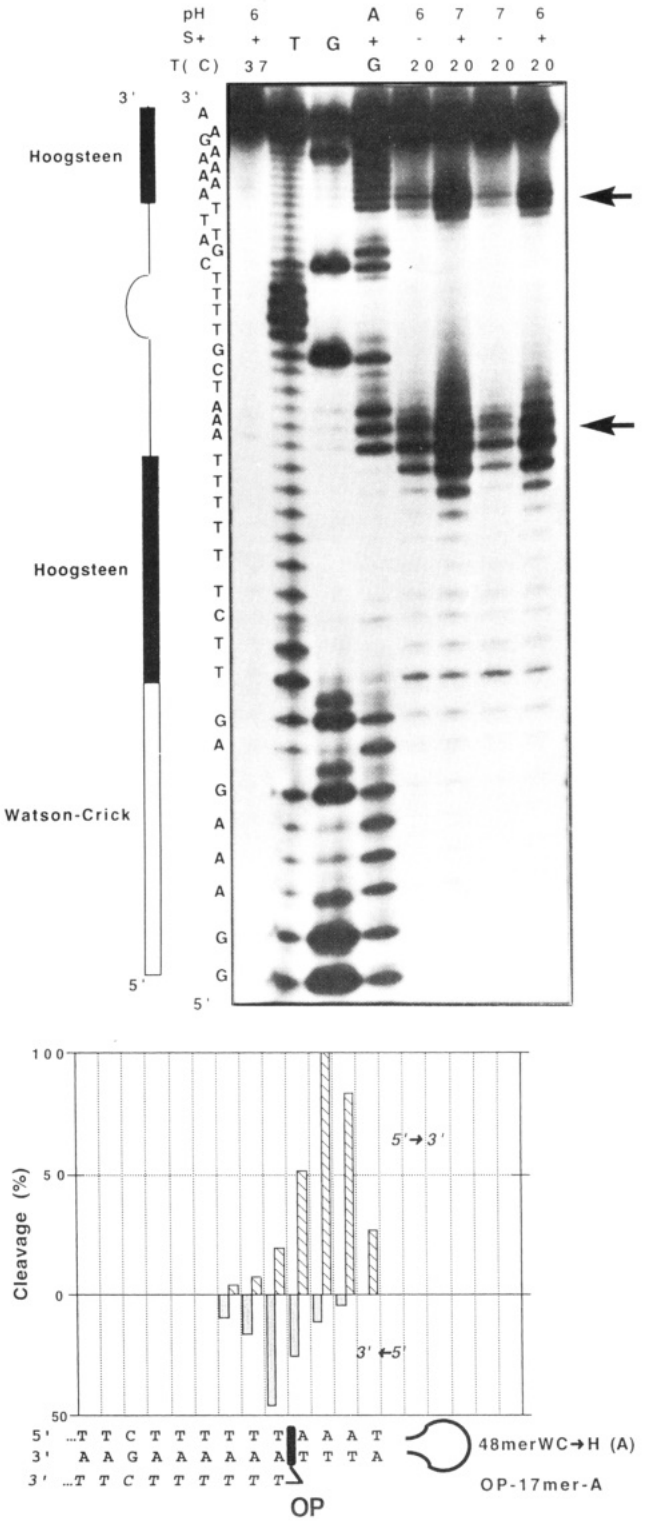


FIGURE 7: Cleavage of hairpin-containing 48merWC→H by a Cu–phenanthroline–oligomer conjugate (OP-17mer-A). (A, top panel) Cleavage conditions are indicated in Materials and Methods. The reaction was carried out at two pH values (6 and 7) in the presence (+) or the absence (−) of 0.2 mM spermine and at two temperatures (37 and 20 °C) as indicated in the upper part of the panel. Cleaved fragments resulting from (T, G+A, G) Maxam–Gilbert reactions are shown. Notice that in the $KMnO_4$ reaction (lane T) unpaired thymines in the T_4 loop are hypersensitive to cleavage. (B, bottom panel) Densitometric analysis of the cleavage pattern obtained at pH 7 in the presence of spermine. Part of the target sequence 48merWC→H(A) is presented from the 5′ end (upper strand) to the 3′ end (lower strand) connected by the T_4 loop. Phenanthroline–oligonucleotide is also indicated in italics with phenanthroline (black rectangle) intercalated at the triplex–duplex junction within the stem structure. Bars represent cleavage intensities observed on the two strands of the stem structure.

DISCUSSION

We have shown that oligonucleotides could be targeted against hairpin structures by formation of both a standard double helix and a triple helix. We have used an oligopyrimidine to analyze the effect of orientation of the double helix versus that of the triple helix. But the double-helical part of the complexes does not need to be an oligopyrimidine–oligopurine sequence. The triple helix-forming portion of the oligonucleotide might consist of an oligopurine or a (G,T)-containing sequence instead of an oligopyrimidine sequence. An oligopurine would bind in an antiparallel orientation with respect to the polypurine sequence of the stem structure (reverse Hoogsteen hydrogen bonding), whereas an oligopyrimidine binds with a parallel orientation (Hoogsteen hydrogen bonding) (Thuong & Hélène, 1993). For (G,T)-containing oligomers the orientation may depend on the sequence (Sun et al., 1991).

In the WC/H complexes, the same oligonucleotide is engaged in the formation of base pairs and base triplets via Watson–Crick and Hoogsteen hydrogen bonds, respectively. The length of the target plays an important role in these structures. To design appropriate oligonucleotides using this alternative approach to recognize nucleic acid structures requires choosing an appropriate length of the Watson–Crick part in order to ensure a sufficient stability of the complex. The Hoogsteen part also needs to be long enough to sustain triple helix formation at physiological pH. A complete Watson–Crick double helix between an oligonucleotide and a single-stranded complementary sequence is more stable than the Watson–Crick/Hoogsteen complexes described in this report. However, the nucleic acid target can be so structured that no complementary oligomer (in the Watson–Crick sense) would disrupt the stem sequence. The Watson–Crick/Hoogsteen oligomer could bind to a structured DNA provided the stem contained an oligopurine–oligopyrimidine sequence, whereas a standard antisense oligonucleotide was not able to disrupt the hairpin structure and to bind to its complementary target.

The WC/H oligonucleotide covalently linked to an active reagent could cleave the hairpin target within the stem structure. Other irreversible (e.g., cross-linking) reactions can also be contemplated. Recently, it was shown that triple helices can be formed either on double-stranded DNA or RNA when the third strand is an RNA or a 2′-*O*-methyl derivative (Shimizu et al., 1992; Roberts & Crothers, 1992; Escudé et al., 1992, 1993; Skoog & Maher, 1993; Han & Dervan, 1993, 1994). Therefore, oligonucleotides can be designed to bind hairpin-containing RNA structures via both Watson–Crick and Hoogsteen hydrogen bonding. These WC/H oligonucleotides might be useful in the antisense strategy by preventing translation of a stabilized hairpin structure. The results presented in the above study have dealt with a hairpin-containing DNA target and an oligodeoxynucleotide ligand. Further studies should aim at investigating the role of DNA or RNA backbone composition on the stability of complexes involving adjacent Watson–Crick and Hoogsteen (reverse Hoogsteen) structures.

ACKNOWLEDGMENT

We thank N. T. Thuong for the synthesis of the 17mer–phenanthroline conjugate. We are also grateful to J. S. Sun for energy minimization calculations and software adaptation for Uvikon 940; J. L. Mergny, M. Rougée, and G. Godard

for helpful discussions; O. Delgado for photographical work; M. Drivon for typing the manuscript; M. T. Bergot for reference searching; and P. King for corrections.

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BI941235O