Sequence-targeted Cleavage of Single- and Double-stranded DNA by Oligothymidylates Covalently Linked to 1,10-Phenanthroline*

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Jean-Christophe François‡, Tula Saison-Behmoaras‡, Marcel Chassignol§, Nguyen T. Thuong§, and Claude Helene‡

From the ‡Laboratoire de Biophysique, Musée National d’Histoire Naturelle, Institut National de la Santé et de la Recherche Médicale U. 201, Centre National de la Recherche Scientifique UA. 481, 43 Rue Cuvier, 75005 Paris, France and the §Centre de Biophysique Moléculaire, Centre National de la Recherche Scientifique, 45071 Oréans Cédex 02 Paris, France

The nuclease activity of 1,10-phenanthroline copper ion was targeted to a specific sequence by attachment of the ligand to the 5' or 3' end of octathymidylates. An acridine derivative was also attached to the other end of the oligothymidylate-phenanthroline conjugate. The duplex formed by the oligothymidylate with its complementary sequence was stabilized by intercalation of the acridine derivative. The reaction induced by 3-mercaptopropionic acid led to a very localized cleavage of a 27-nucleotide-long DNA fragment containing a (dA)₆ sequence. At high NaCl concentration or in the presence of spermine, cleavage of the single-stranded 27-mer fragment occurred on both sides of the target sequences. This was ascribed to the formation of a triple helix involving two 1,10-phenanthroline-octathymidylate strands that adopt an antiparallel orientation with respect to each other.

When a 27-mer duplex was used as a substrate, cleavage sites were observed on both strands. The location of the cleavage sites led us to conclude that the octathymidylate was bound to the (dA)₆-(dT)₆ sequence in a parallel orientation with respect to the (dA)₆-containing strand. This result reflects the ability of thymine to form two hydrogen bonds with adenines already engaged in a Watson-Crick base pair. This study shows that it is possible to design DNA-binding oligodeoxyxynucleotides that could selectively recognize and cleave polypurine-polypyrimidine sequences in double-stranded DNA.

The recent development of antimessenger oligonucleotides provides evidence that the highly specific complexes formed by short oligonucleotides with their complementary sequence can inhibit protein synthesis (1-3). There are several advantages in using short oligonucleotides to inhibit gene expression in a specific way (3). However reduction of oligonucleotide size decreases the stability of the complex formed with a complementary sequence. An increased affinity of oligonucleotides for their target sequence can be achieved by covalent conjugation of a polynucleotide containing a sequence. In all the studies mentioned above, the target was a single-stranded nucleic acid (either DNA or RNA) (12-20). Targeting irreversible reactions to specific sequences on a double-stranded DNA might open new ways for in vivo site-specific mutagenesis or gene inactivation. The possibility of forming a local triple helix has recently been demonstrated using homopyrimidine oligodeoxyxynucleotides covalently linked to azidoprophlavine (19), azidophenacetyl (21), or an EDTA-Fe chelate (22). In the first two cases, irradiation led to cross-linking of the oligonucleotide to both strands of the double helix which was followed by cleavage under alkaline conditions. In the third case OH⁻ radicals generated upon addition of a reducing agent led to cleavage of phosphodiester bonds in the vicinity of the EDTA chelating group.

In this study, we show that an oligodeoxyxymidylate covalently linked to 1,10-phenanthroline on the 5' end and to an acridine derivative on the 3' end can recognize a stretch of A-T base pairs with high specificity. The 27-mer oligonucleotides containing a (dA)₆ and a (dT)₆ sequence will be abbreviated as 27-mer-(dA)₆ and 27-mer-(dT)₆, respectively. Sequences of both 27-mers and oligonucleotides-(OP) used in this report

MATERIALS AND METHODS

The two complementary 27-mer oligodeoxyxynucleotides used as targets for oligonucleotides-(OP)* were synthesized on a Pharmacia LKB Biotechnology Inc. synthesizer and purified by reverse phase chromatography followed by polyacrylamide gel electrophoresis. The 27-mer oligonucleotides containing a (dA)₆ and a (dT)₆ sequence will be abbreviated as 27-mer-(dA)₆ and 27-mer-(dT)₆, respectively. Sequences of both 27-mers and oligonucleotides-(OP) used in this report

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1 The abbreviations used are: (OP), 1,10-phenanthroline; MPA, 3-mercaptopropionic acid; Acr, 2-methoxy-6-chloro-9-aminocaridine; (OP)₂-Cu, 2:1 1,10-phenanthroline-cuprous ion complex; 27-mer-(dA)₆, 5'-TGAGTGAGTAAAAAAAATGAGTGCCAA-3'; 27-mer-(dT)₆, 5'-TTGGGACTCATTCTTACTCACTCA-3'.

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are shown on Fig. 1. End labeling (5' side) was achieved by T4 polynucleotide kinase (Amersham Corp.) using [γ-³²P]ATP (Amersham Corp.).

The synthesis of the octathymidylate attached via its 3' end to (OP), T₄-OP, was described previously (17).

Synthesis of T₄-OP (Scheme 1)—The octathymidylate T₄-OP₂ containing two phenanthrolines attached to the 3' end was synthesized using the nucleoside (3) where thymidine was linked to two groups of bis-cyanoethylphosphate by an ether bond at the 3' position. The nucleoside 3 after detritylation was coupled to an heptathymidylate-3'-phosphodiester (DMTr-T₃) to give the protected oligomer 4. After deprotection and high performance liquid chromatography purification, compound 5 was obtained. Oligomer 5 was then coupled to 5-(iodoacetamido)-1,10-phenanthroline in a solvent containing dimethyl sulfoxide, H₂O, NaHCO₃ 5% in water (2:2:1, v/v) during 24 h to give octathymidylate 6. Chromatography of the purified compound 6 on a silica gel yielded together with the major product a minor product supposed to be a metal complex of compound 6.

Synthesis of (OP)-Ts-Acr—The oligothymidylate containing both an acridine attached at the 3' end and a phenanthroline at the 5' end was synthesized according to Scheme 2. Full details of the synthesis of compound 10 were given elsewhere (23). Thiophosphorylation of compound 10 followed by condensation of the oligomer thiophosphate 11 with an excess of 5-(iodoacetamido)-1,10-phenanthroline afforded compound 13.

Cleavage Reaction—A standard reaction consisted in adding the following reactants in the indicated order in an Eppendorf tube: 10 nM (fragment concentration) of the single-stranded 5'-labeled 27-mer-(dA)₈, 5-20 PM of the oligo-(dT)₈-(OP) derivative and the appropriate amount of sodium phosphate, pH 7.4 (50 mM final concentration), and NaCl (usually 0.1 M final concentration). The cleavage reaction was then initiated by adding cupric sulfate (1 PM final concentration) followed by 3-mercaptopropionic acid (MPA) (2 mM final concentration) to give a standard reaction consisted in adding the following reactants in the indicated order in an Eppendorf tube: 10 nM (fragment concentration) of the single-stranded 5'-labeled 27-mer-(dA)₈, 5-20 PM of the oligo-(dT)₈-(OP) derivative and the appropriate amount of sodium phosphate, pH 7.4 (50 mM final concentration), and NaCl (usually 0.1 M final concentration). The cleavage reaction was then initiated by adding cupric sulfate (1 PM final concentration) followed by 3-mercaptopropionic acid (MPA) (2 mM final concentration) to give a standard reaction consisted in adding the following reactants in the indicated order in an Eppendorf tube.
Sequence-targeted Cleavage of Single- and Double-stranded DNA

RESULTS

Cleavage of the Single-stranded 27-mer-(dA)$_8$ by Oligothymidylates Covalently Linked to 1,10-Phenanthroline

The sequence-specific cleavage of the 27-mer containing a (dA)$_8$ sequence by octathymidylates linked to (OP) is shown on Figs. 2, 4, and 5. The reaction conditions adopted involved the hybridization of the oligonucleotide-(OP) to the 27-mer (dA)$_8$ prior to the addition of cupric ions and 3-mercaptopropionic acid. The concentration of 27-mer-(dA)$_8$ used in these experiments was 10 nM, and the oligonucleotide concentration was 5 μM. Since the stability of the hybrid is higher at low temperatures, the reaction mixtures were kept at 4 °C for 3 h and quenched by addition of 2,9-dimethyl-(OP) (400 μM final concentration). The total volume of the reaction mixture was 10 μl. When indicated, free (OP) was added subsequent to MPA. Before their utilization, distilled water and all solutions other than metal salts were treated with a Chelex 100 resin (Bio-Rad). In some cases, cleavage reactions were also induced by addition of MPA prior to copper sulfate.

The reactions were carried out at 4 °C for 3 h and then quenched by addition of 2,9-dimethyl-(OP) (400 μM final concentration). The samples were lyophilized and then redissolved in Maxam-Gilbert loading buffer (24) (80% deionized formamide containing 0.1% xylene cyanol and 0.1% bromphenol blue). They were heated at 90 °C for 1 min and loaded onto 25% polyacrylamide/7 M urea 29:1 cross-linked gels. Autoradiography was obtained by exposing the gel with a Kodak or Fuji (x-ray) film with an intensifying screen at -70 °C overnight.

The extent of cleavage was determined by comparing the radioactivity of the intact fragment to that of the cleaved fragments by counting the corresponding bands excised from the gel.

The duplex 27-mer was prepared by incubating at 4 °C the 5'-labeled 27-mer-(dA)$_8$ (or 27-mer-(dT)$_8$) with equal concentration of unlabeled 27-mer-(dT)$_8$ (or 27-mer-(dA)$_8$).

The Cleavage Efficiency Depends on the Order of Addition of Coreactants—The extent of 27-mer-(dA)$_8$ cleavage by oligothymidylate-(OP) in the presence of CuSO$_4$ and MPA varied significantly with the order of addition of the different reagents. Maximal degradation was obtained when CuSO$_4$ was added after MPA in a buffered solution containing the 27-mer-(dA)$_8$ and the oligonucleotide-(OP) conjugate. Sequential addition of Ts$_x$-(OP), MPA, and CuSO$_4$ to the buffer containing 27-mer-(dA)$_8$ gave rise to a more efficient cleavage than the order Ts$_x$-(OP), CuSO$_4$ and MPA (Fig. 2, lanes b and c). The same results were obtained when Ts$_x$-(OP)$_2$ was used instead of Ts$_x$-(OP). More efficient degradation was obtained when Cu(II) was added after Ts$_x$-(OP)$_2$ and MPA (Fig. 2, lanes d and e).

Similar observations were reported previously with copper-bleomycin complex (25). This complex cleaves DNA strands in the presence of oxygen and a reducing agent such as dithiothreitol. Maximal degradation was obtained when CuCl$_2$ was added after dithiothreitol in a buffered solution containing target DNA and bleomycin (25). The maximum cleavage obtained with the order oligonucleotide-(OP), MPA, and CuSO$_4$ suggested that reduction of Cu(II) to Cu(I) by MPA preceded metal ion binding by Ts$_x$-(OP) or Ts$_x$-(OP)$_2$.

Kinetics of Cleavage of the 27-mer and Stoichiometry of the Coordination Complex Involved in the Cleavage Reaction—The time course of cleavage of 27-mer-(dA)$_8$ by Ts$_x$-(OP) (5 μM) in the presence of 2 mM MPA at 4 °C is presented on Fig. 3. When free (OP) (5 μM) was added to the reaction mixture containing Ts$_x$-(OP), Cu$^{2+}$, and MPA (for details see Fig. 2, lane f and Fig. 3), the rate of the cleavage reaction was enhanced and 100% of the 27-mer-(dA)$_8$ was cleaved after 90-min incubation. These results suggested that two phenanthrolines were required for the generation of the reactive species. The success in targeting the cleavage in the absence of free (OP) suggested that one of the two phenanthrolines might be contributed by a nonhybridized oligonucleotide-(OP).

Control experiments indicated that cleavage reaction could be obtained only if Ts$_x$-(OP), Cu(II), and MPA were present. For example, addition of MPA to the reaction mixture containing the 27-mer-(dA)$_8$-Ts$_x$-(OP) complex, without Cu(II) did not result in 27-mer-(dA)$_8$ cleavage. No cleavage occurred when MPA and cuprous ion were incubated with the 27-mer-(dA)$_8$ in the absence of the oligonucleotide-(OP) (data not shown). The fact that the cleavage pattern obtained in the presence of free (OP) was similar to that obtained with Ts$_x$-(OP) alone demonstrated that free (OP) interacted with Ts$_x$-(OP) bound to the 27-mer-(dA)$_8$. This was also supported by

![Fig. 2. Effect of mixing order on the efficiency of 27-mer-(dA)$_8$ cleavage by oligothymidylates covalently linked to 1,10-phenanthroline. 5'-32P-labeled 27-mer-(dA)$_8$ (10 nM) was incubated for 3 h at 4 °C with 5 μM octathymidylate-(OP), 1 μM Cu$^{2+}$, and 2 mM MPA in a buffer containing 0.1 M NaCl and 50 mM phosphate, pH 7.4. The order of addition of the different reagents to the 27-mer-(dA)$_8$ solution was as follows: lane b, Ts$_x$-(OP), MPA, Cu(II); lane c, Ts$_x$-(OP), Cu(II), MPA; lane d, Ts$_x$-(OP)$_2$, MPA, Cu(II); lane e, Ts$_x$-(OP)$_2$, Cu(II), MPA; lane f, Ts$_x$-(OP), MPA, Cu(II), (OP) (1 μM). Maxam-Gilbert (G + A) reaction (lane a).](image)

![Fig. 3. Kinetics of cleavage of 27-mer-(dA)$_8$ by Ts$_x$-(OP). 10 nM of 5'-labeled 27-mer-(dA)$_8$ was incubated with 5 μM Ts$_x$-(OP), 1 μM CuSO$_4$, and 2 mM MPA at 4 °C in the absence of free (OP) (■) or in the presence of 5 μM of (OP) added to the reaction mixture prior to the other reagents (▲).](image)
the observation that free (OP), added to the complex formed by 27-mer-(dA)₈ and an octathymidylate, was unable to cleave the 27-mer-(dA)₈ at the same concentration (data not shown).

Cleavage stimulation by addition of free (OP) to an octathymidylate-(OP)-27-mer-(dA)₈ complex strongly suggested the formation of a 2:1 complex between two phenanthrolines and one cuprous ion. Therefore, an octathymidylate covalently linked to two phenanthrolines, Ts-OP), was expected to cleave the 27-mer-(dA)₈ more efficiently than Ts-(OP). Binding of the 1:1 Ts-OP), Cu⁺ complex to the 27-mer-(dA)₈ should occur with lower repulsive effects as compared with that of the 2:1 [Ts-(OP)]₂Cu⁺ complex. As can be seen on Fig. 2, Ts-OP) cleaved the 27-mer-(dA)₈ with higher efficiency than Ts-(OP) when Cu(II) was added prior to MPA (see Fig. 2, lanes c and e). In this experiment, the 27-mer-(dA)₈ concentration was lower than that of the octathymidylate-(OP). Therefore, it was difficult to conclude whether the active species was the 1:1 Ts-OP)₂ Cu⁺ or the 2:1 [Ts-(OP)]₂Cu⁺ complex.

In order to determine the stoichiometry of the complex involved in the cleavage reaction of the 27-mer-(dA)₈ by Ts-(OP), we performed an experiment in which the 27-mer-(dA)₈ was in slight excess with regard to the octathymidylate. The octathymidylates Ts-(OP) and Ts-(OP)₂ (3 μM) cleaved with the same efficiency the 27-mer-(dA)₈ at 5 μM (8% as compared to 20% with 5 μM Ts-(OP) and 10 nM of 27-mer-(dA)₈). Several hypotheses could explain this result. The two linkers used to attach two (OP) to Ts-(OP)₂ could be too short to allow for the formation of a stable complex (OP)₂Cu⁺. Experiments are under way using octathymidylates attached to two (OP) via different linkers with variable lengths. Second, as the two (OP) moieties of Ts-(OP)₂ are able to chelate other cations than cuprous ion, it is possible that the nuclease activity of Ts-(OP)₂ was partially inhibited by these ions despite the fact that all octathymidylates were treated with chelex after their synthesis. Third, when the two (OP)₂ are provided by the same oligonucleotide, the approach of the (OP)₂Cu⁺ complex might be sterically hindered, thereby reducing the efficiency of cleavage. The fact that free (OP) did not enhance the cleavage efficiency of Ts-(OP), whereas it markedly increased that of Ts-(OP), seems to be in favor of this hypothesis.

Analysis of the 27-mer Cleavage Products—Figs. 2, 4, 5, and 6 show polyacrylamide gel electrophoresis analysis of the 27-mer-(dA)₈ single-stranded DNA fragment cleaved by Ts-(OP). Several bands could be observed. The main cleavage products migrated at the same positions as those generated during the Maxam-Gilbert sequencing procedure, indicating formation of 3’-phosphomonoester termini during the 27-mer-(dA)₈ cleavage reaction by Ts-(OP). As we can see on Fig. 4, some species migrated differently from Maxam-Gilbert products, suggesting the formation of other types of products during the cleavage reaction. These products disappeared when cleaved samples were heated with 1 M piperidine at 90 °C during 30 min (Fig. 4, lane c). Recently, it was shown that the (OP)₄-Cu⁺ complex induced single-stranded breaks in DNA and generated different species such as free bases and 3'- and 5'-phosphomonoesters. In addition, 3’-phosphoglycolates could be detected in minor amounts (26). These products were alkali-sensitive and converted into 3’-phosphomonoesters when piperidine was added to the cleavage medium (26, 27). In our experiment, these 3’-phosphoglycolate products were probably the species that migrated differently from the Maxam-Gilbert products. When the cleavage reaction induced by Ts-(OP) was carried out in such a way as to cut a large fraction of the 27-mer-(dA)₈ fragments, several bands were detected on the gels between A₁⁰ and A₁². These products were probably reaction intermediates. Storage of the samples at -20 °C during 1 or 2 days after quenching the reaction by 2,9-dimethyl-(OP) resulted in the conversion of these intermediates into 3’-phosphomonoesters (data not shown).

On Fig. 4 we can observe that piperidine treatment strongly increased the intensity of the fragments with 3’-phosphomonoester termini, but the increase didn’t occur evenly. For example, the intensity of the fragment corresponding to the nucleotide T₉ in Fig. 4, lane c, increased much more than others. The intermediate OH⁻ radicals that have been suggested to be involved in the cleavage reactions mediated by the (OP)₄-Cu⁺ chelate not only react with sugars but also with nucleic acid bases. Some of these reactions lead to alkali-sensitive sites in DNA. The results reported in Fig. 4 show that base reactions are important. Therefore (OP)₄-Cu⁺ induces base damages which are not detected when the gels are run at pH 7.4 but are revealed after piperidine treatment. These damages as well as cleavage reactions should be taken into account when the biological effects of oligonucleotide-(OP) conjugates are investigated.

Triple Helix Formation at High Salt Concentration

Hybridization of complementary oligonucleotides is an ionic strength-dependent process as the presence of cations in the medium should decrease the repulsive effects of the negative charges of the two approaching molecules. An increase in NaCl concentration from 0.1 to 1 M enhanced the efficiency of 27-mer-(dA)₈ cleavage. Fig. 5 shows that the main cleavage sites were located on the 5’ and 3’ end of the (dA)₈ sequence. When the reactive (OP) was attached to the 3’ and 5’ end of the oligothymidylate, respectively. Ts-(OP) gave rise to cleavage products mainly at positions A₇, G₈, T₉, A₁⁰, A₁¹, A₁², (Fig. 5B, a), when (OP)-Ts-Acr exhibited cleavage at A₁⁷, T₈, G₁⁹, A₂⁰. This experiment demonstrated that these oligothymidylylates adopted an antiparallel orientation with respect to the target sequence. The cleavage products
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In our experiments, triple helix formation required the fixation of two (OP)-Ts-Acr molecules on the single-stranded 27-mer-(da)a, one in an antiparallel orientation, the other in a parallel orientation with respect to the (da)a sequence of the 27-mer. To form triple helical DNA, both oligonucleotides-(OP) must overcome the electrostatic repulsion between the 27-mer-(da)a and their own negatively charged phosphodiester backbones. High salt concentration should favor this association. Also, multivalent cations such as polyamines and their derivatives could be used to stabilize triple helical structures (22, 29). Previous x-ray fiber diffraction analyses have shown that the triple-stranded polydeoxyribonucleotide poly(dA)-2 poly(dT) (30, 31) was conformationally similar to poly(A)-2 poly(U) (32). In the latter, each chain had an A' conformation. According to this result, a B to A conformational transition should favor triple helix formation. Therefore, the stability of triple-stranded complexes formed by (OP)-Ts-Acr with the 27-mer-(da)a should increase when this conformational transition is induced in the reaction mixture. Organic solvents, such as ethylene glycol, favor this conformational transition (33).

The data summarized in Fig. 6 demonstrates that (OP)-Ts-Acr formed a triple helix with the single-stranded 27-mer-(da)a in the presence of spermine and ethylene glycol. In this triple helix, each adenine of the (da)a sequence is hydrogen-bonded to two thymines belonging to two octathymidylates-(OP). The two octathymidylates have an antiparallel orientation with respect to each other. Under these conditions Ts-(OP) cleaved the 27-mer-(da)a on both sides of the (da)a target sequence, whereas in the experiment described in Fig. 5 we didn't observe any cleavage on the 5' side of the (da)a sequence. However, the extent of cleavage was high in these experiments (Fig. 5, lane b). Therefore, longer fragments resulting from cleavage on the 3' side of the (da)a sequence might not be observed due to multiple cleavage of the same fragment.

Targeting of an (OP)-Oligonucleotide Reaction to a Double-stranded DNA

In order to determine if a triple helix could be formed by an (OP)-oligonucleotide with double-stranded DNA, the 27-mer duplex was used as a target for (OP)-Ts-Acr. For the reasons mentioned above, the binding of this (OP)-oligonucleotide to a double-stranded DNA should be favored by addition of polyamines and organic solvents. Fig. 7A shows the polyacrylamide gel analysis of the fragments obtained when the 27-mer duplex (10 nM) was reacted with 5 μM (OP)-Ts-Acr in a buffer containing spermine and ethylene glycol. In the absence of spermine, the 27-mer duplex was not cleaved by (OP)-Ts-Acr, indicating the essential role played by the multivalent polyamine in triple helix formation. The reaction rate for the cleavage of the 27-mer duplex by (OP)-Ts-Acr increased upon addition of ethylene glycol (Fig. 7C). Above 20% (by volume) of ethylene glycol, the reaction rate dropped, indicating a possible radical scavenging by this solvent. The

 obtaned with Ts-(OP)2 were located in the same region as Ts-(OP) (see Fig. 5A, lane d).

Althought the main cleavage sites revealed that these oligonucleotides were bound to the 27-mer-(da)a in an antiparallel orientation, a weak cleavage was observed with (OP)-Ts-Acr and Ts-(OP) at the other end of the complementary (da)a sequence. These observations suggested the formation of a triple helix in which two octathymidylates-(OP) were bound with an antiparallel orientation with respect to each other (see Fig. 5A, lane b). These weak cleavage sites were not observed when the concentration of NaCl was lower (0.1 M) (17). Formation of a triple helix has also been described on the same target sequence (27-mer-(da)a) with an oligothymidylate covalently linked to azidophenacyl (21), proflavine (28), or azidoproflavine (19).

Optimal Conditions to Form Triplex Structures

...
Sequence-targeted Cleavage of Single- and Double-stranded DNA

A

G

A

G

A

T

T

A

G

f

5'

B

Acr-TTTTTTTT-COP (3')

(5')

(3')

(5')

(5')-

Acr

T

T

T

T

T

T

T

T

T

T

T

(5')

(3')

(5')

(3')

(5')

(3')

(5')

(3')

(5')

(5')

(3')

(5')

FIG. 6. Triple helix formation: scission of the 27-mer-(dA)$_s$ by (OP)-T$_s$-Acr and T$_s$-(OP). A, lane $b$ shows the cleavage of the 5'-32P-labeled 27-mer-(dA)$_s$ (10 nM) with 5 pM (OP)-T$_s$-Acr, 2.5 pM CuSO$_4$, and 2 mM MPA. The reaction was carried out for 20 h at 4 oC in a buffer containing 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 100 mg/liter tRNA, 1 mM spermine, and 40% (by volume) ethylene glycol. Lane $c$ illustrates the pattern obtained with T$_s$-(OP) (5 pM) under the same conditions as lane $b$. Lane $a$ represents the (G + A) Maxam-Gilbert digests of 5'-labeled 27-mer-(dA)$_s$. B, histograms of the cleavage patterns derived from densitometry of the autoradiograms corresponding to the 27-mer-(dA)$_s$ cleaved by (OP)-T$_s$-Acr (Fig. 6A, lane $b$) and T$_s$-(OP) (Fig. 6A, lane $c$).

location of the cleavage sites (Fig. 7B) demonstrated that (OP)-T$_s$-Acr was bound in a parallel orientation with respect to the (dA)$_s$-containing strand of the duplex. The strand containing the homopurine sequence (dA)$_s$ of the double-stranded 27-mer was cleaved more efficiently than the (dT)$_s$-containing strand. Overall, the reactivity of (OP)-T$_s$-Acr with duplex DNA was less efficient than that observed with a single-stranded fragment (compare Fig. 7, 3, and 5). The maximum extent of cleavage obtained was about 6% on the (dA)$_s$-containing strand of the duplex 27-mer.

DISCUSSION

Oligodeoxythymidylates covalently linked to 1,10-phenanthroline form complexes with a complementary oligodeoxy-

adenylate sequence. Double and triple helices could form, depending on salt concentration. Formation of a triple helix is favored at high salt concentration, because electrostatic repulsion between negatively charged molecules decreases

FIG. 7. Scission of the 27-mer duplex containing a (dA)$_s$-(dT)$_s$ sequence by (OP)-T$_s$-Acr. A, the duplex 27-mer (10 nM) was 5'-labeled either on its (dA)$_s$-containing strand (lanes $a$ and $c$) or its (dT)$_s$-containing strand (lanes $b$ and $d$) (see “Materials and Methods”). It was incubated in the absence (lanes $a$ and $b$) or in the presence of (OP)-T$_s$-Acr (5 pM) (lanes $c$ and $d$), in a buffer containing 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 100 mg/liter tRNA, 1 mM spermine, and 40% (by volume) ethylene glycol. Then, MPA (2 mM) was added, followed by CuSO$_4$ (25 pM). Reactions were carried out at 4 oC for 15 h and quenched by addition of 2,9-dimethyl-(OP). Samples were analyzed on a 20% polyacrylamide/7 M urea gel. B, distribution of cleavage sites obtained with (OP)-T$_s$-Acr bound to the 27-mer duplex. This pattern was obtained by densitometry of the autoradiogram shown in Fig. 7A (lanes $c$ and $d$). C, cleavage efficiency in the presence of ethylene glycol. Bar heights correspond to the relative cleavage efficiencies obtained with (OP)-T$_s$-Acr on the 27-mer duplex, calculated by adding intensities of the four most efficiently cleaved nucleotides (T', A', A'', and A''') using a 5'-labeled (dA)$_s$-containing strand. Numbers given in the box represent the percentage of ethylene glycol (by volume) used in experiments similar to the one described in Fig. 7A. No cleavage occurred in the absence of spermine.
when salt concentration increases.

At low ionic concentration (0.1 M NaCl), the majority of the cleavage reactions occurred on the 3' side of the (dA)$_a$ sequence of the single-stranded 27-mer-(dA)$_a$ with (OP)-T$_a$-Acr and on the 5' side with T$_a$-(OP) and T$_b$-(OP)$_2$. These results are in agreement with the location of the 1,10-phenanthroline in a duplex involving antiparallel orientation of the two strands. The most efficient cleavage of the 27-mer-(dA)$_a$ single strand was observed with T$_a$-(OP). The cleavage reaction was enhanced in a specific manner when free (OP) was added to the reaction mixture. Therefore, two phenanthrolines are required for an efficient generation of the reactive oxidative species, and it is likely that in the absence of free (OP) one of them is contributed by a nonhybridized oligonucleotide-(OP), even though we cannot exclude that a 1:1 (OP)-Cu$^+$ complex might be active in cleaving the target sequence via the involvement of other chelating groups from the target sequence (phosphate, bases).

These results prompted us to investigate whether T$_a$-(OP)$_2$ could induce cleavage with high efficiency without addition of free (OP). No difference in reactivity was observed between T$_a$-(OP) and T$_a$-(OP)$_2$ when the concentration of the oligonucleotide-(OP) conjugate was the same as or lower than that of the target sequence. One explanation for the lack of reactivity differences between T$_a$-(OP) and T$_a$-(OP)$_2$ is that the two linkers of T$_a$-(OP)$_2$ are not long and/or flexible enough to allow for the interconversion between the square planar and the tetrahedral forms of the T$_a$-(OP)$_2$-Cu$^+$ complex. Another explanation rests upon differences in the approach of the (OP)$_2$-Cu$^+$ complex when the two (OP)$_2$s are provided by the same oligonucleotide as compared to that with only one (OP). Experiments are under way using different linkers to tether the two phenanthrolines to the 3' end of an octahydroimidylate.

The cleavage of the 27-mer-(dA)$_a$ by (OP)-oligonucleotides was more efficient when the co-reactants were added in the following order: (OP)-oligonucleotide, MPA, Cu(II), as compared with the order (OP)-oligonucleotide, Cu(II), MPA. This observation suggests that copper has to be reduced first from Cu(II) to Cu(I) before being chelated by (OP) in order to increase the cleavage efficiency.

At high salt concentration (1 M NaCl), cleavage on the opposite sides of the (dA)$_a$ sequence was observed in agreement with triple helix formation. The location of the cleavage sites implied that the two (dT)$_a$s strands in the triple helix have an antiparallel orientation.

Cleavage of the double-stranded 27-mer by (OP)-oligothymidylylates involves local formation of a triple helix, where thymines of the (OP)-oligothymidylylates are hydrogen-bonded to the adenines in the major groove of the DNA double helix. In order to bind to the duplex structure, the (OP)-oligothymidylylate must overcome repulsions between the two anionic chains of the Watson-Crick duplex and its own negatively charged phosphodiester backbone. Optimal cleavage of double-stranded 27-mer by (OP)-T$_a$-Acr was obtained in the presence of 1 mM spermine and 20% ethylene glycol. As it was pointed out previously (22), polyamines stabilize double and triple helical structures of nucleic acids. Spermine as well as organic solvents such as ethylene glycol also favor the B to A conformation transition. No cleavage occurred in the absence of spermine, even at a high concentration of salt (1 M NaCl), which demonstrates the importance of these polycations for triple helix formation.

The cleavage sites on opposite strands are shifted toward the 3' side. Such a shift has been ascribed previously to cleavage by diffusible species generated within the minor groove of DNA. This would mean that OH radicals are produced by the Cu$^+$-phenanthroline complex in the minor groove, even though the oligonucleotide is bound to the major groove. The simplest explanation is that phenanthroline intercalates at the junction of the double helix with the triple helix, bringing the chelating nitrogen atoms in the minor groove. Alternatively, the shift to the 3' side on opposite strands could represent two positions of the phenanthroline with respect to the different grooves in the triple helical region and at the triple duplex junction.

The cleavage efficiency of (OP)-T$_a$-Acr bound to the 27-mer duplex was lower than that observed with a single-stranded 27-mer target. Several factors are involved in determining the oligonucleotide reactivity such as the stability of the triplex structure, the length of the linkers that are used to tether 1,10-phenanthroline to the oligopyrimidine, and the site (5' and 3') of attachment of phenanthroline. We have recently synthesized several oligopyrimidines (11 nucleotides long) which are covalently linked to 1,10-phenanthroline via linkers of different lengths. A pentamethylene linker was shown to induce a cleavage reaction with an efficiency of about 60% on a duplex DNA containing its homopyrimino-homopyrimidine target sequence. These results will be presented elsewhere.

The results presented in the study mentioned above show that T$_a$-Acr covalently linked to (OP) could bind to a double helix containing a (dA)$_a$-(dT)$_a$ sequence. The oligonucleotide is oriented parallel to the (dA)$_a$ strand, and cleavage occurs on both strands. Polyuridine-polyuracil sequences are often present in the control region of prokaryotic or eukaryotic genes and therefore could constitute good targets for site-specific reagents, such as (OP)-oligonucleotides, which could interfere with gene expression.

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