Recognition of tetrahedral 1,10-phenanthroline–cuprous chelates by transcriptionally active complexes does not depend on the sequence of the promoter

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Background: The open complex formed at the initiation site of transcription within the active site of RNA polymerase is unique to actively transcribing genes and is thus an ideal target for the design of transcription inhibitors. Many redox-active tetrahedral cuprous chelates of 1,10-phenanthroline (OP) or derivatives cleave the single-stranded template, principally at sequence positions –7 to –3, whereas the redox-inactive tetrahedral cuprous chelate of 2,9-dimethyl-OP (neocuproine) blocks transcription, but does not cleave. The octahedral $(OP)_3$–Fe^{2+} chelate has no effect. Different promoters can give different cleavage patterns. We therefore searched for structural determinants of the open complex that are important in the cleavage reaction.

Results: Using site-directed mutagenesis, we systematically altered the nucleotides at the cleavage sites of the *Escherichia coli lac* UV-5–RNA polymerase open complex (positions –6 to –4), which are highly variable in *E. coli* promoters. Surprisingly, these changes had little effect on catalytic activity, on transcription inhibition by the cuprous complex of neocuproine and on the cleavage patterns generated by the cuprous chelates of OP derivatives. The scission pattern of a *lac* UV-5 promoter mutant in which the cleavage sites have the sequence of the *trp* EDCBA promoter is that of the *lac* UV-5 promoter, not the *trp* EDCBA promoter.

Conclusions: Nucleotide-specific interactions are not responsible for the observed cleavage patterns. The recognition of the tetrahedral OP chelate must be due to a specific structure of the single-stranded regions, determined by RNA polymerase–DNA interactions in the upstream regulatory region.

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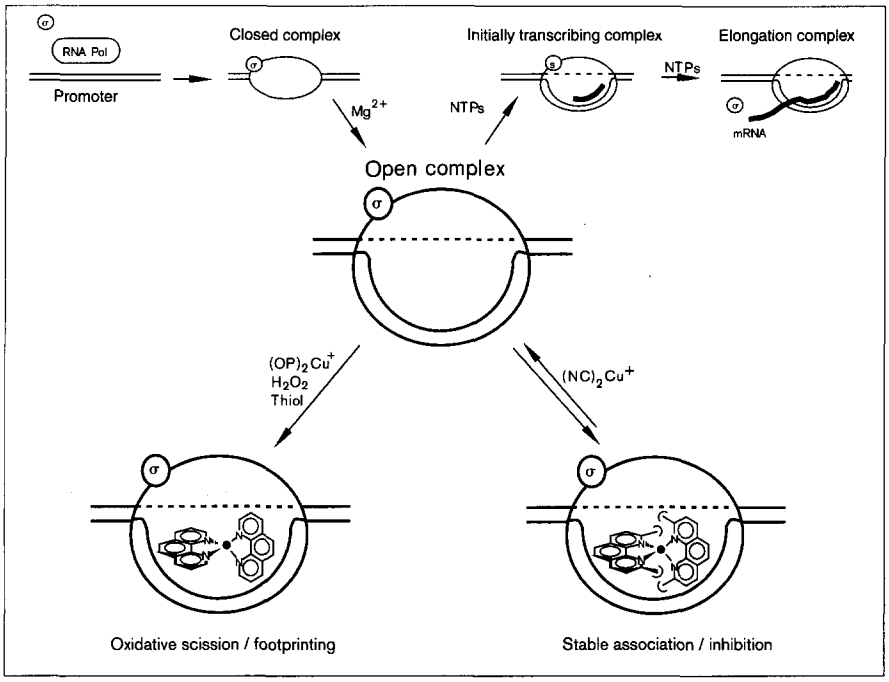
Introduction

The most novel reactivity described for the chemical nuclease *bis*(1,10-phenanthroline) copper(I) [$(OP)_2Cu^+$] is its hydrogen-peroxide-dependent cleavage of the template strand at positions –6 to –3 of *lac* promoters in catalytically competent open transcription complexes [1]. No scission of the non-template strand is observed. Other than the tetrahedral copper chelates of OP and its derivatives, no abiological molecule binds to the single-stranded DNA formed in this essential initial intermediate of transcription [2]. Although hyperreactivity of the nuclease at other prokaryotic and eukaryotic transcription start sites has been reported [3,4], the system that has been most extensively studied is the *Escherichia coli lac* UV-5 promoter and *E. coli* RNA polymerase. The dependence of the cleavage of the open complex on Mg^{2+} indicates that this reaction relies on the catalytic mechanism of RNA polymerase [5].

The kinetic mechanism for the scission of the *lac* promoters can be summarized by a two step scheme (Fig. 1), which indicates that the scission efficiency will depend on the binding affinity of the tetrahedral chelate and the hydrogen-peroxide-dependent cleavage step. Inhibition of the scission reaction by the redox-inert isostere *bis*(2,9-dimethyl-OP) copper(I) ($(NC)_2Cu^+$), but not the octahedral 3:1 complex between OP and Fe^{2+} supports the notion that a reversible complex forms between the chelate and the open complex [2].

Initial studies with the *lac* wild type (wt) promoter and the P_s and UV-5 variants, which differ at sequence positions –8 and –9, indicated that all three promoters gave an identical pattern of scission when they were competent for transcription [1]. For example, the *lac* wt promoter requires the presence of the catabolite activator protein (CAP) for RNA synthesis [6]. In the presence of CAP, but not in its absence, the same sites are observed to be hyperreactive in this promoter as in the *lac* UV-5 promoter, even though this promoter carries out CAP-independent transcription [1]. Since all three *lac* promoters have identical sequences in the region of cleavage, we have investigated the importance of these sequences in defining the cleavage efficiency of the open complex by preparing a series of

Figure 1



Schematic drawing of the binding of the chemical nuclease $(OP)_2Cu^+$ or the redox-inert isostere $(NC)_2Cu^+$ to the open transcription complex. Both compounds bind to the template strand of transcriptionally active complexes. In the presence of hydrogen peroxide, $(OP)_2Cu^+$ cleaves the template strand at positions −6 to −3. $(NC)_2Cu^+$ does not cleave the template strand.

mutants at positions −3 to −6. The cleavage reaction proceeds by deoxyribose oxidation at all four nucleotides [7–10]. If a mutation were to alter the cleavage pattern, this would suggest that specific interactions between the nucleotides and the chelate are essential for recognition of the tetrahedral chelate.

As an additional approach to explore the open-complex-specific reactivity of the chemical nucleases, we have investigated the cleavage of the *trp* EDCBA which regulates the transcription of enzymes involved in tryptophan biosynthesis in *E. coli* [11,12]. We expected that comparing the cleavage of the *trp* EDCBA promoter with that of the *lac* promoter would allow us to determine whether it is the nucleotide sequence at the site of cleavage or the upstream sequence of the promoter that is principally responsible for the binding of the tetrahedral chelates. Our results demonstrate that the reactivity of the open complex shows only a slight dependence on the nucleotide composition and sequence at the cleavage site.

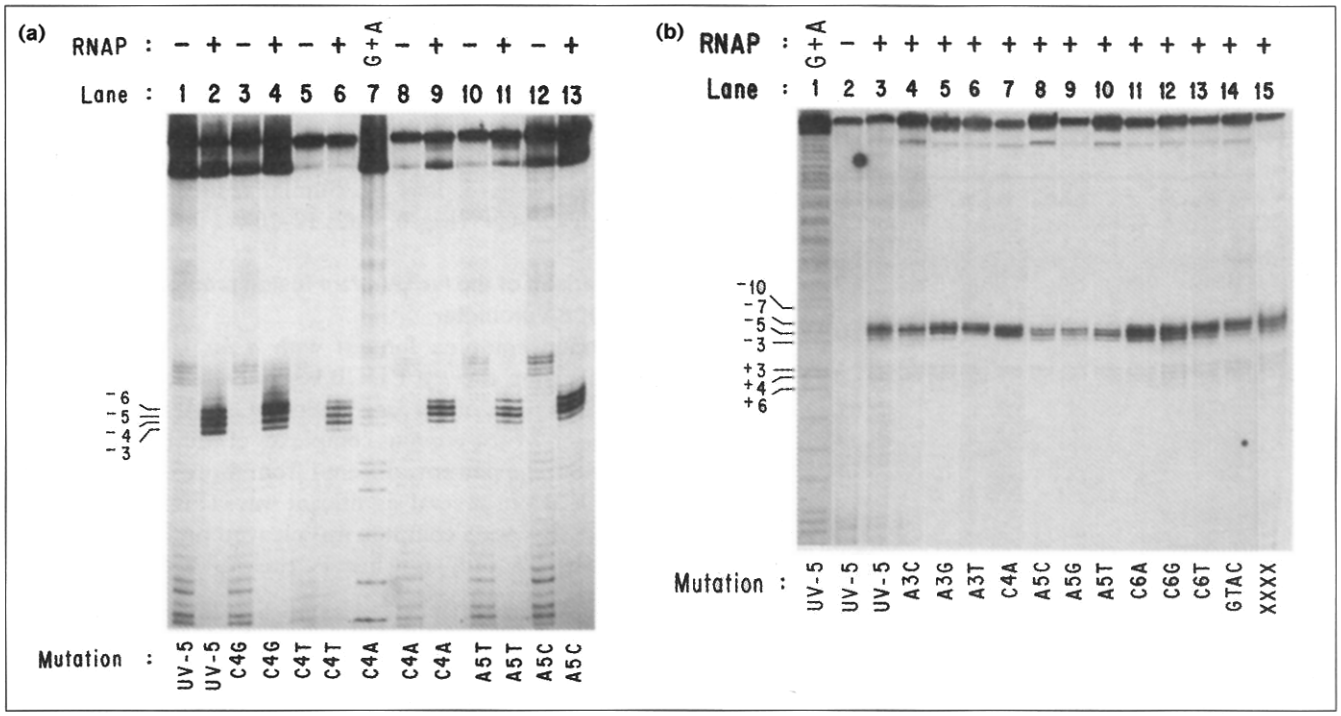
Results

Mutant *lac* promoters

Thirteen variants of the *lac* UV-5 promoter with mutations clustered at sequence positions −6 to −4 were prepared: 12 mutant promoters with single base-pair substitutions at these positions, and one in which the sequence of the *trp* EDCBA promoter from positions −6 to −3, 3'-CATG-5', was substituted for the corresponding sequence of the *lac* UV-5 promoter, 3'-CACA-5'. Gel retardation and transcription assays were used to compare the promoter strength of the variants. For the gel retardation assay, each promoter was 5'-end labeled with ^{32}P on the template strand and was incubated with an excess of RNA polymerase for 20 min. at 37 °C. Following the disruption of nonspecific association of the polymerase with the promoters using heparin, 90 % of the radioactive promoter remained in a retarded complex with polymerase. An assay of transcription revealed no change in catalytic activity. The nucleolytic activity of the cuprous chelates of OP and its derivatives provides a convenient assay for alteration of the binding specificity of the open complex associated with the mutational changes. Results with the chelates of 5-phenyl-OP (5ϕOP) and 3,4,7,8-tetramethyl-OP (Me_4OP) are presented in Figure 2. Although minor perturbations in the scission patterns are apparent for the symmetrically bulky $(Me_4OP)_2Cu^+$, no difference is apparent with $(5\phi OP)_2Cu^+$. There is no obvious relationship between a specific type of base change and altered scission at a particular position.

The redox-inactive cuprous chelates of 2,9-dimethyl-OP (neocuproine, NC) are dead-end inhibitors of transcription, which bind to the single-stranded DNA of open complexes and downstream elongation complexes [13–15]. Their binding specificity mirrors the interaction of the redox-active isosteres. No significant differences were observed in the concentrations of $(NC)_2Cu^+$ needed for 50 % protection of the mutant promoters from scission by $(OP)_2Cu^+$ in their respective open complexes (data not shown). Similarly, inhibition of the synthesis of full-length

Figure 2



Cleavage of the open complexes of the *lac* UV-5 mutant promoters by the cuprous chelates of 5ϕOP and and Me_4OP shows no correlation between a specific base change and altered scission at a particular position. **(a)** $(5\phi OP)_2Cu^+$ at 40 μM. RNAP = RNA polymerase. **(b)** $(Me_4OP)_2Cu^+$ at 15 μM.

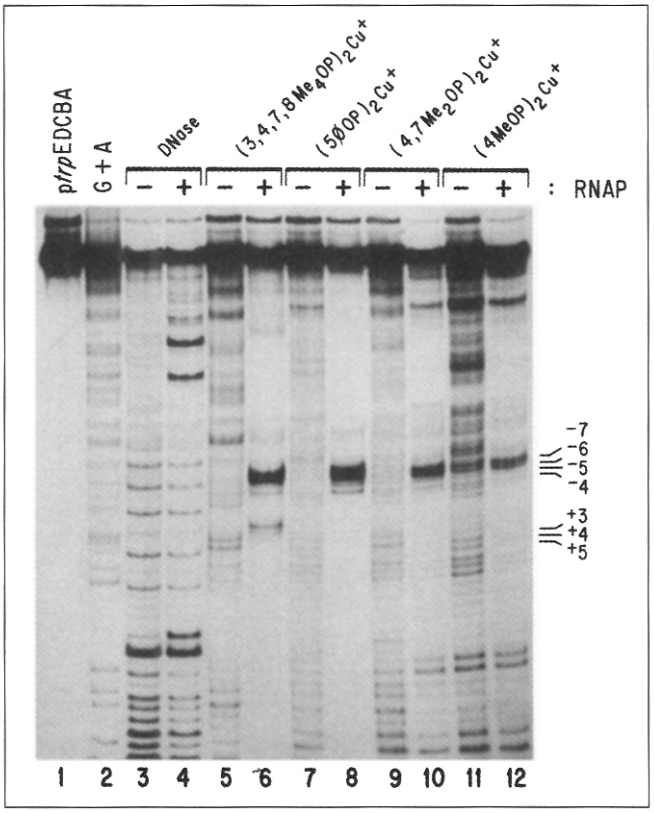
transcripts by $(NC)_2Cu^+$ exhibited similar concentration dependencies with all the mutant promoters.

In contrast to the independence of the cleavage pattern to mutational changes at positions –6 to –3, the cuprous chelates prepared with variously substituted phenanthrolines cleave the open complex composed of *lac* UV-5 and its variants differently [16]. These results suggest that the open complex of the mutant promoters can still differentiate between closely similar tetrahedral structures. This conclusion is reinforced by the dramatically different open-complex-specific reactivity of the cuprous chelates prepared with 5ϕOP, 5ϕCONHOP and $5\phi CONHCH_2OP$. The chelate $(5\phi CONHCH_2OP)_2Cu^+$ cleaves B-DNA more efficiently than $(5\phi OP)_2Cu^+$ or $(5\phi CONHOP)_2Cu^+$. Neither $(5\phi CONHCH_2OP)_2Cu^+$ nor $(5\phi CONHOP)_2Cu^+$ cleave the single-stranded template DNA within the open complex, however, although $(5\phi OP)_2Cu^+$ does cleave efficiently. Similar results are observed with the series $(5\text{-}CH_3OP)_2Cu^+$, $(5\text{-}CH_3CONHOP)_2Cu^+$ and $(5\text{-}CH_3CONHCH_2OP)_2Cu^+$. Open complex scission is only observed with $(5\text{-}CH_3OP)_2Cu^+$. Cuprous chelates of OP bearing carboxamide derivatives at C-5 do not react with open complexes of any of the *lac* UV-5 derived promoters, although their redox-inactive neocuproine analogs remain inhibitory [16].

Cleavage of the open complex of the *trp* EDCBA promoter

Like *lac* UV-5, the *trp* EDCBA promoter does not require activator proteins for transcription and therefore provides a useful comparison for investigating the specificity of the interaction of OP chelates with complexes of RNA polymerase and its DNA substrates [11]. The cuprous chelates of several OP derivatives were used to footprint the *trp* EDCBA open complex. The copper chelates of Me_4OP, 5ϕOP, 4,7-dimethyl-OP and 4-methyl-OP cleave the template strand of the open complex predominantly at sites –6 and –7 (Fig. 3). In the case of Me_4OP and, to a lesser extent, with 5ϕOP, additional scission is observed at sites +3, +4 and +5. The predominant sites of scission of the *lac* UV-5 open complex are at positions –3 to –6. An additional important difference between the two promoters is that two- to five-fold higher concentrations of the cuprous complex of Me_4OP and 5ϕOP are required to effect equivalent scission yields with *trp* EDCBA than are required with *lac* UV-5 under conditions where gel-retardation assays indicate that both are completely bound by RNA polymerase. As with the *lac* UV-5 promoter, no enzyme-induced hypersensitive scission is observed on the non-template strand (data not shown). $(NC)_2Cu^+$ protects the open complex from scission (Fig. 4). Since the scission pattern of free DNA does not reappear upon increased concentration of $(NC)_2Cu^+$, this ligand does not

Figure 3



Footprinting of the open complex of the *trp* EDCBA promoter by several derivatives of OP-copper shows additional sites of cleavage compared to the *lac* UV-5 promoter. Control lanes 1–4: the *trp* EDCBA promoter fragment, Maxam–Gilbert G+A sequencing ladder, and DNase footprint in the absence and presence of RNA polymerase, respectively. Lanes 5 and 6: Me_4OP copper footprinting of *trp* EDCBA in the absence and presence of RNA polymerase, respectively. Lanes 7 and 8: 5ϕOP copper footprinting of *trp* EDCBA in the absence and presence of RNA polymerase, respectively. Lanes 9 and 10: 4,7-dimethyl-OP–copper footprinting of *trp* EDCBA in the absence and presence of RNA polymerase, respectively. Lanes 11 and 12: 4-methyl-OP copper footprinting of *trp* EDCBA in the absence and presence of RNA polymerase, respectively. The concentrations of R-OP and Cu^+ were 50 μM and 10 μM, respectively.

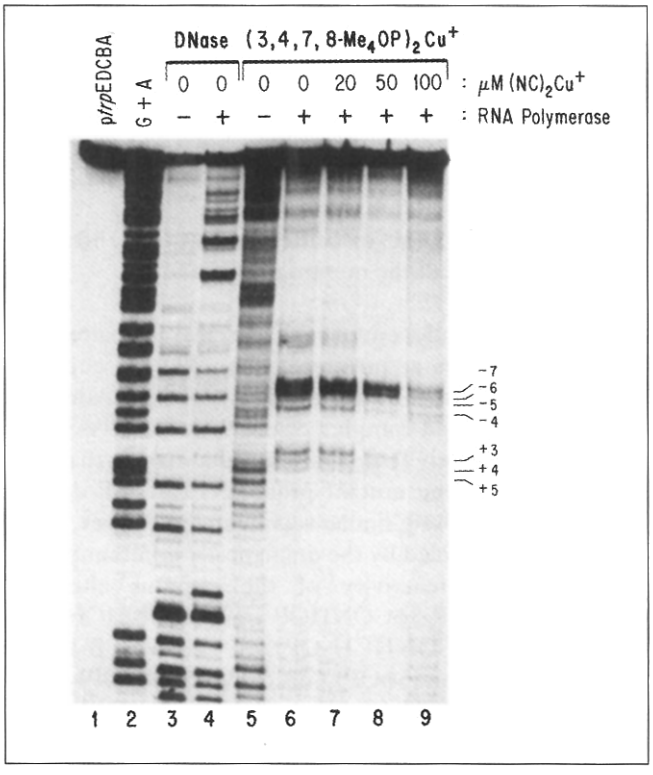
displace RNA polymerase under these conditions. Both $(NC)_2Cu^+$ and two phenyl derivatives, $(4\phi NC)_2Cu^+$ and $(5\phi NC)_2Cu^+$, which gave similar protection, were inhibitors of full length transcription.

In addition to the oxidative footprinting of the open complex, the initially transcribing and initially elongating complexes of *trp* EDCBA are effectively footprinted by the cuprous chelates of both 5ϕOP and Me_4OP (Fig. 5). No progression is observed in the presence of ApA, which represents the formation of the first phosphodiester bond (Fig. 5, lane 7), or when ATP is the initiating nucleotide. When the condensation of GTP with the initiating dinucleotide ApA results in ApApG, the sites of scission shift from –6 and –7 to –4 and –5 (Fig. 5, lane 8). The addition of UTP permits the synthesis of the 5-base oligonucleotide (5-mer) ApApGpUpU. Concomitantly, the scission pattern shifts by one base with scission predominantly observed at –3 and –4. Finally, addition of CTP results in the synthesis of up to a 6-mer, with the sequence ApApGpUpUpC, although no additional shift is observed (Fig. 5, lane 9). Similar results are obtained using 5ϕOP-Cu^+ (Fig. 5, lanes 12–16).

Comparison of the *lac* UV-5/*trp* fusion promoter with the *trp* EDCBA promoter

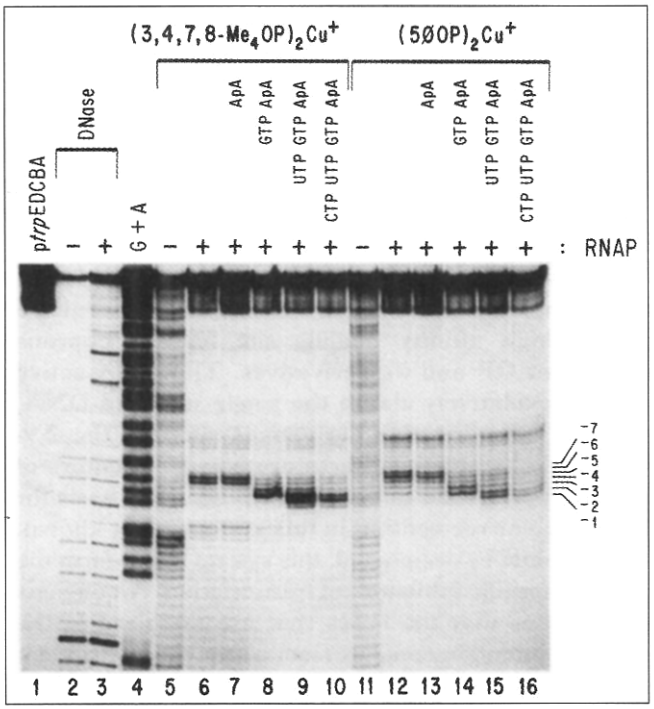
The open complex formed with a *lac* UV-5 promoter incorporating the *trp* EDCBA sequence from –6 to –3, CATG, in place of the *lac* sequence, CACA, was subjected to scission by the cuprous complexes of Me_4OP and 5ϕOP. The cleavage patterns differed from those observed with *trp* EDCBA in several significant ways (Fig. 6). First, the *trp* EDCBA open complex was cleaved predominantly at positions –7 and –6 whereas the *lac* UV-5/*trp* fusion

Figure 4



$(NC)_2Cu^+$ protects the *E. coli* RNA polymerase–*trp* EDCBA open complex from scission by $(Me_4OP)_2Cu^+$. Control lanes 1–4: the *trp* EDCBA promoter fragment, Maxam–Gilbert G+A sequencing ladder, and DNase footprint in the absence and presence of RNA polymerase, respectively. Lanes 5 and 6: $(Me_4OP)_2Cu^+$ footprinting of *trp* EDCBA in the absence and presence of RNA polymerase respectively. Lanes 7–9: Footprinting in the presence of increasing concentration of inhibitor. The concentrations of Me_4OP and Cu^+ were 50 μM and 10 μM, respectively.

Figure 5



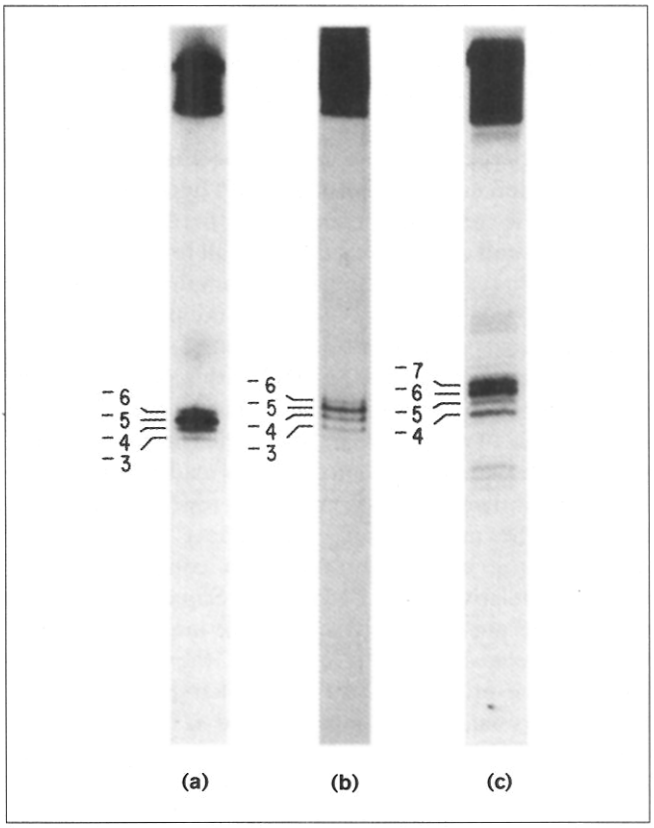
The open complex (OC), initially transcribing complex (ITC), and initially elongating complex (IEC) of the *trp* EDCBA promoter are effectively footprinted with $(Me_4OP)_2Cu^+$ (lanes 5–10) and $(5\phi OP)_2Cu^+$ (lanes 11–16). Control lanes 1–4: the *trp* EDCBA promoter fragment, Maxam–Gilbert G+A sequencing ladder and DNase footprint in the absence and presence of RNA polymerase, respectively. Lanes 5 and 6: $(Me_4OP)_2Cu^+$ footprint of the *trp* EDCBA promoter in the absence and presence of RNA polymerase, respectively. Lane 7: footprint of the OC in the presence of ApA. Lane 8: footprint of the ITC after synthesis of a nascent 3-mer. Lane 9: footprint of the ITC after synthesis of a nascent 5-mer. Lane 10: footprint of the ITC or the IEC after synthesis of a nascent 6-mer. Lanes 11 and 12: $(5\phi OP)_2Cu^+$ footprint of the *trp* EDCBA promoter in the absence and presence of RNA polymerase, respectively. Lane 13: footprint of the OC in the presence of ApA. Lane 14: footprint of the ITC after synthesis of a nascent 3-mer. Lane 15: footprint of the ITC after synthesis of a nascent 5-mer. Lane 16: footprint of the ITC or the IEC after synthesis of a nascent 6-mer.

promoter exhibited the same four cleavage sites from position –6 to–3 as the *lac* UV-5 promoter itself. Second, scission of the promoter at sequence positions +3 to +5 was observed with *trp* EDCBA but not the hybrid promoter. Finally, the concentration of chelates required to observe open complex-specific scission for *trp* EDCBA was higher than that for scission of the hybrid promoter. These results indicate that the open complex of the *lac* UV-5 derived promoter has a higher affinity for the tetrahedral chelates than that formed with *trp* EDCBA.

Discussion

The tetrahedral cuprous chelates of OP react with DNA and RNA via a non-covalent intermediate [17]. This appears to be the case whether the substrate is B-DNA, A-DNA, single-stranded bulges and loops of RNA, or the single-stranded region of DNA in transcriptionally competent complexes. In this study, we have investigated whether specific base–chelate interactions were responsible for the ability of the tetrahedral chelates to bind to the open complex by making systematic mutations at the sites of scission. The results clearly indicate that these interactions do not govern the cleavage pattern. The mutant *lac* UV-5 promoters show little variation in their cleavage patterns despite considerable variation in sequence. This finding is consistent with the ability of the oxidative chemical nucleases to follow the progression of the transcription bubble during RNA synthesis through regions of diverse sequence with both the *lac* UV-5 and *trp* EDCBA promoters [15,16,18]. Despite the apparent insensitivity of the scission pattern to nucleotide sequence, the open complex does exhibit specificity for the substituent on the 5-position. Most remarkable is the barely observable reactivity of $(5\phi CONHCH_2OP)_2Cu^+$ with the open complex even though it is more efficient at

Figure 6



The *lac* UV-5/*trp* fusion promoter is cleaved by $(Me_4OP)_2Cu^+$ in the same pattern as the *lac* UV-5 promoter. **(a)** Cleavage of the open complex of the lac UV-5 promoter by the nuclease at 15 μM. **(b)** Cleavage of the open complex of the *lac* UV-5/*trp* fusion promoter by the nuclease at 15 μM. **(c)** Cleavage of the open complex of the *trp* EDCBA promoter by the nuclease at 10 μM.

cleaving B-DNA than is $(5\phi OP)_2Cu^+$. Similarly, $(5\text{-}CH_3\text{-}CONHCH_2OP)_2Cu^+$ does not exhibit observable reactivity with the open complex although it cleaves B-DNA with comparable efficiency to $(5\text{-}CH_3OP)_2Cu^+$. Molecular recognition by the open complex apparently discriminates against carboxamide groups linked to the phenanthroline ligand, although a molecular explanation for this phenomenon is not available.

Two distinct types of open-complex-specific chemical reagents have now been identified. The first are base-specific reagents such as permanganate [19], dimethylsulfate [20] and diethylpyrocarbonate [21]. They are small, readily diffusible and nucleotide-specific. Permanganate reacts with thymidines, dimethyl sulfate reacts with guanosine and unpaired cytosines, and diethylpyrocarbonate reacts with adenine. Permanganate and dimethylsulfate will react with unpaired bases on either DNA strand if their approach is not sterically hindered. Analysis of the kinetics of modification of the open complex of *lac* UV-5 with diethylpyrocarbonate indicates that at low concentrations (2 mM) the reaction is second order, but at higher concentrations (20 mM) the reaction becomes independent of the modification reagent suggesting that a unimolecular isomerization controls the overall rate. As with the cuprous chelates of phenanthroline, diethylpyrocarbonate does not react with the non-template strand [21].

The second type of open-complex-specific reagents are the tetrahedral cuprous chelates of OP. In contrast to the base specific reagents, they cause oxidation of the deoxyribose and are thus reactive with all four nucleotides [9,10]. They react with the open complex at concentrations of 20 μM or lower. Cleavage of the phosphodiester backbone occurs spontaneously following oxidative attack on the ribose; treatment of the DNA with piperidine is not essential for strand scission. As indicated by the affinity of the redox-inactive cuprous chelates of neocuproine for the open complex and the inability of other oxidative reagents to exhibit similar reactivity, they bind reversibly prior to reaction. The modification sites reflect not only the affinity of the chelate for the open complex but its orientation relative to the two strands. Since the cleavage reaction does not proceed by a diffusible intermediate, the cuprous chelates may not cleave if they bind in an unproductive orientation. In the prokaryotic examples studied thus far, the template strand is preferentially reactive but this finding may not be generally applicable to all transcription units. For both types of reagents, negative results do not provide evidence for the lack of formation of the open complex. Both will fail to react if some component of the transcription machinery sterically blocks access of the reagents to the DNA.

Since the ligand binding properties of the open complex of the hybrid *lac/trp* promoter and *trp* EDCBA are not the same, interaction of upstream regions with RNA polymerase appears crucial in shaping the binding site formed upon transcriptional activation. If the binding determinants of this novel host/guest system can be identified [22], it may be possible to design effective mechanism-based transcription inhibitors that share structural determinants of these chelates but will not be coordination complexes.

Significance

Formation of a single-stranded region of DNA in the open complex at the start of transcription generates a unique, high affinity binding site for the cuprous chelates of OP and its derivatives. The redox-active chelates oxidatively cleave the single-stranded DNA, whereas redox-inactive derivatives such as the 2,9-dimethyl substituted isosteres are potent inhibitors of transcription but do not cause cleavage. The basis for the molecular recognition in this system is not known, but, if it could be deciphered, this system might form the basis for specific inhibitors of transcription. An obvious possibility is that the bases that are unpaired in the promoter initiation complex form a sequence-dependent structure that is important for initial recognition of the tetrahedral OP chelates. In this study, we have examined this possibility using site-directed mutagenesis of the *E. coli lac* UV-5 promoter, and have shown that specific base-chelate interactions in the hypersensitive region (–6 to –3) are not responsible for binding. Since a *lac* UV-5 promoter that has the same sequence as the *trp* EDCBA promoter in the region of scission retains the *lac* UV-5 pattern of cleavage, the sequence of the upstream regulatory region must be involved in shaping the binding pocket. Although the factors that govern the binding affinity of the tetrahedral chelates for open and elongation complexes remain to be determined, the single-stranded DNA formed at the initiation of transcription is an inviting target for the design of gene-specific inhibitors.

Materials and methods

Site-directed mutagenesis of the lac *UV-5 promoter*

Each mutant promoter, spanning the region from –96 to +45, was prepared by PCR amplification using a lac UV-5 promoter template contained in a 206 bp EcoRI restriction fragment of a pUC-derived plasmid. DNA primers were synthesized on a Pharmacia Gene Assembler, deprotected in concentrated aqueous ammonia and purified on a 12 % denaturing polyacrylamide gel. The primer of the non-template strand, corresponding to positions –96 to –77, was 5'-AGCGGGCAGTGAGCGCAACC-3'. The primer of the template strand contained the desired base changes but otherwise corresponded to positions –16 to +45 of the lac UV-5 promoter. The mutant promoters generated by PCR (30 iterative, 2-min cycles with an annealing temperature of 60 to 69 °C; *Taq* DNA polymerase and the nucleotide triphosphates from Gibco BRL) were purified on a 8 % non-denaturing polyacrylamide gel, eluted from the gel using 0.5 M KOAc, precipitated with ethanol, washed with 70 % ethanol, resuspended in water and finally passed through a Sephadex G-50 spin column. The template strand of each promoter was sequenced (using a 20-mer primer) by the

dideoxy method (USB Sequenase DNA Sequencing Kit). Radiolabeled promoters were prepared by 5'-end labeling the template primers (5–50 pmol) used in the PCR reactions with ^{32}P using $\gamma^{32}P$-ATP (ICN, 2000 to 7000 Ci mmol^{-1}) and T4 polynucleotide kinase (Gibco BRL).

The trp *EDCBA promoter*

A 155-bp fragment containing the trp EDCBA promoter and spanning the region from −106 to +49 was prepared by PCR and purified and radiolabeled as described above.

Gel retardation assays

Each ^{32}P-labeled *lac* promoter (~200 fmol) in 10 μl water was incubated with 10 μl of either 2X buffer (80 mM pH 8 TrisHCl, 200 mM KCl, 6 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM dithiothreitol, 200 μg ml^{-1} BSA, 10 % glycerol) or *E. coli* RNA polymerase (Pharmacia, ~4 pmol) freshly diluted into 2X buffer for 10 min at 37 °C. Next, heparin (2 μl, 0.75 mg ml^{-1}) was added to disrupt nonspecific binding of the polymerase to the promoter [5]. One minute later, 25 % Ficoll/0.1 % bromophenol blue (6 μl) was added, and the samples were loaded onto a non-denaturing 8 % polyacrylamide gel. The efficiency of promoter-RNA polymerase complex formation was estimated by scanning an autoradiogram of the gel with a LKB Ultroscan XL laser densitometer.

Footprinting the open complexes with $(OP)_2Cu^+$ *and its derivatives*

Each ^{32}P-labeled *lac* or *trp* promoter (~200 fmol) was incubated with *E. coli* RNA polymerase (~2 pmol) in 10 μl buffer (40 mM TrisHCl, 50 mM KCl, 10 mM $MgCl_2$) at 37 °C for 10 min (*lac* promoters) or 45 min (*trp* promoters). Next, 1 μl of a freshly prepared stock solution of the *bis* cupric complex of OP or its derivatives (4ϕOP, 5ϕOP or Me_4OP) in 95:5 water:ethanol was added. The ratio R-OP:$CuSO_4$ in the stock solutions was either 2.1:1 (*lac* promoters) or 5:1 (*trp* promoters). The cleavage reaction was initiated by adding 1 μl of 58 mM mercaptopropionic acid (MPA), and the reaction was quenched 4 min later by adding 3 μl of 40 mM neocuproine in ethanol. (The cleavage by DNase at 0.83 μg μl^{-1} was allowed to proceed for 1 min and quenched with 1 μl of 0.5 M EDTA.) Calf thymus DNA (1 μl at 1 mg ml^{-1}) was added, and the DNA was precipitated with ethanol (50 μl) or isopropanol (70 μl). The reaction products were resuspended in loading buffer (80 % aqueous formamide with 0.3 % SDS, 150 mM NaOH, 15 mM EDTA, 0.1 % bromophenol blue and 0.1 % xylene cylanol), heated to 90 °C for 5 min, and electrophoresed on a 12 % denaturing polyacrylamide gel.

Protection of the open complex from the OP-Cu footprinting reaction by $(NC)_2Cu^+$ was assayed by modifying the procedure described above as follows. After formation of the open transcription complex, 1 μl of a stock solution of $(NC)_2Cu^+$ was added to each reaction, followed by the MPA. After 4 to 10 min, $(OP)_2Cu^+$ (or one of the other three redox-active chelates) was added. The reactions were quenched 4 min later with neocuproine and treated as described above.

In vitro *transcription*

Each promoter (1 pmol) was incubated with *E. coli* RNA polymerase (2 pmol) in 10 μL buffer (40 mM pH 8 TrisHCl, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5 % glycerol) for 10 min at 37 °C. Transcription was initiated by addition of 1 μl of a mixture containing 1 mM ATP, 1 mM CTP, 1 mM UTP and 200 μM GTP with a specific activity of ~6 Ci mmol^{-1} $\alpha^{32}P$-GTP. After 45 min at 37 °C, transcription was halted by adding 40 μl of deionized formamide containing 23 mM EDTA, 0.1 % SDS, 0.1 % bromophenol blue and 0.1 % xylene cylanol. The reaction mixture was loaded directly onto a 20 % denaturing polyacrylamide gel, and the gel was submitted to autoradiography.

Inhibition of transcription by $(NC)_2Cu^+$

The promoter-polymerase complexes were formed as described above. Then, 1 μl of a series of 10X solutions of $(NC)_2Cu^+$, $(4\phi NC)_2Cu^+$ or $(5\phi NC)_2Cu^+$ (0–1000 μM), serially diluted from a freshly prepared 1 mM stock solution also containing 8 mM ascorbate, was added, and the incubation was continued for 10 min. Next, the mixture of nucleotide triphosphates was added, and the reactions were treated as described above.

Footprinting initially transcribing and elongating complexes of the trp *EDCBA promoter*

For each 10 μl-reaction with the indicated partial complement of nucleotide triphosphates, 1 μl of a 10X stock solution of each of the appropriate nucleotides was evaporated to dryness in a microfuge tube: either 50 μM ApA or 100 μM ATP was used to initiate transcription; the other nucleotides were added to give final concentrations of 100 mM. Then a mixture of the ^{32}P-labeled *trp* promoter (~0.02 μM) was incubated with *E. coli* RNA polymerase (~2 μM) in buffer (40 mM TrisHCl, 50 mM KCl, 10 mM $MgCl_2$) at 37 °C for 10 min. Next, a 10 μl-aliquot of this mixture was added to each of the microfuge tubes containing the partial complements of nucleotide triphosphates. Transcription was allowed to proceed for 45 min before footprinting with the cuprous chelate of 5ϕOP or Me_4OP as described above.

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