Sequence-specific recognition and cleavage of duplex DNA via triple-helix formation by oligonucleotides covalently linked to a phenanthroline–copper chelate

(DNA cleavage/sequence-specific reactions)

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ABSTRACT Homopyrimidine oligodeoxynucleotides recognize the major groove of the DNA double helix at homopurine-homopyrimidine sequences by forming local triple helices. Phenanthroline was covalently attached to the 5' end of an 11-mer homopyrimidine oligonucleotide of sequence d(TCTCTCTC). Simian virus 40 DNA, which contains a single target site for this oligonucleotide, was used as a substrate for the phenanthroline-oligonucleotide conjugate. In the presence of copper ions and a reducing agent, a single specific double-strand cleavage site was observed at 20°C by agarose gel electrophoresis. The efficiency of double-strand cleavage was >70% at 20°C and pH 7.4. Secondary cleavage sites were observed when binding of the oligonucleotide to mismatched sequences was allowed to take place at low temperature. The exact location of the cleavage sites was determined by polyacrylamide gel electrophoresis of denatured fragments by using both simian virus 40 DNA and a synthetic DNA fragment containing the target sequence. The asymmetric distribution of the cleavage sites on the two strands revealed that the cleavage reaction took place in the minor groove even though the phenanthroline linker was located in the major groove. Linkers of different lengths were used to tether phenanthroline to the oligonucleotide and their relative efficacies of DNA cleavage were compared. Based on these comparative studies and on model building, it is proposed that the phenanthroline ring carried by the oligonucleotide intercalates from the major groove and that copper chelation locks the complex in place from within the minor groove where the cleavage reaction occurs.

Oligonucleotides carrying reactive groups have been developed during recent years to direct specific reactions at preselected sequences on single-stranded nucleic acids. Nitrogen mustards covalently attached to oligonucleotides can be used to crosslink the oligonucleotide to a nucleic acid containing the complementary sequence (1). Several metal chelates such as Fe–EDTA (2–6), Cu–phenanthroline (7–10), and Fe–porphyrins (11, 12) induce cleavage reactions at specific sequences when they are tethered to oligonucleotides. It was also reported that an oligonucleotide could be crosslinked to its complementary sequence after light excitation of a photosensitizer covalently linked to one of its ends (13–16).

In all cases mentioned above, a single-stranded nucleic acid was used as a target for derivatized oligonucleotides. The possibility of targeting irreversible reactions to the DNA double helix itself was recently described (13, 14, 17–19).

Homopyrimidine oligonucleotides can bind to the major groove of duplex DNA at homopurine-homopyrimidine sequences. They may be used to bring a reactive group at these specific sites in close proximity to DNA base pairs. Work from our laboratory has shown that photocrosslinking reactions could be induced on each strand of the double helix and that crosslinks could be converted into strand breaks upon alkaline treatment (13, 14). The cleavage reaction induced by Fe–EDTA can be targeted to duplex DNA (6, 17, 18) as well as the crosslinking reaction mediated by a nitrogen mustard (19). The binding of homopyrimidine oligonucleotides to duplex DNA can be monitored by footprinting studies (20) or by gel retardation assays (21). Here we show that a Cu-phenanthroline chelate can be used to cleave both strands of simian virus 40 (SV40) DNA at a well-defined site provided it is tethered to a homopyrimidine 11-mer oligonucleotide. The efficiency depends on the length of the linker used to tether phenanthroline to the oligonucleotide. A very efficient double-strand cut is obtained when a 5'-thiophosphate group of the oligonucleotide is connected to position 5 of the phenanthroline ring via a pentamethylene carboxamide linker. The location of the cleavage sites on the two strands strongly suggests that phenanthroline intercalates within the double helix at the boundary of the triple helix and that cleavage occurs via reactions in the minor groove, even though the oligonucleotide binds to the major groove of DNA.

MATERIALS AND METHODS

The two complementary 32-nucleotide-long oligodeoxynucleotides and the oligopyrimidine used in this study were synthesized on a Pharmacia automatic synthesizer. Their sequences are shown in Fig. 1. They were purified by reverse-phase chromatography followed by polyacrylamide gel electrophoresis. The following chemicals were obtained from commercial sources: 1,10-phenanthroline (Merck Sharp & Dohme); 2.9-dimethyl-1,10-phenanthroline, copper sulfate, and 3-mercaptopropionic acid (MPA) (Janssen Chimica); spermine (Sigma); ethylene glycol (Prolabo, Paris).

Synthesis of the 11-mer Oligodeoxynucleotide-Phenanthroline (OP) Conjugates. Three oligonucleotides (OP)-s-11-mer, (OP)-m-11-mer, and (OP)-l-11-mer carrying 1,10-phenanthroline attached at their 5' end were synthesized (see Fig. 1). Covalent linkage of the phenanthroline derivative to the 5' end of the oligodeoxynucleotide was achieved via a thiophosphate group, which reacted with a 5-(ω-halogenoalkylamido)-1,10-phenanthroline derivative (22).

Abbreviations: SV40, simian virus 40; OP, 1,10-phenanthroline; MPA, 3-mercaptopropionic acid.

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Synthesis of 5-(ω-bromohexanoamido)-1,10-phenanthroline. To a solution of 0.5 mmol of 5-amino-1,10-phenanthroline and 0.58 mmol of diisopropylethylamine in 6 ml of CH3CN was added, with stirring at 20°C, 0.1 ml of 6-bromohexanol chloride. After 2 hr at room temperature, the reaction was quenched with water. The product was extracted with CH2Cl2 and purified by chromatography on aluminum oxide 90 (Merck Sharp & Dohme) (activated, neutral) in CH3Cl2-CH3OH (97:3 vol/vol) (Rf, 0.33; yield, 55%).

Covalent linkage of phenanthroline derivatives to oligonucleotides. Synthesis of the 11-mer oligodeoxynucleotide was carried out on a Pharmacia automatic synthesizer by phosphoramidite chemistry. Bis-2-cyanoethylthiophosphorylation of the 5'-hydroxyl group of the decamer was accomplished by addition of 2 mM MPA and 10 μM CuSO4. The reaction was stopped by adding 100 μM 2,9-dimethyl-1,10-phenanthroline.

The 32-mer duplex was 5'-end-labeled on either its pyrimidine-rich strand or its purine-rich strand. The cleaved 32-mer was mixed (1:1, vol/vol) with 80% deionized formamide containing 0.1% xylene cyanol and 0.1% bromophenol blue and was analyzed by electrophoresis on 20% polyacrylamide/7 M urea (29:1) crosslinked gels. Autoradiograms were obtained by exposing the gel to Kodak or Fuji (x-ray) film at -20°C. The gels were developed in an Agfa-Gevaert automatic film developer. 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. Microdensitometry of autoradiograms was performed on a LKB laser densitometer interfaced with a PC compatible Olivetti microcomputer.

Circular SV40 DNA was linearized with the restriction enzyme Hae II, which cleaves at position 832. The cleavage products were analyzed on 0.8% agarose gels stained with ethidium bromide. In some experiments, linear SV40 DNA was 3'-end-labeled with [α-32P]dATP and the cleaved products were analyzed by autoradiography taking into account the presence of two labels on intact linear DNA and only one in each of the cleaved products. Other conditions are discussed in the legends of Figs. 3–5.

RESULTS

Cleavage of the 32-mer Duplex DNA Fragment. Incubation of the 32-mer duplex with (OP)-m-11-mer and (OP)-l-11-mer in the presence of Cu2+ and MPA induced cleavage reactions at well-defined locations on both strands of the 32-mer duplex (Fig. 1). In contrast, (OP)-s-11-mer did not induce any cleavage reaction. The gel autoradiograms were quantitatively analyzed and the results are shown in Fig. 2, where the bars represent the relative efficiencies of cleavage. Cleavage was slightly more efficient on the purine-rich strand than on the pyrimidine-rich strand. Both the l and m linkers gave the same distribution, with a lower efficiency for the l linker. The cleavage sites on the two strands exhibited an asymmetric distribution. They were shifted toward the 3' end, suggesting

![Fig. 1. Cleavage of the 32-mer duplex (see Fig. 2 for sequences) by phenanthroline-substituted oligonucleotides. The 32-mer duplex was labeled on either the purine-rich strand (Left) or the pyrimidine-rich strand (Right). The first lanes show the (G+A) (Left) and T (Right) sequencing lanes. Lanes l, m, and s, linkers used to tether phenanthroline to the 5' end of the oligonucleotide as indicated (Center). Cleavage reactions were carried out at 20°C in a pH 6 buffer containing 10 mM phosphate, 0.1 M NaCl, 1 mM spermine, and 20% (vol/vol) ethylene glycol.](image-url)
that cleavage took place from the minor groove, as already observed when the free complex Cu(OP)$_2$ cuts duplex DNA (24, 25). The location of the cleavage sites with respect to the target sequence indicated that the oligonucleotide was bound in a parallel orientation with respect to the purine-rich strand, in agreement with major groove recognition via Hoogsteen hydrogen bonding of T and protonated C with Watson–Crick A-T and G-C base pairs, respectively.

We investigated the effect of different experimental conditions on the efficiency of the cleavage reaction induced by (OP)-m-11-mer. No cleavage was observed at 0.1 M NaCl in the absence of spermine. Addition of 1 mM spermine or increasing NaCl concentration to 1 M allowed cleavage to occur. These results are in qualitative agreement with those previously reported either with photocrosslinking agents (13, 14) or with Fe-EDTA–oligonucleotide complexes (6, 17, 18).

**Cleavage of SV40 DNA.** The 32-mer duplex used in the studies presented above is part of the sequence of SV40 DNA (positions 5007–5038) (Fig. 2). SV40 DNA linearized at the Hae II site (position 832) was used as a substrate for the cleavage activity of (OP)-m-11-mer. Two DNA fragments of the expected sizes were obtained after incubation of linear SV40 DNA with the 1,10-phenanthroline-oligopyrimidine conjugate (Fig. 3). Both DNA fragments were the result of a double-strand cleavage by (OP)-m-11-mer at a unique site localized around position 5030. The sizes of the expected fragments were 1045 and 4198 base pairs (bp), in good agreement with the experimental results. In Fig. 3, we had chosen to label SV40 linear DNA at both its 3' ends so as to visualize directly the two DNA fragments obtained by cleavage with (OP)-11-mer. The presence of two labels on intact DNA and of only one in the fragments was taken into account for quantitative analysis. DNA fragments could also be revealed by ethidium bromide staining (see Fig. 5). Quantitative analysis assumed that fluorescence was proportional to the length of the DNA fragments.

The kinetics of SV40 DNA cleavage by (OP)-m-11-mer was studied by these two methods. After incubation for 20 hr at 20°C and pH 7.4, ~70% of SV40 linear DNA was cleaved into two fragments. Both methods gave the same results (see Figs. 3 and 5). Addition of free OP (1 µM) had no effect on the reaction efficiency. No cleavage was observed at neutral pH in the absence of spermine, which is known to stabilize triple-helical structures (17).

The location of the cleavage sites was then analyzed in more detail on an 8% polyacrylamide sequencing gel (Fig. 4). The incubation of (OP)-m-11-mer and 5' 32P-labeled SV40 linearized at the Taq I site (Fig. 4A) or the Hpa II site (Fig. 4B) gave two DNA fragments that were labeled at one extremity. The shorter fragment obtained by incubating (OP)-m-11-mer and SV40 DNA linearized at the Taq I site (position 4739) was 5' 32P-labeled on the homopyrimidine-containing strand of the target sequence (lower strand in Fig. 2). From a semilogarithmic plot, its size was calculated to be 292 ± 3 bases. The cleavage sites induced by oligonucleotide-OP conjugates are spread over 3 or 4 nucleotides in the vicinity of the phenanthroline–copper complex (see above). Even though the 292-bp band in Fig. 4 might represent the superposition of 3 or 4 bands differing in length by 1 nucleotide unit and unresolved under our gel conditions, the location of this band clearly indicated that the oligopyrimidine-(OP) was bound to the 11-bp-long target sequence of double-stranded SV40 in an antiparallel orientation with respect to the homopyrimidine-containing strand. This binding brings the cleaving reagent in close proximity to position 5032 in SV40 linear DNA, and therefore the cleavage reaction leads to two DNA fragments of expected sizes 292 bp (5032–4740) and 4951 bp (5243–292). When Hpa II was used for linearization (Fig. 4B), the two DNA fragments generated by (OP)-m-11-mer were expected to have sizes of 4681 and 562 bp (5243 – 5030 + 349). The homopurine-containing strand of the target sequence was 5' 32P-labeled (upper strand in Fig. 2). The length of the smallest fragment (562 ± 5 bp) was in agreement with triple-helix formation between (OP)-m-11-mer and the SV40 double-stranded target sequence.

To test the temperature dependence of the specificity and efficiency of double-strand cleavage, we performed an experiment described in Fig. 5, in which SV40 DNA was

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**Fig. 2.** Schematic representation of SV40 DNA and the restriction sites used in the present studies. The target sequence for (OP)-11-mer is boxed. Taq I, Hpa II, and Hae II were used for SV40 linearization. Numbers refer to position on the SV40 genome according to ref. 26. Vertical bars above and below sequence represent the extent of cleavage observed when (OP)-11-mer was incubated with a 32-bp DNA fragment (positions 5007–5038 on SV40 DNA): open bars, (OP)-T11-mer; solid bars, (OP)-m-11-mer.

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**Fig. 3.** Site-specific cleavage of linear SV40 DNA by the 11-mer oligopyrimidine covalently linked to 1,10-phenanthroline. SV40 DNA was linearized at the Hae II site (position 832) and 3'-end labeling was performed with [α-32P]dATP. Linear DNA (20 nM) was incubated with (OP)-m-11-mer (20 µM) in a pH 7.4 buffer. To activate the cleavage reaction, MPA (2 mM) and cupric ion (5 µM) were added. The reaction was carried out at 20°C for 1, 4, and 20 hr (lanes b, c, and d). Lane a, control DNA incubated in the absence of oligonucleotide-(OP) but in the presence of CuSO4 and MPA. The samples were then electrophoresed on a horizontal nondenaturing 0.8% agarose gel. Numbers on the left represent DNA size markers (bp) obtained by digestion of PHIX174 with Hae III and of a DNA with BstEII (see Fig. 5).
obtained by digestion with T. containing cleavage sites at 20°C for the DNA concentration, position 4739 labeled at 8%. DNA was linearized with respect to strand. Cleavage sites were then separated by electrophoresis on a non-denaturing 0.8% agarose gel and were analyzed by ethidium bromide staining. When Hae II (position 832) was used for linearization, the two fragments generated by the (OP)-m-11-mer have sizes of 1045 and 4198 bp. Two other fragments (760 and 3440 bp) were generated when the cleavage reaction was carried out at low temperatures. These two fragments could be attributed to a secondary binding site with two mismatches for 5'-d(TTTCCTCCTC)-3' at positions 1592-1602. Lane M, length standards as described in Fig. 3.

the 760 bp was reduced to 50% of its maximum value at 15°C, a temperature much lower than that observed for the fully matched site (30°C) (Fig. 6).

**DISCUSSION**

The results presented in this study show that the nuclease activity of copper–phenanthroline can be targeted to a specific sequence on duplex DNA provided that phenanthroline is covalently attached to a homopurine homopyrimidine oligonucleotide with a linker of appropriate length. This oligonucleotide binds to the major groove of DNA at a homopurine-homopyrimidine sequence as previously shown by footprinting studies. The asymmetric distribution of the cleavage sites on the two strands (Figs. 1 and 2) suggests that the cleavage reaction takes place in the minor groove. In the most efficient (OP)-11-mer, the linker is not sufficiently long to wrap around.

FIG. 4. Autoradiogram of an 8% polyacrylamide gel showing the cleavage products obtained after incubation of SV40 linear DNA and (OP)-m-11-mer. (A) SV40 DNA was linearized at the Taq I site (position 4739) and then labeled at its two 5' ends with [γ-32P]ATP. (B) SV40 DNA was linearized at the Hpa II site (position 346) and labeled at both its 5' ends. The 5' 32P-labeled SV40 DNA (final concentration, 20 nM) was incubated with (OP)-m-11-mer (20 μM) in the same buffer described in Fig. 3. The reaction was carried out at 20°C for 6 and 17 hr (lanes 1 and 2). After quenching, the samples were heated to 90°C for 15 min with 1 vol of loading buffer (80% formamide containing 0.1% xylene cyanol). They were then loaded onto an 8% polyacrylamide/7 M urea (19:1) crosslinked gel. SV40 linear DNA was cleaved by (OP)-m-11-mer at a unique site (main cleavage sites at positions 5030 and 5032 on the lower and upper strand, respectively, as shown in Fig. 2). When Taq I was used for linearization (A), the two fragments generated by the oligonucleotide (OP) should have sizes of 292 and 4951 nucleotides. When Hpa II was used for linearization (B), the two expected fragments were 4681 and 562 nucleotides. The smallest products are shown by arrows. T, position of SV40 linear DNA and the longest cleavage products that are not separated. Lanes M, DNA size markers obtained by digestion of PHIX174 with Hae III.

incubated with (OP)-m-11-mer at several temperatures for 19 hr. This incubation time was chosen to ensure that a plateau was reached at each temperature. The intensity of the specific fragment (1045 bp) observed on the ethidium bromide-stained gel remained constant between 10°C and 20°C (Fig. 6). Above 25°C, the intensity dropped rapidly, indicating a dissociation of the (OP)-11-mer from its double-stranded target sequence. Cleavage was reduced to 50% of its maximum efficiency at ~30°C. At temperatures below 20°C, the incubation of SV40 DNA and (OP)-11-mer resulted in two additional fragments of approximately 3440 and 760 bp. Sequences with partial homologies with the target sequence were searched on the SV40 genome. One site at position 1592–1602 5'-d(AAAGGAGGAAT)-3' contains two mismatches on the 3' side of the (OP)-oligopyrimidine. This secondary binding would bring 1,10-phenanthroline close to position 1591 of SV40 DNA. When Hae II (position 832) was used for linearization, products generated by (OP)-11-mer cleavage at the secondary site should have sizes of 759 bp (1591–832) and 4484 bp (5243–759). The latter fragment can be cleaved by (OP)-11-mer bound to the primary site (position 5030) to give two fragments of 1045 and 3440 bp. The 760- and 3440-bp fragments observed at low temperature are therefore ascribed to cleavage at this secondary site. The intensity of

FIG. 5. Specific cleavage of SV40 DNA by (OP)-m-11-mer as a function of temperature. SV40 DNA was linearized at the Hae II site and then incubated at a final concentration of 20 nM with 20 μM (OP)-m-11-mer in the same buffer as in Fig. 3 except for the removal of ethylene glycol. The reaction was carried out at 0, 10, 15, 20, 25, and 30°C (lanes b–g) for 19 hr in the presence of 5 μM CuSO4 and 2 mM MPA. Lane a, control DNA that was incubated in the absence of (OP)-m-11-mer, Cu2+, MPA, and tRNA. The cleavage products were then separated by electrophoresis on a non-denaturing 0.8% agarose gel and were analyzed by ethidium bromide staining. When Hae II (position 832) was used for linearization, the two fragments generated by the (OP)-m-11-mer have sizes of 1045 and 4198 bp. Two other fragments (760 and 3440 bp) were generated when the cleavage reaction was carried out at low temperatures. These two fragments could be attributed to a secondary binding site with two mismatches for 5'-d(TTTCCTCCTC)-3' at positions 1592–1602. Lane M, length standards as described in Fig. 3.

![Temperature dependence of SV40 DNA cleavage by (OP)-m-11-mer.](attachment:image.png)
either strand of duplex DNA to bring the phenanthroline ring into the minor groove at the position where the cleavage sites are observed. The most likely explanation is that phenanthroline intercalates at the junction between the triple and the double helix. Previous footprinting studies with copper-phenanthroline as an artificial nuclease did not reveal any structural anomaly at the triple–duplex junction on the 5′ side of the oligonucleotide (20). The pyrimidine-purine step (TpA), which is located at the triple–duplex junction, has been previously shown to favor intercalation (23, 27). The phenanthroline ring is tethered to the oligonucleotide via its 5-amino group. Intercalation would bring the nitrogen atoms (positions 1 and 10) in the minor groove where copper binding occurs. The oxidative species generated by the copper-phenanthroline chelate in the presence of a reducing agent would then cleave the two strands in an asymmetric way, with the cleavage sites being shifted toward the 3′ side on both strands as experimentally observed. Model building studies do indicate that it is possible to intercalate the phenanthroline ring at the triple–duplex junction on the 5′ side of the homopyrimidine oligonucleotide. The short linker of (OP)-s-11-mer did not allow for intercalation and, as a matter of fact, no cleavage was observed. The long linker could wrap around the DNA strands but this is unlikely because of the presence of the negatively charged thiosphodiester within the linker. The location of the cleavage sites (Fig. 2) indicates that phenanthroline occupies the same position with both l and m linkers.

The active species responsible for cleavage by copper-phenanthroline chelates has been described as the 1:2 complex Cu(OP)2 (24). Cleavage by (OP)-11-mer was not enhanced by the addition of free phenanthroline. A second phenanthroline could be provided by a second oligonucleotide since a large excess of oligonucleotide over the target was used. However, a strong electrostatic repulsion is expected between the negatively charged oligonucleotide and the duplex–triplex structure. Therefore, the possibility that the active species is a 1:1 complex with, e.g., MPA acting as a second ligand cannot be excluded. The temperature dependence of the cleavage reaction (Fig. 6) shows that 50% of the maximum efficiency is observed at 30°C in a pH 7.4 buffer. This temperature is much higher than expected on the basis of the thermal stability of the 11-mer/32-mer complexes measured by absorption spectroscopy (23). The requirement for cytosine protonation to form a C+C-G+C base triplet makes these complexes unstable at pH 7.4. The model presented above might account for the observed stability of the copper complex. After major groove binding of the oligonucleotide and intercalation of the phenanthroline ring, copper binding would lock the complex in place from within the minor groove. In this model, intercalated phenanthroline acts as a bridge connecting the two grooves of DNA, with the copper complex in the minor groove and the oligonucleotide in the major groove preventing dissociation of the intercalated complex at the specific site. To our knowledge, this is the only reported case of a direct transfer of chemical information between the two grooves of DNA: major groove recognition and intercalation target a chemical reaction to the minor groove.

The experiments carried out with SV40 DNA reveal that cleavage occurs at a single site at high temperature. Double-strand cleavage was demonstrated by the production of two duplex DNA fragments on agarose gels. A mismatched sequence was also cleaved when the temperature was lowered (Fig. 5). If applications of these artificial nucleases are contemplated to cleave long DNA fragments, an optimal temperature should be determined to obtain cleavage only at the fully matched target site.

The results presented here show that it is possible to cleave linear double-stranded DNA at specific sites by homopyrimidine oligonucleotides covalently linked to a phenanthroline–copper chelate. The efficiency is high (70%), much higher than that reported for Fe–EDTA (25%) (17, 18). The efficiency presently attainable is high enough to contemplate biological applications in different fields such as site-directed mutagenesis or the artificial control of gene expression at the DNA level. Such artificial (restriction-like) endonucleases might also be useful for gene mapping on long DNA fragments. By increasing the length of the oligonucleotide and/or attaching phenanthroline at both ends with appropriate linkers, the efficiency of double-strand cleavage might be improved.

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