A Comparison of DNA-Binding Drugs as Inhibitors of E2F1− and Sp1−DNA Complexes and Associated Gene Expression†

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ABSTRACT: In this study, we examined how DNA-binding drugs prevented formation of transcription factor−DNA complexes and influenced gene transcription from the hamster dihydrofolate reductase promoter, which is regulated by E2F1 and Sp1. Gel mobility shift assay data showed that GC-binding drugs (e.g., mitoxantrone) inhibited the DNA binding of both E2F1 and Sp1. In contrast, AT-binding drugs (e.g., distamycin) interfered only with E2F1−DNA complex formation. In an in vitro transcription assay using HeLa nuclear extracts, inhibition of transcription was observed when mitoxantrone or distamycin was added either before or after assembly of the transcription complex on the DNA, although for the latter, higher drug concentrations were needed. Mitoxantrone, which was a stronger inhibitor of transcription factor−DNA complex, was more effective than distamycin at preventing transcript formation. Time course transcription in a cell-free assay with addition of various drug concentrations indicated that high drug concentrations of either mitoxantrone or distamycin completely blocked transcription, while low drug concentrations could delay the synthesis of transcripts.

It is known that the regulation of gene transcription requires both sequence-specific recognition of a gene promoter by transcription factors (TFs)[1] and successive interactions between TFs and associated factors (*1*−*3*). DNA-binding domains of TFs are varied, resulting in the recognition of DNA at GC-rich (e.g., Sp1 and EGR1), AT-rich (e.g., TBP), or GA-rich (e.g., Ets family) sequences (*4*−*7*). Some TFs interact with DNA at more complex sequences such as E2F1, which binds to a core GC sequence flanked by As or Ts (*8*). In addition to TF recognition of regulatory DNA sequences, most specific TFs such as EGR1 bind to the DNA major groove; in contrast, TBP, a core protein of the general transcriptional complex TFIID, binds to the DNA minor groove (*4, 9*). DNA groove binding preferences of TFs are important for the initiation of gene expression since TF binding to DNA can alter the topological conformation of a promoter affecting subsequent associations with additional TFs (*10*).

Certain DNA-binding drugs that resemble TFs in their preference for DNA sequences and groove orientation prevent the binding of TFs to DNA (TF−DNA complex) by directly competing with the TF for DNA binding, or by inducing DNA conformational change (*11, 12*). Our laboratory and others have been interested in targeting DNA-binding drugs toward DNA regulatory elements as a means to interfere with the binding of TFs to their consensus binding sites (*13*−*17*). Only a few studies have been carried out to evaluate the effects of DNA-binding drugs on the inhibition of gene transcription. For example, the studies of Snyder et al. (*18*) and Hardenbol and Van Dyke (*19*) demonstrated that the GC-binding drug mithramycin inhibited c-myc expression from its GC-rich promoter. Another study compared activities of the AT-binding drugs distamycin and tallimustine as inhibitors of TBP binding and basal in vitro transcription (*20*). In these studies, drugs interfered with a single type of TFs (i.e., GC- or AT-binding TFs), resulting in inhibition of transcription.

In the present study, we used a natural promoter, the hamster dihydrofolate reductase (DHFR) promoter which is regulated by two TFs, E2F and Sp1, as a model to evaluate the consequences of inhibition by DNA-binding drugs of both TF−DNA complex formation and the subsequent effect on gene transcription. Studies of transcription of the hamster DHFR gene have shown that Sp1 and E2F are required for efficient gene expression (*21*−*24*). Sp1 binds to a GC-rich site called a GC box (5′-G/TGGGCGGG/AG/AC/T), and E2F1 binds as a protein heterodimer to overlapping E2F-binding sites, 5′-TTTCGCGCCAAA (*5, 25, 26*). We analyzed a panel of GC- and AT-binding drugs for their ability to inhibit E2F1 and Sp1 binding to their consensus binding sites using a gel mobility shift assay. Subsequently, drugs that efficiently prevented TF−DNA complex formation were evaluated for the ability to interfere with gene expression using an in vitro transcription assay. The effectiveness of DNA-binding drugs as inhibitors of individual TF−DNA complexes and transcription is discussed.

MATERIALS AND METHODS

Drugs. Distamycin (Sigma Chemical Co., St. Louis, MO) and netropsin (Boehringer-Mannheim Biochemicals, Indianapolis, IN) were prepared in distilled water. Hedamycin

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[1] Abbreviations: DHFR promoter, dihydrofolate reductase promoter; TFs, transcription factors; E2F1, E2 factor 1; EGR1, early growth response factor 1; TBP, TATA box-binding protein.

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(National Cancer Institute, National Institutes of Health) was prepared in 0.1 N HCl and then neutralized with 0.1 N NaOH. Doxorubicin hydrochloride (Aldrich Chemical Co., Milwaukee, WI), mitoxantrone (Lederle Laboratories, Wayne, NJ), nogalamycin (Upjohn Pharmaceuticals, Kakamazoo, MI), and tilorone (National Cancer Institute, National Institutes of Health) were prepared in dimethyl sulfoxide. All drugs were stored at −20 °C.

Preparation of DNA Probes. A *Sma*I−*Xba*I DNA fragment was prepared from the hamster DHFR promoter (nucleotide positions −103 to −23 relative to the translation start site at +1 and containing the overlapping E2F-binding sites). The DNA fragment was end-labeled with [α-^{32}P]-dNTPs by the Klenow "fill in" method and used for E2F1 gel mobility shift assays (*27*). The Sp1-binding oligonucleotide (5′-GATCGAGGGCGTGGC; GC-oligo) and its complementary strand were synthesized by the Biopolymer facility at Roswell Park Cancer Institute (Buffalo, NY). Gel purification, annealing, and end-labeling of oligonucleotides were performed as described previously (*15*).

Proteins. GST−E2F1 (amino acids 1−437) fusion protein produced in *Escherichia coli* was used in gel mobility shift assays. Preparation of GST−E2F1 fusion protein followed a published procedure (*24*). In general, isopropyl β-D-thiogalactopyranoside-induced bacterial lysate was sonicated in Tris buffer [containing 100 mM NaCl, 1 mM EDTA, 20 mM Tris base (pH 8.0), 0.5% Nonidet P40, and 0.5% nonfat dry milk]. Purification of GST−E2F1 fusion protein was carried out by incubating sonicated bacterial lysate with glutathione−Sepharose beads for 45 min at 4 °C, followed by washing the beads twice with 100 mM Tris base (pH 8.0) and 120 mM NaCl. The fusion protein was eluted with 100 mM Tris base (pH 8.0), 120 mM NaCl, and 6.12 mg/mL glutathione (Sigma). The quantity of purified protein was evaluated by SDS−polyacrylamide gel electrophoresis and Bio-Rad protein assay. Sp1 protein was purchased from Promega Co. (Madison, WI).

Mobility Shift Assays. Association of proteins with their consensus DNA binding sites was examined by gel mobility shift assays. A 20 μL reaction contained 3−5 ng of GST−E2F1 fusion protein and 0.2 nM ^{32}P-labeled DNA fragment in a buffer with 20 mM Hepes−KOH (pH 7.9), 25 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 100 μg/mL bovine serum albumin, 0.5 mM dithiothreitol, 0.8 mM spermidine, 10% glycerol, and 0.025% Nonidet P40. After incubation for 30 min at 30 °C, electrophoresis was performed in a 4% native polyacrylamide gel utilizing TBE buffer (44.5 mM Tris base, 44.5 mM boric acid, and 1 mM EDTA, pH 8.3). The activity of GST−E2F fusion protein binding to its consensus DNA sequence was confirmed by antibody-supershift assay (unpublished data). The assay with Sp1 was carried out under similar conditions except that 5 ng of Sp1 and 0.4 nM ^{32}P-labeled GC-oligo were incubated in a buffer containing 10 mM Tris base (pH 8.0), 50 mM NaCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 1 mg/mL bovine serum albumin, and 0.025% Nonidet P40. Autoradiography and quantitation of protein−DNA complex formation were performed as previously described (*17*). Drugs when present were diluted at the indicated concentrations and incubated with DNA for 30 min at 30 °C prior to the addition of protein. Fifty percent inhibition of protein−DNA complex formation (IC_{50}) was determined by comparing drug-treated samples with untreated controls.

In Vitro Transcription. Gene expression driven by the hamster DHFR promoter was evaluated by an in vitro transcription assay. A *Kpn*I−*Hin*dIII DNA fragment from the hamster DHFR promoter (nucleotide positions −210 to −23 relative to the translation initiation site) which was inserted into a pUC18-CAT vector (pDHF-210CAT) was used as template (*28*). Double CsCl-purified pDHF-210CAT DNA was digested with *Sca*I restriction enzyme, extracted with phenol−chloroform−isoamyl alcohol and chloroform, and used for in vitro transcription as described previously (*22*). Nuclear extracts were prepared from HeLa cells by a modification of the Dignam and Shapiro procedure as described previously (*22*). One microgram of *Sca*I-digested pDHFR-210CAT was incubated with HeLa nuclear extracts (∼ 60 μg) for 15 min at 30 °C followed by adding a mixture of 0.5 μL of each nucleotide (20 mM each of ATP, GTP, and UTP, and 25 μM CTP), 1.0 μL of RNAsin (40 units/μL, Boehringer Mannheim), 1.4 μL of EDTA (2.5 mM), and 10 μCi of [α-^{32}P]CTP (3000 Ci/mmol, NEN) to a final volume of 25 μL. After incubation for 60 min at 30 °C, the reaction was stopped by adding 325 μL of 10 mM Tris base (pH 8.0), 7 M urea, 350 mM NaCl, 10 mM EDTA, 1% SDS, and 50−100 μg of tRNA. T3 transcript (250 bases, Promega Co.) or T7 transcript (350 bases, CloneTech) was used as an internal control. Samples were extracted twice with phenol−chloroform−isoamyl alcohol followed by ethanol precipitation. The pellet was resuspended in 10 μL of formamide-loading buffer, heated at 90 °C for 3 min, and loaded onto a 4% denaturing polyacrylamide gel (containing 7 M urea).

Effects of Drugs on in Vitro Transcription. Three types of experiments were performed to test the effects of drugs on an in vitro transcription. First, a standard assay involved treatment of DNA (*Sca*I-digested pDHF-210CAT vector) with drugs for 30 min at 30 °C prior to the addition of HeLa nuclear extracts and nucleotides (ATP, CTP, GTP, UTP). Second, HeLa nuclear extracts and DNA were preincubated for 15 min followed by a 30 min incubation with drugs. Nucleotides were finally added to individual samples (reverse assay). Examination of transcripts was carried out for 60 min after addition of nucleotides for both standard and reverse assays. Third, the transcription rate was measured by a modification of the procedure of Hawley and Roeder (*29*). Briefly, incubation of HeLa nuclear extracts and DNA for 15 min was followed by addition of drugs and the mixture of four nucleotides. Transcription was examined at various time points (1, 5, 10, 30, and 60 min). In all experiments, the transcriptional activity of individual samples was normalized to an internal control. Inhibition of transcription by drugs was expressed as a percentage of activity of a corresponding untreated control.

RESULTS

Effects of Drugs on TF−DNA Complex Formation. Drugs with various DNA binding motifs (Table 1) were evaluated to determine whether the disruption of TF−DNA complexes on the hamster DHFR promoter correlated with the ability of drugs to influence gene transcription. The activity of individual drugs on E2F1 and Sp1 binding to their regulatory

Table 1: Effects of DNA-Binding Drugs on Formation of the TF−DNA Complex[a]

drug	DNA-binding motif	E2F1−DNA IC_{50} (μM)	Sp1−DNA IC_{50} (μM)
GC-binding drug			
doxorubicin	intercalator	0.035	0.54
hedamycin	intercalator	0.022	ND[b]
mitoxantrone	intercalator	0.015	0.28
nogalamycin	intercalator	0.07	ND
AT-binding drug			
distamycin	minor groove binder	0.05	NE[c]
netropsin	minor groove binder	0.32	ND
tilorone	intercalator	0.18	NE

[a] Effects of DNA-binding drugs on formation of E2F1−DNA or Sp1−DNA complexes are expressed as IC_{50} (50% inhibition of TF-DNA complex). [b] ND, not done. [c] NE, no effect.

elements on the hamster DHFR promoter was first examined by gel mobility shift assays.

The DNA-binding site of E2F1 in the hamster DHFR promoter contains a GC-rich center flanked by ATs at each end. A panel of DNA-binding drugs including GC- and AT-preferential intercalators and AT minor groove binders (Table 1) was tested for their ability to prevent the binding of E2F1 to the hamster DHFR promoter. After incubation of the drug and DNA followed by addition of E2F1, gel mobility shift assays were performed. Representative gel mobility shift assays in the presence of drugs are shown in Figure 1. The GC intercalator mitoxantrone blocked E2F1−DNA complex formation in a concentration-dependent manner (Figure 1A). Complex formation was completely inhibited at a drug concentration of 0.5 μM (Figure 1A, lane 6) whereas complex was not blocked at a lower drug concentration of 0.001 μM (Figure 1A, lane 9).

Inhibition of E2F1−DNA complex by distamycin, an AT-binding minor groove binder, was also concentration-dependent, but required higher drug concentration compared with mitoxantrone (Figure 1B). Five micromolar distamycin was required to prevent complex formation by 90% (Figure 1B, lane 3). The E2F1−DNA complex was partially blocked when the drug concentration was reduced to 0.1 μM, and no inhibition of the complex was seen $\leq$0.001 μM (Figure 1B, lanes 4−8). Quantitative data for mitoxantrone and distamycin as inhibitors of the DNA binding of E2F1 are shown in Figure 1C.

Additional GC- and AT-binding drugs were tested, and the results, expressed as IC_{50} (drug concentration that inhibits complex formation by 50%), are summarized in Table 1. The most potent inhibitor of E2F1−DNA complex formation among the GC-specific agents was mitoxantrone with an IC_{50} of 0.015 μM; among the AT-specific agents, distamycin was most potent with an IC_{50} of 0.05 μM. The abilities of GC-binding drugs at inhibiting complex formation by 50% was in the order mitoxantrone > hedamycin > doxorubicin > nogalamycin; the rank order for AT-binding drugs was distamycin > tilorone > netropsin.

Subsequently, we determined the ability of GC-binding mitoxantrone and doxorubicin and AT-binding distamycin and tilorone to inhibit the binding of Sp1 which binds to the GC box on the hamster DHFR promoter. Mobility shift assays were performed in which the DNA binding of Sp1 was determined following drug treatment. Quantitative results in the presence of mitoxantrone or distamycin are

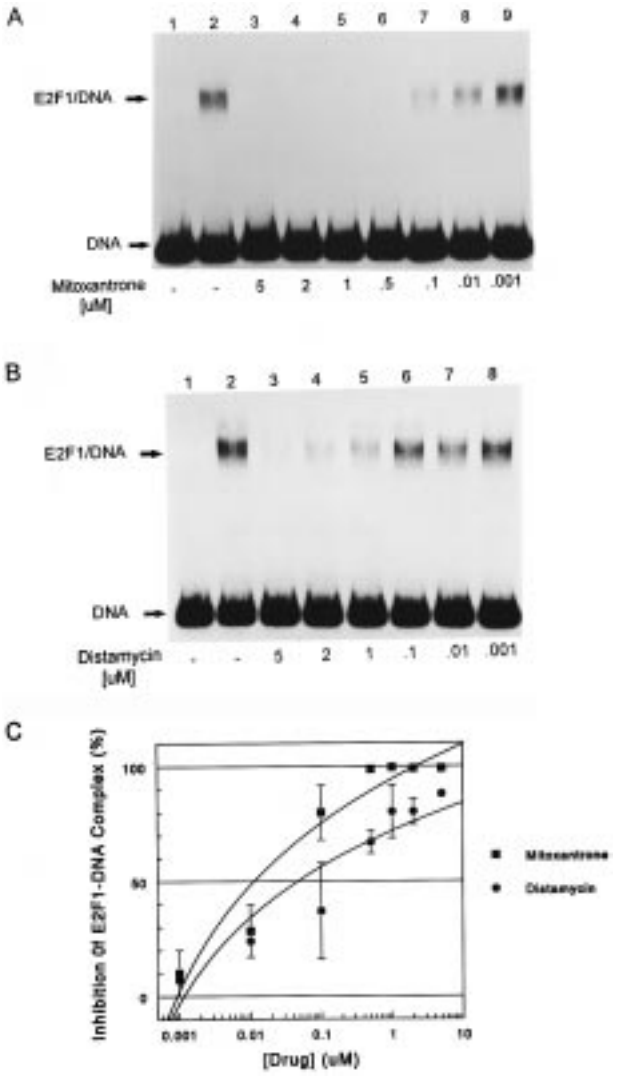

FIGURE 1: Effects of drugs on E2F1−DNA complex formation. Mobility shift assays were used to evaluate the ability of mitoxantrone (A) and distamycin (B) to inhibit E2F1−DNA complex formation. Drugs at the indicated dilutions were incubated with ^{32}P-labeled DNA (from the hamster DHFR promoter containing sequences from positions −103 to −23 from the translation starting site) for 30 min at 30 °C, and then E2F1 protein (a GST-fusion protein) was added to the reaction. After a 30 min incubation at 30 °C, electrophoresis was performed in a 4% native polyacrylamide gel running in a buffer containing 44.5 mM Tris base, 44.5 mM boric acid, and 1 mM EDTA. Autoradiography was performed by exposing the dried gel to Kodak film. Lane 1, free labeled DNA probe; lane 2, E2F1−DNA complex; lanes 3−9, reactions of protein−DNA in the presence of drugs at the indicated concentrations. (C) Quantitative data showing the percentage of inhibition of E2F1−DNA complexes by mitoxantrone (■) and distamycin (●). The results represent the mean of at least three separate experiments (mean values ± SD)

shown in Figure 2. Formation of the Sp1−DNA complex was blocked by 90% when treated with 2 μM mitoxantrone, while at 0.1 μM complex formation was reduced by 20%. As seen in Table 1, the GC-binding drugs doxorubicin and mitoxantrone at 0.54 and 0.28 μM blocked Sp1 from binding DNA by 50% (IC_{50}). In contrast, the AT-binding drugs distamycin and tilorone had no effect on the binding of Sp1 (Figure 2; Table 1).

Inhibition of in Vitro Transcription from the Hamster DHFR Promoter by DNA-Binding Drugs. In experiments to this point we have examined the ability of GC- and AT-

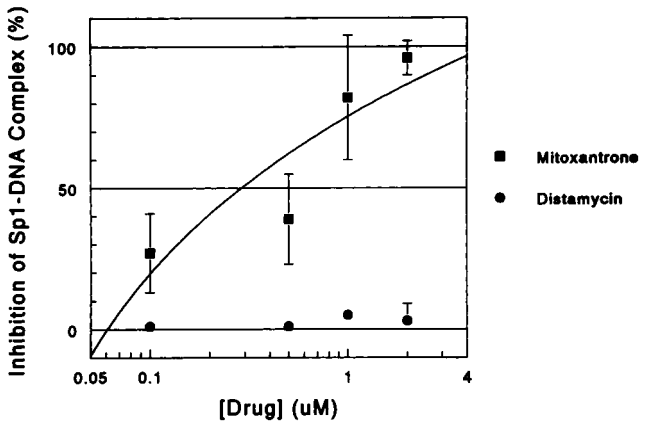

FIGURE 2: Effects of drugs on Sp1−DNA complex formation. The ability of mitoxantrone (■) and distamycin (●) to inhibit DNA binding of Sp1 was examined by mobility shift assays as described for Figure 1 except that a 15-mer oligonucleotide containing one GC box (5′-GATCGAGGGCGTGGC) was used as probe for the binding of Sp1 which was purchased from Promega. The results represent the mean of at least three separate experiments (mean values ± SD).

binding drugs to prevent E2F1 and Sp1 binding to their consensus binding sites on the hamster DHFR promoter by gel mobility shift assays. While maximal transcription requires both E2F1 and Sp1, Sp1 is known to be absolutely required for transcription from the DHFR promoter (*21, 22*). For example, previous findings demonstrated that no transcription was initiated when Sp1-binding sites were mutated (*21, 28*). Thus, drugs which block not only E2F1−DNA complex but also Sp1−DNA complex would be expected to strongly inhibit transcription. The effect of drugs (i.e., mitoxantrone and distamycin) on the transcription from the hamster DHFR promoter was then examined.

In vitro transcription assays were performed by incubating DNA and drug prior to the addition of HeLa nuclear extracts (standard assay) as described under Materials and Methods. A representative result with mitoxantrone is shown in Figure 3A. In the untreated control, the major transcript of 736 bases and the minor transcript of 762 bases were synthesized as previously published (Figure 3A, lane 1) (*22*). Effects of drugs on the more abundant 736-base transcript were evaluated. When mitoxantrone was added to the reaction, a concentration-dependent reduction of the major transcript was obtained (Figure 3A, lanes 2−5). In Figure 3B, quantitative data indicated that 20 μM mitoxantrone inhibited transcription by 70% whereas 9 μM drug reduced transcription by 50%. Similarly, a transcription assay with distamycin was carried out to compare its effectiveness with that of mitoxantrone at inhibiting transcription from the hamster DHFR promoter. When distamycin was used, 20 μM drug inhibited transcript formation by about 65% and 10 μM drug blocked transcription by 50%. Reducing the concentration of both drugs to 1 μM resulted in no inhibition of transcription (Figure 3B).

In the previous experiments, the effects of drugs on transcription from the hamster DHFR promoter were examined in a standard assay where DNA was treated with drugs prior to the addition of nuclear extracts. Whether drugs could function efficiently to inhibit transcription when DNA was mixed with nuclear extracts prior to the addition of drugs (reverse assay) was tested next. In such transcription assays,

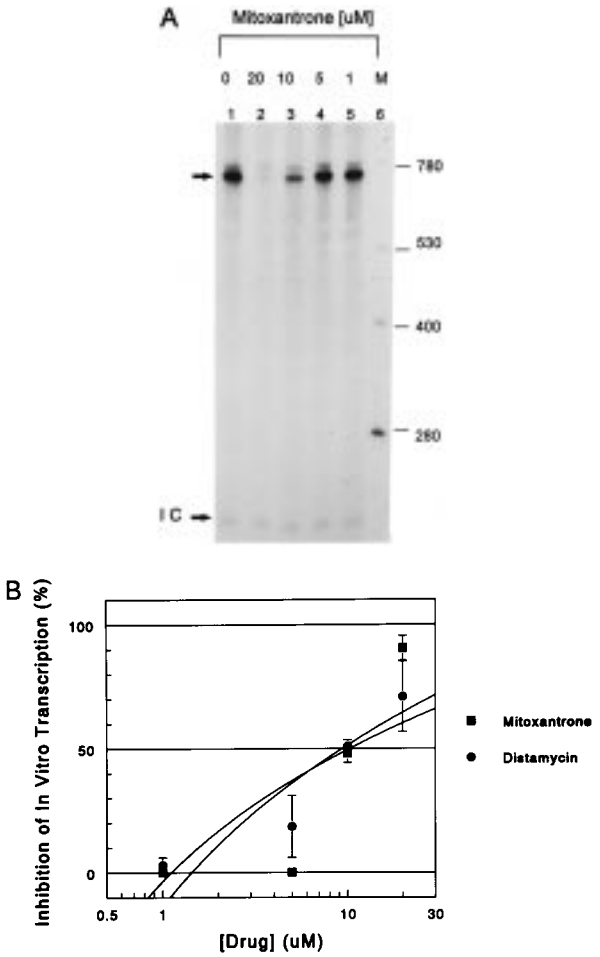

FIGURE 3: Influence of drugs on in vitro transcription from the hamster DHFR promoter. Mitoxantrone and distamycin were examined for their ability to interfere with transcription from the hamster DHFR promoter. *Sca*I-digested pDHF-210CAT was used as a DNA template, and in vitro transcription assay was performed as described under Materials and Methods. (A) A representative in vitro transcription assay in the presence of mitoxantrone. Lane 1, in vitro transcription in the absence of drug; lanes 2−5, in vitro transcription in the presence of mitoxantrone at concentrations of 20, 10, 5, and 1 μM; lane 6, RNA marker. The arrow indicates the position of the major 736-base transcript. IC, internal control. (B) Inhibition of in vitro transcription determined by comparing samples which were treated with mitoxantrone (■) or distamycin (●) with the control. The results represent at least three separate experiments (mean values ± SD).

HeLa nuclear extracts and DNA were incubated for 15 min, and then drugs were added to the reaction. Data shown in Figure 4 demonstrated that both mitoxantrone and distamycin were capable of inhibiting transcription. At a concentration of 40 μM, mitoxantrone and distamycin inhibited the formation of transcript by 90 and 60%, respectively. When 20 μM mitoxantrone and distamycin were used, inhibition of transcription was decreased to 70 and 40%, respectively. No significant difference was observed between the drugs with drug concentrations lower than 5 μM.

Time Course of Transcription in the Presence of Drugs. The mobility shift and in vitro transcription assays demonstrated that mitoxantrone and distamycin resulted in inhibition of TF−DNA complex formation with concomitant inhibition of transcription. Examination of transcription at 60 min indicated that transcript quantity was decreased but there was no evidence of prematurely terminated transcripts (i.e.,

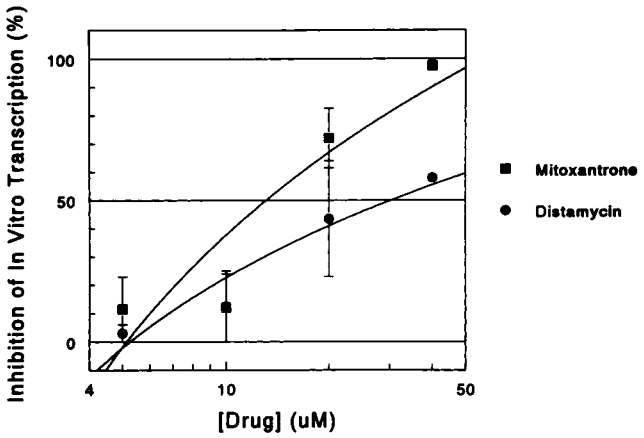

FIGURE 4: Effect of the order of addition of drugs on in vitro transcription (reverse in vitro transcription assay). Mitoxantrone (■) and distamycin (●) were examined for their ability to interfere with in vitro transcription where incubation of *Sca*I-digested pDHF-210CAT DNA and HeLa nuclear extracts for 15 min was followed by addition of individual drugs at the indicated concentrations. The in vitro transcription assay was carried out as described under Materials and Methods. The results represent three separate experiments (mean values ± SD).

specific blocks to elongation). These experiments do not address whether drugs decreased transcript production by interfering with initiation or decreased the rate of elongation, which would result in discrete shorter transcripts at early time points. To distinguish between these possibilities, transcription in the absence and presence of mitoxantrone or distamycin was examined at several time points during the reaction.

DNA template and HeLa nuclear extracts were preincubated, followed by the addition of mitoxantrone and nucleotides. In the absence of drug treatment, transcript was obtained by 5 min and gradually increased in abundance at longer incubation times up to 60 min (Figure 5A, lanes 1, 3, 5, 7, and 9). The effect of 40 or 8 μM mitoxantrone on transcript production is shown in Figure 5A,B. These data are included in the quantitative analyses of these concentrations along with others in Figure 5C. When 40 μM mitoxantrone was added to the reaction, no transcript was synthesized over the entire period of the reaction. Synthesis of transcript was delayed when reactions were exposed to 8 μM mitoxantrone where 50% of the control level was reached after 60 min. The lowest concentration of mitoxantrone (4 μM) inhibited transcript formation by more than 50% within 10 min but had little effect on transcript formation after 30 min.

We next examined how the AT-binding drug distamycin would affect the pattern of transcription. DHFR template and nuclear extract were preincubated, followed by addition of distamycin. At a concentration of 80 μM, distamycin essentially abolished transcription. Synthesis of transcript was delayed when reactions were exposed to 8 μM, where 70% of the control level was obtained at 30 min but transcription approached the control level at 60 min (Figure 6).

DISCUSSION

Whether drugs that target TF-regulatory binding sites cause sequential biological effects on TF-regulated gene expression has recently been investigated. In this study, we used a cell-free transcription assay, with HeLa nuclear extracts and the hamster DHFR promoter, to compare how DNA-binding drugs interfered with the formation of TF−DNA complexes and inhibited transcription.

A group of drugs with distinct DNA sequence preferences (i.e., GC- or AT-rich sites) and binding motifs (i.e., intercalators and minor groove binders) were initially examined for their ability to inhibit the binding of E2F1 and Sp1 to their consensus binding sites on the hamster DHFR promoter. The gel mobility shift data demonstrated that both GC- and AT-binding drugs prevented E2F1 from binding to the DNA-binding site, 5′-TTTCGCGCCAAA-3′ (Table 1). Mitoxantrone was the most potent drug at inhibiting formation of the E2F1−DNA complex. Mitoxantrone is an intercalator that is structurally related to doxorubicin but lacks an amino sugar moiety (*30*) and preferentially binds to the consensus sequences 5′-(A/T)CA and 5′-(A/T)CG (*31*). In comparison to mitoxantrone, doxorubicin and nogalamycin, which contain the aglycon chromophore with one or two sugar residues attached at either or both ends, were relatively weak inhibitors of the E2F1−DNA complex formation (*32*−*35*), suggesting that drug activities were related to differences in drug structure.

Unlike GC intercalators that interact with GC sequences that are located at the central part of E2F1-binding site, AT-binding drugs (i.e., the minor groove binders distamycin and netropsin, and the intercalator tilirone) are relatively weak inhibitors which bind to AT sequences at either end of the E2F1-binding site. In agreement with a previous study showing that mutation of the GC sequence abolishes E2F1−DNA binding whereas mutation of the AT sequence at either end of the overlapping E2F site decreases the stability of E2F1 binding (*26*), GC-binding intercalators are in general more effective than AT-binding drugs at inhibiting E2F1−DNA complex formation. However, in a recent study, we examined the ability of a new class of DNA-binding drugs, microgonotropens, for their ability to inhibit E2F1−DNA complex formation (*36*). Microgonotropens contain a tripyrrole peptide, and polyamine chains where the tripyrrole peptide bind to AT regions of the DNA minor groove and adjacent G, and the polyamine chain extends to the negative phosphodiester linkages in the major groove. Results showed that microgonotropens were extremely effective inhibitors of the E2F1−DNA complex. Subsequent studies will be undertaken to evaluate their effectiveness as transcription inhibitors.

In a standard transcription assay, where DNA was exposed to drugs prior to the addition of HeLa nuclear extracts, mitoxantrone and distamycin both affected transcription comparably, although mitoxantrone was a slightly stronger inhibitor than distamycin at higher drug concentration. When drugs were added to reactions where transcription complexes were preformed on the DNA (reverse assay), mitoxantrone was again more potent than distamycin at inhibiting promoter function. Greater drug concentrations were required for mitoxantrone and distamycin to prevent transcription in a reverse transcription assay as compared with a standard transcription assay, indicating that the order of addition of drugs could alter drug activities at inhibiting transcription.

Distamycin, which blocks E2F1 binding but not Sp1, completely inhibited transcription activity from the hamster

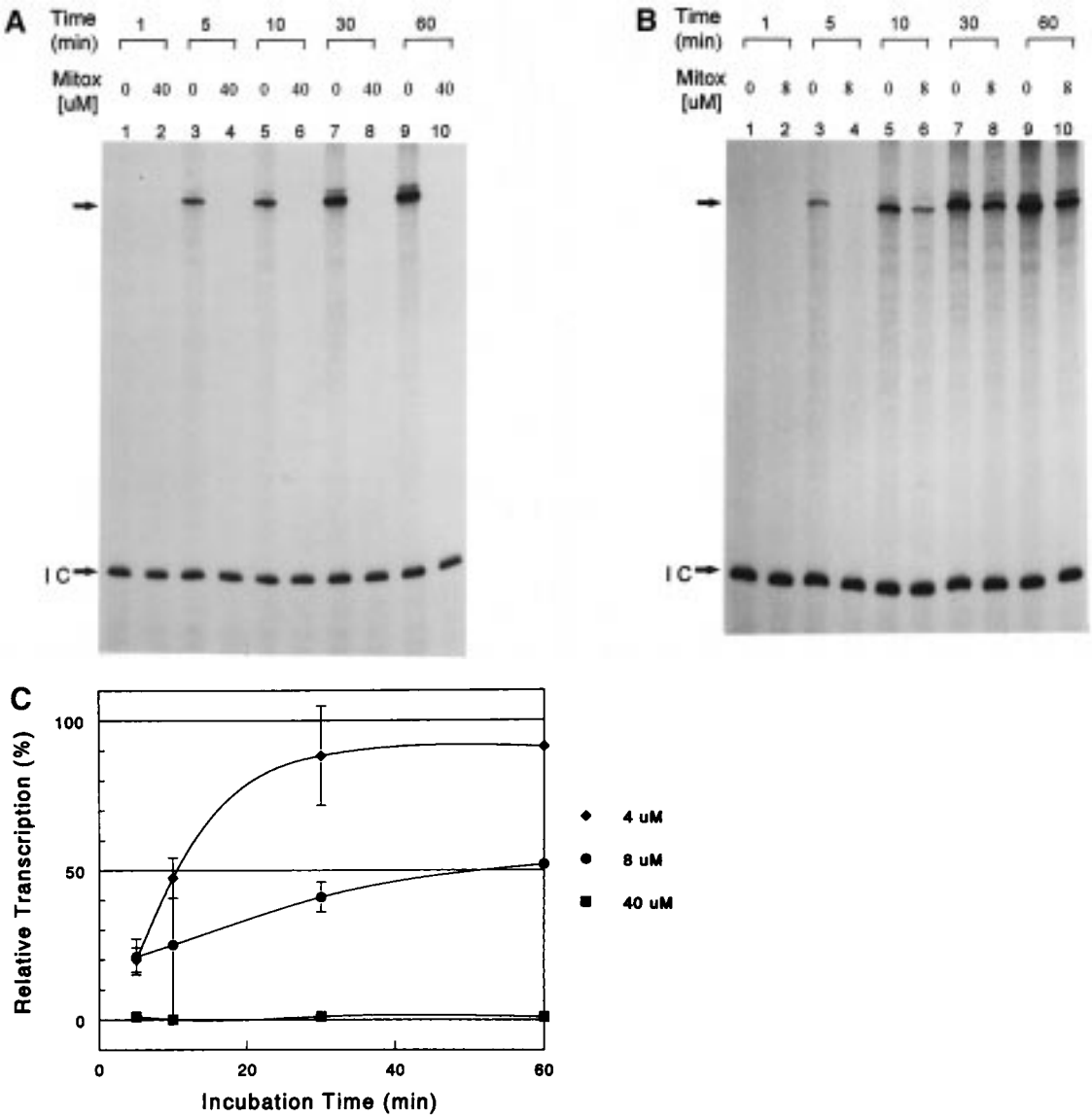

FIGURE 5: In vitro transcription time course in the presence of mitoxantrone. The time course of the in vitro transcription assay was performed by preincubating *Sca*I-digested pDHF-210CAT DNA with HeLa nuclear extract for 15 min followed by adding simultaneously a mixture of the four nucleotide triphosphates and drugs at the indicated concentrations. Transcription was stopped at incubation times of 1, 5, 10, 30, and 60 min by addition of 10 mM Tris base (pH 8.0), 350 mM NaCl, 10 mM EDTA, 7 M urea, 1% SDS, and 50 μg of tRNA. (A) A representative time course for the in vitro transcription assay in the presence of 40 μM mitoxantrone. Lanes 1, 3, 5, 7, and 9, transcription at incubation times of 1, 5, 10, 30, and 60 min, respectively, in the absence of drug; lanes 2, 4, 6, 8, and 10, in vitro transcription with treatment of mitoxantrone performed at the indicated incubation times. (B) A representative in vitro transcription assay time course in the presence of 8 μM mitoxantrone. Transciption in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of mitoxantrone was analyzed at the indicated time points. The arrow indicates the position of the major 736-base transcript. IC, internal control. (C) Quantification of in vitro transcription in the presence of mitoxantrone at concentrations of 4 (◆), 8 (●), and 40 (■) μM compared with non-drug-treated control. The results represent three separate experiments (mean values ± SD).

DHFR promoter. Earlier studies demonstrated that Sp1 binding was essential for hamster DHFR transcription, whereas binding of E2F1 enhanced the activity of the promoter (*21*, *22*). It is likely that the interaction of distamycin with AT nucleotides within the E2F1-binding site interferes with the basal transcription machinery. This is based upon our observation that TBP−TFIIA binds to a 27-mer oligonucleotide derived from the hamster DHFR promoter containing the E2F1-binding site (5′-TTTCGCGC-CAAA) (unpublished data). Moreover, we have shown that TBP−TFIIA binding to an AT-rich sequence was readily blocked by distamycin (*15*, *17*). Further studies are needed to confirm that TBP or other transcription initiation-related factors interact with DNA around the E2F1 site.

To determine whether drug inhibition of transcription is due to drugs blocking and/or delaying initiation from the hamster DHFR promoter, time course transcription assays were carried out at various concentrations of mitoxantrone and distamycin. If the drugs interacted with specific DNA sequences within the transcribed region, initiated transcripts would be expected to prematurely terminate at the drug-binding sites, resulting in formation of shorter transcripts. Our results demonstrated that (i) the patterns of transcripts formed were the same throughout the incubation time when DNA was treated with mitoxantrone or distamycin. Under the assay conditions tested, no shorter transcripts were formed, suggesting that E2F1- and Sp1-binding sites within the DHFR promoter were preferential binding sites for

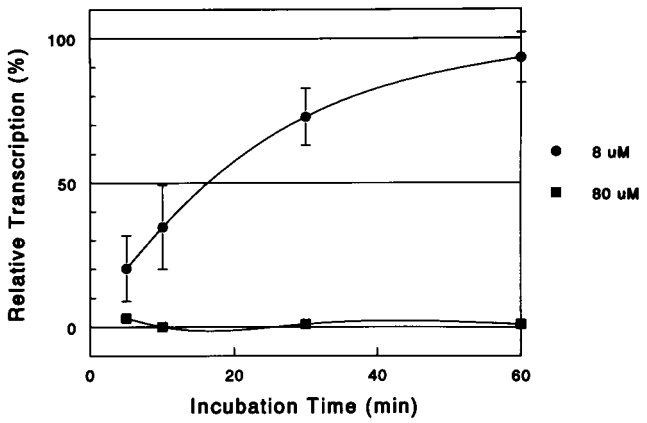

FIGURE 6: In vitro transcription time course in the presence of distamycin. A time course for the in vitro transcription from the hamster DHFR promoter in the presence of distamycin was carried out as described in Figure 5. The relative level of synthesis of in vitro transcription treated with 8 (●) or 80 (■) μM distamycin was determined by comparing drug-treated samples with non-drug-treated control at different incubation times. The results represent three separate experiments (mean values ± SD).

mitoxantrone and distamycin. (ii) When drug concentrations were increased to 40 μM for mitoxantrone or 80 μM for distamycin, transcription of the DHFR transcript was completely inhibited at the early incubation time, suggesting that drugs constantly occupy the promoter, resulting in no transcription formation. (iii) In contrast, at low drug concentrations (4 or 8 μM of mitoxantrone or distamycin), the initiation of transcription was delayed in that less transcript was synthesized at the early incubation time (30 min) but the amount of transcript was almost identical with and without drug treatment after a 60 min incubation (Figures 5 and 6). It is possible that drugs partially occupy the promoter, allowing some initiation of transcription. It is also noted that the control level of the transcript is only increased by 20% from 30 to 60 min.

In this paper, we have examined the relationships between drugs that inhibited the formation of TF−DNA complexes and transcription under cell-free conditions. Drugs that were stronger inhibitors of TF complex formation were more efficient at inhibiting transcription. In addition, efficient inhibition of gene transcription was strictly concentration-dependent. Information from these studies will be useful for development of drugs that effectively block expression of specific genes.

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