Biochemistry

 Volume 29, Number 39 October 2, 1990

Perspectives in Biochemistry

Chemical Nucleases†

David S. Sigman*

Department of Biological Chemistry, School of Medicine, and Molecular Biology Institute, Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024

Received February 16, 1990; Revised Manuscript Received June 12, 1990

"Chemical nucleases" are defined in this perspective as redox-active coordination complexes that nick nucleic acids under physiological conditions by oxidative attack on the *ribose* or *deoxyribose* moiety. The 1,10-phenanthroline–copper complex was the first synthetic coordination complex demonstrated to have an efficient nucleolytic activity and is the primary focus of this brief review (Sigman et al., 1979; Sigman, 1986). Other examples of chemical nucleases are derivatives of ferrous–EDTA (Hertzberg & Dervan, 1982; Schultz et al., 1982; Tullius & Dombroski, 1986), various metalloporphyrins (Ward et al., 1986; Le Doan et al., 1986; Groves & Farrell, 1989), and octahedral complexes of 4,7-diphenyl-1,10-phenanthroline (Barton, 1986) (Figure 1). Bleomycin, an antibiotic derived from *Streptomyces verticillus*, nicks DNA via a metal ion and oxygen dependent reaction and has provided important precedents in the development of research on chemical nucleases (Stubbe & Kozarich, 1987; Kozarich et al., 1989).

The ribose-directed reactivity distinguishes chemical nucleases from other chemical modification reagents which are widely used in nucleic acid chemistry. For example, dimethyl sulfate, diethyl pyrocarbonate, osmium tetraoxide, and permanganate react preferentially with the various bases and do not cause strand scission without subsequent base treatment (e.g., piperidine as in Maxam–Gilbert sequencing). In contrast, chemical nucleases can cut DNA directly under physiological conditions at all sequence positions regardless of the nucleotide linked to the deoxyribose oxidized.

Applications of Chemical Nuclease

Research with chemical nucleases has developed in several directions. Their most widespread use has been as footprinting reagents. Although pancreatic DNase I and dimethyl sulfate probably are the preeminent tools for the study of protein–DNA interactions, chemical nucleases provide certain advantages such as better definition of protected sequences (Van Dyke & Dervan, 1983), the potential for inferring minor and major groove contacts (Kuwabara et al., 1986), greater sensitivity to protein-induced changes in DNA structure (Spassky & Sigman, 1985), and the capability of reacting within an acrylamide gel matrix (Kuwabara & Sigman, 1987). Another application is the characterization of sequence-dependent variation in DNA and RNA conformation (Yoon et al., 1988; Barton, 1986; Veal & Rill, 1988, 1989). To be most useful in this context, the reactivity of these reagents should be calibrated with reference structures solved by X-ray crystallography or NMR. Finally, since chemical nucleases are unique in their ability to cleave the phosphodiester backbone of nucleic acids under physiological conditions, linking them to ligands with specificity for some aspect of nucleic structure has permitted the synthesis of new scission reagents which may prove to be useful in the dissection of complex DNAs (Dervan, 1986). These targeted nucleases also provide a unique method for studying the binding of the carrier ligand itself.

Chemistry of Scission

Kinetics of Chemical Nucleases. Since chemical nucleases, including OP–Cu, generate highly reactive oxidative species, productive attack on a target DNA requires that the oxidative intermediate be generated near the surface of the DNA. Reactive species generated remote from the DNA are likely to be quenched by a buffer component (e.g., Tris). Chemical nucleases will therefore be most efficient and sequence specific if their reactions are funneled through a reversible complex as illustrated for OP–Cu in eq 1.

$$(OP)_2Cu^+ + DNA \overset{K_a}{\leftrightarrow} (OP)_2Cu^+DNA \xrightarrow{H_2O_2\ (k_2)} \text{nicked products} \quad (1)$$

In a sense, chemical nucleases are similar to photoaffinity labels used to explore the binding sites of enzymes and proteins (Chowdhry & Westheimer, 1979). The intermediates generated are reactive and nonselective; specificity is provided by the binding of the carrier. Unlike true enzymes, the binding

† This work was supported by the National Institutes of Health and the Office of Naval Research.

* Address correspondence to the author at the Molecular Biology Institute, UCLA, Los Angeles, CA 90024-1750.

0006-2960/90/0429-9097$02.50/0

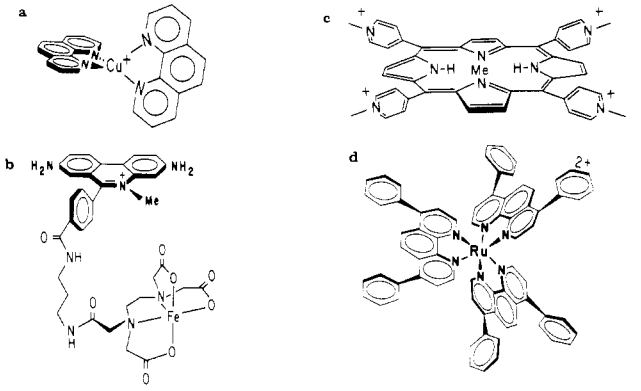


FIGURE 1: Structures of chemical nucleases: (a) 2:1 1,10-phenanthroline–cuprous complex; (b) methidiumpropyl-EDTA–iron; (c) metalloporphyrin; (d) Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium.

of a chemical nuclease does not activate the covalent chemistry which proceeds equally efficiently free in solution or on the surface of the nucleic acid. This parallel to photoaffinity labeling is underscored by the photoactivated nucleolytic activity of rhodium complexes (Barton, 1986; Uchida et al., 1989). The nucleolytic activities of 1,10-phenanthroline–copper, ferrous–EDTA, and metalloporphyrins are activated by reducing agents (e.g., thiol or ascorbic acid) in the presence of molecular oxygen or hydrogen peroxide.

Intermediate complexes are central to two types of experiments that have been carried out with the nuclease activity of OP–Cu. In the first, the tetrahedral 2:1 1,10-phenanthroline–cuprous complex is the ligand that binds reversibly to DNA. This hydrophobic cation has shown remarkable specificities in its binding and scission of RNA and DNA that will be summarized below. However, its reactivity is not predictable and must be empirically determined. In the second type of experiment, the nucleolytic activity is covalently linked to a carrier ligand with intrinsic affinity for the DNA which directs scission adjacent to its binding site (Chen & Sigman, 1986, 1987, 1988).

Reactions involving ferrous–EDTA similarly proceed through the kinetic scheme of eq 1 when tethered to ligands with affinity for DNA (e.g., methidium). The free negatively charged ferrous–EDTA shows subtle sequence variability in its reactivity, which probably reflects the accessibility and reactivity of hydroxyl radicals to the ribose and deoxyribose moieties (Tullius & Dombroski, 1986; Tullius, 1988; Latham & Cech, 1989; Celander & Cech, 1990) but which may also be due to weak binding of the coordination complex to the nucleic acid (Jezewska et al., 1989, 1990). The nucleolytic activity of ferrous–EDTA has provided a chemical criterion for solvent-inaccessible regions of a folded RNA; sequence positions protected from cutting are presumed to be located in the interior of the nucleic acid (Latham & Cech, 1989).

Reaction Mechanism of OP–Cu. The nuclease activity of OP–Cu was discovered during an investigation of the mechanism of 1,10-phenanthroline inhibition of *Escherichia coli* DNA polymerase I. It had been postulated that this enzyme and other polymerases were all inhibited by 1,10-phenanthroline because they contained a tightly bound zinc ion (Springgate et al., 1973). Its mechanistic role might be the activation of the 3′-hydroxyl group of the growing end for nucleophilic attack on the α–β pyrophosphate bond of the next nucleotide triphosphate (Sigman et al., 1972).

The 1,10-phenanthroline inhibition of *E. coli* DNA polymerase I proved difficult to characterize accurately. Multiple variables—including thiol concentration and structure, the time

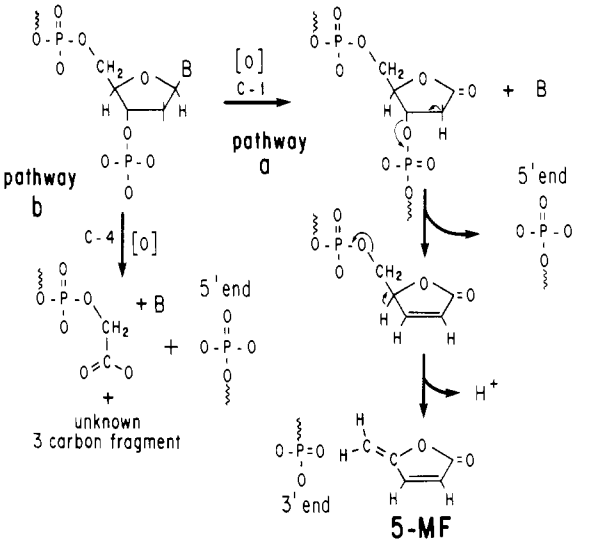


FIGURE 2: Chemical mechanism for the scission of DNA by the coreactants 1,10-phenanthroline–cuprous complex and hydrogen peroxide.

of incubation of 1,10-phenanthroline with the primer-template poly(dA-T), and cupric ion—influenced the extent of inhibition (D'Aurora et al., 1977, 1978). The discovery of the nucleolytic activity of 1,10-phenanthroline–copper in the presence of thiol and endogenously generated hydrogen peroxide suggested that this reaction might be responsible for these unexpected findings and prompted a detailed analysis of the products of the reaction to identify the actual inhibitors of Pol I.

The following stable reaction products were identified: 5′-phosphorylated termini, 3′-phosphorylated termini, free bases, and 5-methylenefuranone, as well as minor amounts of 3′-phosphoglycolate termini (Pope et al., 1982; Kuwabara et al., 1986; Goyne & Sigman, 1987). Since exonuclease III and alkaline phosphatase relieved the inhibition of *E. coli* Pol I by OP–Cu (Pope et al., 1982), 3′-phosphorylated termini are responsible for the observed inhibition of *E. coli* DNA polymerase I (and presumably all other polymerases) by 1,10-phenanthroline. Earlier work had shown that the 3′-phosphorylated ends produced by micrococcal nuclease digestion were inhibitors of Pol I (Englund et al., 1969).

A possible reaction scheme that would accommodate the formation of these products is summarized in Figure 2. Pathway a, which accounts for 80–90% of the scission events, involves initial oxidative attack at the C-1 hydrogen of the deoxyribose by the DNA-bound coordination complex. The predicted metastable intermediate at the 3′-terminus has been trapped with DNA labeled at the 5′-end (Kuwabara et al., 1986). All stable products incorporated in this scheme have been isolated, including 5-methylenefuranone (Goyne & Sigman, 1987). Consistent with the early elimination of the 5′-phosphate terminus in the scission reaction, no transient intermediate is observed if 3′-labeled DNA is used as substrate. 5-Methylenefuranone has been identified as a scission product of two other nucleolytic activities: the potassium monopersulfate dependent scission by manganese porphyrin (Bernadou et al., 1989) and the alkali-sensitive lesion produced by UV irradiation (Urata et al., 1989).

The cleavage chemistry of OP–Cu is distinct from that of bleomycin where the major site of oxidative attack is the C-4 hydrogen and the primary stable product at the 3′-end is the phosphoglycolate (Stubbe & Kozarich, 1987; Kozarich et al., 1989). This is a minor product in the OP–Cu reaction (pathway b) and a significant product (ca 50%) for the MPE-dependent scission (Hertzberg & Dervan, 1984). Base

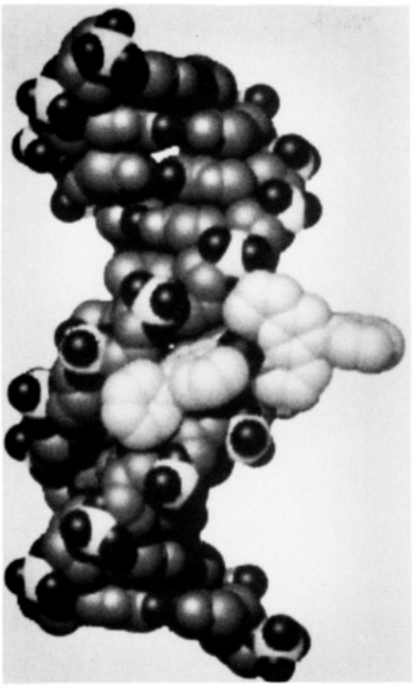


FIGURE 3: Possible model for the noncovalent intermediate formed between the 2:1 5-phenyl-1,10-phenanthroline–cuprous complex and B-DNA.

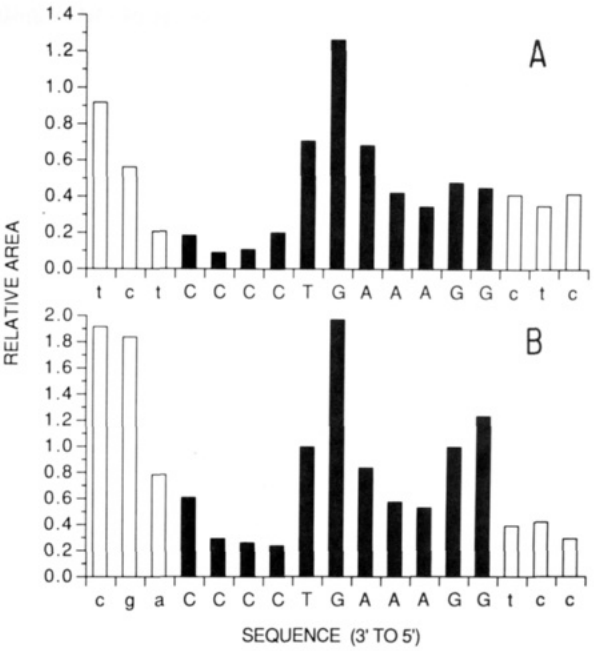


FIGURE 4: OP–Cu scission pattern of the recognition sequence of the regulatory protein NFkB in the κB light chain (A) and in the human immunodeficiency light chain LTR (B).

propenal is produced by bleomycin in amounts equivalent to that of the 3′-glycolate but is not detectable in MPE or OP–Cu digests. The fragments of the deoxyribose or ribose produced by ferrous–EDTA oxidation have not yet been identified. However, it has been reported that the relative yield of phosphomonesters and phosphoglycolate at the 3′-terminal is a function of hydrogen peroxide (Hertzberg & Dervan, 1984). Although neocarzinostatin also attacks the C-1 hydrogen of the deoxyribose, its primary site of attack is the C-5 hydrogen (Kappen et al., 1990).

Stereochemical Implications of the Chemical Mechanism of OP–Cu Scission. The identification of the C-1 hydrogen as the site of oxidation by OP–Cu requires that the oxidative reaction be initiated within the minor groove. Additional evidence for minor groove binding of the tetrahedral 2:1 1,10-phenanthroline–cuprous complex is provided by studying its reactivity with DNA–ligand complexes of known structure (Kuwabara et al., 1986). For example, netropsin, which binds in the minor groove of A-T-rich regions, blocks scission at the expected residues. In contrast, proteins that bind exclusively in the major groove, such as *Eco*RI, have only minimal effect on the OP–Cu scission pattern. A possible model for the binding of the tetrahedral complex within the minor groove is presented in Figure 3 with 5-phenyl-1,10-phenanthroline as the ligand (Thederahn et al., 1990).

The preferential reactivity of OP–Cu for B-DNA relative to A-DNA (i.e., 4-fold) must reflect the relative stability of the essential noncovalent complex (eq 1). The shallow minor groove of the A-structure is more accessible to solvent and the tetrahedral $(OP)_2Cu^+$, but its shape must make it a poorer host than the narrower and deeper minor groove of the B-structure for the coordination complex. Moreover, Z-DNA, whose minor groove bears little resemblance to that of B-DNA, is not measurably cleaved under conditions where B-DNA is completely digested (Pope & Sigman, 1984). Therefore, the binding specificity of the tetrahedral cuprous complex stands in marked contrast to that of octahedral 4,7-diphenyl-1,10-phenanthroline complexes where the λ isomer binds preferentially to Z-DNA (Barton et al., 1984; Barton, 1986; Tamilarasan et al., 1990).

Sequence-Dependent Variability of B-DNA Scission by OP–Cu

The pioneering X-ray crystallographic studies by Dickerson and Drew (1981a,b) on the self-complementing dodecamer 5′-CGCGATTCGCG-3′ provided the first unambiguous evidence for sequence-dependent conformational variability in B-DNA. This structural heterogeneity is reflected in OP–Cu scission patterns. Although the reaction is not specific for the nucleotide at the site of scission, its rate does depend on local sequence (Law et al., 1987; Veal & Rill, 1988, 1989; Yoon et al., 1988, 1990). Analysis of over 1600 scission events using statistical techniques has led to the conclusion that the most important influence on the intensity of cutting OP–Cu at any sequence position is the neighboring 5′-nucleotide (Yoon et al., 1990). One interesting consequence of this dependence is that two identical recognition sequences will have different scission patterns if their 5′-flanking sequences are dissimilar. This is the case for the DNA binding site of the lymphocyte-specific regulatory protein NFkB in the immunoglobulin κ light chain enhancer region and in the human immunodeficiency virus LTR (Figure 4). Since these sequences exhibit different affinities for NFkB, the structural determinants that affect the binding of the coordination complex may also influence the binding of the protein. Flanking sequences may therefore serve as a "fine-tuning" mechanism for regulating gene expression.

In several bacterial transcription units, mutational changes leading to altered promoter strength also cause changes in the OP–Cu scission pattern. As in the case of NFkB binding, this correlation suggests that at least one consequence of mutational changes is to alter the preexisting DNA conformation and affect the binding affinity of a protein such as RNA polymerase (Sigman et al., 1985; Spassky & Sigman, 1985; Spassky et al., 1988).

One novel application of chemical nucleases is in the analysis of branched DNA molecules which serve as model recombinational intermediates (Guo et al., 1990). MPE, ferrous–

EDTA, and OP–Cu have been used to determine the symmetry of these four-stranded structures which possess structural discontinuities at the crossover points or junctions. It is of interest that the tetrahedral 2:1 1,10-phenanthroline–cuprous complex, consistent with its previously demonstrated multifaceted binding potential, exhibits the greatest reactivity at the anomalous junction. The chemical nucleases also provide a sensitive probe for monitoring the binding of other DNA ligands such as propidium iodide and actinomycin D at this region (Guo et al., 1990).

Chemical Nucleases as Footprinting Reagents

A variety of reagents have been used to define the sequence-specific contacts of a protein on DNA by footprinting analysis. As noted above, the most widely used reagents are dimethyl sulfate and DNase I. Chemical nucleases are increasingly employed in footprinting applications (Tullius et al., 1987; Tullius, 1989). The advantage of MPE, ferrous–EDTA, uranyl acetate (Nielsen et al., 1988a; Jeppesen & Nielsen, 1989), and bis(phenanthrenequinone diimine)(bipyridyl)rhodium(III) {$Rh(phi)_2(bpy)^{3+}$} (Uchida et al., 1989) is that their scission patterns are very even; therefore, they give readily identifiable zones of protection. Protection patterns obtained with OP–Cu and DNase I are usually sufficiently well-defined to identify protected regions even though both nucleolytic agents digest free DNA in a sequence-dependent manner.

Footprinting reagents usually probe different aspects of the structure of a protein–DNA complex. For example, the primary site of reaction of dimethyl sulfate with B-DNA is the N-7 of guanine which is positioned in the major groove. Thus, those guanines in intimate contact with the protein will be protected from alkylation. However, if a protein or ligand primarily makes contact with a DNA sequence in the minor groove, dimethyl sulfate may not be a useful reagent to visualize this contact. On the other hand, OP–Cu will reveal minor groove interactions. OP–Cu will detect binding in the major groove when its approach to its minor groove binding site is sterically blocked or if the interaction of the ligand in the major groove substantially alters the minor groove structure. As the scission chemistry of the different chemical nucleases is better understood, it will be possible to pinpoint more precisely within a recognition sequence the stereochemistry of a ligand–DNA interaction. In a valuable review, Tullius has compared footprinting patterns obtained with ferrous–EDTA to those obtained with OP–Cu and MPE (Tullius et al., 1987).

There are two novel features of OP–Cu as a footprinting reagent. The first takes advantage of its small size and the ready diffusibility of the reaction components: 1,10-phenanthroline, copper ion, 3-mercaptopropionic acid, and hydrogen peroxide (molecular oxygen). These features permit the coupling of OP–Cu footprinting with the widely used gel retardation assay to study DNA–protein interactions (Kuwabara & Sigman, 1987). In gel retardation assays, the binding of a protein to a labeled restriction fragment alters the migration of the DNA in a nondenaturing acrylamide gel (Garner & Revzin, 1986; Crothers, 1987). Even in a complex mixture of proteins, it is possible to separate a protein that binds to a labeled DNA. To identify the sequence-specific contacts between the protein and DNA, the footprinting reaction with OP–Cu can be carried out *directly* in the gel (Figure 5). Deoxyoligonucleotide products are then eluted from the gel and analyzed on sequencing gels.

In addition to convenience, merging these two methodologies has additional advantages. First, their combination ensures that the footprinting reaction is carried out on a discrete

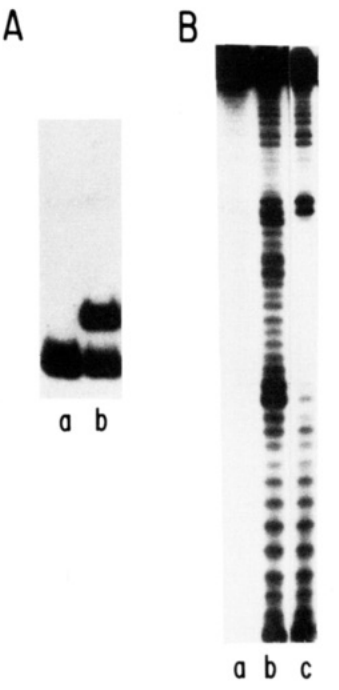


FIGURE 5: Interaction of cro protein with OR-3. (Panel A) Gel retardation assay: (a) no protein added; (b) *E. coli* extract containing cro in the pUC119 plasmid. (Panel B) Scission of retarded band within the gel: (a) control DNA; (b) free DNA cleaved by OP–Cu; (c) cro–DNA complex cleaved by OP–Cu.

electrophoretic species. Second, unstable complexes are more readily detected because the background is reduced by the separation of unbound DNA from the protein–DNA complex. In addition, the gel matrix stabilizes the protein–DNA complex by reducing the dissociation rate of the protein from its binding site on DNA.

It is of interest to compare this experimental procedure with the "missing nucleotide experiment", which provides a novel method to study protein–DNA interactions (Hayes & Tullius, 1989). In this method, a DNA recognition sequence is nicked with ferrous–EDTA. The randomly nicked DNA is then incubated with binding protein and then analyzed by nondenaturing gel electrophoresis. Following electrophoretic separation, the DNA of the retarded complex is analyzed on a sequencing gel. No scission will be apparent at the sequence positions of the nucleotides which are essential for the binding affinity of the protein. Usually, footprinting with nucleolytic agents such as DNase I, OP–Cu, and MPE provides complete protection with a recognition sequence whether or not binding to particular nucleotides provides substantial stabilization to the complex. The missing nucleotide experiment therefore provides important new insight in studying protein–DNA complexes because it permits an ordering of the strength of interaction of the binding protein with the different nucleotides within the recognition sequence. A possible disadvantage of the procedure however is the conclusions are only strictly applicable to the binding of the protein to nicked DNA (Hayes & Tullius, 1989).

A second novel feature of OP–Cu as a footprinting reagent is its ability to detect protein-induced changes in DNA (Spassky & Sigman, 1985). This unanticipated feature of the reactivity of OP–Cu probably arises from the hydrophobic cation's affinity not only for DNA but also for protein surfaces. For example, single-stranded DNA formed at the active site of *E. coli* RNA polymerase of the transcriptionally competent open complex is efficiently cut on the template strand by OP–Cu and even more effectively by the copper complex of

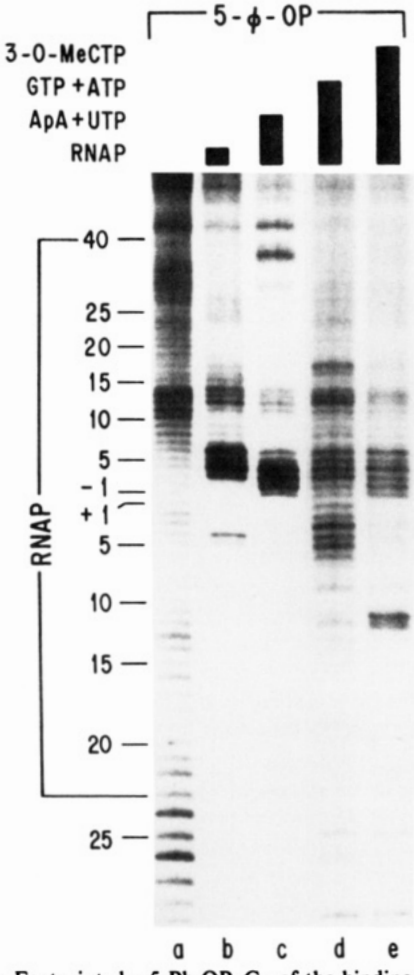


FIGURE 6: Footprints by 5-Ph-OP–Cu of the binding of RNA polymerase to 5′-lac UV5 as a function of added nucleotides. Components of each reaction mixture are reflected by the size of the bar on top of each lane: (a) DNA scission pattern in absence of any addition; (b) in the presence of RNA polymerase; (c) ApA and UTP; (d) nucleotides ApA, UTP, GTP, and ATP; (e) chain-terminating 3-*O*-methyl-CTP. Brackets to left are the limits of the footprint of RNA polymerase observed with DNase I. Note the doublet in lanes b and e corresponding to double-stranded DNA at the leading edge of the transcription bubble.

5-phenyl-1,10-phenanthroline (5-Ph-OP-Cu) (Thederahn et al., 1990). Both of these reagents can be used to detect the series of intermediates formed during the initiation of transcription with the lac UV-5 promoter; however, 5-Ph-OP-Cu is more sensitive (Spassky & Sigman, 1985; Spassky, 1986; Spassky et al., 1988). In fact, 5-Ph-OP-Cu can detect double-stranded DNA at the leading edge of a transcription bubble both in the open and in the elongation complexes (Figure 6, lanes b and e) (Thederahn et al., 1990). The inference that DNA downstream of the positions of nucleotide triphosphate incorporation is double stranded relies on the observation of nicking on both strands. Scission sites on the template strand upstream of the site of nucleotide triphosphate incorporation are not associated with any cutting on the nontemplate strand. Methylation of cytosine at N-4 with dimethyl sulfate has demonstrated that this region is single stranded (Kirkegaard et al., 1983). Detection of these strained intermediates appears to be a unique feature of the phenanthroline–copper complexes. Neither MPE nor DNase I can detect polymerase-induced changes at the start of transcription. The combination of gel retardation/OP–Cu footprinting methodology has shown that Mg^{2+} is essential for the enzyme-induced melting of the double-stranded DNA but not for the stability of the polymerase–DNA complex (Kuwabara & Sigman, 1987).

RNase Activity of OP–Cu

Although RNA is 750 times more stable than DNA to acid-catalyzed base loss, RNA and DNA strands are equivalently susceptible to digestion by OP–Cu if they are placed in the same orientation with respect to the binding site of the coordination complex (Pope & Sigman, 1984; Chen & Sigman, 1988). To establish that the ribose and deoxyribose moieties reacted similarly, a heteroduplex was prepared from poly(dT) and poly(rA) (Pope & Sigman, 1984). In this A-helix, both the RNA and DNA strands are adjacent to the weak binding sites of the coordination complex in the minor groove, and both were cleaved at the same rates.

In addition to its unexpected reactivity toward single-stranded DNA bound at the active site of RNA polymerase, OP–Cu exhibited another unanticipated reaction preference when assayed with t-RNA^{Phe} and a fragment of lac RNA (Murakawa et al., 1989). Rather than preferentially cutting the double-helical stem of stem–loop structures as has been observed with DNA (Drew, 1984), the reagent demonstrated enhanced reactivity for the loop regions of RNA. The structure of the noncovalent intermediate that could account for this type of scission has not yet been determined. Octahedral complexes of ruthenium and rhodium nick t-RNA^{Phe} nonrandomly, but the structural determinants that influence their reactivity have not yet been identified (Chow & Barton, 1990).

Since most ribonucleases are either nucleotide or secondary structure specific (or both), chemical nucleases may prove valuable as a footprinting reagent in studying RNA–protein interactions. Useful results have already been obtained with uranyl acetate (Gaynor et al., 1989) and ferrous–EDTA (Wang & Padgett, 1989), neither of which is sensitive to secondary structure in their scission reaction. OP–Cu has been used to identify the ribosome binding site on lac mRNA (Murakawa & Nierlich, 1989).

Targeted Nuclease Activities

The tetrahedral, hydrophobic cuprous complex of 1,10-phenanthroline exhibits diverse, and not readily predictable, binding patterns with both RNA and DNA. As noted above, the first example of a targeted nucleolytic activity was provided by Schultz and Dervan (1982), who used a distamycin derivative to direct the scission by ferrous–EDTA. Subsequent studies have used deoxyoligonucleotides derivatized with ferrous–EDTA hybridized in Watson–Crick duplexes (Chu & Orgel, 1985; Dreyer & Dervan, 1985) or in triplexes (Moser & Dervan, 1987; Strobel et al., 1988) to achieve site-specific scission.

Targeting Scission of OP–Cu with Oligonucleotides. Initial experiments in targeting the nuclease activity of OP–Cu utilized deoxyoligonucleotides as carrier ligands. Two methods for derivatizing the 5-position of a deoxyoligonucleotide involve the formation of a phosphoramidate or the alkylation of thiophosphoryl group attached to the 5′-end with polynucleotide kinase and ATP-γ-S. A deoxyoligonucleotide corresponding to positions +1 to +21 of the template strand has been used to cleave a complementary DNA and RNA strand (Figure 7). As would be anticipated from the results obtained in the scission of poly(rA)·poly(dT), the target DNA and RNA were cleaved with equivalent rates and specificity (Chen & Sigman, 1988). Although two ligands per one copper ion are essential for productive cleavage with unsubstituted 1,10-phenanthroline, a single phenanthroline is sufficient for scission if linked to a carrier with high affinity for the target DNA.

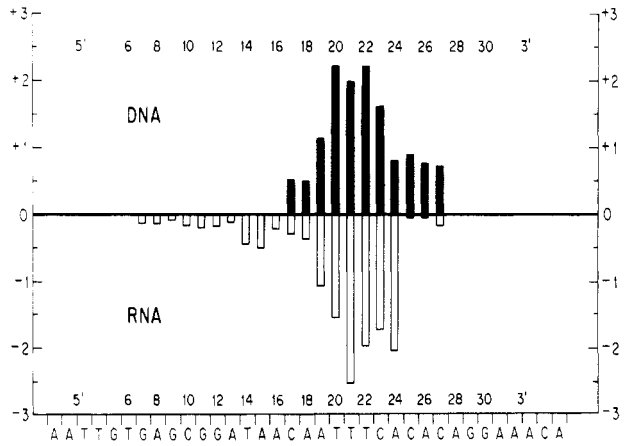


FIGURE 7: Scission patterns of DNA and RNA of identical sequence cut by the same OP-linked deoxyoligonucleotide.

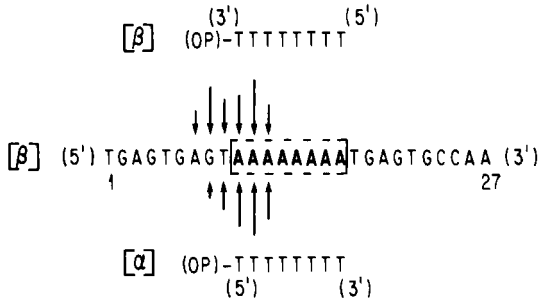


FIGURE 8: Comparison of the pattern of scission observed with α- and β-deoxythymidylates.

Helene and colleagues have made creative use of the scission chemistry of OP–Cu in defining novel forms of hybridization. α-Deoxyoligonucleotides provide an interesting approach for the chemical synthesis of antisense reagents because they are hydrolytically stable yet are still capable of sequence-specific recognition (Sun et al., 1988). To determine the directionality of the double helix formed with a target sequence, α-octadeoxythymidylate was derivatized at the 5′-end with OP–Cu and its scission of a complementary sequence examined. The pattern of cleavage observed indicates a parallel orientation relative to the purine strand of the phosphodiester backbone in the double helix formed (Figure 8).

Triple-helix formation is an active area of investigation because it provides an opportunity to synthesize sequence-specific scission reagents that might be appropriate in the analysis of complex DNAs. In addition, the inhibition of expression of a target gene may be possible by blocking the binding of proteins essential for transcription (Maher et al., 1989; Francois et al., 1989b). The most stable triple helices appear to be formed between two pyrimidine-rich strands and one purine-rich strand, in which a double helix using Watson–Crick base pairing is the host and the second pyrimidine-rich strand is the guest binding to the major groove through Hoogsteen pairing in a parallel orientation with respect to the purine-rich strand (Arnott & Selsing, 1974). Elegant NMR experiments have provided strong support for this structural model (Rajagopal & Feigon, 1989). Scission of a target duplex via triple-helix formation with a pyrimidine-rich strand derivatized with ferrous–EDTA or OP–Cu has also supported the proposed parallel orientation of the third strand with respect to the purine-rich strand (Moser & Dervan, 1987; Griffin & Dervan, 1989; Francois et al., 1989). The 3′-stagger in the scission pattern for the OP–Cu-targeted scission via triple-helix formation (Figure 9) is diagnostic of minor groove cutting. Although the third strand binds in the major groove, by linking OP–Cu to the pyrimidine strand via a linker arm,

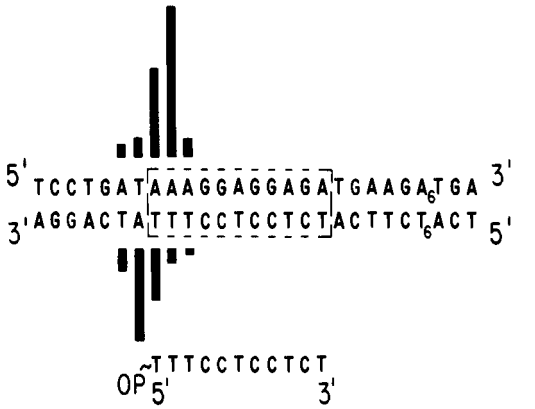


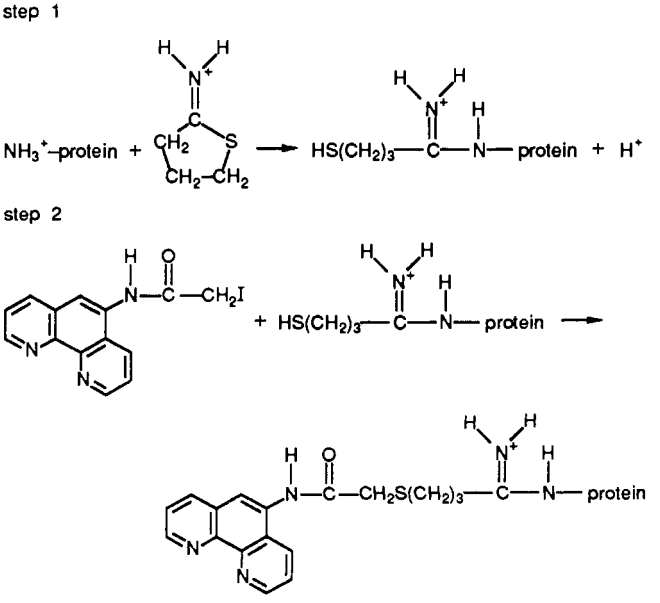
FIGURE 9: Scission pattern of B-DNA via triple-helix formation by OP–Cu. The bars indicated the intensity of cutting by OP–Cu targeted by the indicated pyrimidine strand.

it is able to enter the minor groove and achieve scission (Francois et al., 1989).

The formation of a triple helix can also be inferred by OP–Cu footprinting even though the third strand binds in the major groove. The inhibition of the scission by the minor groove specific OP–Cu can be attributed to the transformation of the host double-stranded helix to an A-like helix, which does not provide stable binding sites for the tetrahedral 2:1 1,10-phenanthroline–cuprous complex (Francois et al., 1988).

Targeting the Nuclease Activity with DNA Binding Proteins. Using DNA binding proteins to target the scission of OP–Cu is of interest from two perspectives. First, the specificity of sequence recognition typically ranges from 12 to 18 base pairs. Since double-helical DNA is recognized by proteins, hybridization strategies (essential when a deoxyoligonucleotide is the carrier) are obviated. The second motivation for interest in protein targeting is that the scission patterns obtained provide a test for models of protein–DNA interaction proposed by crystallographic or model-building studies.

The first example of the conversion of a DNA binding protein into a site-specific nuclease involved the *E. coli* trp repressor (Chen & Sigman, 1987), a protein possessing a helix–turn–helix format interacting in the major groove of the target sequence (Schevitz et al., 1985; Zhang et al., 1987; Otwinowski et al., 1988). In these experiments, the protein was derivatized with 1,10-phenanthroline according to the two-step modification procedure involving iminothiolane:



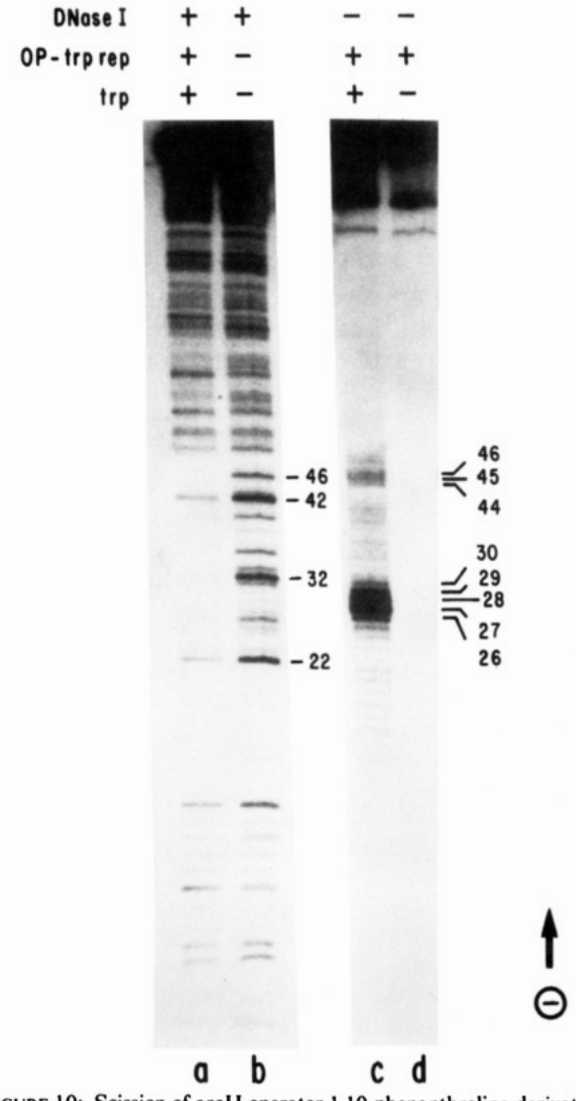


FIGURE 10: Scission of aroH operator 1,10-phenanthroline-derivatized *E. coli* trp repressor. The modified protein retains binding as measured by DNase I footprinting; the corepressor L-tryptophan is essential for sequence-specific scission. In lanes c and d, 3-mercaptopropionic acid and cupric ion have been added to activate the scission reaction.

Four 1,10-phenanthrolines were incorporated per subunit. Although there are four lysyl residue per subunit, one site of modification could be the amino terminus. The chemical modification did not measurably alter the protein's binding affinity as determined by DNase I footprinting. Scission is observed on both strands of *E. coli* operators regulated by the trp repressor upon addition of cupric ion and 3-mercaptopropionic acid (Figure 10). Cutting is only observed in the presence of L-tryptophan, which serves as a corepressor and is essential for the sequence-specific binding of the trp repressor (Arvidson et al., 1986; Marmorstein et al., 1987).

The OP-derivatized trp repressor can complement other approaches in developing a detailed understanding of the protein's interaction with its operator. For example, footprinting experiments with DNase I and dimethyl sulfate indicate that the trp repressor binds to three successive major grooves of the aroH operator and four successive major grooves of the trp EDCBA operator (Kumamoto et al., 1987). The OP-derivatized trp repressor supports this conclusion because distinct scission patterns of these targets have been obtained. Two other aspects are under investigation. The most recent crystallographic studies of the complex of the trp repressor with its recognition sequence have suggested unanticipated interactions which could be attributable to the conditions essential for crystal formation. Examining the solvent dependence of the scission reaction can address this question. In addition, since nonspecific binding is intrinsic to all DNA binding proteins, the modified protein may provide a novel approach for determining the relative affinities of these interactions for the trp repressor.

The minor groove directed chemistry of OP–Cu may be a crucial feature in the success of protein targeting. Any chemistry that depended on major groove attack would be blocked by the carrier protein. With the nuclease activity of OP–Cu, protein–DNA interactions are preserved without hindering the nucleolytic reaction.

The elegant studies of Ebright and colleagues provide an interesting example of the chemical modification of a DNA binding protein by 5-(iodoacetyl)-1,10-phenanthroline to yield a discrete site-specific cleavage reagent (Ebright et al., 1990). In these experiments, cysteine-178 of the *E. coli* catabolite activating protein (CAP), which corresponds to amino acid 10 of the helix–turn–helix domain, is the site of alkylation. Although the affinity of the modified protein is 400-fold less than the wild protein (1×10^8 M^{-1} vs 4×10^{10} M^{-1}), it reacts efficiently with its 22 base pair recognition sequence on both strands at precisely the sites that are anticipated from examination of the molecular model of the protein–DNA complex. It can also accomplish the double-strand scission of a 7164-bp fragment containing this recognition sequence and yield discrete products. Like the trp repressor, a small-molecule effector (e.g., cyclic AMP) is essential for the binding of the parent protein and for the scission reaction. The conversion of helix–turn–helix DNA binding proteins into site-specific nucleases by comparable modifications could provide a valuable array of nucleolytic agents useful for chromosomal mapping.

A different approach to the protein targeting of the nuclease activity of OP–Cu has involved the bacteriophage λ cro repressor. Pioneering crystallographic studies with cro by Matthews and co-workers have suggested that a helix–turn–helix format is responsible for sequence recognition and that the C-terminus of this protein can contribute to the stability of the interaction by binding within the minor groove (Anderson et al., 1981; Ohlendorf et al., 1982; Brennan & Matthews, 1989). To test this suggestion, the C-terminal alanine residue of cro has been converted to a cysteine by site-directed mutagenesis, forming an altered repressor, cro A66C, which is then alkylated by 5-(iodoacetyl)-1,10-phenanthroline. The 1,10-phenanthroline-modified cro has high binding affinity and is an efficient nuclease because the 1,10-phenanthroline is lodged in the minor groove, the site of oxidative attack (Bruice et al., 1990). Activation of the nuclease activity by the addition of cupric ion and thiol leads to scission on either strand within the recognition sequence. These results provide strong support for the proposed model of cro binding.

Conformationally stable, chemically synthesized peptides derived from the DNA binding domains of hin recombinase and the yeast transcriptional activator GCN4 have been used to target the oxidative scission of their respective recognition sequences. With the hin recombinase, ferrous–EDTA and the copper complex of the tripeptide Gly-Gly-His were the two oxidative nucleolytic activities used (Sluka et al., 1987; Mack et al., 1988). The tripeptide ligand system is of special interest because it would allow the biosynthetic incorporation of a nucleolytic activity. The work with the 56 amino acid long peptide derived from GCN4 provided novel chemical support for the model of DNA binding for the transcriptional activators

of the leucine zipper motif (Oakley & Dervan, 1990).

SUMMARY/PROSPECTIVE

Chemical nucleases are to nucleic acid chemistry what affinity labels and active site directed inhibitors are to proteins and enzymes. They provide a chemical approach for analyzing conformation and specificity of a biomolecule in solution, but their value is enhanced when linked to other experimental approaches. At present, chemical nucleases provide a useful variety of footprinting reagents. Further work is required before they can serve as reliable tools for analyzing structural variation of nucleic acid structures. The targeting of their scission with proteins and deoxyoligonucleotides has provided interesting insights into the binding of the carrier ligands. As yet, targeted nucleolytic agents have not reached the efficiency and specificity required for a reliable method of cleaving chromosomal DNA, but that time may be rapidly approaching.

ACKNOWLEDGMENTS

I appreciate the contributions of my co-workers cited in publications from our laboratory.

Registry No. $(OP)_2Cu^+$, 17378-82-4; nuclease, 9026-81-7.

REFERENCES

Anderson, W. F., Ohlendorf, D. H., Takeda, Y., & Matthews, B. W. (1981) *Nature 290*, 754–758.

Arnott, S., & Selsing, E. (1974) *J. Mol. Biol. 88*, 509–521.

Arvidson, D. N., Bruce, C., & Gunsalus, R. P. (1986) *J. Biol. Chem. 261*, 238–243.

Barton, J. K. (1986) *Science 233*, 727–734.

Barton, J. K., Danishefsky, A. T., & Goldberg, J. M. (1984) *J. Am. Chem. Soc. 106*, 2172–2176.

Bernadou, J., Lauretta, B., Pratviel, G., & Meunier, B. (1989) *C. R. Acad. Sci., Ser. 3 309*, 409–414.

Brennan, R. G., & Matthews, B. W. (1989) *Trends Biochem. Sci. 13*, 286–290.

Bruice, T. W., Wise, J., & Sigman, D. S. (1990) *Biochemistry 29*, 2116–2121.

Celander, D. W., & Cech, T. R. (1990) *Biochemistry 29*, 1355–1361.

Chen, C.-H. B., & Sigman, D. S. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7147–7151.

Chen, C.-H. B., & Sigman, D. S. (1987) *Science 237*, 1197–1201.

Chen, C.-H. B., & Sigman, D. S. (1988) *J. Am. Chem. Soc. 110*, 6570–6572.

Chow, C. S., & Barton, J. K. (1990) *J. Am. Chem. Soc. 112*, 2839–2841.

Chowdhry, V., & Westheimer, F. H. (1979) *Annu. Rev. Biochem. 48*, 293–325.

Chu, B. C. F., & Orgel, L. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 963–967.

Crothers, D. M. (1987) *Nature 325*, 464–465.

D'Aurora, V., Stern, A. M., & Sigman, D. S. (1977) *Biochem. Biophys. Res. Commun. 78*, 170–176.

D'Aurora, V., Stern, A. M., & Sigman, D. S. (1978) *Biochem. Biophys. Res. Commun. 80*, 1025–1032.

Dervan, P. B. (1986) *Science 232*, 464–471.

Drew, H. R. (1984) *J. Mol. Biol. 176*, 535–557.

Drew, H. R., & Dickerson, R. E. (1981a) *J. Mol. Biol. 152*, 723–736.

Drew, H. R., & Dickerson, R. E. (1981b) *J. Mol. Biol. 151*, 535–556.

Dreyer, G. B., & Dervan, P. B. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 968–972.

Ebright, R. H., Ebright, Y. W., Pendergrast, P. S., & Gunasekera, A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2882–2886.

Englund, P. T., Kelly, R. B., & Kornberg, A. (1969) *J. Biol. Chem 244*, 3045–3052.

Francois, J.-C., Saison-Behmoras, T., & Helene, C. (1988) *Nucleic Acids Res. 16*, 11431–11440.

Francois, J.-C., Saison-Behmoaras, T., Barbier, C., Chassignol, M., Thuong, N. T., & Helene, C. (1989a) *Proc. Natl Acad. Sci. U.S.A. 86*, 9702–9706.

Francois, J.-C., Saison-Behmoaras, T., Thuong, N. T., & Helene, C. (1989b) *Biochemistry 28*, 9617–9619.

Garner, M. M., & Revzin, A. (1986) *Trends Biochem. Sci. 11*, 395–396.

Gaynor, R., Soultanakis, E., Kuwabara, M., Garcia, J., & Sigman, D. S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4858–4863.

Goyne, T. E., & Sigman, D. S. (1987) *J. Am. Chem. Soc. 109*, 2846–2848.

Griffin, L. C., & Dervan, P. B. (1989) *Science 245*, 967–971.

Groves, J. T., & Farrell, T. P. (1989) *J. Am. Chem. Soc. 111*, 4998–5000.

Guo, Q., Lu, M., Seeman, N. C., & Kallenbach, N. R. (1990) *Biochemistry 29*, 570–578.

Hayes, J. J., & Tullius, T. D. (1989) *Biochemistry 28*, 9521–9527.

Hertzberg, R. P., & Dervan, P. B. (1982) *J. Am. Chem. Soc. 104*, 313–315.

Hertzberg, R. P., & Dervan, P. B. (1984) *Biochemistry 23*, 3934–3945.

Jeppesen, C., & Nielsen, P. (1989) *Nucleic Acids Res. 17*, 4947–4956.

Jezewska, M. J., Bujalowski, W., & Lohman, T. M. (1989) *Biochemistry 28*, 6161–6164.

Jezewska, M. J., Bujalowski, W., & Lohman, T. M. (1990) *Biochemistry 29*, 5220.

Kappen, L. S., Goldberg, I. H., Wu, S. H., Stubbe, J., Worth, L., Jr., & Kozarich, J. W. (1990) *J. Am. Chem. Soc. 112*, 2797–2798.

Kirkegaard, K., Spassky, A., Buc, H., & Wang, J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2544–2548.

Kozarich, J. W., Worth, L., Jr., Frank, B. L., Christner, D. F., Vanderwall, D. E., & Stubbe, J. (1989) *Science 245*, 1396–1399.

Kumamoto, A. A., Miller, W. G., & Gunsalus, R. P. (1987) *Genes Dev. 1*, 556–564.

Kuwabara, M. D., & Sigman, D. S. (1987) *Biochemistry 26*, 7234–7238.

Kuwabara, M., Yoon, C., Goyne, T. E., Thederahn, T., & Sigman, D. S. (1986) *Biochemistry 25*, 7401–7408.

Latham, J. A., & Cech, T. R. (1989) *Science 245*, 276–282.

Law, R., Kuwabara, M. D., Briskin, M., Fasel, N., Hermanson, G., Sigman, D. S., & Wall, R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 9160–9164.

Le Doan, T., Perrouault, L., Helene, C., Chassignol, M., & Thuong, N. T. (1986) *Biochemistry 25*, 6736–6739.

Mack, D. P., Iverson, B. L., & Dervan, P. B. (1988) *J. Am. Chem. Soc. 110*, 7572–7574.

Maher, L. J., III, Wold, B., & Dervan, P. B. (1989) *Science 245*, 725–730.

Marmorstein, R. Q., Joachimiak, A., Sprinzl, M., & Sigler, P. B. (1987) *J. Biol. Chem. 262*, 4922–4927.

Moser, H. E., & Dervan, P. B. (1987) *Science 238*, 645–650.

Murakawa, G. J., & Nierlich, D. P. (1989) *Biochemistry 28*, 8067–8072.
Murakawa, G. J., Chen, C.-B., Kuwabara, M. D., Nierlich, D., & Sigman, D. S. (1989) *Nucleic Acids Res. 17*, 5361–5369.
Nielsen, P. E., Jeppesen, C., & Buchardt, O. (1988) *FEBS Lett. 235*, 122–124.
Oakley, M. G., & Dervan, P. B. (1990) *Science 248*, 847–850.
Ohlendorf, D. H., Anderson, W. F., Fisher, R. G., Takeda, Y., & Matthews, B. W. (1982) *Nature 298*, 718–723.
Otwinowski, Z., Schevitz, R. W., Zhang, R. G., Lawson, C. L., Joachimiak, A., Marmorstein, R. Q., Luisi, B. F., & Sigler, P. B. (1988) *Nature 335*, 321–329.
Pope, L. E., & Sigman, D. S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3–7.
Pope, L. M., Reich, K. A., Graham, D. R., & Sigman, D. S. (1982) *J. Biol. Chem. 257*, 12121–12128.
Rajagopal, P., & Feigon, J. (1989) *Nature 339*, 637–640.
Schevitz, R. W., Otwinowski, Z., Joachimiak, A., Lawson, C. L., & Sigler, P. B. (1985) *Nature 317*, 782–786.
Schultz, P. G., Taylor, J. S., & Dervan, P. B. (1982) *J. Am. Chem. Soc. 104*, 6861–6863.
Sigman, D. S. (1986) *Acc. Chem. Res. 19*, 180–186.
Sigman, D. S., Wahl, G. M., & Creighton, D. J. (1972) *Biochemistry 11*, 2236–2242.
Sigman, D. S., Graham, D. R., D'Aurora, V., & Stern, A. M. (1979) *J. Biol. Chem. 254*, 12269–12272.
Sigman, D. S., Spassky, A., Rimsky, S., & Buc, H. (1985) *Biopolymers 24*, 183–197.
Sluka, J. P., Horvath, S. J., Bruist, M. F., Simon, M. I., & Dervan, P. B. (1987) *Science 238*, 1129–1132.
Spassky, A. (1986) *J. Mol. Biol. 188*, 99–103.
Spassky, A., & Sigman, D. S. (1985) *Biochemistry 24*, 8050–8056.
Spassky, A., Rimsky, S., Buc, H., & Busby, S. (1988) *EMBO J. 7*, 1871–1879.
Springgate, C. F., Mildvaan, A. S., Abramson, R., Engle, J. L., & Loeb, L. A. (1973) *J. Biol. Chem. 248*, 5987–5993.
Strobel, S. A., Moser, H. E., & Dervan, P. B. (1988) *J. Am. Chem. Soc. 110*, 7927–7929.
Stubbe, J., & Kozarich, J. W. (1987) *Chem. Rev. 87*, 1107–1136.
Sun, J. S., Francois, J. C., Lavery, R., Saison-Behmoaras, T., Montenay-Garestier, T., Thuong, N. T., & Helene, C. (1988) *Biochemistry 27*, 6039–6045.
Tamilarasan, R., Ropartz, S., & McMillin, D. R. (1990) *Inorg. Chem.* (in press).
Thederahn, T., Kuwabara, M. D., Spassky, A., & Sigman, D. S. (1990) *Biochem. Biophys. Res. Commun. 168*, 756–762.
Tullius, T. D. (1988) *Nature 332*, 663–664.
Tullius, T. D. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 213–237.
Tullius, T. D., & Dombroski, B. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5469–5473.
Tullius, T. D., Dombroski, B. A., Churchill, M. E. A., & Kam, L. (1987) *Methods Enzymol. 155*, 537–558.
Uchida, K., Pyle, A. M., Morii, T., & Barton, J. K. (1989) *Nucleic Acids Res. 17*, 10259–10279.
Urata, H., Yasmamoto, K., Akagi, M., Hiroaki, H., & Uesugi, S. (1989) *Biochemistry 28*, 9566–9569.
Van Dyke, M. W., & Dervan, P. B. (1983) *Nucleic Acids Res. 11*, 5555–5567.
Veal, J. M., & Rill, R. L. (1988) *Biochemistry 27*, 1822–1827.
Veal, J. M., & Rill, R. L. (1989) *Biochemistry 28*, 3243–3250.
Wang, X., & Padgett, R. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7795–7799.
Ward, B., Skorobogaty, A., & Dabrowiak, J. C. (1986) *Biochemistry 25*, 6875–6883.
Yoon, C., Kuwabara, M. D., Law, R., Wall, R., & Sigman, D. S. (1988) *J. Biol. Chem. 263*, 8458–8463.
Yoon, C., Kuwabara, M. D., Spassky, A., & Sigman, D. S. (1990) *Biochemistry 29*, 2116–2121.
Zhang, R. G., Joachimiak, A., Lawson, C. L., Schevitz, R. W., Otwinowski, Z., & Sigler, P. B. (1987) *Nature 327*, 591–597.