Detailed Study of Sequence-Specific DNA Cleavage of Triplex-Forming Oligonucleotides Linked to 1,10-Phenanthroline†

Miho Shimizu,‡ Hideo Inoue, and Eiko Ohtsuka*

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

Received August 4, 1993; Revised Manuscript Received October 26, 1993®

ABSTRACT: We introduced eight bases, including four base analogs, into 15-mer triplex-forming oligonucleotides (TFOs) [d-psTTTCTTTNTTTTCTT; ps = thiophosphate; N = A, G, C, T, 2′-deoxyinosine (I), 2′-deoxyxanthosine (X), 5-methyl-2′-deoxycytidine (m^5C), or 5-bromo-2′-deoxyuridine (br^5U)] to investigate the Hoogsteen-like hydrogen bonding to the base in the target 34-mer strand (d-TGAGTG-AGTAAAGAAARAAAAGAATGAGTGCCAA·d-TTGGCACTCATTCTTTTYTTTCTTTACT-CACTCA; RY = AT, GC, TA, or CG). We examined the thermal stability of 15-mer triplexes in buffer containing 100 mM sodium acetate and 1 M NaCl at pH 5.0. The triplexes with typical triplets of T·AT (51.3 °C), br^5U·AT (52.4 °C), C^+·GC (66.7 °C), and m^5C^+·GC (66.8 °C) at the central position showed relatively higher T_m values, as expected. The relatively high stability of the X·AT triplex (39.8 °C) was observed. Among the N·TA triplets, G·TA (44.8 °C) was thermally the most stable, and moreover, the data showed that the N·TA triplet was also stabilized by I in the N position (40.7 °C). Furthermore, the TFOs were converted to DNA -cleaving molecules by introducing a newly synthesized 1,10-phenanthroline (OP) derivative on the thiophosphate group at the 5′ end. Cleavage reactions of the ^{32}P -labeled DNA (34-mer) were carried out. The cleavage efficiencies were compared to the T_m values of triplexes with or without an OP derivative. Results showed that the increased cleavage yields reflect the higher thermal stability of the triplex formed in most cases, but a few exceptional cases existed. Especially, the G -containing TFO did not show the above correlation between thermal stability and cleavage yield. The possibility of the binding of free Cu^{2+} ion to a G base or the formation of the 8-oxo-G base could explain the results. The influence of reducing agents on cleavage efficiency was also examined.

Techniques of targeting and cleaving double-stranded DNA by triple-helix -forming oligonucleotides (TFOs)[1] with nuclease activity may be very attractive for mapping the large megabase-sized human genome (Dervan, 1992). The major advantages of using modified oligonucleotides are the more diverse and longer recognition sequences, compared to small DNA-binding drugs, other chemicals (Sigman & Chen, 1990), and restriction enzymes.

In the pyrimidine·purine·pyrimidine DNA triplex, a TFO consisting of homopyrimidine nucleotides binds parallel to a homopurine sequence in the major groove of a homopurine·homopyrimidine duplex DNA through Hoogsteen bond formation (Moser & Dervan, 1987; Hélène & Toulmé, 1990). The pyrimidine·purine·pyrimidine triplexes contain T·AT and C^+·GC triplets. A nonstandard G·TA triplet is included in the above triple helix (Griffin & Dervan, 1989). Another type of triple helix, a purine·purine·pyrimidine DNA triplex, has also been reported. In the triplex, a TFO binds antiparallel to the purine strand of the Watson–Crick duplex (Kohwi & Kohwi-Shigematsu, 1988; Cooney et al., 1988).

In targeting double-helical DNA by TFOs with a DNA-cleaving molecule, we considered two important questions. First, because it is obvious that triple helix formation is mostly limited to the T·AT and C^+·GC hydrogen bonds in the pyrimidine·purine·pyrimidine type triplex, it is desirable to find a specific binding mode for all four natural bases in the target strands. For this purpose, some nonnatural bases have been designed for targeting a pyrimidine base positioned in the purine strand (Kiessling et al., 1992; Koh & Dervan, 1992; Krawczyk et al., 1992). Second, affinity cleaving methods through triple-helix formation have been widely used for sequence-specific cleavage of a single target site on duplex DNA (Moser & Dervan, 1987; François et al., 1989a,b; Povsic et al., 1992; Povsic & Dervan, 1990; Shaw et al., 1991; Takasugi et al., 1991; Giovannangeli et al., 1992), but little is known about the relationship between the thermal stability of the oligonucleotide-bound triple helix and the cleavage efficiencies of the targeted strands in those containing a mismatched Hoogsteen-type hydrogen bond (Singleton & Dervan, 1992).

In addition to the four natural bases, namely, 2′-deoxyadenosine (A), 2′-deoxyguanosine (G), 2′-deoxycytidine (C), and thymidine (T), we introduced four base analogs into the 15-mer pyrimidine strand (TFO) to investigate another Hoogsteen-like hydrogen bonding to the base in the target 34-mer strand: 2′-deoxyinosine (I), 2′-deoxyxanthosine (X), 5-methyl-2′-deoxycytidine (m^5C), and 5-bromo-2′-deoxyuridine (br^5U). We examined the thermal stability of 15-mer triplexes containing various triplets in the central position of the complex (32 combinations; Figure 1). Furthermore, the TFOs were converted to DNA-cleaving molecules by

† This research was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

* To whom correspondence should be addressed.

‡ Present address: Institute of Biosciences and Technology, Texas A&M University, Houston, TX 77030.

® Abstract published in *Advance ACS Abstracts,* December 15, 1993.

[1] Abbreviations: TFO, triple-helix-forming oligonucleotide; A, 2′-deoxyadenosine; G, 2′-deoxyguanosine; C, 2′-deoxycytidine; T, thymidine; X, 2′-deoxyxanthosine; br^5U, 5-bromo-2′-deoxyuridine; m^5C, 5-methyl-2′-deoxycytidine; I, 2′-deoxyinosine; OP, 1,10-phenanthroline; $(OP)_2$–Cu, 2:1 1,10-phenanthroline–copper complex; MPA, β-mercaptopropionic acid; As, ascorbic acid; DTT, dithiothreitol; Cys, L-cysteine; GSH, glutathione, reduced form; 8-oxo-G, 8-oxo-2′-deoxyguanosine; NPE, (4-nitrophenyl)ethyl; EDTA, ethylenediaminetetraacetic acid (disodium salt); OP–N, 1,10-phenanthroline-linked d(TTTCTTTNTTTTCTT); PAGE, polyacrylamide gel electrophoresis.

0006-2960/94/0433-606$04.50/0

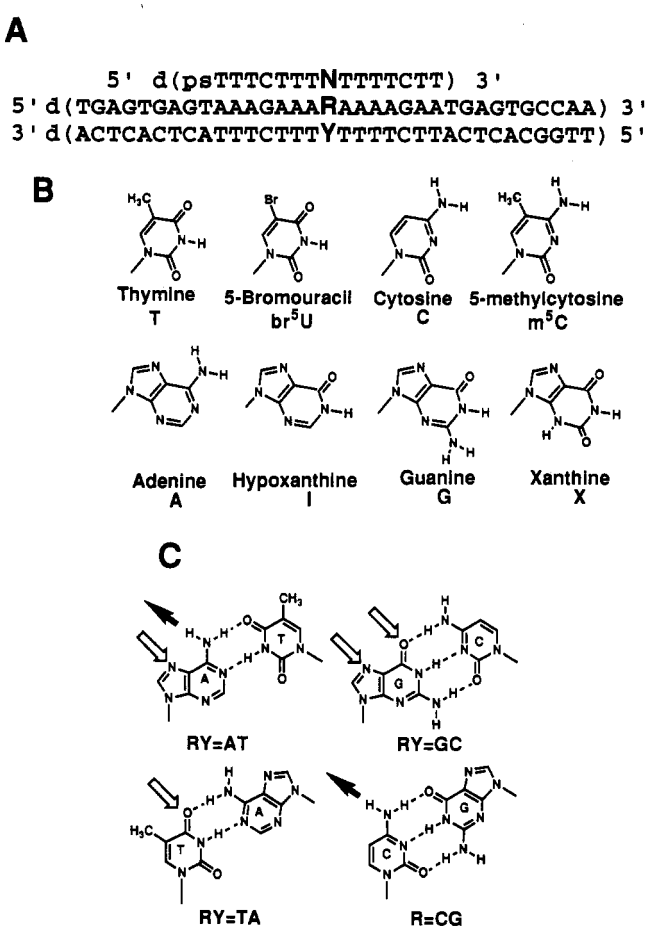


FIGURE 1: Triple helix containing various bases at the central positions of the complex. (A) Sequence of the triple helix containing a 15-mer TFO and the 34-mer target duplex. Abbreviations for 34-mer duplexes in the text: AT34 (RY = AT), GC34 (RY = GC), TA34 (RY = TA), and CG34 (RY = CG). (B) Structures of nucleobases introduced into the N position of the TFO. (C) Watson–Crick base pairs in the N·RY triplexes. Hydrogen donors are indicated by the solid arrows, and hydrogen acceptors are indicated by the open arrows.

introducing a newly synthesized 1,10-phenanthroline (OP) derivative on the thiophosphate group at the 5′ end. Cleavage reactions of the ^{32}P-labeled DNA (34-mer) were carried out. The cleavage efficiencies were compared to the respective T_m values.

Although the kinetic mechanism of the nuclease activity of a 1,10-phenanthroline–copper complex [$(OP)_2$–Cu] was demonstrated (Thederahn et al., 1989), there have been few mechanistic studies of OP-linked oligonucleotides (François et al., 1989a,b). In general, the cleavage reaction has been initiated by *in situ* generation of hydrogen peroxide. β-Mercaptopropionic acid (MPA) is generally used for this purpose (Sigman et al., 1991; François et al., 1989a,b). Recently, cleavage was optimized by using ascorbic acid (As) as a reducing agent (Veal et al., 1991). We compared the influence of the reducing agents on the cleavage reactions by TFOs with OP. A non-thiol reagent, namely, As, and four thiol reagents, MPA, dithiothreitol (DTT), L-cysteine (Cys), and glutathione, reduced form (GSH), were examined. We observed that a broad range of cleavage products were obtained when ascorbic acid was used.

MATERIALS AND METHODS

^{1}H-NMR spectra of the protected nucleosides and the phenanthroline derivatives were measured at 270 MHz with a JEOL GX270 spectrometer. ^{31}P-NMR spectra were measured at 36.25 MHz with a JEOL FX90Q spectrometer.

Chemicals. Oligodeoxynucleotides were synthesized on an Applied Biosystems (ABI) 394 DNA/RNA synthesizer using the standard phosphoramidite method (Sinha et al., 1983) with commercially available reagents (ABN for br^{5}U and ABI for others). The 3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) derivative of X and the 8-oxo-2′-deoxyguanosine-containing (8-oxo-G-containing) oligonucleotide were prepared as described below. The synthesis of the phosphoramidite derivative of N^4-benzoyl-5-methyl-2′-deoxycytidine was based on reported procedures (Divakar & Rees, 1982; Bhat et al., 1989). Oligodeoxynucleotides were purified by reverse-phase HPLC using a Inertsil ODS-2 column (10 × 250 mm; GL Science Inc.). Further purification was done by anion-exchange HPLC using a TSK gel DEAE-2SW column (4.6 × 250 mm; Tosoh Co.). The following chemicals were obtained from commercial sources: (2-cyanoethoxy)-[2-[[2-[(4,4′-dimethoxytrityl)oxy]ethyl]sulfonyl]ethoxy](*N*,*N*-diisopropylamino)phosphine (Clontech Laboratories), 5-nitro-1,10-phenanthroline (Aldrich), and *N*-[(γ-maleimidobutyryl)oxy]succinimide (Dojindo Laboratories).

Preparation of a Phosphoramidite Derivative of Protected X. The synthesis of an X phosphoramidite derivative was generally based on the reported procedure (Eritja et al., 1986). A (4-nitrophenyl)ethyl (NPE) group was introduced into 3′,5′-di-*O*-acetyl-2′-deoxyguanosine at the O-6 position without protection of the amino group (Schulz & Pfleiderer, 1985). This O-6 -protected 2′-deoxyguanosine was deaminated with nitrous acid, followed by deacylation of the sugar residue to produce O-6-protected X. The hydroxyl function at the 5′ position was protected by a 4,4′-dimethoxytrityl group. The nucleoside was phosphitylated using 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite: ^{31}P-NMR ($CDCl_3$; trimethyl phosphate was used as an internal reference) δ 146.46, 143.03 ppm.

Deprotection of the Oligodeoxynucleotide Containing X. The 15-mer oligonucleotide containing X at the N position was deprotected as follows. After automated DNA synthesis, the support (1-μmol CPG column) was treated with 2 mL of 0.5 M 1,8-diazabicyclo[5.4.0]undec-7-ene in anhydrous pyridine (2 h, room temperature) to remove the NPE group before the oligonucleotide was detached from the support with concentrated aqueous ammonia. The side product, which was presumably produced by nucleophilic substitution at the O-6 position, was observed when the NPE group was removed after deprotection of other protecting groups by heating with concentrated aqueous ammonia (55 °C, 5 h) as described previously (Kamiya et al., 1992).

Synthesis of the OP-Linked 15-mer TFOs. The synthetic route is shown in Figure 2. The newly synthesized thiol-specific OP derivative was attached to the oligodeoxynucleotides via a thiophosphate group at the 5′ end.

Preparation of 5-[[N-(4-Maleimido-1-oxobutyl)glycyl]-amino]-OP. 5-Nitro-1,10-phenanthroline was converted to 5-(glycylamino)-OP by the method described (Sigman et al., 1991). Ten milligrams (0.04 mmol) of 5-(glycylamino)-OP was suspended in a mixture containing dimethyl sulfoxide (DMSO) (50 μL), 1 M $NaHCO_3$ (pH 8.0, 10 μL), and H_2O (40 μL). The mixture was cooled in ice. To this was added an equimolar amount of *N*-[(γ-maleimidobutyryl)oxy]succinimide (dissolved in 100 μL of DMSO). The reaction mixture then became clear, and it was shaken gently at room temperature for 3 h. The entire reaction mixture was applied to a column of LH20 (Pharmacia, 18 × 400 mm) and eluted

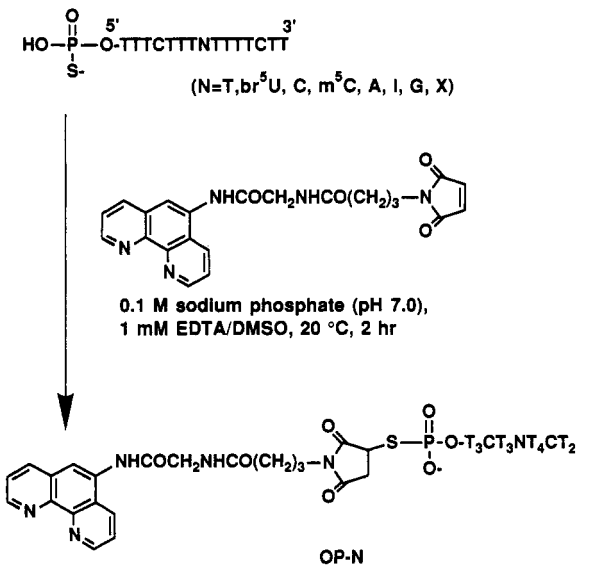


FIGURE 2: Attachment of the maleimido derivative of phenanthroline to oligonucleotides. Abbreviations used for OP–Ns in the text: OP–T (N = T), OP–br^{5}U (N = br^{5}U), OP–C (N = C), OP–m^{5}C (N = m^{5}C), OP–A (N = A), OP–I (N = I), OP–G (N = G), and OP–X (N = X).

with methanol at 4 °C. The yellow-colored fractions were analyzed separately by thin-layer chromatography (Kiesel gel 60F_{254} plates, Merck; 3% aqueous ammonia in methanol). The product showed faster mobility than 5-(glycylamino)-OP. Fractions containing pure material were pooled and dried under vacuum. ^{1}H-NMR (DMSO-d_6; TMS was used as an internal reference) δ 1.77, 2.20, and 3.44 (6H, m, $(CH_2)_3$), 4.05 (2H, d , 5-NHCOCH_2), 7.00 (2H, s, maleimide), 8.11 (1H, s, H-6), 8.31 (1H, t, CH_2NHCO), 7.79, 8.52, and, 9.08 (6H, m, Ar), 10.14 ppm (1H, s, 5-*NH*CO).

Synthesis of TFOs Carrying a Phosphorothioate at the 5′ End. At the last coupling step of the automated oligonucleotide synthesis on the support, (2-cyanoethoxy)[2-[[2-[(4,4′-dimethoxytrityl)oxy]ethyl]sulfonyl]ethoxy](*N*,*N*-diisopropylamino)phosphine was allowed to react with the 5′-hydroxyl group. The intermediate phosphite was oxidized with 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (0.05 M in acetonitrile) for 30 s in place of I_2 in H_2O–pyridine (Iyer et al., 1990a,b).

Synthesis of OP-Linked Oligodeoxynucleotides. The 5′-thiophosphorylated oligodeoxynucleotides (2 A_{260} units) were treated with a slight excess of 5-[[*N*-(4-maleimido-1-oxobutyl)glycyl]amino]-OP in a solution containing 0.1 M sodium phosphate (pH 7.0), 1 mM EDTA (20 μL), and DMSO (20 μL). The reaction mixture was placed in a 1.5-mL Eppendorf tube at 20 °C for 2 h under an argon atmosphere. The oligodeoxynucleotides were separated from the unreacted OP reagents by gel filtration (Sephadex G-50, Pharmacia, 18 × 400 mm) twice in H_2O. The oligodeoxynucleotides were then analyzed and purified by anion-exchange HPLC with a linear gradient of ammonium formate (from 2.5% to 5% in 20 min) in 20% aqueous acetonitrile followed by gel filtration. Finally the products were passed through a column of Chelex 100 (Bio-Rad) to remove trace metals. The pure materials were obtained in the range from 10% to 20% on the basis of the 5′-thiophosphorylated oligodeoxynucleotides.

UV Melting Studies. Thermal denaturation profiles were performed in a buffer containing 100 mM sodium acetate (pH 5.0) and 1.0 M NaCl. We employed a relatively higher salt condition (1 M NaCl) to confirm the formation of the triplex containing mismatched base pairs. The 15-mer oligodeoxynucleotides with a thiophosphate group at the 5′ end and without a OP derivative were used for hybridization to 34-mer duplex DNAs. The oligodeoxynucleotide concentration was 1 μM single strands. The minimum absorbance was approximately 0.7 at 260 nm. Thermal denaturation curves were recorded at 260 nm on a Gilford Response II UV-vis spectrophotometer using the temperature programming. The cell path length was 1 cm. The mixture of target duplexes and 15-mer single strands was first heated to 85 °C and then cooled to 5 °C over 40 min. The temperature was then increased to 85 °C at a rate of 0.4 °C/min. The total run time was 200 min. The cuvette-holding chamber was flushed with N_2 gas for the duration of the run at the lower temperature (between 0 and 20 °C). Thermal denaturation profiles were evaluated by identifying the temperatures (T_m values) at which $d(A_{260})/d(T)$ *versus* T (°C) plots (first-derivative plots) reached their maxima.

End Labeling of a 34-mer Strand at the 5′ or 3′ Site. The purine-rich strand was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Takara Shuzo). The pyrimidine-rich strand was labeled with 3′-[α-^{32}P]dATP and terminal deoxyribonucleotidyltransferase (DuPont) and purified on a NENSORB 20 column (DuPont). The efficiency of the phosphorylation and the purity of the labeled strands were analyzed by homochromatography on DEAE-cellulose thin-layer plates with Homo-mix I (Jay et al., 1974). The yields of the 5′-end-labeling reactions were more than 90%. The labeled strand was annealed with the unlabeled complementary strand by heating at 80 °C for 10 min and then slowly cooling to room temperature to prepare 0.25 μM (single strands) stock solution.

Cleavage Reactions. Typically, a 4-μL solution of target 34-mer duplex DNA (0.5 pmol) and OP–oligonucleotide (10 pmol) in a buffer containing 12.5 mM sodium acetate (pH 5.0), 125 mM NaCl, 1.25 mM spermine·4HCl, and carrier tRNA was heated at 50 °C for 10 min and slowly cooled to 4 °C for triple-helix formation. Ten micromolar copper sulfate (0.5 μL) was added, and the mixture was preincubated at 37 °C for 1 h. The cleavage reaction was initiated by adding 100 mM MPA (0.5 μL). The solutions of copper ion and MPA were freshly prepared in ultrapure water purified by a Milli-Q system (Millipore) and by TORAY's ion-exchange fiber (TORAY PURE LV-08). The reaction mixture was incubated at 37 °C for 24 h. The cleavage reaction was stopped by adding 100 μM neocuproine (1 μL in ethanol). This solution was mixed (1:2, v/v) with 80% deionized formamide containing 0.1% xylene cyanol and 0.1% bromophenol blue. Samples were heated for 1 min at 90 °C and chilled in ice for denaturing. They were then loaded onto a 20% polyacrylamide gel (29:1, acrylamide:bis(acrylamide); 40 cm long) containing 7 M urea. The gel was run at constant voltage (1600 V). The gel was dried and exposed to an imaging plate. The radioactivity was analyzed by using a bioimaging analyzer, FUJI BAS2000. Autoradiography of the gel was done at −80 °C using Kodak X-Omat RP film with an intensifying screen.

Synthesis of 8-Oxo-G Containing TFO. The phosphitylation of N^2-acetyl-8-methoxy-5′-*O*-(monomethoxytrityl)-2′-deoxyguanosine (Kuchino et al., 1987) was carried out according to the general procedure. The 15-mer TFO 5′-d(TTTCTTTNTTTTCT) (N = N^2-acetyl-8-methoxy-2′-deoxyguanosine) was synthesized via the standard phosphoramidite method. Purification of the oligonucleotide was done by the method described above. The conversion of the 8-methoxyguanine base into the 8-oxoguanine base in the TFO was accomplished as follows: The oligonucleotide was treated with thiophenol in the presence of triethylamine at 50 °C for

Table 1: Thermal Dissociation Temperatures (°C) of Hoogsteen Bonds in the N·RY Triplexes (Figure 1A) at pH 5.0 in 100 mM Sodium Acetate Containing 1 M NaCl

	N							
RY	T	br^{5}U	C	m^{5}C	A	I	G	X
AT	51.3	52.4	31.7	34.5	34.7	33.2	27.5	39.8
GC	47.1	48.9	66.7	66.8	49.2	42.8	38.9	35.1
TA	31.8	31.5	31.5	32.0	34.7	40.7	44.8	30.9
CG	43.8	42.0	42.5	42.0	31.9	35.3	38.0	37.1

two days in an inert gas. The TFO containing 8-oxo-G was isolated by anion-exchange HPLC and desalted by gel filtration.

RESULTS AND DISCUSSION

UV Melting Studies on Triplex Formation. The effects of the various N-position nucleotides in the TFOs on the stability of a triple helix were evaluated by the T_m measurements. Temperature-dependent thermal denaturation studies were performed on the 32 combinations of the triplex (Figure 1). We used a buffer containing a relatively higher salt concentration (1 M) to confirm the formation of the triplex containing one mismatched base triplet. In all cases, the melting profiles showed a biphasic dissociation that was typical of a triple helix. The second transition corresponding to the dissociation of the 34-mer duplex had almost same T_m values (RY = AT, 78.4 °C; GC, 79.0 °C; TA, 78.1 °C; CG, 79.4 °C). The T_m values of the TFOs from the target duplex are summarized in Table 1.

In the case of RY = AT and RY = GC, the underlying duplexes contain entire purine/pyrimidine strands. The triplexes with typical triplets of T·AT (51.3 °C), br^{5}U·AT (52.4 °C), C$^+$·GC (66.7 °C), and m^{5}C$^+$·GC (66.8 °C) at the central position exhibited relatively higher T_m values, as expected. The relatively high stability of the X·AT triplex (T_m = 39.8 °C) was observed among the 6-oxopurine series (X, I, G), which might form two hydrogen bonds, NH-1(X)·N-7(A) and O-6(X)·NH-6(A). On the other hand, triplexes that contained the underlying duplexes with nonhomogeneous strands (RY = TA and CG) displayed lower T_m values than the above cases.

Among the N·TA triplets, G·TA (Tm = 44.8 °C) was thermally the most stable. This result agreed with the observation reported previously (Griffin & Dervan, 1989). The data showed that the N·TA triplet was also stabilized by I in the N position (T_m = 40.7 °C). This stability might reflect the formation of a hydrogen bond between NH-1(I) and O-4(T).

It was expected that the 2-keto acceptor of X could form a single hydrogen bond with the 4-amino group in a CG base pair. Unfortunately, the X·CG triplet (T_m = 37.1 °C) did not contribute to triple-helix stabilization, and it was found that the N·CG triplex became comparatively stable when the pyrimidine bases were introduced into the N position; the T·CG triplex was the most stable (T_m = 43.8 °C) and was followed by C$^+$·CG, br^{5}U·CG, and m^{5}C$^+$·CG. These results were similar to those reported recently (Mergny et al., 1991).

Cleavage of Target Duplex DNAs by OP–15-mers. OP–oligonucleotides containing various bases at the central position of the 15-mer strand (OP-N: OP-d(TTTCTTTNTTTTCTT)) were prepared (Figure 2) as described in Materials and Methods. To find optimal conditions for obtaining higher DNA cleavage efficiency, we examined the effect of the amount of OP–N relative to that of the target strands. In this experiment, we used OP–T as the cleavage reagent and AT34

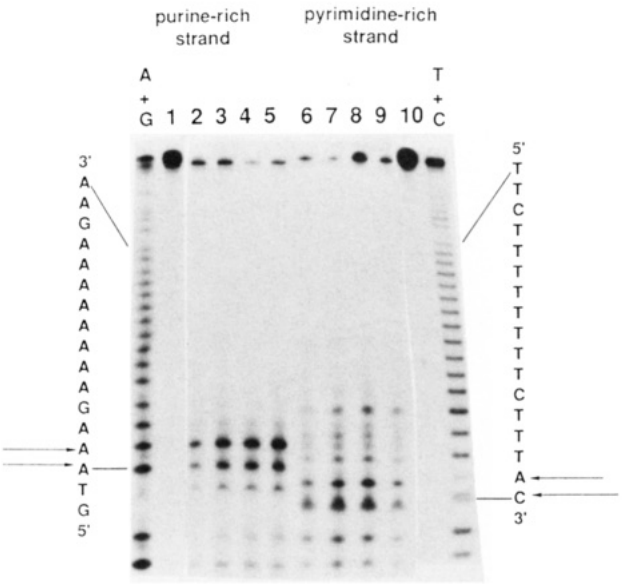


FIGURE 3: Site-specific cleavage of AT34 by OP–T on either ^{32}P-labeled strand: purine-rich strand (lanes 1–5); pyrimidine-rich strand (lanes 6–10). Different concentrations of the OP–T were used: 0 μM (lanes 1 and 10); 1 μM (lanes 2 and 9); 2 μM (lanes 3 and 8); 10 μM (lanes 4 and 7); 20 μM (lanes 5 and 6). A+G and T+C indicate the products of Maxam–Gilbert chemical sequencing reactions.

as the target strand. The concentrations of OP–T ranged from 0.1 to 20 μM with a constant substrate concentration (0.1 μM). MPA was used as a reducing agent.

After an incubation for 24 h at 37 °C in the pH 5.0 buffer, the reaction mixture was analyzed by denaturing 20% polyacrylamide gel electrophoresis (PAGE) (Figure 3). The cleavage site was identified from the comigrated Maxam–Gilbert sequencing ladders (Maxam & Gilbert, 1980) of AT34, namely, A+G ladders for the ^{32}P-labeled purine-rich strand (upper strand of AT34) and T+C ladders for the ^{32}P-labeled pyrimidine-rich strand (lower strand of AT34). The reaction yielded two main labeled products from each strand. The cleavage sites were assigned as A10 and A11 (numbered from the 5′ end of the strand) in the purine-rich strand and C8 and A9 in the complementary pyrimidine-rich strand. These results showed that OP–T was bound to the purine stretch of AT34 in a parallel orientation to form the local triple helix.

By the determination of the cleavage efficiency, it was found that 20 equiv of OP–T (2 μM) was sufficient to obtain cleavage products with a 60–70% yield (Figure 3, lanes 3 and 8). The yield was decreased when 200 equiv of OP–T was used (Figure 3, lanes 5 and 6). Presumably, aggregation of the oligonucleotides might occur to prevent the triplex formation. The optimal ratio of copper to OP was examined, in a range from 0.2 to 20 μM. At concentrations above 0.5 μM, the reaction gave almost the same cleavage yield. From this result we concluded that the concentration of copper ion is more important than having an excess OP to copper ratio in our reaction system. We used 1 μM copper ion/2 μM OP–N in all reactions described below.

Influence of the Reducing Agents on Cleavage Efficiency. To cleave DNA by an OP complex, Cu^{2+} and a reducing agent are the essential coreactants. Typically, MPA was used as a reducing agent for the DNA scission chemistry of the $(OP)_2$–Cu and the OP-linked oligonucleotide. A recent report stated that As enhanced the rate of DNA cleavage in the $(OP)_2$–Cu system, mainly because it is a poorer chelating agent than MPA (Veal et al., 1991). We tested As as a reducing agent in our OP–oligonucleotide system. The

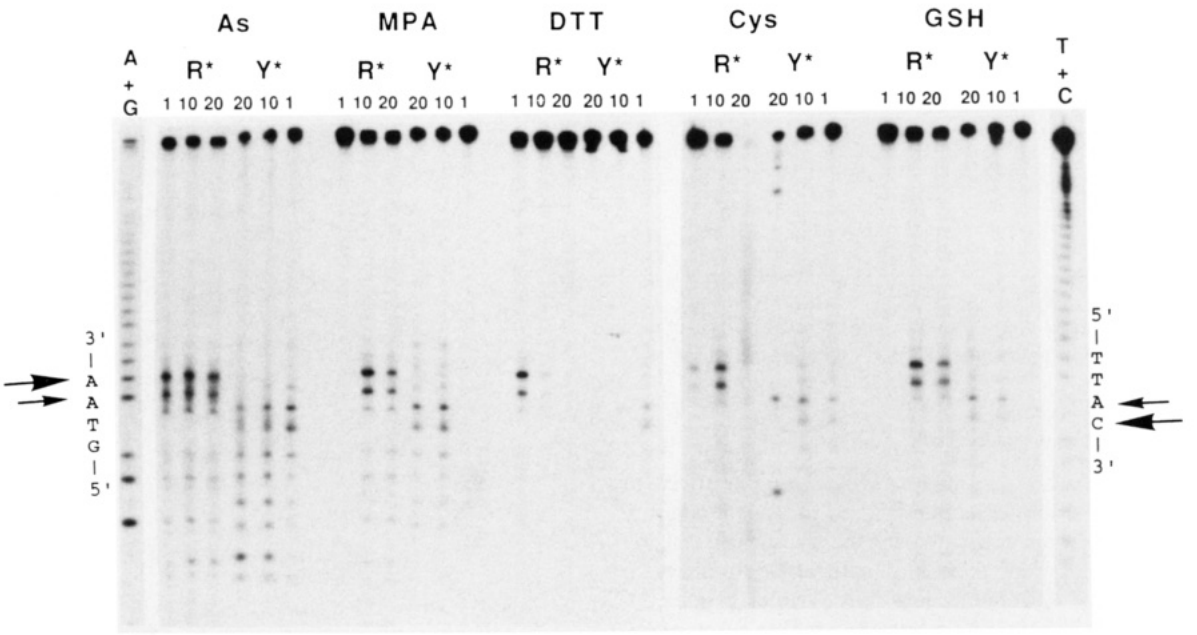


FIGURE 4: Influence of reducing agents (As, MPA, DTT, Cys, and GSH) on the efficiency of AT34 cleavage by OP–T. The cleavage reaction was carried out under the conditions described in Materials and Methods, but with varying reducing agents. ^{32}P-Labeled strands are indicated by an asterisk, either R* (purine-rich strand) or Y* (pyrimidine-rich strand). The final concentration (mM) of the reducing agent is shown at the top of each lane. A+G and T+C are the products of chemical sequencing reactions, as described in the caption of Figure 3.

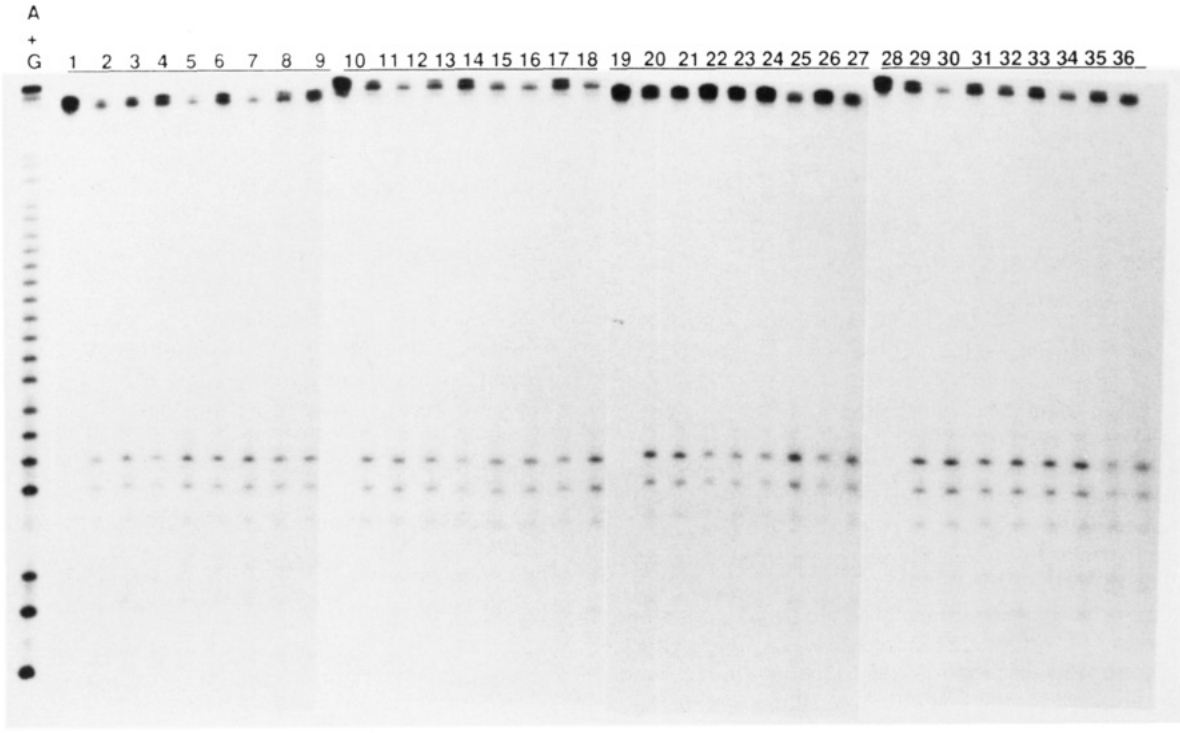
targeted duplex DNA (AT34, 0.1 μM) was incubated with OP–T (2 μM) in the presence of copper ion (1 μM) and As at various concentrations (1, 10 and 20 mM). When the purine-rich labeled DNA strand was targeted (indicated as R* in Figure 4), a rather broad range of cleavage products were observed. On the other hand, localized cleavage products were observed when MPA was used as a reducing agent (Figure 4). We identified the major product as a 3′-phosphomonoester, which arises from an oxidative attack at C1′ in the minor groove of the target duplex DNA, because its mobility was similar to Maxam–Gilbert sequencing products in a denaturing gel (Sigman, 1986).

Other thiols, such as DTT, Cys, and GSH, were examined. Gel analysis revealed that the termini of the cleavage products were 3′-phosphomonoesters, the same as when MPA was used as a reducing agent (Figure 4). The overall cleavage efficiency of the target duplex decreased depending on the higher chelating ability and the greater bulkiness of the compounds (As > MPA ≫ DTT, Cys, GSH). As the chelating ability of the reducing agents increased, the cleavage efficiency, especially on the pyrimidine-rich strands, decreased (indicated as Y* in Figure 4). These results show that the OP moiety was located closer to the purine-rich strands. In addition, the structures of the active, copper-chelated OP complexes may vary with different reducing agents. In other words, the possibility that thiols coordinate to the copper–OP–TFO in the minor groove of DNA should be considered, as described (Veal et al., 1991). In addition, the thiols coordinated to the copper and/or to free thiols would prevent the diffusion of the generated hydroxyl radicals to the far site, the pyrimidine strand, due to their quenching. The active species in the scission chemistry of OP–TFO were not determined previously (François et al., 1989a,b). We considered that a 1:1 complex of copper–OP linked to TFO may exist, rather than a 2:1 complex that contained Cu-free OP–TFO as the second chelating group, in the cases where a thiol (MPA, DTT, Cys, GSH) was used as a reducing agent. Thiols are good chelating agents for copper ions (Veal et al., 1991), and the cleavage efficiency did not change in our experiments, in either the presence or the absence of the Cu-free OP–TFO (conditions of predominant and lesser amounts of copper ion to OP). We concluded that MPA is the reducing agent of choice to obtain a localized cleavage site with a reasonable cleavage yield.

Cleavage Reactions of Local Triple-Helix-Containing N·RY Triplets in the Central Position. Using 20 equiv of OP–N and 1 μM copper ion, we examined the effect of various triplets on cleavage efficiency. Reactions were carried out with both canonical (T·AT, br^{5}U·AT, C$^+$·GC, m^{5}C$^+$·GC) and noncanonical triplets (X·AT, G·TA, C$^+$·CG, etc.) in 32 combinations. The target duplex contained four natural Watson–Crick base pairs in the central position (RY = AT, GC, TA, or CG), and the reagents were eight kinds of OP–Ns (N = A, G, T, C, br^{5}U, m^{5}C, X, or I). A 5′-end-labeled purine-rich strand was used in the cleavage reactions. We assessed the cleavage efficiency of all the combinations in the pH 5.0 buffer at 37 or 47 °C.

In all cases, the cleavage reaction occurred at the same position (Figure 5A), but the efficiencies were different with the respective triplets (Figure 5B). The triple helices that contained canonical base triplets (lanes 2, 5, 12, and 13) showed relatively higher cleavage efficiencies as expected. The cleavage yields were around 60% for the maximal case (br^{5}U·AT, C$^+$·GC, C$^+$·CG, I·GC, G·AT, G·GC, G·TA, G·CG) at 37 °C. At the higher temperature (47 °C), the cleavage efficiencies decreased to around 20%, respectively. These results should reflect the thermal stability of each triplex. The TA34 duplex was cleaved effectively not only by OP–G but also by OP–I, as predicted by the higher T_m values of the G·TA and I·TA triplexes (Table 1). As predicted from the T_m values, relatively higher cleavage yields were obtained when the RY = CG mixed sequence was used.

Relationship between Cleavage Efficiencies of Target Duplexes by OP–Ns and T_m Values of Triplexes Containing a Variety of N·RY Triplets. To assess the sequence specificities of the cleavage reactions, the relationship between the cleavage efficiencies (Figure 5B) and the T_m of triplexes (Table 1), which would be mainly influenced by the thermal stability of the N·RY triplets, is considered. In the case of the T·RY triplets, the T·AT triplex containing a canonical triplet showed the highest cleavage yield and the highest T_m value among the



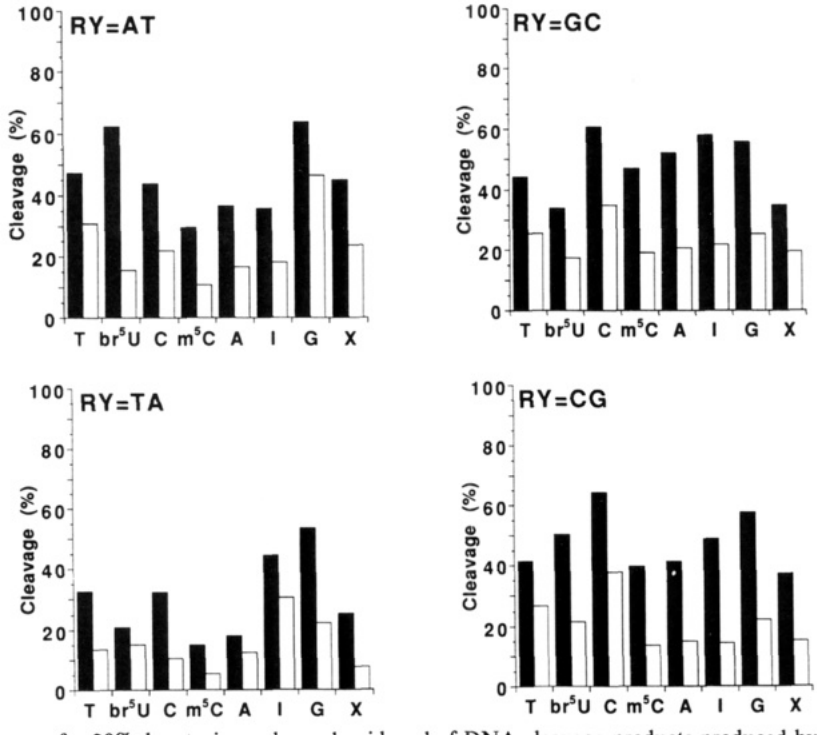


FIGURE 5: (A) Autoradiogram of a 20% denaturing polyacrylamide gel of DNA cleavage products produced by OP–N at 37 °C for 24 h: AT34 (lanes 1–9), GC34 (lanes 10–18), TA34 (lanes 19–27), and CG34 (lanes 28–36); without OP–N (lanes 1, 10, 19, 28), OP–T (lanes 2, 11, 20, 29), OP-C (lanes 3, 12, 21, 30), OP–m^5C (lanes 4, 13, 22, 31), OP–br^5U (lanes 5, 14, 23, 32), OP–A (lanes 6, 15, 24, 33), OP–G (lanes 7, 16, 25, 34), OP–X (lanes 8, 17, 26, 35), or OP–I (lanes 9, 18, 27, 36). (B) Histograms representing the absolute cleavage yields. The bars depict the total cleavage products of the respective cleavage reactions. Cleavage reactions were carried out at 37 °C for 24 h (solid bars, data from Figure 5A) and at 47 °C for 24 h (open bars).

series (T·GC, T·TA, and T·CG). Similar results were obtained in the br^5U·AT, C^+·GC, and m^5C^+·GC triplets. In other cases with the mixed pyrimidine TFOs (N = purines), the results also showed that the cleavage efficiencies and the thermal stabilities of the N·RY triplexes, except N = G, had a positive correlation. The diagrams for OP–G are essentially opposite. In particular, the least stable triplex, which contained the G·AT triplet (T_m = 27.5 °C), showed the highest cleavage yield of the triplets (Figure 5B).

Little has been practically determined about the relationship between the thermal stability of the oligonucleotide-bound triple helix and the cleavage efficiencies of the targeted strands (Singleton & Dervan, 1992). It is particularly interesting to consider why the OP–G cleaved the target AT34 duplex with higher efficiency, in spite of the relatively lower thermal stability of the G·AT triplex (27.5 °C).

There are two possible stabilizing effects of the G·AT triplet in the reaction. One possibility is that the binding of free

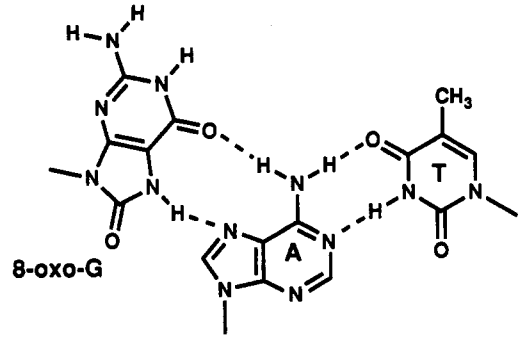


FIGURE 6: Postulated 8-oxo-G·AT triplet.

Cu^{2+} ion to the guanine base at the O-6 and N-7 positions is expected (Pezzano & Podo, 1980; Kornilova et al., 1991). If the OP–G single strand is considered, a Cu^{2+}-mediated interaction (Pezzano & Podo, 1980; Kornilova et al., 1991) in addition to Watson–Crick and Hoogsteen hydrogen bonds may occur in triplex formation, thereby increasing the thermal stability of the OP–G triplex. This hypothesis is partially supported by the T_m measurements of the triplex consisting of the 15-mer TFO (N = G in Figure 1A) and AT34, in either the presence or the absence of 1 μM copper ion in the same buffer as the cleavage reaction (10 mM sodium acetate, 100 mM NaCl, and 1 mM spermine, pH 5.0). In the presence of copper ion, the T_m of the Hoogsteen bonds of the triplex containing a G·AT triplet increased about 14 °C (from 37.2 to 51.1 °C) without an alteration of the thermal stability of the Watson–Crick hydrogen bonds (73.2 °C). On the other hand, the T_m of the third strand did not change when the triplex of N = T and RY = AT (Figure 1A) was measured with or without copper ion (T_m = 65.5 °C).

Second, in the presence of a reducing agent used in the DNA cleavage reaction, an additional event might also happen when copper ion forms a chelate of the N-7–Cu^{2+}–O-6 type with G. The reduction of Cu(II) to Cu(I) may occur with the generation of hydroxyl radicals, which could attack the C-8 of G to lead to the formation of 8-hydroxyguanine (8-oxo-G). It has been reported that the Cu^{2+}, hydrogen peroxide, and ascorbic acid system produced base modification in DNA, and the major base product was 8-oxo-G (Aruoma et al., 1991). To obtain more information about this possibility, we prepared the TFO of N = 8-oxo-G (without the thiophosphate at the 5′ end in Figure 1) and measured the thermal stability of triplexes containing 8-oxo-G·AT and other triplets in the same buffer as that of Table 1. When the TFO containing 8-oxo-G was annealed with AT34, the T_m of the Hoogsteen bonds was 46.9 °C. This value was high, like that of the triplex containing the T·AT triplet (T_m = 51.3 °C), and the increased stability compared to that of the G·AT triplet was Δ19.4 °C. This stability may be due to the two hydrogen bonds between O-6(8-oxo-G) and NH_2(A) as well as NH-7(8-oxo-G) and N-7(A) (Figure 6). Although the C8 oxidation of the TFO (N = G) remains to be clarified, the experiment indicates that a triple helix containing 8-oxo-G·AT is thermally stable. Recently, it was reported that N^6-methyl-8-oxoadenine (Krawczyk et al., 1992) and 8-oxoadenine (Miller et al., 1992) could form the hydrogen bonds to the G in the purine strand of a triplex. Our results suggest that 8-oxo-G may be used for recognition of A in the AT base pair. The thermal stability of the 8-oxo-G·AT triplex was high enough to distinguish other triplexes: 8-oxo-G·GC (T_m = 39.5 °C), 8-oxo-G·TA (T_m = 40.0 °C), and 8-oxo-G·CG (T_m = 37.8 °C).

In conclusion, the cleavage of duplex DNAs by OP–oligonucleotide reagents was examined. The results showed that the increased cleavage yields reflect the higher thermal stability of the triplex formed in most cases, but there existed a few exceptional cases. In particular, the G-containing TFO did not show the above correlation between thermal stability (in the absence of copper ion) and cleavage yield. Our results demonstrate that some exceptional cases exist in the sequence-specific cleavage of duplex DNA by OP-linked TFOs.

ACKNOWLEDGMENT

We thank Dr. David S. Sigman for a detailed protocol for synthesis of 5-amino-1,10-phenanthroline. We also thank Dr. Serge L. Beaucage for synthesis advice and for handling the 3*H*-1,2-benzodithiol-3-one 1,1-dioxide.

REFERENCES

Aruoma, O. I., Halliwell, B., Gajewski, E., & Dizdaroglu, M. (1991) *Biochem. J. 273*, 601–604.

Baht, V., Ugarkar, B. G., Sayeed, V. A., Grimm, K., Kosora, N., Domenico, P. A., & Stocker, E. (1989) *Nucleosides Nucleotides 8*, 179–183.

Cooney, M., Czernuszewicz, G., Postel, E. H., Flint, J. S., & Hogan, M. E. (1988) *Science 241*, 456–459.

Dervan, P. B. (1992) *Nature 359*, 87–88.

Divakar, K. J., & Reese, C. B. (1982) *J. Chem. Soc., Perkin Trans. 1*, 1171–1176.

Eritja, R., Horowitz, D. M., Walker, P. A., Ziehler-Martin, J. P., Boosalis, M. S., Goodman, M. F., Itakura, K., & Kaplam, B. E. (1986) *Nucleic Acids Res. 14*, 8135–8153.

François, J.-C., Saison-Behmoaras, T., Chassignol, M., Thuong, N. T., & Hélène, C. (1989a) *J. Biol. Chem. 264*, 5891–5898.

François, J.-C., Saison-Behmoaras, T., Barbier, C., Chassignol, M., Thuong, N. T., & Hélène, C. (1989b) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9702–9706.

Giovannangeli, C., Rougee, M., Garestier, T., Thuong, N. T., & Hélène, C. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8631–8635.

Griffin, L. C., & Dervan, P. B. (1989) *Science 245*, 967–971.

Hélène, C., & Toulmé, J.-J. (1990) *Biochim. Biophys. Acta 1049*, 99–125.

Iyer, R. P., Egan, W., Regan, J. B., & Beaucage, S. L. (1990a) *J. Am. Chem. Soc. 112*, 1253–1254.

Iyer, R. P., Phillips, L. R., Egan, W., Regan, J. B., & Beaucage, S. L. (1990b) *J. Org. Chem. 55*, 4693–4699.

Jay, E., Bambara, R., Padmanabhan, R., & Wu, R. (1974) *Nucleic Acids Res. 1*, 331–353.

Kamiya, H., Shimizu, M., Suzuki, M., Inoue, H., & Ohtsuka, E. (1992) *Nucleosides Nucleotides 11*, 247–260.

Kiessling, L. L., Griffin, L. C., & Dervan, P. B. (1992) *Biochemistry 31*, 2829–2834.

Koh, J. S., & Dervan, P. B. (1992) *J. Am. Chem. Soc. 114*, 1470–1478.

Kohwi, Y., & Kohwi-Shigematsu, T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3781–3786.

Kornilova, S. V., Sorokin, V. A., Blagoi, Yu. P., Valeev, V. A., & Arutyunyan, S. G. (1991) *Mol. Biol. (Moscow) 25*, 648–657.

Krawczyk, S. H., Milligan, J. F., Wadwani, S., Moulds, C., Froehler, B. C., & Matteucci, M. D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3761–3764.

Kuchino, Y., Mori, F., Kasai, H., Inoue, H., Iwai, S., Miura, K., Ohtsuka, E., & Nishimura, S. (1987) *Nature 327*, 77–79.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–599.

Mergny, J.-L., Sun, J.-S., Rougee, M., Montenay-Garestier, T., Barcelo, F., Chomilier, J., & Hélène, C. (1991) *Biochemistry 30*, 9791–9798.

Miller, P. S., Bhan, P., Cushman, C. D., & Trapane, T. L. (1992) *Biochemistry 31*, 6788–6793.

Moser, H. E., & Dervan, P. B. (1987) *Science 238*, 645–650.
Perrouault, L., Asseline, U., Rivalle, C., Thuong, N. T., Bisagni, E., Giovannangeli, C., Doan, T. L., & Hélène, C. (1990) *Nature 344*, 358–360.
Pezzano, H., & Podo, F. (1980) *Chem. Rev. 80*, 365–401.
Povsic, T. J., & Dervan, P. B. (1990) *J. Am. Chem. Soc. 112*, 9428–9430.
Povsic, T. J., Strobel, S. A., & Dervan, P. B. (1992) *J. Am. Chem. Soc. 114*, 5934–5941.
Schulz, B. S., & Pfleiderer, W. (1985) *Tetrahedron Lett. 26*, 5421–5424.
Shaw, J.-P., Milligan, J. F., Krawczyk, S. H., & Matteuci, M. (1991) *J. Am. Chem. Soc. 113*, 7765–7766.
Sigman, D. S. (1986) *Acc. Chem. Res. 19*, 180–186.
Sigman, D. S., & Chen, C.-h. B. (1990) *Annu. Rev. Biochem. 59*, 207–236.
Sigman, D. S., Kuwabara, M. D., Chen, C.-h. B., & Bruice, T. W. (1991) *Methods Enzymol. 208*, 414–433.
Singleton, S. F., & Dervan, P. B. (1992) *J. Am. Chem. Soc. 114*, 6957–6965.
Sinha, N. D., Biernat, J., & Köster, H. (1983) *Tetrahedron Lett. 24*, 5843–5846.
Takasugi, M., Guendoz, A., Chassignol, M., Decout, J. L., Lhomme, J., Thuong, N. T., & Hélène, C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5602–5606.
Thederahn, T. B., Kuwabara, M. D., Larsen, T. A., & Sigman, D. S. (1989) *J. Am. Chem. Soc. 111*, 4941–4946.
Veal, J. M., Merchant, K., & Rill, R. L. (1991) *Nucleic Acids Res. 19*, 3383–3388.