Modulation of DNA-protein interactions in the P1 and P2 *c-myc* promoters by two intercalating drugs

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(Received 22 September 1997) – EJB 97 1358/2

Regulation of transcription from the oncogene *c-myc* has an important role in the genesis of various tumors. Therefore, *c-myc* is a potential target for chemotherapy by drugs which are able to modify its activity directly. In this article, we identify the binding sites in the P1 and P2 promoter regions of *c-myc* for the intercalating antibiotics actinomycin D and elsamycin A. Gel retardation experiments indicate that actinomycin D or elsamycin A binding can inhibit the formation of several DNA-protein complexes. However, relatively low concentrations of elsamycin A, but not actinomycin D, appear to increase the level of binding to the P1 promoter of a protein factor. Using pure Sp1 transcription factor and an oligonucleotide containing the Sp1 putative binding site, we determined that the binding enhancement induced by small amounts of elsamycin was on the Sp1-DNA complex. Run-off transcription experiments *in vitro* showed that the effect of elsamycin A on Sp1 binding is followed by the maintenance or a relative rise in transcription levels from the P1 promoter of *c-myc*, while actinomycin D always inhibited the transcription from the P1 *c-myc* promoter in a concentration-dependent manner. Higher concentrations of elsamycin acted as an inhibitor of the transcription from the P1 start site but not from the P2.

Keywords: *c-myc*; transcription; actinomycin D; elsamycin A.

The control of *c-myc* expression appears to be essential for normal cellular proliferation, and its failure may lead to the genesis of various tumours [1–5]. Transcription of the *c-myc* gene is initiated from two major start sites termed P1 and P2, which are located 161 bp apart in the human gene [1, 2]. In normal and resting cells P2 is the pivotal promoter producing up to 90% of the *c-myc* mRNA, while P1 provides up to 25% of *c-myc* transcripts in normal cells, but may predominate in malignant cells [1–3]. Regulation has been shown to occur at multiple levels and several positive and negative cis-acting elements within a 2.3-kb region upstream of the promoters coordinate the *c-myc* expression in response to various physiological stimuli [1, 6, 7]. P1 and P2 appear to be regulated by a combination of positive and negative *trans*-acting factors binding within this region.

The antitumor antibiotics actinomycin D (dactinomycin) and elsamycin A (elsamitrucin) [8, 9] are two DNA-binding drugs which intercalate preferentially into G+C-rich steps [10–14], and they are able to inhibit the transcription *in vitro* from prokaryotic and phage genes [15, 16]. However, there is an increasing need to complement these studies with additional information on the effect of other antitumor drugs on specific genes, and in particular on *c-myc*, which plays a crucial role in malignant cell differentiation.

We have sought to verify the notion that some drugs that bind specifically to G+C-rich DNA might achieve their chemo-

therapeutic action through their interaction with the *c-myc* oncogene [17–19]. In order to identify the mechanism by which some drugs inhibit the expression of the *c-myc* gene, we analyzed the binding of actinomycin D and elsamycin A within the region –101 to +212 of the human *c-myc* promoter region. We found that the presence of intercalating drugs can modulate the binding of several protein factors to the P1 and P2 promoter regions, especially the Sp1 *trans*-acting factor, which also recognizes a consensus G+C-rich tract in DNA [20]. We have also identified the binding sites for these drugs in the promoters, and show that certain discrete DNA-protein complexes behave differentially in the presence of either actinomycin D or elsamycin A since some complexes are blocked by the presence of either drug, while others seem to be insensitive, or even somewhat enhanced by the drugs. In general, impedence of the formation of DNA-protein complexes by the intercalating drugs produces the inhibition of *c-myc* promoter transcription *in vitro*, though P1 and P2 are differentially sensitive to the presence of drug concentrations.

MATERIALS AND METHODS

Protein extracts and drugs solutions. HeLa cell nuclear extracts were purchased from Promega; the protein concentration was between 3.6–8.3 µg/ml depending on the lot used. Pure Sp1 transcription factor was obtained from Promega. Actinomycin D (Serva) and elsamycin A (a gift of Dr A. M. Casazza, Bristol-Myers Squibb) were freshly prepared as 250 µM stock solutions in 10 mM Tris/HCl, pH 7.4, 20 mM KCl (containing 25% methanol for elsamycin), and diluted to the desired concentrations using the same buffer without methanol prior to the experiments.

Labeling and isolation of DNA fragments. The pGEM-myc1 plasmid, which contains the *Sma*I–*Sst*II fragment of the

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Enzymes. DNase I (EC 3.1.21.1); Klenow enzyme (DNA polymerase I, large fragment) (EC 2.7.7.7); T4 polynucleotide kinase (EC 2.7.1.78).

human *c-myc* [18], was kindly provided by Dr R. Ray (University of St Louis). Two restriction fragments from this plasmid were purified, *SmaI*–*XhoI* to create pVP1 and *XhoI*–*NaeI* to create pVP2, and inserted between the *SmaI*–*SalI* sites of pBluescribe (Stratagene), grown in *Escherichia coli* TG1 and purified using CsCl and standard protocols. Hence, the pVP1 plasmid contains, in its *SmaI*–*XhoI* fragment, the *c-myc* region from –110 to +68, including the P1 initiation site (fragment SX, see Fig. 1); while pVP2 contains, in its *XhoI*–*NaeI* fragment, the *c-myc* region from +68 to +212 including the P2 initiation site (fragment XN, see Fig. 1). DNA fragments (SX and XN) to be used in the gel retardation (band-shift) and footprinting assays were prepared by digestion with *EcoRI* and *HindIII* followed by labeling at their 3'-ends using [α - 32 P]dATP and the Klenow enzyme. The fragments were further separated from the remainder of the plasmids by running on a non-denaturing 6% polyacrylamide gel.

An oligonucleotide containing the consensus Sp1 binding site [20], whose sequence (upper strand) is: 5'-ATTTCGATCGGGGCGGGCGAGC3' (purchased from Promega) was also employed to analyze the effect of the intercalating drugs on Sp1 binding. It was labelled at its 5' ends using [γ - 32 P]ATP and T4 polynucleotide kinase.

Gel retardation assays. The gel retardation (band shift) assays were performed as described elsewhere [18] with a minor modification: the binding buffer contained 2% glycerol and 0.02% Nonidet P-40 (Sigma). In reactions containing actinomycin or elsamicin, the DNA fragments were incubated with different concentrations (see legends to figures) of the drugs before the addition of the different amounts of protein extract, except where otherwise noted. A typical reaction contained 4–12 μ g HeLa extract or 25 ng pure Sp1 protein and 1500–3000 cpm (about 1.5 nmol in bp) of end-labelled DNA or oligonucleotide, in the presence of 1–3 μ g poly[d(I-C)] (Boehringer Mannheim). The samples were analyzed on 4.5% non-denaturing polyacrylamide gels containing 45 mM Tris/borate, 1 mM EDTA pH 8.3. After running at low voltage (12 V/cm), the gels were soaked in distilled water, dried under vacuum and subjected to autoradiography. Quantitative analysis of the complex formation was performed with a Molecular Dynamics computing densitometer using the ImageQuant 3.2 software. Each gel track, from four independent experiments, was normalized with respect to the total amount of loaded radioactivity and represented as the mean value $\pm$ SD.

DNase I footprinting. Samples containing incubated complexes of the end-labelled SX or XN fragments of the human *c-myc* (3000 cpm, around 10 pmol in bp) and different concentrations of actinomycin, or elsamicin, both in the presence and the absence of pure Sp1 transcription factor, at the concentrations stated in the legend of Fig. 7, were digested with DNase I (Boehringer Mannheim) at a final concentration of 0.01 U/ml for 2 min at 25°C, and the reaction stopped by adding a solution containing 85% formamide 10 mM EDTA and 0.02% bromophenol blue. Samples were heated at 95°C for 2 min prior to electrophoresis. The footprints were resolved by high-voltage electrophoresis in 90 mM Tris/borate, 2 mM EDTA pH 8.3 using 6% or 8% polyacrylamide gels containing 8 M urea, together with a dimethylsulphate-piperidine marker specific for guanine. After running, the gels were soaked in distilled water, dried under vacuum and the bands were observed by autoradiography.

In vitro transcription. The effect of actinomycin and elsamicin on the transcription of the human *c-myc* P1 and P2 promoters was measured using *SmaI*-linearized pGEMmyc1 plasmid digested with *FokI*. Run-off transcription assays *in vitro* were performed in a final volume of 25 μ l and contained 30 mM Hepes pH 7.9, 7 mM MgCl₂, 5 μ M ZnSO₄, 1 mM dithiothreitol,

Fig. 1. Schematic representation of the human *c-myc* P1 and P2 promoters. The putative Sp1 binding site, as well as the P1 and P2 start sites, are indicated (top). The indicated restriction enzymes were used to generate the two fragments: SX (*SmaI*–*XhoI*) and XN (*XhoI*–*NaeI*). The lower part displays the nucleotide sequences of the upper strand of these restriction fragments.

0.2 mM EDTA, 2% poly(ethyleneglycol)8000 (Sigma), 300 μ M each of ATP, CTP and GTP, 1 μ M UTP and 20 μ Ci [α - 32 P]UTP, in the presence of the amounts of actinomycin D or elsamicin A indicated in the legend to Fig. 8. Transcription was allowed to proceed for 60 min at 30°C and the samples were phenol-extracted before gel loading. Transcription using HeLa whole nuclear extracts, in either the presence or absence of additional Sp1 transcription factor (25 ng pure protein), renders two transcripts of 348 nucleotides (P1 promoter) and 187 nucleotides (P2 promoter). In the transcription experiments an internal standard for recovery and gel loading was used (a 230-nucleotide RNA kindly provided by Dr J. Bernués). Transcripts were analyzed by high-voltage electrophoresis in 90 mM Tris/borate, 2 mM EDTA pH 8.3 using 8% polyacrylamide gels containing 7 M urea. After running, the gels were soaked in distilled water, dried under vacuum and the bands were observed by autoradiography. The relative amounts of transcripts from both promoters, in the presence or absence of drugs, was quantified using a Molecular Dynamics computing densitometer.

RESULTS

The influence of actinomycin D and elsamicin A on the binding of protein factors, from nuclear HeLa extracts, to the human (–110 to +212) *c-myc* promoter region was examined using the gel retardation technique on two restriction fragments (designated SX and XN, see Fig. 1), which contain the P1 and P2 promoters respectively together with the surrounding regions. These two DNA fragments were used to identify specific protein complexes as well as their sensitivity to the intercalating ligands actinomycin D and elsamicin A.

Actinomycin D and elsamicin A bind differentially to the P2 promoter of *c-myc*, and compete with protein factors for their binding sites. Fig. 2 shows a gel retardation experiment using the XN restriction fragment of *c-myc*, whose sequence is displayed in Fig. 1. Four different complexes were observed

Fig. 2. Effect of actinomycin D and elsamicin A on the binding of a HeLa nuclear extract to the P2 promoter of *c-myc*. The experiments were performed using the XN restriction fragment, whose nucleotide sequence is displayed in Fig. 1. Lane 1, control DNA (1.5 nmol in bp); lanes 2–5, gel retardation analysis of the protein binding in the presence of increasing amounts of actinomycin D (ACTD; 1, 5, 10 and 20 μ M); lanes 6–9, analysis of the protein binding in the presence of increasing amounts of elsamicin A (ELSA; 5, 15, 50 and 150 μ M); lane 10, 8 μ g HeLa nuclear extract plus DNA.

when this restriction fragment was incubated with HeLa extract (designated B1–B4). All of them were differentially sensitive to actinomycin and elsamicin. The presence of the B1 protein–DNA complex, the slowly migrating one in Fig. 2, was clearly inhibited by the presence of either antibiotic, though the exact concentration of drug required to inhibit the formation of the complex completely was higher in the case of elsamicin (compare lanes 5 and 9 in Fig. 2). Nevertheless, B2 and B3 were quite insensitive to the presence of either drug. In fact, concentrations of elsamicin above 100 μ M were required to inhibit the formation of such a complex. The most rapidly migrating complex (B4) was very sensitive to actinomycin while elsamicin was required at concentrations about 100 μ M to prevent the formation of this DNA–protein complex completely (compare lanes 3 and 7 in Fig. 2). A similar result, with differential modulation of DNA–protein complexes in the human *c-myc* P2 promoter, has been reported previously using mithramycin [18], a minor-groove-binding drug [21]. Several nuclear protein binding sites have been identified within the *c-myc* promoters [1, 2, 22]. Although our band-shift results are consistent with the presence of such binding sites, the detection of a different number of protein–DNA interactions might originate from the different protein composition or stability of the HeLa nuclear extracts, since two different preparations containing the same amount of proteins do not necessarily contain the same number of active protein factors. In any case, the behavior of the different complexes in the presence of elsamicin and actinomycin was consistent with the binding of these drugs to G+C-rich regions [10, 11] as it is in the experiments with mithramycin [18]. Notwithstanding, there were differences in the concentration dependence of the inhibition by the three drugs which may have arisen from a more pronounced effect of the intercalators on DNA structure, or the respective binding constants of the intercalating ligands.

At some concentrations elsamicin A might relatively enhance the binding of Sp1 protein factor to the P1 promoter of *c-myc*. A band shift analysis of the SX fragment, which contains the P1 start site, is displayed in Fig. 3A. At least six different protein–DNA complexes were detected when this restriction fragment was used under the experimental conditions described in Materials and Methods. They were differentially sensitive to

Fig. 3. Analysis of the effect of actinomycin D and elsamicin A on the binding of a HeLa nuclear extract to the P1 promoter of *c-myc*. (A) Effect of actinomycin D and elsamicin A on the binding of a HeLa nuclear extract to the P1 promoter of *c-myc* (SX restriction fragment, whose nucleotide sequence is displayed in Fig. 1). Lane 1, control DNA (1.5 pmol in bp); lanes 2 and 8, DNA plus drugs; lanes 3 and 9, DNA plus HeLa extract; lanes 4–7, analysis of the protein binding (8 μ g of HeLa extract) in the presence of four concentrations of actinomycin D (5, 15, 45 and 90 μ M); lanes 10–13, analysis of the protein binding in the presence of four concentrations of elsamicin A (5, 15, 45 and 90 μ M). (B) Quantitative analysis of the effect of actinomycin D and elsamicin A on the binding of some transcription factors to the P1 promoter (displayed in A), measured as the percentage of complex formation in the presence of the drugs. (Δ) A4 complex plus elsamicin A; ($\square$) A4 complex plus actinomycin D; ($\circ$) A5 complex plus actinomycin D. The data represent the mean of four independent experiments like those in A (mean value $\pm$ SD).

elsamicin and actinomycin in a concentration-dependent manner. All the complexes were observed in the protein–DNA control experiment (lanes 3 and 9) whereas they were not observed in the control of DNA alone (lane 1). The rapidly migrating protein–DNA complex, named A5, was clearly dissociated by actinomycin D at concentrations above 15 μ M, while it remained quite insensitive to elsamicin up to the same concentration. The presence of actinomycin produced a diminution in the intensity of the A2 and A4 complexes in a concentration-dependent manner. The incubation of the labelled DNA with the protein extract and either actinomycin D or elsamicin A added simultaneously also resulted in the inhibition of most of the DNA–protein complexes (results not shown). Elsamicin A produced a relative increase of the A4 band at concentrations between 5–15 μ M (lanes 10 and 11), although the shifted band of the complex was always less intense than in the absence of the drug (compare lanes 8 and 11 in Fig. 3A). At higher amounts of elsamicin a concentration-dependent disassociation of the DNA–protein

Fig. 4. Displacement of the complex A4 by increasing amounts of the oligonucleotide 5'-ATTTCGATCGGGGCGGGGCGAGC-3' containing the Sp1 consensus sequence. The end-labelled SX fragment of *c-myc* was incubated with increasing amounts of unlabelled oligonucleotide, in the presence of 8 µg HeLa extract. Lane 1, DNA fragments; lane 2, DNA plus 25 ng pure Sp1; lane 3, DNA plus HeLa extract; lanes 4–6, 0.15, 0.28 and 0.84 ng oligonucleotide, respectively. The band displaced by competitive binding is indicated with an arrow.

complex occurred as for actinomycin. The behavior of the A4 protein-DNA complex was reasonably reproducible in four independent experiments. The plot in Fig. 3B shows a quantitative analysis of the complex formation in these experiments. The interference of elsamicin and actinomycin on the formation of the A4 complex was determined from densitometric scans (see Materials and Methods). The dose/response curves in Fig. 3B compare drug-treated samples with those obtained in the absence of any drug. They substantiate, within experimental error, the enhancement of the A4 band due to the binding of elsamicin, after a previous decay. A further probe of this enhancement was obtained using pure protein (see below).

To evaluate the effectiveness of both drugs as inhibitors of DNA-protein complex formation, we established conditions of the assay to maximize complex formation. The temperature for incubation and electrophoresis were optimized to enhance the stability of the DNA-protein complexes (see Materials and Methods). Due to the peculiar behavior of complex A4, we attempted to identify the relationship between this particular band and some putative protein factors that have been described to bind to this region of *c-myc* [2]. Fig. 4 shows a competition experiment between increased amounts of an unlabelled oligonucleotide, which contains the consensus sequence for Sp1 binding [20], and the SX end-labelled fragment. One of the radioactive bands, which appears to be the same as complex A4 (cf. Figs 3A and 4), clearly decreased in the presence of the unlabelled competing oligonucleotide while the intensity of the other bands remained practically unaltered. This led us to identify the band, the intensity of which was modulated in direct proportion to the amount of elsamicin added, as produced by Sp1 binding (lanes 10–13 in Fig. 3A). A putative Sp1 binding site is located upstream the P1 promoter of *c-myc* (see Fig. 1). It consists of two overlapping Sp1 binding sites, which can bind either a single Sp1 (giving the electrophoretic band that runs ahead) or a slowly migrating band due to the binding of two Sp1 mole-

Fig. 5. Effect of actinomycin D and elsamicin A on Sp1 binding to an end-labelled oligonucleotide containing its consensus binding sequence. Lanes 1–4, control lanes for DNA, protein, and drug complexes; lanes 5–7, Sp1 binding in the presence of increasing amounts of actinomycin D (ACTD; 2, 6, 15 µM); lanes 8–10, Sp1 binding in the presence of increasing amounts of elsamicin A (ELSA; 2, 6, 15 µM).

cules (upper band in Fig. 4, lane 2). It seems that when Sp1 is displaced by the specific oligonucleotide, there is also a diminution in the intensity of the slowly migrating band, although it is not completely removed at the concentrations used to displace the A4 band. We verified our interpretation that the two bands observed should correspond to Sp1 binding as a monomer or homodimer complex, respectively, by the presence of a unique electrophoretic band in experiments performed with a labelled oligonucleotide containing a unique Sp1 binding site (Fig. 5).

To seek new insight into the effect of actinomycin D and elsamicin A on the binding of Sp1 to its recognition site, we performed band-shift experiments with pure Sp1 protein on both the oligonucleotide (5'-ATTTCGATCGGGGCGGGGCGAGC-3', as a double strand) and the SX fragment that contains the putative Sp1 binding site [20] in the *c-myc* promoter. Illustrated in Fig. 5 are the effects of different drug concentrations on the binding of Sp1 to the oligonucleotide containing its consensus sequence. The retarded electrophoretic band, which corresponds to the analyzed DNA-protein complex (lane 2 in Fig. 5), was sensitive to both actinomycin and elsamicin in a concentration-dependent manner as detected by a titration of the previously formed protein-DNA complex using increasing amounts of actinomycin D (lanes 5–7) or elsamicin A (lanes 8–10). 6 µM actinomycin produced more than 90% inhibition of the DNA complex, while at the same concentration, or even at smaller ones, elsamicin completely prevented the formation of an Sp1-oligo complex. These results agree with a competition between the two drugs, which are G+C sequence-selective binding ligands [10, 23], and the purified Sp1 protein factor binding to the promoter regulatory element containing a G+C-rich tract.

At first sight, the results obtained with elsamicin A (Fig. 5), seem to be at variance with the relative increase of the complex that was observed when total HeLa extract and the SX restriction fragment described above were employed (Fig. 3). In order to investigate the modulation of Sp1 binding to the *c-myc* promoter by elsamicin A, and to gain further information on the effect of some antitumor antibiotics on the promoter region of *c-myc*, we also performed experiments with purified human Sp1 transcription factor and the SX fragment. In Fig. 6 the Sp1-DNA electrophoretic band was sensitive to actinomycin D with concentrations of around 5 µM preventing the formation of DNA-protein complexes. However, while Sp1-DNA complex formation seemed to be fairly blocked around 1.5 µM elsamicin A (Fig. 6B, lane 6), at higher concentrations (lanes 7–9) the

Fig. 6. Analysis of the effect of actinomycin D on Sp1 binding to the P1 promoter (SX restriction fragment whose nucleotide sequence is displayed in Fig. 1). (A) Effect of actinomycin D on Sp1 binding to the P1 promoter (SX restriction fragment whose nucleotide sequence is displayed in Fig. 1). Lane 1, DNA alone; lane 2, DNA plus 25 ng pure Sp1; lane 3, DNA plus 15 μ M actinomycin D (ACTD); lane 4, DNA plus 15 μ M elsamicin A (ELSA); lanes 5–9, DNA-Sp1 complex incubated with increasing concentrations of actinomycin D (0.1, 0.5, 1, 5 and 15 μ M). (B) Effect of elsamicin A on Sp1 binding to the same fragment containing the P1 promoter. Lane 1, DNA alone; lane 2, DNA plus 5 μ M elsamicin A; lane 3, DNA plus 25 ng pure Sp1; lanes 4–10, DNA-Sp1 complex incubated with increasing concentrations of elsamicin A (0.5, 1, 1.5, 2, 2.5, 5 and 15 μ M).

electrophoretic band increased slightly, in agreement with the observations using total nuclear HeLa extract described above (compare Figs 3 and 6B). There is also a clear effect on the slowly migrating band, which would correspond to the binding of two Sp1 molecules as described above. When higher concentrations (around 15 μ M) were used there was an inhibition of the complex formation. These experiments were also performed using different concentrations of elsamicin and the results were unambiguously reproducible. In any case, the DNA concentration in the band shifts was about 1.5 nmol (in bp), so that there was a molar excess of both purified Sp1 and drug molecules. As a result, under our experimental conditions, the amount of drug that can block (or enhance) the binding of the transcription factor to DNA (or compete for the G+C-rich zones in DNA) was limited by the equilibrium binding constant of the reaction rather than by the drug/DNA stoichiometric ratio.

Footprinting analysis showed that Sp1 and elsamicin A can compete for their overlapping binding sites. DNase I footprinting experiments were performed to characterize binding of the pure Sp1 protein to the P1 promoter of *c-myc*, in the presence and absence of actinomycin and elsamicin. By using this approach, it is feasible to assess the overlapping binding sites of Sp1 and either actinomycin or elsamicin A to the *c-myc* P1 promoter. In all respects, binding of these drugs to the SX restriction fragment, in the absence of the protein factor, produced a footprinting pattern (Fig. 7) which resembled those obtained for these antibiotics on other DNA fragments [10, 11]. The reactions containing Sp1 showed a clear protection in a wide region of the P1 promoter. The presence of 5 μ M actinomycin D or elsamicin A produced a clear footprint in the region which overlapped the Sp1 footprint. We consider that either actinomycin or elsamicin can compete with the protein binding to its putative site in the *c-myc* P1 promoter in a concentration-dependent way. The experimental fact that elsamicin and actinomycin bind extensively to the same sites as Sp1 suggests that spacious overlapping of the binding sites is required to inhibit the binding of the protein factor, in agreement with the footprinting pattern generated by mithramycin binding to the Sp1 binding site [18]. In the

Fig. 7. DNase I footprinting of Sp1 and actinomycin D or elsamicin A binding to the P1 promoter region. Lane 1, dimethylsulphate-piperidine markers specific for guanine; lane 2, DNase I cleavage in the presence of 5 μ M elsamicin A (ELSA) plus 50 ng pure human Sp1; lane 3, in the presence of 5 μ M elsamicin A; lane 4, in the presence of 150 ng pure Sp1; lane 5, labelled DNA alone (control); lane 6, 5 μ M actinomycin D (ACTD); lane 7, 5 μ M actinomycin D plus 50 ng Sp1. The region corresponding to the Sp1 footprint is indicated on the figure.

Fig. 8. Effect of actinomycin D and elsamicin A on *in vitro* run-off transcription from the *c-myc* P1 and P2 promoters by HeLa extract. Lane 1, a size marker (end-labelled *HinfI* digest of ϕ X174 DNA) used to locate the transcripts; lane 2, transcripts from the *c-myc* P1 and P2 promoters (control); lane 3, transcripts obtained using whole HeLa extract plus added pure Sp1 (25 ng); lanes 4–6, effect of increasing amounts of actinomycin D (ACTD; 0.5, 1, 5 μ M) on the run-off transcription; lanes 7–9, effect of increasing amounts of elsamicin A (ELSA; 0.5, 1, 5 μ M) on the run-off transcription. Note that in these transcription experiments an internal standard for recovery and gel loading was used (indicated by an asterisk).

footprinting studies, we did not find any symptom of increased binding of Sp1 by added elsamicin, as described in the band-shift studies (Figs 3–6) or the transcription assays *in vitro*, described below; undigested material is obviously difficult to observe in a region that is already protected from DNase I cleavage, though the phenomenon might exist. In Fig. 7, the differences between the DNase I cleavage enhancements in lanes 2 (elsamicin + Sp1) and 7 (actinomycin + Sp1) support this interpretation. An undigested region at the 3' site of the *c-myc* P1 promoter (upper part of the gel) is observed in the presence of elsamicin A, or mithramycin [18], but not actinomycin D. It may correspond, at least in part, to a distortion of the DNA structure after Sp1 binding, since Sp1 binding can induce significant structural distortions at the 3' site of G+C-rich regions [24]. It is worth mentioning that this region contains the TATA box of P1 (see Fig. 1), which does not coincide with a preferred binding site for the drugs studied here.

Elsamicin A changes the affinity of Sp1 protein for the P1 promoter while it can either inhibit or enhance the transcription from P2 *c-myc* promoter. To detect the effect of actinomycin D and elsamicin A on the transcription activity from the P1 and P2 promoters of *c-myc* *in vitro*, we undertook a run-off transcription assay using the pGEMmyc1 plasmid (see Materials and Methods). P1 and P2 transcripts (348 and 187 nucleotides) are clearly visible in the control (no drug added, Fig. 8, lanes 2 and 3). Lane 3 shows the result of the transcription using HeLa extract plus added Sp1 protein (25 ng) to check the effect of elsamicin A and actinomycin D on its binding through the indirect effect on transcription of the *c-myc* promoter by whole HeLa extract. Lane 3 shows that adding pure Sp1 to a HeLa extract (which already contains Sp1) produced an enhancement of the amount of transcript from P1 but not from P2 promoter, as would be expected from the relative location of the Sp1 bind-

Fig. 9. Quantitative analysis of the effect of elsamicin A (ELSA) or actinomycin D (ACTD) on transcription from the *c-myc* promoter. (□) P1 promoter; (■) P2 promoter. The bars correspond to the relative amount of transcript obtained in comparison to the basal transcription (lane 2 in Fig. 8), and considering 100% for the *c-myc* transcription from P1. Sp1 produces a clear activation of its proximal promoter (P1, see Fig. 1) but not P2. The densitometric profiles used to generate the plot were corrected for any gel loading artifact using the band (marker) indicated by an asterisk in Fig. 8. The transcripts obtained in the presence of 1 μ M actinomycin D (lane 5 in Fig. 8) were not considered in this quantitative analysis since the sample was deemed to be unevenly loaded.

ing site in the *c-myc* promoter (see Fig. 1). Actinomycin D strongly inhibited the transcription from P1 at all the concentrations tested (Fig. 8, lanes 4–6) while transcription from the P2 remains almost unaltered (lane 5 in Fig. 8 should not be taken in consideration at this point since it seems to show an unevenly loaded sample). In the presence of 0.5 μ M elsamicin, the transcription was active from both the P1 and the P2 start sites (lane 7). From P1 it even appeared to be fairly enhanced at concentrations around 1 μ M. Higher concentrations of elsamicin were required to completely inhibit transcription from P1. Fig. 9 shows a quantitative analysis of the transcription from P1 and P2 promoters in which the amount of transcription has been normalized using a standard for recovery and loading (indicated with an asterisk in Fig. 8). The P2 transcript was clearly enhanced in the presence of 5 μ M elsamicin while transcription from P1 was strongly inhibited (Figs 8 and 9). These results are consistent with the general inhibition, by actinomycin D, of *c-myc* transcription from the P1 promoter in a concentration-dependent manner. They also agree with the relative increment of Sp1 binding in the presence of some concentrations of elsamicin A, described in the band-shift assays and the correct assembly of the initiation complex on the P1 promoter at low concentrations of elsamicin (1 μ M). These might result in the maintenance of transcription levels and a slight increase in the yield of total transcript formed from the P1 start site (see the quantitative analysis in Fig. 9) but not from the P2 start site, which is further from the Sp1 binding site than the P1 promoter [2]. Surprisingly, once P1 is inhibited (Fig. 8, lane 9) there is a manifest increase in transcript yield from the P2 promoter. Tentatively, we suggest that this situation arises from a redistribution of some basal *trans*-acting factors due to the stronger binding of the drug in a region close to P1. Some effects of drugs in the relative formation of multimeric protein complexes have been described, as for example some intercalators that affect the interaction between a growth response factor and the TATA-box binding protein [25].

DISCUSSION

While, in general, the major groove of DNA is recognized by many, but not all, *trans*-acting protein factors, the minor groove is employed by several drugs, including many intercalating agents which are known to exert antitumor activity. The experimental approaches presented in this article indicate that two such intercalators, actinomycin D and elsamicin A, might induce a specific release of some proteins which selectively bind to the human *c-myc* promoter. Hence, it is feasible to inhibit the binding of regulatory proteins to DNA using small ligands [26, 27]. This effect may be responsible for the differential modulation of the transcription of a particular gene by intercalating agents (*c-myc* should be considered an example).

By exploring the band-shift assays (Figs 2 to 6) and the DNase I footprinting (Fig. 7), we can observe several DNA-protein complexes that can be completely inhibited or/and dissociated. The results presented in this article accord with the view that low-molecular-mass ligands are able to displace proteins bound to DNA [25–28]. A possible explanation is that the competition observed is caused by a change in the local DNA conformation. This may be mediated by the unwinding of the polynucleotide induced by the intercalation. Nevertheless, it is worth noting that minor-groove binding ligands, which are considered to distort DNA to a lesser extent, can also inhibit the formation of DNA-protein complexes [22, 28].

Experiments using pure Sp1, and either the P1 promoter or a consensus oligonucleotide, indicate that actinomycin D and elsamicin A overlap the Sp1 binding site, thus preventing protein binding (at any actinomycin concentration and relatively high concentrations of elsamicin). Under certain concentration-dependent conditions, the protein binding to the preferred sequence is maintained and sometimes it appears to be relatively enhanced in the vicinity of the P1 promoter. This enhancement is not observed when Sp1 is bound to a synthetic oligonucleotide that lacks the flanking sequences in the *c-myc* promoter. Since DNase I footprinting establishes that both drugs bind preferentially to C+G-rich regions in DNA [10, 11] including the *c-myc* promoters (see Fig. 7), the relative enhancement in the Sp1-DNA complex in Figs 3 and 6 (in the presence of some concentrations of elsamicin, but not in the presence of any concentration of actinomycin) deserves further discussion. We tentatively consider that elsamicin A intercalates at DNA regions located within or in the vicinity of the Sp1 binding site using the unoccupied minor groove and changing DNA to a more-favored conformation for Sp1 binding, while actinomycin might occupy all the C+G-rich regions and perhaps other weaker binding sites [14], thus making some of the DNA-protein complexes more sensitive than others. The binding of elsamicin is somewhat driven by the flanking sequences since the phenomenon is not observed on the consensus oligonucleotide (Figs. 5 and 6). The conformational change produced by elsamicin binding could increase the affinity constant of the Sp1 protein for its consensus sequence. In the P1 promoter of *c-myc* there are two overlapping sequences with eight bases identical with the consensus sequence [2, 18]. The ability of elsamicin A to form a ternary complex, with Sp1 and the P1 promoter DNA, may lead to the stability of the protein-DNA complex at moderate drug concentrations, but not at higher ones. The run-off transcription experiments (Figs 8 and 9) agree with such an interpretation. There was a subtle sustenance of the amount of transcript from P1 in the presence of low elsamicin concentration, but not of actinomycin. In any case, the yield of transcript from P1 was below the amounts obtained without any drug. Additional experiments, using other promoters, will be required to check whether this behavior is widely observable or due only to the peculiar charac-

teristics of the *c-myc* P1 promoter or elsamicin A. However, since we added some pure Sp1 in the run-off experiments, it was unambiguously established that the changes in the yield of RNA from P1 were intimately related to the levels of Sp1 binding to the P1 promoter region (see the quantitative analysis in Fig. 9). At the same time, there was a general inhibition of the transcription due to the binding of the drugs to other C+G-rich regions. Several lines of evidence also show that elsamicin and actinomycin might behave differentially in transcription from phage genes while still acting as transcription inhibitors [16]. The effects of actinomycin and elsamicin are not due to their direct interaction with the Sp1 protein alone, as indicated by incubating drug and protein together first (details not reported).

In the run-off transcription experiments using human *c-myc* (Fig. 8), a slight increment of transcripts from P1 at 1 μ M elsamicin is evident (after inhibition at lower concentrations), in agreement with the effect mediated through Sp1 recognition of its consensus sequence observed in the band-shift assays. Nevertheless, the drug somewhat retains the transcription to a level similar to that the transcription obtained using HeLa extract alone, but without reaching the amounts for the HeLa+Sp1 (lanes 2 and 3 in Fig. 8). It is likely that the binding of elsamicin to other C+G-rich sites in the promoter acts as an inhibitor of transcription, as observed with actinomycin (Fig. 8, lanes 4–6). Hence, the effect on Sp1 binding is partially masked, yet it is quantitatively evident in Fig. 9. The abilities of small ligands to both augment and arrest transcription by RNA polymerase II in a concentration-dependent fashion does not seem to be a peculiarity of the drugs and/or promoters used in this article, since the ability of some intercalating drugs as well as minor groove binders to enhance and/or impede RNA elongation has been described elsewhere [19, 28, 29]. Notwithstanding, with elsamicin the inhibition of the transcription from P1 was concomitant with the increase in transcript yield from P2, which might be explained by considering that the some protein factors released from the P1 promoter bind to the P2 promoter, thus activating the initiation from the P2 start site (Fig. 8, lane 9, and Fig. 9). Nevertheless, our results point out the occurrence of this dual effect in the transcription of an oncogene in the presence of clinically useful drugs under physiologically relevant concentrations.

So far as our results go, it seems likely that the concentration-dependent response of *c-myc* promoter to elsamicin A is mediated by changes in the affinity of Sp1. The concentrations of elsamicin required to observe the relative enhancement of protein binding (Fig. 3) or to increase the transcription yield (Fig. 8) are fairly different. They possibly reflect the distinct experimental conditions in the band-shift and run-off transcription experiments. RNA polymerase II needs to read through natural pause sites, in which the polymerase proceeds slowly [7, 30]. Elsamicin might act by allowing these pauses to be overridden. This explanation is consistent with the role assigned to the levels of Sp1 in eliminating the promoter proximal pausing of RNA polymerase in *c-myc* [7]. A direct link between the capacity of elsamicin to modulate *c-myc* expression and to influence biological processes will require further experiments *in vivo*. The results described here represent a new step towards the design of such experiments.

We are thankful to Dr J. Bernués for helpful discussions and Dr R. Ray (University of St Louis) for a gift of pGEMmyc1. Elsamicin A was kindly provided by Dr A. M. Casazza (Bristol-Myers Squibb). This work was financed by grants from the Spanish *Dir. Gen. Enseñanza Superior* (PB 95-0064) and *Dir. Gen. de Investigación Científica y Técnica* (PB93/102). The support of the *Generalitat de Catalunya* through its *Centre de Referencia en Biotecnologia* is also acknowledged.

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