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Nonenzymatic Sequence-Specific Cleavage of Duplex DNA via Triple-Helix Formation by Homopyrimidine Phosphorothioate Oligonucleotides

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Abstract—Phenanthroline was attached covalently to the 5′-terminus of the unmodified and modified (3′-terminal phosphorothioate) oligonucleotide sequences, TTTTTTCTTCTCTTTCC (OP-17 mer) and TTTTTTTCTTCTCTTTCsC (OPRp-17 mer or OPSp-17 mer) via a phosphoramidite bond. Simian virus 40 DNA contains a single target site for these oligonucleotides. In the presence of copper ions, the efficient double-stranded cleavage at 37 °C and pH 7.0 was observed by agarose gel electrophoresis. 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 linker was located in the major groove. Of particular interest are the 3′-terminal phosphorothioate oligonucleotide-phenanthroline derivatives (Rp or Sp), which were found to have cleavage activities of the same order as for the oligonucleotide phenanthroline (OP-17 mer). Furthermore, the OPSp-17 mer was intact after incubation in 10% fetal bovine serum for 24 h, whereas, the OPRp-17 mer was slightly more unstable than the OPSp-17 mer. However, the OP-17 mer was completely degraded. An increased resistance to nucleases has been observed by the introduction of phosphorothioate groups on the 3′-terminus of oligonucleotide-phenanthroline derivatives. This stabilization should help us to design much more efficient chemical recognition enzymes and antisense nucleic acid based anti-viral therapies, which could be used as tools in cellular biology. Copyright © 1996 Elsevier Science Ltd

Introduction

Homopyrimidine–homopurine sequences have been mapped to several sites in the regulatory regions of eukaryotic genes, and have been found to be hypersensitive to single-strand-specific nucleases, such as S1.[1–3] These sequences have been suspected to exhibit unusual DNA structures, as they are known to undergo a transition to an underwound state in plasmids, under conditions of moderately acidic pH and negative supercoiling. Studies of these systems appear to support a model consisting of a triple-strand (pyr-pur-pyr) plus a single-stranded structure called H-DNA.[4–7] Triple-helix formation also has been exploited as a strategy for DNA recognition and site-specific double-stranded cleavage,[8–19] and has generated considerable excitement in recent years. The strategy would utilize these homopyrimidine oligonucleotides with metal complexes covalently linked to a single position, to form pyr-pur-pyr triplexes, which would oxidatively cleave double-stranded DNA at recognition sites. 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. Several metal chelates, such as Fe–EDTA[8,9] and Cu–phenanthroline,[14,15,20,21] induce cleavage reactions in duplex DNA when they are connected to homopyrimidine oligonucleotides. Among the most elegant examples in this area are the 'chemical recognition enzymes', which cleave nucleic acids in a sequence-directed manner. Such triplex techniques to isolate large segments of genomic DNA for mapping and sequencing may have important implications for human genetics. Furthermore, artificial enzymes of this kind should be valuable tools in the fields of biotechnology and antisense DNA therapy.

In this paper, we show that oligonucleotides covalently linked to a phenanthroline–copper chelate can be used to cleave both strands of simian virus 40 (SV40) DNA at a well-defined site. We also show that the double strands cleaved by the 3′-terminal phosphorothioate oligonucleotide-phenanthroline derivatives (OPRp-17 mer or OPSp-17 mer) are strongly resistant to 3′-exonucleases.[22–24] The cleavage sites on the double strands suggest that the phenanthroline intercalates within the double helix at the boundary of the triple helix, and that the oligonucleotide binds to the major groove of the DNA. In particular, the 3′-terminal phosphorothioate oligonucleotide-phenanthroline derivatives may be useful in antisense nucleic acid based antiviral therapies with triple-helix approaches, both in vitro and in vivo.

Results and Discussion

Analysis of binding specificity by affinity cleavage

The relative affinities of the homopyrimidine oligonucleotide-phenanthroline derivatives for Watson–Crick base pairs within a pyrimidine-purine-pyrimidine triple-helix motif were examined by affinity cleavage. The homopyrimidine oligonucleotide-phenanthroline derivatives (OP-17 mer, OPRp-17 mer, and OPSp-17

mer) were prepared (Fig. 1) as described in the Experimental section. The homopyrimidine oligonucleotides were separated by reverse phase HPLC. The P—S bonds were characterized by ^{31}P NMR spectroscopy (see Experimental section). When the 5′-labeled purine-rich strand or the 3′-labeled pyrimidine-rich strand 30 mer oligonucleotides containing the target sequence were incubated in the presence of Cu^{2+}, cleavage reactions were induced at well-defined locations on both strands of the 30 mer duplex (Figs 2 and 3). The amount of labeled DNA in the bands at the target cleavage site was measured from reference phosphor autoradiograms. The vertical bars indicate the nucleotide positions that are cleaved most efficiently by the oligomers (OP-17 mer, OPRp-17 mer, and OPSp-17 mer) (Fig. 3), and the lengths of the vertical bars are proportional to the relative cleavage efficiencies by oligomers (the 5′-labeled purine-rich strands, OP-17 mer, OPRp-17 mer, and OPSp-17 mer: 7dC-8dC = 17, 14, and 14%; 8dC-9dA = 16, 12, and 12%; 9dA-10dA = 15, 13, and 12%; the 3′-labeled pyrimidine rich strands, OP-17 mer, OPRp-17 mer, and OPSp-17 mer: 6dA-7dG = 18, 10, and 14%; 7dG-8dG = 20, 13, and 16%; 8dG-9dT = 20, 11, and 15%). The cleavage was slightly more efficient on the pyrimidine-rich strand than on the purine-rich strand. The cleavage sites on the two strands exhibited an asymmetric distribution. This result suggests that the cleavage reaction takes place in the minor groove. A similar conclusion was made, based on recent results that showed that the free complex $Cu^{+}(OP)_2$ cuts the duplex DNA in the minor groove.[25,26] From these results, major groove binding of the oligonucleotide and intercalation of the phenanthroline group occurred, and the phenanthroline–copper chelation locked the complex in place from within the minor groove, where the cleavage reaction occurred.

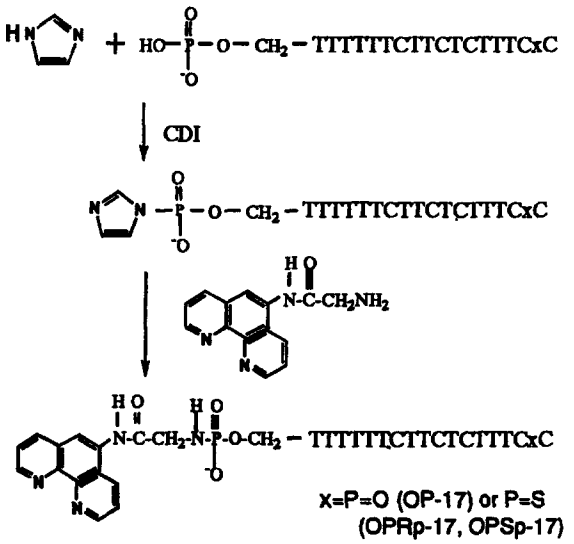

Figure 1. Synthesis of 5′-phenanthroline-P-17 mers.

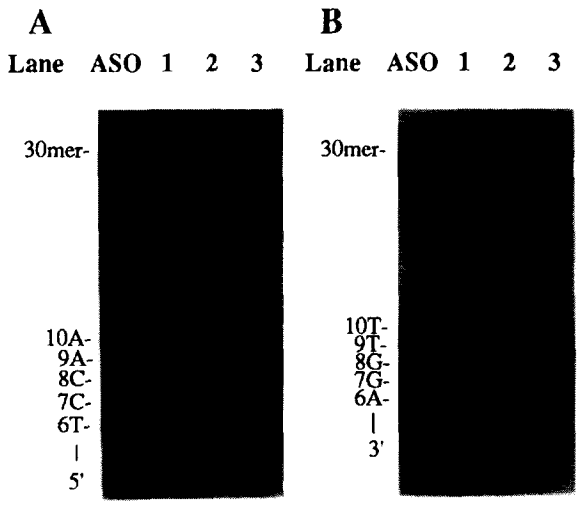

Figure 2. Cleavage of the 30 mer duplex DNA by phenanthroline-substituted oligonucleotides (OP-17 mer, OPRp-17 mer, and OPSp-17 mer). (A) The 30 mer duplex DNA was the 5′-labeled-purine-rich strand [lanes 1 (OP-17 mer), 2 (OPRp-17 mer), and 3 (OPSp-17 mer)]. (B) The 30 mer duplex DNA was the 3′-labeled-pyrimidine-rich strand [lanes 1 (OP-17 mer), 2 (OPRp-17 mer), and 3 (OPSp-17 mer)]. ASO: 5′- or 3′-labeled authentic sample oligomers.

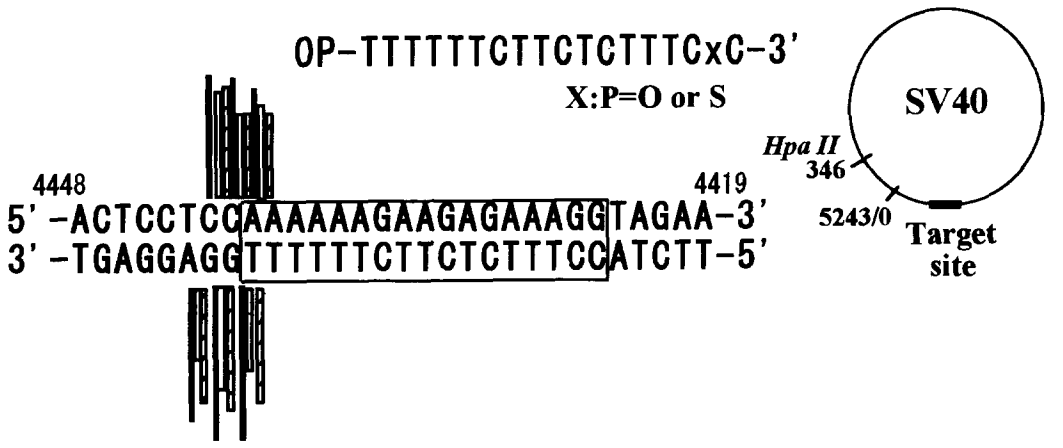

Figure 3. Schematic representation of SV40 DNA. The sequence of the 17 mer target site (shaded box) is shown below, along with the relative position of the target site within the radiolabeled duplex. Vertical bars indicate the nucleotide positions that are cleaved most efficiently by oligonucleotides (OP-17 mer, OPRp-17 mer, and OPSp-17 mer), and the lengths of the vertical bars are proportional to the relative cleavage efficiencies of the oligonucleotides: solid bars, OP-17 mer; open bars, OPRp-17 mer; shaded bars, OPSp-17 mer.

Site-specific double-stranded cleavage of SV40 DNA

The ability of homopyrimidine-oligonucleotide-phenanthroline–Cu^{2+} complexes with 3′-terminal unmodified and modified (P=S) nucleotides to cause site-specific double-stranded breaks at all four naturally occurring base pairs in SV40 DNA was examined under physiologically relevant pH and temperature conditions. The 30 mer duplex used in the studies presented above is part of the SV40 DNA sequence (Fig. 3). SV40 DNA was digested with *Hpa* II (position 346) and was used as a substrate to assay the cleavage activity of the homopyrimidine-oligonucleotide-phenanthroline derivatives. DNA fragments of the expected sizes were obtained after an incubation of linear SV40 DNA with the 3′-terminal phosphorothioate oligonucleotide-phenanthroline derivatives (OPRp-17 mer, OPSp-17 mer, and OPRpSp-17mer) (Fig. 4A, B). The sizes of the expected fragments were 1150 and 4090 base pairs, and were in good agreement with the results of the experiment. The DNA fragments were visualized by ethidium bromide staining and quantitative analysis, assuming that the fluorescence intensities were proportional to the lengths of the DNA fragments. When linear SV40 DNA was incubated with the OPRp-17 mer and OPSp-17 mer for 20 h at 37 °C and pH 7.0, 58 and 54% of the SV40 DNA, respectively, was cleaved into two fragments (Fig. 4A). On the other hand, the *R,S* mixture of the phosphorothioate oligonucleotide-phenanthroline derivative (OPRpSp-17 mer) showed lower cleavage activity than the OPRp-17 mer and the OPSp-17 mer (Fig. 4B). The diastereoisomer factor of the OPRpSp-17 mer is due to the destabilization of triple-helix formation. This suggests that a diastereoisomer factor plays an important role in triplex formation. Furthermore, to test the pH dependence of the efficiency of double-stranded cleavage, we carried out a reaction with SV40 DNA and the OPRp-17 mer and the OPSp-17 mer, for 20 h at pH 7.8 and at 37 °C (data not shown). The cleavage was reduced to 15% of its maximum efficiency. This indicates that, at this pH, the 3′-terminal phosphorothioate oligonucleotide-phenanthroline derivatives dissociated from the double-stranded target sequence. However, the result presented above might also be a consequence of the 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. The intercalated phenanthroline directly transfers chemical information between the two DNA grooves: major groove recognition and intercalation target the chemical reaction in the minor groove. The active species responsible for the cleavage by OP-Cu^{2+} has been described as the 1:2 complex of $Cu^{+}(OP)_2$.[15] However, only one linked phenanthroline–copper is essential for cleavage. A second phenanthroline, unlinked to a carrier, is necessary to increase the affinity of the coordination complex for DNA. Furthermore, the oligonucleotide-phenanthroline derivative (OP-17 mer) and the 3′-terminal phosphorothioate oligonucleotide-phenanthroline derivatives (OPRp-17 mer and OPSp-17 mer) were tested for their relative cleavage activities under the same conditions as in Figure 4. Figure 5 shows that the OP-17 mer (60%) had similar cleavage activity as the OPRp-17 mer (62%) and the OPSp-17 mer (61%).

Finally, the exonuclease resistance of the single-strand of the 3′-terminal phosphorothioate oligonucleotide-

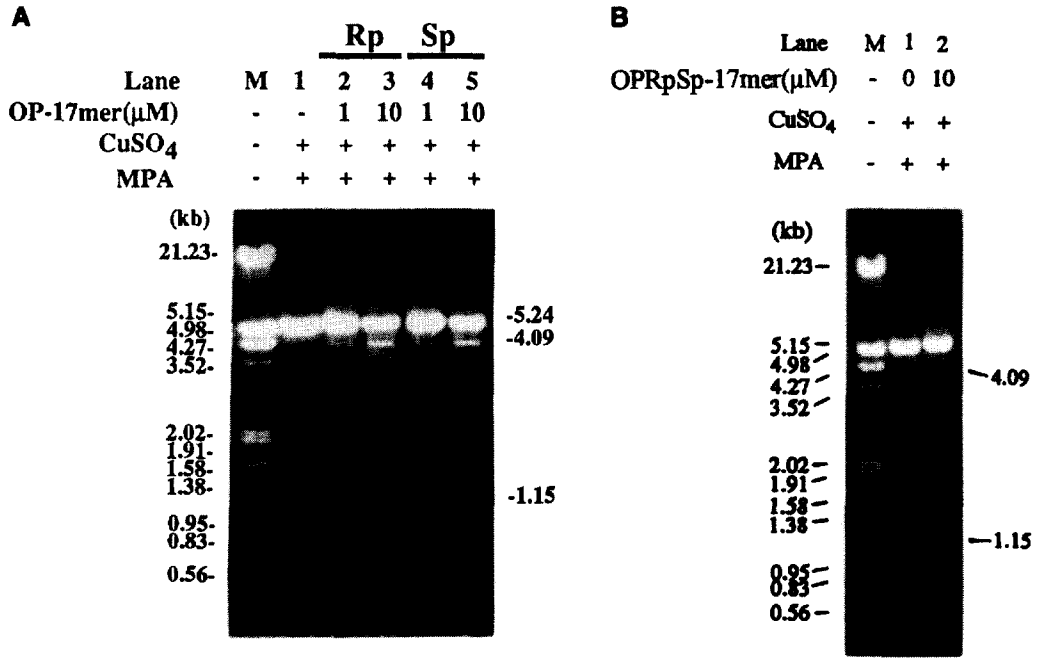

Figure 4. Specific cleavage of SV40 DNA by OPRp-17 mer, OPSp17 mer, and OPRpSp-17 mer. SV40 DNA was linearized at the *Hpa* II site, and was then incubated at a concentration of 10 nM with 0–10 μM of the OPRp-17 mer, OPSp-17 mer, and OPRpSp-17 mer under the same conditions as in Figure 2. Lane M: DNA size markers obtained by digestion of λDNA with *Hind* III and *Eco* R 1. (A) OPRp-17 mer and OPSp-17 mer. (B) OPRpSp-17 mer.

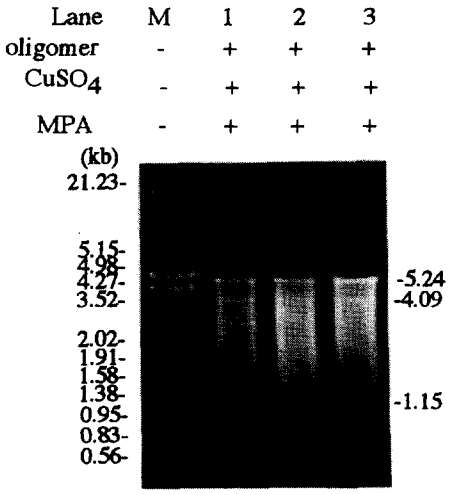

Figure 5. Specific cleavage of SV40 DNA by OP-17 mer, OPRp-17 mer, and OPSp-17 mer. Linear SV40 DNA (10 nM) was incubated with 10 μM of the oligonucleotide-phenanthroline derivatives under the same conditions as in Figure 2. Lane 1: OP-17 mer; Lane 2: OPRp-17 mer; Lane 3: OPSp-17 mer.

phenanthroline derivatives (OPRp-17 mer or OPSp-17 mer) was examined. Burgers et al.[27] and Potter et al.[28] found that snake venom phosphodiesterase hydrolyzes only the Rp isomer, and nucleases P1 and S1 hydrolyze only the Sp isomer. Our results suggest that the OPSp-17 mer is more resistant to 3′-exonucleases than the unmodified oligonucleotide, the OP-17 mer, and the modified phosphorothioate oligonucleotide, the OPRp-17 mer. The results obtained by incubating the oligonucleotides (OP-17 mer, OPRp-17 mer, and OPSp-17 mer) in fetal bovine serum provided additional evidence of their relative stability (Fig. 6). The OPSp-17 mer was stable after 24 h of incubation, whereas the OPRp-17 mer was slightly degraded. The OP-17 mer was completely degraded. An increased resistance to nuclease degradation has been observed by incubation of the the 3′-terminal phosphorothioate oligonucleotide-phenanthroline derivatives with 3′-exonucleases. The OPSp-17 mer has increased nuclease resistance. This stabilization should help us to design much more efficient chemical recognition enzymes, which could be used as tools in cellular biology. Furthermore, the results presented here suggest applications in different biological fields, such as site-directed mutagenesis and the artificial control of gene expression at the DNA level.

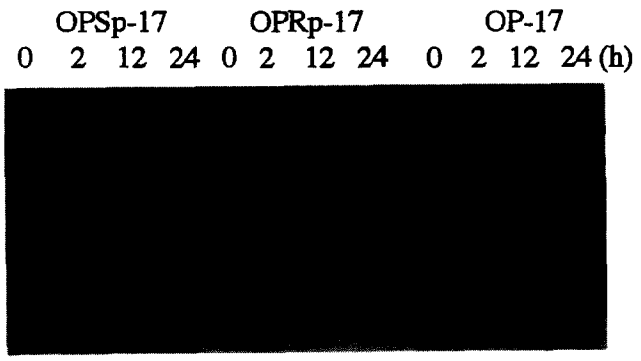

Figure 6. Stability of oligonucleotide-phenanthroline derivatives (OP-17 mer, OPRp-17 mer, and OPSp-17 mer) in the presence of 10% fetal bovine serum at 37 °C for 24 h.

Experimental

Oligonucleotide synthesis

The two complementary oligonucleotide 30 mers and the oligopyrimidine used in this study were synthesized on an Applied Biosystems DNA/RNA synthesizer model 392, by the phosphoramidite method, as described, by the manufacturer's protocol (Applied Biosystems). These sequences are shown in Figure 1. Furthermore, we synthesized the 5′- or 3′-labeled authentic oligonucleotides to determine the nucleotide cleavage positions. They were purified by reverse phase chromatography, followed by polyacrylamide gel electrophoresis.

Circular SV 40 DNA was linearized with the restriction enzyme *Hpa*II, which cleaves at position 346. The cleavage products were separated on 0.8% agarose gels. The products were eluted from the gel, and were recovered from the eluate by ethanol precipitation.

The following chemicals were obtained from commercial sources: 1,10-phenanthroline (Merck Sharp & Dohme), copper sulfate, and 3-mercaptopropionic acid (MPA) (Aldrich).

Synthesis of the 17-mer oligonucleotide-phenanthroline (OP) conjugates

Three oligonucleotides, (OP)-17-mer, (OPRp)-17-mer, and (OPSp)-17-mer, bearing 1,10-phenanthroline attached to the 5′-end, were synthesized (see Fig. 1). Covalent linkage of the phenanthroline derivative to the 5′-end of the oligonucleotide was achieved via the phosphoramidite bond of the 5-(glycylamido)-1,10-phenanthroline derivative.

Covalent linkage of phenanthroline derivatives to oligonucleotides

Synthesis of the 17-mer oligonucleotides was carried out on an Applied Biosystems DNA/RNA synthesizer, model 392, by the phosphoramidite method. Sulfuration for the phosphorothioate bonds was performed after the coupling step by using 0.5 M tetraethylthiuram disulfide (TETD) dissolved in acetonitrile. The phosphorylation of the 5′-hydroxyl group of the 17 mer oligonucleotides attached to the support was carried out according to the procedure recently described for the synthesis in solution, using a 5′-terminal modifier $(DMTrO\text{-}(CH_2)_2SO_2(CH_2)_2OP\text{-}N(iPr)_2\text{-}(OCH_2CH_2CN)$ followed by oxidation of the intermediate phosphite.[29] After synthesis, the support was treated with concd aq NH_3 for 12 h at 40 °C to cleave the oligonucleotide derivatives from the support. The unmodified 5′-P-17

mer was purified by reverse phase HPLC on an oligo-DNA column. The Rp and Sp phosphorothioate bond containing oligonucleotides were separated and purified by reverse phase HPLC on an oligo-DNA column, with a linear gradient of 0–30% acetonitrile in 0.1 M triethylammonium acetate (pH 7.0) during 50 min. The retention times of the Rp-17 mer and the Sp-17 mer substrates were 43.1 min (Rp) and 44.0 min (Sp), respectively. The P—S bonds were characterized by ^{31}P NMR [(85% H_3PO_4 in D_2O as an external reference) Rp-17 mer; 55.78, −1.27 ppm; Sp-17 mer; 54.83, −1.79 ppm] spectroscopy. The 5′-P-17 mer oligonucleotides were first converted to the 5′-phosphoroimidazolides (5′-Im-P-17 mer oligonucleotides) by treatment with 0.12 M 1-ethyl-3,3-dimethylaminopropyl carbodiimide in 0.1 mL of 0.1 M imidazole–HCl buffer at pH 6.1, for 1 h at room temperature.[30] The products were obtained in 78–83% yields and were separated from the 5′-P-17 mer oligonucleotides by HPLC on an oligo-DNA column. The 5′-Im-17 mer oligonucleotides (0.5 OD) were treated with 6 mg (24 μM) of 5-(glycylamido)-1,10-phenanthroline[31] and 10 μL of 2,6-lutidine in 90 μL of H_2O at 50 °C for 12 h. The oligonucleotide-phenanthroline derivatives were separated by 20% polyacrylamide/7 M urea gel electrophoresis. The products were eluted from the gel, and were recovered from the eluate by ethanol precipitation.

Cleavage reactions

Cleavage of the 30-mer duplex DNA fragment and the SV40 linear DNA was carried out in a buffer containing 10 mM sodium phosphate, 0.1 M NaCl, 1 mM spermine, 20% (vol/vol) ethylene glycol, and tRNA (0.1 g/L). The pH was adjusted to a value between 7.0 and 7.8. The substrate concentration (30-mer or SV40) was 10 nM. The concentration of the phenanthroline-substituted oligonucleotides was usually 10 μM. The cleavage reaction was started by the addition of 20 mM MPA and 10 μM $CuSO_4$. The reaction mixture was incubated at 37 °C for 20 h, and was stopped by adding 1 mM 2,9-dimethyl-1,10-phenanthroline. The cleavage products were separated by nondenaturing 0.8% agarose gel electrophoresis and were analyzed by ethidium bromide staining on a Millipore BioImage 60 S (Fig. 3).

The 30-mer duplex was 5′- or 3′-end-labeled on either its pyrimidine-rich strand or its purine-rich strand. The purine-rich strand was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The pyrimidine-rich strand was labeled with [α-^{32}P]ATP and terminal deoxyribonucleotidlytransferase. The cleaved 30-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) gels. Autoradiograms were obtained by exposing the gel to Fuji (X-ray) film at −20 °C. The extent of cleavage was determined by comparing the radioactivity of the intact fragment to that of the cleaved fragments with a BioImage analyzer BAS 2000 (Fuji Medical Systems USA) (Fig. 2).

Exonuclease stability for exonuclease of oligonucleotide-phenanthroline derivatives

The oligonucleotides (0.2 A_{260}) were incubated with 200 μL of culture medium containing 10% fetal bovine serum for 24 h at 37 °C. Aliquots were taken at 0, 2, 4, 8, and 24 h, and were analyzed by PAGE (20% polyacrylamide containing 7 M urea). Densitomeric analysis of gels stained with silver nitrate was performed on a Millipore BioImage 60 S.

Acknowledgements

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References

1. Larsen, A.; Weintraub, H. *Cell* **1982**, *29*, 609.
2. Nickel, J. M.; Felsenfeld, G. *Cell* **1983**, *35*, 467.
3. Elfin, S. C. R. *Nature* **1984**, *309*, 213.
4. Hun, H.; Dahberg, J. E. *Science* **1988**, *241*, 1791.
5. Johnston, B. H. *Science* **1988**, *241*, 1800.
6. Lee, J. S.; Woodsworth, M. L.; Latimer, L. P.; Morgan, A. R. *Nucleic Acids Res.* **1984**, *12*, 6603.
7. Lyamichev, V. I.; Miklin, S. M.; Frank-Kamenetskii, M. D. *J. Biomol. Struct. Dyn.* **1986**, *3*, 667.
8. Mosher, C.; Dervan, P. B. *Science* **1987**, *238*, 645.
9. Strobe, S. A.; Moser, H. E.; Darvan, P. B. *J. Am. Chem. Soc.* **1988**, *110*, 7927.
10. Le Doan, T.; Perrouault, L.; Praseuth, D.; Habhoub, N.; Decout, J.-L.; Though, N. T.; Lhomme, J.; Hélène, C. *Nucleic Acids Res.* **1987**, *15*, 7749.
11. Praseuth, D.; Puerility, L.; Le Doan, T.; Chassignol, M.; Though, N. T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1349.
12. Povsic, T. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 3059.
13. Maher, L. J.; Wold, III B.; Dervan, P. B. *Science* **1989**, *245*, 725.
14. François, J.-C.; Saison-Behmoatas, T.; Chassignol, M.; Though, N. T.; Hélène, C. *J. Biol. Chem.* **1989**, *264*, 5891.
15. François, J.-C.; Saison-Behmoatas, T.; Barbier, C.; Chassignol, M.; Though, N. T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9702.
16. Horne, D. A.; Dervan, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 2435.
17. Perrouaut, L.; Asseline, U.; Rivalle, C.; Though, N. T.; Bisagni, E.; Giovannangeli, C.; Lye Joan, R.; Hélène, C. *Nature* **1990**, *344*, 358.
18. Distefano, M. D.; Shin, J. A.; Dervan, P. B. *J. Am. Chem. Soc.* **1991** *113*, 5901.
19. Singleton, S.; Dervan, P. B. *Biochemistry* **1992**, *31*, 10995.

20. Sigman, D. S.; Chen, C.-B.; Gorin, M. B. *Nature* **1993**, *363*, 474.

21. Shimizu, M.; Inoue, H.; Ohtsuka, E. *Biochemistry* **1994**, *33*, 606.

22. Kim, S.-G.; Tsukahara, S.; Yokoyama, S.; Takaku, H. *FEBS Lett.* **1992**, *314*, 29.

23. Tsukahara, S.; Yamakawa, H.; Takai, K.; Takaku, H. *Nucleosides Nucleotides* **1994**, *13*, 1617.

24. Hacia, J. G.; Wold, B. J.; Dervan, P. B. *Biochemistry* **1994**, *33*, 5367.

25. Thederahn, T. B.; Kuwabara, M. D.; Larsen, T. A.; Sigman, D. S. *J. Am. Chem. Soc.* **1989**, *111*, 4941.

26. Veal, J. M.; Rill, R. L. *Biochemistry* **1989**, *28*, 3243.

27. Burgers, M.; Eckstein, F. *Biochemistry* **1979**, *18*, 592.

28. Potter, B. V. L.; Connolly, B. A.; Eckstein, F. *Biochemistry* **1983**, *22*, 1369.

29. Yamakawa, H.; Ishibashi, T.; Nakashima, H.; Yamamoto, N.; Takai, K.; Takaku, H. *Nucleosides Nucleotides* **1995**, *14*, 1149.

30. Chu, B. C. F.; Orgel, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 963.

31. Sigman, D. S.; Kuwabara, M. D.; Chen, C.-H. B.; Bruice, T. W. *Meth. Enzymol.* **1991**, *208*, 414.

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