Evaluation of the Effectiveness of DNA-Binding Drugs To Inhibit Transcription Using the c-fos Serum Response Element as a Target†

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ABSTRACT: Previous work has demonstrated that sequence-selective DNA-binding drugs can inhibit transcription factors from binding to their target sites on gene promoters. In this study, the potency and effectiveness of DNA-binding drugs to inhibit transcription were assessed using the c-fos promoter's serum response element (SRE) as a target. The drugs chosen for analysis included the minor groove binding agents chromomycin A_3 and Hoechst 33342, which bind to G/C-rich and A/T-rich regions, respectively, and the intercalating agent nogalamycin, which binds G/C-rich sequences in the major groove. The transcription factors targeted, Elk-1 and serum response factor (SRF), form a ternary complex (TC) on the SRE that is necessary and sufficient for induction of c-fos by serum. The drugs' abilities to prevent TC formation on the SRE in vitro were nogalamycin > Hoechst 33342 > chromomycin. Their potencies in inhibiting cell-free transcription and endogenous c-fos expression in NIH3T3 cells, however, were chromomycin > nogalamycin > Hoechst 33342. The latter order of potency was also obtained for the drugs' cytotoxicity and inhibition of general transcription as measured by [^{3}H]uridine incorporation. These systematic analyses provide insight into how drug and transcription factor binding characteristics are related to drugs' effectiveness in inhibiting gene expression.

Drugs that bind to DNA can act as template poisons by inhibiting interactions between cellular proteins and their DNA targets. The activity of RNA polymerases, DNA polymerases, and topoisomerases I and II can all be affected by drug treatment of their DNA templates (*1*−*3*). Our studies focus on evaluating DNA reactive agents as inhibitors or disruptors of transcription factor (TF)[1] binding to target sequences in gene promoters and their resultant effects on gene expression. Successful initiation of transcription requires specific binding of TFs to their cognate promoter sequences (reviewed in *4*). Interfering with these specific TF/DNA interactions may therefore lead to decreased expression of a target gene of interest. The ability to selectively prevent TFs from binding to desired DNA targets has implications in the study of the molecular regulation of gene expression and, ultimately, in the development of therapeutics (reviewed in *5*).

DNA-binding agents can be classified according to both their mode of binding as well as their sequence preference. Members of the reversible minor groove binding family (MGBs), such as distamycin, netropsin, and the Hoechst compounds, exhibit preferential binding to A/T tracts at least 4 base pairs long (reviewed in *6*). Their curved structure allows them to interact favorably with base pairs on the floor and wall of the minor groove via hydrogen bonds and van der Waal forces. The sequence selectivity of these agents may be influenced by subtle, sequence-dependent variations in DNA structure. The inherent differences in groove width and flexibility that result from neighboring base pair effects are factors that influence these agents' ability to optimally recognize and bind to a particular sequence (reviewed in *7*). This generally holds true for other drugs as well. Anticancer antibiotics, such as chromomycin A_3 and mithramycin, are MGBs that prefer G/C-rich elements. Unlike the A/T-selective MGBs, which primarily widen the minor groove, chromomycin can unwind the double helix by about 11° and cause more extensive DNA structural alterations (*8*).

Intercalators, such as nogalamycin, doxorubicin, and hedamycin, possess aromatic moieties that are inserted between base pairs, resulting in unwinding and extension of the DNA helix. Their binding is therefore more disruptive to DNA structure than MGBs. While intercalators generally prefer binding to wider-grooved G/C-rich sequences, their binding selectivity may be influenced by surrounding base pairs, as has been demonstrated for nogalamycin (*9*). Footprinting studies have shown that this agent selectively binds to regions of alternating purines and pyrimidines, and notably prefers 5′TpG steps (*10*). Nogalamycin is also

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[1] Abbreviations: SRE, serum response element; SRF, serum response factor; TC, ternary complex; TF, transcription factor; MGB, minor groove binding drug; EMSA, electrophoretic mobility shift assay; bp, base pair(s).

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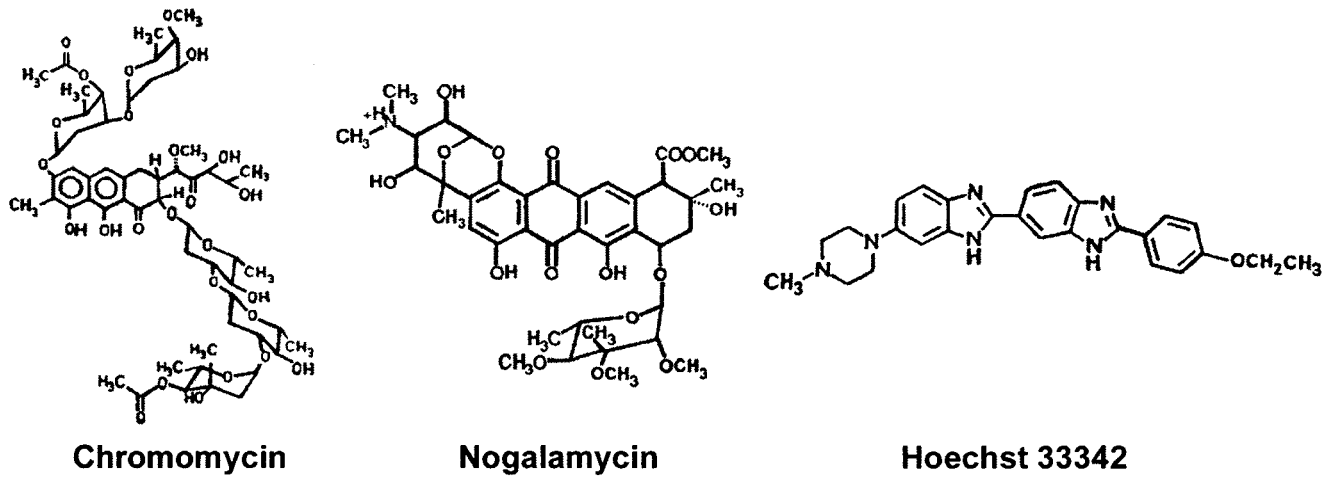

FIGURE 1: Structures of chromomycin A_3, nogalamycin, and Hoechst 33342.

somewhat unique because of its extremely slow dissociation kinetics, which likely stem from its characteristic threading mode of intercalation (*11*).

There has been considerable interest in targeting TFs using each of these drug classes. TFs can be classified into families according to their conserved DNA-binding domains. While TFs in each family can recognize and bind to specific consensus sequences, their precise recognition of promoter sites is often dependent on the composition and conformation of neighboring DNA base pairs. Electrophoretic mobility shift assays (EMSAs) using a variety of TF and DNA targets have established the potential of DNA-binding drugs as selective disruptors of target gene function. These previous studies have demonstrated that inhibition of TF/DNA complexes by DNA-binding drugs in vitro is more effective if the TFs and drugs analyzed share binding selectivities and characteristics. For example, the MGB distamycin is a potent inhibitor of TATA box binding protein (TBP) association with its A/T-rich target sequence in the minor groove (*12*). Distamycin's distortion of DNA groove conformation has also been implicated in its ability to disrupt TFs bound to A/T-rich sites in the major groove, such as homeodomain peptides (*13*).

Further investigations have suggested a relationship between the ability of drugs to disrupt TF binding to DNA in the simpler EMSAs and their ability to inhibit cell-free transcription under more complex conditions using a nuclear lysate. Distamycin was an effective inhibitor of both TBP complex formation in EMSAs as well as TBP-driven cell-free transcription (*14*). Mithramycin and chromomycin inhibited the binding of nuclear factors to G/C-rich Sp-1-binding sites on the long terminal repeat (LTR) of the HIV-1 promoter and were also able to inhibit HIV-1 LTR-directed transcription in a cell-free system (*15*). In such studies, higher drug concentrations were often needed to observe inhibition of transcription. In regards to novel drug development, these studies emphasize the need to maximize drug effectiveness in simpler systems before proceeding with testing in more complex assays or whole cells.

Evaluating DNA reactive compounds and developing paradigms for drug targeting in increasingly complex assay environments will help to improve drug specificity and potency. The c-fos promoter's serum response element (SRE) (reviewed in *16*) has characteristics that make it appropriate for this type of drug evaluation. The c-fos gene, which has been rigorously studied and characterized due to its importance in growth control, is an immediate-early response gene that is tightly regulated at the level of transcription (*17*). The SRE is necessary and sufficient for the rapid and transient induction of c-fos by serum (*18*). Two transcription factors bind to adjacent sites in this promoter sequence and mediate c-fos expression. A homodimer of SRF binds to the CArG box, an A/T-rich region (*19*). SRF binding is required for efficient recruitment of the ternary complex factor Elk-1 to its *ets* motif immediately upstream (*20*) (see Figure 2). Like other members of the *ets* family, Elk-1 binds to an invariant GGA core sequence in the major groove of DNA, but makes contacts with the phosphate backbone of unique flanking nucleotides in the minor groove (*21*). The TC, consisting of SRF and Elk-1, is constitutively bound to the SRE. Activation of this complex and upregulation of transcription are achieved as part of the cellular response to serum. Activation of signal transduction pathways by growth factors results in the phosphorylation of these factors by various kinases, most notably members of the mitogen-activated protein kinase (MAPK) family (*22*, *23*).

The A/T-rich and mixed sequences present in the SRE are well suited for the study of a wide variety of DNA reactive agents. Also, the fact that Elk-1 contacts both grooves makes it an interesting target for drugs that bind in one groove or the other. Moreover, using this sequence as a target allows drug effects on more than one TF to be studied in a single system. Another advantage is that in addition to EMSAs and cell-free transcription assays, use of the c-fos SRE allows drug effects on endogenous c-fos mRNA production to be assessed. The rapid and transient expression of c-fos that follows serum induction provides a facile way of determining immediate or short-term drug effects on transcription in whole cells.

Here, we investigate the effectiveness of drugs as inhibitors of TF/DNA interactions in increasingly complex systems using the c-fos SRE as a target. Representative agents from the drug classes discussed above were chosen based upon their contrasting sequence preferences, and modes of DNA-binding (drug structures are shown in Figure 1). The MGBs chromomycin and Hoechst 33342, which do not radically distort DNA, have G/C- and A/T-rich preferences, respectively. They were compared to nogalamycin, which has less

sequence specificity but which causes greater helical distortion. The agents' abilities to affect TF/DNA interactions in vitro were evaluated in EMSAs, a simple system consisting of purified proteins and short oligonucleotides. The cell-free transcription assay, which uses a nuclear lysate to drive transcription from a plasmid, was used to analyze the drugs' effects in a more complicated environment. Finally, effects of these agents on endogenous c-fos expression in whole cells were assessed using Northern blots. Cytotoxicity and RNA synthesis assays also provided insight into how these agents were affecting cells in general.

MATERIALS AND METHODS

Drugs. Stocks of 5 mM chromomycin A_3 (Sigma, St. Louis, MO) and 5 mM nogalamycin (Pharmacia Upjohn Corp.) were prepared in dimethyl sulfoxide. A 20 mM stock of Hoechst 33342 (Aldrich Chemical Co.) was prepared in distilled water. All drugs were stored in the dark at −20 °C and diluted into water immediately before use.

Oligonucleotides. Two 24-mer oligonucleotides and their complementary strands were synthesized by the Biopolymer facility at Roswell Park Cancer Institute (Buffalo, NY) and purified on a Poly-Pak column. The first oligo (5′-ACACAGGATGTCCATATTAGGACA-3′), designated "SRE", contained the −301 to −324 sequence of the human c-fos promoter SRE. The second oligo (5′-GATACCGGAAGTCCATATTAGGAC-3′), designated "E74", was similar, but contained the high-affinity *ets*-binding site from the E74 *Drosophila* promoter (underlined), based on the consensus sequence published by Urness et al. (*24*). Oligos were reannealed according to Lee et al. (*25*). These double-stranded oligos were 5′-end-labeled with [γ-^{32}P]ATP (10 mCi/mL) and T4 Polynucleotide Kinase. Unincorporated nucleotides were removed using a Sephadex G-25 microspin column (Amersham Pharmacia Biotech).

Protein Purification. pILASRF, a plasmid encoding the SRF protein with an N-terminal His-tag, was developed in the Nordheim laboratory (Institut fuer Zellbiologie, Universitaet Tuebingen, Tuebingen, Germany). Expression of this protein and its purification were achieved following the protocol by Heidenreich et al. (*26*), but with two changes. First, the bacterial pellet was resuspended in PBS and lysed using three freeze/thaw cycles. The lysate was sonicated and pelleted at 10000*g* for 25 min at 4 °C before being combined with Ni−NTA beads (Qiagen, Inc.). Second, after transfer to a column, the beads were initially rinsed 4 times with 2 column volumes of PBS. pAS278, a plasmid encoding full-length Elk-1 with a C-terminal His-tag (*27*), was generously provided by Dr. Andrew Sharrocks (University of Newcastle, Newcastle upon Tyne, England). Following expression in BL21-pLysS bacteria, the protein was purified under native conditions using Ni−NTA beads, following manufacturer's instructions (Qiagen).

Electrophoretic Mobility Shift Assays. In general, experiments were performed as follows: drug, radioactively end-labeled oligo, and binding buffer were combined and allowed to incubate at room temperature for 30 min. Purified transcription factors were diluted into binding buffer. Following addition of purified protein(s), the reactions were allowed to incubate an additional 30 min at room temperature before being electrophoresed on a 5% polyacrylamide gel. These incubation times were based on time course experiments that established 30 min as sufficient time to achieve equilibrium of complex formation. Specifically, for the Elk-1 EMSAs, a binding buffer containing 25 mM Hepes−KOH, pH 7.9, 10 mM $MgCl_2$, 10 mM EDTA, 10 mM spermidine, 10 mM dithiothreitol, 7.5 μg/μL bovine serum albumin, and 20% glycerol was used. Ten nanograms of purified Elk-1 was added to 1 nM ^{32}P-end-labeled E74 oligo in these reactions. For the SRF EMSAs, the binding buffer contained 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.05% milk, 10 mM DTT, and 5% glycerol. Twenty-five nanograms of purified SRF was added to 1 nM ^{32}P-end-labeled SRE in these reactions. For ternary complex formation, the SRF binding buffer was used. Twenty-five nanograms of SRF and 6.25 ng of Elk-1 were added to 1 nM ^{32}P-end-labeled SRE. In samples containing chromomycin, Mg^{2+} was added to a final concentration of 10 mM in the binding reactions. For all EMSAs, a 5% native polyacrylamide gel was pre-run at room temperature at 200 V in 0.5× TBE buffer (44.6 mM Tris base, 44.5 mM boric acid, and 10 mM EDTA). Reactions were loaded onto the gel and electrophoresed for a maximum of 2 h. Adequate separation of free and complexed DNA on the Elk-1 and SRF MSAs was achieved by as little as 30 min of electrophoresis. Dried gels were exposed to Kodak Biomax Scientific Imaging film. Quantitation of free and complexed DNA was carried out by scanning the resulting autoradiogram on a computing laser densitometer (Molecular Dynamics) and analyzing the results with the manufacturer's ImageQuant program. Fifty percent inhibition of complex formation by the drugs (IC_{50}) was calculated by comparing drug-treated samples to controls. Where specified, purified proteins were added to the oligo before drug. These "reverse" experiments were electrophoresed as detailed above.

Cell Culture. Murine NIH3T3 fibroblast cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4500 mg/mL) and sodium pyruvate (110 mg/mL) and supplemented with 10% calf serum. Cells were maintained at 37 °C and 5% CO_2.

NIH3T3 Nuclear Lysate Preparation for Cell-Free Transcription. NIH3T3 cells were grown in 175 cm^2 flasks until approximately 60% confluent before being starved overnight in starvation media (DMEM containing 0.5% calf serum). In general, a minimum of 10 flasks was needed to see adequate lysate activity. Cells were induced by adding induction media (DMEM with 15% calf serum) for 30 min, rinsed with room temperature PBS, and scraped into ice-cold PBS. Nuclear lysates were then prepared essentially as described by Blake et al. (*28*). All centrifugations were performed at 4 °C. In brief, cells were pelleted by centrifugation, washed, and repelleted in 5 pellet volumes of Buffer A [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)/KOH, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 1 mM dithiothreitol (DTT)], and then allowed to swell on ice in the same volume of Buffer A for approximately 20 min. Cells were dounced on ice until 95% cell lysis was achieved, and then centrifuged until 31000*g* was reached. As soon as this speed was obtained, the centrifuge was turned off and the rotor was allowed to come to a halt. The pelleted nuclei were

resuspended in Buffer C [20 mM Hepes/KOH, pH 7.9, 20% glycerol, 0.2 mM EDTA, 2 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, 2 mM DTT, and 1 mM phenylmethanesulfonyl fluoride (PMSF)] to a final minimum concentration of 5×10^8 nuclei/mL. An equal volume of Buffer C + NaCl (same composition as buffer C, but with 0.75 M NaCl) was then added dropwise, with swirling. The lysate was rocked at 4 °C for 30 min, and then centrifuged at 214000*g* for 45 min. Supernatants were pooled, loaded into a Slide-A-Lyzer dialysis cassette (Pierce) with a 10 000 molecular weight cut-off, and dialyzed against 500 volumes of buffer D (20 mM Hepes/KOH, pH 7.9, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.2 mM EGTA, 12.5 mM $MgCl_2$, 2 mM DTT, and 1 mM PMSF) at 4 °C for 3 h, with a buffer change after the first 1.5 h. Dialyzed extract was cleared by a 15 min spin at 31000*g*. Aliquots of the resulting supernatant were immediately frozen on dry ice and stored at −80 °C.

Cell-Free Transcription Assay. The template, pFosLuc19, containing a human c-fos promoter fragment (−711 to −3) upstream of a luciferase reporter gene, was developed in the Nordheim laboratory. Digestion of this plasmid with *Sph*I prior to use in the assay yields a transcript approximately 750 bases in length. For drug studies, 0.5 μg of this plasmid was combined with drug and 5 μL of Buffer D for a total volume of 9 μL, and allowed to incubate 30 min at 30 °C. Approximately 15 μg of NIH3T3 nuclear lysate was added, the total volume was brought to 19 μL with Buffer D, and the reaction was allowed to incubate for 15 min at 30 °C. The subsequent reaction and transcript purification steps were carried out as described by Chiang et al. (*29*). A T3 transcript of pGEM4z (Promega), 250 bases long, was used as an internal control. Quantitation following autoradiography was as described for the EMSAs, and the visualized transcripts were normalized to the internal controls.

Whole Cell Drug Treatment and Northern Blot Analysis. For typical drug treatments prior to Northern blot analysis, 2.5×10^5 NIH3T3 cells were plated in 60 mm dishes and allowed to grow for 48 h until approximately 60% confluent. Growth medium was removed, the cells were rinsed with PBS, and starvation medium (DMEM containing 0.5% calf serum) was added. Appropriate dilutions of drugs were made into sterile water, and 20 μL of these dilutions was added directly to the plates. Solvent controls in which no drug was added were also prepared. Cells were generally starved for 16 h at the growth conditions detailed above. Induction of c-fos was accomplished by adding calf serum directly to the plates to a final concentration of 15%, followed by incubation at 37 °C for 30 min.

RNA Isolation/Northern Blot Analysis. Following serum induction of NIH3T3 cells, total RNA was isolated using TRIzol (GIBCO BRL). In brief, 20 μg of total RNA was loaded onto 1.5% denaturing agarose gel (2.2 M formaldehyde, 40 mM MOPS, pH 7.0, 10 mM sodium acetate, and 10 mM EDTA) and electrophoresed in 1× MOPS buffer (40 mM MOPS, 10 mM sodium acetate, and 10 mM EDTA) at 80 V for 4.5 h. The gel was rinsed in ddH_2O, and the RNA was transferred to GeneScreen (NEN Life Science Products) overnight. Following UV cross-linking of the RNA, the membrane was pre hybridized for 1 h at 60 °C in pre-hybe buffer [0.5 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA, 1% bovine serum albumin (BSA)]. A plasmid containing 4.8 kb of the murine c-fos coding sequence, pGEM4z-Fos (Loftstrand Labs, Ltd.), was linearized with *Hin*dIII before being radioactively labeled using a DecaPrime II kit (Ambion Inc.) and [α-^{32}P]dCTP (10 mCi/mL) for use as a probe. A phagemid containing the coding sequence for human glyceraldehyde-3-phosphate dehydrogenase (G3) (American Type Culture Collection) was also linearized with *Hin*d III and similarly labeled. Hybridization with the radiolabeled probes was overnight at 60 °C. Membranes were washed twice with wash buffer A (40 mM sodium phosphate, pH 7.2, 5% SDS, 1 mM EDTA, and 0.5% BSA) and twice with wash buffer B (20 mM sodium phosphate, pH 7.2, 1% SDS, and 1 mM EDTA) at 60 °C (each wash was for 20 min). The blot was exposed in a phosphorimager cassette (Molecular Dynamics) and scanned with a Molecular Dynamics phosphorimager.

Cytotoxicity Assay: Colony Formation Using NIH3T3 Cells. A total of 1×10^5 NIH3T3 cells were plated in 35 mm dishes and allowed to grow for 48 h until approximately 50% confluent. Growth medium was removed, and 1 mL of fresh medium containing the desired drug concentration was added. Following a 4 h drug exposure, the cells were trypsinized, serially diluted, and replated into 60 mm dishes. The total number of cells plated per dish ranged from 1×10^4 to 1×10^2. Cells were incubated at normal growth conditions for 10 days. The medium was then removed, and the cells were stained using 2 mL of methylene blue staining solution (7 mg/mL methylene blue in 70% EtOH) per dish for 30 min. Following removal of the staining solution, the dishes were rinsed in lukewarm water and air-dried. Colonies, designated as groups of 50 or more cells, were counted under a stereo microscope. Plating efficiencies were calculated by dividing the number of colonies by the total number of cells plated. Relative plating efficiencies for the drug treatments were then calculated by dividing the plating efficiencies of the drug-treated samples by the plating efficiencies of the controls.

RNA Synthesis ([^{3}H]Uridine Incorporation Assay). A total of 6.6×10^5 NIH3T3 cells were plated in 60 mm dishes and allowed to grow for 48 h until approximately 85% confluent. Drugs were diluted appropriately and added directly to the growth medium for a 4 h exposure under normal growth conditions. Then 2 μCi of [5-^{3}H]uridine (15 mCi/mmol) and unlabeled uridine to a final concentration of 50 μM were added to each dish. Following a 30 min pulse, the cells were rinsed with cold PBS, and then each dish was scraped into 1 mL of ice-cold 0.5 M perchloric acid (PCA) to begin precipitation of nucleic acids; 0.5 mL of the resuspension was transferred to prechilled eppendorf tubes, 1 mL of cold 0.5 M PCA was added, and the tubes were incubated on ice for 30 min. The samples were pelleted at 2800 rpm at 4 °C and washed 2 times in 0.4 M PCA before 0.5 mL 0.5 M PCA was added. The tubes were then heated to 70 °C for 1 h, and the counts in 0.5 mL were measured on a scintillation counter.

RESULTS

c-fos Components. The c-fos SRE sequence targeted in this study is shown in Figure 2A. This sequence is located approximately 300 bp upstream of the c-fos transcription

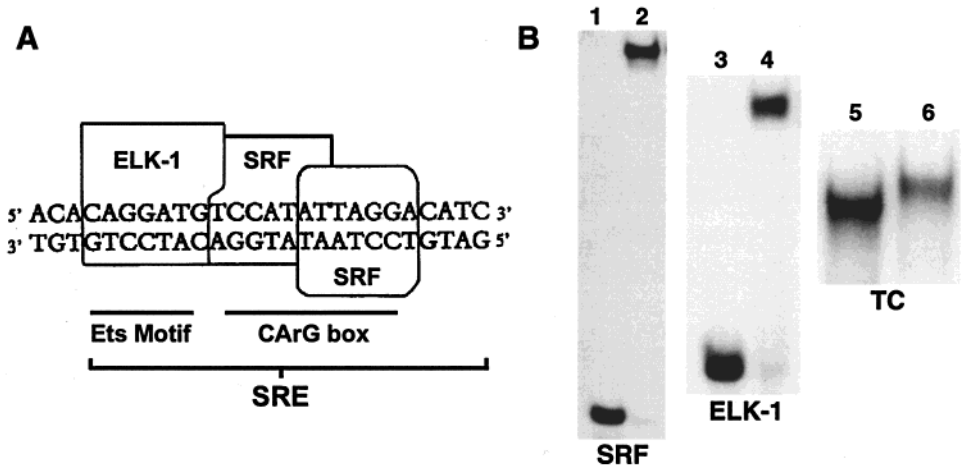

FIGURE 2: Components of the c-fos promoter. (A) TFs bound to the human c-fos promoter's SRE. Binding of the homodimer of SRF is required for the recruitment of Elk-1. Together, these TFs make up the ternary complex (TC). (B) Binding of SRF and Elk-1 to radiolabeled probes in EMSAs. ^{32}P-labeled oligonucleotides were incubated with purified proteins for 30 min at room temperature before being electrophoresed on a 4% polyacrylamide gel and autoradiographed. The 24 bp oligonucleotides contained sequences from the c-fos SRE or the *Drosophila* E74 promoter. Lane 1, free SRE probe; lanes 2 and 5, SRE plus SRF protein; lane 3, free E74 probe; lane 4, E74 plus Elk-1 protein; lane 6, SRE plus SRF and Elk-1 forms the TC. In lanes 5 and 6, the gel was electrophoresed longer to maximize shift differences. The free SRE probe ran off the gel under these conditions.

initiation site. Binding sites for the ternary complex factor Elk-1 and the homodimer of SRF are indicated. The TC is formed when binding of SRF to the CArG box recruits Elk-1 to the *ets* motif immediately upstream. SRF and Elk-1 were expressed as 6-Histidine-tagged proteins in bacteria and purified for use in the EMSAs as described in detail under Materials and Methods. While the SRE can be used to study drug effects on SRF binding and ternary complex formation on the SRE, it cannot be used to assess drug effects on Elk-1 binding alone, since this protein cannot bind to the SRE without SRF present (*30*). However, it can bind to the high-affinity *ets* motif in the *Drosophila* E74 promoter (*31*). Therefore, we made use of this promoter to study drugs' influence on Elk-1 association with DNA. Recombinant proteins were combined with 24 bp radiolabeled oligonucleotides containing the targeted promoter regions, and the complexes were electrophoresed on a polyacrylamide gel. Free SRE and E74 probe was successfully shifted by the addition of SRF or Elk-1 alone (Figure 2B, compare lanes 1 and 3 with lanes 2 and 4). In lanes 5 and 6, the gel was electrophoresed longer in order to maximize the difference in shift evident when SRF and Elk-1 were combined to form the TC (lane 6) as compared to SRF alone (lane 5). Under these conditions, the free probe ran off the gel. In subsequent experiments, where the complexes were electrophoresed under standard conditions as in Figure 2B (lanes 1−4), we noted that the TC was still distinguishable from the SRF complex on the basis of a slight difference in mobility (see Figure 3A, compare lanes 8 and 10 with lane 9).

Inhibition of TF Binding to the SRE in EMSAs. The ability of drugs to prevent TF binding to their specific promoter sites was assessed using the components of the SRE system. Three drugs were chosen on the basis of their different binding properties. The structures of chromomycin and Hoechst 33342, minor groove binding agents with G/C and A/T preferences, respectively, and nogalamycin, an intercalating drug that prefers G/C-rich regions, are shown in Figure 1. When incubated with the radiolabeled probes prior to protein addition, these drugs prevented TF binding in a dose-dependent manner. Representative results are shown in Figure 3A for chromomycin. As the amount of chromomycin added to the E74 promoter decreases in lanes 3−7, the amount of Elk-1 complexed with the probe increases back to control levels (lanes 1 and 2, no drug addition). Chromomycin also yields a dose-dependent inhibition of the TC as seen in lanes 11−15, as compared to controls in lanes 8 and 10. Similar inhibition was also achieved for SRF complex formation (data not shown).

Quantitation of the free and shifted DNA in drug-treated samples, and comparison to non-drug-treated controls, allowed percent inhibition of complex formation to be calculated. Representative dose−response curves for chromomycin (Figure 3B) show that this agent exhibits different potencies on the three TF complexes analyzed. The Elk-1 complex is by far the most sensitive to this drug, exhibiting a steep dose−response curve that plateaus by 10 μM. Formation of the SRF complex is less sensitive and yields a more gently sloping curve that starts to level off around 30 μM. When Elk-1 and SRF are combined to form the TC, the dose−response curve falls between those obtained for the individual factors, but retains the rather steep increase of the Elk-1 curve.

The drug concentration needed to inhibit complex formation by 50% (IC_{50}) was determined from dose−response curves plotted for each agent analyzed and used to compare the drugs' effectiveness in preventing TF binding (Figure 3C). Hoechst 33342 exhibited a trend similar to that observed for chromomycin: it was not particularly potent in preventing the SRF complex from forming, but was much more effective in inhibiting TC formation. Notably, Hoechst 33342 was approximately twice as potent as chromomycin in preventing TC formation. Unfortunately, the effects of Hoechst 33342 on Elk-1 complexes could not be determined, since binding of this drug to the E74 probe alone resulted in its retention in the well of the gel and excessive smearing, making quantitation impossible. Of the three drugs tested, nogalamycin was by far the most potent agent; IC_{50}s for all complexes fell well below 5 μM. Its inhibition profile differed from chromomycin in that SRF, rather than Elk-1, was the most sensitive target. Like chromomycin, however,

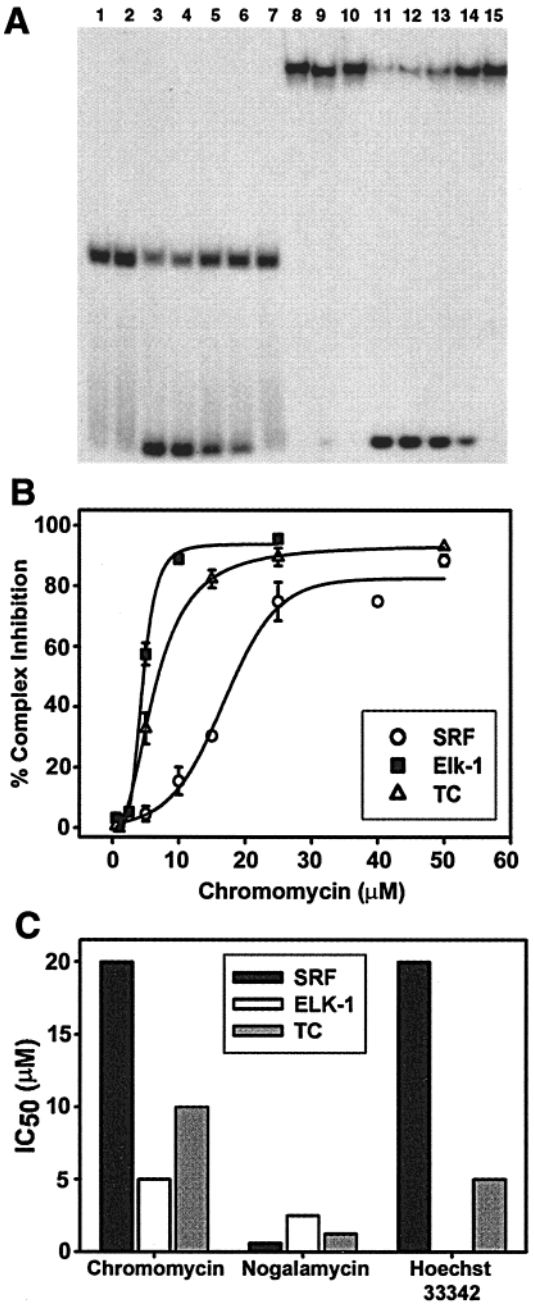

FIGURE 3: Effect of drugs on preventing complex formation in EMSAs. (A) Chromomycin's effect on Elk-1 and SRF complex formation. Increasing amounts of drug were incubated with the probes for 30 min before addition of purified proteins and electrophoresis as described in Figure 2. Lanes 1−7 contain Elk-1 complexes. Lanes 1−2, Elk-1 complex controls, no drug; lanes 3−7 contain 50, 25, 10, 5, and 1 μM chromomycin, respectively. Lanes 8 and 10−15 contain TC bound to the SRE. Lanes 8 and 10, TC controls, no drug; lane 9, SRF complex control, no drug; lanes 11−15 contain 100, 50, 25, 10, and 1 μM chromomycin, respectively. (B) Quantitation of chromomycin's inhibition of complex formation. Percent probe shifted in each drug treatment was compared to non-drug-treated controls to yield percent complex inhibition for SRF (○), Elk-1 (■), and TC (△). Results are the mean of four experiments (mean value ± standard error). (C) Comparison of drugs' potency in preventing complex formation in the EMSAs. Drug concentrations needed to prevent each complex formation by 50% (IC_{50}s) were calculated using graphs as shown in panel B.

the IC_{50} for TC inhibition fell between the IC_{50}s for the individual factors' complexes. In addition to its higher potency and unique inhibition profile in comparison to the MGBs, nogalamycin possessed another distinct characteristic. Reverse assays, in which each of the drugs was added after the TC was already formed on the SRE, demonstrated that only nogalamycin required significantly higher concentrations to disrupt complex formation (data not shown).

Drug Effects on c-fos Promoter-Driven Cell-Free Transcription. Upon observing different potencies among the drugs in the EMSAs, we wished to determine if similar levels of effectiveness could be maintained in a more complex, cell-free environment containing additional nuclear proteins and larger amounts of DNA with greater sequence complexity. The cell-free transcription assay makes use of a linearized plasmid containing the c-fos promoter. Upon addition of nuclear lysate from serum-induced NIH3T3 cells and the proper mix of nucleotides, the c-fos promoter drives the production of a transcript of a known length of 750 bp (Figure 4A, control lanes 3 and 8 marked by asterisks, top arrow). Preincubation of the plasmid with drug before nuclear lysate addition results in a dose-dependent inhibition of transcript production, as is seen for representative results following chromomycin treatment (lanes 1, 2, and 4−7). At 7.5 μM chromomycin (lanes 1 and 2), transcript appearance is abolished. At lower drug concentrations (5 and 2.5 μM in lanes 4−5 and 6−7, respectively), the intensity of the transcript is diminished, but there is no change in transcript size. The lack of detectable shorter transcripts suggests that transcriptional elongation is not being affected by the drug treatment. Quantitation of the bands followed by normalization to a 250 bp internal standard (Figure 4A, bottom arrow), and comparison to controls, yields percent inhibition of transcription. Dose−response curves, as seen in the representative graph for chromomycin in Figure 4B, were plotted for each drug treatment. The IC_{50}s for this assay were then used to compare the drugs in Figure 4C. Chromomycin and nogalamycin showed a level of potency that was only about 3 times higher than Hoechst 33342. The trend evident in the EMSAs, where an unusually high level of chromomycin was required to inhibit TC formation, therefore did not hold true in the cell-free transcription assays. While the IC_{50}s for nogalamycin and Hoechst 33342 increased approximately 4-fold from inhibition of the TC to inhibition of cell-free transcription, the respective IC_{50}s for chromomycin's inhibition actually decreased by a factor of 2.5.

Use of Northern Blots To Measure c-fos mRNA Induction. After observing that the drugs' potencies in the EMSAs and cell-free transcription assays were comparable, we next wished to assess the drugs' effectiveness in inhibiting c-fos expression in whole cells using Northern blots. The serum inducibility of the c-fos gene and its quick mRNA turnover (*17*) are advantageous in analyzing drug effects on endogenous c-fos transcription because complications arising from preexisting levels of c-fos mRNA are minimized. Optimal conditions established for c-fos induction in NIH3T3 cells are shown in Figure 5A. Unsynchronized, logarithmically growing cells have undetectable levels of c-fos mRNA (Figure 5A, lane 1). Inducing these cells with 15% serum for 30 min results in its detectable upregulation (lane 2). Starving the cells overnight (16 h) in media containing 0.5% serum downregulates c-fos (lane 3), and a subsequent 30 min induction of these cells with serum results in optimal expression (lane 4), as determined in time course studies (data not shown). The housekeeping gene, glyceraldehyde-3-

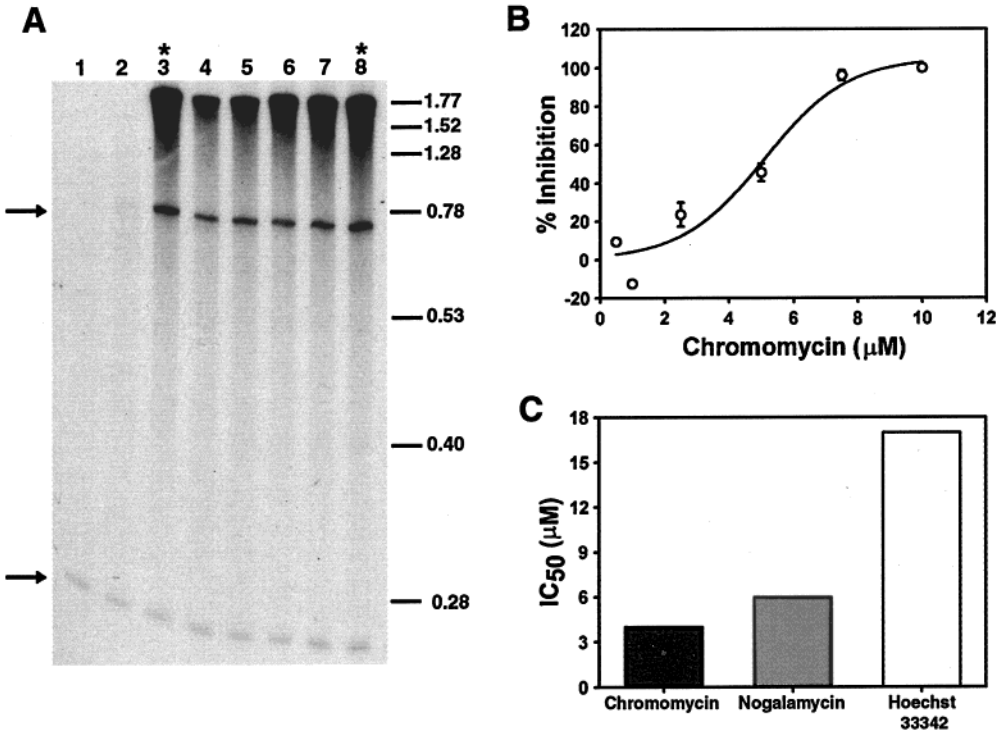

FIGURE 4: Effect of drugs on cell-free c-fos promoter-driven transcription (A) Chromomycin's effect on cell-free transcription. *Sph*I-linearized pFosLuc, a plasmid containing the human c-fos promoter upstream of a luciferase gene, was incubated with varying concentrations of drug for 30 min. Nuclear lysate from serum-induced NIH3T3 cells was then added for 15 min before the addition of nucleotides and [^{32}P]CTP. After a 1 h incubation, the resultant RNA transcripts were isolated and electrophoresed on a 4% denaturing polyacrylamide gel. Top arrow: the expected pFosLuc transcript at approximately 750 bases; lower arrow, internal control: a T3 transcript from pGEM4z at approximately 250 bases. Lanes 1−2, 4−5, and 6−7 contain 7.5, 5, and 2.5 μM chromomycin, respectively. Lanes 3 and 8, marked by asterisks: controls, with no drug treatment. Positions of size markers in a typical RNA ladder, in kilobases, are indicated. (B) Quantitation of chromomycin's inhibition of cell-free transcription. As in Figure 3B, comparison of drug-treated samples to controls yielded percent inhibition of transcription. Results are the mean of three experiments (mean ± standard error). (C) Comparison of drugs' effectiveness as inhibitors of cell-free transcription. IC_{50}s for each agent were calculated from graphs as shown in panel B.

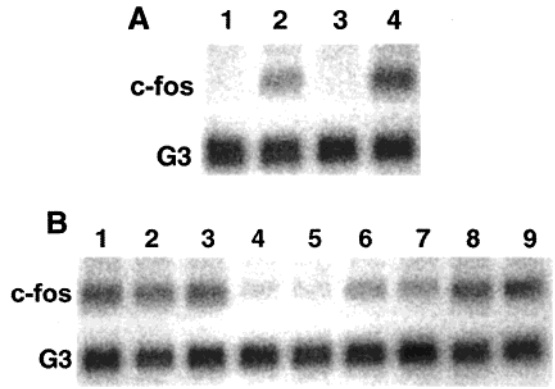

FIGURE 5: Representative Northern blot results. (A) Characteristics of c-fos expression in NIH3T3 cells. Following various treatments, 20 μg total RNA was electrophoresed on formaldehyde-containing agarose gels, transferred to a nylon membrane, and hybridized with radiolabeled probes for c-fos and G3. Lanes 1−2, normally growing cells; lanes 3−4, 16 h starvation in 0.5% serum; lanes 2 and 4, cells induced by raising serum concentration to 15% for 30 min. (B) Representative results on c-fos expression following exposure of NIH3T3 cells to chromomycin. After cells were starved for 16 h, drug was added for 1 h, and then cells were induced for 30 min as in (A). Lanes 1−3, controls, no drug treatment; lanes 4−5, 6−7, and 8−9 were exposed to 1, 0.5, and 0.25 μM chromomycin, respectively.

phosphate dehydrogenase (G3), is used in these blots as a loading control.

Analysis of Drug Effects on c-fos Expression in Whole Cells. For drug treatments, cells were starved for 16 h, exposed to varying drug concentrations for 1 h, and subsequently induced with serum. Chromomycin, as shown in Figure 5B, was capable of decreasing absolute c-fos mRNA levels in a dose-dependent manner (lanes 4−9), as compared to non-drug-treated controls (lanes 1−3). An approximately 30% decrease in c-fos expression is noted at only 0.5 μM (lanes 6 and 7), and the message is almost completely eliminated with a 1 μM treatment (lanes 4 and 5). There is no significant effect on G3 mRNA after a 1 h treatment at any drug concentration. In addition, as was noted in the cell-free transcription assay, there were no detectable levels of shorter transcripts following drug exposure.

Nogalamycin and Hoechst 33342 were analyzed in the above manner to see if they were also capable of inhibiting c-fos expression. Quantitation of the resulting hybridized signals allowed dose−response curves to be plotted (Figure 6). From these curves, it is evident that the drugs differ in their effectiveness: a 1 h exposure to 1 μM chromomycin results in 80% inhibition of c-fos expression, compared to no substantial inhibition for nogalamycin or Hoechst 33342 at the same concentration (compare panels A to B and C). At 2.5 μM Hoechst 33342 or nogalamycin, expression is inhibited approximately 40%, compared to greater than 95% inhibition for chromomycin. Chromomycin is therefore about an order of magnitude more potent than nogalamycin or Hoechst 33342 in inhibiting c-fos expression after a 1 h treatment. This is in contrast to the results obtained in the cell-free transcription assay, where chromomycin and nogala-

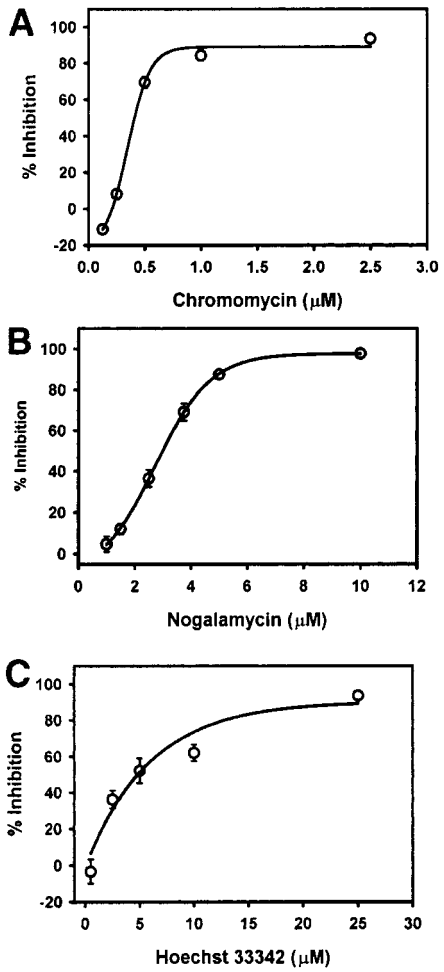

FIGURE 6: Quantitation of drugs' effects on endogenous c-fos expression in NIH3T3 cells following 1 h exposures. Cells were starved for 16 h before being exposed to a range of drug concentrations, in μM, for 1 h and induced with 15% serum for 30 min as described in Figure 5. Total RNA was analyzed in Northern blots through hybridization to radiolabeled c-fos and G3 probes, and the blots were visualized following autoradiography. Quantitation of the bands and comparison of the drug-treated lanes to controls yielded percent inhibition for each drug: (A) chromomycin; (B) nogalamycin; and (C) Hoechst 33342. Results are the mean of five experiments (mean $\pm$ standard error). The IC_{50}s calculated from these graphs are presented in Figure 8.

mycin exhibited similar potencies that were only 3 times greater than Hoechst 33342.

Chromomycin's Effects on c-fos Expression over a Shorter Time Course. After observing very effective inhibition of c-fos expression following exposure of cells to low levels of chromomycin after only 1 h, we wished to further characterize this drug's effect during shorter exposures. NIH3T3 cells were therefore starved for 16 h in low-serum media before being exposed to 1 μM chromomycin for various times (0−60 min). For the zero time point, drug was added to cells immediately before serum induction. Cells were then induced for 30 min before RNA isolation as discussed above. A concentration of 1 μM was chosen for this time course because of its substantial effect on c-fos expression (~80% inhibition after a 1 h exposure). As seen in Figure 7, there is a slight induction of c-fos mRNA after a 15 min exposure to chromomycin, but inhibition begins by 30 min and increases fairly rapidly to reach nearly 80% after an hour.

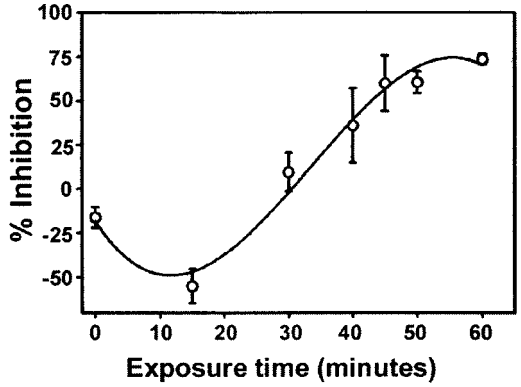

FIGURE 7: Chromomycin's inhibition of endogenous c-fos expression in NIH3T3 cells over time. Cells were starved for 16 h before being exposed to 1 μM chromomycin for various lengths of time. They were then induced with 15% serum for 30 min. Analysis using Northern blots and quantitation was carried out as previously described in Figure 6. Results are the mean of three experiments (mean $\pm$ standard error).

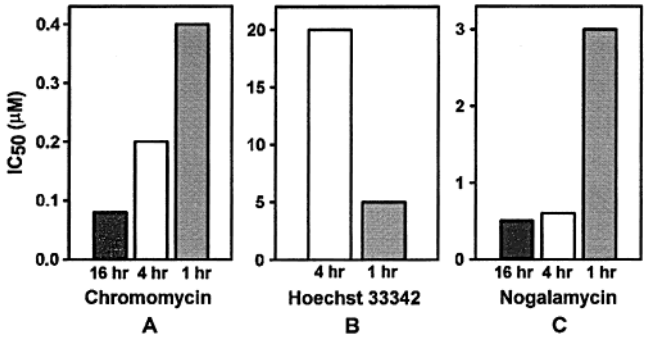

FIGURE 8: Effect of time on drug inhibition of endogenous c-fos expression. One hour drug exposures (gray bars) following 16 h starvation of NIH3T3 cells were performed as described in Figure 5. The 4 h exposures (white bars) were also performed after the cells were starved 16 h. For the 16 h exposures, (black bars), cells were starved and exposed to drug simultaneously. Northern blot analysis following hybridization to c-fos and G3 probes and subsequent quantitation yielded IC_{50} values. These values are plotted for each time point for (A) chromomycin, (B) Hoechst 33342, and (C) nogalamycin.

Drugs' Abilities To Inhibit c-fos Expression Over Time. Clearly, chromomycin is a rapid inhibitor of c-fos expression, but is this effect maintained over time to result in a continued decrease in expression? Longer exposures of 4 and 16 h were carried out to determine if each drug was able to maintain its effectiveness in the cellular environment. Like the 1 h time points, the 4 h drug exposures followed a 16 h overnight starvation period. However, for 16 h treatments, cells were starved and exposed to drug concurrently. The IC_{50} values were then used to compare the time course results as well as to compare drugs to one another. For chromomycin (Figure 8A), there was a continued increase in drug potency over time. That is, less drug was needed to obtain 50% inhibition of c-fos expression following a 16 h exposure compared to a 1 h exposure. Interestingly, Hoechst 33342 yielded a pattern of inhibition that was completely opposite. While a 16 h exposure to Hoechst 33342 yielded no consistent inhibition (not shown), the drug was more effective at shorter time points, with the IC_{50} dropping 4-fold between 4 and 1 h (Figure 8B). The pattern exhibited by nogalamycin (Figure 8C) was similar to that obtained for chromomycin. However, 5 times more drug was needed to obtain an

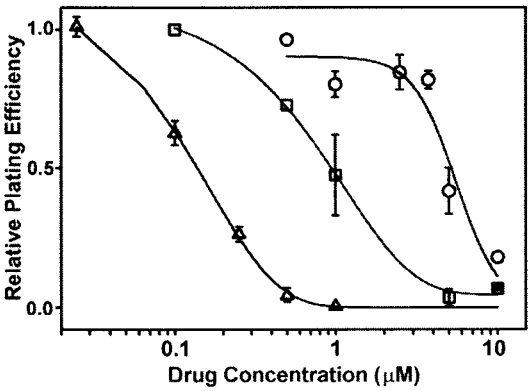

FIGURE 9: Cytotoxic effect of drugs on NIH3T3 cells. Cells were exposed to a range of drug concentrations for 4 h before being serially diluted, replated, and grown for 10 days. The cells were then stained and fixed in a solution of methylene blue and ethanol. Groups of more than 50 cells were deemed colonies and counted under a stereo microscope. Plating efficiencies were calculated by dividing the number of colonies by the number of cells plated. Relative plating efficiencies were then calculated by dividing the plating efficiency of each drug treatment by the control plating efficiency. The results shown for chromomycin (△), nogalamycin (□), and Hoechst 33342 (○) are the mean of three experiments (mean ± standard error).

equivalent level of inhibition at 1 h as compared to 4 h, after which more time did not result in any substantial further increases in drug effectiveness.

Drugs' Effects on Cell Survival and RNA Synthesis. The Northern blot results demonstrated that the drugs chosen are capable of affecting endogenous c-fos expression in a dose- and time-dependent manner. However, the complexity of the cellular environment necessitates that these results be interpreted in the context of the agents' other biological activities and whole cell effects. Therefore, to further characterize the drugs' effects in whole cells, NIH3T3 cells were exposed to a range of drug concentrations for 4 h before being plated in a colony formation assay. The colonies formed after 10 days of growth were counted and used to calculate relative plating efficiencies for each drug treatment as compared to non-drug-treated controls (Figure 9). Chromomycin was found to be the most toxic agent, causing 50% cell death at only 0.125 μM. The amount of Hoechst 33342 required to obtain the same level of toxicity (4.5 μM) was over an order of magnitude greater. Nogalamycin's toxicity fell between these values, with an IC_{50} of 1 μM. The cytotoxicity of these compounds may be reflected by their ability to shut down general transcription in cells, since they are capable of binding to many regions on DNA. To get a sense of how general transcription was being affected, RNA synthesis in NIH3T3 cells, as quantitated by [^{3}H]uridine incorporation, was measured following 4 h drug exposures (data not shown). The IC_{50}s from this assay are summarized in Table 1, along with IC_{50}s for the 4 h drug treatments in the cytotoxicity and Northern blot assays. The order of potency for drugs in the cellular assays was chromomycin > nogalamycin > Hoechst 33342. For all agents, similar drug concentrations were needed to achieve equivalent levels of activity in each assay.

Table 1: IC_{50} Values for Selected Whole Cell Assays[a]

drug	cytotoxicity	c-fos mRNA synthesis	total RNA synthesis
chromomycin	0.125	0.2	0.1
nogalamycin	1.0	0.6	0.5
Hoechst 33342	4.5	20	7.5

[a] Concentrations of drug, in μM, needed to inhibit the measured activity by 50%. The values for each assay, as described under Materials and Methods, were obtained after exposing NIH3T3 cells to drug for 4 h.

DISCUSSION

This study has compared and contrasted the ability of various classes of DNA-binding agents to inhibit TF/DNA interactions and resultant gene expression using a defined target gene promoter sequence, the c-fos SRE. To our knowledge, this is the first study that systematically analyzes the effectiveness of drugs possessing different sequence selectivities and modes of DNA binding to target two different TF-binding motifs in increasingly complex assays. The drugs' abilities to inhibit TF binding to target sequences in EMSAs were compared to their potencies in inhibiting cell-free transcription as well as their abilities to inhibit cellular gene expression. The drugs selected for analysis, chromomycin, nogalamycin, and Hoechst 33342, exhibited different potencies in each assay. Interesting differences were also noted when the drugs' effectiveness was compared between assays. These variations may stem from many factors including the DNA-binding characteristics of the compounds as well as their overall stability in cells.

In the EMSAs, the overall order of decreasing potency for inhibiting SRF and Elk-1 complexes was nogalamycin > Hoechst 33342 > chromomycin. Potential binding sites for nogalamycin, consisting of alternating purines and pyrimidines, are located near the *ets* motif of the Elk-1 binding site and in the CArG box of the SRF binding site. As an intercalator that makes contacts in both the major and minor grooves (*32*), and in comparison to the MGBs, nogalamycin dissociates from DNA very slowly (*11*), favoring more effective inhibition of TF binding under equilibrium conditions. It was anticipated that since SRF is required for Elk-1 binding, the IC_{50}s for inhibition of TC and SRF binding would be equivalent. However, 2 times more nogalamycin was needed to inhibit TC formation by 50% as compared to SRF binding. Structural studies and circular permutation analyses have shown that binding of a homodimer of SRF bends DNA 72° (*33*), while DNA bound to the TC has increased flexibility and is bent approximately 50° (*34*). This alteration in DNA conformation upon Elk-1 binding and its effects on neighboring DNA structure may therefore influence a drug's ability to optimally recognize and bind its target sequences.

In contrast to nogalamycin, chromomycin was more potent in inhibiting TC formation as compared to SRF binding but exhibited the highest potency in inhibiting Elk-1 complexes. While the E74 oligo used in the EMSAs contains four contiguous G/C base pairs in the *ets* motif that may be an appropriate chromomycin binding site (5′-CCGG-3′), the SRE lacks such a sequence. However, this drug was still able to prevent SRF and TC binding with lower potency, suggesting that if a consensus sequence is not available, the drug will associate with other sequences by default. Similar results have been obtained using chromomycin to inhibit TBP association with its A/T-rich binding site (*35*). The higher concentration of chromomycin required to inhibit TC forma-

tion as compared to Elk-1 complex formation may be due to SRF's stabilization of Elk-1 binding. Analogous results were seen in previous evaluations of drug effects on TBP binding. When TBP's binding was stabilized by addition of TFIIA, higher concentrations of distamycin were needed to disrupt complex formation (*12*).

Analysis of Hoechst 33342's inhibition of Elk-1 complexes was limited by well retention of the oligo when complexed to drug, regardless of whether protein was present (data not shown). This effect has been seen previously in our lab with other drug/oligonucleotide combinations. Despite its A/T preference, Hoechst 33342 was not a potent inhibitor of SRF binding to the A/T-rich CArG box on the SRE. Sequence selection studies have demonstrated that other bisbenzimidazole-based drugs, such as Hoechst 33258, greatly prefer some A/T sites over others, and surrounding sequences appear to influence the drug's affinity and optimal binding ability (*36*). In particular, the presence of 5′TpA steps greatly decreases the affinity of this Hoechst dye for DNA (*37*). The two 5′TpA steps present in the CArG box may therefore be contributing to the low potency observed in the SRF complex analysis. Given its high IC_{50} value, it is possible that Hoechst 33342 is binding to the target oligo by default, much like chromomycin.

When EMSA analyses using the TC were carried out, drugs never solely inhibited Elk-1 binding, which would have resulted in the appearance of the SRF complex. This suggests that these agents are generally disruptive and that they apparently alter the conformation of the DNA through bending or groove widening so that neither TF is able to bind. This is supported by previous studies that investigated long-range effects of drug binding on DNA. For example, the MGB distamycin can alter DNA allosterically up to 100 bp away from its binding site (*38*). Furthermore, the alterations in local DNA structure following binding of distamycin or the intercalator actinomycin produce changes in DNase I cleavage patterns at flanking sites (*39*).

In reverse EMSAs, where drug was added to preformed TF/DNA complexes, only nogalamycin required higher concentrations to achieve equivalent levels of TC inhibition. The equilibrium conditions required to inhibit complex formation were therefore altered, since more drug was needed to disrupt the TC if it was added after the proteins were bound. Dissociation studies carried out for the TC showed that this complex was stably bound for over 2 h under assay conditions (data not shown). SRF and Elk-1 primarily contact the SRE in the major groove. Since nogalamycin binds in a similar manner, the drug's association with DNA may be hindered by the presence of TFs at or near its binding site. In contrast, drugs such as chromomycin or Hoechst 33342 may be more effective in inhibiting TF binding to the major groove in reverse assays because they can approach DNA from the opposite, minor groove (*35*).

Drug activity was maintained in a more complex milieu of nuclear proteins and plasmid DNA in the cell-free transcription assay. IC_{50}s obtained in this assay were not substantially different from the EMSA IC_{50}s for TC inhibition, but the order of potency was chromomycin > nogalamycin > Hoechst 33342. As noted in Figure 4C, there was only a 4-fold decrease in potency for nogalamycin and Hoechst 33342 in this assay compared to the EMSA results. In contrast, chromomycin was about 2.5 times more effective in inhibiting transcription than in preventing TC formation. Overall, the presence of additional proteins and a higher amount of DNA with greater sequence complexity do not seem to greatly interfere with the ability of these drugs to inhibit cell-free transcription. This is in contrast to previous studies in which the MGB distamycin was used to inhibit the TF E2F from binding to the dihydrofolate reductaste (DHFR) promoter and to inhibit DHFR promoter-driven cell-free transcription (*29*). Here, 200 times more drug was needed to inhibit transcription as compared to inhibition of E2F/DHFR promoter complex formation. However, studies carried out using mithramycin (which is chemically related to chromomycin) demonstrated that similar levels of drug were needed to inhibit both TF complex formation and cell-free transcription from the c-myc promoter (*40*). The ability of any given drug to inhibit TF complex formation and to maintain an equivalent level of activity in a cell-free environment may therefore be dependent on the particular TFs studied and the DNA sequence used as a target. The results obtained here may also stem from the drugs' effects on other sequences in the c-fos promoter. By interfering with the binding of other TFs, recruitment of a functional RNA polymerase complex may be inhibited and levels of transcription will drop. The TFs bound to the c-fos promoter may be more sensitive to chromomycin than the other drugs analyzed. This may explain chromomycin's greater potency in inhibiting cell-free transcription and endogenous c-fos expression, as discussed below. None of the drugs tested resulted in the detectable production of shorter transcripts, which suggests that these agents are acting on the level of transcription initiation, rather than elongation. This is supported by previous work where transcriptional elongation inhibition was not observed except at high drug concentrations in some systems (*14*, *41*).

The serum inducibility of the c-fos promoter and the rapid turnover of its mRNA facilitated assessment of immediate or short-term drug effects on gene expression in NIH3T3 cells. In Northern blots, the order of the drugs' potency in inhibiting endogenous c-fos transcription following serum induction was chromomycin > nogalamycin > Hoechst 33342. The time course study, which demonstrated an approximately 40% inhibition after only 40 min of treatment with 1 μM chromomycin, demonstrates the fast-acting nature of this drug and suggests that it is able to enter cells quickly and effectively. These rapid effects, in addition to the cell-free transcription data discussed above, suggests that this drug is acting on the level of DNA by inhibiting TF association with the c-fos promoter. Higher concentrations of chromomycin were needed at shorter time points in order to achieve an equivalent level of inhibition. This drug's effects may therefore depend, at least in part, on accumulation within the cell. The fact that the drug still exhibits inhibitory effects after 16 h in cells suggests that it is relatively stable. Similar results were obtained in studies using mithramycin and chromomycin to inhibit the expression of a stably transfected c-myc gene in NIH3T3 cells (*41*). Here, expression of an exogenous gene was very effectively inhibited after a 24 h exposure to 1 μM of either drug.

Nogalamycin's pattern of inhibition of gene expression was similar to that obtained using chromomycin. Hoechst 33342, however, was more effective at shorter time points as evidenced by a lower IC_{50} for 1 h exposures. The

inhibition seen following 16 h exposures, although variable, was marginal at best (data not shown). Hoechst 33342 may therefore become unstable or inactivated in these cells over time. Because there are undetectable levels of c-fos mRNA prior to serum induction and because shorter transcripts cannot be detected, the presence of drug is likely preventing the initiation of transcription. No decrease in absolute G3 mRNA levels was noted after any drug treatment. In short-term drug exposures, this may be due to G3's longer mRNA half-life (8 h for G3 compared to 30 min for c-fos) (*42*). In addition, the lack of detectable shorter G3 transcripts following drug treatment of cells is again consistent with the drugs' inhibition of transcription initiation. If the drugs were causing transcript degradation or inhibition of transcription elongation, partial transcripts or smearing of the RNA samples would have been visible.

The IC_{50}s for drug inhibition of c-fos expression as measured in the Northerns were less than the IC_{50}s calculated for the cell-free transcription assay. The largest difference of an order of magnitude was obtained for chromomycin. The process of transcription is far more complex on an endogenous promoter, where many other proteins and long-range changes in DNA conformation come into play to provide an intricately regulated cellular response. These drugs' abilities to bind to many other regions on the c-fos promoter is likely contributing to their increased potency in the whole cell environment. The complexity of the drugs' effects is further evidenced by the results obtained in the other whole cell analyses. The similarity in IC_{50}s for the cytotoxicity, RNA synthesis, and Northern blot assays following 4 h drug exposures suggests that the toxicity of these agents is reflected by their inhibition of general transcription.

Drug association with many sequences in the genome and the resultant toxic effects could potentially be avoided by developing more specific DNA-binding compounds. Designing more effective and potent DNA-binding drugs with the goal of disrupting specific TF/DNA complexes is an essential step in developing potentially therapeutic compounds. Our results demonstrate how the properties and DNA-binding characteristics of classical DNA-binding compounds are related to their biological consequences on a particular gene. Structural modification of classical drugs has yielded novel agents, such as microgonotropens and polyamides (*43*, *44*). These agents will next be analyzed using the c-fos SRE target to determine which modifications result in potent and specific DNA-binding drugs with significant cellular activity.

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