Nuclease Activity of 1,10-Phenanthroline–Copper Ion. Conformational Analysis and Footprinting of the *lac* Operon†

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ABSTRACT: The nuclease activity of 1,10-phenanthroline–copper [$(OP)_2Cu^+$] preferentially nicks the wild-type, Ps, and L8-UV-5 *lac* promoters in the conserved promoter specific sequence (Pribnow box). The preferred sites of attack of the wild-type fragment within this region are at positions −13 and −12 on the template strand. When the comparable fragment from the Ps promoter, which differs from the wild type at position −9 (T instead of C), is cleaved with $(OP)_2Cu^+$, a new strong band at position −10 in the gel patterns is clear. An apparent increase in cutting at position −11 can also be observed. The conversion of the Ps promoter to the L8-UV-5 promoter (a change from an A to a T at position −8 and a change from a C to a T at position −66) results in alteration of the relative intensities of the four prominent bands at positions −13 to −10. Most notably, the intensity at position −10 is attenuated in L8-UV-5. The hypersensitivity of the Pribnow box region to the coordination complex is also apparent if the cutting of the missense strand is analyzed. The region of strong nicking in this case ranges from positions −11 to −3, and the relative intensities of the bands depend on the primary sequence of the promoters. These data suggest that a single base change induces local variation in the DNA structure. This new structure may be responsible for the notable difference in the efficiency of the promoters. Pancreatic deoxyribonuclease I (DNase I) does not preferentially cleave the Pribnow box relative to other regions of the sequence. The only observed difference in the DNase pattern between the wild-type promoter and the stronger Ps and L8-UV-5 promoters is the attenuation of a relatively intense band at position −8. The nuclease activity of 1,10-phenanthroline–copper is useful as a footprinting reagent. Digestion of *lac* promoter–*Escherichia coli* RNA polymerase complexes in which the sense strand is 5′-labeled reveals that positions −13 to −10 are protected from cleavage as expected but that new hypersensitive sites at positions −6 to −3 appear. These new sites of attack, which previous studies with dimethyl sulfate have indicated are single-stranded in the enzyme–substrate complex, are only apparent in kinetically competent open transcription complexes. For example, they are observed with the strong L8-UV-5 promoter in the absence of the cyclic AMP binding protein but require the protein effector with the weaker wild-type promoter. $(OP)_2Cu^+$ therefore provides a convenient and precise method for determining the productive occupancy of a promoter.

The 1,10-phenanthroline–cuprous ion complex [$(OP)_2Cu^+$][1] with hydrogen peroxide as a coreactant nicks DNA in a reaction that requires the binding of the coordination complex to nucleic acid (Sigman et al., 1979; Marshall et al., 1981). In studies with synthetic polynucleotides, the artificial nuclease has demonstrated a clear secondary structural specificity (Marshall et al., 1981; Pope & Sigman, 1984). B-DNA is cleaved more readily than A-DNA while Z-DNA and noncomplementing single strands are nicked substantially slower, if at all. When native B-form DNA is digested by the artificial nuclease, cleavage is observed at all four bases but hypersensitive regions are readily apparent (Sigman et al., 1985). This nonuniform cleavage presumably reflects local structure variation in the DNA which creates binding sites of variable affinity for the coordination complex.

Recent studies of *lac* operon control regions have indicated that the conserved promoter sequence (Pribnow, 1975) is hypersensitive to the nuclease activity and suggest that the conformation of the DNA in this region possesses a characteristic three-dimensional structure. Mutational changes that result in tighter RNA polymerase binding increase the hypersensitivity of the region to the 1,10-phenanthroline–cuprous complex on the template strand. For example, the Ps and L8-UV-5 mutants, which are stronger promoters and less cyclic AMP dependent (Reznikoff & Abelson, 1978; de Crombrugghe et al., 1984), not only exhibit the hypersensitive sites characteristic of the wild type but also demonstrate susceptible sites at two additional adjacent loci in the Pribnow box region (Sigman et al., 1985). The changes in reactivity at sites other than the positions of the base changes indicate that local conformational differences rather than any intrinsic alteration of chemical reactivity affect the pattern of scission.

In this paper, we compare the action of DNase I to $(OP)_2Cu^+$ with this series of promoters. We also demonstrate

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[1] Abbreviations: OP, 1,10-phenanthroline; $(OP)_2Cu^+$, the 2:1 1,10-phenanthroline–cuprous ion complex (endogenously generated hydrogen peroxide must be present as a coreactant); RNP, *Escherichia coli* RNA polymerase; CRP, cyclic AMP binding protein (presence of cyclic AMP bound to the protein is implied); DNase I, deoxyribonuclease I; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; bp, base pair.

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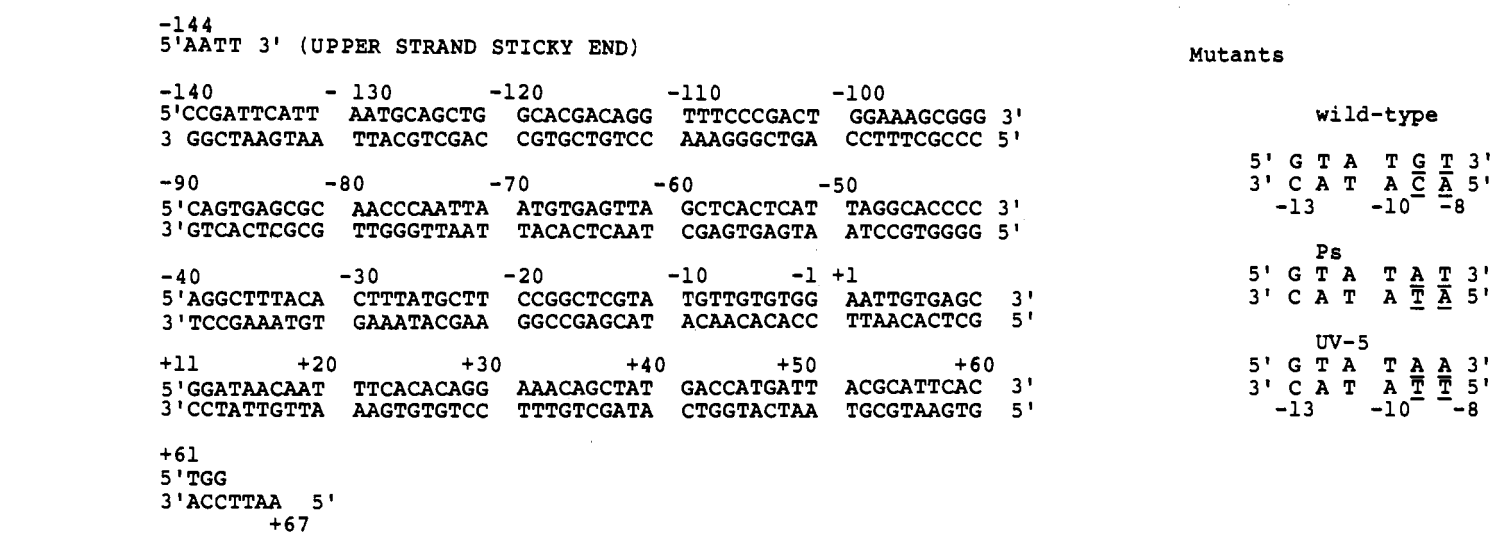


FIGURE 1: Primary sequence of the 203-bp *lac* control region.

that 3′-labeled missense strands also exhibit hyperreactivity in the promoter region extending from position −11 to the start of transcription. Although not as apparent as with the 5′-labeled strands, the cleavage patterns with the 3′ strands show distinct differences between the three different promoters. Finally, we show that $(OP)_2Cu^+$ is a valuable footprinting reagent that can reveal interesting details of RNA polymerase binding to the *lac* promoter that are not apparent when DNase I is used in a parallel way. Both nuclease activities therefore provide complementary information regarding protein–DNA complexes.

Experimental Procedures

Materials. The labeled 186 base pair fragments were purified from the corresponding 203 base pair fragments by methods that have been previously described by H. Buc and colleagues (Schaeffer et al., 1982; Spassky et al., 1984; Kolb et al., 1983). 5′ labeling of the sense strand was carried out by the exchange reaction catalyzed by polynucleotide kinase followed by cleavage with *Pvu*II. 3′ labeling of the missense strand was accomplished with the Klenow fragment and [α-^{32}P]dATP, also followed by cleavage with *Pvu*II.

The general conditions for the cleavage of DNA have been previously described (Spassky et al., 1984). Methods used to analyze DNase I digestions and footprinting experiments are generally applicable for $(OP)_2Cu^+$ in preparing samples for electrohoretic analysis. One modification in the use of $(OP)_2Cu^+$ during the course of a footprinting experiment is that buffers should not contain millimolar amounts of dithiothreitol or EDTA. Trial cleavage reactions should be attempted if specific components must be included in the incubation mixtures. General conditions for cleavage involve the addition of 10 μL of a stock solution containing either OP (2 mM) and Cu^{2+} (0.15 mM) or OP (2 mM) and Cu^{2+} (0.45 mM) to a 100-μL solution of labeled DNA in a 40 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$ and 100 mM KCl. The reaction is initiated by the addition of 10 μL of a mercaptopropionic acid solution (0.058 M). Incubation times range from 3 s to 2 min. The reaction is quenched by the addition of 10 μL of 28 mM 2,9-dimethyl-1,10-phenanthroline in ethanol. Then 15 μL of a stop solution containing 220 μg/μL tRNA, 2 M sodium acetate, and 10 mM EDTA is added. After phenol extraction, the DNA is precipitated with ethanol. Generally the precipitate was allowed to form overnight at −20 °C. The precipitate was collected by centrifugation in an Eppendorf microfuge for 10 min and then loaded on a 10% sequencing gel that was calibrated with G+A lanes as described by Maxam & Gilbert (1980).

Results

The 1,10-phenanthroline–cuprous complex is a small structurally constrained tetrahedral chelate that can potentially interact with DNA by intercalation of the phenanthroline moiety and/or by interaction with the minor groove. Since the binding of the coordination complex should be restricted to three base pairs, the complex should be more sensitive to local conformational changes than DNase I, which like most hydrolases must possess an extended binding site (Dickerson & Geis, 1969). The sequences of the *lac* promoter control region of the wild-type, Ps, and L8-UV-5 mutations are presented for reference in Figure 1 (Reznikoff & Abelson, 1978). The sense strand is the lower strand and can be uniquely labeled at the 5′ ends as noted under Experimental Procedures. As presented, the adenosine residue at position 67 bears the ^{32}P-labeled phosphate. Figure 2 compares the cleavage of the three homologous restriction fragments by $(OP)_2Cu^+$ and DNase I. The most striking feature of the $(OP)_2Cu^+$-mediated cleavage is that the wild-type cAMP-dependent promoter possesses two strong bands at the boundary of the Pribnow box while the cyclic AMP independent promoters Ps and L8-UV-5 possess three strong bands at positions −13, −12, and −10 and an enhanced band at position −11. Corresponding changes are not apparent in the digestion of the promoter region by DNase I although a weak band at position −9 is present in Ps and L8-UV-5 but not in wild type.

There are three additional differences between $(OP)_2Cu^+$ and DNase I in the cleavage of the fragments. The most apparent is that the promoter sequence is one of a limited set that is preferentially nicked by the coordination complex. This preference is not shared by DNase I, which tends to exhibit equivalent reactivity over the entire fragment. Another clear difference between the activities is that, in the alternating sequence 3′GTGTGT5′ (e.g., positions 23–28), DNase I preferentially nicks 5′ to the thymidines while $(OP)_2Cu^+$ effectively nicks 5′ to the guanine residues as well. The preferential cutting by DNase I 5′ to the pyrimidines in alternating copolymers has been previously noted (Scheffler et al., 1968). Finally, $(OP)_2Cu^+$ exhibits reduced reactivity with runs of pyrimidines (e.g., positions 28–33) while DNase I nicks effectively at positions 30 and 31.

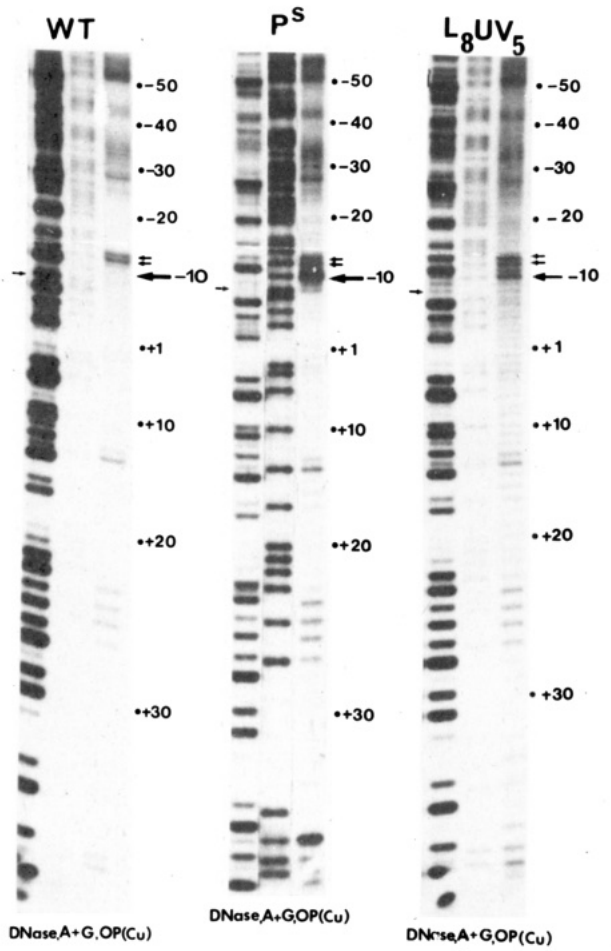


FIGURE 2: 5′-Labeled sense strand of wild-type (WT), Ps, and UV-5 *lac* promoters. Strand scission by DNase I and $(OP)_2Cu^+$ is compared by analysis on a sequencing gel using A+G Maxam–Gilbert sequencing for calibration. Position 1 corresponds to the 5′ end of mRNA.

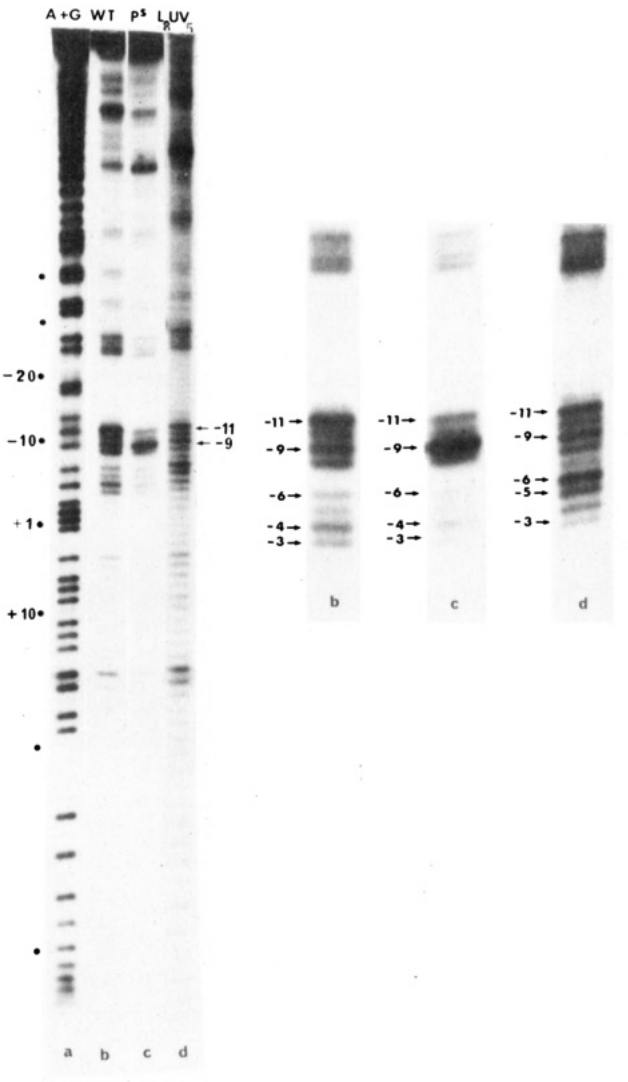


FIGURE 3: $(OP)_2Cu^+$ cleavage of wild-type, Ps, and UV-5 *lac* promoters 3′-labeled on the upper or nontemplate strand. Lanes: a, A+G sequencing reaction; b, wild type; c, Ps; d, L8-UV-5.

The Pribnow box region is hyperreactive to $(OP)_2Cu^+$ in the 3′-labeled upper or missense strand as well as in the lower or sense strand of the three *lac* promoter regions (Figure 3). In contrast, the position 24–27 sequence, which was a preferred region of cutting by the coordination complex in the sense strand (Figure 2), is barely reactive in the 3′-labeled missense strand (Figure 3). The reason for the low reactivity of the upper strand in the sequence

$$\begin{array}{lcl} 5' & \text{A–C–A–C–A} & 3' \\ 3' & \text{T–G–T–G–T} & 5' \\ & 24 \quad\quad 28 & \end{array}$$

is not clear. As indicated in Figure 4, where the 3′-labeled Ps fragment was used, the 24–28-positions remain a preferred site of attack of DNase I.

The 3′-labeled upper strand shows two clusters of hyperreactive bands in the regions −11 to −8 and −6 to −3. (Figure 3). Although less dramatic at first glance than the results with the lower strand, the cleavage pattern of the upper strand is different for the three promoters. For example, the bands at positions −11 to −8 show similar relative intensity in the wild type and L8-UV-5. In contrast, Ps shows a strong band at position −9 that is significantly more intense than the others in the position −11 to −8 region. The strong L8-UV-5 promoter shows more extensive hypersensitivity than the wild-type and Ps promoters at positions −6 and −5, two positions in the sequence that are identical in all three promoters. Ps seems generally less susceptible in the position −6 to −3 region although the relative intensities of the bands parallel those of the wild type (i.e., the band at position −4 is among the most intense in this region).

Use of $(OP)_2Cu^+$ as a Footprinting Reagent. The nuclease activity of $(OP)_2Cu^+$ has obvious potential as a footprinting reagent because it cleaves DNA under physiological conditions (Schmitz & Galas, 1978; Galas & Schmitz, 1979). It is also smaller than DNase I and might be expected to detect structural changes that DNase I may be unable to sense. Further, since it produces predominantly 3′-phosphorylated and 5′-phosphorylated ends as cleavage products, gels can be accurately calibrated with the Maxam–Gilbert sequencing reactions.

In order to test the utility of $(OP)_2Cu^+$ as a footprinting reagent, the interaction of the L8-UV-5 promoter with *Escherichia coli* RNA polymerase was examined. This promoter, which supports CRP-independent transcription, permits the study of the binding of the polymerase wtihout CRP and the binding of CRP in the presence of RNA polymerase. In Figure 5, data obtained with both DNase I and $(OP)_2Cu^+$ as footprinting reagents are compared. Lanes b and d again contrast the action of DNase I and $(OP)_2Cu^+$ on DNA (cf.

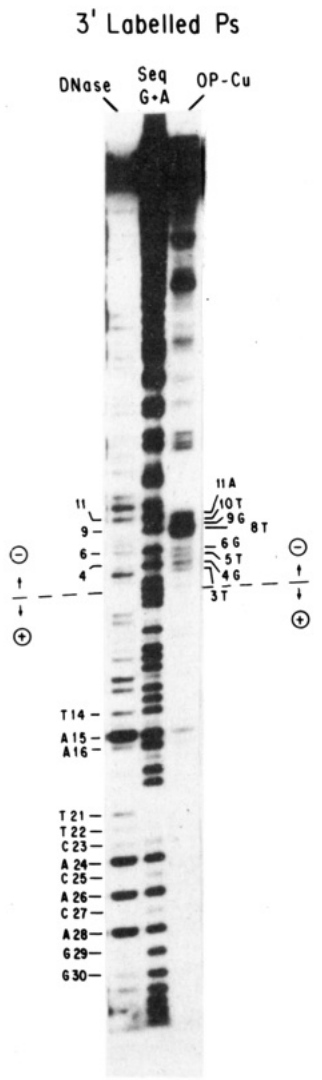
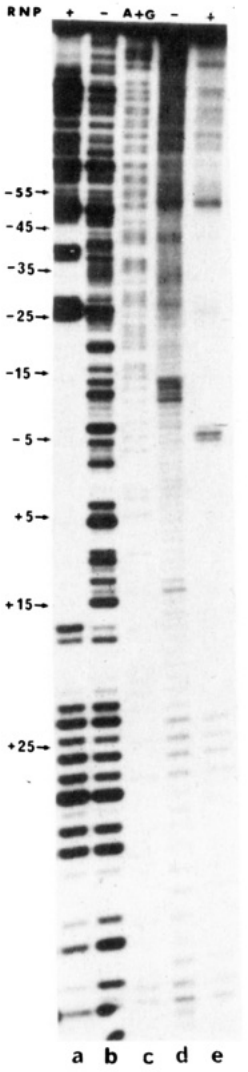


FIGURE 4: Comparison of the cleavage pattern by DNase and $(OP)_2Cu^+$ of the 3′-labeled upper strand of the *lac* Ps promoter. Lanes as indicated.



TEMPLATE STRAND

FIGURE 5: Footprint pattern of RNA polymerase binding of the L8-UV-5 *lac* promoter 5′-labeled in the sense strand developed with DNase I and $(OP)_2Cu^+$. RNP (100 nM) was incubated with the restriction fragment (1–2 nM) in 40 mM Tris buffer, pH 8.0, containing 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM dithiothreitol, and 5% glycerol. Lanes a and b: DNase I digestion in the presence and absence of RNA polymerase. Lane c: A+G sequencing reaction. Lanes d and e: $(OP)_2Cu^+$ cleavage in the absence and presence of RNA polymerase.

Figure 2), showing that the $(OP)_2Cu^+$ reagent more actively seeks out the Pribnow box region than DNase I relative to other positions in the sequence.

Important and complementary changes of the digestion patterns are observed when *E. coli* RNA polymerase is added to the 5′-labeled fragment prior to the addition of DNase I or $(OP)_2Cu^+$. Consistent with previous studies, the addition of RNA polymerase to DNA blocks the DNase I hydrolysis from base positions −20 to 16 and enhances the hydrolysis at positions −25 and −38 (Galas & Schmitz, 1978; Schmitz & Galas, 1979). The inhibition of DNase hydrolysis does not necessarily mean that all the positions of the sequence are in contact with RNA polymerase but only that the access of DNase I to the DNA strand is inhibited. The pattern observed with $(OP)_2Cu^+$ in the presence of RNA polymerase is fully consistent with this view. Although protection from cleavage is observed in the same general region, including the hyperreactive Pribnow box region, the most striking observation is the appearance of new hypersensitive bands at positions −6 to −3. Previous studies have identified this region as single-stranded in the *lac* UV-5 promoter–RNA polymerase complex using a method based on the differential reactivities of cytosine with dimethyl sulfate in single- and double-stranded structures (Kirkegaard et al., 1984). This work has shown that the cytosine residues at positions −6, −4, −2, and −1 of the UV-5 promoter are unpaired. The new sites that are hypersensitive to $(OP)_2Cu^+$ are therefore in a single-stranded region that is formed by RNA polymerase in order to initiate transcription. If the parallel experiment is carried out with the 3′-labeled missense strand, no equally obvious new hypersensitive sites are apparent (Figure 6). This interesting asymmetry shows that the accessibility of the coordination complex is restricted and favors the site that is accessible to the incoming nucleotide triphosphates.

The pattern of hyperreactive bands at positions −6 to −3 in the sense strand of the UV-5 promoter requires the presence of the polymerase (Figure 7). In the case of L8-UV-5 promoter, the only additional changes observed when CRP is added with polymerase to the strong promoter are those in the position −75 to −50 region, where CRP binds. With the L8 mutation, the binding of CRP is not detectable under our experimental conditions in the absence of polymerase. For the wild-type promoter, binding of CRP can be observed, but

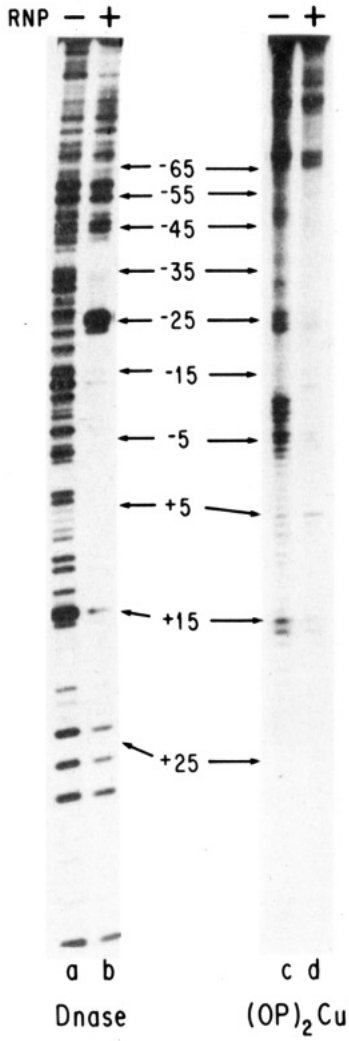


FIGURE 6: Footprint pattern of RNA polymerase binding of the L8-UV-5 promoter on the 3′-labeled missense strand developed with DNase I and $(OP)_2Cu^+$. Incubation conditions were as described in Figure 5. Lanes a and b: DNase digestion in the absence and presence of RNA polymerase. Lanes c and d: $(OP)_2Cu^+$ digestion in the absence and presence of RNA polymerase.

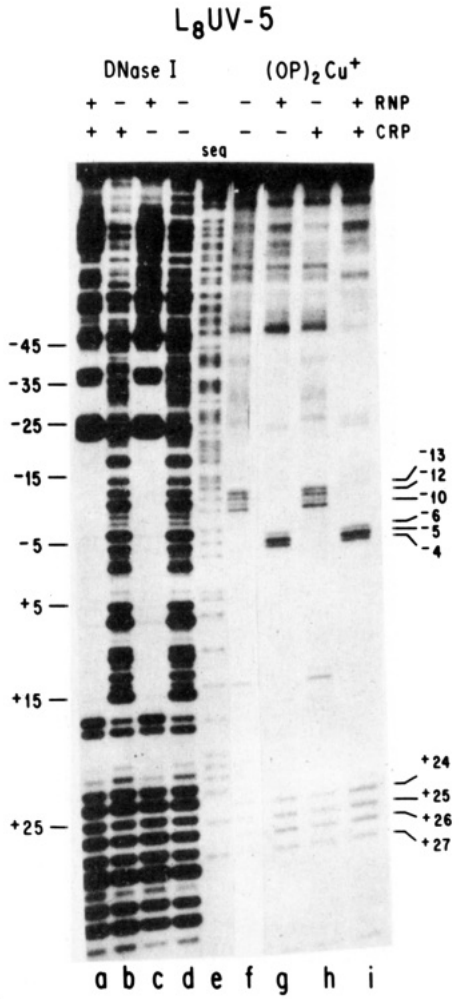


FIGURE 7: Influence of CRP binding digestion patterns of DNase I and $(OP)_2Cu^+$ on 5′-labeled L8-UV-5 fragments. CRP (50 nM) and cyclic AMP (20 μM) were incubated with DNA (1–2 nM) for 10 min at 37 °C before addition of polymerase and then incubated for an additional 10 min prior to the addition of DNase I or $(OP)_2Cu^+$. Lanes a–d: DNase I digestion in the presence of (a) RNA polymerase and CRP, (b) CRP only, (c) RNA polymerase, or (d) no addition. Lane e: G+A sequencing reaction. Lanes f–i: $(OP)_2Cu^+$ digestion in the presence of (f) no addition, (g) RNA polymerase only, (h) CRP only, or (i) RNA polymerase and CRP.

it does not produce a change from the wild-type two-band pattern to the four-band pattern of the stronger promoters (data not shown). In addition, the new hypersensitive sites (positions −6 to −3) in the case of the wild-type DNA can only be detected in the presence of CRP and RNA polymerase. Since this pattern of bands is the same for all three promoters, it must be characteristic of kinetically competent open complexes (Figure 8). The experiment presented in Figure 8 has been carried out with a concentration of CRP insufficient to activate fully the wild-type and Ps promoters. As a result, the intensities of the bands from positions −6 to −3 vary inversely to those of positions −13 to −10 as would be expected if the bands in this upstream reaction are not protected by polymerase unless they are bound in an "open" complex ready for transcription. Comparison of the relative intensity of these two families of bands provides a chemical method to determine the ratio of transcription initiation sites that are kinetically competent relative to those that are inactive.

Discussion

Single-crystal X-ray structures of synthetic oligonucleotides have indicated sequence-dependent conformational variability in DNA structure within a prevailing B-helix format (Dickerson et al., 1982). The potential importance of this structural heterogeneity would be to provide a mechanism by which a ligand that binds DNA might search for a specific recognition sequence. However, sensitive methods that can identify unique conformational features using naturally occurring DNAs are not available. These tools would be essential in characterizing unusual structures and determining if these structures have a characteristic function. The results presented in this paper indicate that $(OP)_2Cu^+$ is a reagent that preferentially attacks an important highly conserved region of a bacterial promoter.

This preference suggests that the conformation of the DNA in this region provides a more favorable structure for the binding of the coordination complex than other regions of the DNA and therefore must be structurally distinct. The unusual conformation, which can be detected by the small constrained coordination complex, is not particularly recognized by pancreatic DNase I. Because of the greater susceptibility of this region to $(OP)_2Cu^+$, changes generated in the Pribnow box region of the *lac* promoter by the Ps and UV-5 "up" mutations are readily detected by the coordination complex but not by DNase I. Although an additional band corresponding to

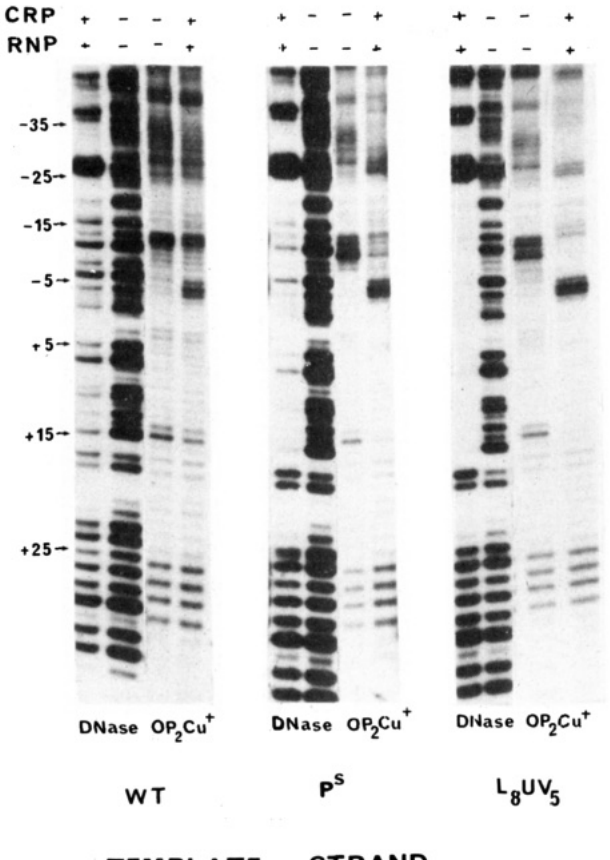


FIGURE 8: Comparison of the footprinting patterns of the three promoters in the presence of RNA polymerase and CRP. See Figure 7 for reaction conditions. Lanes as indicated.

position −9 disappears in the DNase I digest of these two promoters when a thymidine is substituted for a cytosine, this general region remains unexceptional in its reactivity to the enzyme. The relative intensity of this band shows no further change when the base at −8 changes from T to A in the conversion of the Ps mutation into UV-5. In contrast, the reactivity of the promoter to the coordination complex is sensitive to the mutational change. This hyperreactivity extends at least two positions from the mutational changes. This distance dependence excludes the intrinsic reactivity of the base as an explanation for the altered pattern and strongly indicates that the three-dimensional structure is the cause of the substantial change in reactivity.

The precise structural determinants in the promoter region that enhance the sensitivity of the promoters to the coordination complex as well as increase the affinity for RNA polymerase cannot be specified in terms of the helical parameters of B-DNA. Since pronounced changes are observed when C is changed to T in the conversion from wild type to Ps, the treatments of Calladine (1982) and Dickerson (1983), which do not distinguish between pyrimidines, cannot explain the altered reactivity.

The observation made by Drew & Travers (1984), on the basis of their studies of the $(OP)_2Cu^+$ cleavage of the *E. coli* tyr tRNA gene, that the positions of the cuts on each strand are staggered by three bases provides a useful guideline but may not be strictly obeyed. On the basis of their observations, these workers suggested that the complex binds in the minor groove and releases hydroxyl radicals to cause cleavage at opposing bases across the minor groove. Our work shows that there is an apparent correlation between the sites of nicking on the 5′-labeled sense strand and the 3′-labeled missense strand. It is most notable in the Pribnow box where both strands are rapdily cleaved. However, the sluggish reactivity of the position 24–27 region in the 3′-labeled strand relative to its reactivity in the sense strand is a notable exception. Both strands are efficient substrates of DNase I in this region. Our work shows that diffusible hydroxyl radicals, if formed, have a short or restricted diffusive path.

$(OP)_2Cu^+$ has proved to be an excellent probe to examine ligand binding in the region of the conserved promoter region. This can be attributed to its high specificity for the conserved sequence. It provides an ideal method for examining the binding of the protein to this region as has been demonstrated by the data presented in Figure 5. An unexpected advantage of $(OP)_2Cu^+$ as a reagent is its apparent ability to detect phenomena that are inaccessible to DNase I. For example, $(OP)_2Cu^+$ cleaves single-stranded DNA formed at the active site of RNA polymerase. It therefore detects kinetically competent origins of transcription. No hypersensitive sites can be observed in the position −6 to −3 region in the lower or sense strand unless the proteins essential for transcription are present. The reactivity of this enzyme-bound single-stranded DNA is not inconsistent with our previous results which have demonstrated that the artificial nuclease activity reacts more slowly with single-stranded DNA. Free single-stranded DNA does not present an appropriate substrate for the binding of the coordination complex. However, when the DNA is bound to an enzyme active site, the potential for binding of the coordination complex nearby clearly exists. The ability of the coordination complex to cleave both the B and A forms indicates that no single conformation is necessary for the chemical events responsible for phosphodiester bond cleavage.

Previous studies have emphasized the lack of specificity of the reagent with respect to base (Marshall et al., 1981). No feature of the individual bases appears essential for the reaction to proceed. Strong cleavage is observed at all base positions (e.g., position −13 C, position −12 A, position 24 T, and position 25 G). If reactions are carried out under single-hit conditions, a sequence-dependent set of hypersensitive regions is apparent. Our goal is to understand the structural determinants that create these hypersensitive regions. The artificial nuclease activity of $(OP)_2Cu^+$ might then be able to specify more precisely characteristic local structural variations in DNA.

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Activation of Porcine Factor VIII:C by Thrombin and Factor Xa†

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ABSTRACT: The activation of porcine factor VIII:C by thrombin and by factor Xa was studied by a chromogenic substrate assay and by sodium dodecyl sulfate–polyacrylamide gel radioelectrophoresis of ^{125}I-labeled factor VIII:C activation products. In the chromogenic assay, the kinetics of factor VIII:C dependent activation of factor X by factor IXa in the presence of calcium and phosphatidylserine/phosphatidylcholine vesicles were measured with *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine *p*-nitroanilide (S2222) as substrate. Substrate dependence of initial rates of the reaction at fixed factor IXa, factor VIII:C, lipid, and calcium obeyed Michaelis–Menten kinetics. At fixed factor IXa, factor X, lipid, and calcium the initial rates of the reaction varied linearly with lower factor VIII:C concentrations and plateaued at higher concentrations. The linear initial rate dependence formed the basis of a rapid, plasma-free assay of activated factor VIII:C. The activation of factor VIII:C by thrombin or factor Xa and the enzyme-independent rate of spontaneous inactivation were studied under conditions of excess enzyme. A model of the activation kinetics was developed and fit to the data by a nonlinear least-squares technique. From the model, the catalytic efficiencies (k_{cat}/K_m) of factor VIII:C activation by thrombin and factor Xa were 5.0×10^6 M^{-1} s^{-1} and 1.1×10^6 M^{-1} s^{-1}, respectively. By comparison with published values of the catalytic efficiencies of several other coagulation enzymes for various substrates, both thrombin and factor Xa are efficient enzymes toward factor VIII:C. Additionally, the model allows calculation of the relative cofactor activities of thrombin-activated factor VIII:C (factor VIII:Ca_{IIa}) vs. factor Xa activated factor VIII:C (factor VIII:Ca_{Xa}). The ratio of cofactor activities (VIII:Ca_{IIa}/VIII:Ca_{Xa}) is 3.0. This indicates that significantly more activity is generated when factor VIII is fully activated by thrombin than when factor VIII is fully activated by factor Xa. The formation of cofactor activity by both enzymes is closely paralleled by proteolysis of factor VIII:C polypeptides although thrombin and factor Xa give distinctly different products.

Activated factor VIII:C (antihemophilic factor) is a protein that is a cofactor for the activation of factor X by factor IXa and is necessary for normal hemostasis in vivo. Factor VIII:C circulates as a procofactor and requires activation for coagulant activity. Activation of human, bovine, and porcine factor VIII:C occurs after thrombin-catalyzed limited proteolysis of the procofactor molecule (Vehar & Davie, 1980; Hoyer & Trabold, 1981; Knutson & Fass, 1982; Fulcher & Zimmerman, 1982; Weinstein & Chute, 1984). Enzymes that are known to activate factor VIII:C in addition to thrombin include factor Xa (Davie et al., 1975; Vehar & Davie, 1980; Griffith et al., 1982; Hultin, 1982; Hultin & Jesty, 1982) and factor IXa (Rick, 1982). The relative catalytic efficiencies of these enzymes have not been reported, and the physiological activator(s) of factor VIII:C remains (remain) unknown. In this report we show that thrombin is a more efficient activator than factor Xa in a synthetic lipid system. Factor IXa is without effect at the concentrations used. Additionally, the thrombin-activated cofactor has more activity than the factor Xa activated cofactor. Factor Xa, however, is a relatively efficient catalyst when compared to other coagulation enzyme–substrate reactions, and since significant amounts of factor Xa formation may precede thrombin formation, factor Xa may participate in factor VIII:C activation in vivo.

Experimental Procedures

Materials. *N*-Benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine *p*-nitroanilide (S2222) was purchased from Kabi Diagnostica, Stockholm, Sweden. L-α-Phosphatidylcholine (PC), type III-E, and L-α-phosphatidylserine (PS) were purchased

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