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Targeting of the Sp1 Binding Sites of HIV-1 Long Terminal Repeat with Chromomycin

DISRUPTION OF NUCLEAR FACTOR • DNA COMPLEXES AND INHIBITION OF *IN VITRO* TRANSCRIPTION

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ABSTRACT. Sequence selectivity of DNA-binding drugs has recently been reported in a number of studies employing footprinting and gel retardation approaches. In this paper, we studied the biochemical effects of the sequence-selective binding of chromomycin to the long terminal repeat of the human immunodeficiency type I virus. Deoxyribonuclease I (E.C.3.1.21.1) footprinting, arrested polymerase chain reaction, gel retardation and *in vitro* transcription experiments have demonstrated that chromomycin preferentially interacts with the binding sites of the promoter-specific transcription factor Sp1. Accordingly, interactions between nuclear proteins and Sp1 binding sites are inhibited by chromomycin, and this effect leads to a sharp inhibition of *in vitro* transcription. BIOCHEM PHARMACOL 52;10:1489–1498, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. DNA-binding drugs; HIV-1; chromomycin; transcription factors; DNase I footprinting; *in vitro* transcription

Both constitutive and tissue-specific regulation of gene expression is operated on the transcriptional level by the interaction between nuclear proteins (transcription factors) and promoter regions containing DNA elements (transcription signals) that exhibit specific nucleotide sequences [1–5]. Recent reviews dealing with both nucleotide sequences of transcription signals and the relative binding proteins are available [2, 6–8].

DNA-binding drugs displaying sequence selectivity [9] can exhibit differential effects on the interactions between DNA and different DNA-binding proteins, including TFIID†/TBP, EBNA-1, Oct-I, NIL2α, EGR1, WT1, NFE-1 and Sp1 [10–20]. Therefore, sequence-selective DNA-binding drugs could be considered as potential anticancer and antiviral drugs [9, 21]. In the light of these considerations, a large number of investigations on sequence selectivity of DNA-binding drugs have recently been published, most of them employing footprinting experiments and gel retardation studies [21–30]. A+T selective DNA-binding drugs, including distamycin and distamycin analogues, inhibit the interaction between DNA and TF-IID, Oct-I and EBNA-1 [10, 13, 16]; these tools are ineffective on Sp1/DNA interactions [13]. By contrast, mithramycin preferentially binds to G+C rich sequences and inhibits the interaction between Sp1 and the Sp1 binding sites of SV40 and c-myc promoters [19, 20]. In this respect, mithramycin and structurally related minor groove ligands could be of great interest as potential antiviral agents, e.g. in the experimental therapy of AIDS. It has recently been demonstrated that transcription of HIV-1 depends on interactions between the cellular transcription factor Sp1 and three sites of the LTR [3, 31–34]. These results prompted us to study the effects of mithramycin and chromomycin on protein/DNA interaction and HIV-1 transcription. Mithramycin and chromomycin are good candidates for selective binding to the C+G rich regions of the HIV-1 LTR recognized by the transcription factor Sp1. To confirm sequence-selective binding to the HIV-1 LTR, both DNase I footprinting [13, 35] and arrested PCRs [36, 37] were performed.

The effects on the molecular interactions between nuclear factors isolated from human T-cell lines and oligonucleotides containing the Sp1 binding sites were studied by gel retardation [13, 38]. The effects on HIV-1 transcription were analysed using an *in vitro* transcription system [31]. In some experiments, distamycin was used as a control

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† *Abbreviations:* TFIID, transcription factor IID; NF-kB, nuclear factor kB; Sp1, promoter-specific transcription factor Sp1; NF-Y, nuclear factor Y; EBNA-1, Epstein-Barr nuclear antigen type 1 virus; TBP, TATA-binding protein; NIL2α, negative regulator of interleukin-2; EGR1, early growth response gene-1; WT1, Wilms tumor suppressor gene-1; Oct-I, octamer binding protein-1; NFE-1, nuclear erythroid-specific factor-1; ER, estrogen receptor; AIDS, acquired immunodeficiency syndrome; HIV-1, human immunodeficiency type 1 virus; LTR, long terminal repeat; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; DNase I, deoxyribonuclease I; EMSA, electrophoretic mobility shift assay; TFO, triple helix-forming oligonucleotide.

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because this DNA-binding drug exhibits, unlike chromomycin and mithramycin, prefer A+T-rich DNA sequences [13].

MATERIALS AND METHODS

DNA-Binding Drugs

Distamycin, mithramycin and chromomycin were obtained from Sigma (St. Louis, Missouri, USA). Stock solutions of distamycin (9.6 mM), mithramycin (0.5 mM) and chromomycin (0.84 mM) were each stored at −20°C in the dark and diluted immediately before use.

Target DNA, Oligonucleotide Primers and PCR

The locations of the primers (HIV-1-F, HIV-1-R, HIV-1-R1 and HIV-1-R2) used for PCR [38, 39] are reported in Fig. 1. The nucleotide sequences of the primers (purchased from Pharmacia, Uppsala, Sweden) were HIV-1-F, 5′-ATTTCATCACATGGCCCGAG-3′; HIV-1-R, 5′-CAGGATCTGAGGGCTCGCC-3′; HIV-1-R1, 5′-AGGCTCAGATCTGGTCTAACCAG-3′; and HIV-1-R2, 5′-AGGCAAGCTTTATTGAGGCT-3′. For the PCR-mediated amplification of the 5′ region of the HIV-1 LTR, the target DNA was the plasmid pT_ZIIICAT [13].

The nucleotide sequence of a 3.2-Kb genomic region located upstream of the ER sequence, originally designated exon 1, was investigated and described as containing a T+A-rich sequence with a $(TA)_{26}$ stretch located approximately 1.4-Kb upstream from exon 1 [40]. For the amplification of the 5′ region of the human ER gene, the target DNA was either the pBLCAT8ERCAT1 plasmid [37, 40] or human genomic DNA, and the primers used were 5′-GACGCATGATATACTTCACC-3′ and 5′-GCAGA-ATCAAATATCCAGATG-3′. To confirm specificity of ER PCR product, hybridization was performed with a [^{32}P]-labeled ER probe.

Taq DNA polymerase (DYNAZYME™, E.C.2.7.7.7, Finnzymes Oy, Espoo, Finland) was added at 2 U/25 μL final concentration. PCR conditions were denaturation, 92°C, 45 sec; annealing, 55°C (ER) and 60°C (HIV-1), 1 min; and elongation, 72°C, 1 min (30 cycles). Amplified DNA was analysed by electrophoresis on 2.5% agarose in TAE (0.04 M Tris-acetate, 0.001 M EDTA), 0.5 μg/mL ethidium bromide.

The effects of chromomycin, mithramycin and distamycin were analysed after incubating target DNA at room temperature for 5 min, with increasing amounts of DNA-binding drugs, as reported in the text, followed by PCR.

Preparation of [^{32}P]-End-Labelled HIV-1 Footprinting Probe

The 259-bp DNA fragment mimicking a region of the HIV-1 LTR was prepared by PCR [38, 39] by using 10 ng of the plasmid pT_ZIIICAT as template DNA [13] and 150 ng of HIV-1-F and HIV-1-R2 primers (see Fig. 1A for location of the primers within the HIV-1 LTR). For preparation of the footprinting probe, amplification by PCR was performed by using a [^{32}P]-labelled HIV-1-R2 PCR primer (Pharmacia). PCR was performed in 25 μL of 50 mM KCl, 10 mM Tris-HCl, pH 8.8, 2.5 mM $MgCl_2$ and 0.1% Triton X-100 by using 2 U/reaction of Taq DNA polymerase (DYNAZYME™). The PCR cycles were denaturation, 1 min, 94°C; annealing, 1 min, 60°C; and elongation, 1 min, 72°C. The [^{32}P]-labelled amplified fragment was analysed by electrophoresis on 2% agarose gel, purified by phenol-chloroform extraction, washed through Microcon 30 (Amicon Inc-Grace Company, Beverly, MA, USA) with 400 μL of water and resuspended in 100 μL of water.

PCR and Preparation of the Fluorescein-Labelled HIV-1 LTR Probe

For preparation of the nonradioactive footprinting probe, PCR amplification was performed by using fluorescein-end labelled HIV-1-R2 PCR primer. The 5′ fluorescein-end-labelled HIV-1-R2 primer was purchased from Pharmacia. PCR was performed as described for the preparation of the [^{32}P]-labelled amplified HIV-1 LTR footprinting probe. The amplified fragments were analysed by electrophoresis on 2% agarose gel and washed through Microcon 30 with 200 μL of water.

Preparation of Nuclear Factors from Jurkat T Cells

Nuclear extracts were purified as described elsewhere [41, 42]. T-lymphoid Jurkat cells were harvested from cell culture media by centrifugation for 10 min at 2000 rpm and the pelleted cells suspended in five volumes of 4°C phosphate buffered saline and collected by centrifugation. Subsequent steps were performed at 4°C. The cells were suspended in five packed cell pellet volumes of buffer A (buffer A: 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT) and allowed to stand for 10 min. The cells were collected by centrifugation and suspended in two packed pellet volumes of buffer A (volume prior to the initial wash with buffer A) and lysed by 15 strokes of a Kontes all-glass Dounce homogenizer (B type pestle) [42]. The homogenate was collected by centrifugation for 10 min at 3000 rpm to pellet nuclei. The pelleted nuclei were suspended in one packed cell pellet volume of buffer C (buffer C: 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 10 mM KCl and 0.5 mM DTT) and lysed by 15 strokes of a Kontes all-glass Dounce homogenizer (B-type pestle). The homogenate was mixed for 30 min at 4°C. The homogenate was collected by centrifugation for 30 min at 16,000 rpm and the supernatant dialysed 5–8 hr against 20 volumes of buffer D (buffer D: 20 mM

HEPES pH 7.9, 20% glycerol, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 0.1 M KCl and 0.5 mM DTT). The dialysed supernatant was collected by centrifugation for 10 min at 12,000 rpm to remove membrane residuals. The supernatant, designated the nuclear extract, was frozen as aliquots in liquid nitrogen and stored at −80°C.

Footprinting Reactions

Footprinting conditions were the same for both fluorescein-end-labelled and [^{32}P]-end-labelled DNA probes. Footprinting reactions were carried out in 50 μL containing [^{32}P]-end-labelled or fluorescein-labelled DNA, 5% glycerol, 20 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT and 0.01% Triton X-100. Fifty microliters of 10 mM $MgCl_2$, 5 mM $CaCl_2$ were added 1 min before the addition of DNase I. DNase I (E.C.3.1.21.1) was purchased from Promega (Madison, WI, USA) at 1 U/μL stock solution, stored in aliquots at −20°C and diluted in 10 mM Tris-HCl, pH 8, to working concentrations immediately before use. DNA was incubated with the DNA-binding drugs for 5 min at room temperature before the reaction with DNase I [13]. The footprinting reactions were blocked at room temperature by adding 90 μL of 200 mM NaCl, 30 mM EDTA, 1% sodium dodecyl sulfate and 100 μg/mL yeast RNA [13, 14]. Reactions were phenol-extracted and precipitated by adding 2.5 volumes of ethanol. The pellets were washed in 70% ethanol, air dried, resuspended in 3 μL of loading dye (96% formamide, 6 mg/mL dextran blue), denatured for 2 min at 90°C, ice-cooled for 1 min and layered onto a 6% polyacrylamide (acrylamide/bis-acrylamide ratio, 19:1), 7-M urea sequencing gel. Analysis of the nonradioactive footprinting reactions were performed by using the ALF Automated Sequencing System (Pharmacia). Control footprinting experiments were performed in the absence of DNA-binding drugs. Molecular weight markers were obtained by G+A sequencing reactions of the footprinting probes [13].

Electrophoretic Mobility Shift Assay

The EMSA [13, 41] was performed by using double-stranded synthetic oligonucleotides containing the target DNA sequences of transcription factor Sp1 [13] (5′-ATTCGATCGGGGCGGGGCGAGC-3′). The synthetic oligonucleotides were purchased from Promega and were 5′-end-labelled using [^{32}P]-γ-ATP. Binding reactions were set up as described elsewhere [13, 14] in binding buffer (10% glycerol, 0.05% NP-40, 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5 mM DTT) in the presence of the nonspecific competitor poly(dI:dC) · poly(dI:dC) (Pharmacia) (1 μg), 1 μg of crude nuclear extract isolated from the human T-lymphoid Jurkat cell line and 0.25 ng of labelled oligonucleotide, in a total volume of 25 μL. After 30-min binding at room temperature of protein factors to synthetic oligonucleotides, samples were electrophoresed at constant voltage (200 V for 1.5 hr) under low ionic strength conditions (0.35 × TBE buffer; 1 × TBE = 0.089 M Tris-borate, 0.002 M EDTA) on 6% polyacrylamide gels until the tracking dye (bromophenol blue) reached the end of a 16-cm slab. Gels were dried and exposed with intensifying screens at −80°C. Unless otherwise stated, addition of the reagents was as follows: (a) poly(dI:dC) · poly(dI:dC); (b) labelled Sp1 mer; (c) DNA-binding drug; (d) binding buffer; and (e) crude nuclear extracts.

In Vitro *Transcription*

In vitro transcription was performed by using an HeLa nuclear extract *in vitro* transcription system (Promega) [31]. The template DNA was generated from NcoI digestion of the pT_2IIICAT plasmid, containing the CAT gene under the control of the HIV-1 LTR. One hundred nanograms of HIV-1 LTR template was incubated in the absence or presence of chromomycin for 5 min at room temperature. After this binding reaction, transcription was initiated by adding 2 μL of HeLa nuclear extracts (6.3 mg/mL) in a total volume of 25 μL in a buffer containing 2 mM Hepes, pH 7.9, 10 mM KCl, 0.02 mM EDTA, 0.05 mM DTT, 2% glycerol, 1 mM ATP, CTP and UTP, and 0.4 mM GTP in the presence of [^{32}P]-α-GTP. After 60 min, transcription was terminated by adding 175 μL of 0.3 M Tris-HCl, pH 7.4, 0.3 M sodium acetate, 0.5% SDS, 2 mM EDTA, and 3 μg/mL of yeast tRNA; the transcription reaction was then phenol-chloroform extracted, precipitated with 500 μL of 100% ethanol and the transcription products analysed by electrophoresis in 6% acrylamide, 7-M urea and TBE 1× buffer. To show the requirement of Sp1/DNA interactions, HIV-1-LTR-directed transcription was inhibited in control experiments by adding a triple helix forming oligonucleotide (HIV-1/Sp1-TFO, 5′TGGGTGGGGTGGGGTGG-GGGGTGTGGGGTGTGGGGTG-3′) [31] and an excess of cold HIV-1-F/HIV-1-R1 PCR product (80 ng/reaction).

RESULTS

Chromomycin Inhibits PCR-Mediated Amplification of HIV-1 LTRs Sequences Containing the Sp1 Binding Sites

The location of the two PCR primers (HIV-1-F and HIV-1-R) used in this experiment are shown in Fig. 1A. The results of the arrested PCR are shown in Fig. 1B–E. In this experiment, the pT_2IIICAT plasmid was used as target DNA (10 ng/reaction). In the presence of increasing amounts of chromomycin, mithramycin and distamycin, a clear differential effect of these DNA-binding drugs on PCR-mediated amplification of the HIV-1 LTR region containing the Sp1 binding sites was observed. When the effects of chromomycin and mithramycin are compared (Fig. 1B, C), it is evident that suppression of generation of HIV-1 PCR products occurred in the presence of 8–10 μM chromomycin (Fig. 1B), whereas the mithramycin concen-

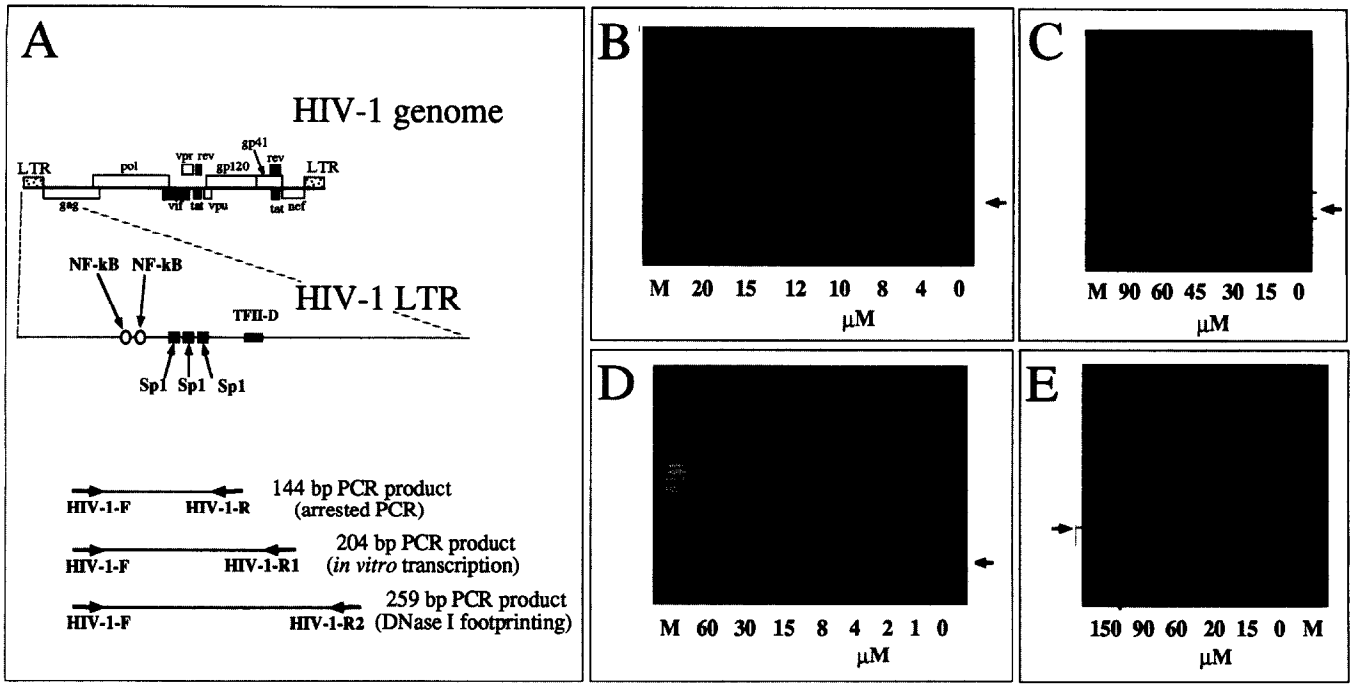

FIG. 1. (A) Structure of the HIV-1 genome (upper part of the panel) and location of the primers used (arrows) for chromomycin-mediated arrested PCR, competition in the *in vitro* transcription and DNase I footprinting. The location of the binding sites of the nuclear factor kB (open circles), the promoter-specific transcription factor Sp1 (black boxes) and the transcription factor IID (TF-IID) (gray box) is indicated. Effects of chromomycin (B,E), mithramycin (C) and distamycin (D) on PCR-mediated amplification of the HIV-1 LTR region containing the Sp1 binding sites (B–D) and the 5′ region of human estrogen receptor gene (E). PCR was performed in the presence of the indicated concentrations of DNA-binding drugs by using the $pT_zIIICAT$ plasmid (B–D) or the pBLCAT8ERCAT1 plasmid (E) as target DNA. For PCR-mediated amplification of HIV-1 LTR, the oligonucleotides HIV-1-F and HIV-1-R were used as PCR primers. M, molecular weight marker (Hae III-restricted pBR322 DNA). The HIV-1 (B–D) and the ER (E) PCR products are indicated by arrows.

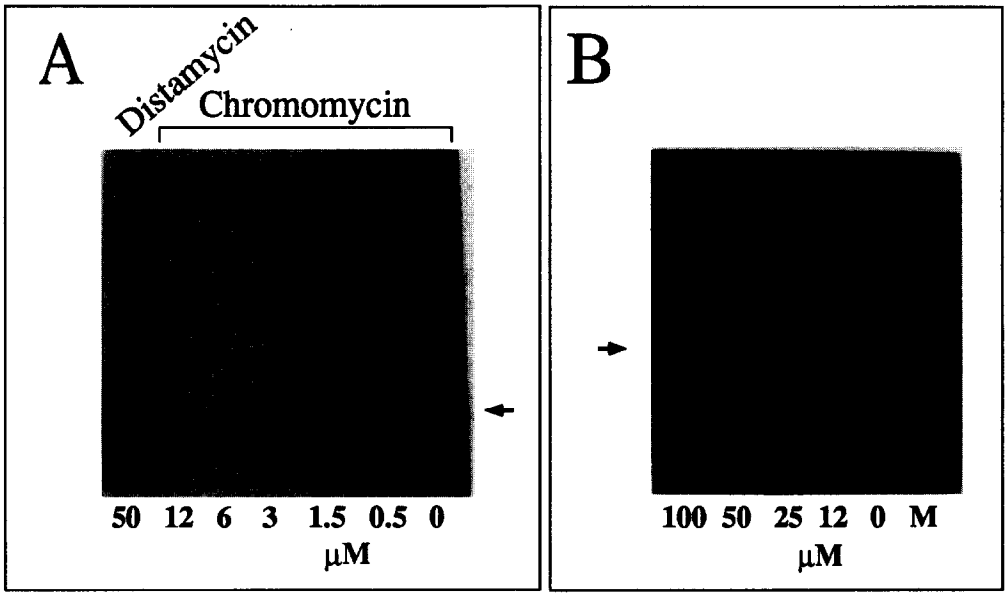

FIG. 2. Effects of chromomycin (A, B) and distamycin (A) on PCR-mediated amplification of the HIV-1 LTR region containing the Sp1 binding sites (A) and the 5′ region of human estrogen receptor gene (B). PCR was performed in the presence of the indicated concentrations of DNA-binding drugs by using human genomic DNA as target DNA. The HIV-1 PCR products were Southern blotted and hybridised with a [^{32}P]-labelled Sp1 oligonucleotide to demonstrate specificity of PCR amplification from genomic DNA. For PCR-mediated amplification of HIV-1 LTR, the oligonucleotides HIV-1-F and HIV-1-R were used as PCR primers. M, molecular weight marker (Hae III-restricted pBR322 DNA). The HIV-1 (A) and the ER (B) PCR products are indicated by arrows.

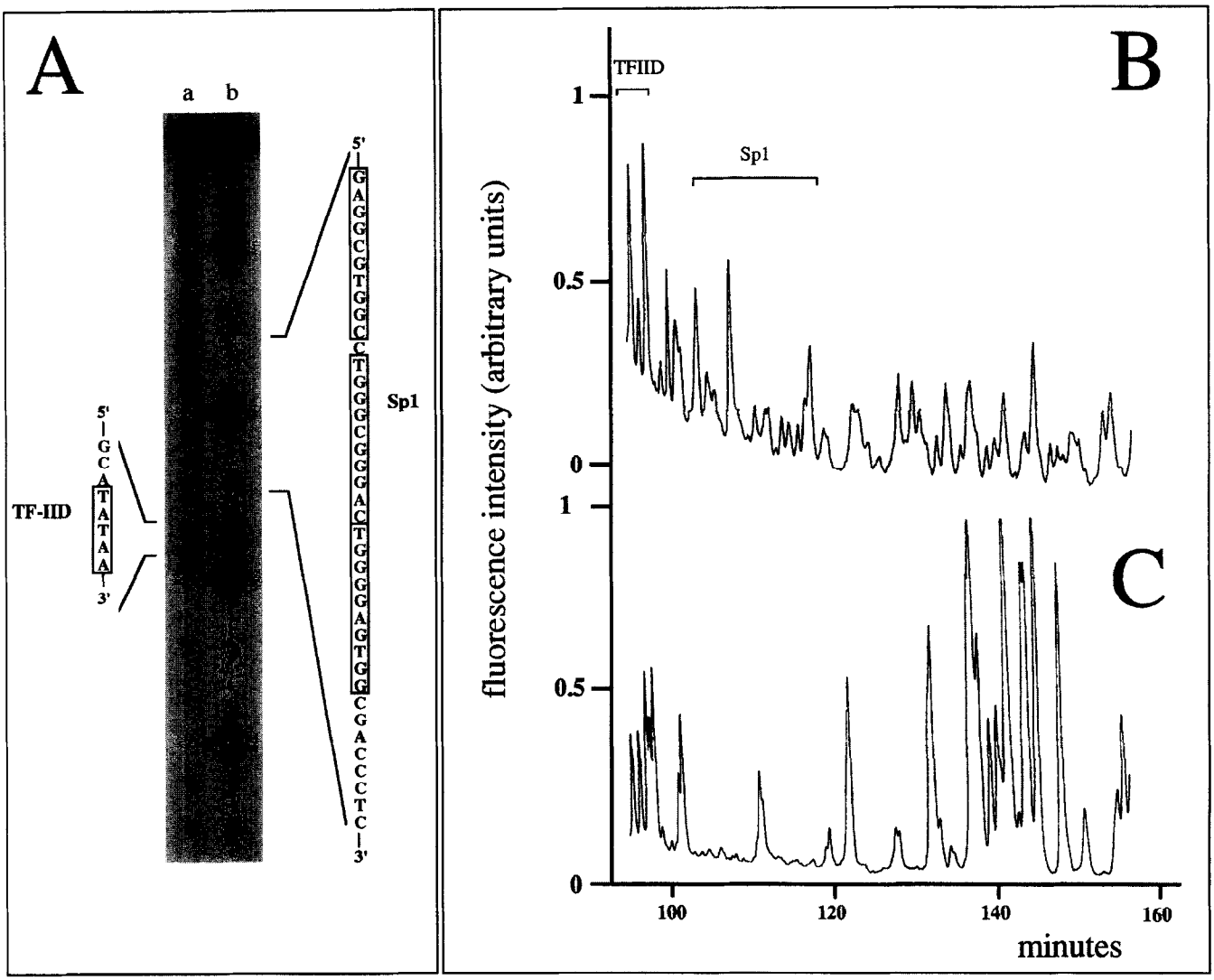

FIG. 3. Effects of chromomycin on DNase I footprinting. The nucleotide sequences relative to the peaks corresponding to the binding sites of Sp1 and TFIID have been deduced from G+A Maxam-Gilbert sequencing reactions and are indicated. Footprinting reactions were carried out in the absence (A, lane a; B) or in the presence (A, lane b; C) of 12 μM chromomycin. The DNase I-generated fragments corresponding to TFIID and Sp1 binding sites are indicated in A and B. (A) A radioactive experiment employing [^{32}P]-labelled oligonucleotide as PCR primer to generate the HIV-1 footprinting probe is shown. (B, C) A nonradioactive experiment employing the ALF-DNA Sequencing System (Pharmacia). B, control DNase I cleavage; C, DNase I footprinting pattern generated by 12 μM chromomycin.

tration needed to reach suppression of PCR-mediated amplification was greater than 30 μM (Fig. 1C). By contrast, no inhibition by distamycin treatment was observed even when the DNA-binding drug was added at a final concentration of 60 μM (Fig. 1D). Interestingly, distamycin binds to A+T-rich DNA sequences, whereas chromomycin and mithramycin bind to G+C-rich DNA sequences [13, 24–27]. These results are in agreement with previous observations that indicate a differential effect of distamycin, mithramycin and chromomycin on PCR-mediated amplification of A+T-rich ER and G+C-rich Ha-ras gene sequences [36, 37, 40]. In addition, Fig. 1E demonstrates that 15 and 90 μM chromomycin did not lead to inhibition of PCR-mediated amplification of A+T-rich human ER sequences, whereas only a partial inhibition of PCR was obtained in the presence of 150 μM chromomycin. This result clearly indicates that the inhibitory effects of chromomycin on HIV-1 PCR are related to the nucleotide sequences of the PCR products rather than to a simple inhibition of enzyme function (Fig. 1D, E). By sharp contrast, treatment with 15–150 μM distamycin leads to a sharp inhibition of A+T-rich PCR products, such as those generated by PCR-mediated amplification of the human ER gene [37, 40]. These results suggest that chromomycin displays the highest activity for sequence-driven inhibition of the generation of HIV-1 PCR products containing the three Sp1 binding sites. Accordingly, chromomycin was selected for further studies employing PCR on human genomic DNA, DNase I

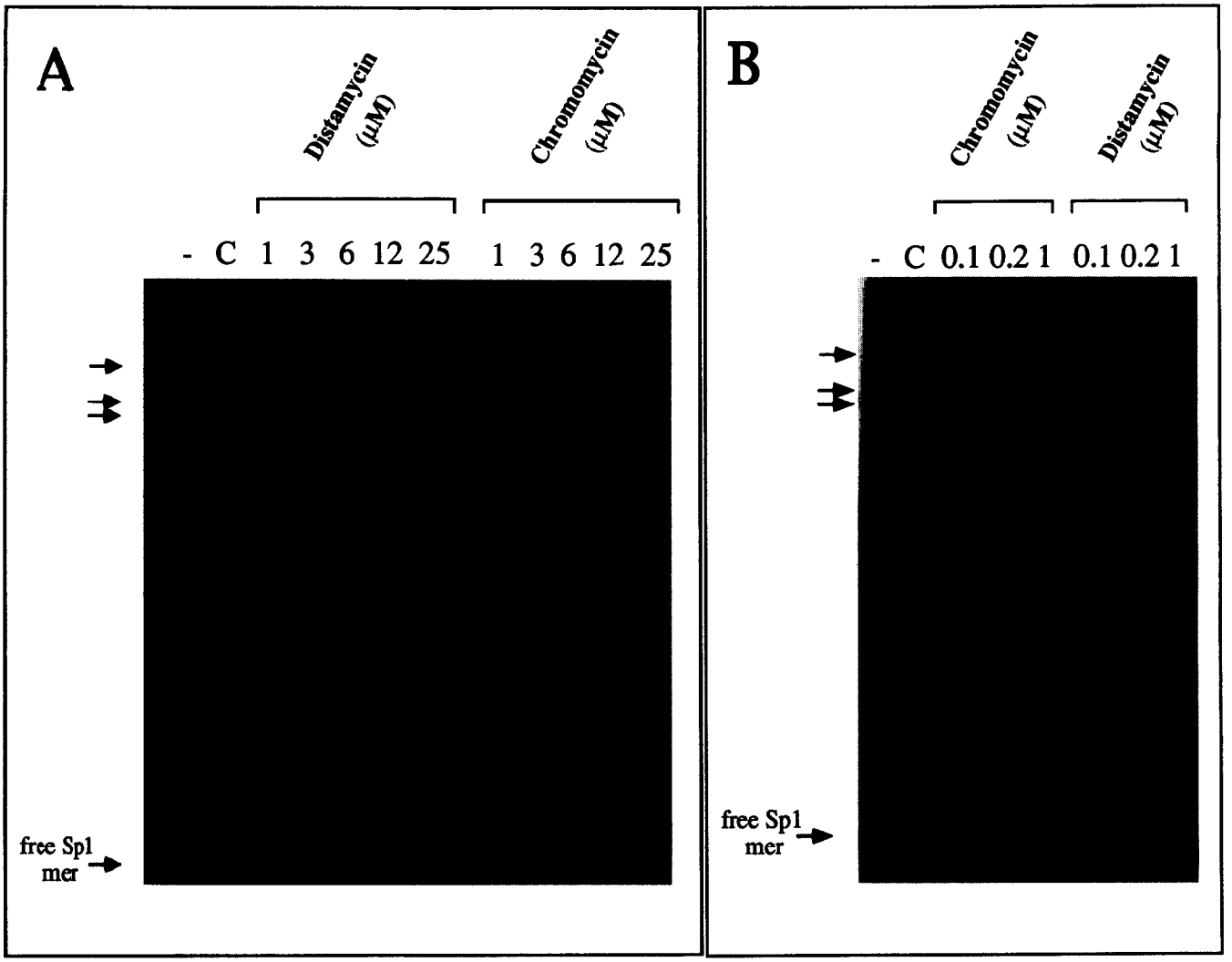

FIG. 4. Effects of chromomycin and distamycin on interactions between nuclear factors from Jurkat cells and the [^{32}P]-labelled Sp1 mer. The effects of high (1–25 μM) and low (0.1–1 μM) concentrations of DNA-binding drugs are shown in A and B, respectively. Protein–DNA complexes are indicated by arrows in the upper part of the panels. The free [^{32}P]-labelled Sp1 mer is also indicated. Binding reactions performed in the absence of DNA-binding drugs have been layered on lane C. -, electrophoretic migration of [^{32}P]-labelled Sp1 mer after incubation in the absence of both nuclear factors and DNA-binding drugs.

footprinting, gel retardation assays and *in vitro* transcription.

Figure 2 shows that chromomycin inhibited amplification of HIV-1 LTR sequences containing the Sp1 binding sites even when human genomic DNA was used as target DNA (Fig. 2A). Here, genomic DNA from the human T-cell line H938, containing an integrated HIV-1 LTR, was used. Figure 2A shows that inhibition of PCR-generated amplification of HIV-1 LTR sequences was obtained with 3 μM chromomycin. To demonstrate conclusively the specificity of the PCR product, the PCR-amplified DNA was Southern blotted and hybridized with a [^{32}P]-labelled Sp1 oligonucleotide. Figure 2B demonstrates a lack of suppression of PCR-mediated amplification of the ER A+T-rich genomic sequences even in the presence of 100 μM chromomycin. The specificity of the human ER PCR product has been established [40].

DNase I Footprinting Reactions Suggest That Chromomycin Interacts with the Sp1 Binding Sites of the HIV-1 LTR

In a second set of experiments, the [^{32}P]-labelled HIV-1 footprinting probe (the HIV-1-F/HIV-1-R2 259-bp PCR product; see Fig. 1A) was incubated in the presence of increasing amounts of chromomycin to determine whether this DNA-binding drug interacts with the Sp1 binding sites present within the HIV-1 LTR. After binding of chromomycin to the HIV-1 DNA, DNase I was added and the digestion products analysed on a sequencing gel. The results indicate that chromomycin bound to the 259-bp HIV-1 fragment in a sequence-specific manner (Fig. 3A). In particular, chromomycin protected a number of DNA stretches from DNase I cleavage, including those corresponding to the binding sites of the promoter-specific tran-

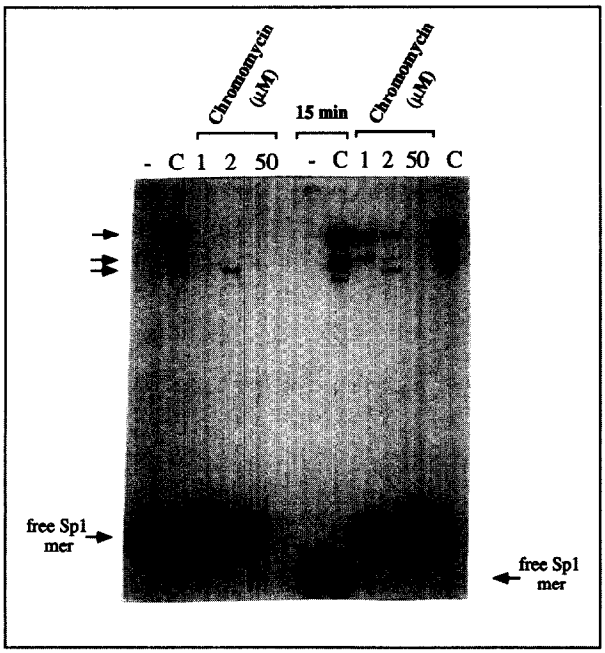

FIG. 5. Effects of chromomycin on preformed protein–DNA complexes. –, migration of the free [^{32}P]-labelled Sp1 mer; C, control (complexes generated in the absence of chromomycin). Left: Control experiments show the effects of the indicated concentrations (1–50 μM) of chromomycin on interactions between nuclear factors and the [^{32}P]-labelled Sp1 mer. In this experiment, DNA–protein binding was allowed to take place for 30 min. Right: Displacement by the indicated amounts (1–50 μM) of chromomycin of preformed protein–DNA complexes generated after 15 min of binding. After 15 min of binding of nuclear factors to the ^{32}P-labelled Sp1 mer, the reaction was divided into four aliquots: one was immediately electrophoresed (middle region of the panel, control binding, C, 15 min) and the others were incubated with chromomycin for an additional 15 min. The apparently faster migration of control samples in the middle of the panel is due to the fact that these two samples were immediately electrophoresed after 15 min of incubation. Nuclear factors were isolated from the human Jurkat cell line.

scription factor Sp1. By contrast, no protection was detected at the level of other HIV-1 LTR regions, including those corresponding to the binding sites of TF-IID (boxed in Fig. 3A and indicated in Fig. 3B) and NF-kB (corresponding to fragments present in the upper portion of the gel shown in Fig. 3A).

The nonradioactive automated footprinting study (Fig. 3B) confirmed these data. In this experiment, the 259-bp DNA fragment was prepared by PCR with a fluorescein-labelled HIV-1-R2 primer.

Chromomycin-Mediated Inhibition of Interactions Between Nuclear Factors and the Sp1 mer

When the [^{32}P]-labelled double-stranded oligonucleotide mimicking the Sp1 binding sites was mixed with nuclear factors from human Jurkat T-cells, three major retarded bands were observed (Fig. 4A, control lane C). These results are in line with the fact that Sp1 interacts with a variety of eukaryotic nuclear factors, including NFY, tat, NF-kB and p53 [3, 32–34]. Accordingly, these three retarded bands are likely to be different combinations of protein complexes interacting with the Sp1 binding sites. When the band-shift experiment was conducted in the presence of different amounts of chromomycin (0.1–25 μM), suppression of retarded bands was obtained (Fig. 4A, B), indicating chromomycin-mediated inhibition of the interactions between nuclear factors and Sp1 binding sites. By sharp contrast, distamycin was not able to suppress the binding of nuclear factors to the Sp1 mer when added at 6 μM. When distamycin was used at 12–25 μM, only inhibition of the faster migrating band was detectable. These results demonstrate that when these DNA-binding drugs were used at 1–6-μM concentrations only chromomycin inhibited the binding of nuclear factors to the Sp1 mer. It is interesting to note that inhibition of these protein–DNA interactions occurred at chromomycin concentrations that induce a complete supershift of the free Sp1 mer (Fig. 4B; 1 μM chromomycin), indicating that all the molecules of the [^{32}P]-labelled Sp1 oligonucleotide had been recognized by the DNA-binding ligand. These results were confirmed in four independent experiments. Similar results were obtained when recombinant Sp1 was used (data not shown). In addition, control experiments demonstrated that distamycin did not inhibit Sp1/DNA interactions between recombinant Sp1 and target DNA sequences even when added at 100 μM, being effective in inhibiting TFIID/DNA interactions when added at 25 μM (data not shown) [13].

Disruption of Preformed Protein/Sp1 mer Complexes by Chromomycin

A second set of experiments (Fig. 5) was performed to determine whether chromomycin was able to disrupt preformed complexes between nuclear factors and the [^{32}P]-labelled double-stranded Sp1 oligonucleotide. Before addition of the indicated concentrations of chromomycin, the [^{32}P]-labelled Sp1 mer was mixed with Jurkat nuclear factors for 15 min. Then the band-shift reactions were divided into five aliquots. The first was layered on the top of the electrophoretic gel and electrophoresis was performed; the others were treated without or with 1, 2 and 50 μM chromomycin and incubation was continued for a further 15 min. Control band-shift reactions done by adding chromomycin before nuclear extracts were also carried out as controls (left side of Fig. 5).

The results of this experiment clearly indicate that (a) after 15 min binding nuclear factors · DNA complexes were generated at a level similar to complexes produced in 30-min reactions (compare central lane C, 15 min, with lane C of the right side of the panel shown in Fig. 5) and (b) that inhibition of the interactions between nuclear factors and the Sp1 mer was achieved even if chromomycin was added 15 min after binding of nuclear extracts to the [^{32}P]-labelled double-stranded Sp1 oligonucleotide. Complete disruption

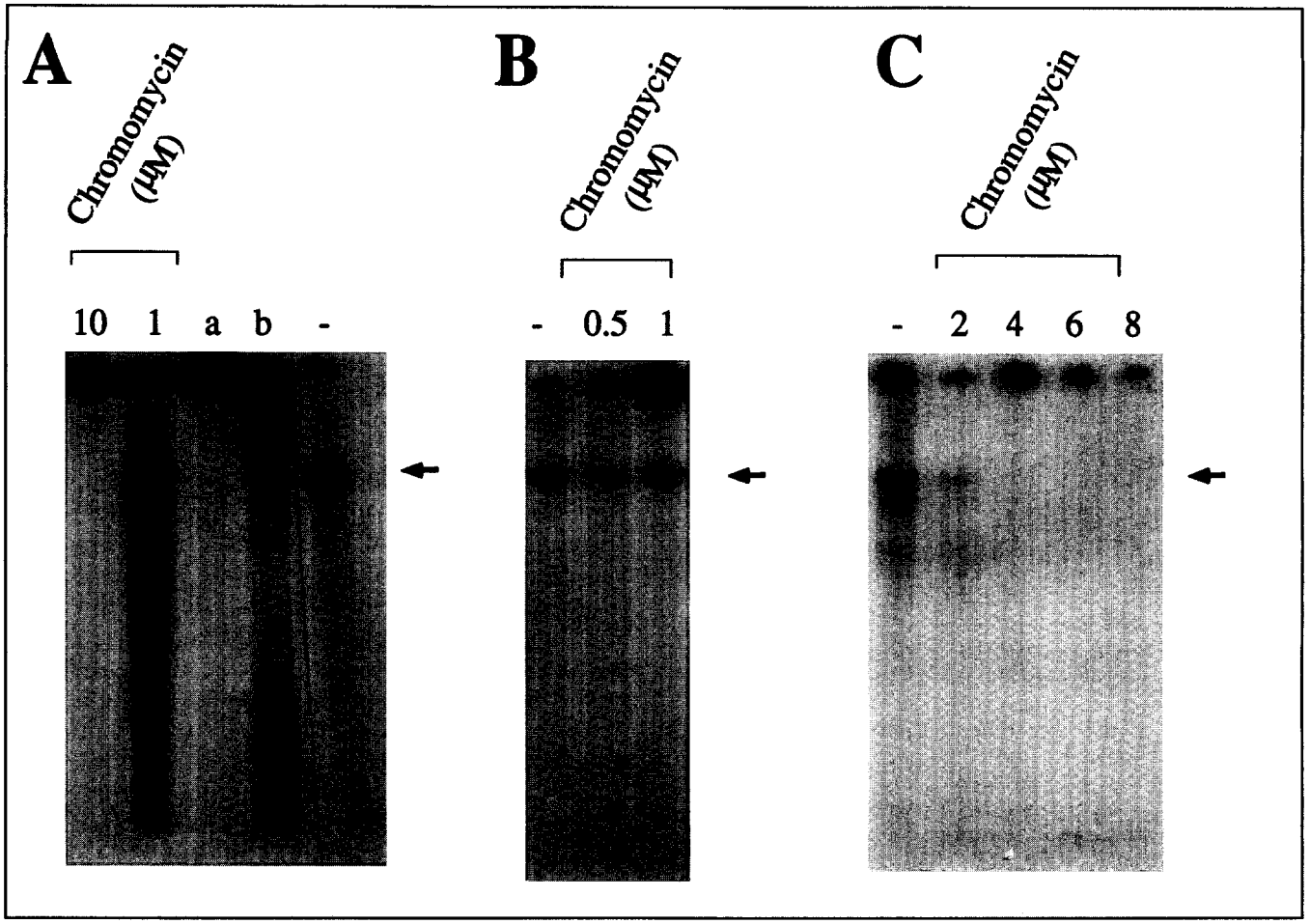

FIG. 6. Effects of chromomycin on HIV-1 *in vitro* transcription. In the three experiments reported, *in vitro* transcription was done in the absence (−) or in the presence of the indicated concentrations (A, 1 and 10 μM; B, 0.5 and 1 μM; C, 2–8 μM) of chromomycin. The *in vitro* transcription product is indicated by arrows. (A) Lane a shows the effects of the addition of 1 μg of the HIV-1/Sp1 TFO on transcription. Lane b shows effects of the addition of 80 ng of HIV-1-F/HIV-1-R1 PCR products on transcription.

of preformed protein/Sp1 mer complexes was achieved when chromomycin was added at 50 μM; at lower concentrations of chromomycin (1 and 2 μM), only partial disruption of preformed protein/Sp1 mer complexes was obtained (Fig. 5). This inhibition was more than 75%, as judged by scanning the autoradiograms through a BIO-RAD densitometer (data not shown).

Inhibition of protein–DNA interactions does not necessarily lead to transcription inhibition [42–44]. In the case of HIV-1, different members of the Sp1 multigene family (such as Sp3) exert opposite effects on LTR-directed transcription [43]. Therefore, an analysis of the effects of chromomycin on HIV-1 transcription was performed.

Effects of Chromomycin on HIV-1 LTR-Directed In Vitro *Transcription*

In vitro transcription was studied by using an HIV-1 LTR-directed *in vitro* transcription system and nuclear extracts from HeLa cells [31]. The $pT_ZIIICAT$ plasmid was cleaved with NcoI (Promega) to generate a DNA template able to produce a sizable RNA transcript of approximately 600 bp (arrows in Fig. 6). Preliminary experiments demonstrated that binding of Sp1 was required for transcription because 1 μg of a TFO recognizing the Sp1 binding sites was able to suppress transcription fully (Fig. 6A, lane a). Control experiments demonstrated that 80 ng of the HIV-1-F/HIV-1-R1 PCR products were fully able to suppress *in vitro* transcription directed by the HIV-1 LTR (Fig. 6A, lane b).

When *in vitro* HIV-1 transcription was performed in the presence of increasing amounts of chromomycin (0.5–10 μM), inhibitory effects were clearly detectable: 50% inhibition of the HIV-1 directed transcription was obtained at 2 μM chromomycin (Fig. 6C). Suppression of *in vitro* transcription was obtained at 4–10 μM chromomycin (Fig. 6A, C). By contrast, chromomycin was not able to reduce transcription when added at a concentration of 0.5 and 1 μM (Fig. 6A, B). Control experiments demonstrated that distamycin did not suppress HIV-LTR-directed transcription, even when added at a concentration of 10 μM (data not shown). In addition, chromomycin was found to be significantly less efficient in inhibiting *in vitro* transcription di-

rected by promoters lacking Sp1 binding sites (data not shown).

DISCUSSION

A number of recent reports have suggested that DNA-binding compounds might be used as antiviral and antitumor drugs because they modulate the formation of DNA/nuclear protein complexes (reviewed in [21]).

We [13–15] and others [10–12, 16–20] have reported that distamycin and chromomycin are strong inhibitors of the interaction between nuclear factors and target DNA sequences. Accordingly, these and related DNA-binding drugs have been reported to inhibit DNA synthesis and RNA transcription [19–21, 36, 37, 42].

The first conclusion that can be drawn from the experiments reported in the present paper is that chromomycin affects PCR-mediated amplification of gene regions and DNase I cleavage in a sequence-dependent manner. Chromomycin, but not distamycin, was found to (a) inhibit PCR-mediated amplification of the HIV-1 LTR region containing the G+C-rich sequences of the Sp1 binding site and (b) protect these gene regions from DNase I cleavage. In particular, we emphasise the results obtained by arrested PCR experiments performed on human genomic DNA, which suggest that chromomycin binds to the Sp1 binding sites of HIV-1 LTR integrated in the human genomic DNA. This result is of particular relevance because previous reports using TFOs have clearly demonstrated that synthetic compounds recognising the Sp1 binding sites of the HIV-1 LTR are potential inhibitors of HIV-1 replication in infected cells [31].

The second conclusion suggested by our paper is that chromomycin disrupts preformed complexes between nuclear factors and the Sp1 mer. Although the effects of the analogue mithramycin have been reported by others [19, 20], few data are available on disruption of preformed protein–DNA complexes. Our data suggest that this minor groove ligand is able to induce the displacement of nuclear factors (likely including Sp1 and/or factors belonging to the Sp1 family) to a synthetic oligonucleotide mimicking the Sp1 binding sites.

Inhibition of protein–DNA interactions might lead to different effects on transcription. Transcription inhibition could be obtained if the DNA-binding drug inhibits the interactions between promoters and transcriptional activators. By contrast, DNA-binding drugs could lead to an activation of transcription, especially when they inhibit the interactions between promoter regions and sequence-specific transcription repressors [30]. With respect to this point, transcription directed by the HIV-1 LTR depends on the activity of transcription factor Sp1 [31–33] (Fig. 6, lanes a and b). Accordingly, we demonstrated an inhibitory activity of chromomycin on HIV-1 transcription. Control experiments showed that (a) distamycin does not efficiently inhibit HIV-1-LTR-directed transcription and (b) chromomycin is significantly less efficient in inhibiting *in vitro* transcription directed by promoters lacking Sp1 binding sites (data not shown).

The results reported in the present paper have theoretical and practical implications. From the theoretical point of view, these data support the hypothesis that certain DNA-binding drugs might be sequence-selective modifiers of DNA/protein interactions, possibly leading to specific alterations of biological functions. From a practical point of view, our results suggest the possible use of chromomycin as a pharmacological tool to interfere with the biological functions of promoters (such as the HIV-1 LTR) whose activity is potentiated by interactions with Sp1.

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