Precision Sequence-Specific Cleavage of a Nucleic Acid by a Minor-Groove-Directed Metal-Binding Ligand Linked through N-2 of Deoxyguanosine

Donald E. Bergstrom* and Norman P. Gerry

Department of Medicinal Chemistry and Pharmacognosy
Purdue University, West Lafayette, Indiana 47907

Received December 14, 1993
Revised Manuscript Received September 6, 1994

We have found that it is possible to obtain relatively precise cuts in a short DNA target with sequence-specific oligonucleotides containing phenanthroline ligands bound through N-2 of deoxyguanosine. Although reports on the use of metal complex linked oligonucleotides as sequence-specific nucleases have been relatively numerous, both precision and extent of cleavage have typically been limited.[1] Redox active metal complexes, which function as sequence-specific chemical nucleases when linked to oligonucleotides, include phenanthroline copper,[2−4] iron−EDTA,[5−7] iron−bleomycin,[8] iron−porphyrins,[9,10] iron−2,6-pyridinedicarboxylate, iron−2,2′-dipicolylamine,[11] and [tris(*N*-methylpyridino)porphyrinato]manganese(III).[12] In contrast to previous studies, in which typically the ligand was appended at the 5′-end of an oligonucleotide, the modified nucleoside constructed for this study was designed to position a metal-binding ligand specifically within the minor groove of a duplex nucleic acid. By linking phenanthroline to N-2 of deoxyguanosine and C-2 of phenanthroline via a $-CH_2CH_2CH_2O-$ tether, the ligand is forced to project along the minor groove passing close to abstractable hydrogen atoms (H-1′, H-4′, and H-5′,5″) of the target strand deoxyribosyls. The phenanthroline can reach either the deoxyribose of the deoxycytidine paired to the modified dG or the deoxyribose of the nucleoside directly below that dC (in the 3′-direction). Phenanthroline binds most metal ions known to undergo redox reactions that lead to abstraction of hydrogen atoms from deoxyribose in DNA.

To test the concept, a series of oligonucleotides (15-mers) containing the phenanthroline-modified deoxyguanosine at four different positions were synthesized and cleavage studies of a complementary 31-mer in the presence of ferrous ion and dithiothreitol carried out. The modified deoxyguanosine, **G***, (Chart 1), was synthesized from 9-β-D-2′-deoxyribofuranosyl-2-fluoro-6-[(*p*-nitrophenyl)ethoxy]purine[13] by reaction with 2-(3-aminopropoxy)phenanthroline[14] (**G***). Transformation to the 5′-

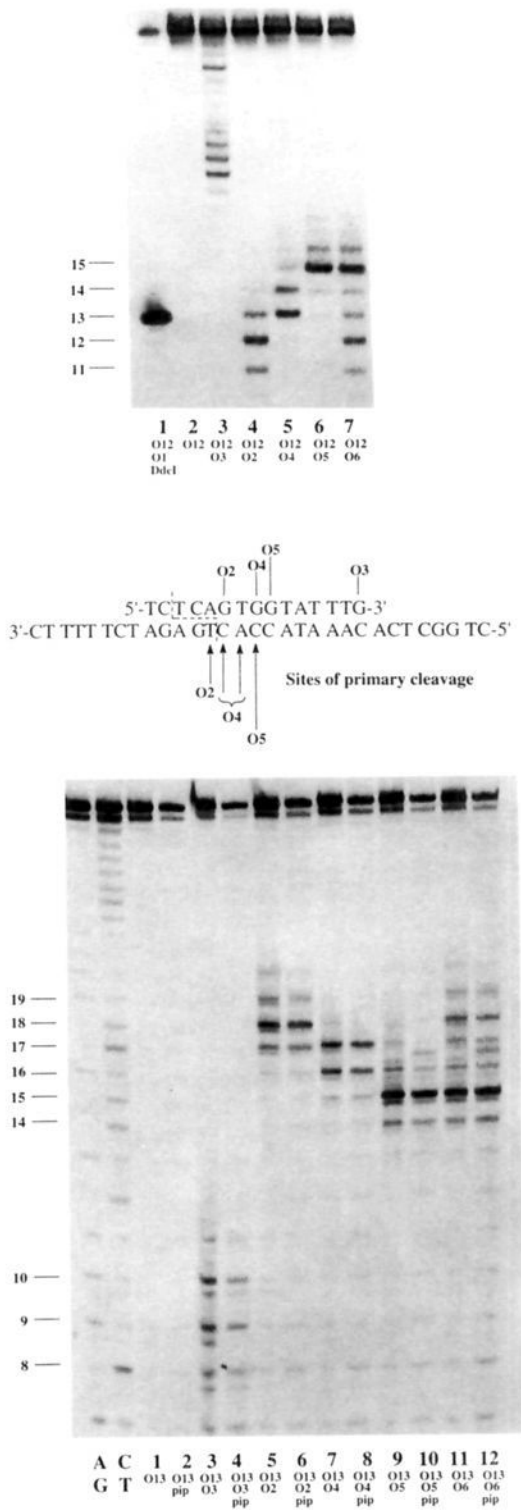

Figure 1. Polyacrylamide gels of cleavage reactions. (A) Cleavage products of 3′-biotin-labeled **O12:** lane 1, 13-mer fragment from endonuclease *Dde*I cleavage of **O12**; lane 2, **O12** control; lane 3, **O12/O3**; lane 4, **O12/O2**; lane 5, **O12/O4**; lane 6, **O12/O5**; lane 7, **O12/O6**. The ratio of target (**O12**) to phenanthroline-modified oligonucleotide (**O2**−**O6**) was 1:5 in each experiment. (B) Cleavage products of 5′-^{33}P-labeled **O13**: lane 1, **O13** control; lane 2, **O13** following treatment with piperidine; lane 3, **O13/O3**; lane 4, **O13/O3** following treatment with piperidine; lane 5, **O13/O2**; lane 6, **O13/O2** following treatment with piperidine; lane 7, **O13/O4**; lane 8, **O13/O4** following treatment with piperidine; lane 9, **O13/O5**; lane 10, **O13/O5** following treatment with piperidine; lane 11, **O13/O6**; lane 12, **O13/O6** following treatment with piperidine. Maxam−Gilbert sequencing lanes are shown on the left-hand side of the gel.

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0002-7863/94/1516-12067$04.50/0

Chart 1

Synthetic DNA targets for model cleavage studies

5 10 15 20 25 30

O12 5'-CT GGC TCA CAA ATA CCA CTG AGA TCT TTT T^BC-3'

O13 5'-^{33}P-CT GGC TCA CAA ATA CCA CTG AGA TCT TTT TC-3'

T^B = biotin linked thymidine analog

Ligand modified oligonucleotides

O1 5'-TCTCAGTGGTATTTG-3'

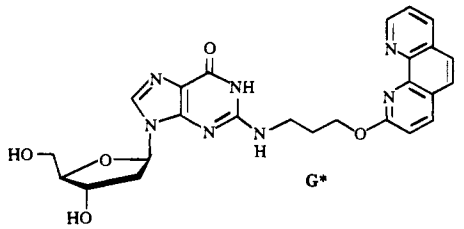
O2 5'-TCTCAG*TGGTATTTG-3'

O3 5'-TCTCAGTGGTATTTG*T-3'

O4 5'-TCTCAGTG*GTATTTG-3'

O5 5'-TCTCAGTGG*TATTTG-3'

O6 5'-TCTCAG*TGG*TATTTG-3'



O-dimethoxytrityl 3'-phosphoramidite was accomplished by established procedures.[15]

Oligonucleotides were synthesized using standard phosphoramidite chemistry (1 μM scale) and deprotected by treatment with 0.5 M DBU/pyridine for 24 h before cleavage from the column with concentrated ammonia. Two target oligonucleotides, one labeled at the 3'-penultimate T by biotin (**O12**) and the other labeled at the 5'-end by ^{33}P (**O13**), were prepared by standard procedures. Following purification by HPLC on a C-18 column eluting with an aqueous/acetonitrile gradient, and desalting, the dimethoxytrityl protecting groups were removed by treatment with aqueous acetic acid. HPLC analysis of an enzymatic digestion (snake venom phosphodiesterase and bacterial alkaline phosphatase at pH 7.5)[16] established that each oligonucleotide had the appropriate composition and showed that the modified base was successfully incorporated and deprotected.

Aqueous solutions of the oligonucleotides (0.9 pmol of target/4.5 pmol of phenanthroline-modified oligonucleotide) were combined and evaporated to dryness. The oligonucleotides were redissolved in 8 μL of 50 mM TRIS pH 7.4, 50 mM NaCl and annealed by heating to 90 °C for 5 min, followed by cooling at room temperature for 30 min. The cleavage reaction was initiated by addition of 1 μL of 0.1 mM ferrous ammonium sulfate followed by 1 μL of 40 mM dithiothreitol (both solutions freshly prepared). The reaction was quenched by the addition of 1 μL of 0.011 M EDTA (pH 8) after 5 h at 22 °C. The solution was then evaporated to dryness, redissolved in loading buffer (formamide/10× TBE, 9:1), heat denatured, and run on a 0.4 mm 20% denaturing polyacrylamide gel at 1800 V for 3.5 h. Biotin-labeled oligonucleotide was visualized by the chemiluminescence protocol specified by the manufacturer (Millipore). ,The ^{33}P-labeled oligonucleotides were visualized by capillary blotting of the gel to a nylon membrane for 15 min, drying, and then exposure to X-ray film.

The results of a cleavage study of oligonucleotides **O12** and **O13** by oligonucleotides **O2**, **O3**, **O4**, **O5**, and **O6** are shown in Figure 1. When the phenanthroline was located near the 3'-end of the oligonucleotide (**O3**), with **G*** paired to C9, iron-mediated cleavage occurred over at least a 10 nucleotide region (lane 3, Figure 1A). On the other hand sequences in which the phenanthroline was tethered near the middle of the oligonucleotide led to *highly specific cleavage concentrated at one or two sites*. It appears that **O2** cuts predominantly at T19 but cleavage is also observed at G20 and C18 (**G*** base pairs to C18). **O4** cuts both at C18 and A17 (**G*** base pairs to C16). **O5** cuts with high specificity at C16 (**G*** base pairs to C15). The sequence **O6** contains two **G***s (located opposite C18 and C15) and cuts at C16 and T19. The cleavage at C16 especially predominates. The results were completely reproducible. Virtually identical cleavage patterns were obtained from reactions repeated at the same reactant concentrations as well as reactions run with different concentrations of the reactants. Iron-mediated cleavage of **O13** allowed visualization of the 5'-fragments and determination of the nature of the 3'-terminus. Significantly, more 3'-phosphate- than 3'-phosphoglycolate-terminated oligonucleotide was obtained, a result very similar to that reported by Kuwabara et al. for a copper–phenanthroline-mediated cleavage reaction.[17] The extent of cleavage of the ^{33}P-labeled 31-mer **O13** was quantitated on a phosphorimager. **O5** gave the most specific cleavage; 38% of the 31-mer was cleaved, with 70% of the cleavage occurring at C16 yielding a 15-mer 5'-fragment. When a portion of the cleavage reaction was treated with piperidine, 54% of the 31-mer was cleaved, with 67% of the cleavage occurring at C16.

We believe that the results reported here illustrate the potential for the further development of redox-based chemical nucleases. Second generation modified nucleosides with more precisely positioned ligands (by way of more conformationally restricted linkers) that could increase specificity and decrease self-damage, which may be the key to increasing the cleavage yield and may eventually make catalytic turnover possible, are currently under development.

Acknowledgment. The National Institutes of Health is gratefully acknowledged for support of this research through NIH Grants AI-26029 and CA-09634. We also gratefully acknowledge support by the Walther Cancer Institute.

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