Sequence-specific recognition of the HIV-1 long terminal repeat by distamycin: a DNAase I footprinting study

Giordana FERIOTTO,* Carlo MISCHIATI* and Roberto GAMBARI*†‡
*Biochemistry Institute, and †Biotechnology Center, Ferrara University, Via L. Borsari 46, 44100 Ferrara, Italy

Pharmacological modulation of the interaction between transcription factors and target DNA sequences of cellular and viral genes could have important effects in the experimental therapy of a large variety of human pathologies. For instance, alteration of the DNA/protein interaction might be among the molecular mechanisms of action of DNA-binding drugs, leading to an inhibition of the expression of genes involved in the control of *in vitro* and *in vivo* growth of neoplastic cells and virus DNA replication. Natural oligopeptides, such as distamycin, are powerful inhibitors of the interaction between nuclear factors and target DNA sequences and, therefore, have been proposed as compounds retaining antibiotic, antineoplastic and antiviral properties. In this study we performed DNAase I footprinting analysis using a PCR product mimicking a region of the long terminal repeat (LTR) of the human immunodeficiency type 1 (HIV-1) retrovirus. The data obtained suggest that distamycin binds to different regions of the HIV-1 LTR depending on the DNA sequence. Electrophoretic mobility shift assays using both crude nuclear extracts from the Jurkat T-lymphoid cell line and the recombinant proteins transcription factor IID and Sp1 suggest that distamycin differentially inhibits the interaction of these two proteins with their specific DNA target sequences, in good agreement with the results obtained by DNAase I footprinting analysis.

INTRODUCTION

The transcriptional regulation of the expression of both eukaryotic and viral genes is mediated by complex interactions of *trans*-regulatory proteins with target DNA elements exhibiting defined nucleotide sequences [1]. For instance, DNA-binding proteins belonging to the jun family recognize the TGAGTCA consensus sequence [2]; members of the Oct family bind to the ATTTGCAT octamer motif [3]; Sp1 recognizes the consensus CGGGGCGGGGC [4]; and AP2 binds to CCCAGGC [5]. Most of these and similar DNA elements are present in the promoter of cellular genes whose expression is strictly controlled at the transcriptional level [1,6–8]. Interactions between nuclear transcription factors and proximal promoter and/or enhancer elements of cellular genes is the major step controlling a large number of molecular processes involved in development, differentiation and progression of eukaryotic cells through the different phases of cell cycle [9,10]. In addition, it is well known that interactions between transcription factors and promoters are also involved in human pathologies, including neoplastic transformation and tumour progression to a metastatic phenotype [11].

Furthermore, the long terminal repeat (LTR) of retroviruses contains many DNA motifs recognized by transcription factors [12]. This has been suggested as one of the most important features of the organization of retrovirus genomes. A number of cellular nuclear proteins, together with virus-encoded transcription factors, are indeed known to play a crucial role in trans-activating retroviral genomes. For instance, in the LTR of the human immunodeficiency type I (HIV-1) retroviral genome are present DNA motifs that are recognized by a variety of eukaryotic transcription factors, such as nuclear factor (NF)-κB, Sp1, glucocorticoid receptor (GR), upstream stimulatory factor (USF), transcription factor IID (TFIID) and others (for a review see [12]).

From these considerations, it is clear that pharmacologically mediated modulation of DNA/nuclear protein complex formation could represent one promising approach to control expression of cellular genes of eukaryotic cells as well as viral infectivity [13–15]. With respect to this goal, DNA-binding drugs appear to be of great interest, because they could interfere with DNA/protein interactions [16–19]. In addition, DNA-binding drugs displaying sequence selectivity could exert differential effects on the binding between DNA and different transcription factors, depending on the sequences recognized by the proteins.

Natural oligopeptides, such as distamycin, are known to interfere with proteins capable of associating with DNA, such as restriction enzymes, topoisomerase II, DNA ligase, RNA polymerase, DNA polymerases and transcription factors [14,15,20–22]. The effects of distamycin on the interaction between nuclear transcription factors and target DNA sequences are supported by a number of reports describing gel retardation experiments employing crude nuclear extracts or purified proteins and target DNA sequences containing motifs recognized by different DNA-binding nuclear factors, including octamer transcription factor-1 (OTF-1) [15], nuclear factor erythroid-1 (NFE-1) [15], GTATA/interferon-γ [14], *antennapedia* homeodomain [20] and *fushi-tarazu* homeodomain [20].

In order to obtain more detailed information on the sequence-selectivity of the binding of distamycin to DNA, we have analysed the possible sequence-mediated interaction of distamycin with the HIV-1 LTR. In the present paper the sequence specificity of distamycin was analysed by DNAase I footprinting experiments and the effects of distamycin on DNA/protein interactions by means of gel retardation assays.

Abbreviations used: LTR, long terminal repeat; HIV-1, human immunodeficiency virus type 1; TFIID, transcription factor IID; NF-κB, nuclear factor κB.

‡ To whom correspondence should be addressed: Instituto di Chimica Biologica, Università di Ferrara, Via L. Borsari n. 46, 44100 Ferrara, Italy.

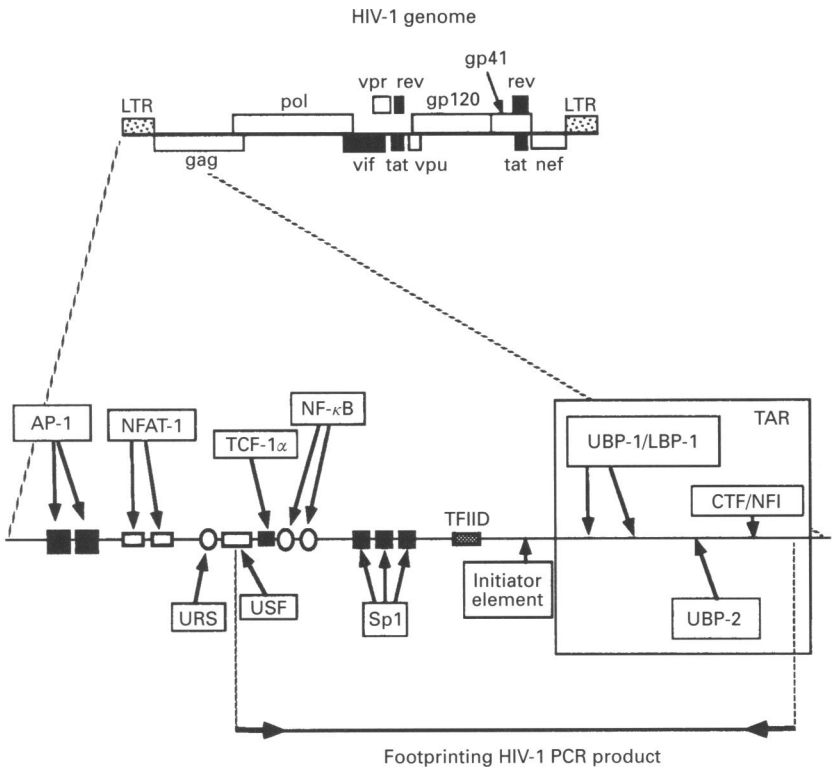

Figure 1 Structure of the HIV-1 genome

In the lower part of the Figure the LTR is shown, including the location of some sequences recognized by transcription factors. The location of the two primers used to generate the 259 bp HIV-1 LTR fragment used in the footprinting assays is also shown.

MATERIALS AND METHODS

Drugs and enzymes

Distamycin was obtained from Sigma. Doxorubicin was obtained from Menarini Laboratories (Pomezia, Italy). DNAase I was purchased from Promega (Madison, WI, U.S.A.) as a 1 unit/μl stock solution, stored in aliquots at -20 °C and diluted in 10 mM Tris/HCl, pH 8, to working concentration immediately before use.

PCR protocol

The 259 DNA fragment mimicking a region of the HIV-1 LTR was prepared by PCR [23,24] using as DNA template the DNA plasmid pT$_z$IIICAT. In each PCR reaction, 10 ng of DNA plasmid containing the HIV-1 LTR was amplified by *Taq*I polymerase using the two PCR primers whose location is depicted in Figure 1. The sequences of the primers were 5′-ATTTCAT-CACATGGCCCGAG-3′ (forward) and 5′-AGGCAAGCTTT-ATTGAGGCT-3′ (reverse). PCR was performed in 25 μl of 10 mM KCl, 10 mM Tris/HCl, pH 8.3, and 2.5 mM $MgCl_2$ by using 2 units of *Taq* polymerase (Perkin–Elmer)/reaction. The reverse primer was used after 5′-end-labelling with [γ-^{32}P]ATP in order to produce a PCR product suitable for DNAase I footprinting studies. The PCR cycles were as follows: denaturation, 1 min, 94 °C; annealing, 1 min, 60 °C; elongation, 1 min, 72 °C. The PCR-amplified HIV-1 LTR fragment was analysed by PAGE and purified with Microcon 30 (Amicon).

The amplified HIV-1 LTR region contains the DNA motifs for a variety of transcription factors, including TFIID, Sp1 and NF-κB (see Figure 1).

Footprinting assays

The experimental conditions for footprinting assays were as follows. Footprinting reactions were carried out in 50 μl containing 10000 c.p.m. of ^{32}P-end-labelled DNA, 5% glycerol, 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol and 0.01% Triton X-100; 50 μl of 10 mM $MgCl_2$ and 5 mM $CaCl_2$ was added 1 min before the addition of DNAase I. The footprinting reaction was blocked at room temperature by adding 90 μl of 200 mM NaCl, 30 mM EDTA, 1% SDS and 100 μg/ml yeast RNA. Reactions were phenol-extracted and precipitated by adding 2.5 vol. of ethanol. The pellets were re-suspended in 3 μl of loading dye, denatured for 2 min at 90 °C, ice-cooled and layered on to a 8% polyacrylamide/7 M urea sequencing gel. After electrophoresis, gels were vacuum-dried and exposed with Kodak X-Omat films. Maxam–Gilbert G+A sequencing reactions [25] were performed in 10 μl of TE buffer (10 mM Tris/HCl, pH 8, 1 mM EDTA), using 3.6 ng of ^{32}P-end-labelled DNA and 1 μg of calf thymus DNA; 1 μl of 4% formic acid, pH 2, was added and the reaction mixture was incubated for 25 min at 37 °C. After the addition of 150 μl of 1 M piperidine and a further incubation for 30 min at 90 °C, the reaction mixtures were extracted with 1 ml of butanol. The pellets were washed with 150 μl of 1% SDS and 1 ml of butanol. After two additional washes with butanol, the pellets were dried, re-

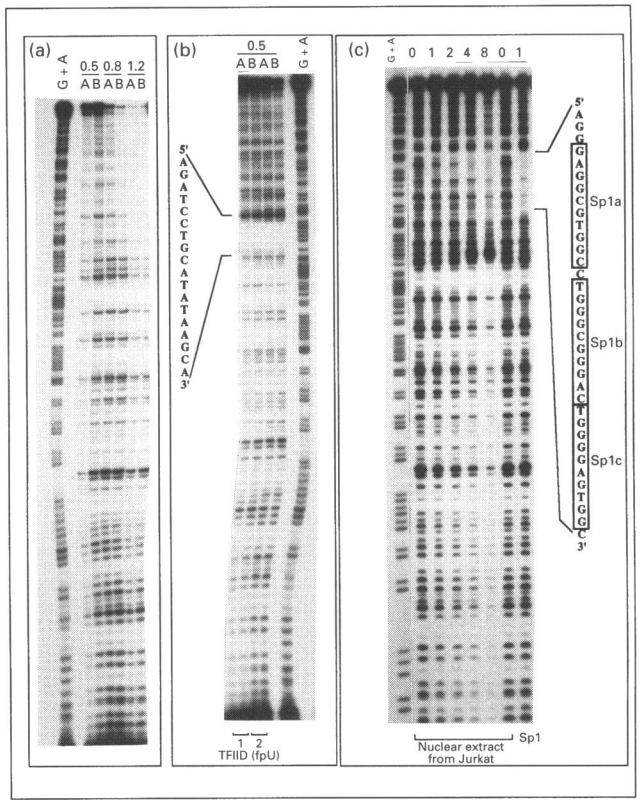

Figure 2 DNAase I footprinting of the HIV-1 LTR

(**a, b**) DNAase I footprinting patterns of the 259 bp HIV-1 LTR fragment. DNAase I was used at 0.5, 0.8 and 1.2 units/reaction, as indicated, for 1 (A) and 2 (B) min in the absence (**a**) or in the presence (**b**) of 1 or 2 fpU of recombinant TFIID. (**c**) DNAase I footprinting patterns generated by addition of 1–8 μg of crude nuclear extracts from Jurkat cells or 1 fpU of recombinant Sp1; 0 = footprinting patterns generated in the absence of DNA-binding proteins. Maxam–Gilbert G+A sequence reactions are shown on the sides of the panels.

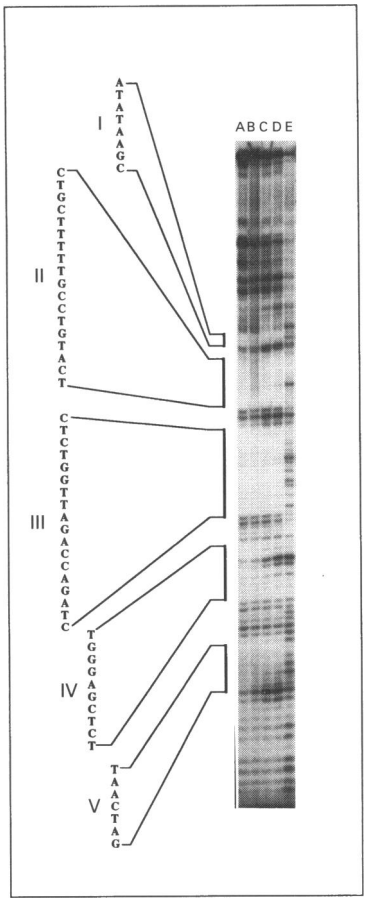

Figure 3 DNAase I footprinting patterns in the absence and in the presence of distamycin

Concentrations used: lane A, 25 μM; B, 12 μM; C, 6 μM; D, 3 μM; E, zero. In this experiment the 259 bp ^{32}P-labelled HIV-1 LTR fragment was incubated using the experimental conditions described at a DNAase I concentration of 0.5 unit/reaction for 1 min. I–V indicate the DNA sequences corresponding to the footprints generated by distamycin.

suspended in loading dye and analysed by electrophoresis on the 8% polyacrylamide/7 M urea sequencing gel.

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay [26,27] was performed by using double-stranded synthetic oligonucleotides containing the target DNA sequences of transcription factors TFIID [28] (5′-GCAGAGCATATAAGGTGAGGTAGGA-3′) and Sp1 [4] (5′-ATTCGATCGGGGCGGGGCGAGC-3′). The synthetic oligonucleotides were purchased from Promega and 5′-end-labelled using [γ-^{32}P]ATP. Binding reactions were set up as described elsewhere [28] in binding buffer (10% glycerol, 0.05% Nonidet P-40, 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.5 mM dithiothreitol), in the presence of poly(dI-dC)·poly(dI-dC) (Pharmacia, Uppsala, Sweden), 1 μg of crude nuclear extract from Jurkat cells [29] or 1 unit of recombinant factor (Sp1 or TFIID), and 0.25 ng of labelled oligonucleotide, in a total volume of 20 μl. Nuclear extracts were purified from Jurkat cells as described in detail elsewhere [30,31].

After 30 min of binding of protein factors to synthetic oligonucleotides at room temperature, samples were electrophoresed at constant voltage (300 V for 1 h) through a low ionic strength buffer (0.35 × TBE; 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 vacuum-dried and exposed with Kodak X-Omat films.

Addition of the reagents was as follows: (1) poly(dI-dC)·poly(dI-dC); (2) labelled TFIID or Sp1 mers; (3) DNA-binding drug; (4) binding buffer; (5) crude nuclear extracts or recombinant TFIID and Sp1 proteins. The recombinant Sp1 and TFIID proteins used in some of the band-shift experiments performed were purchased from Promega.

RESULTS

Effects of distamycin on DNAase I digestion of the HIV-1 LTR

For footprinting studies we have used a PCR-generated DNA fragment produced with two PCR oligonucleotide primers able to amplify a LTR HIV-1 region containing the DNA sequences recognized by a number of transcription factors, including USF, NF-κB, Sp1, TFIID, leader binding protein (LBP), untranslated binding protein-1 (UBP-1), CAAT-box transcription factor/nuclear factor I (CTF/NFI) and tat. The 259 bp HIV-1 LTR PCR-generated product (see Figure 1 for the structure of the HIV-1 genome and location of PCR primers) was digested with increasing amounts (0.5, 0.8 and 1.2 units/reaction) of DNAase I, which generated sizeable DNA fragments (Figure 2a). In the

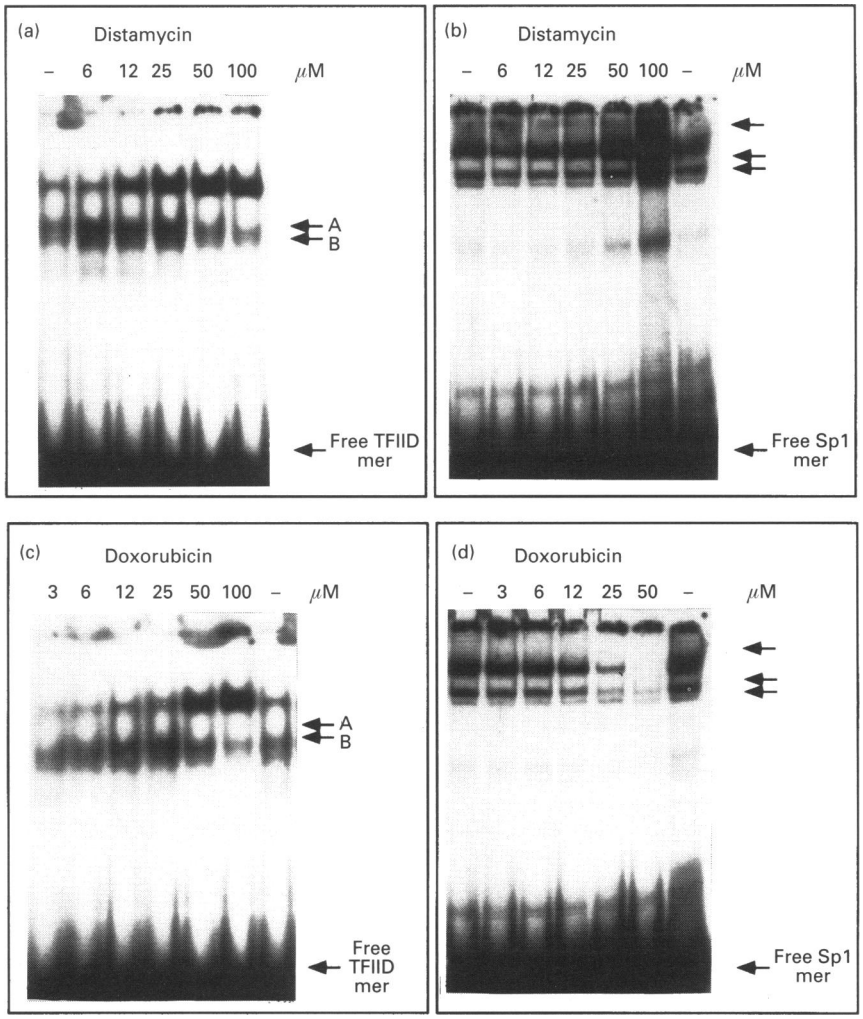

Figure 4 Effects of distamycin (a, b) and doxorubicin (c, d) on the binding of nuclear proteins from Jurkat cells to ^{32}P-labelled synthetic oligonucleotides containing the target sequences of the transcription factors TFIID and Sp1

The electrophoretic migration of the free TFIID and Sp1 mers is indicated. Retarded protein/DNA complexes of interest are arrowed in the upper part of each gel.

preliminary experiment shown in Figure 2(a), digestion with DNAase I was performed for 1 min and 2 min (lanes A and B respectively). G+A sequencing reactions [25] were routinely run in parallel to identify DNAase I-generated DNA fragments. Figures 2(a) and 2(b) show that the 259 bp HIV-1 PCR product is useful for determining possible inhibition of DNAase I digestion following DNA/protein interactions. For instance, when the HIV-1 PCR product was incubated with 1 and 2 fpU of the recombinant TFIID protein before DNAase I digestion (0.5 unit/reaction), a major footprint was generated (Figure 2b), corresponding to the DNA region containing the sequence 5′-ATATAA-3′ (the 'TATA box' of the HIV-1 LTR). Furthermore, Figure 2(c) shows that when nuclear extracts from the T-lymphoid cell line Jurkat (from 1 to 8 μg/reaction mixture) were preincubated with the ^{32}P-labelled 259 bp HIV-1 PCR product, a number of footprints were detectable after digestion with DNAase I and gel sequencing analysis, corresponding to DNA sequence targets of a number of cellular and viral transcription factors involved in the trans-activation of the HIV-1 genome (see Figure 1). As expected, a major footprint was detected, corresponding to DNA sequences containing the three Sp1-binding sites (Sp1a, Sp1b and Sp1c; boxed in Figure 2c). This region is also protected from DNAase I digestion when recombinant Sp1 protein is used (Figure 2c).

Following these preliminary observations, the PCR-generated HIV-1 LTR fragment was incubated in the presence of increasing amounts of distamycin, in order to determine whether this DNA-binding drug protects DNA from DNAase I digestion in a sequence-dependent manner.

The results obtained are shown in Figure 3, and clearly indicate that distamycin protects the 259 bp LTR HIV-1 fragment from DNAase I digestion in a sequence-specific manner. For instance, distamycin appeared to bind at low concentration (6 μM) to the ATATAAGC DNA sequence, containing the target site of TFIID. By contrast, at both low and high distamycin concentrations only weak effect were detectable on the Sp1-binding sequence. The major footprints I, II and III were generated at low concentrations of distamycin, while IV and V were generated at higher concentrations. Parallel experiments were conducted with an HIV-1 LTR fragment obtained by digestion of the pT$_2$IIICAT LTR plasmid DNA with the restriction enzymes *Hpa*II and *Mse*I. This HIV-1 LTR DNA fragment allows the study of the DNA region 5′ to the ATATAA TFIID site. The results obtained showed that the sequences

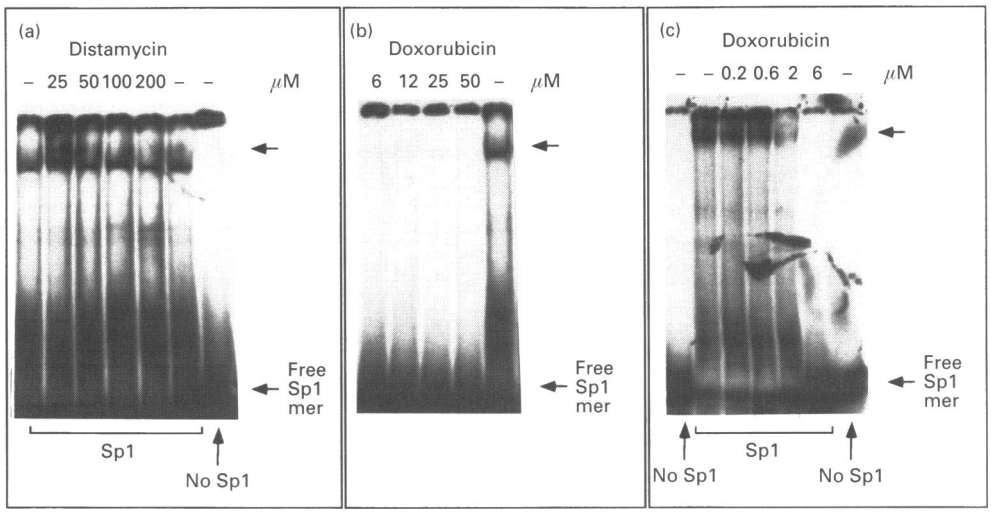

Figure 5 Effects of distamycin (a) and doxorubicin (b, c) on the interaction between recombinant Sp1 and a ^{32}P-labelled synthetic oligonucleotide containing the Sp1 target motif (Sp1 mer)

Free Sp1 mer and Sp1/Sp1 mer complexes are arrowed; –, indicates no drug added in the binding reaction.

CTGATATCGAG (interferon-γ region), CTACAAGGGACT-TTCCGCTG (containing one NF-κB site) and CCAGGGAG were protected from DNAase I digestion at intermediate concentrations (12 μM) of distamycin (results not shown). At higher concentrations (100–200 μM) of distamycin, the sequence selectivity of DNA-distamycin interactions was lost and large footprints could be detected within the entire HIV-1 LTR region (results not shown).

Differential effects of distamycin on the interactions of the nuclear factors TFIID and Sp1 with target DNA sequences

The electrophoretic mobility shift assay was employed to determine whether the differential DNAase I footprinting pattern exhibited by distamycin on the HIV-1 LTR fragment used in our experiments could be associated with possible differential effects of this drug on the interaction between nuclear factors and synthetic oligonucleotides containing target motifs of different transcription factors. In accordance with the results shown in Figure 3, two synthetic oligonucleotides, one containing the target sequence of TFIID and the other containing the Sp1-binding motif, were employed. In this experiment, total nuclear extracts from the Jurkat T-lymphoid cell line were used.

The results obtained are reported in Figures 4(a) and 4(b), and show that distamycin did not efficiently inhibit the interaction(s) between nuclear factors and the Sp1-mer (Figure 4b). On the contrary, distamycin was effective in inhibiting the intensity of some of the retarded bands generated by the interactions between nuclear factors and the synthetic oligonucleotide containing the TFIID-binding site. In more detail, distamycin inhibited the formation of complexes A and B (Figure 4a). According to Buratowski et al. [32] these complexes are produced by the interaction between the synthetic oligonucleotide and TFIID + TFIIA (complex A) or TFIID + TFIIA + TFIIB (complex B), while complexes exhibiting the lowest mobility rates contain also RNA polymerase II.

Control experiments were performed by using DNA-binding drugs, such as doxorubicin and daunomycin, that are known to bind to DNA by a mechanism different to that of distamycin [33,34]. As shown in Figures 4(c) and 4(d), doxorubicin was active in inhibiting the interactions between nuclear factors and both the Sp1 and TFIID synthetic oligonucleotides.

As is clearly evident from the band-shift data shown in Figure 4, multiple bands interacting with both TFIID and Sp1 oligonucleotides are present when total crude nuclear extracts are used, due to multiple interactions between Sp1, TFIID and other DNA-binding proteins involved in transcriptional activation of eukaryotic genes [35,36]. The patterns shown in Figure 4 are in good agreement with band-shift analyses reported by our research groups [37]. Accordingly, interactions of Sp1 with TFIID and transcriptional co-factors are well described phenomena [4,8–10, 35–38] and, therefore, it was not surprising to obtain a complex pattern of gel retardation when crude nuclear extracts from the Jurkat T-lymphoid cells were employed. However, in order to further confirm the data shown in Figure 4, commercially available Sp1 and TFIID recombinant proteins were employed.

Differential effects of distamycin and doxorubicin on the interactions between recombinant Sp1 and its target sequence

The results of the experiment employing recombinant Sp1 proteins are reported in Figure 5, and show that distamycin, unlike doxorubicin, does not efficiently inhibit Sp1/DNA interactions. Figure 5(a) shows that treatment with distamycin, even when used at 200 μM, did not lead to a decrease in the intensity of the retarded band generated by the interaction between the recombinant Sp1 and the ^{32}P-labelled Sp1 mer. On the contrary, addition of doxorubicin to the gel retardation incubation mixture resulted in inhibition of the interaction between the recombinant Sp1 and Sp1 mer (Figures 5b and 5c); 50% inhibition was obtained when doxorubicin was used at 2 μM (Figure 5c).

Both distamycin and doxorubicin inhibit the interaction between TFIID and the target sequence present in the HIV-1 LTR

Figure 6 shows that both distamycin and doxorubicin inhibited the formation of DNA/protein complexes when recombinant

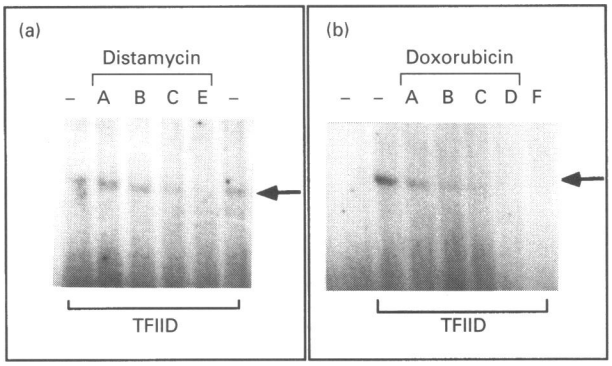

Figure 6 Effects of distamycin (a) and doxorubicin (b) on the interaction between recombinant TFIID and a ^{32}P-labelled synthetic oligonucleotide containing the TFIID target motif (TFIID mer)

TFIID/TFIID mer complexes are arrowed. Concentrations of the drugs were as follows: A, 3 μM; B, 12 μM; C, 50 μM; D, 100 μM; E, 200 μM; F, band shift in the presence of an excess (2 pmol) of unlabelled TFIID oligonucleotide; –, no drug added in the binding reaction.

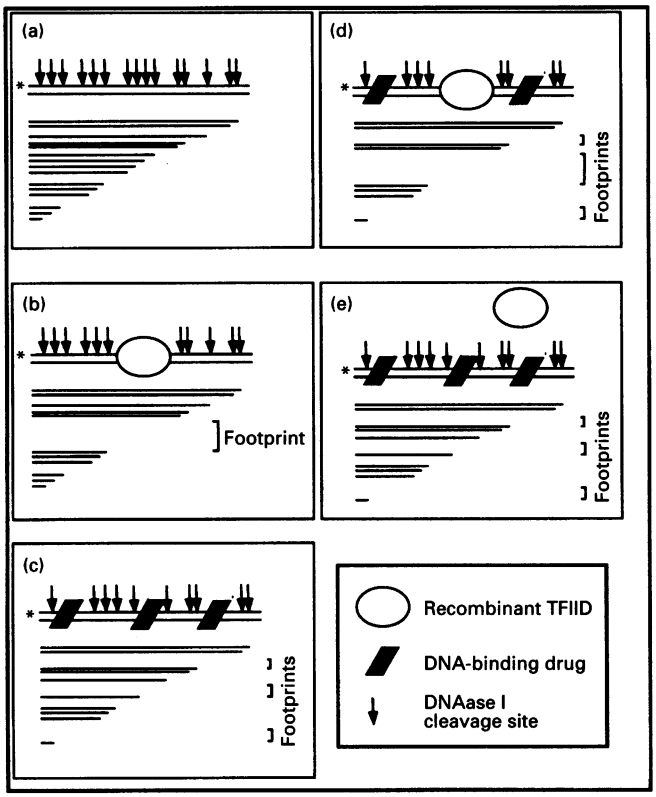

Figure 7 Scheme outlining the footprinting approach to determine the effects of DNA-binding drugs on the interaction between TFIID and PCR products mimicking a portion of the HIV-1 LTR

*, ^{32}P-labelled 5′-end. (**a**) DNAase I cleavage; (**b**) DNAase I cleavage in the presence of TFIID; (**c**) DNAase I cleavage in the presence of a DNA-binding drug; (**d**) DNAase I cleavage expected if the DNA-binding drug does not inhibit TFIID/DNA interactions; (**e**) DNAase I cleavage expected if the DNA-binding drug inhibits TFIID/DNA interactions.

TFIID and the relative target oligonucleotide were used in electrophoretic mobility shift assays. Therefore, in order to better evaluate this effect at the DNA sequence level, both DNA-binding drugs were incubated in footprinting assays with the HIV-1 LTR by using the experimental approach described in detail in Figure 7. Briefly, the HIV-1 LTR PCR product, DNA-binding drug and TFIID were all present in the footprinting reaction mixture. Therefore DNAase I will cleave the HIV-1 LTR depending on the extent of the binding of TFIID and/or DNA-binding drug (see scheme in Figure 7). If TFIID binds to the DNA target sequences in the presence of DNA-binding drug, the pattern generated will be the sum of the footprints generated by TFIID and the DNA-binding drug used singularly (compare Figures 7(b), 7(c) and 7(d)). In contrast, if the interaction of the DNA-binding drug with the DNA interferes with TFIID/DNA binding, the DNAase I footprinting pattern will be similar to that generated by the DNA-binding drug alone (compare Figures 7c and 7e).

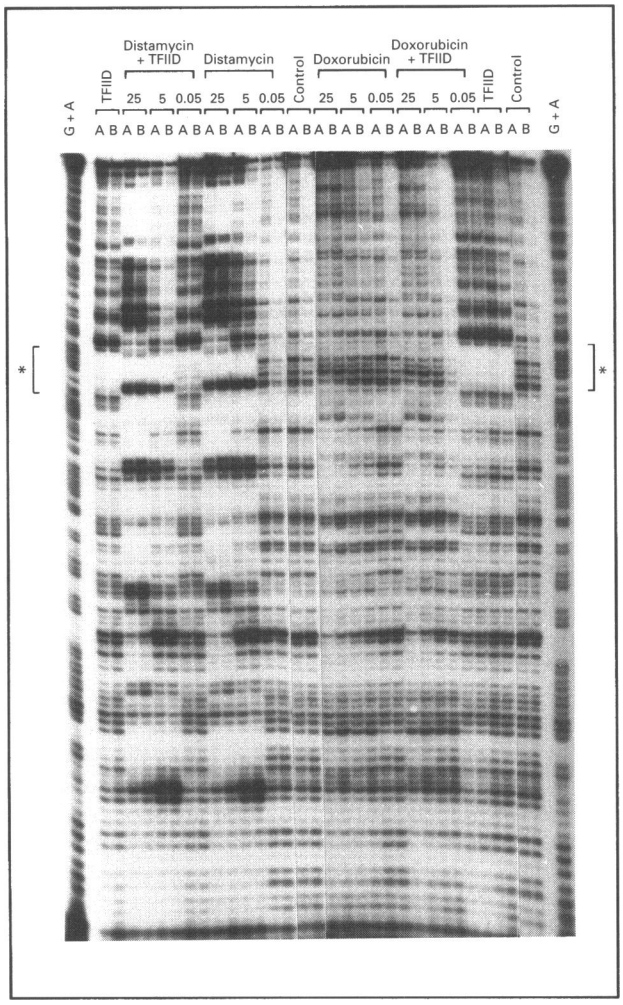

Figure 8 DNAase I footprinting patterns in the absence (control) or in the presence of TFIID, distamycin, TFIID + distamycin, doxorubicin and TFIID + doxorubicin

The labels 0.05, 5 and 25 indicate the concentrations (μM) of the drugs used. Incubation with DNAase I was performed for 1 (A) and 2 (B) min. Maxam–Gilbert G+A sequence reactions are shown on the two sides of the panel. Asterisks indicate the TFIID DNAase I footprint.

The results of this experiment are shown in Figure 8. When distamycin and doxorubicin were used at 5 and 25 μM, the DNAase I footprints generated in the presence of the DNA-binding drug (either distamycin or doxorubicin) and TFIID are

similar to those generated by the DNA-binding drug alone, and sharply different from the footprint generated by TFIID in the absence of drugs. These data suggest that TFIID does not bind to the HIV-1 LTR PCR product in the presence of 5 or 25 μM distamycin or doxorubicin. In contrast, TFIID binds to the HIV-1 LTR in the presence of very low concentrations of distamycin and doxorubicin (e.g. 0.05 μM) that do not affect the DNAase I footprinting pattern (Figure 8 and results not shown). Under these experimental conditions, typical TFIID footprints were generated when the footprinting reactions were performed in the presence of TFIID and 0.05 μM doxorubicin, while the DNAase I footprinting patterns generated in the presence of 0.05 μM distamycin and TFIID showed relatively little evidence of radioactive DNAase I-generated DNA fragments within the expected TFIID footprint. These latter data are consistent with a 10–15% inhibition of the interaction between TFIID and the HIV-1 LTR, even in the presence of these low concentrations of distamycin. Complete TFIID-mediated protection from DNAase I cleavage was obtained with lower concentrations of distamycin (results not shown).

DISCUSSION

The transcriptional regulation of the expression of the HIV-1 genome is mediated by complex interactions of *trans*-regulatory proteins with target DNA elements exhibiting defined nucleotide sequences and located in the LTR [1]. In the HIV-1 LTR are, for instance, present DNA sequence targets of a number of cellular and viral transcription factors, including Sp1, TFIID, NF-κB and tat. These factors recognize different DNA motifs. Sp1 binds to GC-rich regions (the Sp1 binding motifs in HIV-1 LTR are 5′-GAGGCGTGGC-3′, 5′-TGGGCGGGAC-3′ and 5′-TGGGG-AGTGG-3′), while TFIID recognizes an AT-rich DNA motif (5′-CATATAAGCA-3′ in HIVHXB2CG). NF-κB recognizes the DNA motif 5′-GGGACTTTCC-3′. The activity of some of the cellular transcription factors recognizing the HIV-1 LTR is required for the transcriptional activation of HIV-1.

A number of recent reports suggested that DNA-binding compounds could be proposed as antiviral drugs acting by modulating the formation of DNA/nuclear protein complexes (reviewed in [14]). We [22] and others [15,20] have recently reported that distamycin is a strong inhibitor of the interaction between nuclear factors and target DNA sequences. The main aim of the present study was to determine (a) whether distamycin does bind to the HIV-1 LTR and (b) whether this binding is sequence-specific. We thus compared the protection from DNAase I digestion by distamycin with (a) that of nuclear extracts from the Jurkat T-lymphoid cell line and (b) that of the recombinant proteins Sp1 and TFIID.

The results obtained suggest that distamycin binds to the HIV-1 LTR in a sequence-specific manner, e.g. to the TFIID and TAR region. Other regions, such as the Sp1-binding sites, are not protected by distamycin from DNAase I digestion. The footprinting data are in good agreement with the results obtained in the electrophoretic mobility shift assays (see Figures 4, 5 and 6). Distamycin does not inhibit Sp1/Sp1 mer interactions, while being effective in inhibiting interactions between TFIID and the CATATAAGC HIV-1 target sequence. Therefore the major conclusion of our paper is that distamycin exhibits sequence selectivity, recognizing different regions of the HIV-1 LTR and this might have functional implications, possibly leading to differential effects of this DNA-binding drug on DNA/protein interactions.

We suggest that molecular analyses similar to those described in the present paper should be undertaken in order to determine possible links between *in vitro* effects of DNA-binding drugs and biological activity on intact cells and/or in experimental animals. This could be very important, especially in studies focusing on distamycin-like compounds that could display differential sequence selectivity and/or activity with transcription factors [38].

An analysis of the effects of antitumour and antiviral DNA-binding drugs could be of some interest, in relation to the fact that transcriptional regulation of viral and cellular genes is a very complex phenomenon. For instance, negative regulation has been also described, such as that involving albumin negative factor (ANF) (sequence recognized CTTTATCTGG) [39], GC-binding factor (GCF) (sequence recognized C/G-CG-C/G-C/G-C/G-C) [40] and plasmocytoma repressor factor (PCF) (sequence recognized AGAAAGGGAAAGGA) [41], protein factors which are responsible for the negative regulation of the albumin, epidermal growth factor and *c-myc* genes respectively. In these cases it is reasonable to hypothesize that, after treatment with DNA-binding drugs, the inhibition of the interaction between a negative transcription factor and its target sequence could lead to an increased expression of certain genes. This is a very important point for retrovirus genomes, since the LTRs of retroviruses usually display regions which are recognized by negative transcription factors [42]. For instance, when the negative regulatory element (NRE) is deleted from the HIV-1 LTR, an increase in the transcription of the HIV-1 genome occurs. The NRE region is recognized by a number of nuclear proteins, as judged by footprinting assays.

When cells expressing these factors are treated with DNA-binding drugs, activation of genes that are negatively regulated could occur. In HIV-infected cells, treatment with DNA-binding drugs could lead to activation of transcription. In line with this hypothesis, when CAT assays were performed on cells stably transfected with the CAT gene under the control of the HIV-1 LTR, induction of CAT activity was obtained following treatment with DNA-binding drugs [43,44].

Footprinting analyses, electrophoretic mobility shift assays, *in vitro* transcription and transfection of target cells with suitable recombinant clones could provide very useful data in the evaluation of the effects of DNA-binding drugs at the molecular level.

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