

## Nuclease activity of 1,10-phenanthroline-copper: Sequence-specific targeting

(artificial DNase/1,10-phenanthroline/oxidative scission)

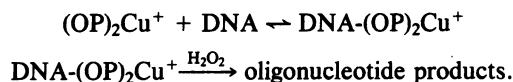
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**ABSTRACT** The nuclease activity of 1,10-phenanthroline-copper ion can be targeted to specific DNA sequences by attachment of the ligand to the 5' end of complementary deoxyoligonucleotides via a phosphoramidate linkage. To synthesize the adduct, the phosphorimidazolidine of the deoxyoligonucleotide is prepared using a water-soluble carbodiimide and is then coupled to 5-glycylamido-1,10-phenanthroline. After hybridization to the target DNA, sequence-specific cleavage is observed upon the addition of cupric ion and 3-mercaptopropionic acid. Two methods of assaying the cutting of the operator sequence of the *lac* operon have been employed using the oligonucleotide 5'-AATTGTTATCCGCTCACAAT-T-3' representing sequence positions 21-1 of the template strand. In the first, the single-stranded DNA of the phage M13mp8 was the target, and cuts were detected using a primer-extension assay. In the second, the substrate was an *EcoRI* fragment 3' labeled in the nontemplate strand. After denaturation and reannealing to the oligonucleotide-1,10-phenanthroline adduct, cupric ion and 3-mercaptopropionic acid were added, and the products were analyzed directly on a sequencing gel. With the phenanthroline moiety attached to position 21 of the oligonucleotide carrier, cutting was observed at positions 20-25 using both assays.

The 1,10-phenanthroline-cuprous complex [(OP)<sub>2</sub>Cu<sup>+</sup>] with hydrogen peroxide as a coreactant cleaves DNA by oxidatively degrading the deoxyribose moiety (1, 2). The proposed mechanism for the cleavage reaction involves the reversible binding of the coordination complex to the minor groove prior to the one-electron oxidation by hydrogen peroxide to produce the hydroxyl radical species directly responsible for the scission reaction as in the following equation:



Products of the reaction include 5'- and 3'-monophosphate ester termini, free bases, and minor amounts of 3'-phosphoglycolate (3, 4).

The artificial nuclease activity is conformation dependent even though the oxidation of the deoxyribose is independent of the base linked to it. The B-DNA strand of poly(dA-T) is the preferred substrate for the nuclease activity. A-DNA in heteroduplexes composed of poly(rA)·poly(T) is cut at one-third the rate of B-DNA, but poly(dG-dC) in the Z structure and noncomplementing single-stranded DNAs are not good substrates (2, 5). The sequence-dependent reactivity preferences that have been observed in a restriction fragment derived from the *lac* operon control region correlate with biochemical function. For example, the promoter conserved

region (Pribnow box) is hyperreactive, reflecting a minor groove geometry distinct from the majority of base pairs in the restriction fragment (6-8). Single base changes in the promoter region, which increase promoter strength, dramatically enhance reactivity toward the coordination complex at up to four sequence positions away from the mutational change. These results have demonstrated that mutations in the promoter region can influence biochemical activity by changing DNA conformation as reflected by reactivity to the minor groove-specific chemical nuclease (7).

The reaction specificity of (OP)<sub>2</sub>Cu<sup>+</sup> is attributable to the preferential binding of the coordination complex near the site of the deoxyribose attack. In contrast, diffusible hydroxyl radicals, generated by <sup>60</sup>Co irradiation, do not demonstrate the specificity observed in (OP)<sub>2</sub>Cu<sup>+</sup>-mediated reactions (4). The utility of the oxidative nuclease activity would be enhanced if the specificity could be targeted. In this communication, we report our first attempts to direct the (OP)<sub>2</sub>Cu<sup>+</sup> nuclease activity. We have attached the 1,10-phenanthroline (OP) moiety to the 5'-phosphorylated terminus of an oligonucleotide and achieved site-specific cutting of a complementary DNA following hybridization.

### MATERIALS AND METHODS

[ $\gamma$ -<sup>32</sup>P]ATP (4500 Ci/mmol; 1 Ci = 37 GBq) and [ $\alpha$ -<sup>32</sup>P]dATP (3000 Ci/mmol) were purchased from ICN, iodo[<sup>3</sup>H]acetic acid (100 mCi/mmol) was from New England Nuclear, and dNTPs were from Pharmacia P-L Biochemicals. The sequence 5'-AATTGTTATCCGCTCACAATT (21-mer lower strand) was the generous gift of R. E. Dickerson and Mary Kopka of the Molecular Biology Institute, U.C.L.A. Single-stranded phage M13mp8 was prepared according to standard methods (9). The *EcoRI* restriction fragment containing the wild-type promoter was kindly provided by Annick Spassky from the Institut Pasteur, Paris. HPLC was done with a polyethyleneimine column using an ammonium sulfate gradient (10).

**Synthesis of Iodo[<sup>3</sup>H]acetamido-1,10-Phenanthroline.** A solution of iodo[<sup>3</sup>H]acetic acid (186 mg, 1 mmol; specific activity 1 mCi/mmol) and dicyclohexylcarbodiimide (93 mg, 0.45 mmol) in 3 ml of ethyl acetate (dried over calcium hydride and distilled before use) was stirred at room temperature for 3 hr. First, the resulting urea was removed by filtration; then the solution was concentrated to dryness by rotary evaporation, and the residue was redissolved in 1.5 ml of acetonitrile (dried over calcium hydride and distilled before use). The solution thus obtained was then added to 2 ml of dry acetonitrile containing 5-amino-OP (30 mg, 0.12

Abbreviations: OP, 1,10-phenanthroline; (OP)<sub>2</sub>Cu<sup>+</sup>, 1,10-phenanthroline-cuprous complex (2:1); 21-mer lower strand, oligonucleotide 5'-AATTGTTATCCGCTCACAATT; 5'-P-21-mer, the 5' monophosphate ester of the 21-mer; glycyl-OP, 5-glycylamido-1,10-phenanthroline; *lac*, wild-type *lac* operator; OP-DNA, the glycyl-OP adduct of the 21-mer.

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mmol). After the solution was stirred overnight at room temperature, the product was collected by centrifugation and washed once with cold 5% sodium bicarbonate, once with water, and dried in a desiccator under vacuum overnight. TLC (alumina, 95% ethanol) showed a new spot moving slightly faster than 5-amino OP. All the starting material had been transformed. (Yield: 36.3 mg;  $0.75 \times 10^8$  cpm).

**Synthesis of [ $^3\text{H}$ ]Glycylamido-1,10-Phenanthroline.** The conversion of the 5-iodoacetamido-1,10-phenanthroline to 5-glycylamido-1,10-phenanthroline (glycyl-OP) was accomplished by adding 6 ml of concentrated ammonium hydroxide to 30 mg ( $0.66 \times 10^8$  cpm) of the iodoacetyl derivative in dimethyl formamide and stirring overnight at room temperature. TLC using silica and 95% ethanol demonstrates the disappearance of the starting material and the formation of a new spot. The mixture was then concentrated to dryness, redissolved in methanol, and purified by chromatography on LH-20 eluting with methanol/water, 3:1 (vol/vol).

**Reaction Scheme.** The reaction scheme used for the synthesis of the OP-bearing oligonucleotide is indicated in Fig. 1 and relies on the specific formation of phosphorimidazolide in aqueous solution recently reported by Orgel and coworkers (11).

**Phosphorylation of the Operator (Lower Strand) 21-mer.** The 5' phosphorylated 21-mer lower strand was prepared by treating the 21-mer (3.0 OD units) with [ $\gamma\text{-}^{32}\text{P}$ ]ATP (330 Ci/mol) and T4 polynucleotide kinase (20 units) in 100  $\mu\text{l}$  of kinase buffer at 37°C for 1.5 hr. After phenol extraction and desalting by chromatography on a C-18 column (12), the conversion of the 21-mer to the 5'-monophosphate ester of the 21-mer (5'-P-21-mer) was followed by HPLC on a polyethyleneimine column.

**Synthesis of the Glycyl-OP Adduct of the 21-mer.** The first step in the synthesis of the glycyl-OP-DNA adduct was the conversion of the oligonucleotide to its imidazolide. This was accomplished by allowing the 5'-P-21-mer to stand in 100  $\mu\text{l}$  of 0.1 M imidazole-hydrochloride buffer that contains 3-dimethylaminopropyl carbodiimide (0.012 M) at room temperature for 2 hr as previously reported (11). After all the 5'-P-21-mer was converted to phosphorimidazolide as shown by HPLC, 10  $\mu\text{l}$  of 2,6-lutidine, followed by 6 mg of glycyl-OP, was added to give a solution of 0.22 M of glycyl-OP at pH 7.5. The resulting mixture was heated at 50°C for 1 hr. The adduct was purified by gel electrophoresis using a 20% polyacrylamide gel, 1:19 cross-linked in 8.3 M urea. The major band, which moved slightly more slowly than the 5'-P-21-mer, was isolated by elution from the gel with buffer overnight at 37°C. The eluant was desalted using a C-18 column (12). Proof of the presence of the OP-moiety was provided by the use of [ $^3\text{H}$ ]glycyl-OP and the demonstration of its presence in stoichiometric amounts in the product by its radioactivity.

**3'-End Labeling of the Wild Type.** The wild-type promoter of the *lac* control region obtained from the digestion of plasmid pBR322 with *Eco*RI was 3'-labeled with Klenow

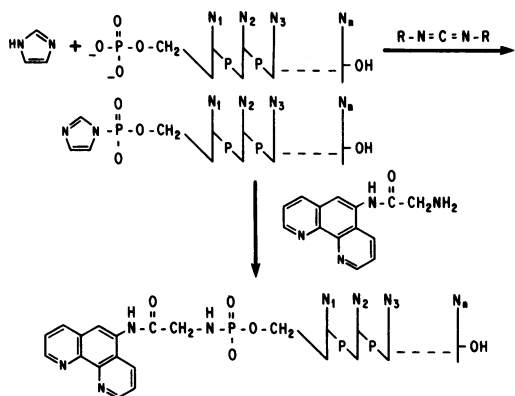


FIG. 1. Synthetic route for OP-oligonucleotide.

fragment of DNA polymerase I and [ $\alpha\text{-}^{32}\text{P}$ ]dATP. After a second restriction digestion with *Pvu* II, the wild-type fragment was isolated by gel electrophoresis on a 10% polyacrylamide 19:1 cross-linked gel.

**Cleavage Reactions. Single-stranded M13mp8.** A solution of phage M13mp8 single-stranded DNA (0.9 pmol) and OP-DNA (48 pmol) in 50 mM Tris-HCl, pH 7.4/50 mM NaCl was heated to 90°C for 5 min and then allowed to stand at room temperature for 30 min for the hybridization to occur. The cleavage reaction was then initiated by adding cupric sulfate (20 pmol), followed by 3-mercaptopropionic acid (50 nmol). The final volume was 20  $\mu\text{l}$ . After 75 min at room temperature, the reaction was quenched by adding 2,9-dimethyl-OP. The sample was then desalted by passing through a Sephadex G-50 spin column. The eluant collected was then dried, resuspended in a small amount of water, and used as a template in a primer extension reaction to visualize the nicks. The primer extension reaction was carried out in the same manner as that used in the dideoxy sequence analysis, except that dATP, dTTP, dCTP, and dGTP were used. The samples were then extracted with phenol/chloroform, 1:1 (vol/vol), dried, and redissolved in Maxam-Gilbert loading buffer (80% deionized formamide containing 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue) (13). They were then loaded onto a 10% polyacrylamide/8.3 M urea 19:1 cross-linked gel, electrophoresed at 45 W for 2 hr and 45 min. The gel was exposed to Kodak x-ray film at -70°C with an intensifying screen. Labeling of the primer was done by methods previously reported from our laboratory (4).

**Double-stranded wild-type *lac* operator.** A solution of 3'-[ $^{32}\text{P}$ ]labeled *lac* (0.18 pmol/100,000 cpm) and OP-DNA (200 pmol) in Tris-HCl, pH 7.4/50 mM NaCl was heated to 90°C for 4 min, then chilled on ice for 10 min. After the solution was warmed to room temperature, cupric sulfate (20 pmol) was added, followed by mercaptopropionic acid (50 nmol) to start the cleavage reaction. The final volume was 10  $\mu\text{l}$ . This reaction was carried out at room temperature for 75 min and then quenched by the addition of 2,9-dimethyl-OP. The sample was then lyophilized, resuspended in Maxam-Gilbert loading buffer, and analyzed by 10% polyacrylamide gel as earlier described.

## RESULTS

**Site of Phenanthroline Substitution.** The symmetric OP moiety offers the 2, 3, 4, and 5 positions as potential sites for attachment to a carrier. The 5 position was chosen as the site of linkage because the copper complex formed by OP derivatives altered at this locus exhibits efficient nuclease activity and generates the same pattern of cleavage as the parent ligand when a restriction fragment is degraded. In addition, the synthesis of glycyl-OP from the readily available 5-nitro derivative proceeds efficiently and allows the introduction of radioactive ligand without difficulty. In contrast, position 2, although readily derivatized (14), was excluded since the copper complexes of phenanthrolines substituted at this position neither catalyze the oxidation of thiols by molecular oxygen nor exhibit nuclease activity. Facile synthetic methods for the 4- and 3-substituted derivatives are not yet available.

**Choice of the *lac* Operon Test System.** The *lac* operon control regions provide a convenient experimental system to test the feasibility of the oligonucleotide-directed scission by OP-copper complex. The double-stranded restriction fragment containing the control region and the amino terminus of galactosidase had been previously used by us in studying the nuclease activity of unsubstituted OP-copper complex (6, 7). The sequence of this restriction fragment is presented in Fig. 2 for reference (15). The oligonucleotide derived from the template, or lower strand, which has been used as the carrier, is underlined. The single-stranded plus (+) strand of the

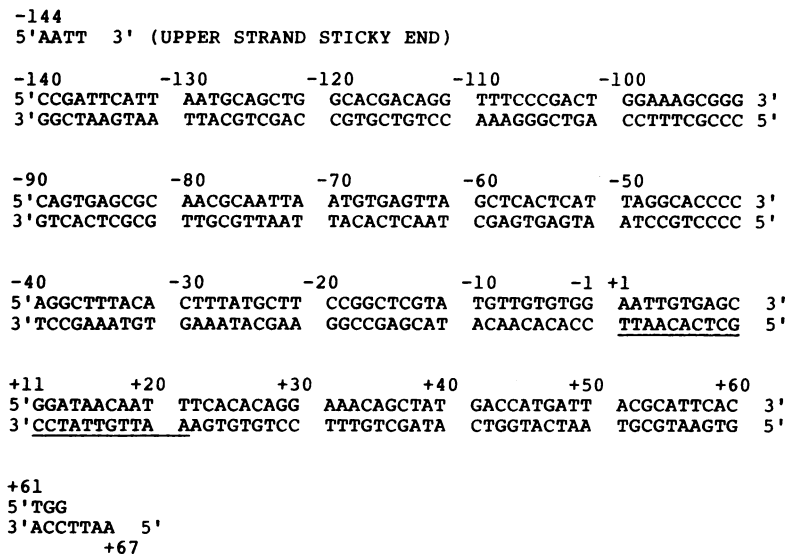


FIG. 2. Sequence of the *EcoRI* fragment containing the *lac* control region. Underlined sequence is the oligonucleotide carrier for 1,10-phenanthroline moiety. Numbering system relates to sequence of *lac*-coded mRNA. The number +1 corresponds to the first base of the *lac* mRNA.

cloning vector M13mp8 contains the upper, or noncoding region, of this restriction fragment of the *lac* operon (16).

This experimental system therefore provides two methods for assaying the scission reaction. In the first, the oligonucleotide derived from the template strand can be hybridized to the M13 DNA. To assay the sequence-dependent cutting, a primer extension assay was used in which the primer was 5'-labeled with <sup>32</sup>P by polynucleotide kinase (17). The overall design of the experiment is summarized in Fig. 3. An important strength of this test system was that it minimized problems attendant with the initial hybridization of the OP-DNA, because a competitive and longer complementary strand was not present. In the second, a terminally labeled restriction fragment can be used. Following heat denaturation and then reannealing in the presence of the oligonucleotide bearing the terminal phenanthroline moiety, cutting can be directly observed.

**Sequence Specific Cutting of Cloning Vector M13mp8 DNA.**

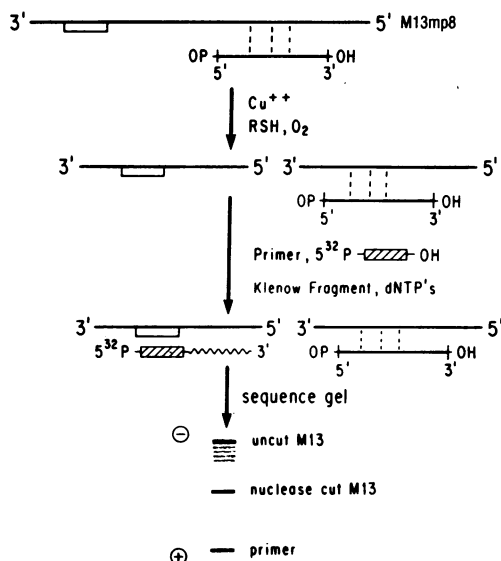
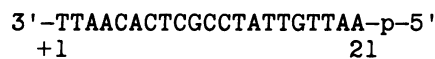


FIG. 3. Scheme for directed cleavage of vector M13mp8 using OP-oligonucleotide.

The OP moiety was attached to the 5'-phosphorylated end of the oligonucleotide



of the template strand using the chemistry outlined in *Materials and Methods* (Fig. 1).

The reaction conditions adopted involved the hybridization of the OP-DNA prior to the addition of the cupric ion and mercaptopropionic acid in order to initiate the reaction. The concentration of vector M13mp8 DNA used was 0.045 μM, and the oligonucleotide concentration was 2.4 μM. Cupric ion was added last, because of the possibility that it would be chelated by two phenanthrolines to form a complex structure that would not hybridize efficiently.

The data summarized in Fig. 4 clearly demonstrate that in the presence of OP-DNA, 1.0 μM cupric ion, and 2.5 mM mercaptopropionic acid, the primer extension assay reveals a family of bands that would be consistent with sequence-specific cutting. Comparable bands are not observed if mercaptopropionic acid is omitted nor if the same oligonucleotide without the OP moiety is incubated with the target DNA in the presence of thiol and cupric ion. However, in the absence of added copper, a faint pattern of bands was apparent (data not shown), suggestive of sequence-specific cutting. This likely reflects trace amounts of cupric ion that must be present in our buffer and can sustain the cleavage chemistry. With the exception of a very faint band at position +9, not readily visible in reproductions of the gels, all other bands are present in the control reactions.

**Nicking of Restriction Fragment.** The primer-extension assay, although sensitive and readily performed, however, does not necessarily assay strand scission. If the OP-DNA, with copper and thiol, oxidizes the bases, or causes another form of damage (e.g., depurination), the catalysis by the Klenow fragment used in the primer-extension assay might be blocked, and products of these lengths could accumulate without strand scission. To demonstrate unambiguously that the products that are generated in the primer-extension assay are the result of phosphodiester bond cleavage, we labeled the upper strand derived from the *EcoRI* restriction at the 3' terminus with the Klenow fragment and [ $\alpha$ -<sup>32</sup>P]dATP (6, 7). This fragment was then secondarily restricted with *Pvu II* as

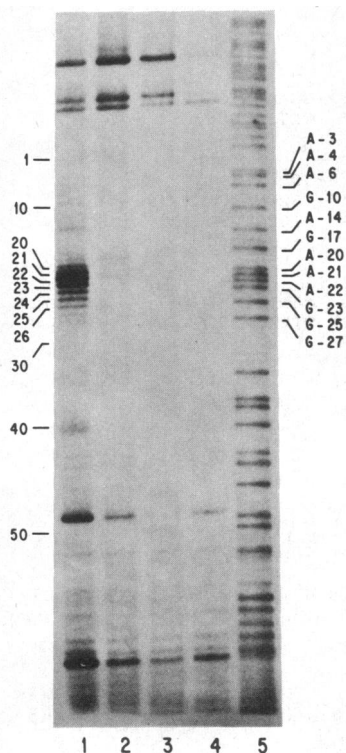
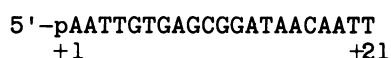


FIG. 4. Primer-extension analysis of reaction of OP-oligonucleotide with M13. The primer-extension products when (lane 1) 45 nM M13mp8 was hybridized with 2.4  $\mu$ M OP-oligonucleotide and then treated with 1  $\mu$ M  $\text{CuSO}_4$  and 2.5 mM mercaptopropionic acid before extension, (lane 2) vector M13mp8 was treated with OP-oligonucleotide as lane 1 before extension, but without 3-mercaptopropionic acid, (lane 3) unsubstituted oligonucleotide (21-mer) was used for hybridization, then treated similarly as shown in lane 1. Lane 4, control, no treatment, before primer extension. Lane 5, the G+A Maxam-Gilbert digests of products obtained from primer extension using M13 as a template.

previously described (6, 7) and used as substrate for cleavage by the lower strand OP-oligonucleotide.

Following hybridization and then addition of cupric ion and thiol, sequence-specific strand cleavage of the 3'-labeled upper strand is observed (Fig. 5). The pattern of scission products closely corresponds to the oligonucleotides produced in the primer-extension assay, suggesting that in the latter experiments the accumulated products represented strand scission. The predominant products are formed at positions 20, 21, 22, 23, and 24. Although no reaction is apparent when mercaptopropionic acid is omitted, a slow but measurable rate of cleavage is observed in the lane lacking exogenously added copper. As in the case of the experiment reported in Fig. 4, trace levels of copper are capable of sustaining the cleavage reaction to a minor extent. The band at position 29, which is present in all lanes, is an impurity in the labeled DNA.

Comparable experiments to those presented in Fig. 5 have been done with an OP-oligonucleotide prepared from the nontemplate strand and hybridized to the 5'-labeled template strand. In this case a pattern of four prominent bands was apparent. With the phenanthroline moiety attached to the 5'-terminus of the oligonucleotide sequence



cleavage sites were observed at  $-1$ ,  $+1$ ,  $+2$ , and  $+3$ .

**Hydrogen Peroxide Concentrations.** The reaction conditions used to achieve the sequence-specific cutting are

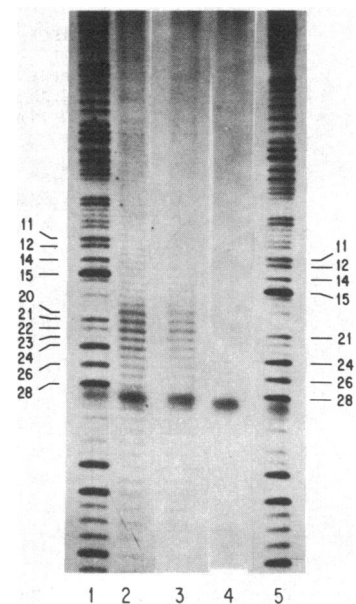


FIG. 5. Scission of 3'-labeled upper strand of *lac* operon by OP-oligonucleotide. Lane 2 shows the cleavage of 18 nM of *lac* operon fragment with 20  $\mu$ M OP-oligonucleotide, 2  $\mu$ M  $\text{Cu}^{2+}$ , and 5 mM mercaptopropionic acid. Lanes 3 and 4 illustrate the patterns obtained under the same conditions as lane 2, but in the absence of cupric ion and mercaptopropionic acid respectively. Lanes 1 and 5 are DNase I digests of the specifically labeled fragment.

dramatically different from those that have been used in the reaction of the unsubstituted coordination complex with the restriction fragment of the *lac* control region. The concentrations of phenanthroline and added copper ion are at least 1/10 to 1/50 less than used previously (2, 7). As a result, one significant factor controlling the reaction may be the concentration of hydrogen peroxide that can be generated. Exogenous hydrogen peroxide was not added in order to avoid the formation of the *N*-oxide, which does not support the cleavage chemistry.

Residual levels of copper ion in the buffer (1  $\mu$ M or less) are sufficient to sustain the oligonucleotide-directed scission reaction. Previous work has shown that these low levels of copper ion are also sufficient to support the cleavage of poly(dA-T) by OP in the presence of thiol (3, 18).

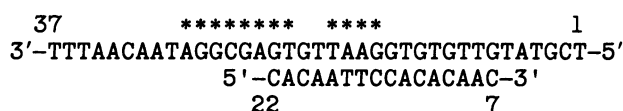
## DISCUSSION

Previous studies have demonstrated that the 2:1 complex of OP-cuprous ion, or  $(\text{OP})_2\text{Cu}^+$ , is the reactive nucleolytic form of the OP-copper chelates, while the 1:1 complex is inert (2). As a result, the feasibility of targeting of the nuclease activity of OP-copper ion was not apparent. From our earlier work, it was not clear whether both phenanthrolines were important for binding the copper complex to the minor groove of the DNA, or if they were essential for generating the reactive oxidative species. Examination of space-filling models indicates that the tetrahedral complex can bind to the minor groove if one ring lies in the groove with its plane roughly perpendicular to the helical axis, while the plane of the second phenanthroline, which extends outside the wall of the minor groove, is parallel to the helical axis. The success in targeting the cleavage tends to support the view that one of the phenanthrolines is essential to facilitate the binding of the coordination complex to the DNA and can be replaced by a carrier molecule. Two phenanthrolines do not appear essential for the generation of the reactive oxidative species. However, it is difficult to exclude the possibility that one phenanthroline is contributed by a nonhybridized OP-oligonucleotide.

The close correspondence of the results obtained in the reaction with vector M13mp8 and the cleavage of the double-stranded restriction fragment emphasizes the specificity of the cleavage chemistry. The primer-extension assay, although intended to measure nicks in single-stranded DNA, could also detect damaged bases that no longer are competent to serve as a template for the Klenow fragment (17). The similar pattern of products in the primer-extension assay and in the electrophoretic analysis of terminally labeled restriction fragments, which only measures strand scission, emphasizes that the bases are not targets of the  $(OP)_2Cu^+$  chemistry. The exclusive site of attack is the deoxyribose.

Subsequent to the discovery of the nuclease activity of  $(OP)_2Cu^+$  (1), methidiumpropyl-EDTA-iron (II) (MPE) was also demonstrated to nick DNA via a hydroxyl radical-like species (19). Continuing work on both nucleolytic activities has identified two salient differences in their modes of action. MPE has proven to be more sequence neutral in its action than  $(OP)_2Cu^+$  (20–22). This is consistent with the intercalative mode of binding of MPE (23) and the minor-groove mode of binding of  $(OP)_2Cu^+$  (4). Generally, small organic ligands that bind to the minor groove (e.g., netropsin) exhibit sequence-dependent binding (24, 25), whereas intercalating agents, such as ethidium bromide, do not (26). The second important difference between the two reactions is that MPE produces equivalent amounts of 3'-phosphoglycolate and 3'-phosphomonester termini, whereas  $(OP)_2Cu^+$  predominantly generates 3'-phosphomonesters, although 3'-phosphoglycolates are detectable (4). Moreover, the relative yield of the two termini produced by  $(OP)_2Cu^+$  depends on the DNA sequence. The product distribution suggests that the hydroxyl radical-like species generated by  $(OP)_2Cu^+$  is less freely diffusible than that produced by ferrous-EDTA, which appears equivalent to the hydroxyl radicals generated by <sup>60</sup>cobalt radiation (27, 28).

Both nucleolytic activities have now been attached to oligonucleotide carriers and have achieved sequence-specific cutting. The pattern of nicking with both targeted reagents has reflected the results obtained with the parent reagents. For example, Chu and Orgel (29) have hybridized a 16-mer bearing a ferrous-EDTA on the 5'-terminus to a 37-unit long oligonucleotide:



(\* implies cutting). Although the 16-mer spanned sequence positions 7–22 from the 5' end of the 37-mer (the 5'-end of the 16-mer was hydrogen-bonded to position 22 of the 37-mer), pronounced scission was observed at positions 28–21 and a lesser cluster of bands was observed from position 15 to 18. Thus, whereas the OP-oligonucleotide reagent caused cleavage over a 6-base sequence range approximately centered at the sequence position of the ligand, the ferrous-EDTA reagent nicked over a 14-base span in two clusters with the maximum of each cluster 4 or 5 sequence positions away from the 5'-oligonucleotide bearing the hydroxyl radical generator.

Dreyer and Dervan (30) have also linked a ferrous-EDTA to an oligonucleotide but have used a different approach in their synthesis. Rather than attaching it to the 5'-end of the carrier, they linked it to a modified thymidine residue that was then incorporated synthetically into position 10 of a 19-unit oligonucleotide. The oligonucleotide was then hybridized to a complementary sequence contained in a 167-base-pair restriction fragment, and the cleavage reaction was initiated by the addition of dithiothreitol. Scission was observed in an interval of 16 bases centered at the modified

thymidine with upstream and downstream families of bands each centered four sequence positions from the active center.

The more localized nicking sites observed with the OP-oligonucleotide are in agreement with observations that indicated that the "hydroxyl" radical produced by  $(OP)_2Cu^+$  and  $H_2O_2$  is not freely diffusible. Although it is unlikely that any modification of the reaction system will permit a unique site of attack on the DNA analogous to a restriction enzyme, the targeting of the nuclease activity of  $(OP)_2Cu^+$  by hybridization provides a method to tailor single-stranded sequences, as well as to analyze DNA and RNA for single-stranded domains.

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