Interactions of Transcription Inhibitors with the *Escherichia coli* RNA Polymerase–*lacUV5* Promoter Open Complex†

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ABSTRACT: The interactions of transcription inhibitors with the open complex composed of *Escherichia coli* RNA polymerase and the *lacUV5* promoter have been studied using gel retardation, the chemical nuclease activity of the cuprous complexes of 1,10-phenanthroline (OP) and its derivatives, and steady-state kinetics. Gel retardation shows that two inhibitors, the 2:1 2,9-dimethyl-1,10-phenanthroline–cuprous complex [$(2,9\text{-Me}_2\text{OP})_2\text{Cu}^+$] and rifampicin, bind stably to the open-complex. $(2,9\text{-Me}_2\text{OP})_2\text{Cu}^+$ blocks scission by the chemical nuclease by interfering with the binding of its redox-active isosteres. Rifampicin does not block scission by the cuprous complexes of 3,4,7,8-tetramethyl-OP, 4-phenyl-OP, and OP but does perturb scission by the cuprous complex of 5-phenyl-OP. Organic ligands including intercalating agents and groove binders (e.g., daunomycin, di(amidinophenyl)indole (DAPI), actinomycin D, distamycin, 9-aminoacridine, mithramycin, and chromomycin A_3), which bind to free DNA with high affinity, do not form stable ternary complexes with the open-complex. Gel retardation experiments demonstrate that they promote dissociation of the enzyme from the promoter. The greater sensitivity of enzymatic catalysis to inhibitor concentration relative to polymerase binding suggests that these ligands form metastable, catalytically inactive ternary complexes with RNA polymerase and the promoter.

A wide variety of structurally dissimilar molecules inhibit transcription. The mechanisms by which they exert their kinetic effects are of interest because these may provide insight into the multistep reaction pathway catalyzed by RNA polymerase and could possibly lead to the design of new therapeutic agents. Most kinetic schemes for transcription include the following steps: (a) the reversible binding of the polymerase to the promoter to form the "closed complex", (b) its isomerization to the kinetically competent "open complex", (c) initiation of transcription by incoming nucleoside triphosphates, (d) the processive elongation of the RNA, and (e) termination of transcription (Buc, 1987; Buc & McClure, 1985; Daube & von Hippel, 1992; Suh *et al.*, 1992, 1993; von Hippel & Yager, 1992). Any of these enzyme–substrate intermediates is a potential target for a transcription inhibitor.

$$E + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{CLOSED COMPLEX}}{E\text{-}P} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{OPEN COMPLEX}}{EP_{\text{OPEN}}} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \underset{\text{INITIATING COMPLEX}}{EP_{\text{INITIATING}}} \xrightarrow[\sigma]{\text{NTPs}} \text{RNA} \quad (1)$$

Because of the complexity of the scheme, kinetic measurements themselves are not sufficient for defining inhibition mechanisms. However, two experimental approaches, gel retardation analysis and chemical nuclease footprinting with the cuprous complexes of 1,10-phenanthroline and its derivatives (Sigman & Chen, 1990), can supplement kinetic studies since they provide complementary information. Gel retardation analysis (Crothers, 1987; Garner & Revzin, 1986) can determine if the inhibitor forms a stable ternary complex with the open complex or promotes its dissociation. 1,10-Phenanthroline–copper footprinting can provide information on the conformation and accessibility to solvent of the single-stranded DNA of the open complex.

Two mechanisms are possible for describing the interaction of inhibitors with the open complex. In the first (eq 2), the inhibitor (I) forms a ternary complex with one of the steady-state intermediates (EP_x). If this complex is stable, K_e is lower than the dissociation constant of the enzyme from the promoter in the absence of inhibitor. If this complex is metastable, K_e is higher than the corresponding dissociation constant in the absence of inhibitor and enzyme binding is destabilized.

$$E\text{-}P_x + I \overset{K_i}{\leftrightarrow} EP_x\text{-}I \overset{K_e}{\leftrightarrow} E + PI \quad (2)$$

$$K_i = \frac{(EP_x)(I)}{(EP_xI)} \qquad K_e = \frac{(E)(PI)}{(EP_xI)}$$

In the second mechanism, no ternary complex is formed. Inhibitor only binds to the DNA (eq 3b) or the enzyme (eq 3c) after the dissociation of the intermediate (eq 3a).

$$EP_x \leftrightarrow E + P \quad (3a)$$

$$P + I \leftrightarrow PI \quad (3b)$$

$$E + I \leftrightarrow E\text{-}I \quad (3c)$$

Only the mechanism summarized in eq 2 is relevant to inhibitors which form stable ternary complexes with polymerase and promoter. Both mechanisms (eqs 2 and 3) may contribute to the ligand-induced dissociation of the polymerase from the promoter.

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The extensively studied *Escherichia coli lacUV5* promoter was chosen as the model transcription unit because its kinetic properties are well-known (Spassky & Sigman, 1985; Buc, 1987; Buc & McClure, 1985; Thederahn *et al.*, 1990). Gel retardation analysis has demonstrated that the 2:1 2,9-dimethyl-1,10-phenanthroline–cuprous complex [$(2,9\text{-}Me_2\text{-}OP)_2Cu^+$] (Mazumder *et al.*, 1993) and rifampicin (Carpousis & Gralla, 1980), which bind free DNA weakly, are the only transcription inhibitors examined in this study which stably associate with the open complex. $(2,9\text{-}Me_2OP)_2Cu^+$ blocks chemical nuclease scission of the open complex; rifampicin perturbs the scission by the cuprous complex of 5-phenyl-1,10-phenanthroline but not that of the other cuprous complexes at kinetically relevant concentrations. The other transcription inhibitors studied bind DNA tightly by diverse mechanisms and include daunomycin (Chaires *et al.*, 1987; Marky *et al.*, 1983; Wang *et al.*, 1987), actinomycin D (Phillips & Crothers, 1986; Sobell, 1985; Trask & Muller, 1988), di-(amidinophenyl)indole (DAPI) (Straney & Crothers, 1987), distamycin (Kopka *et al.*, 1985), 9-aminoacridine (Ferguson & Denny, 1991), chromomycin A_3 (Gao & Patel, 1989), and mithramycin (Banville et al., 1990). Gel retardation analyses show that they do not form stable ternary complexes. Although both mechanisms for promoting dissociation of the enzyme are probably operative, the first mechanism (eq 2) predominates because enzymatic activity is decreased at lower ligand concentrations than polymerase binding.

MATERIALS AND METHODS

5′-Labeling of Template Strand. Forty micrograms of the plasmid pBVM87 is restricted with 80 units of *Eco*RI (Gibco–BRL) for 3 at 37 °C to generate a 203 base pair fragment. This fragment is then treated with 5 units of calf intestinal phosphatase (CIP) (Boehringer-Mannheim). The CIP is inactivated by heating at 72 °C for 2 h in the presence of 100 mM NaCl, 50 mM Tris, pH 8, 5 mM EDTA, and 1% SDS. The reaction is then subjected to phenol–chloroform extraction and loaded on an 8% nondenaturing polyacrylamide gel. The gel is run at 250 V for 1 h. The gel is stained with ethidium bromide and the 203 base pair fragment is excised and eluted overnight at 37 °C in PAGE elution buffer (0.5 M ammonium acetate, pH 7, and 1 mM EDTA). After 12 h, the solution containing the DNA is extracted with butanol, ethanol-precipitated, washed, dried, and resuspended in 100 μL, of autoclaved, deionized water. Fifty microliters of the fragment (about 5 μg) is 5′-end-labeled using T4 kinase (Gibco–BRL) and [γ-^{32}P]ATP (Amersham). The labeled fragment is run through a Sephadex G-50 spin column and the eluant is subjected to a *Pvu*II digest to generate a singly labeled 186-bp fragment. The reaction is electrophoresed on an 8% nondenaturing polyacrylamide gel for 1 h at 250 V. Autoradiography is performed to localize the 186-bp fragment, which is then excised and eluted in PAGE elution buffer. The labeled DNA is then ethanol-precipitated, washed, dried, and resuspended in a final volume to give a specific activity of about 15 000–30 000 cpm/μL.

Open Complex Formation and Scission. One microliter of RNA polymerase (Pharmacia) is diluted to a final volume of 50 μL in 2× transcription buffer (80 mM Tris, pH 8, 100 mM KCl, and 20 mM $MgCl_2$) to give a stock concentration of 400 nM. Five microliters of 5′-labeled *lacUV5* fragment is incubated with 5 μL of either 2× transcription buffer (for the DNA alone control) or 1:50 RNA polymerase in 2× transcription buffer at 37 °C for 10 min. Two microliters of a stock solution of 465 μM OP/233 μM $CuSO_4$ is then added, followed by 1.5 μL of 69 mM 3-mercaptopropionic acid (MPA) to make the solution 17.3 μM in $(OP)_2Cu^+$ (or related chelate). The scission chemistry is allowed to proceed for 4 min at 37 °C. The reaction is quenched by the addition of 1.5 μL of 0.5 M EDTA to a final concentration of 50 mM, 2 μL of 3 M NaOAc, and 50 μL of ethanol. The reactions are then ethanol-precipitated at −80 °C for 40 min and the precipitates are washed twice with 70% ethanol, evaporated to dryness, and redissolved in deionized formamide dye (80% deionized formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, and 1 mM EDTA). The reactions are then heat-denatured and electrophoresed on a 10% denaturing gel run at 60 W for 2 h 20 min. Autoradiography is performed overnight at −80 °C.

Gel Retardations. For gel retardations, a 5% nondenaturing polyacrylamide gel is used. The RNA polymerase stock solution (1:50 dilution in 2× transcription buffer) is added to the 5′-labeled *lacUV5* fragment and allowed to bind to the promoter for 10 min at 37 °C. For reactions with inhibitor, 2 μL of the drug is added. The reaction is incubated at 37 °C for an additional 5 min. One-half microliter of 2 mg/mL heparin is added and the incubation is continued at 37 °C for 2 min. Four microliters of Ficoll/0.1% bromophenol blue is added and the reaction is loaded on the gel. The gel is then run at 25 mA for 2 h.

Blockage of Scission by a Ligand or Antibiotic. Five microliters of 5′-labeled *lacUV5* is incubated with 5 μL of RNA polymerase diluted 1:50 in 2× transcription buffer for 10 min at 37 °C. After 10 min, 2 μL of either water (for the no-inhibitor control) or the 7.75× stock of the ligand or antibiotic is added and the reaction is incubated at 37 °C for an additional 2 min. The cleavage reaction and electrophoresis are then carried out as described above.

Transcription Reactions. Unlabeled *lacUV5* DNA is generated by *Eco*RI restriction digest of the pBVM87 plasmid containing the *lacUV5* promoter. The fragment is dissolved in water at a final concentration of 0.1 nmol/mL. For each transcription reaction, 16 μL of this template is mixed with 3 μL of 10× transcription buffer (400 mM Tris-HCl, pH 8, 500 mM KCl, and 100 mM $MgCl_2$), 1 μL of 1 mM DTT, and 10 μL of 1:50 RNA polymerase. This reaction is incubated at 37 °C for 10 min. Three microliters of the drug is added, and the reaction is incubated for an additional 3 min. One microliter of [α-^{32}P]UTP (specific activity 400 Ci/mmol) and 3 μL of the nucleotide mixture (1.5 mM ATP, 0.625 mM UTP, 0.625 mM GTP, and 0.625 mM CTP) are added. The transcription is allowed to proceed for 30 min at 37 °C. The reaction is then quenched with 2 μL of 0.5 M EDTA and 8 μL of deionized formamide dye and heated at 95 °C for 5 min. Ten microliters of each reaction is loaded on a 20% denaturing polyacrylamide gel and electrophoresed at 60 W for 2 h. Autoradiography is performed at −80 °C with an intensifying screen.

RESULTS

Inhibitors Which Bind to the Open Complex: (A) (2,9-Me_2OP)-Cu^+. The cuprous complexes of 1,10-phenanthroline and its 5-phenyl-, 5-methyl-, and 5-bromo derivatives all cleave the template strand of the open complex of the *lacUV5* promoter (Thederahn *et al.*, 1990). However, all the scission patterns are not identical. For example, the cuprous complex of 5-phenyl-1,10-phenanthroline [$(5\phi OP)_2Cu^+$] is particularly efficient in cleaving the open complex. It cuts not only upstream of the start of transcription at sequence positions −6 to −3 but also weakly downstream at sequence positions +3

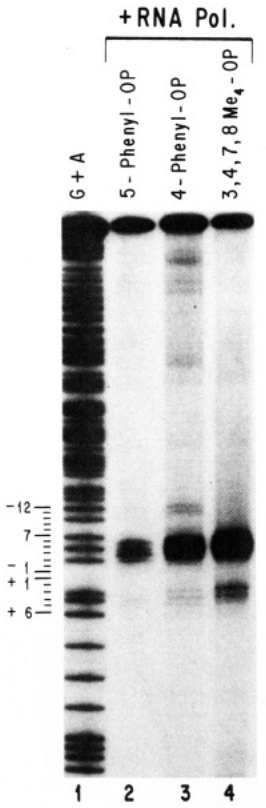

FIGURE 1: Scission of *lacUV5–E. coli* RNA polymerase by copper complexes of 1,10-phenanthroline and its derivatives. Lane 1: Maxam–Gilbert G + A sequencing lane. Lanes 2–4: Scission of the open complex by $(5\phi OP)_2Cu^+$ (lane 2), $(4\phi OP)_2Cu^+$ (lane 3), or $(3,4,7,8\text{-}Me_4OP)_2Cu^+$ (lane 4).

to +5. In order to generate the broadest range of probes for the molecular architecture of the open complex, we have now examined the reactivity of the copper complexes of 4-phenyl-1,10-phenanthroline [$(4\phi OP)_2Cu^+$] and 3,4,7,8-tetramethyl-1,10-phenanthroline [$(3,4,7,8\text{-}Me_4OP)_2Cu^+$]. Both yield scission patterns that are different from those observed with $(OP)_2Cu^+$ and $(5\phi OP)_2Cu^+$ (Figure 1). In addition to generating the bands from –6 to –3 characteristic of all the 1,10-phenanthroline–copper chemical nucleases, the $(4\phi OP)_2Cu^+$ digest (lane 3) gives products at –11 and –12 and +3 to +5. These scission sites probably define the lagging and leading edges of the transcription bubble. The $(3,4,7,8\text{-}Me_4OP)_2Cu^+$ digest (lane 4) is similar to that produced with $(5\text{-}\phi OP)_2Cu^+$ except that the downstream scission sites at positions +3 to +5 are more intense than the corresponding sites with $(5\text{-}\phi OP)_2Cu^+$.

In previous studies, we have demonstrated that the 2:1 $(2,9\text{-}Me_2OP)_2Cu^+$ complex inhibits transcription from the *lacUV5* promoter and blocks scission by $(OP)_2Cu^+$ with an I_{50} of approximately 20 μM and a Hill coefficient of 1.8 (Mazumder *et al.*, 1993). $(2,9\text{-}Me_2OP)_2Cu^+$ binding to the open complex can also be visualized using the "reporter" nuclease activities of $(3,4,7,8\text{-}Me_4OP)_2Cu^+$ or $(5\text{-}\phi OP)_2Cu^+$ (Figure 2). The protection of scission by the $(2,9\text{-}Me_2OP)_2Cu^+$ indicates that this redox-stable coordination complex interferes with the binding of the cleavage-competent coordination complex. Since the cleavage-competent coordination complexes must bind adjacent to the DNA of the transcription bubble for transcription to be observed, $(2,9\text{-}Me_2OP)_2Cu^+$ must bind at this locus as well.

(*B*) *Rifampicin.* The prokaryotic-specific transcription inhibitor rifampicin inhibits the synthesis of the second phosphodiester bond in an *in vitro* transcription reaction

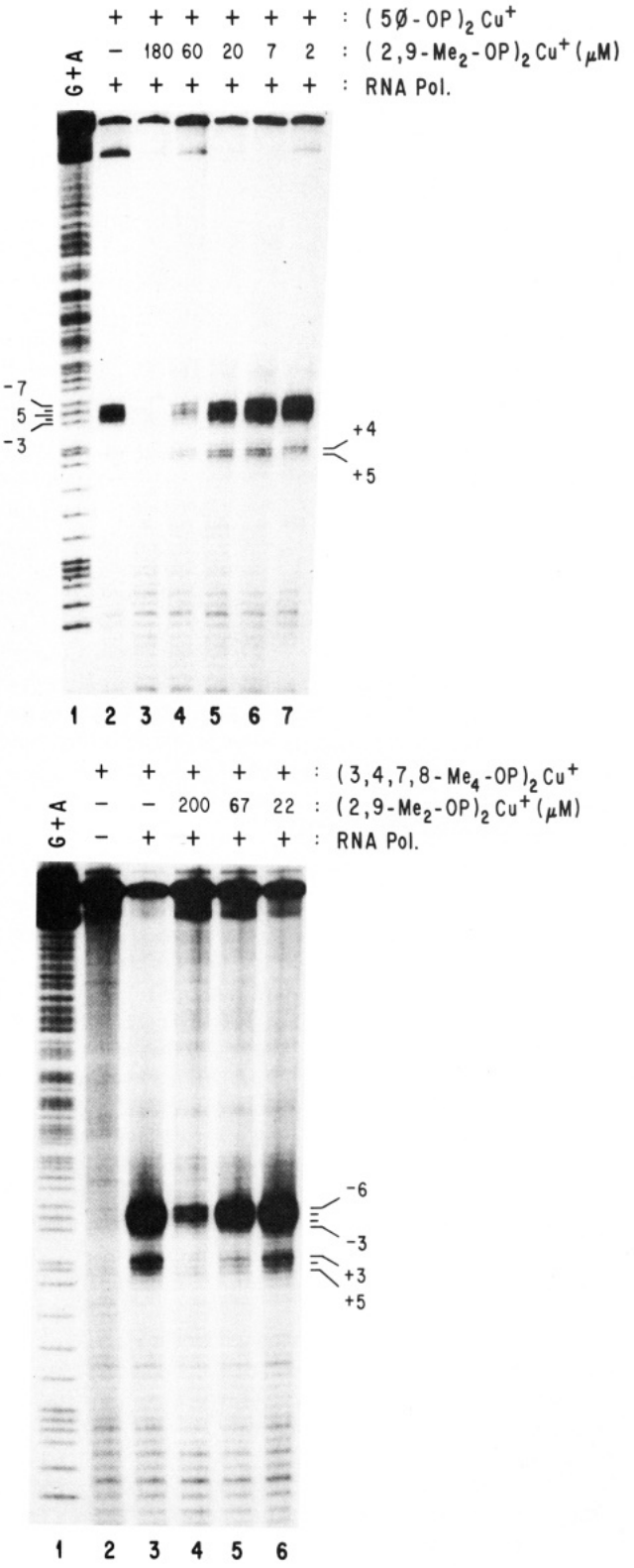

FIGURE 2: Protection of the *lacUV5* open complex scission by $(5\phi OP)_2Cu^+$ and $(3,4,7,8\text{-}Me_4OP)_2Cu^+$ by 2:1 $(2,9\text{-}Me_2OP)_2Cu^+$ complex. (A, top panel) $(5\phi OP)_2Cu^+$. Lane 1: Maxam–Gilbert G + A sequencing lane. Lane 2: Scission of the open complex by $(5\phi OP)_2Cu^+$. Lanes 3–7: Protection of scission at –6 to –3 by 2,9-Me_2-1,10-phenanthroline–copper at 180 μM (lane 3), 60 μM (lane 4), 20 μM (lane 5), 7 μM (lane 6), or 2 μM (lane 7). (B, bottom panel) $(3,4,7,8\text{-}Me_4OP)_2Cu^+$. Lane 1: G + A. Lane 2: Scission of DNA only. Lane 3: Scission of the open complex. Lanes 4–6: Protection of scission by 2,9-Me_2-1,10-phenanthroline–copper at 200 μM (lane 4), 67 μM (lane 5), or 22 μM (lane 6).

(Carpousis & Gralla, 1980). We investigated the interaction of this inhibitor with the open complex of *lacUV5* and RNA

polymerase using gel retardation assays and the chemical nuclease activity of $(5\phi OP)_2Cu^+$. As with $(2,9\text{-}Me_2OP)_2Cu^+$, gel retardation assays demonstrated that rifampicin did not displace RNA polymerase from the *lacUV5* promoter at concentrations where it inhibited the enzymatic activity completely (Figure 3A).

Rifampicin's influence on the scission patterns of the 1,10-phenanthroline-derived chemical nucleases is consistent with the formation of stable ternary complexes. No perturbation of the $(OP)_2Cu^+$ cleavage pattern is observed even at concentrations in excess of 20 μM, where complete transcription inhibition is observed (Figure 3B). In contrast, 20 μM rifampicin perturbs the scission pattern of $(5\phi OP)_2Cu^+$ both upstream and downstream of the start of transcription. Scission at –3 and –4 is enhanced, while cleavage from –6 to –5 is diminished (Figure 3C). Cleavage at the downsteam sites +4 to +5 is also attenuated. In contrast to the results obtained with $(OP)_2Cu^+$, the rifampicin-induced changes in the pattern of scission by $(5\phi OP)_2Cu^+$ provides positive evidence for the formation of a ternary complex composed of RNA polymerase, *lacUV5* and the inhibitor. Rifampicin's ability to perturb the pattern of cleavage of the open complex by $(5\phi OP)_2Cu^+$ probably reflects the different orientations that the tetrahedral cuprous chelates can assume within the open complex. This factor must also contribute to the distinctive scission patterns of the various chemical nucleases. With the data presently available, it is not possible to determine if rifampicin affects scission by steric hindrance or by the induction of conformational changes as a result of its binding to the β subunit of RNA polymerase.

Inhibition by DNA Ligands. Neither $(2,9\text{-}Me_2OP)_2Cu^+$ nor rifampicin binds to free DNA with high affinity or sequence specificity (Graham & Sigman, 1984; Liu et al., 1993), even though they bind stably to the open complex. However, ligands like 9-aminoacridine, ethidium bromide, actinomycin D, daunomycin, distamycin, DAPI, mithramycin, and chromomycin A_3, which bind to DNA with high affinity, are also effective transcription inhibitors. Gel retardation, $(OP)_2Cu^+$ footprinting, and inhibition kinetics indicate that these ligands form unstable ternary complexes with the polymerase and DNA which promote the dissociation of the enzyme.

Intercalating Agents. A variety of hydrodynamic and spectroscopic methods have indicated that intercalating agents and groove binders perturb the conformation of DNA (Feigon et al., 1984; Waring, 1979). Structural alterations can also be detected by the chemical nuclease activity of $(OP)_2Cu^+$. When the binding of ethidium bromide to the *lacUV5* promoter is studied using $(OP)_2Cu^+$ as the footprinting reagent and the products are analyzed on a sequencing gel, the concentration-dependent attenuation of $(OP)_2Cu^+$ scission as a function of ethidium bromide concentration is observed. No specific sequence is protected from $(OP)_2Cu^+$ cutting. This inhibition of scission can be explained by the reaction mechanism of $(OP)_2Cu^+$, which involves the reversible binding of the tetrahedral cuprous complex to the minor groove of the target DNA. Cleavage is inhibited by ligands which either bind to the minor groove, block access to it, or perturb the DNA structure so that the minor groove loses its affinity for the tetrahedral complex. Intercalating agents (e.g., 9-aminoacridine, proflavin, and ethidium bromide) must block scission by the latter mechanism because they lengthen the DNA and alter the dimensions of the minor groove.

Correspondingly, the global changes in DNA caused by intercalating agent will also interfere with the binding of RNA polymerase. This is demonstrated by the displacement of the

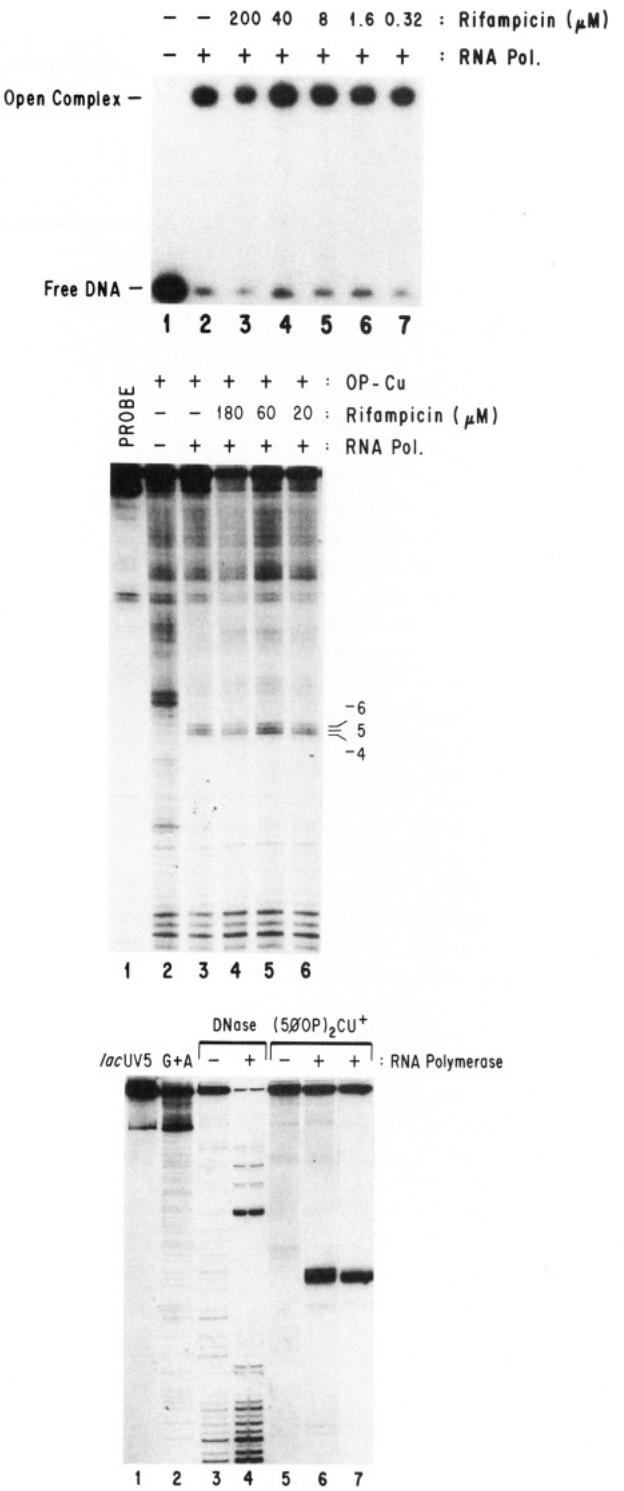

FIGURE 3: Binding of rifampicin to *lacUV5* open complex. (A, top panel) Stability of the open complex in the presence of rifampicin. Lane 1: 5′-labeled *lacUV5* DNA alone. Lane 2: Gel retardation of the open complex in the absence of rifampicin. Lanes 3–7: Gel retardation in the presence of rifampicin at 200 μM (lane 3), 40 μM (lane 4), 8 μM (lane 5), 1.6 μM (lane 6), or 0.32 μM (lane 7). (B, middle panel) Scission of the open complex by $(OP)_2Cu^+$ in the presence of rifampicin. Lane 1: *lacUV5* DNA alone. Lane 2: OP-Cu [$(OP)_2Cu^+$] scission of *lacUV5*. Lane 3: Scission of open complex. Lanes 4–6: Scission of the open complex in the presence of 180, 60, and 20 μM rifampicin. (C, bottom panel) Scission of the open complex by $(5\phi OP)_2Cu^+$ in the presence of rifampicin. Lane 1: Uncut *lacUV5* DNA. Lane 2: G + A calibration. Lane 3: DNase I digest. Lane 4: DNase I footprint of open complex. Lane 5: $(5\phi OP)_2Cu^+$ scission of *lacUV5* DNA. Lane 6: $(5\phi OP)_2Cu^+$ scission of open complex. Lane 7: $(5\phi OP)_2Cu^+$ scission of open complex in the presence of 20 μM rifampicin.

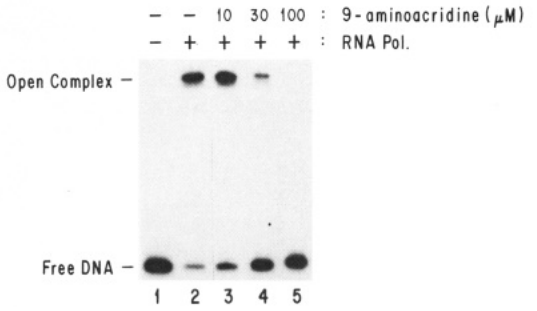

FIGURE 4: Displacement of *E. coli* RNA polymerase from *lacUV5* by 9-aminoacridine measured by gel retardation. Lane 1: 5′-Labelled *lacUV5* DNA alone. Lane 2: Gel retardation of the open complex in the absence of 9-aminoacridine. Lanes 3–5: Gel retardation in the presence of 10 μM (lane 3), 30 μM (lane 4), or 100 μM (lane 5) 9-aminoacridine.

enzyme from the *lacUV5* promoter by 9-aminoacridine using a gel retardation assay (Figure 4). $(OP)_2Cu^+$ can also be used to monitor the dissociation of the open complex caused by intercalating agents. Hypersensitive bands at –6 to –3 are lost and the strong cleavage sites of the Pribnow box characteristic of unbound *lacUV5* DNA are restored with increasing concentration of intercalating agent.

Groove Binders. In contrast to intercalating agents, ligands which bind exclusively (e.g., distamycin) or partially (actinomycin D) to the minor groove are sequence-specific in their interaction. Their binding sites can be identified by $(OP)_2Cu^+$ footprinting. For example, the scission of the Pribnow box by $(OP)_2Cu^+$ is protected by netropsin, Hoechst 33258, and distamycin (Figure 5A). Certain ligands induce hyperreactive $(OP)_2Cu^+$ sites in the DNA substrate. In particular, the scission patterns of $(OP)_2Cu^+$ on the template strand of the *lacUV5* promoter in the presence of mithramycin and actinomycin D contain strong cleavage sites which are not present in the control at sequence positions +12 to +20 (Figure 5A). They also increase the cleavage rates of $(OP)_2Cu^+$ within the Pribnow box.

These DNA ligands can therefore inhibit transcription in two ways. First, they can weaken RNA polymerase binding by inducing conformational changes in the DNA binding site of the enzyme. Second, they can compete with the enzyme at a variety of sequences over the extended binding site of RNA polymerase. The effectiveness of actinomycin D, DAPI, and daunomycin in displacing the polymerase from the promoter is clearly evident from the gel retardation analysis (Figure 5B). The dependence of enzymatic activity, enzyme binding, and $(OP)_2Cu^+$ scission on the concentration of DAPI, distamycin, daunomycin, and mithramycin are summarized in Figure 6. In all cases, enzyme activity is inhibited at lower inhibitor concentrations than $(OP)_2Cu^+$ scission or RNA polymerase binding (Figure 6). These observations are consistent with the formation of a metastable ternary complex in which the precise orientation of catalytic groups is disrupted by the binding of the high-affinity DNA ligands. None of these ligands attenuate the upstream scission site of $(OP)_2Cu^+$ without displacing the enzyme from the promoter. There is no evidence that they interact with the single-stranded DNA within the open complex.

DISCUSSION

Chemical nuclease footprinting with the copper complexes of 1,10-phenanthroline and its derivatives and gel retardation analyses provide convenient experimental tools to characterize the interaction of transcription inhibitors with the open

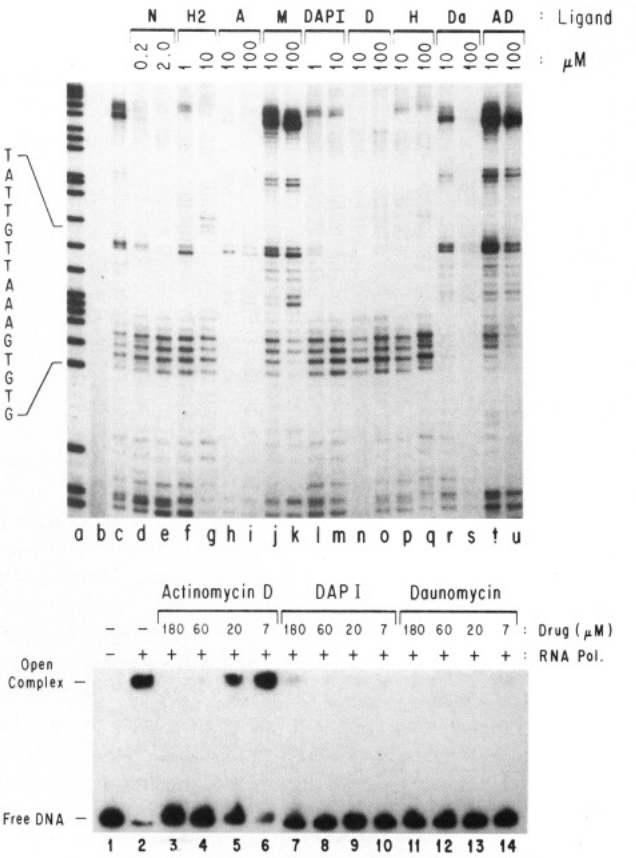

FIGURE 5: Interaction of groove binders with the *lacUV5* promoter and its open complex. (A, top panel) $(OP)_2Cu^+$ Footprinting of the binding of groove binders to the *lacUV5* promoter. The binding of the following DNA ligands to the template strand of the *lacUV5* promoter was assayed using $(OP)_2Cu^+$ as the footprinting reagent. Abbreviations: N, netropsin; H2, Hoechst 33258; A, adriamycin; M, mithramycin; D, distamycin; H, Hoechst 33342; Da, daunomycin; Ad, actinomycin D. The concentrations of ligands in the reaction mixtures are indicated. (B, bottom panel) Stability of open complexes assayed by gel retardation. Lane 1: 5′-Labeled *lacUV5* DNA alone. Lane 2: Gel retardation of the open complex in the absence of any ligand. Lanes 3–6: Gel retardation in the presence of 180 μM (lane 3), 60 μM (lane 4), 20 μM (lane 5), or 7 μM (lane 6) actinomycin D. Lanes 7–10: Gel retardation in the presence of 180 μM (lane 7), 60 μM (lane 8), 20 μM (lane 9), or 7 μM (lane 10) DAPI. Lanes 11–14: Gel retardation in the presence of 180 μM (lane 11), 60 μM (lane 12), 20 μM (lane 13), or 7 μM (lane 14) daunomycin.

complex formed between *E. coli* RNA polymerase and the *lacUV5* promoter. With these complementary approaches, two categories of inhibitors have been identified. The first category includes ligands such as $(2,9\text{-}Me_2OP)_2Cu^+$ and rifampicin. Although they do not bind to the free DNA tightly, they stably associate with the open complex. The second category of ligands bind to DNA with high affinity and defined stereochemistry. They inhibit transcription by destabilizing the binding of RNA polymerase and/or directly competing with the binding of the enzyme to the promoter. Ligands of this class are 9-aminoacridine, distamycin, actinomycin D, DAPI, mithramycin, and chromomycin A_3.

Inhibitors Which Bind to the Open Complex. The tetrahedral 2:1 $(2,9\text{-}Me_2OP)_2Cu^+$ complex, which is a redox-inactive analog of the chemical nuclease $(OP)_2Cu^+$, inhibits transcription by binding to the single-stranded DNA formed in the open complex. Gel retardation analyses have demonstrated that the complex does not displace the enzyme from the promoter (Mazumder et al., 1993). In fact, at high concentrations it appears to stabilize the binding of the enzyme and to alter its electrophoretic migration in a nondenaturing

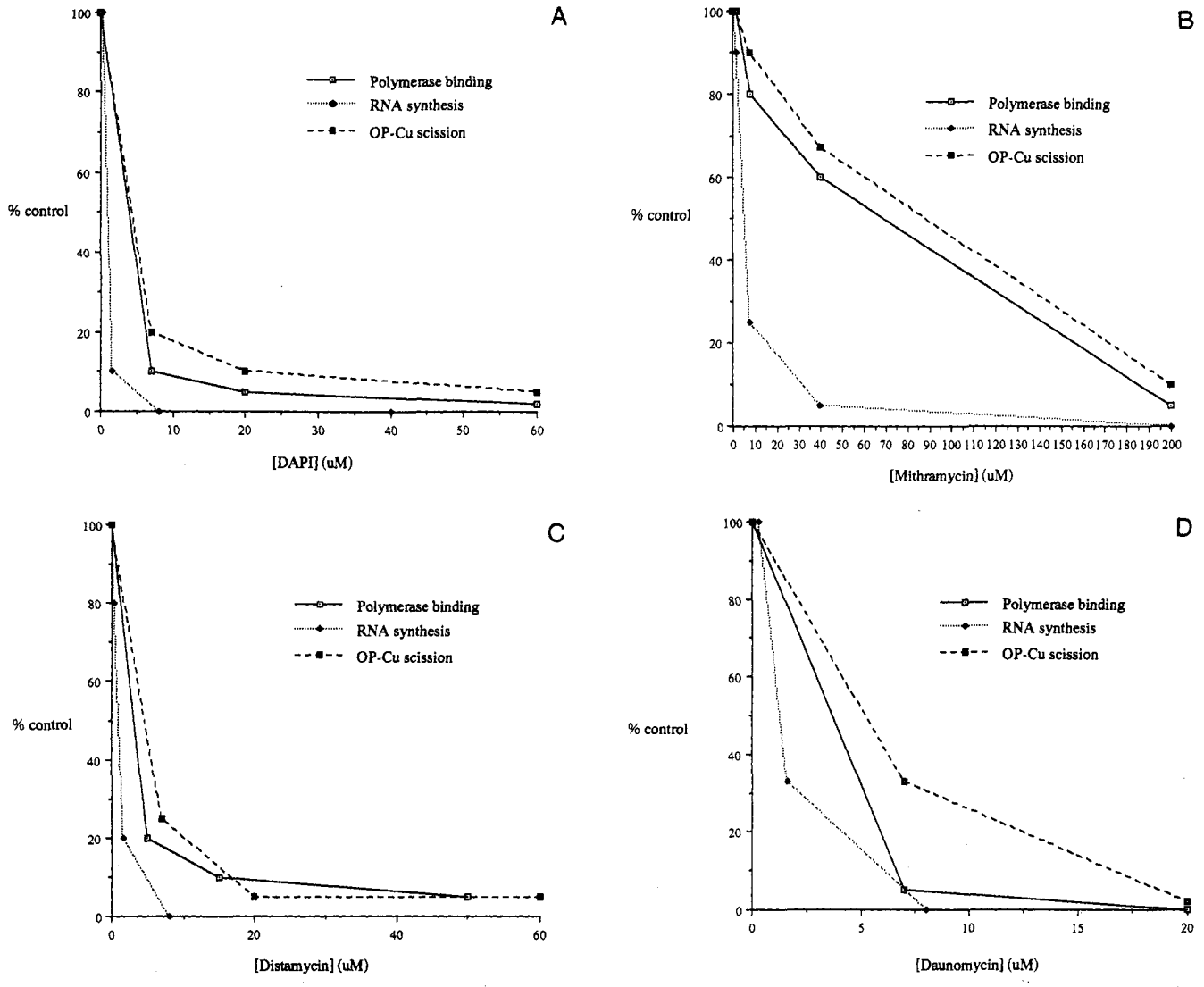

FIGURE 6: Dependence of open complex stability, transcription inhibition, and blockage of $(OP)_2Cu^+$ Scission on ligand concentration. Gel retardation, inhibition of transcription, and protection of the open complex from scission by 1,10-phenanthroline–copper were used to assay the interaction of (A) DAPI, (B) daunomycin, (C) distamycin, and (D) mithramycin with the open complex of *lacUV5*. All experiments were carried out at 37 °C, pH 8.0.

acrylamide gel (Mazumder et al., 1993). $(2,9\text{-}Me_2OP)_2Cu^+$ is the only known inhibitor of transcription which binds to the melted DNA bubble of the open complex. This site of binding is *created by the interaction of the protein with DNA*. In this sense the mechanism of inhibition resembles the binding of quinolone antibacterials with gyrase–DNA intermediates (Shen *et al.*, 1989).

Rifampicin is another inhibitor which binds to the open complex. Gel retardation analyses demonstrate that rifampicin does not displace RNA polymerase from *lacUV5*. Since rifampicin-resistant mutants of *E. coli* have been isolated which have alterations in their β subunits (Jin *et al.*, 1988a,b; Jin & Gross, 1988, Jin & Gross, 1991), the primary site of action of rifampicin must be on the protein, a conclusion supported by biophysical studies as well (Kumar & Chatterji, 1990; Kumar *et al.*, 1992). Therefore, in contrast to $(2,9\text{-}Me_2OP)_2Cu^+$, the formation of the open complex does not generate the rifampicin binding site. The failure of rifampicin to affect the scission of the open complex by $(OP)_2Cu^+$ at concentrations at which it completely blocks transcription is consistent with this model. The perturbation by rifampicin of the $(5\phi OP)_2Cu^+$ scission sites provides positive evidence for ternary complex formation although steric clash between the antibiotic and the chelate or structural changes in the open complex due to the binding of rifampicin to the β subunit of RNA polymerase may be responsible for the observed effect.

Inhibitors Which Destabilize the Open Complex. The other transcription inhibitors examined in this study differ from $(2,9\text{-}Me_2OP)_2Cu^+$ and rifampicin in having a high affinity for DNA in the absence of RNA polymerase. These drugs include 9-aminoacridine (Ferguson & Denny, 1991), distamycin (Kopka et al., 1985), actinomycin D (Phillips & Crothers, 1986; Sobell, 1985; Trask & Muller, 1988), and DAPI (Straney & Crothers, 1987). They inhibit enzymatic activity at lower concentrations than those at which they displace the enzyme or block the scission of the transcription bubble by the chemical nuclease. This discrepancy argues for the formation of a catalytically inactive metastable ternary complex. In these complexes, the structural changes associated with their binding to DNA, reflected in the footprinting experiment with $(OP)_2Cu^+$ (Figure 5A), are likely to be capable of disrupting the catalytic competence of the open complex and destabilizing the binding of RNA polymerase to the promoter. However, the details of the structural alterations for each of the inhibitors are not yet known.

Many DNA ligands are cytotoxic and pharmacologically useful (Denny, 1989; Hurley & Boyd, 1988; Zunino & Capranico, 1990). The purpose of these studies was to

investigate their mechanism of transcription inhibition of the well-characterized *lacUV5* promoter. A surprising result was that $(2,9\text{-}Me_2OP)_2Cu^+$ is the only transcription inhibitor which interacts specifically with the single-stranded DNA formed at the open-complex during transcription initiation. The unexpected cytotoxicity of this chemically unreactive and exchange-inert copper complex may arise from its ability to inhibit transcription in a variety of cell types (Antic *et al.*, 1977; Mohindru *et al.*, 1983). The inhibition of DNA replication might be an alternative mode of action of this complex given the reactivity of its isostere $(OP)_2Cu^+$ at replication origins (Diffley & Cocker, 1992). The discovery that intercalating agents and groove binders with distinct binding specificities inhibit transcription by the relatively nonspecific mechanism of disrupting and/or displacing the polymerase from the promoter was unexpected.

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REFERENCES

Antic, B. M., van der Goot, H., Nauta, W. T., Balt, S., De Bolster, W. G., Stouthamer, A. H., Verheul, H., & Vis, R. D. (1977) *Eur. J. Med. Chem. 12*, 573–575.

Banville, D. L., Keniry, M. A., & Shafer, R. H. (1990) *Biochemistry 29*, 9294–9304.

Buc, H. (1987) in *Nucleic Acids and Molecular Biology* (Eckstein, F., & Lilley, D. M. J., Eds.) Vol. 1, pp 186–195, Springer-Verlag, Berlin and Heidelberg, Germany.

Buc, H., & McClure, R. (1985) *Biochemistry 24*, 2714–2723.

Carpousis, A. J., & Gralla, J. D. (1980) *Biochemistry 19*, 3245–3253.

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

Daube, S. S., & von Hippel, P. H. (1992) *Science 258*, 1320–1324.

Denny, W. A. (1989) *Anti-Cancer Drug Des. 4*, 241–263.

Diffley, J. F. X., & Cocker, J. H. (1992) *Nature 357*, 169–172.

Feigon, J., Denny, W. A., Leupin, W., & Kearns, D. R. (1984) *J. Med. Chem. 27*, 450–465.

Ferguson, L. R., & Denny, W. A. (1991) *Mutat. Res. 258*, 123–160.

Gao, X.; & Patel, D. J. (1989) *Biochemistry 28*, 751–762.

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

Graham, D. R., & Sigman, D. S. (1984) *Inorg. Chem. 23*, 4188–4191.

Hurley, L. H., & Boyd, F. L. (1988) *Trends Pharmacol. Sci. 9*, 402–407.

Jin, D. J., & Gross, C. A. (1988) *J. Mol. Biol. 202*, 45–58.

Jin, D. J., & Gross, C. A. (1991) *J. Biol. Chem. 266*, 14478–14485.

Jin, D. J., Walter, W. A., & Gross, C. A. (1988a) *J. Mol. Biol. 202*, 245–253.

Jin, D. J., Cashel, M., Friedman, D. I., Nakamura, Y., Walter, W. A., & Gross, C. A. (1988b) *J. Mol. Biol. 204*, 247–261.

Kopka, M. L., Yoon, C., Goodsell, D., Pjura, P., & Dickerson, R. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1376–1380.

Kumar, K. P., & Chatterji, D. (1990) *Biochemistry 29*, 317–322.

Kumar, K. P., Reddy, P. S., & Chatterji, D. (1992) *Biochemistry 31*, 7519–7526.

Liu, F., Meadows, K. A., & McMillin, D. R. (1993) *J. Am. Chem. Soc. 115*, 6699–6704.

Marky, L. A., Snyder, J. G., Remeta, D. P., & Breslauer, K. J. (1983) *J. Biomol. Struct. Dyn. 1*, 487–507.

Mazumder, A., Perrin, D. M.; Watson, K. H., & Sigman, D. S. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8140–8144.

Mohindru, A., Fisher, J. M., & Rabinovitz, M. (1983) *Biochem. Pharmacol. 32*, 3627–3632.

Phillips, D. R., & Crothers, D. M. (1986) *Biochemistry 25*, 7355–7362.

Sigman, D. S., & Chen, C.-h. B. (1990) *Annu. Rev. Biochem. 59*, 207–236.

Sobell, H. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5328–5331.

Spassky, A., & Sigman, D. S. (1985) *Biochemistry 24*, 8050–8056.

Straney, D. C., & Crothers, D. M. (1987) *Biochemistry 26*, 1987–1995.

Suh, W.-C., Leirmo, S., & Record, M. T., Jr. (1992) *Biochemistry 31*, 7815–7825.

Suh, W.-C., Ross, W., & Record, M. T., Jr. (1993) *Science 259*, 358–361.

Thederahn, T., Spassky, A., Kuwabara, M. D., & Sigman, D. S. (1990) *Biochem. Biophys. Res. Commun. 168*, 756–762.

Trask, D. K., & Muller, M. T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1417–1421.

von Hippel, P. H., & Yager, T. D. (1992) *Science 255*, 809–812.

Wang, A. H. J., Ughetto, G., Quigley, G. J., & Rich, A. (1987) *Biochemistry 26*, 1152–1163.

Waring, M. (1979) *Annu. Rev. Biochem. 50*, 159–192.

Zunino, F., & Capranico, G. (1990) *Anti-Cancer Drug Des. 5*, 307–317.