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Footprinting DNA-Protein Complexes in Situ following Gel Retardation Assays Using 1,10-Phenanthroline-Copper Ion: *Escherichia coli* RNA Polymerase-*lac* Promoter Complexes†

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ABSTRACT: Protein-DNA complexes isolated in gel retardation assays can be digested within the acrylamide matrix by the nuclease activity of 1,10-phenanthroline-copper ion (OP-Cu). When the oligonucleotide products are eluted and analyzed on a sequencing gel, a footprint of the DNA-protein complex is obtained. Therefore, any protein-DNA complex isolated by the widely used gel retardation technique can be defined in terms of sequence-specific interactions by this simple methodology. The binding of the *lac* repressor and *Escherichia coli* RNA polymerase to an *Eco*RI fragment containing the *lac* control region has been studied by the combined gel retardation-1,10-phenanthroline-copper ion footprinting procedure. Footprints of *lac* repressor binding correspond to those obtained in solution with OP-Cu and DNase I and verify the experimental procedures. In studying *E. coli* RNA polymerase-promoter complexes, we have found that magnesium ion is required to form single-stranded DNA structures characteristic of kinetically competent open transcription complexes.

Gel retardation assays are a practical and powerful method for studying DNA-protein interactions (Garner & Revzin, 1981, 1982, 1986; Revzin et al., 1986; Fried & Crothers, 1981; Crothers, 1987). In this paper, we extend the utility of this technique by demonstrating that protein-DNA complexes can be footprinted within the gel matrix using the nuclease activity of 1,10-phenanthroline-copper ion (Sigman et al., 1979; Marshall et al., 1981; Sigman, 1986). It is therefore possible to define rapidly and accurately the DNA sequences directly interacting with protein in any complex that can be detected by the retardation assay.

Using the *lac* repressor-operator as a model system, we demonstrate that footprints obtained by this technique are consistent with those obtained in solution with DNase I (Galas & Schmitz, 1978). The advantages of combining gel retardation and OP-Cu[1] footprinting in a single procedure are illustrated by studying the interaction of the *lac* UV5 promoter with *Escherichia coli* RNA polymerase (Spassky & Sigman, 1985; Spassky, 1986). Using this method, we demonstrate that magnesium ion is essential for the formation of the kinetically competent open complex.

Experimental Procedures

Materials

The following reagents were purchased from the indicated suppliers: *E. coli* RNA Polymerase (Pharmacia); 1,10-phenanthroline and 2,9-dimethyl-1,10-phenanthroline (G. F. Smith); cupric sulfate (Mallinckrodt); 3-mercaptopropionic acid (Aldrich). The *Eco*RI fragment containing the *lac* L8-UV5 promoter was isolated and uniquely labeled as previously described (Spassky & Sigman, 1985, Kuwabara et al., 1986). *lac* repressor was a generous gift of Dr. Phillip Pjura.

Methods

Gel Retardation Assays. Slab gels were used for all gel retardation assays. The general conditions of Fried and Crothers (1981) were used for experiments with the *lac* repressor and of Straney and Crothers (1985) for gel retardation assays with *E. coli* RNA polymerase.

Footprinting in Solution. 5'-Labeled L8-UV5 186-bp DNA (10^5 cpm; 0.03 pmol) was incubated with 50 ng of *lac* repressor in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 0.2 mg/mL BSA in 10 μL for 10 min at 37 °C. For DNase I footprinting, 1 μL of 40 units/mL DNase I was added and incubated for 2.0 min at 37 °C. Digests were stopped by addition of a 10-μL aliquot of the digestion solution to 10 μL of a mixture composed of the following: 300 mg of sucrose, 840 mg of urea, 40 μL of 0.5 M EDTA, 100 μL of 0.1% bromophenol blue, and 100 μL of 0.1% xylene cyanol. In order to obtain 10 μL of a homogeneous mixture, the latter was liquified by heating

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[1] Abbreviations: OP, 1,10-phenanthroline; 2,9-dimethyl-OP, 2,9-dimethyl-1,10-phenanthroline; MPA, 3-mercaptopropionic acid; OP-Cu, 1,10-phenanthroline-copper ion complex; ApA, adenylyl(3'-5')adenosine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

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to 90 °C. For OP–Cu footprinting, 1 μL of 1 mM OP/0.23 mM $CuSO_4$ and 1 μL of 58 mM MPA were added to the 10-μL mixture and incubated for 5.0 min at 37 °C. Digests were quenched by first adding 1 μL of 28 mM 2,9-dimethyl-OP and then adding 10 μL of the reaction mixture to the sucrose–urea mixture as above. The quenched solutions containing the sample were then heated at 90 °C for 5 min and loaded directly onto 10% sequencing gels and run at 45 W for 3 h. Gels were exposed to X-ray film at −20 °C.

Footprinting Reaction in the Acrylamide Matrix. The same four reaction components are necessary for the OP–Cu footprinting reaction in the acrylamide matrix as in solution: 1,10-phenanthroline (OP), 2,9-dimethyl-1,10-phenanthroline (2,9-dimethyl-OP), cupric sulfate, and 3-mercaptopropionic acid (MPA). Prepare the following solutions just prior to use. Mix equal volumes of 40 mM OP (in 100% ethanol) with 9.0 mM $CuSO_4$ (in water). Dilute 1/10 with water to 2.0 mM OP/0.45 mM $CuSO_4$ (solution A). Dilute neat MPA 1/200 with water to 58 mM MPA (solution B).

Two methods of carrying out the footprinting reaction within the acrylamide matrix have been used. In the first, the wet retardation gel is exposed to X-ray film for 30 min at room temperature until the retarded bands are visible. The bands of interest are then excised with a razor and immersed in 100 μL of 50 mM Tris-HCl, pH 8.0. Then, 10 μL of solution A is added followed by 10 μL of solution B. The digestion is carried out for 10 min at room temperature and then quenched by addition of 10 μL of 28 mM 2,9-dimethyl-OP (in 100% ethanol) and 270 μL of a solution containing 0.5 M ammonium acetate and 1 mM EDTA. The DNA is then eluted overnight at 37 °C. The eluted DNA is ethanol precipitated (carrier DNA is unnecessary), resuspended in 80% (v/v) formamide, 10 mM NaOH, 1.0 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol, and loaded on a 10% sequencing gel.

An alternate method of carrying out the footprinting within the gel takes advantage of the ready availability of the reagents and involves immersion of the whole gel in a 1,10-phenanthroline–copper solution without the prior identification of the retarded bands. The first step of this alternative procedure is the immersion of the slab gel into 200 mL of Tris-HCl, pH 8.0, buffer. The gel is removed from its glass plate. Then 20 mL of solution A and 20 mL of solution B are added, and the digestion is allowed to proceed for 10 min at room temperature. To quench the reaction, 20 mL of 28 mM 2,9-dimethyl-OP is added, and the resulting solution is allowed to stand for 2.0 min. The gel is rinsed with distilled water and exposed to X-ray film for 30 min at room temperature. Bands of interest are cut from the gel and eluted overnight at 37 °C in 0.5 M ammonium acetate/1 mM EDTA as above. Eluted DNA is ethanol precipitated, resuspended in 80% (v/v) formamide, 10 mM NaOH, 1.0 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol, and loaded on a 10% sequencing gel. The significant advantage of this procedure is that if a long exposure is essential to identify weak bands, the footprint will not be perturbed by protein denaturation. Reagents are not reusable because thiol is consumed and the 1,10-phenanthroline is oxidatively destroyed.

Results

The binding of the *lac* repressor to the *lac* operator provides a useful test system to assess the validity of OP–Cu footprinting following gel retardation assays. This equilibrium has been extensively studied with footprinting reagents (Gilbert et al., 1975; Galas & Schmitz, 1978) and was among the first studied by gel retardation (Fried & Crothers, 1981). Initial studies using gel retardation to analyze the *lac* repressor–operator interaction revealed a series of retarded bands composed of 1:1 to 8:1 protein molecules:restriction fragment upon incubation of increasing concentrations of the repressor protein with the target DNA prior to electrophoresis (Fried & Crothers, 1981). With repressor protein preparations available to us, complexes with stoichiometries of 1:1 and 2:1 could be detected. Acrylamide plugs containing the free DNA and 1:1 and 2:1 protein–DNA complexes were excised from the acrylamide slab gel and then reacted with OP–Cu.

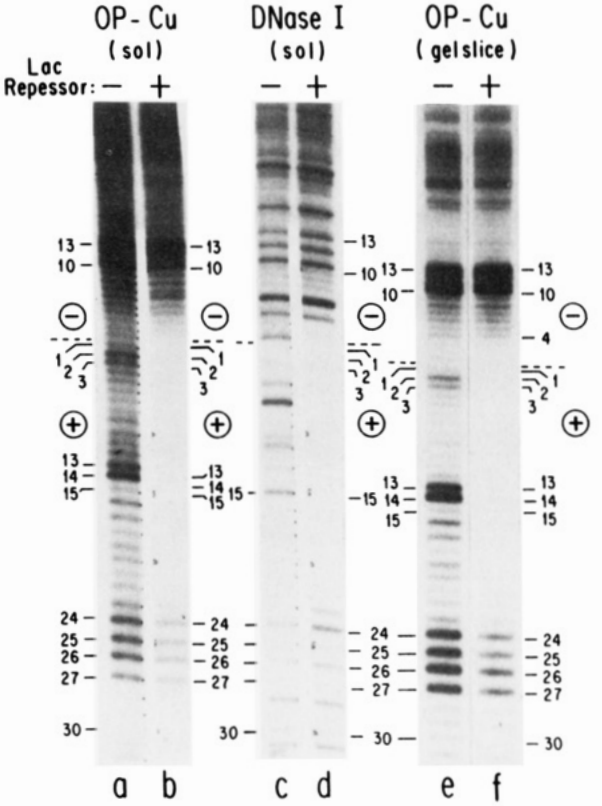

FIGURE 1: Comparison of the OP–Cu and DNase I footprints of *lac* repressor–operator binding in solution with the 1:1 complex in the acrylamide matrix following separation in a gel retardation assay. The footprinting experiment was carried out an acrylamide plug that was excised from the gel. The 1:1 complex was isolated from an incubation mixture containing 9.4 nM L8-UV5 DNA (2.5×10^6 cpm) and 19 nM *lac* repressor. (Lanes a and b) OP–Cu digestion in solution: (a) free DNA; (b) repressor DNA–complex: (Lanes c and d) DNase I digestion in solution: (c) free DNA; (d) repressor–DNA complex. (Lanes e and f) OP–Cu digestion within the gel slice: (e) unbound DNA from gel retardation assay; (f) 1:1 repressor–DNA complex from gel retardation assay. See text for details of footprinting.

Important features of the footprinting method can be inferred from a comparison of digestion patterns obtained within the gel matrix to those obtained in solution (Figure 1). First, the OP–Cu digestion pattern of free DNA cut within the acrylamide gel matrix (Figure 1, lane e) is the same as that of DNA digested in solution (Figure 1, lane a). The acrylamide matrix therefore does not perturb the DNA conformation. For example, the prominent quartet of bands of the Pribnow box at positions −13, −12, −11, and −10 and the strong set of four bands corresponding to the sequence 3′-TGTG-5′ at sequence positions +24 to +27 were clearly evident (Sigman et al., 1985; Spassky & Sigman 1985). No difference was observed between DNA that had been incubated with the repressor and then separated from it electrophoretically and control DNA that had been electrophoresed without prior incubation with any protein.

The footprint of the 1:1 complex within the acrylamide plug (Figure 1, lane f) revealed a protected region from position

−1 to 20 fully consistent with results obtained in solution using OP–Cu (Figure 1, lane b) and DNase I (Figure 1, lane d). The prominent feature of the OP–Cu footprint is the suppression of the prominent cutting sites at positions +13 and +14 (compare lanes f and b of Figure 1). Since comparable results are obtained whether the footprinting reaction is performed on an excised gel slice or on the entire retardation gel, the gel must be readily permeated by the reagents and the quenching solutions.

The digestion pattern of the 2:1 repressor–DNA complex within the plug is comparable to that presented for the 1:1 complex in the sequence positions −1 to 20. But an interesting and important difference in the footprints of the two retarded bands is observed in the region of the pseudooperator site O-3, which extends from sequence positions −70 to −90 (Gilbert et al., 1975). The digestion patterns of free DNA and the 1:1 complex are similar in this region. In contrast, the 2:1 complex shows a partial protection at sequence positions −70 to −75 that is not observed in the free DNA or 1:1 repressor–DNA complex (data not shown). Incomplete protection of this region is consistent with the intermediate affinity of the pseudooperator for the repressor relative to the true operator and nonhomologous DNA segments (Winter & Von Hippel, 1981). Two explanations are possible for the partial protection of O-3. The first is that the repressor binding at O-3 partially dissociates during the footprinting procedure. The second is that the 2:1 complex, isolated as a discrete band by gel retardation, is not a homogeneous species. One repressor molecule is bound at the high-affinity operator; but, the second repressor molecule, while favoring O-3, also binds in a nonfootprintable manner along the length of the DNA molecule. Nonspecific binding must be responsible for the isolation of complexes with three or more repressor molecules per restriction fragment.

RNA Polymerase–lac UV5 Promoter Complex. The strong *lac* UV5 promoter binds to *E. coli* RNA polymerase without the requirement for the cyclic AMP binding protein that serves as a positive effector protein. Gel retardation assays have been reported that show two retarded bands (designated O_u and O_l for the slower and faster moving band, respectively) that are kinetically competent for transcription (Straney & Crothers, 1985). A third, slower moving diffuse band, likely representing the formation of aggregates, is detectable and can be preferentially decreased by the addition of calf thymus DNA. The intensities of both bands can be enhanced if the enzyme and restriction fragment are preincubated with the dinucleotide ApA and UTP in the presence of magnesium ion (Figure 2), conditions that lead to the synthesis of the tetranucleotide ApApUpU (Spassky, 1986).

Previous use of OP–Cu to footprint the binding of RNA polymerase to the *lac* UV5 fragment in solution has revealed a new family of bands at sequence positions −6, −5, −4, and −3 whose intensities vary inversely with the hyperreactive bands of the Pribnow box (Spassky & Sigman, 1985). Alkylation experiments with dimethyl sulfate have indicated that these sequence positions correspond to single-stranded DNA formed at the active site of RNA polymerase in kinetically competent open transcription complexes (Kirkegaard et al., 1983). The displacement of these bands two sequence positions downstream upon the initiation of transcription with the addition of ApA and UTP is fully consistent with their assignment as a kinetically competent transcription bubble (Spassky, 1986).

Surprisingly, footprints of the two retarded bands, O_u and O_l, formed in the presence or absence of ApA and UTP carried out in the gel slice did not contain the bands at positions −6 through −3 characteristic of the open complex in the absence

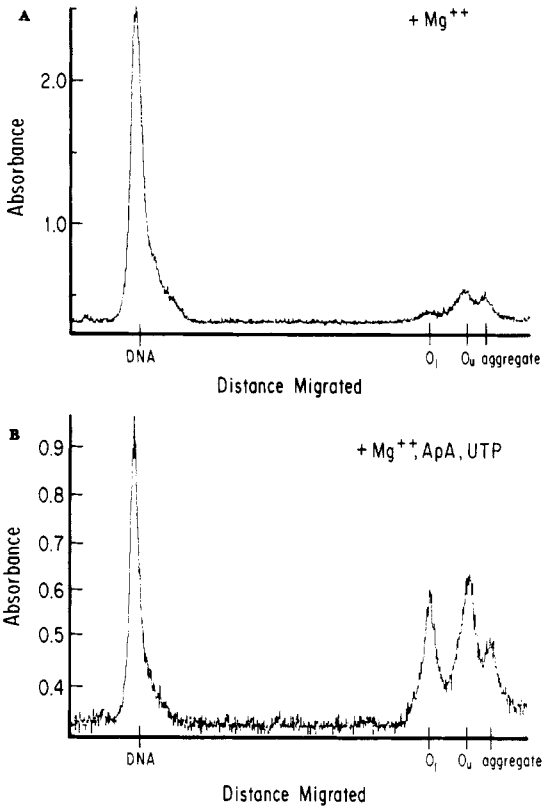

FIGURE 2: Gel retardation assay of RNA polymerase binding to *lac* UV5. 5′-Labeled 186 base pair *lac* UV5 (4.1×10^5 cpm) was incubated for 10 min with 0.46 μg of RNA polymerase with 10 mM magnesium chloride in the presence and absence of 500 μM ApA and 50 μM UTP. Buffer conditions: 40 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM DTT, 0.1 mM EDTA, and 5% glycerol in a reaction volume of 7 μL. After 0.06 μg of calf thymus DNA was added to the incubation mixture, 2 μL of 30% (v/v) glycerol solution containing 0.25% bromophenol blue and 0.25% xylene cyanol was added, and the reaction mixtures were loaded on a 4% acrylamide gel (41:1 cross-linking) and electrophoresed at 20 V/cm for 2.5 h in 89 mM Tris–borate and 1 mM EDTA. Direction of migration from right to left; positive pole at left. (A) Incubation with magnesium ion; (B) incubation with magnesium ion, ApA, and UTP.

of added nucleotides (Figure 3, lane c) or the pattern of bands at positions −4 through −2 indicative of a displaced transcription bubble (Figure 3, lane e). The decreased cutting within the Pribnow box sequences relative to the landmark bands at positions +24 to +27 nevertheless revealed that the enzyme was positioned on the restriction fragment in a sequence-specific manner, consistent with the assignment of this band in the retardation gel as a "closed" complex (Schleif, 1986).

This apparent dilemma was resolved with the recognition that the OP–Cu cleavage reaction was carried out in magesium ion free buffers after it was electrophoresed in a buffer composed of Tris, borate, and EDTA. When the gel slice is equilibrated with buffers containing magnesium ion and the polymerase–DNA complexes footprinted, the bands attributable to the formation of single-stranded DNA can be readily detected (Figure 3, lane d). Moreover, the positions of the bands are shifted when the nucleotides ApA and UTP and magnesium ion are simultaneously added (Figure 3, lane h), indicating that structural changes occurring in the DNA characteristic of a transcriptionally active complex are retained within the acrylamide matrix. Therefore, in order to obtain footprints characteristic of a catalytically active complex, magnesium ion must be added. In the absence of magnesium ion, the footprint of the closed complex was obtained (Figure 3, lanes c and e).

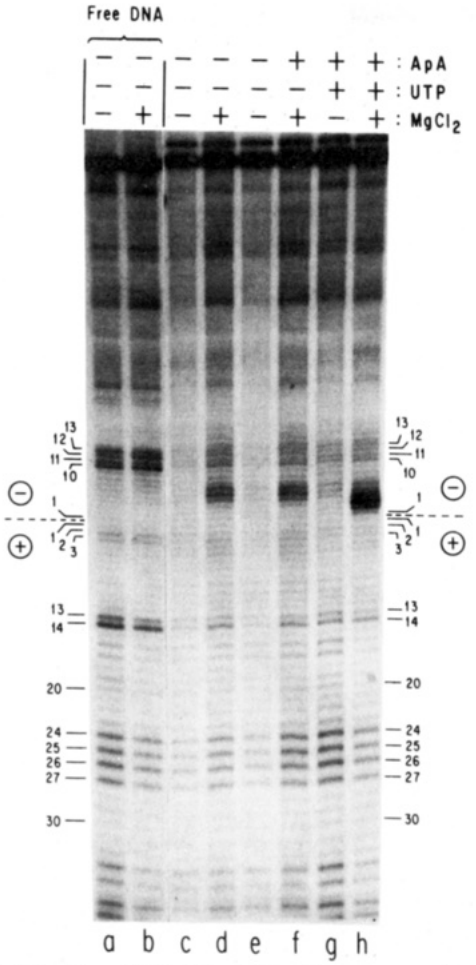

FIGURE 3: Digestion of L8-UV5–RNA polymerase complexes within a gel slice. A gel slice containing O_u or unbound DNA was excised from a retardation gel in which the RNA polymerase–*lac* fragment was incubated with magnesium ion only (Figure 2A) or with magnesium ion, ApA, and UTP (Figure 2B). The gel slices containing the DNA or DNA–protein complexes were immersed in 50 mM Tris-HCl buffer, pH 8.0, containing the indicated additional components. When included, $[Mg^{2+}] = 10$ mM, [ApA] = 500 μM, and [UTP] = 50 μM. (Lanes a–d) Obtained from magnesium ion only incubation; (Lanes e–h) Obtained from magnesium ion, ApA, and UTP incubation. Digestion pattern of free DNA is the same from all incubation conditions.

The requirement for the addition of the nucleotide substrates and magnesium ion to regenerate the abortive transcription products indicates that during the gel retardation assay small molecules, such as ApA, UTP, and the product ApApUpU, can diffuse from the migrating DNA–protein complex. Combining the two procedures of gel retardation and OP–Cu footprinting allows distinction between ionic conditions that stabilize the formation of a complex from those essential for catalytic activity.

Discussion

Coupling in situ OP–Cu footprinting with gel retardation assays provides a rapid, accurate, and convenient method to define the sequence-specific binding sites of proteins on DNAs. Several advantages relative to existing methodologies are apparent. Protein–DNA complexes formed in solution may often be heterogeneous. For the simple case of *lac* repressor binding, gel retardation assays indicate that up to eight species may be generated depending on the relative concentrations of protein to DNA. Footprints carried out in solution will not correspond to a single species but instead reflect the multiple species present. However, footprinting the complexes subsequent to a gel shift experiment permits the examination of discrete species. Since the 2:1 repressor–restriction fragment complex is separated from the 1:1 complex, background cutting due to the 1:1 complex or uncomplexed DNA is minimized. As a result, subtle phenomena, such as the weak binding of the *lac* repressor to O-3, can be observed.

The experiments presented here indicate that the structural and functional properties of DNA are not altered by entrapment in the acrylamide matrix. For example, the *Eco*RI restriction fragment of the *lac* control region studied here exhibits identical sequence-dependent reactivity with OP-Cu as it does in solution. The structure of the DNA, as reflected by the minor groove reactivity of OP–Cu, is not altered by incorporation into the gel.

Gel retardation assays have been carried out subsequent to methylation or DNase I footprinting experiments and have provided useful sequence information on protein binding sites (Hendrickson & Schleif, 1985; Singh et al., 1986). However, since the footprinting reaction described here with OP–Cu is carried out subsequent to the separation of the protein–DNA complexes, it is possible not only to obtain a footprint of the retarded complexes, as in the case of *lac* repressor, but to identify structural changes associated with the binding of diffusible ligands, as in the case of magnesium ion binding to the RNA polymerase–promoter complex. Studying changes induced by substrates or effectors in an OP–Cu footprint of functional protein–DNA complexes of defined stoichiometry enmeshed in solid phase may prove to be a powerful method to probe the dynamics of protein–DNA interactions. In particular, factors that influence the activity of complexes are distinguishable from those that influence their stability.

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Emil Kakkis made the insightful suggestion that reacting the entire gel with OP–Cu might prove useful. Dr. C. B. Chen, Dr. R. Wall, T. Thederahn, and R. Law have made valuable comments during the course of this work.

Registry No. OP–Cu, 15891-89-1; Mg, 7439-95-4; RNA polymerase, 9014-24-8.

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Effects of Functional Group Changes in the *Eco*RI Recognition Site on the Cleavage Reaction Catalyzed by the Endonuclease†

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ABSTRACT: Oligodeoxynucleotides have been prepared that contain changes in the functional group pattern present in the *Eco*RI recognition site. These changes involve "functional group deletions", "functional group reversals", and "displaced functional groups". Steady-state kinetic parameters have been used to characterize the interaction of these modified recognition sites with the *Eco*RI endonuclease. Changes in the functional group pattern have varying effects upon the cleavage reaction. Both the exocyclic amino groups of the two adenine residues and the methyl groups of the thymine residues appear to interact with the endonuclease quite differently. In both cases efficient catalysis was observed when these functional groups were present at the "outer" dA-dT base pair. Selectivity was decreased by over an order of magnitude largely via increases in K_m when these functional groups were deleted. Similar modificatons at the "inner" dA-dT base pair did not alter the kinetic parameters significantly from those observed with the native sequence. Addition of an amimo group to the minor groove at the outer dA-dT base pair resulted in a modified recognition site that interacted with the enzyme, on the basis of observed competitive inhibition kinetics, but was not cleaved.

Sequence specific protein-nucleic acid recognition appears to be a general feature of many biological processes. In order to understand such interactions between macromolecules, it is necessary to examine them at a functional group level. It has been proposed that an important aspect of the recognition process between proteins and nucleic acids involves hydrogen-bond formation between functional groups of the protein amino acid side chains and nucleobase functional groups available in the major and minor grooves of the nucleic acid (Seeman et al., 1976). Complementary patterns of hydrogen-donating and -accepting functional groups would define a particular hydrogen-bonding pattern and assist in determining the affinity of the protein for a given nucleic acid sequence. One approach to decipher a characteristic functional group pattern present in the nucleic acid recognition site involves the sequential deletion or modification of single functional groups from the recognized sequence and the examination of subsequent effects upon protein binding and/or catalysis.

The effects of base analogue substitutions in DNA on restriction endonuclease activity have been reported (Kaplan & Nierlich, 1975; Berkner & Folk, 1977, 1979; Mann & Smith, 1977; Mann et al., 1978; Marchionni & Roufa, 1979; Bodnar et al., 1983). One disadvantage with this approach is that the analogue is introduced at multiple positions, which can complicate subsequent binding and/or catalytic analyses. Oligodeoxynucleotides prepared by chemical and/or enzymatic methods allow the creation of recognition sites with single functional group deletions or modifications. Base analogue substitution in oligodeoxynucleotides has seen some success in the examination of binding and/or catalysis with restriction (Dwyer-Hallquist et al., 1982; Ono et al., 1984; Yolov et al., 1985; Seela & Driller, 1986; Jiricny, et al., 1986; Fliess et al., 1986; Brennan et al., 1986a) and modification (Brennan et al., 1986b) enzymes as well as DNA binding proteins such as the lac repressor (Yansura et al., 1977, 1979; Goeddel et al., 1977, 1978; Fisher & Caruthers, 1979, 1980) and most recently with RNA polymerase-promoter sequence recognition (Dubendorff et al., 1987).

The interaction of restriction endonucleases with their DNA recognition sites [for recent reviews see Jack et al. (1981), Wells et al. (1982), and Modrich (1982)] is an attractive system for studying this phenomenon. Restriction endonucleases recognize a relatively short DNA sequence (commonly four or six base pairs) and do so with a high degree of selectivity. This high sequence selectivity suggests that functional group contacts between the protein and DNA occur at each base pair within the recognition site.

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