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Sequence-specific recognition of the major groove of DNA by oligodeoxynucleotides via triple helix formation. Footprinting studies

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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. The oligonucleotide is bound parallel to the homopurine strand of the duplex. This binding can be revealed by a footprinting technique using copper-phenanthroline as a cleaving reagent. Oligonucleotide binding in the major groove prevents cleavage by copper-phenanthroline . The clea vage patterns on opposite strands of the duplex at the boundaries of the triple helix are asymmetric. They are shifted to the 3'-side, indicating that the copper-phenanthroline chelate binds in the minor groove of the duplex structure. Binding of the chelate at the junction between the triple and the double helix is not perturbed on the 5'-side of the bound homopyrimidine oligonucleotide. In contrast, a strong enhancement of cleavage is observed on the purine-containing strand at the triplex-duplex junction on the 3'-side of the homopyrimidine oligonucleotide.

INTRODUCTION

The selective recognition of nucleic acid base sequences remains a central problem in molecular biology. Several regulatory proteins have been shown to recognize the major groove of DNA (1). Many DNA-binding drugs, by contrast, binds in the minor groove (2). Intercalating agents usually bind via the minor groove (3) even though there are reported cases where intercalation occurs from the major groove (4). Watson-Crick base pairs in DNA still possess several hydrogen bonding sites which are accessible in both the minor and major grooves (1). In the major groove thymine can form two hydrogen bonds with an adenine already engaged in a Watson-Crick A.T base pair. Protonated cytosine can form two hydrogen bonds with a guanine base involved in a Watson-Crick G.C base pair. It is thus possible to form local triple helices with two polypyrimidine strands hydrogen bonded to a polypurine (5,6). An oligopyrimidine should also be able to bind into the major groove of DNA at a homopurine.homopyrimidine sequence according to the rules described above for the formation of base triplets. We recently showed that an oligothymidylate could

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bind into the major groove of DNA at an $(A)_n.(T)_n$ sequence (7,8). When the oligothymidylate carries a photoactive group at one of its ends it is possible to induce photocrosslinking reactions on either strand of the double helix. We made use of azidoproflavine (7) or para-azidophenacyl (8) as photocrosslinkers and showed that cleavage reactions could be induced at the crosslinked sites upon treatment of the irradiated samples with piperidine at high temperature. The location of the crosslinked sites allowed us to demonstrate that the oligothymidylate was bound parallel to the purine-containing strand of the double helix. This conclusion was reached independently of whether the oligothymidylate was synthesized with the natural [β] anomers or the synthetic [α] anomers of nucleoside units. Moser and Dervan (9) showed that cleavage reactions could be targeted to specific homopurine-homopyrimidine DNA sequences using an EDTA-Fe chelate tethered to an oligopyrimidine oligonucleotide.

Binding of a ligand to the DNA double helix may perturb the reaction of a nuclease along the DNA strands. This method - known as footprinting - has been used to detect the binding sites of proteins (10) or drugs (11). It provides information on the region of DNA which is made inaccessible to the nuclease and on the conformational changes that may be induced at a distance from the ligand binding site. Metal chelates such as EDTA-Fe (12) or $(phenanthroline)_2Cu$ (abbreviated as $Cu(OP)_2$) (13) can induce cleavage reactions in the phosphodiester backbone of nucleic acids in the presence of reducing agents. Therefore they can be used as artificial nucleases to probe ligand binding on DNA (12,14). The EDTA-Fe complex does not bind to DNA but generates OH• radicals that diffuse and attack deoxyribose (mostly at the 1' and 4' positions) (12). The $Cu(OP)_2$ complex binds to DNA and induces cleavage reactions that might involve Cu-oxo intermediates (13). These reactions depend on the base sequence and are sensitive to the local conformation of DNA which modulates phenanthroline binding (15). The $Cu(OP)_2$ chelate has been previously used to probe conformational changes that occur, e.g., in promoters during transcription (16,17). In the present study we show that binding of a homopyrimidine oligonucleotide to the DNA double helix can be revealed by footprinting studies using copper-phenanthroline as an artificial nuclease.

MATERIAL AND METHODS

Oligonucleotides

The two complementary 32 nucleotide-long oligodeoxynucleotides and the oligopyrimidines used in this report were synthesized on a Pharmacia synthesizer (see figure 1 for the sequences). They were purified by polyacry-

lamide reverse phase chromatography followed by gel electrophoresis. The following chemicals were obtained from commercial sources : 1,10-phenanthroline (Merck) ; 2,9-dimethyl-1,10-phenanthroline, copper sulfate, and 3-mercaptopropionic acid (Janssen Chimica) ; spermine (Sigma) ; ethylene glycol (Prolabo).

Footprinting by copper-phenanthroline

The duplex 32-mer used in footprinting studies was prepared by incubating equal concentrations of the two complementary oligodeoxynucleotides in H_2O at 20°C. One strand was 5'-labelled by T_4-polynucleotide kinase (Amersham) using (γ-P^{32}) ATP. Distilled water and all solutions other than metal salts were treated with a Chelex 100 resin (Biorad).

Footprinting reactions were performed in Eppendorf tubes containing 10 nM (fragment concentration) of the 5'-labelled 32-mer duplex, 10 µM of the oligopyrimidine, 20 µM of 1,10-phenanthroline, 5 µM $CuSO_4$ and 2 mM 3-mercaptopropionic acid. Cleavage reactions were carried out at 4°C for 3 hours in a pH 6 buffer containing 100 mM NaCl, 10 mM phosphate, 1 mM spermine, 20 percent (by volume) ethylene glycol. Cleavage reactions were stopped by addition of 100 µM 2,9-dimethyl 1,10-phenanthroline. The samples were then dried, resuspended in Maxam-Gilbert loading buffer (18) (80 % deionized formamide containing 0.1 % xylene-cyanol and 0.1 % bromophenol blue) and analyzed by electrophoresis on 20 % polyacrylamide-7 M urea 29 : 1 cross-linked gels. Autoradiograms were obtained by exposing the gels to Kodak or Fuji (X-ray) films at -20°C. The films were developed in a 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. Densitometric methods were used to analyze the cleavage pattern induced by $Cu(OP)_2$ on the 32-mer duplex in the absence or in the presence of oligopyrimidines. Microdensitometry of autoradiograms was performed on an Helena densitometer.

RESULTS

Footprint of an 11-mer oligopyrimidine bound to a DNA double helix

The $Cu(OP)_2$ complex has been shown to bind into the minor groove of DNA. In agreement with this hypothesis the cleavage sites on opposite strands of the double helix are shifted towards the 3'-side (15). Binding of a homopyrimidine oligonucleotide in the major groove of DNA is expected to induce conformational changes of the minor groove and thereby alter the $Cu(OP)_2$

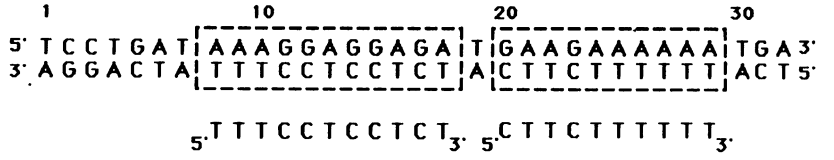

Figure 1 : Sequences of the 32-mer duplex and of two homopyrimidine oligodeoxynucleotides $d^{5'}(TTTCCTCCTCT)^{3'}$ and $d^{5'}(CTTCTTTTTT)^{3'}$ which bind to the 32-mer duplex in a parallel orientation with respect to the purine-containing strand.

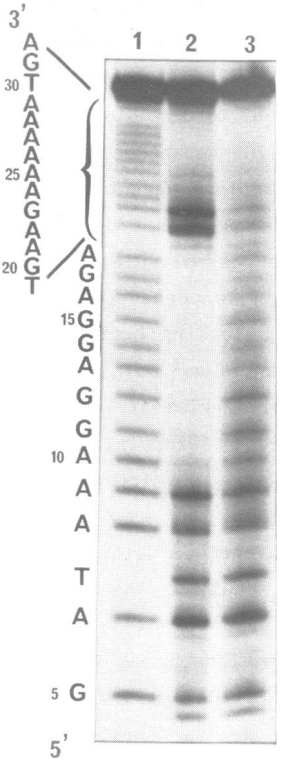

Figure 2 : Footprinting of the 11-mer oligopyrimidine $d^{5'}(TTTCCTCCTCT)$ bound to the 32-mer duplex. The 32-mer duplex was 5'-labelled on its purine-containing strand. Digestion by $Cu(OP)_2$ was performed in the absence (lane 3) or in the presence of 10 µM of the 11-mer oligonucleotide (lane 2). Conditions of cleavage are described in Materials and Methods. Lane 1 represents the (G+A) Maxam-Gilbert digests of the 5'-labelled single-stranded 32-mer containing the homopurine sequence.

cleavage reactions. We used an 11-mer oligopyrimidine of sequence d5'(TTTCCTCCTCT)3' which is expected to bind to the homopurine.homopyrimidine sequence (referred to in the following as $(pu)_{11}.(py)_{11}$) of the 32-mer duplex shown in figure 1. Treatment of the 32-mer duplex with $Cu(OP)_2$ in the presence of mercaptopropionic acid led to cleavage reactions along the two strands with an efficiency that depended on the local base sequence (figures 2 and 3). The oligo$(dA)_6$ sequence located at the 3' end of the purine-containing strand was only very weakly cut by $Cu(OP)_2$. In the presence of the homopyrimidine oligonucleotide, the cleavage reactions were inhibited in a well-defined re-

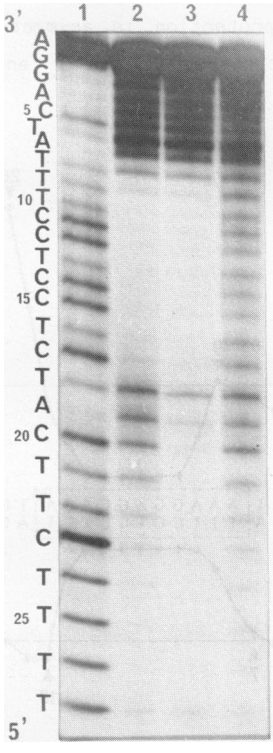

Figure 3 : Footprinting of homopyrimidine oligonucleotides bound to the 32-mer duplex. The duplex 32-mer was 5'-labelled on its pyrimidine-containing strand. It was incubated in the absence (lane 4) or in the presence of d5'(TTTCCTCCTCT)3' (10 µM) (lane 2). Lane 3 represents the footprint observed on the 32-mer duplex in the presence of two oligopyrimidines (see figure 1 for the sequences). Lane 1 shows the (C+T) Maxam-Gilbert products of the 5'-labelled single-stranded 32-mer containing the homopyrimidine sequence.

gion on each strand (figures 2 and 3). In addition enhanced cleavage was observed at a few sites on the 3'-side of the purine-containing strand. This effect was obtained independently of the extent of cleavage indicating that it was not due to a redistribution of $Cu(OP)_2$ complexes along the 32-mer duplex. No enhancement was observed on the pyrimidine-containing strand.

Quantitative analysis of the footprinting pattern

On figure 4 is plotted the ratio of the intensities of every DNA fragment produced by cleavage in the presence and in the absence of the homo-pyrimidine oligonucleotide. Several observations can be made : i) the protected region clearly involves the $(pu)_{11}.(py)_{11}$ sequence expected to bind the oligonucleotide, ii) the protection is asymmetric, cleavage being observed within the $(pu)_{11}.(py)_{11}$ sequence at the 5' end on each strand. Protection

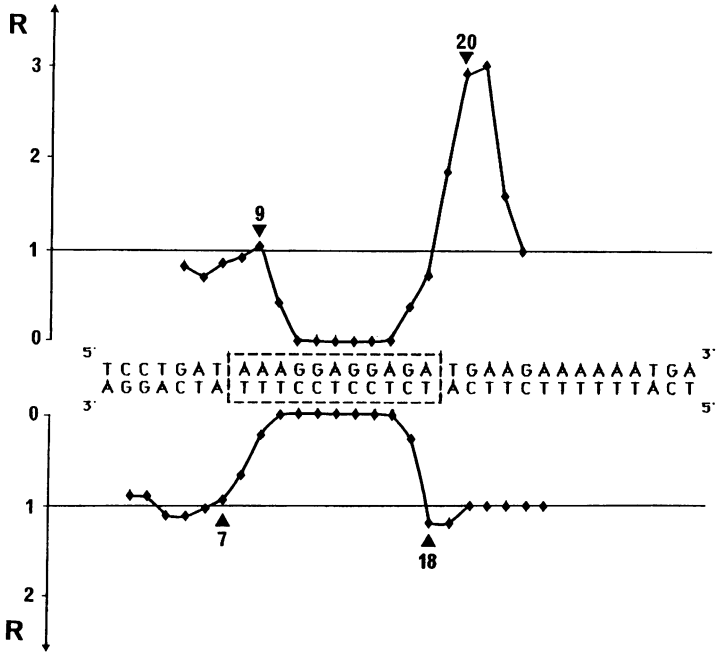

Figure 4 : Quantitative analysis of the footprinting data of figures 2 and 3. The intensities of the different bands observed on the autoradiograms were measured after cleavage of each of the two strands of the 32-mer by $Cu(OP)_2$ in the presence and in the absence of the 11-mer oligopyrimidine. R is the ratio of the intensities obtained for each band in the presence and in the absence of the oligonucleotide. R = 1 means no protection ; R = 0 : full protection ; R > 1 : enhancement of cleavage.

occurs between base-pairs T.A (7) and A.T (18) on the pyrimidine-containing strand and between base pairs A.T (9) and A.T (18) on the purine containing strand ; iii) the cleavage efficiency is enhanced on the 3' side of the purine-containing strand (base pairs 19 to 22).

There is no protection on the purine-containing strand up to position 9 and on the pyrimidine-containing strand up to position 7 (notice that the purine-containing strand is numbered from 5' to 3' while the pyrimidine-containing strand is numbered from 3' to 5'). Such a shift of two nucleotides towards the 3'-side has been previously observed when $Cu(OP)_2$ cuts double-stranded DNA (15).

Due to enhanced cleavage on the 3'-side of the purine-containing strand it is difficult to ascribe a well-defined limit to the protected region on this side of the triple helix. Protection terminates at nucleotide T-18 on the pyrimidine-containing strand. The corresponding position on the complementary strand is G-20. Therefore, a shift of two nucleotides towards the 3'-side is also suggested on this side of the homopyrimidine oligonucleotide binding site with a preferential binding of $Cu(OP)_2$ close to the purine-containing strand.

Simultaneous binding of the two neighbouring oligodeoxynucleotides

On figure 3 is presented the footprint observed on the pyrimidine-containing strand of the 32-mer duplex in the presence of two oligodeoxynucleotides whose sequences are $d^{5'}(TTTCCTCCTCT)^{3'}$ and $d^{5'}(CTTCTTTTTT)^{3'}$ (see figure 1). The first oligonucleotide is identical to that described above ; the second one is expected to bind to the $(pu)_{10}.(py)_{10}$ sequence also present on the 32-mer duplex. The footprinting experiment presented on figure 3 reveals that this second oligonucleotide binds to the double helix in the presence of the first one and protects the pyrimidine-containing strand against the $Cu(OP)_2$ cleavage reactions. Protection of the purine-containing strand was also observed (data not shown).

When the two homopyrimidine oligonucleotides are bound to the 32-mer duplex, cleavage by $Cu(OP)_2$ is still observed at the junction between the two oligonucleotides. The main cleavage sites are at T-18 on the pyrimidine-containing strand (figure 3) and at A-21 on the purine-containing strand. These cleavage sites on opposite strands are shifted to the 3'-side and reflect the binding of $Cu(OP)_2$ into the minor groove of duplex DNA between the two triplex structures. It should be noted that there is only one base pair (T.A (19))) which is not involved in triplex formation. This is sufficient to allow $Cu(OP)_2$ binding and cleavage.

DISCUSSION

Footprinting experiments using $Cu(OP)_2$ as an artificial nuclease clearly show that a homopyrimidine oligonucleotide binds to a DNA double helix at a homopurine.homopyrimidine sequence. $Cu(OP)_2$ does not bind to the triple helix. The asymmetry of the cleavage patterns induced on opposite strands at the junctions between the triplex and duplex structures indicates that $Cu(OP)_2$ binds in the minor groove of duplex DNA, in agreement with previous results (13-15). It has been postulated that one of the two phenanthrolines of $Cu(OP)_2$ intercalates in DNA. Although there is no available data reporting intercalation of free phenanthroline, N-methylphenanthrolinium cations have been shown to intercalate in DNA (19,20).

Binding of a ligand in the major groove of duplex B-DNA is not sufficient to prevent $Cu(OP)_2$ cleavage. EcoRI endonuclease which binds in the major groove but leaves the minor groove accessible does not protect duplex DNA against $Cu(OP)_2$ attack (21). The inhibition of DNA cleavage when an oligopyrimidine binds in the major groove reveals an important conformational change that prevents binding of $Cu(OP)_2$ into the minor groove. Fiber diffraction studies have shown that a triple helix such as poly(dT).poly(dA).poly(dT) or poly(rU).poly(rA).poly(rU) adopts an A-like conformation (5). Even though A-like structures such as poly(rA).poly(rU) or poly(rA).poly(dT) bind intercalators such as ethidium bromide or propidium iodide (22,23), the triple helix poly(rA).2poly(rU) binds ethidium weakly (24) and ethidium binding induces a conversion of the triple helix poly(dA).2poly(rU) to the double helix poly(dA).poly(rU) (22). The inhibition of DNA cleavage by $Cu(OP)_2$ when a triple helix is formed might reflect the decreased ability of phenanthroline to intercalate. Alternatively the shallow minor groove of A-DNA might not fit the $Cu(OP)_2$ chelate structures as well as the deeper minor groove of B-DNA. It should be noted that an A-like structure such as poly(rA).poly(dT) is cleaved by $Cu(OP)_2$ much more slowly than B-DNA (25) even though efficient intercalation occurs in such a structure (22,23).

Triple helix formation does not prevent $Cu(OP)_2$ binding to the duplex at the boundaries of the triple helix. The shift of the cleavage sites towards the 3'-side is characteristic of an attack via the minor groove. In addition the local formation of the triple helix induces a conformational change on the 3'-side of the bound oligonucleotide that is reflected in an enhanced cleavage efficiency on the purine-containing strand. Binding of $Cu(OP)_2$ on this side of the triple helix is clearly asymmetric as revealed by a large difference in the reactivity of the two strands that is not observed

on the double helix in the absence of the oligonucleotide. It should be noted that there is an intrinsic asymmetry in the efficiency of $Cu(OP)_2$-induced cleavage on opposite strands of a double helix (15). This probably reflects the asymmetry of $Cu(OP)_2$ binding into the minor groove which brings the radical-generating copper ion closer to one of the two strands. This property depends on the local base sequence. The formation of a local triple helix might be expected to change this asymmetric binding at its boundaries. This is not observed on the 5'-side of the oligopyrimidine but this is clearly the case on its 3'-side where the purine-containing strand is more efficiently cut whereas no change is observed on the pyrimidine-containing strand.

In conclusion the studies presented in this report show, for the first time, that it is possible to reveal the binding of an oligonucleotide into the major groove of a double helix using the footprinting technique that has proved so useful in detecting specific protein-nucleic acid interactions. The $Cu(OP)_2$ chelate was used as an artificial nuclease to probe conformational changes induced in the double helix at the site of oligonucleotide binding. The footprinting studies have also revealed differences at the junctions between the triple helix and the duplex structure. Whereas the boundary on the 5'-side of the oligopyrimidine is not perturbed, that on the 3'-side clearly shows a preference for binding of $Cu(OP)_2$ to the purine-containing strand. These observations are important in designing oligonucleotides that bind more strongly in the major groove of DNA, e.g., via the covalent attachment of intercalating agents at the 3' and/or 5'-end (26). Further studies along these lines are presently under way.

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REFERENCES

1. Hélène, C. and Lancelot, G. (1982) Prog. Biophys. Mol. Biol. **39**, 1-68.
2. Zimmer, C. and Wähnat, U. (1986) Prog. Biophys. Mol. Biol. **47**, 31-112.
3. Neidle, S. and Abraham Z. (1984) C.R.C. Crit. Rev. Biochem. **17**, 73-121.
4. Delbarre, A., Delepierre, M., Garbay, C., Igolen, J., Le Pecq, J.B. and Roques , B.P. (1987) Proc. Natl. Acad. Sci. USA **84**, 2155-2159.
5. Arnott, S. and Selsing, E. (1974) J. Mol. Biol. **88**, 509-521.
6. Lee, J.S., Woodsworth, M.L., Latimer, L.J.P. and Morgan A.R. (1984) Nucleic Acids Res. **12**, 6603-6614.
7. Le Doan, T., Perrouault, L., Praseuth, D., Habhoub , N., Decout, J.L.,

Thuong, N.T., Lhomme, J. and Hélène, C. (1987) Nucleic Acids Res. **15**, 7749-7760.
8. Praseuth, D., Perrouault, L., Le Doan, T., Chassignol, M., Thuong, N.T. and Hélène, C. (1988) Proc. Natl. Acad. Sci. USA **85**, 1349-1353.
9. Moser, H.E. and Dervan, P.B. (1987) Science **238**, 645-650.
10. Galas, D.J. and Schmitz, A. (1978) Nucleic Acids Res. **5**, 3157-3170.
11. Van Dyke, M.W., Hertzberg, R.P. and Dervan, P.B. (1982) Proc. Natl. Acad. Sci. USA **79**, 5470-5474.
12. Tullius, T.D., and Dombroski, B.A. (1985) Science **230**, 679-681.
13. Sigman, D. (1986) Accounts Chem. Res. **19**, 180-186.
14. Kuwabara, M.D. and Sigman, D.S. (1987) Biochemistry **26**, 7234-7238.
15. Veal, J.M. and Rill, R.L. (1988) Biochemistry **27**, 1822-1827.
16. Spassky, A. and Sigman, D.J. (1985) Biochemistry **24**, 8050-8056.
17. Spassky, A. (1986) J. Mol. Biol. **188**, 99-103.
18. Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. **65**, 497-559.
19. Gabbay, E.J., Scofield, R.E. and Baxter, C.S. (1973) J. Am. Chem. Soc. **95**, 7850-7857.
20. Jain, S.C., Bhandary, K.K., and Sobell, H.M. (1979) J. Mol. Biol. **135**, 813-840.
21. Kuwabara, M.D., Yoon, C., Goyne, T., Thederahn, T. and Sigman, D.S. (1986) Biochemistry **25**, 7401-7408.
22. Bresloff, J.L. and Crothers, D.M. (1981) Biochemistry **20**, 3547-3553.
23. Chou, W.Y., Marky, L.A., Zanczkowski, D. and Breslauer, K.J. (1987) J. Biomol. Struct. Dyn. **5**, 345-359.
24. Le Pecq J.B. and Paoletti C. (1965) C.R. Acad. Sci. Paris **260**, 7033-7036.
25. Pope, L.E. and Sigman, D.S. (1984) Proc. Natl. Acad. Sci. USA **81**, 3-7.
26. Asseline, U., Delarue, M., Lancelot, G., Toulmé, F., Thuong, N.T., Montenay-Garestier, T. and Hélène, C. (1984) Proc. Natl. Acad. Sci. USA **81**, 3297-3301.