Inhibitors of *Escherichia coli* RNA Polymerase Specific for the Single-Stranded DNA of Transcription Intermediates. Tetrahedral Cuprous Chelates of 1,10-Phenanthrolines†

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ABSTRACT: Single-stranded DNA of the *lac*UV-5 promoter formed at the active site of *Escherichia coli* RNA polymerase during transcription is specifically cleaved by the redox active tetrahedral cuprous chelates of 1,10-phenanthroline and its derivatives. The cleavage sites are observed in the open, initiating, and elongating complexes. Redox-inert, tetrahedral cuprous chelates of neocuproine (2,9-dimethyl-1,10-phenanthroline) and its 5-phenyl and 4-phenyl derivatives protect the template strand of DNA from scission within these steady state intermediates and inhibit transcription. Although these cuprous chelates of neocuproine bind at multiple sites within three distinct enzyme intermediates, the highest affinity site is within the elongation complex. The I_{50} of 5 μM for the 2:1 5-phenylneocuproine cuprous complex ($(5\phi NC)_2Cu^+$) in runoff transcription therefore primarily reflects its interaction with this intermediate. The neocuproine cuprous chelates are novel transcription inhibitors because they bind to single-stranded DNA sites generated during the course of catalysis by RNA polymerase.

Most low molecular weight inhibitors of transcription are high-affinity ligands for B-DNA which associate with DNA by two, well-studied interactions: minor groove binding or intercalation or both (Bailly et al., 1992; Dervan & Becker, 1978; Kopka et al., 1985a,b; Lehrman & Crothers, 1977; Waring, 1965). These ligands inhibit the processive catalysis of RNA polymerase by destabilizing the binding of the enzyme to DNA (Mazumder et al., 1994). Two antibiotics: streptolydigin and rifampicin are exceptions and bind to RNA polymerase and block transcription initiation without dissociating the enzyme from the promoter (Jin et al., 1988; Kumar & Chatterji, 1990; Severinov et al., 1993). Our work with the chemical nuclease 1,10-phenanthroline copper has led to the discovery that the tetrahedral cuprous chelates of 1,10-phenanthroline (OP) and 2,9-dimethyl-1,10-phenanthroline (neocuproine = NC) bind to single-stranded DNA formed at the active site of RNA polymerase during catalysis.

Messenger RNA synthesis by DNA-dependent RNA polymerases (E) is a multistep enzymatic reaction which includes at least five discrete kinetic intermediates: (a) the closed complex (CC); (b) the open complex (OC); (c) the initially transcribing complex (ITC); (d) the initially elongating complex (IEC); and e) the elongation complex (EC) shown in Scheme 1 (Carpousis & Gralla, 1985; Gralla, 1991; Krummel & Chamberlin, 1992; McClure, 1985; Chamberlin, 1992−1993). In the transition from the CC to the OC, the double-stranded DNA (P) is "melted" into regions of single-stranded DNA to permit the faithful transcription of the template strand of DNA in an isomerization that depends on Mg^{2+} (Roe et al., 1985; Kuwabara & Sigman, 1987). In the case of eucaryotic systems, this protein induced transition in the DNA structure requires various transcriptional activating factors (Wang et al., 1992; Buratowski et al., 1989; Hori & Carey, 1994). Incorporation of four or fewer nucleotides results in little or no progression of polymerase along the DNA, while incorporation of 5−8 nucleotides is accompanied by a progression of the polymerase along the DNA resulting in the ITC. Incorporation of 9−11 nucleotides results in further progression of the polymerase along the DNA, giving rise to the IEC. This intermediate is characterized by both dissociation of the σ-subunit required for initiation and by loss of rifampicin sensitivity (Carpousis & Gralla, 1985; Gralla, 1991; Krummel & Chamberlin, 1989, 1992).

These transitions can be monitored with chemical modification reagents such as dimethyl sulfate and permanganate that react with the single-stranded bases of both DNA strands (Gralla, 1985; Kirkegaard et al., 1983; Sasse-Dwight & Gralla, 1988,1989). In contrast, the single-stranded template DNA of the OC of the *lac*UV-5 *Escherichia coli* RNA polymerase is exclusively and efficiently cleaved by the oxidative chemical nuclease, 1,10-phenanthroline copper $(OP)_2Cu^+$ (Spassky & Sigman, 1985; Kuwabara & Sigman, 1987). Unlike other footprinting reagents which are base specific either modifying the N7 of G and the O4 of C in the case of alkylating agents like DMS, or the C5 and C6 of T in the case of oxidizing agents like $KMnO_4$, $(OP)_2Cu^+$ reacts with deoxyribose (Kuwabara et al., 1986; Goyne & Sigman, 1987).

The cuprous chelate of 5-phenyl-1,10-phenanthroline (5ϕOP) is especially reactive with the template strand of the open complex formed with the *lac*UV-5 promoter (Thederahn

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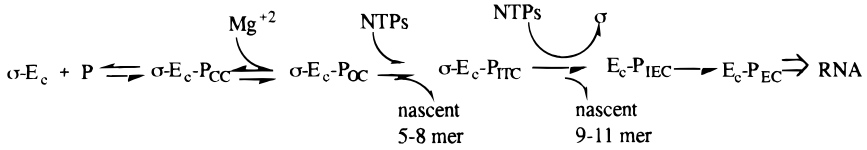
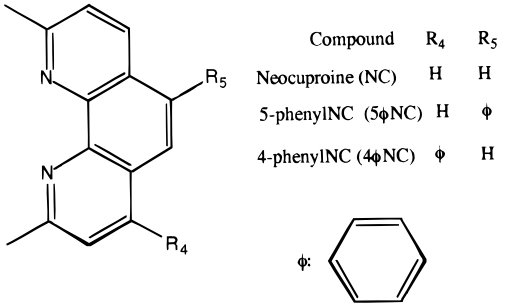
Scheme 1



et al., 1990). This chelate, as well as $(OP)_2$ Cu^+, has been used to map the initial progression of the transcription bubble upon addition of an incomplete complement of nucleotides (Spassky, 1986; Thederahn et al., 1990). In all of the intermediate complexes, the strong scission sites of $(OP)_2Cu^+$ and its derivatives lagged the sequence position at which the nascent phosphodiester bond was synthesized. The footprint of elongation complexes was obtained when 3-*O*-methylCTP was added as the sole source of cytosine since this nucleotide does not appear in the mRNA until position 10 of the sequence (Thederahn et al., 1990). In this case, the hyper-reactive sites appeared at sequence positions +11 and +12 in advance of the site of phosphodiester bond synthesis (Thederahn et al., 1990). The hyper-reactivity of the open complex to $(OP)_2Cu^+$ has been attributed to the generation of a high-affinity binding site for this tetrahedrally coordinated hydrophobic cation. This hypothesis has been supported by the demonstration that the redox-inactive isosteric 2:1 neocuproine−cuprous chelate [$(NC)_2Cu^+$; NC = 2,9-dimethyl-1,10-phenanthroline] is an effective inhibitor of transcription from the *lac*UV-5 promoter and blocks the scission of the open complex by $(OP)_2Cu^+$ (Mazumder et al., 1993a) (Scheme 2).

In this communication, the mechanism of inhibition of transcription from *lac*UV-5 by derivatives of $(NC)_2Cu^+$ has been examined. These studies have been undertaken because $(NC)_2Cu^+$ is a global inhibitor of transcription blocking RNA synthesis from both eucaryotic and procaryotic promoters (Perrin et al., 1994). For example, $(NC)_2Cu^+$ inhibits transcription from several bacterial promoters, the HIV-LTR promoter, and TATA-containing and TATA-less initiator (Inr) promoters, as well as a synthetic eucaryotic TATA promoter derived from the adenovirus E4 promoter containing multiple binding sites for the chimeric viral-yeast transcriptional activator, GAL4-VP16 (Perrin et al., 1994). Our results indicate that the cuprous chelates of neocuproine and its derivatives bind not only to the open complex of *lac*UV-5 as initially demonstrated (Mazumder et al., 1993a) but also to other transcriptional intermediates as well. These conclusions are based on the lower I_{50} observed for runoff transcription relative to abortive synthesis and the protection of the single-stranded DNA of the various steady state intermediates from scission by $(OP)_2Cu^+$ and its derivatives.



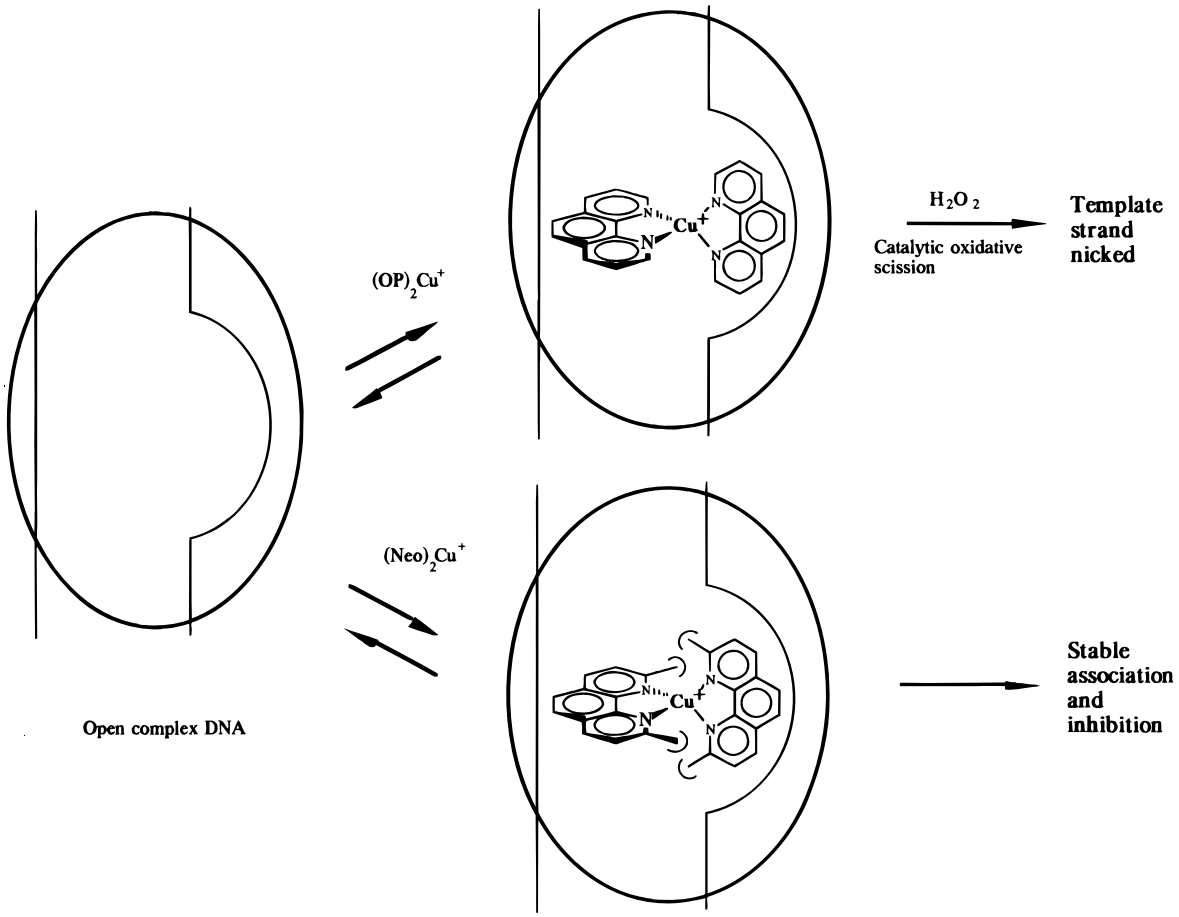
EXPERIMENTAL PROCEDURES

Materials. Phenanthroline derivatives were obtained from G. F. Smith Chemical Company. Organic reagents, 1,4-diazabicyclo[2.2.2]octane (DABCO), methyllithium, and MnO_2 were obtained from Aldrich. Reagent grade organic solvents were obtained from Fisher. A 203 bp *Eco*RI fragment containing the *lac*UV-5 promoter is overexpressed on a pUC-derived high copy number plasmid, restricted, and purified by 8% PAGE. This fragment was used for inhibition of full-length transcript. For footprinting studies, it was then dephosphorylated with calf intestinal phosphatase and 5′ labeled by T4 polynucleotide kinase (Gibco-BRL) in the presence of ^{32}P-γ-ATP. *Pvu*II digestion of the 203 bp fragment yields a 186 bp fragment uniquely labeled on the template strand at the 5′ end [see Spassky and Sigman (1985) for complete sequence]. The promoter was also prepared by PCR using 5′-GCGGGCAGTGAGCGCAACCC-3′ as the primer of the nontemplate strand and 5′-CCAGTGAATGCGTAATCATGGTCATAGC-3′ as the primer of the template strand. This generates a 158 bp fragment spanning positions −110 to +48 of the *lac*UV-5 promoter. Following purification by 8% PAGE, this abbreviated fragment behaved indistinguishably from the longer fragment both in footprinting and transcription assays.

E. coli RNA polymerase (4 000−10 000 units/μL) was purchased from Pharmacia. Its active site normality was titrated with the *lac*UV-5 promoter by gel retardation and was determined to be 1 pmol/10 μL. Nucleotide triphosphates were obtained from Gibco BRL, and ^{32}P-γ-ATP and ^{32}P-α-UTP were purchased from ICN. Acrylamide was purchased from National Diagnostics.

Synthesis of 5-Phenylneocuproine (5φNC). Methyl lithium (12.3 mL of 1.3 M in ether) was slowly added to a slurry of 1.05 g (4 mmol) of 5-phenyl-OP and 1.8 g (16 mmol) of DABCO in 50 mL of dry benzene at −78 °C under a dry nitrogen atmosphere (Case, 1968; Engbersen, 1986). The slurry turned red upon addition of the methyllithium. The mixture warmed up to room temperature and was vigorously stirred for 2 h. It was then poured onto ice whereupon it turned yellow. The organic phase was decanted, and the aqueous layer was further extracted with methylene chloride. The organic extracts were combined and washed by 5 mL of water and dried over magnesium sulfate. After the solvent was removed *in vacuo*, the residue was dissolved in 30 mL of methylene chloride and was stirred with 10 g of activated MnO_2 at room temperature for 2 h. The yellow color faded. The MnO_2 was removed by filtration over a 20 mL Celite column. The MnO_2, now on the column, was washed by a large amount of methylene chloride. After the solvent was removed, a 1:1 mixture of 5-phenyl-2-methyl-OP and 6-phenyl-2-methyl-OP containing a small amount of 5-phenyl-NC (5ϕNC) was obtained in almost quantitative yield and used in the next step without further purification. The same procedure was repeated to give the 5ϕNC. Contaminating amounts of monomethylated OP were difficult to remove. After several recrystallizations from hot benzene, the product was pure with a final recovery of 200 mg. Mp: 158−159 °C. ^{1}H NMR in $CDCl_3$: 8.23 (2H, d, J = 8.4 Hz), 7.71 (1H, s), 7.25−7.61 (7H, m), 3.05 (3H, s), 3.01 (3H, s). ^{13}C NMR: 159, 137.84, 135.6, 129.9, 128.6, 128.0, 125.4, 124.5,

Scheme 2: Redox Active and Inactive Isosteres Bound to the Open Complex



123.9, 20.6. $C_{20}H_{16}N_2$: calcd., 284.13135; found, 284.13190.

Synthesis of 4-Phenylneocuproine (4φNC). The above procedure was employed with the only change being that 4-phenyl-1,10-phenanthroline was used for starting material. ^{1}H NMR in $CDCl_3$: 8.04 (1H, d, J = 8.24 Hz), 7.74 (1H, d, J = 9.0 Hz), 7.56 (1H, d, J = 9.0 Hz) 7.46 (5H, s) 7.44 (1H, d, J = 8.21 Hz), 7.37 (1H, s), 2.906 (3H, s), 2.90 (3H, s). ^{13}C NMR: 159.4, 158.7, 148.6, 138.4, 136.0, 129.7, 128.5, 128.3, 126.4, 125.1, 125.0, 123.8, 123.6, 123.2, 26.00, 25.91. M^+/Z parent ion at 284.1309 corresponds to the product with an error of <2 ppm.

Preparation of Neocuproine−Cuprous Chelates. Solutions of appropriate concentrations are prepared daily in 4 mM sodium ascorbate.

DNase and OP−Copper Footprinting. The open complex is generated from labeled *lac*UV-5 restriction fragment (1000−3000 cpm/μL) and 0.2 units of RNA polymerase/mL in 1× transcription buffer (50 mM KCl, 40 mM Tris HCl, pH 7.9, and 10 mM $MgCl_2$) and incubated for 20 min at 37 °C. DNase footprinting: cutting proceeds for 1 min at 37 °C (0.83 mg of final DNase/mL) and is quenched with 1 μL of 0.5 M EDTA. Where indicated, the open complex or elongation complexes formed for 10−15 min at 37 °C followed by addition of 1 μL of the preformed $(NC)_2Cu^+$ to give a final concentration as indicated. Samples were incubated for an additional 5 min before oxidative footprinting was initiated. 5φ/4φOP-Cu footprinting: final concentrations of 21 μM 5φ/4φOP, 10 μM $CuSO_4$, and 5.8 mM mercaptopropionic acid (MPA) are used, and 1 μL of 100 μM $(5\phi/4\phi OP)_2Cu^{2+}$ is added followed by 1 μL of a 58 mM MPA. Cutting proceeds for 4 min at 37 °C before quenching with 3 μL of 40 mM ethanolic neocuproine and precipitating with 70 μL of isopropanol. The samples are pelleted for 30 min at 14 000 rpm. The pellets are then washed with 70% ethanol, dried, resuspended in 25 μL of loading solution (deionized formamide, 0.1% bromphenol blue), heated to 90 °C for 5 min, and analyzed by 12% urea denaturing PAGE. The gel is run at 55 W constant in 1× TBE until the bromphenol blue has just run off the gel.

Full and Partial Complement Transcription Assays, Autoradiography, and Analysis. (A) *In Vitro Runoff Transcription Inhibition Assay (Full Length).* Unlabeled 203 bp fragment (1 pmol) was added to RNA polymerase in a buffer made of 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, and 50 mM KCl. Inhibitor solutions [1 mM $(4\phi NC)_2Cu^+$, 1 mM $(5\phi NC)_2Cu^+$] were added to the RNA polymerase/promoter mixture to give the indicated final concentrations. Tubes were incubated at 37 °C for 15 min. Nucleotide triphosphates (final concentration of 100 μM; one million cpm of ^{32}P-α-ATP) were added, and incubation was carried out at 37 °C for 45 min. Transcription was quenched with formamide, EDTA, and loading dye, and transcripts were analyzed by 20% denaturing PAGE.

(B) *In Vitro Transcription Inhibition Assay with Incomplete Complement of Nucleotides and ^{32}P-γ-ATP.* Unlabeled 158 bp fragment obtained from PCR was added to RNA polymerase in a buffer of final concentration 30 mM Tris, pH 7.5, 100 mM KCl, and 3 mM $MgCl_2$. A 1 μL amount of appropriately diluted $(5\phi NC)_2Cu^+$ was added to the promoter/polymerase mix to give final concentrations of 0, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 24 μM. These solutions were incubated at 37 °C for 10 min. A reaction

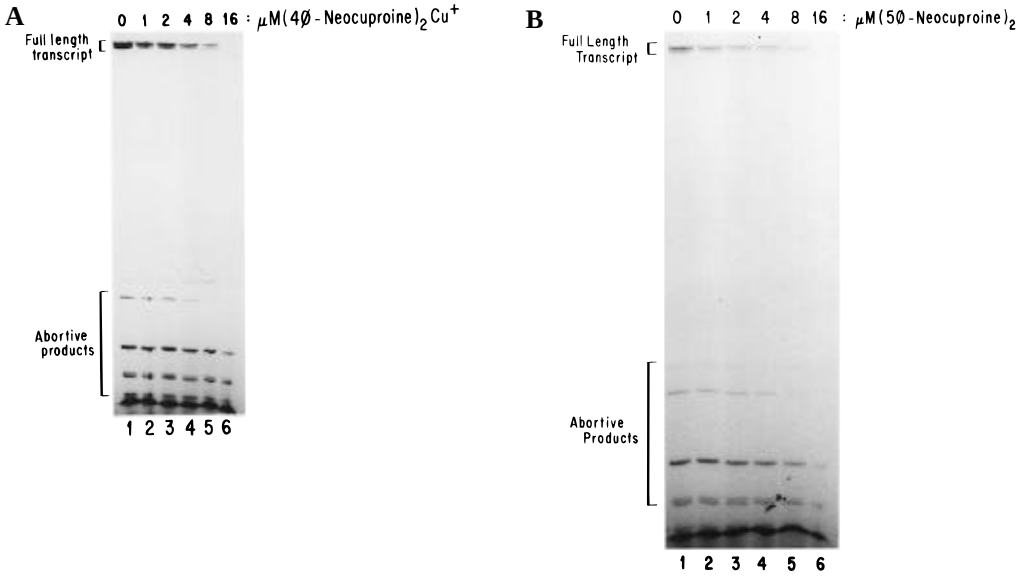


FIGURE 1: (A) $(4\phi NC)_2Cu^+$ inhibits transcription of full-length message. Lane 1: 66 nt full-length message. Lanes 2−6: Increasing concentrations of $(4\phi NC)_2Cu^+$: 1, 2, 4, 8, 16 and μM. (B) $(5\phi NC)_2Cu^+$ inhibits transcription of full-length message. Lane 1: 66 nt full-length message. Lanes 2−6: Increasing concentrations of $(5\phi NC)_2Cu^+$: 1, 2, 4, 8, 16 and μM.

mixture of NTP's was added to result in a final concentration of 100 μM each ATP and UTP and one million cpm per lane each of ^{32}P-γ-ATP and ^{32}P-α-UTP. Transcription proceeded at 37 °C for 45 min and was quenched with deionized formamide, 1 mM EDTA, and 1% SDS. The reaction mixture was loaded onto a denaturing polyacrylamide gel composed of 20% Sequagel supplemented with additional 1% bis and 1% glycerol. Gels were placed on Whatman filter paper and dried. At each corner, a pin prick was made through the filter paper onto the film. After the film was developed, the pin holes on the film were lined up with the pinpricks placed there prior to exposure and the bands were cut out, placed in scintillation vials containing 2 mL of 3% H_2O_2 in 1 M NaOH, and counted after overnight solubilization. Three gel slices corresponding to the same positions as the bands representing the three abortive transcripts were also obtained to control for background. The value from the background count was subtracted from the counts of all other bands migrating at the same position. Values for amount of product (pmol) were calculated from the specific activity. Autoradiograms were densitometrized where indicated.

RESULTS

Comparison of I_{50} for Runoff Transcription and Open Complex Binding. The cuprous chelates of neocuproine and its derivatives inhibit runoff transcription from the model *lac*UV-5 promoter (Mazumder et al., 1993; Perrin et al., 1994). The most inhibitory chelates are prepared from 4ϕ- and 5ϕ-neocuproine; both inhibit runoff transcription with an I_{50} of approximately 5 μM (Figure 1A,B). Generally, the neocuproine derivatives whose 1,10-phenanthroline -copper chelates most effectively cleave the open complex are the most potent inhibitors of transcription. Neocuproine analogs of 1,10-phenanthroline that do not cleave the open complex are not inhibitory. For example, the cuprous chelate of 4,7-diphenyl-1,10-phenanthroline does not cleave the open complex, and the cuprous chelate of the 4,7-diphenylneocuproine analog is not inhibitory.

The affinities of $(4\phi NC)_2Cu^+$ and $(5\phi NC)_2Cu^+$ for the open complex formed with the *lac*UV-5 promoter were investigated using a footprinting protection assay. In these experiments, the *lac*UV-5 open complex was footprinted with either $(4\phi OP)_2Cu^+$ or $(5\phi OP)_2Cu^+$ in the presence of increasing concentrations of the corresponding bisneocuproine cuprous chelate after a 10 min incubation. Figure 2A demonstrates the ability of $(4\phi NC)_2Cu^+$ to protect against $(4\phi OP)_2Cu^+$-directed scission, while Figure 2B demonstrates the ability of $(5\phi NC)_2Cu^+$ to protect against $(5\phi OP)_2Cu^+$-directed scission. Similarly, $(5\phi NC)_2Cu^+$ protects the OC from cleavage by $(4\phi OP)_2Cu^+$ and $(4\phi NC)_2Cu^+$ protects the OC from cleavage by $(5\phi OP)_2Cu^+$. In each case, half-maximal protection is observed at roughly 15 μM for $(4\phi NC)_2Cu^+$ and 20 μM for $(5\phi NC)_2Cu^+$.

Although these results are consistent with $(5\phi OP)_2Cu^+$ and $(4\phi NC)_2Cu^+$ competing for the same binding sites as their redox active phenanthroline cuprous isosteres, alternate possibilities for the protection must be excluded. For example, the attenuation of the cleavage sites cannot be due to chelation of copper by neocuproine since the neocuproine is added as the preformed cuprous chelate. In addition, since the cuprous chelates of neocuproine and its derivatives are exchange stable, the inhibition of scission cannot be due to the formation of an inactive 1:1:1 phenanthroline−neocuproine−cuprous chelate. The specificity of the protection is supported by the failure of noninhibitory chelates such as the tetrahedral cuprous complex of 4,7-diphenylneocuproine (bathocuproine) and the octahedral 3:1 1,10-phenanthroline ferrous chelate to protect against scission of the open complex. Inhibitory neocuproine chelates are the only transcription inhibitors which protect the characteristic upstream cleavage sites of $(OP)_2Cu^+$ and its derivatives in the open complex and do not displace the enzyme from the promoter. For example, although the intercalating agent 9-aminoacridine blocks cleavage at these sites, it displaces the enzyme from the promoter at its inhibitory concentrations as is indicated by the restoration of the signature hypersensitive bands on free DNA of $(OP)_2Cu^+$ and its derivatives on the template strand at positions −13 to −9 (Spassky & Sigman, 1985). In contrast, these characteristic upstream signature bands are not restored upon addition of the neocuproine chelates at their inhibitory concentrations (Ma-

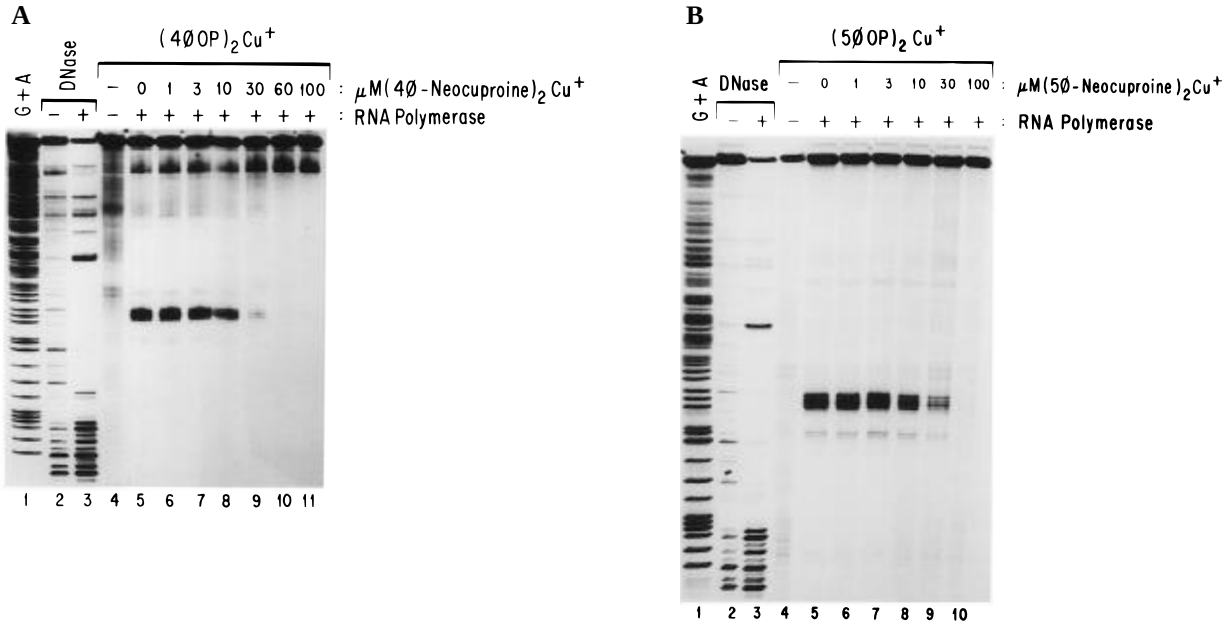


FIGURE 2: (A) $(4\phi NC)_2Cu^+$ association is assayed by protection of the open complex from cleavage by $(4\phi OP)_2Cu^+$. Lane 1: Maxam−Gilbert G+A sequencing ladder. Lanes 2 and 3: DNase footprint in the absence and presence of RNA polymerase, respectively. Lane 4: 4ϕOP-Cu^+ digestion of the *lac*UV-5 promoter. Lanes 5−11: 4ϕOP-Cu^+ footprint of the open complex in the presence of increasing concentrations of $(4\phi NC)_2Cu^+$: 1, 3, 10, 30, 60, and 100 μM. (B) $(5\phi NC)_2Cu^+$ association is assayed by protection of the open complex from cleavage by $(5\phi OP)_2Cu^+$. Lane 1: Maxam−Gilbert G+A sequencing ladder. Lanes 2 and 3: DNase footprint in the absence and presence of RNA polymerase, respectively. Lane 4: 5ϕOP-Cu^+ digestion of the *lac*UV-5 promoter. Lanes 5−10: 5ϕOP-Cu^+ footprint of the open complex in the presence of increasing concentrations of $(5\phi NC)_2Cu^+$: 1, 3, 10, 30, and 100 μM.

zumder et al., 1993). DNaseI footprinting and mobility shift assays have also been used to demonstrate that the neocuproine chelates do not displace the enzyme from the promoter at their inhibitory concentrations whereas 9-aminoacridine does (data not shown).

The dependence of the footprinting protection and transcriptional inhibition assays on the concentration of the chelates was compared. These correlations are shown in Figure 3A,B. Half-maximal inhibition of transcription occurs at 5 μM $(5\phi NC)_2Cu^+$, whereas half-maximal protection of scission is observed at approximately 20 μM. A similar discrepancy occurs for $(4\phi NC)_2Cu^+$.

Inhibition of Tetranucleotide Synthesis. This lack of congruence suggested that binding of the neocuproine chelate to the open complex must not be sufficient to account for the transcription inhibition observed with this ligand. In order to identify the reason for this discrepancy, the $(5\phi NC)_2Cu^+$ inhibition of RNA polymerase in abortive transcription was examined. This investigation parallels that used to characterize the kinetics of inhibition by rifampicin, which binds to RNA polymerases and inhibits the synthesis of the second phosphodiester bond (McClure et al., 1978; McClure & Jovin, 1975). Having demonstrated that both $(4\phi NC)_2Cu^+$ and $(5\phi NC)_2Cu^+$ bind to the open complex, a kinetic analysis using a partial complement of nucleotides was undertaken to determine if these chelates inhibit the formation of all intermediates in abortive synthesis to the same extent under one single set of experimental conditions (Figure 4). If the neocuproine chelates inhibit the rate of internucleotide bond formation by a partially noncompetitive mechanism where the inhibitor reduced but did not block catalysis, then all products would be inhibited to the same extent (Dixon & Webb, 1979). Alternatively, if differential inhibition of the products is observed, then the chelates must bind to the various steady state intermediates with varying affinities.

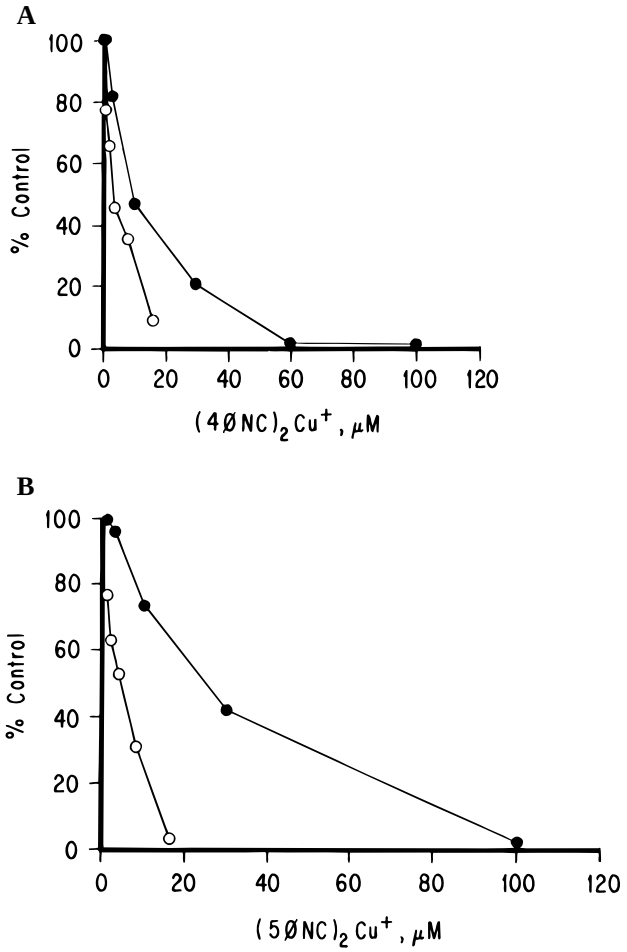


FIGURE 3: Correlation of footprint protection with transcription inhibition by $(4\phi NC)_2Cu^+$ (A) and $(5\phi NC)_2Cu^+$ (B). ○, % activity; ●, % scission.

The sequence of the first four nucleotides incorporated into the mRNA encoded by the *lac*UV-5 promoter is

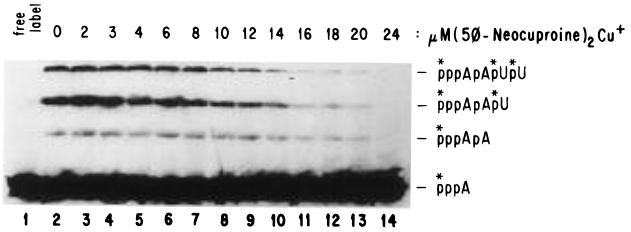


FIGURE 4: $(5\phi NC)_2Cu^+$ inhibits abortive transcription. Lane 1: Free label (background). Lanes 2−14: Increasing concentrations of $(5\phi NC)_2Cu^+$: 0, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24 and μM.

ApApUpU. In addition to pppApApUpU, pppApApU and pppApA are abortive products formed if only ATP and UTP are present in the incubation mixture. To assay the inhibition of di-, tri-, and tetra-nucleotide formation, ^{32}P-γ-ATP and ^{32}P-α-UTP at a concentration of 100 μM each were used in the presence of increasing amounts of $(5\phi NC)_2Cu^+$. Direct quantitation of products instead of densitometry was used to analyze the rates of formation of products following denaturing PAGE. The gel was dried on paper and autoradiographed. The autoradiogram was realigned over the paper such that the bands could be marked, cut out, resolubilized, and counted. Background counts were subtracted in each case. The number of picomoles of product was calculated from the adjusted specific radioactivity of the two substrates and plotted against inhibitor concentration (Figure 5A). The synthesis of pppApA is clearly inhibited. Since the first step is inhibited, the quantities of all subsequent products will be diminished. However, the question then remains whether further steps are also inhibited or whether inhibition of pppApA synthesis is sufficient to explain diminished amounts of pppApApU and pppApApUpU.

To address this question, the effect of the inhibitor on the first step must be differentiated from its effect on subsequent steps. This is accomplished by measuring the ratios of consecutive products as a function of inhibitor concentration. If the amounts of product are inhibited at a single step, then this ratio should be independent of inhibitor concentration. If the inhibitor acts at both steps, then the ratio of both products will decrease as function of inhibitor concentration. The relative amounts of pppApApU, normalized for inhibited amounts of pppApA, and the relative amounts of pppApApUpU, normalized for inhibited amounts of pppApApU, are plotted in Figure 5B. Since the ratio of pppApApU to pppApA decreases as a function of inhibitor concentration, the chelate blocks the synthesis of the trinucleotide from the dinucleotide. In contrast, the synthesis of pppApApUpU from pppApApU is not inhibited as reflected by the independence of the ratio of these two products as a function of increasing chelate concentration.

In summary, $(5\phi NC)_2Cu^+$ inhibits the syntheses of both pppApA and pppApApU. The K_I's for the synthesis of pppApApU (as well as that for the uninhibited synthesis of the extended pppApApUpU) are approximately 10 μM, whereas the I_{50} for the synthesis of pppApA is approximately 16 μM. Since the new sites formed during the synthesis of the short oligonucleotides have a similar affinity for the tetrahedral cuprous chelates as the open complex, they cannot account for the discrepancy between open complex binding and runoff transcription inhibition presented in Figure 3A,B.

Binding of $(5\phi NC)_2Cu^+$ to Intermediates in the Synthesis of Nine-Nucleotide Long Transcript. These studies prompted us to search for binding sites with even greater affinity for

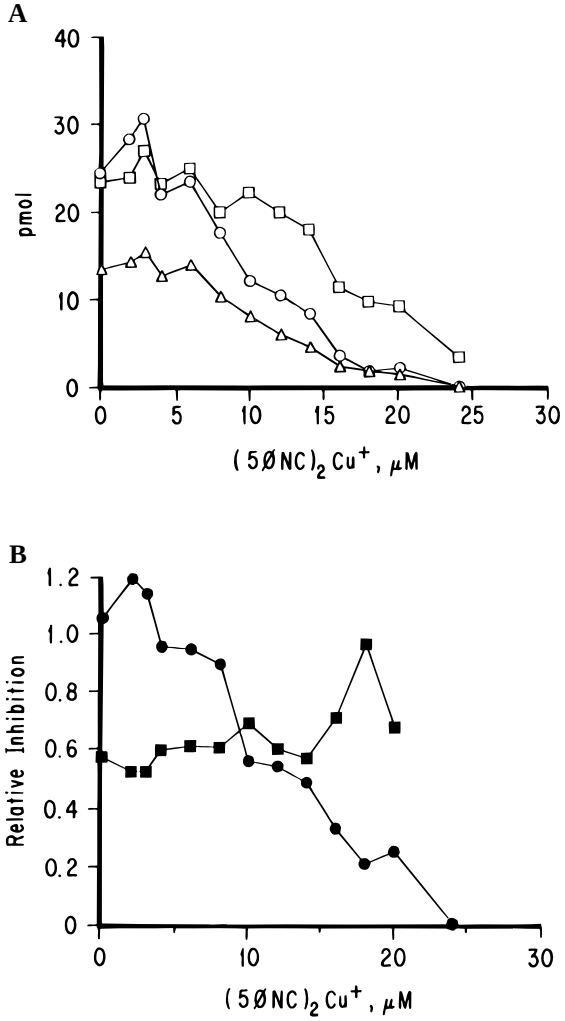


FIGURE 5: (A) Quantitation of inhibition of di-, tri-, and tetranucleotide synthesis from autoradiogram from Figure 4. □, pmol of pppApA; ○, pmol of pppApApU; △, pmol of pppApApUpU. (B) $(5\phi NC)_2Cu^+$ inhibits the synthesis of both the dinucleotide and trinucleotide but not that of the tetranucleotide. ■, $^4/_3$; ●, $^3/_2$.

the chelate formed during the synthesis of longer ribooligonucleotides. As a result, we monitored the binding of $(5\phi NC)_2Cu^+$ to intermediates formed during the synthesis of the nine-base long (9mer) C-less transcript. Upon the synthesis of the 9mer, the processive elongation complex forms and the polymerase holoenzyme loses its σ-subunit and its rifampicin sensitivity. Intermediates in this reaction can be formed by manipulating the nucleotide composition of the reaction mixture and then detected by oxidative footprinting with $(5\phi OP)_2Cu^+$ (Figure 6). The cleavage patterns indicate that all steady state intermediates of transcription possess binding sites for the tetrahedral cuprous chelates of OP and its derivatives. In Figure 6, lanes 6, 8, 11, 14, and 17 demonstrate the footprinting of the following respective conformations: the OC, the OC−ITC transition, the ITC, and the ITC−IEC transition. Since the footprinting reaction is carried out under steady state conditions, a population of steady state intermediates is simultaneously sampled. The very intense scission characteristic of the static open complex is diminished because the concentration of this steady state intermediate is decreased in favor of intermediates formed at more downstream sites.

The addition of $(5\phi NC)_2Cu^+$ at each of the designated concentrations inhibits the scission reactions in the presence of the different complements of NTPs. For example,

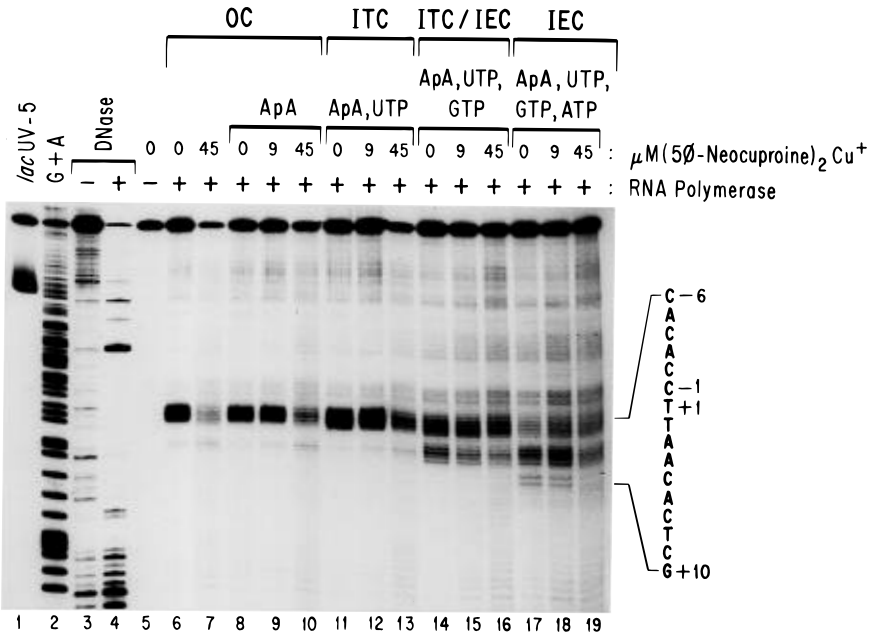


FIGURE 6: $(5\phi NC)_2Cu^+$ associates with elongation complexes as assayed by protection from $(5\phi OP)_2Cu^+$ footprinting. The sequence presented at the right margin is that of the template strand. Lane 1: *lac*UV-5 promoter fragment. Lane 2: Maxam−Gilbert G+A sequencing ladder. Lanes 3 and 4: DNase footprint in the absence and presence of RNA polymerase, respectively. Lanes 5 and 6: $(5\phi OP)_2Cu^+$ footprint in the absence and presence of RNA polymerase, respectively. Lane 7 (open complex): $(5\phi NC)_2Cu^+$ protection of the open complex from scission. Lanes 8−10 (open complex): $(5\phi OP)_2Cu^+$ footprint in the presence of ApA and 0, 9, and 45 μM $(5\phi NC)_2Cu^+$, respectively (no synthesis possible). Lanes 11−13 (initially transcribing complex): synthesis of the tetranucleotide ApApUpU is observed; $(5\phi OP)_2Cu^+$ footprint in the presence of ApA and UTP and 0, 9, and 45 μM $(5\phi NC)_2Cu^+$, respectively. Lanes 14−16 (initially transcribing complex−initially elongating complex): synthesis of the ribonucleotide ApApUpUpG with this complement of NTPs: $(5\phi OP)_2Cu^+$ footprint in the presence of ApA, UTP, and GTP and 0, 9, and 45 μM $(5\phi NC)_2Cu^+$, respectively. Lanes 17−19 (initially elongating complex): synthesis of ApApUpUpGpUpGpApG is observed; $(5\phi OP)_2Cu^+$ footprint in the presence of ApA, UTP, GTP, and ATP and 0, 9, and 45 μM $(5\phi NC)_2Cu^+$, respectively.

$(5\phi NC)_2Cu^+$ protects the ITC and the IEC from $(5\phi OP)_2Cu^+$-directed scission. In general, all of these sites are protected with increasing concentrations of $(5\phi NC)_2Cu^+$, from 9 to 45 μM. However, upon closer examination, not all sites are protected equally. It is noted that in lanes 14−16, where the ITC-IEC transition is footprinted, the sites at +3 and +4 are preferentially protected while those characteristic of the OC−ITC transition at −3 to −6 remain virtually unaffected even at 45 μM $(5\phi NC)_2Cu^+$. This is in contrast to the protection observed in lanes 7 and 10 where the OC and the OC−ITC transition are protected at positions −3 to −6. The fact that the sites from −3 to −6 are no longer protected by $(5\phi NC)_2Cu^+$ in the presence of the initiating dinucleotide ApA and two nucleotides UTP and GTP (lanes 14−16) indicates that the OC might not be the major intermediate present under these conditions. Therefore, the sites from −3 to −6 at the ITC−IEC transition have a lower affinity for $(5\phi NC)_2Cu^+$ relative to the new sites created at +3 and +4. The same effect is seen in lanes 17−19, where scission at all sites is inhibited but the two sites at +9 and +10 are preferentially protected. Figure 6 indicates that as the polymerase isomerizes from the OC to the ITC and then to the IEC, it creates new sites for $(5\phi NC)_2Cu^+$ association. Since these sites have higher affinity than the open complex, the lack of correspondence of the I_{50} for full-length transcript (5 μM) with the binding affinity measured by open complex footprinting (20 μM) can be explained. Previous work has demonstrated that the cuprous chelates of neocuproine do not bind B-DNA with the micromolar dissociation constants characteristic of their interaction with transcription complexes nor do they protect B-DNA from cleavage by their redox active isosteres (Graham & Sigman, 1984; Sigman et al., 1985).

DISCUSSION

The most unique reactivity of the nuclease activity of the cuprous complex of 1,10-phenanthroline and its derivatives is its cleavage of single-stranded template DNA formed in transcriptionally competent complexes of RNA polymerases with their promoters. This enhanced reactivity was initially discovered with *E. coli* RNA polymerase and the *lac*UV-5 bacterial promoter (Spassky & Sigman, 1985); subsequent studies with this promoter have shown that magnesium ion is essential for observing the hypersensitive sites −3 to −6 upstream of the start of transcription (Kuwabara & Sigman, 1987). Although the cleavage patterns vary in detail depending on the 1,10-phenanthroline derivative used to form the redox active cuprous chelate, all of the digests share common features. The most important is that all the redox active chelates react exclusively with the template strand but not the nontemplate strand. These chemical nucleases are therefore useful only for the footprinting of inhibitors which bind at or near the single-stranded template strand.

Two chelates, $(OP)_2Cu^+$ and $(5\phi OP)_2Cu^+$, have been used to visualize the downstream progression on the *lac* promoter from the open complex (OC) to the initial transcribing complex (ITC) and in turn to the initial elongating complex (IEC; Spassky, 1986; Thederahn et al., 1990). Footprints of the initial closed complex (CC) by the chemical nuclease in the absence of magnesium do not exhibit the hypersensitive sites but do reveal the position of the enzyme on the promoter (Kuwabara & Sigman, 1987). The sequence positions at which scission is observed vary for the different initiating complexes. In the open complex and the initial transcribing complex, the cleavage sites lag the position of phosphodiester bond synthesis. However, if the nucleotide

complement added to the incubation mixture allows the synthesis of a 9mer, then cleavage at sequence positions corresponding to the positions of phosphodiester bond synthesis is observed. Other redox active coordination chelates do not exhibit enhanced reactivity with the single-stranded DNA intermediates formed during transcription (Craig et al., 1995). Minor perturbations are observed with ferrous-EDTA but the progression of transcription cannot be readily detected either with this reagent or with methidium−propyl EDTA−ferrous ion (Thederahn et al., 1990; Craig et al., 1995).

Hyper-reactivity to $(OP)_2Cu^+$ has also been observed in eucaryotic transcription units. A series of cleavage sites centered upstream around position −8 relative to the start of transcription is observed in the case of the open complex formed by the adenovirus major late promoter, yeast RNA polymerase, and purified factors of basal transcription (Buratowski et al., 1991). However, not all bacterial and eucaryotic promoters exhibit this enhanced reactivity. Failure to observe these cleavage patterns may reflect the steady state level of the open complex, productive orientation of the bound redox active chelate relative to the oxidatively sensitive deoxyribose, or possibly experimental difficulties such as the low yields of transcriptionally active species and sequestration of copper by impure protein preparations especially in the more complex eucaryotic systems.

The hyper-reactivity of the open complexes to $(OP)_2Cu^+$ and its derivatives has been attributed to the generation of a binding site for the tetrahedral chelates in transcriptionally active complexes. This model accounts for the unique reactivity of the tetrahedral chelates and the variation of the cleavage patterns with 1,10-phenanthroline structure. This hypothesis has been supported by the demonstration that chelates formed between cuprous ion and neocuproine are potent transcription inhibitors and protect the open complex from scission (Scheme 2) (Mazumder et al., 1993b). In the present studies, we have demonstrated that the chelates prepared from 5ϕNC and 4ϕNC bind not only to the open complex but also to the single-stranded DNA of steady-state intermediates formed during catalysis. These studies have relied on the use of the characteristic patterns cleavage by $(5\phi OP)_2Cu^+$ of the different transcription intermediates to monitor the binding of the chelates directly. The affinity of the chelates for the binding sites in the downstream intermediates is greater than that for the open complex. Our results show that $(5\phi NC)_2Cu^+$ can bind to all of the intermediates formed in the synthesis of the C-less 9mer from the *lac* UV-5 promoter even in the presence of 100 μM nucleotide triphosphates.

The mechanism of transcription inhibition by $(5\phi NC)_2Cu^+$ is fundamentally different from of a series of well-studied antibiotics which bind with high affinity to DNA. They strongly destabilize polymerase binding and promote its dissociation as indicated by gel retardation and by both $(OP)_2Cu^+$ and DNase footprinting (Mazumder et al., 1994). In contrast, neocuproine chelates form ternary complexes with the enzyme and promoter and bind to the single-strand DNA characteristic of kinetically competent transcription complexes at lower concentrations than these chelates bind to free B-DNA. Although these results suggest that $(5\phi NC)_2Cu^+$ or $(4\phi NC)_2Cu^+$ binds to single-stranded regions of DNA on the template strand upstream of the start of transcription at sites −3 to −6, the chelates do not necessarily stabilize the binding of the promoter to the RNA polymerase.

Consider the formation of the ternary complex composed of RNA polymerase (E), promoter (DNA), and the chelate (C) to be represented by eqs 1a,b.

$$\text{DNA}-\text{E} + \text{C} \leftrightarrow \text{DNA}-\text{E}-\text{C} \quad (1a)$$

$$K_a = (\text{DNA}-\text{E})(\text{C})/(\text{DNA}-\text{E}-\text{C}) \quad (1b)$$

If this ternary complex is unstable, it can dissociate into binary complexes by two paths (eqs 1c−f).

$$\text{DNA}-\text{E}-\text{C} \leftrightarrow \text{DNA}-\text{C} + \text{E} \quad (1c)$$

$$K_b = (\text{DNA}-\text{C})(\text{E})/(\text{DNA}-\text{E}-\text{C}) \quad (1d)$$

or

$$\text{DNA}-\text{E}-\text{C} \leftrightarrow \text{E}-\text{C} + \text{DNA} \quad (1e)$$

$$K_c = (\text{E}-\text{C})(\text{DNA})/(\text{DNA}-\text{E}-\text{C}) \quad (1f)$$

If K_b/K_{DNA}, where $K_{DNA} = (\text{E})(\text{DNA})/(\text{DNA}-\text{E})$, is much less than 1, then C will appear to displace DNA competitively from E even if the binding sites of DNA and the chelate do not overlap. If this ratio is slightly less than 1, then C will have a measurable residence time in the open complex and binding will be detectable as has been observed here. As demonstrated by earlier studies with horse liver alcohol dehydrogenase (Sigman, 1967; Frolich et al., 1978), the dissociation rate constant of the ternary complex for the competitive pattern is higher than that observed with a metastable complex. Although gel retardation experiments have indicated that incubation of the neocuproine chelates with the open complex for 10 min does not induce the significant dissociation of the promoter from the RNA polymerase, prolonged incubation (45 min) does result in polymerase dissociation as would be expected if the ternary complexes are metastable. Two experiments indicate that this slow chelate-induced dissociation is not responsible for the observed kinetic effects. First, if dissociation were responsible for the inhibition, then the synthesis of all products would be inhibited equivalently. But this is not the case (Figure 5B). Secondly, the binding of $(5\phi NC)_2Cu^+$ to several transcription intermediates indicates that this chelate does not promote the dissociation of the enzyme from the DNA (Figure 6).

The determinants of the enzyme-bound single-stranded DNA responsible for binding the 1,10-phenanthroline and neocuproine chelates to the open complex are under investigation. Although reactive with single- stranded RNAs (Murakawa et al., 1989), the chelates do not cleave single-stranded DNA unless hairpin structures form (Marshall et al., 1981; Sigman et al., 1985). RNA polymerase may not directly interact with the chelate, but by stabilizing the single-stranded regions in the presence of Mg^{2+} it contributes to the formation of the binding site. Rifampicin and its derivatives, alone among transcription inhibitors, also form stable ternary complexes, but they bind to the protein and inhibit synthesis of the second phosphodiester bond presumably by inducing conformational changes in the protein (Jin & Gross, 1988; Kumar et al., 1992; McClure & Cech, 1978). Rifampicin does not abolish, but does alter, the scission of the template DNA observed with $(5\phi OP)_2Cu^+$ (Mazumder et al., 1994).

Two models for the mechanism of transcription elongation have been proposed. In one model, the hybridization of the

recently synthesized RNA to the template DNA stabilizes the elongation complex (von Hippel et al., 1984; Daube & von Hippel, 1994). In the other model, the binding of the product RNA to the enzyme stabilizes the elongation complex (Chamberlin, 1993). Although the tetrahedral chelates described react differently with initiating and elongation complexes, these compounds cannot distinguish the two models with the results presently available. For example, loss of upstream reactive sites could be due to the formation of an unreactive heteroduplex or the rehybridization and protection of the recently copied template DNA. However, given the high-affinity binding sites generated for the tetrahedral chelates in the open and elongation complexes, domains of RNA polymerase neighboring these two types of chelate binding sites can be identified using either the protease activity of the redox active coordination chelate (Wu et al., 1995) or the stable neocuproine chelate as a platform for photoreactive chemical modification reagents (Chowdhry & Westheimer, 1979). These chemical approaches could prove useful in distinguishing the competing models.

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