Targeting of the HIV-1 long terminal repeat with chromomycin potentiates the inhibitory effects of a triplex-forming oligonucleotide on Sp1–DNA interactions and *in vitro* transcription

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We have studied the effects of chromomycin and of a triple-helix-forming oligonucleotide (TFO) that recognizes Sp1 binding sites on protein–DNA interactions and HIV-1 transcription. Molecular interactions between chromomycin, the Sp1 TFO and target DNA sequences were studied by gel retardation, triplex affinity capture using streptavidin-coated magnetic beads and biosensor technology. We also determined whether chromomycin and a TFO recognizing the Sp1 binding sites of the HIV-1 long terminal repeat (LTR) inhibit the activity of restriction enzyme *Hae*III, which recognizes a sequence (5′-GGCC-3′) located within these Sp1 binding sites. The effects of chromomycin and the TFO on the interaction between nuclear proteins or purified Sp1 and a double-stranded oligonucleotide containing the Sp1 binding sites of the HIV-1 LTR were studied by gel retardation. The effects of both chromomycin and TFO on transcription were studied by using an HIV-1 LTR-directed *in vitro* transcription system. Our results indicate that low concentrations of chromomycin potentiate the effects of the Sp1 TFO in inhibiting protein–DNA interactions and HIV-1-LTR-directed transcription. In addition, low concentrations of chromomycin do not affect binding of the TFO to target DNA molecules. The results presented here support the hypothesis that both DNA binding drugs and TFOs can be considered as sequence-selective modifiers of DNA–protein interactions, possibly leading to specific alterations of biological functions. In particular, the combined use of chromomycin and TFOs recognizing Sp1 binding sites could be employed in order to abolish the biological functions of promoters (such as the HIV-1 LTR) whose activity is potentiated by interactions with the promoter-specific transcription factor Sp1.

INTRODUCTION

A number of studies have suggested that triple-helix-forming oligonucleotides (TFOs) might be considered as potential inhibitors of the transcription of cellular [1–9] and viral [10] genes. The proposed mechanism of action of TFOs is based on the recognition of homopurine/homopyrimidine sequences frequently found in gene regulatory regions [1–3]. This recognition involves the formation of Hoogsteen hydrogen bonds between the TFO and the purines of Watson–Crick base pairs [3,4].

In the case of the modulation of gene expression of the human immunodeficiency type I virus (HIV-1), McShan et al. [10] have demonstrated that a TFO specifically recognizing the Sp1 binding sites of the HIV-1 long terminal repeat (LTR) inhibits Sp1–DNA interactions and LTR-directed transcription. This oligonucleotide was designed to bind to the regulatory Sp1 elements of the HIV-1 LTR, by forming G:GC and T:AT triplets, in a parallel orientation [10], in accordance with studies [11–13] investigating DNA triplexes with mixed purine/pyrimidine targets and containing mostly G:GC triplets. The finding that Sp1–DNA interactions could be inhibited by TFOs recognizing Sp1 binding sites of the HIV-1 LTR is of relevance, because the transcription factor Sp1 is involved, together with other DNA binding proteins, in the control of LTR-directed transcription [14–16] leading to activation of the HIV-1 provirus [17–19].

The effects of TFOs on transcription were confirmed by other investigators in a variety of experimental systems, involving genes such as c-*myc* [5,6], *HER-2/neu* [9], interleukin-2Rα [7] and Ha-*ras* [8]. Consistently, TFOs directed against the promoter sequences of these genes were found to inhibit the interaction between DNA and transcription factors, leading to suppression of transcriptional initiation and elongation [5–9].

Additional modifiers of transcription have been identified, such as sequence-selective DNA binding drugs, including distamycin, berenil, chromomycin and mithramycin [20–36]. Some of these DNA binding drugs exhibit preferences for G+C-rich regions [31–34]. For instance, we have demonstrated that chromomycin recognizes the Sp1 binding sites of the HIV-1 LTR, but displays low efficiency in interacting with the transcription factor IID binding region [26,35]. Accordingly, chromomycin and the analogue mithramycin selectively inhibit the interaction between Sp1 and the C+G-rich target sequences [22,33,34] present in the promoters of the collagen α1(I) [22] and Ki-*ras* [22,34] genes. No effects of mithramycin were reported on interactions between nuclear factor I and DNA [22].

Since TFOs have been consistently demonstrated to bind to the major groove of DNA [1–3], whereas many DNA binding drugs (such as chromomycin and mithramycin) bind to the minor groove [22], it is possible that these two classes of transcription inhibitors could bind simultaneously to the target DNA sequences, perhaps leading to an increase in their ability to inhibit the biological functions of specific genes [36]. Nevertheless, the effects of DNA binding drugs on triple-helix formation are not well characterized [36]. Stabilization of triple-helical DNA by a benzo[f]pyridoindole analogue has been reported [37]. Furthermore, the A+T-specific minor-groove ligand berenil

Abbreviations used: TFO, triple-helix-forming oligonucleotide; LTR, long terminal repeat; Sp1(HIV-1)TFO, TFO recognizing the Sp1 binding sites of the HIV-1 LTR; DTT, dithiothreitol; SPR, surface plasmon resonance; BIA, biospecific interaction analysis.

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binds with high efficiency to the DNA triple helix [38]. Highly efficient binding to the DNA triple helix was also reported for ethidium [39] and coralyne [40]. Finally, Vigneswaran et al. [36] presented data demonstrating that the G+C-rich minor-groove ligand mithramycin binds preformed triplex DNA. However, high concentrations of this DNA binding drug were found to cause displacement of a TFO from double-stranded DNA [23,36].

We were interested in determining the effects of G+C-selective DNA binding drugs and TFOs when used at low concentrations. As an *in vitro* experimental model system we employed the HIV-1 LTR and performed experiments aimed at studying protein–DNA interactions and transcription when chromomycin was added *in vitro* together with a TFO recognizing the Sp1 binding sites of the HIV-1 LTR [Sp1(HIV-1)TFO]. Since triple-helix formation blocks restriction enzyme cleavage [41,42], we first determined whether Sp1(HIV-1)TFO inhibits the activity of the restriction enzyme *Hae*III, which recognizes a sequence (5′-GGCC-3′) from the Sp1 binding site of the HIV-1 LTR. The effects of both chromomycin and TFO on Sp1–DNA interactions were studied by gel retardation using crude nuclear extracts from the T-lymphoid Jurkat cell line as well as purified transcription factor Sp1 [43]. Effects on transcription were studied by using an LTR-directed *in vitro* transcription system [10].

MATERIALS AND METHODS

DNA binding drugs and synthetic oligonucleotides

Chromomycin was obtained from Sigma. Stock solutions (0.84 mM) were stored at −20 °C in the dark and diluted immediately before use.

Sp1(HIV-1)TFO (5′-TGGGTGGGGTGGGGTGGGGGG-GTGTGGGGTGTGGGGTG-3′) and other synthetic oligonucleotides were purchased from Pharmacia (Uppsala, Sweden). The nucleotide sequences of the HIV-1 Sp1a mer and Sp1b mer are shown in Figure 1.

Triplex affinity capture

A ^{32}P-end-labelled double-stranded HIV-1 Sp1 target oligonucleotide (see Figure 1 for nucleotide sequence) (50 ng) was incubated in the presence or in the absence of chromomycin with 2 μg of biotinylated Sp1(HIV-1)TFO in 50 μl of a binding buffer containing 100 mM NaCl, 1 mM dithiothreitol (DTT), 50 mM Tris, pH 7.4, and 20 mM $MgCl_2$. After a 1 h incubation, streptavidin-coated magnetic beads (40 μg of Dynabeads Streptavidin M-280; DYNAL, Great Neck, NY, U.S.A.) were added and a further 30 min incubation was performed. Complexes between the TFO and the target DNA were separated by a magnetic particle concentrator [44–46], and radioactivity present in the pellets was determined.

The stability of complexes generated in parallel control reaction mixtures was evaluated after resuspending the pellets in binding buffer in the presence of increasing concentrations of chromomycin. After a further 5 min incubation, the remaining TFO–DNA complexes were separated by the magnetic particle concentrator [44–46] and the radioactivity of the pellets was determined.

Biospecific interaction analysis (BIA) using biosensor technology

A BIAcore-1000® instrument (Pharmacia Biosensors) was used in all experiments [47–50]. Sensor chip SA5 (research grade),

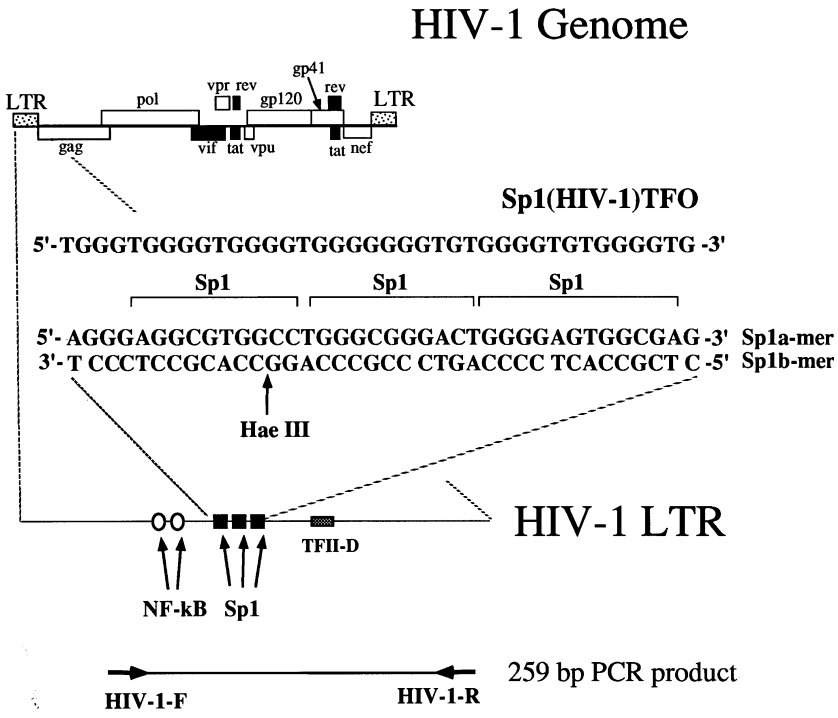

Figure 1 Structure of the HIV-1 genome, location of the primers used for PCR and sequence of Sp1(HIV-1)TFO

The locations of the primers used for PCR (HIV-1-F and HIV-1-R) are indicated by arrows. The Sp1a mer and the Sp1b mer nucleotide sequences are also shown. The binding sites for nuclear factor κB (NF-kB; open circles), Sp1 (black boxes) and transcription factor II-D (TFII-D; grey box), and the region recognized by the Sp1(HIV-1)TFO, are indicated. The location of the sequence recognized by *Hae*III is also shown.

precoated with streptavidin, was from Pharmacia Biosensors. The experiments were conducted at 25 °C, at a flow rate of 4 μl/min. The protocol for generation of the double-stranded target Sp1 mer was as follows. One 30 μl pulse of biotinylated Sp1 mer (nucleotide sequence 5′-CCCCGCCCCCGCCCCG-CCCC-3′) was performed to give streptavidin-mediated capture of 700–1000 resonance units of single-stranded Sp1 DNA. Double-stranded DNA was obtained by injecting 30 μl of the complementary Sp1 mer. The effects of chromomycin on the formation of complexes between TFO and double-stranded Sp1 target DNA were monitored after the injection of 30 μl of chromomycin, followed by 30 μl of 1 μM Sp1(HIV-1)TFO. The running buffer was 100 mM NaCl, 50 mM Tris, pH 7.4, and 20 mM $MgCl_2$. The generation of double-stranded Sp1 target DNA sequences was performed in HBS buffer (10 mM Hepes, pH 7.4, 0.15 M NaCl, 3.4 mM EDTA and 0.05 % Surfactant P2) (Pharmacia Biosensors).

Inhibition of restriction enzyme-dependent cleavage

*Hae*III and *Mbo*I were used in these assays as previously described [43]. The activity of the restriction enzymes was determined in the absence or in the presence of chromomycin and Sp1(HIV-1)TFO, as described in the Results section. The target DNA was a PCR-generated fragment [51] containing the three Sp1 binding sites of the HIV-1 LTR. The locations of the primers (HIV-1-F, HIV-1-R) used for PCR are reported in Figure 1. The nucleotide sequences of the primers were: HIV-1-F, 5′-ATTTCATCACA-TGGCCCGAG-3′; HIV-1-R, 5′-AGGCAAGCTTTATTGAG-GCT-3′. *Taq* DNA polymerase (DYNAZYME®) was added at a final concentration of 2 units/25 μl. The target DNA was the $pT_zIIICAT$ plasmid, containing the chloramphenicol acetyltransferase gene under the control of the HIV-1 LTR [25,26]. Restriction enzyme reactions were performed in 10 % (w/v) glycerol, 0.05 % Nonidet P-40, 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT and 5 mM $MgCl_2$ for 16 h at 37 °C [43]. The reactions were analysed by electrophoresis on 8 % polyacrylamide gels. In control experiments, *Hae*III digestion reactions were performed in the presence of 34 μM of an unrelated oligonucleotide (5′-TAGCAGAATAGGCGTTA-3′).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays [25] were performed by using double-stranded synthetic oligonucleotides containing target DNA sequences for transcription factor Sp1 [10] (see Figure 1 for nucleotide sequences). The synthetic oligonucleotides were 5′-end-labelled using [γ-^{32}P]ATP. Binding reactions were set up as described elsewhere [25] in binding buffer [10 % (w/v) glycerol, 0.05 % Nonidet P-40, 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 15 mM $MgCl_2$, 0.5 mM DTT] in the presence of 1 μg of poly(dI,dC) (Pharmacia), 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. Nuclear extracts were purified as described in detail elsewhere [25,43]. In the case of binding reactions using purified Sp1 (Promega), 0.5 footprinting units were used for each reaction.

After binding of DNA binding proteins to the synthetic target oligonucleotides for 30 min at room temperature, samples were electrophoresed at constant voltage (200 V for 1.5 h) at low ionic strength (0.25 × TBE buffer; 1 × TBE = 0.089 M Tris/borate and 0.002 M EDTA) on 6 % (w/v) polyacrylamide gels until tracking dye (Bromophenol Blue) reached the end of a 16 cm slab. Gels were dried and exposed to intensifying screens at −80 °C. Addition of the reagents took place in the following order: (1) poly(dI,dC); (2) Sp1 mers; (3) binding buffer; (4) crude nuclear extracts or purified Sp1; (5) chromomycin and/or Sp1(HIV-1)TFO.

Binding of chromomycin to ^{32}P-labelled DNA was evaluated by gel mobility shift assays as described elsewhere [36]. Usually, the concentration of double-stranded ^{32}P-labelled DNA in each binding reaction was 10 nM. Binding was carried out in 10 % (w/v) glycerol, 0.05 % Nonidet P-40, 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 15 mM $MgCl_2$ and 0.5 mM DTT. The electrophoresis buffer comprised 10 mM Tris/HCl, pH 7.5, 50 mM NaCl and 15 mM $MgCl_2$.

In vitro transcription

In vitro transcription was performed using HeLa nuclear extracts (Promega) [10,43]. The template DNA was generated from *Nco*I digestion of the $pT_zIIICAT$ plasmid. A 100 ng portion of HIV-1 LTR template or control template containing the cytomegalovirus immediate-early promoter was incubated in the absence or in the presence of chromomycin for 5 min at room temperature. Transcription was initiated by adding 2 μl of HeLa nuclear extract (6.3 mg/ml) in a total volume of 25 μl of a buffer containing 20 mM Hepes, pH 7.9, 100 mM KCl, 20 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 20 % (w/v) glycerol, 1 mM each of ATP, CTP and UTP and 0.4 mM GTP in the presence of [α-^{32}P]GTP. After 60 min, transcription was terminated by adding 175 μl of buffer containing 0.3 M Tris/HCl, pH 7.4, 0.3 M sodium acetate, 0.5 % SDS, 2 mM EDTA and 3 μg/ml yeast tRNA. The transcription reaction was extracted with phenol/chloroform (1:1, v/v) and precipitated with 500 μl of 100 % (v/v) ethanol, and the transcription products were analysed by electrophoresis in 6 % polyacrylamide, 7 M urea and 1 × TBE buffer.

RESULTS

Effects of chromomycin on the interaction between Sp1(HIV-1)TFO and double-stranded Sp1 mer

Figure 1 shows the nucleotide sequence of the HIV-1 LTR region that is recognized by Sp1(HIV-1)TFO. The effects of chromomycin on the interaction between Sp1(HIV-1)TFO and double-stranded Sp1 mer were first analysed by magnetic capture of double-stranded HIV-1 Sp1 mer with biotinylated Sp1(HIV-1)TFO. Figure 2(A) shows a schematic representation of the experimental approach. Figure 2(B) shows the relationship between chromomycin concentration and binding of biotinylated Sp1(HIV-1)TFO to the ^{32}P-labelled double-stranded Sp1 mer. The results were obtained by performing magnetic capture of complexes between Sp1 mer and Sp1(HIV-1)TFO using streptavidin-coated Dynabeads Streptavidin M-280 [44–46]. ^{32}P-labelled HIV-1 Sp1 mer was incubated in the absence or in the presence of 0.5, 1, 2, 5 or 100 μM chromomycin with 2 μg of biotinylated Sp1(HIV-1)TFO in 50 μl of a binding buffer containing 100 mM NaCl, 1 mM DTT, 50 mM Tris, pH 7.4, and 20 mM $MgCl_2$. After a 1 h incubation, the magnetic beads were added and a further 30 min incubation was performed. Complexes between the TFO and target DNA were separated by a magnetic particle concentrator [44–46], and radioactivity present in the pellets was determined. The data obtained demonstrate that neither 0.5 nor 1 μM chromomycin caused inhibition of binding of Sp1(HIV-1)TFO to the target sequence (Figure 2B). Furthermore, biotinylated Sp1(HIV-1)TFO was able to capture ^{32}P-labelled target double-stranded DNA even in the presence of 2 μM chromomycin (only 15 % inhibition was found under these experimental conditions).

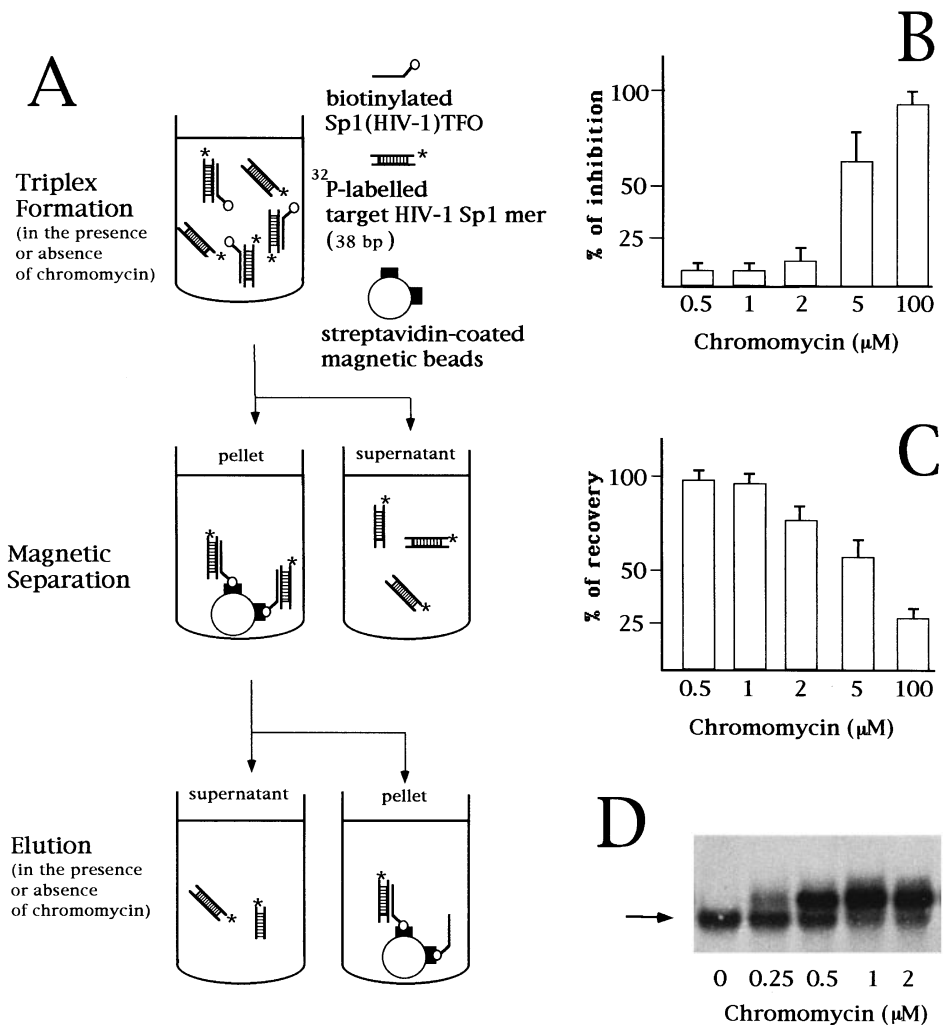

Figure 2 Effects of chromomycin on the generation and stability of TFO–target-DNA complexes

(**A**) Schematic representation of triplex affinity capture using biotinylated Sp1(HIV-1)TFO and ^{32}P-labelled HIV-1 Sp1 mer (Sp1a/Sp1b; see Figure 1 for nucleotide sequences). (**B**) Chromomycin-mediated inhibition of the formation of TFO–target-DNA complexes. In this experiment the triplex-formation reaction was performed for 60 min in the absence or in the presence of 0.5, 1, 2, 5 or 100 μM chromomycin by incubating Sp1(HIV-1)TFO and ^{32}P-labelled HIV-1 Sp1 mer target DNA in 50 μl of binding buffer (see the Materials and methods section). After magnetic capture and three washes in binding buffer, the radioactivity present in the pellet was determined and compared with that in control binding reactions conducted in the absence of chromomycin. Inhibition (%) = [(radioactivity of control binding reaction) − (radioactivity of chromomycin-treated samples)] × 100/(radioactivity of control binding reaction). (**C**) Chromomycin-mediated disruption of preformed TFO–target-DNA complexes. After a 60 min incubation of biotinylated Sp1(HIV-1)TFO with ^{32}P-labelled HIV-1 Sp1 mer, magnetic separation was performed, and the pellets were washed three times and gently resuspended in binding buffer in the absence or in the presence of 0.5, 1, 2, 5 or 100 μM chromomycin; after a further 5 min time period, magnetic separation was performed and the residual radioactivity in the pellets was determined and compared with that in control reactions conducted in the absence of chromomycin. Recovery (%) = (radioactivity in chromomycin-treated samples) × 100/(radioactivity in control binding reaction). Results in (**B**) and (**C**) represent means ± S.D. of four separate experiments. (**D**) Chromomycin-induced shift in the electrophoretic mobility of ^{32}P-labelled double-stranded HIV-1 Sp1 target DNA. Target ^{32}P-labelled Sp1 DNA (10 nM) was incubated in the absence (0 μM) or in the presence of 0.25–2.0 μM chromomycin in binding buffer (see the Materials and methods section). After 30 min, electrophoresis was performed using a 16% (w/v) polyacrylamide gel in 90 mM Tris/HCl, pH 8, 90 mM boric acid and 10 mM $MgCl_2$. Higher concentrations of chromomycin reproducibly induced the supershift observed with 0.25–2.0 μM chromomycin [36].

In a second set of experiments, the stability of TFO–DNA complexes generated in the absence of chromomycin was evaluated after resuspending the pellets in binding buffer in the presence of increasing concentrations of chromomycin. After a further 5 min incubation, the remaining TFO–DNA complexes were separated by the magnetic particle concentrator [44–46] and the radioactivity of the pellets was determined. The results suggest that chromomycin at 0.5 and 1 μM does not disrupt preformed complexes between biotinylated Sp1 mer and Sp1(HIV-1)TFO (Figure 2C). In addition, the data obtained show that 81% of complexes between double-stranded Sp1 DNA and Sp1(HIV-1)TFO were recovered after treatment with 2 μM chromomycin. It should be noted that gel-retardation experiments show that a shift in the electrophoretic mobility of ^{32}P-labelled double-stranded Sp1 mer occurs even at low chromomycin concentrations (0.5 and 1 μM) (Figure 2D), suggesting that chromomycin binds to the majority of the Sp1 mer molecules. As expected, an electrophoretic mobility shift similar to that found at 1 μM chromomycin was observed reproducibly when higher concentrations of the drug were employed (e.g. 2, 10 and 50 μM) (Figure 2D; and results not shown). In contrast, chromomycin does not alter the electrophoretic migration of single-stranded DNA (results shown).

Thus higher concentrations of chromomycin (5 and 100 μM)

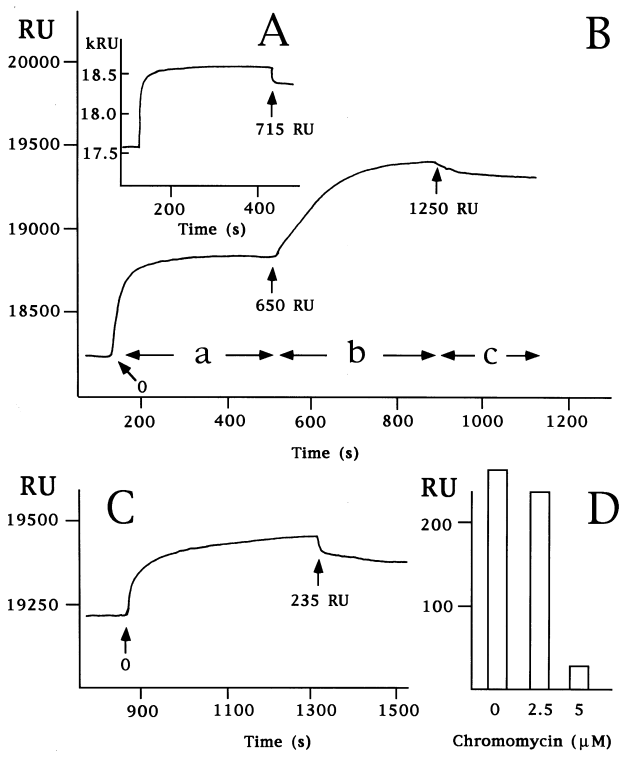

Figure 3 BIA of the effects of chromomycin on the generation of TFO–target-DNA complexes

Shown are the increases in the SPR response [in resonance units (RU)] after injection of 30 μl of 1 μM single-stranded Sp1 mer (**A**), followed by injection of 30 μl of 1 μM complementary Sp1 mer (**B**, part a), followed by injection of 30 μl of 2.5 μM chromomycin (**B**, part b). The chromomycin–DNA complex appears to be fairly stable after injection of 20 μl of binding buffer (**B**, part c). (**C**) Increase in the SPR response caused by the injection of 30 μl of 1 μM Sp1(HIV-1)TFO on a sensor chip containing chromomycin–DNA complexes (see **B**, parts b and c). (**D**) Increase in the SPR response when Sp1(HIV-1)TFO was injected on a sensor chip containing double-stranded Sp1 target DNA complexed with chromomycin (injection of 30 μl of binding buffer containing 0, 2.5 or 5 μM chromomycin, as indicated). The running and binding buffer comprised 100 mM NaCl, 50 mM Tris, pH 7.4, and 20 mM $MgCl_2$. The generation of double-stranded Sp1 target DNA sequences was performed in HBS buffer (10 mM Hepes, pH 7.4, 0.15 M NaCl, 3.4 mM EDTA and 0.05% Surfactant P2).

were able to suppress interactions between Sp1(HIV-1)TFO and double-stranded DNA (Figure 2B) and to disrupt preformed complexes (Figure 2C). In contrast, low concentrations of chromomycin do not inhibit TFO–DNA interactions and do not disrupt preformed complexes between TFO and double-stranded DNA. This conclusion could be supported by (i) extending the analysis to other experimental systems (e.g. TFOs recognizing the Sp1 binding sites of the Ha-*ras* promoter; results not shown), and (ii) employing other molecular techniques, such as gel retardation and, more importantly, BIA using surface plasmon resonance (SPR) technology and the biosensor BIAcore-1000® (Pharmacia). The latter approach allows the real-time monitoring of a variety of molecular interactions, including protein–protein, protein–DNA and DNA–DNA interactions [47–50].

The description of BIA methodology and its use for studying triple-helix formation has been reported previously [47–50]. We first produced a sensor chip containing Sp1 target double-stranded DNA [Figures 3A and 3B (part a)], and then we studied chromomycin binding to the DNA (Figure 3B, part b). Finally, we determined whether Sp1(HIV-1)TFO was able to interact under these experimental conditions (Figure 3C).

In order to obtain efficient capture of the HIV-1 Sp1 DNA on the sensor chip, the interaction between streptavidin and biotin was employed [47]. The sensor chip SA5 was used in order to capture 5′-biotinylated Sp1 oligonucleotide (Figure 3A) that was injected over the surface. The data shown demonstrate that rapid capture of about 700 resonance units of a complex between biotin and Sp1 single-stranded DNA was observed within 4–6 min. Double-stranded Sp1 target DNA was generated by the injection of 1 μM complementary Sp1 mer in 20 μl (Figure 3B, part a).

The experimental results obtained by performing BIA were in broad agreement with data of Figure 2. We first demonstrated an ability to analyse molecular interactions between TFOs and biotinylated double-stranded DNA using SPR technology (results not shown; and C. Rutigliano and R. Gambari, unpublished work). These interactions require $MgCl_2$ and are disrupted by the addition of HBS (containing 3.5 mM EDTA); this treatment does not affect Watson–Crick base pairs stabilizing the double-stranded Sp1 target DNA immobilized on the SA5 sensor chip (results not shown). Secondly, we found that the interactions between Sp1(HIV-1)TFO and the biotinylated double-stranded Sp1 target DNA immobilized on the SA5 sensor chip were not affected by the previous injection of 2.5 μM chromomycin (Figure 3C). Pre-injection of increasing concentrations of chromomycin (up to 5 μM) caused a sharp inhibition of Sp1(HIV-1)TFO binding to double-stranded Sp1 mer immobilized on the chip (Figure 3D).

In conclusion, the results presented in Figures 2 and 3 confirm that complexes between TFOs and target DNA sequences can be detected by (i) magnetic capture using streptavidin-coated magnetic beads [44] and (ii) SPR and biosensor technology. The data obtained consistently suggest that stable triple-helix complexes between Sp1(HIV-1)TFO and target DNA are generated in the presence of low concentrations of chromomycin. These data confirm and further extend the results of Vigneswaran et al. [36] showing that high (100 μM) concentrations of the chromomycin analogue mithramycin suppress TFO–DNA complex-formation and disrupt preformed TFO–DNA complexes.

Inhibition of *Hae*III-dependent cleavage

Figure 1 shows the HIV-1 LTR region recognized by the Sp1(HIV-1)TFO and the location, within this region, of the site recognized by the restriction enzyme *Hae*III (sequence recognized: 5′-GGCC-3′). It should be noted that the site recognized by *Hae*III forms part of the Sp1 binding region of the HIV-1 LTR. Therefore we first determined whether chromomycin inhibits *Hae*III cleavage of this Sp1 binding region. As control, *Mbo*I, a restriction enzyme that recognizes a sequence (5′-GATC-3′) located outside the Sp1 binding site, was employed. The sequences within and surrounding the *Mbo*I site are not G+C-rich and therefore should not be recognized by Sp1(HIV-1)TFO and chromomycin. The 259 bp PCR-generated HIV-1 LTR fragment that was used in our experiments is also shown in Figure 1.

Figure 4 shows the effects of chromomycin and Sp1(HIV-1)TFO on digestion by *Hae*III of this 259 bp PCR product. Densitometric scans of the ethidium bromide-stained polyacrylamide gel shown in Figure 4 demonstrated that 50% inhibition was obtained when 1.5 μM chromomycin was added to the restriction enzyme mixture (Figure 4, lane c). When 15 μM Sp1(HIV-1)TFO was used, no more than 10% inhibition of cleavage by *Hae*III was observed (Figure 4, lane d). Control experiments demonstrated that (i) *Mbo*I also cleaved the HIV-1

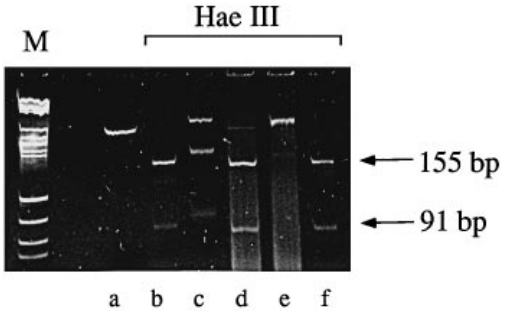

Figure 4 Effects of chromomycin and Sp1(HIV-1)TFO on *Hae*III digestion of the HIV-1 LTR PCR product

Lane M contains molecular size markers (*Hae*III-digested pBR322 plasmid). Portions of 300 ng of the 259 bp HIV-1 LTR PCR product (see Figure 1) were digested in 50 μl of reaction buffer in the absence (lane b) or in the presence of 1.5 μM chromomycin (lane c) or 15 μM Sp1(HIV-1)TFO (lane d). Lane a represents the control undigested HIV-1 PCR product. Lane e contains the restriction digestion performed in the presence of both 1.5 μM chromomycin and 15 μM of Sp1(HIV-1)TFO. No inhibition of *Hae*III-dependent cleavage was obtained by the addition of 34 μM of an unrelated oligonucleotide (lane f). The restriction products are arrowed.

PCR fragment in the presence of chromomycin and Sp1(HIV-1)TFO (results not shown), and (ii) higher concentrations of chromomycin or Sp1(HIV-1)TFO suppressed *Hae*III-dependent cleavage fully (results not shown).

Figure 4 (lane e) clearly shows that 1.5 μM chromomycin and 15 μM Sp1(HIV-1)TFO added together fully suppressed cleavage by *Hae*III; this suggests that the combined use of these two DNA binding molecules leads to an increase in the inhibition of *Hae*III restriction enzyme cleavage of the Sp1 binding region of the HIV-1 LTR. The shift in the electrophoretic mobility of chromomycin-treated DNA fragments was due to chromomycin–

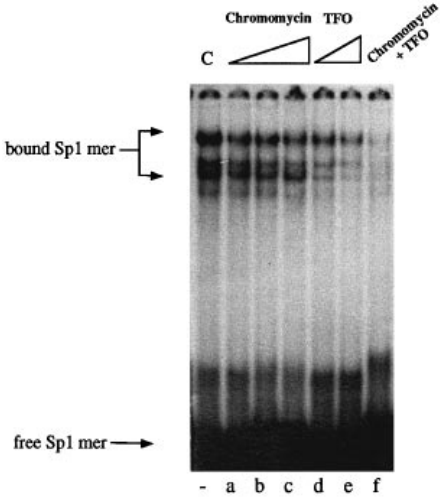

Figure 5 Effects of chromomycin and Sp1(HIV-1)TFO on interactions between nuclear factors and ^{32}P-labelled Sp1 mer

Nuclear extracts from Jurkat cells (2 μg) were incubated with $\sim$ 5 nM ^{32}P-labelled double-stranded Sp1 mer in the presence of 0.5 (lane a), 1 (lane b) or 2 (lane c) μM chromomycin; 0.15 (lane d) or 0.3 (lane e) μM Sp1(HIV-1)TFO; or 2 μM chromomycin plus 0.15 μM Sp1(HIV-1)TFO (lane f). Lane C contains a control binding reaction carried out in the absence of chromomycin and Sp1(HIV-1)TFO. The protein–DNA complexes are arrowed. The free ^{32}P-labelled Sp1 mer is also arrowed.

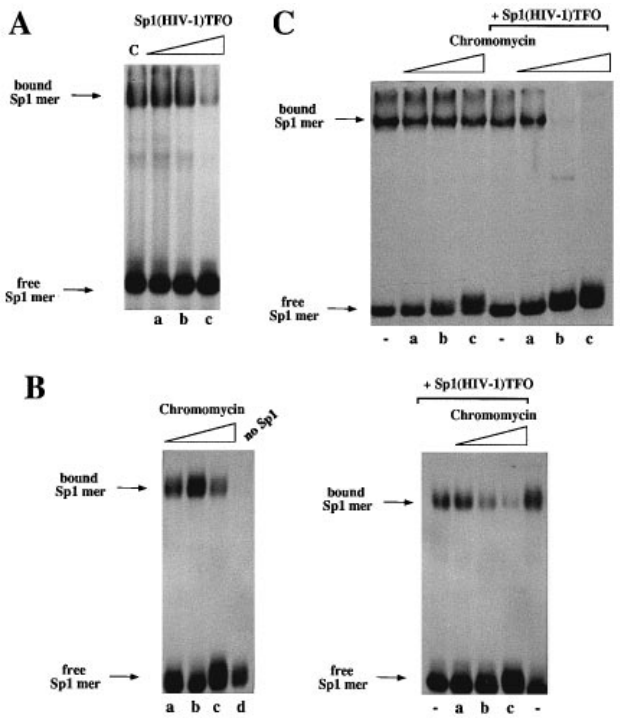

Figure 6 Effects of chromomycin and Sp1(HIV-1)TFO on interactions between purified Sp1 and ^{32}P-labelled Sp1 mer

(**A**) and (**B**) (left-hand panel) show the effects of increasing concentrations of (**A**) Sp1(HIV-1)TFO (lane a, 0.15 nM; lane b, 3 nM; lane c, 15 nM) and (**B**) chromomycin (lane a, 0.25 μM; lane b, 0.5 μM; lane c, 1.0 μM) on the generation of molecular interactions between ^{32}P-labelled double-stranded Sp1 mer and purified Sp1; lane d represents a negative control (^{32}P-labelled Sp1 mer alone). In the right-hand panel of (**B**), binding reactions were performed in the presence of 3 nM Sp1(HIV-1)TFO plus 0.25 (lane a), 0.5 (lane b) or 1 (lane c) μM chromomycin. (**C**) Effects on interaction between ^{32}P-labelled Sp1 mer and purified Sp1 of 0.25 (lane a), 0.5 (lane b) and 1 (lane c) μM chromomycin in the absence (left) or in the presence (right) of 3 nM Sp1(HIV-1)TFO. Each binding reaction was performed with 0.5 footprinting units of purified Sp1 and $\sim$ 5 nM ^{32}P-labelled Sp1 mer. Lanes — indicate binding reactions performed in the absence of chromomycin (left) or in the absence of both chromomycin and TFO (right). Sp1–DNA complexes and free ^{32}P-labelled Sp1 mer are arrowed.

DNA interactions, in agreement with results reported elsewhere [26,36]. Control experiments demonstrated that an unrelated single-stranded oligonucleotide did not affect *Hae*III cleavage of the HIV-1 LTR PCR product (Figure 4, lane f). Taken together, these data indicate that the simultaneous addition of chromomycin and Sp1(HIV-1)TFO increases the inhibitory effects of these drugs on DNA cleavage by *Hae*III.

Inhibition of interactions between Sp1 and Sp1 mer by chromomycin and Sp1(HIV-1)TFO

When a ^{32}P-labelled double-stranded oligonucleotide mimicking the Sp1 binding site of the HIV-1 LTR was mixed with nuclear factors from Jurkat cells, a limited number of major retarded bands were observed (Figure 5, lane C). This is in agreement with the observation that Sp1 interacts with a variety of eukaryotic nuclear factors [6,16]. When the band-shift experiment was conducted at low concentrations of chromomycin (0.5, 1 and 2 μM), only a small inhibition of protein–DNA interactions was observed (Figure 5, lanes a–c). Higher concentrations of chromomycin fully suppressed the binding of nuclear factors to the Sp1 mer (results not shown; and [35]). Figure 5 (lanes d–e) shows that

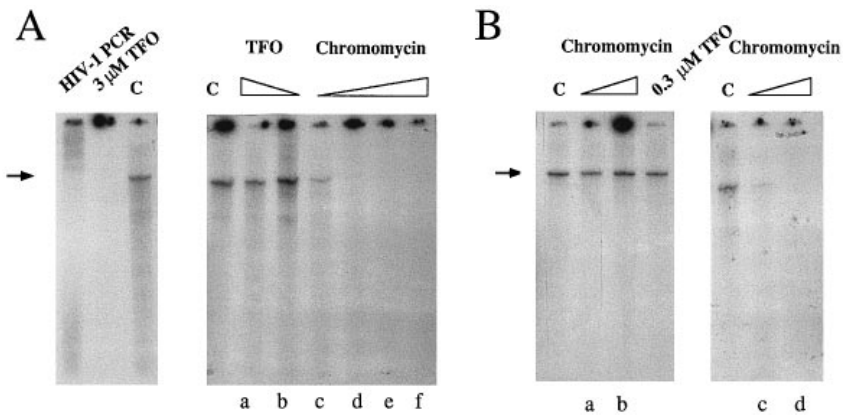

Figure 7 Effects of chromomycin and Sp1(HIV-1)TFO on HIV-1-directed *in vitro* transcription

(**A**) *In vitro* transcription was carried in the absence of both chromomycin and Sp1(HIV-1)TFO (lanes C) or in the presence of Sp1(HIV-1)TFO (left-hand panel, 3 μM; lanes a and b, 0.3 and 0.03 μM respectively) or chromomycin (lane c, 2 μM; lane d, 4 μM; lane e, 8 μM; lane f, 10 μM). The effect of the addition of 80 ng of HIV-1 PCR product is also shown in the left-hand panel. (**B**) *In vitro* transcription reactions performed in the absence of both chromomycin and Sp1(HIV-1)TFO (lanes C) or in the presence of 0.3 μM Sp1(HIV-1)TFO; 0.5 μM (lane a) or 1 μM (lane b) chromomycin; or 0.3 μM Sp1(HIV-1)TFO plus 0.5 μM (lane c) or 1 μM (lane d) chromomycin. The HIV-1-LTR-directed RNA transcript is arrowed.

0.15–0.3 μM Sp1(HIV-1)TFO (50–100 ng in the binding reaction) only partially inhibited the generation of protein–DNA complexes. Control experiments performed with unrelated double-stranded oligonucleotides demonstrated no inhibition of protein–DNA interactions (results not shown).

Figure 5 (lane f) clearly demonstrates that addition of both 2 μM chromomycin and 0.15 μM Sp1(HIV-1)TFO resulted in marked inhibition of the binding of nuclear factors to the Sp1 mer. These results suggest that the combined use of chromomycin and Sp1(HIV-1)TFO increases the inhibitory activities of these drugs on the interaction between Sp1 and Sp1 mer.

This conclusion is further supported by the experiments shown in Figure 6, in which purified Sp1 was incubated for 15 min in the presence of ^{32}P-labelled Sp1 mer. After binding, increasing concentrations of Sp1(HIV-1)TFO were added in the absence (Figure 6A, lanes a–c) or in the presence (Figure 6B, right-hand panel, lanes a–c) of increasing amounts of chromomycin. Under these experimental conditions, 50 nM and 0.1 μM Sp1(HIV-1)TFO were found to fully suppress interactions between Sp1 and Sp1 mer (results not shown), 15 nM TFO was able to partially inhibit binding (Figure 6A, lane c), and concentrations of 0.15–0.3 nM were ineffective (Figure 6A, lanes a and b). In the absence of TFO, 0.25–1 μM chromomycin did not suppress these interactions (Figure 6B, left-hand panel, lanes a and b). Interestingly, the combined use of 0.5 or 1 μM chromomycin plus 3 nM Sp1(HIV-1)TFO strongly inhibited interactions between Sp1 and Sp1 mer (Figure 6B, right-hand panel, lanes b and c), confirming the results obtained with crude nuclear factors. These results were supported in a similar experiment (Figure 6C). No inhibition of the Sp1–Sp1-mer interaction occurred in the presence of 0.25–1 μM chromomycin unless 3 nM Sp1(HIV-1)TFO was added. The results shown in Figures 6(B) and 6(C) were reproduced in two similar independent experiments (results not shown).

Taken together, the data shown in Figures 5 and 6 strongly suggest that the combined use of low concentrations of chromomycin and Sp1(HIV-1)TFO increases the inhibitory activities of these drugs on the interaction between Sp1 and Sp1 mer compared with their effects when used alone.

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 [10,43]. The pT$_z$IIICAT plasmid was cleaved with *Nco*I, in order to generate a DNA template able to produce a sizeable RNA transcript (arrowed in Figure 7). In agreement with results published elsewhere [10], binding of Sp1 was required for transcription, as 3 μM Sp1(HIV-1)TFO completely suppressed transcription (Figure 7A, left-hand panel). Control experiments demonstrated that 80 ng of the HIV-1-F/HIV-1-R PCR product was also able to fully suppress *in vitro* transcription directed by the HIV-1 LTR (Figure 7A, left-hand panel). In contrast, no inhibition of transcription was obtained when 0.03–0.3 μM Sp1(HIV-1)TFO was used (Figure 7A, lanes a and b). When *in vitro* HIV-1 transcription was performed in the presence of increasing amounts (2–10 μM) of chromomycin (Figure 7A, lanes c–f), inhibitory effects were clearly detectable. However, it should be noted that suppression of *in vitro* HIV-1-LTR-directed transcription was obtained at 4–10 μM chromomycin (Figure 7A, lanes d–f), whereas 60–75% inhibition was obtained at 2 μM chromomycin (Figure 7A, lane c). In contrast, chromomycin was not able to inhibit transcription when added at 0.5 or 1 μM (Figure 7B, lanes a and b).

When 0.5 or 1 μM chromomycin and 0.3 μM Sp1(HIV-1)TFO were added together in the *in vitro* transcription assay, suppression of HIV-1-LTR-directed transcription was observed, indicating that TFO and DNA binding drugs can co-operate in inhibiting biological functions (Figure 7B, lanes c and d).

DISCUSSION

A number of reports have suggested that DNA binding drugs (such as berenil, CC-1065, distamycin, mithramycin and chromomycin) inhibit the formation of complexes between DNA and nuclear proteins (reviewed in [28]) and therefore can be considered as potential modifiers of transcription. For instance, distamycin and its analogues DAPI, Hoechst 33258 and netropsin inhibit the interaction between transcription factor II-D and DNA, as determined by gel-retardation assays and DNase I footprinting

[25]. Of the DNA binding drugs studied, mithramycin and chromomycin were found to bind G+C-rich sequences of the Sp1 binding site and protect these promoter regions from cleavage by DNase I in a variety of experimental systems [26,33–35]. Interestingly, these sites are also the molecular target of a TFO which, as already reported, is able to inhibit HIV-1 transcription [10].

In agreement with the pivotal role of transcription in the activation of viral genes and of cellular genes involved in human pathologies [16,51–55], including neoplastic diseases [56], DNA binding drugs are potential anti-viral and anti-tumour agents [20,28]. Chromomycin has been described as a minor-groove-binding drug [57,58], whereas TFOs bind to the major groove of the DNA [1–3].

In the present study we determined whether chromomycin affects the activity of a TFO that recognizes the Sp1 binding sites of the HIV-1 LTR. Our results demonstrate that low concentrations of chromomycin able to bind to double-stranded Sp1 sites of the HIV-1 LTR do not interfere with the generation of complexes between double-stranded Sp1 DNA and Sp1(HIV-1)TFO. This was firmly established by experiments employing (i) magnetic capture of the Sp1 triple helix and (ii) BIA using SPR and biosensor technologies. In particular, 2.5 μM chromomycin allowed the formation of complexes between HIV-1 Sp1 target DNA sequences and the Sp1(HIV-1)TFO. In contrast, high levels of chromomycin inhibited triplex formation and affected triplex stability, as recently reported by Vigneswaran et al. [36].

The main conclusion of our experiments is that low concentrations of chromomycin potentiate the effects of an Sp1-specific TFO in inhibiting both Sp1–DNA interactions (Figures 5 and 6) and HIV-1-LTR-directed transcription (Figure 7). Different amounts of Sp1(HIV-1)TFO were required to inhibit molecular activities such as cleavage by restriction enzymes (Figure 4), interactions between nuclear factors and Sp1 mer (Figure 5), interactions between purified Sp1 and Sp1 mer (Figure 6), and *in vitro* transcription (Figure 7). This is not unexpected, since the experimental conditions are clearly different. However, chromomycin was always able to potentiate the activity of the Sp1(HIV-1)TFO. These data could be explained by a co-operative effect of chromomycin and TFO; neverthless, additive effects could not be ruled out.

The results presented here have both theoretical and practical implications. From the theoretical point of view, our data support the hypothesis that both DNA binding drugs and TFOs can be considered as sequence-selective modifiers of DNA–protein interactions, possibly leading to specific alterations in biological functions. From the practical point of view, our results suggest that the combined use of chromomycin and TFOs recognizing Sp1 binding sites could be proposed in order to abolish the biological functions of promoters (such as the HIV-1 LTR) whose activity is dependent on the interactions of the promoter-specific transcription factor Sp1. As far as the latter point is concerned, it should be underlined that, in addition to target genes, most housekeeping genes also exhibit functional Sp1 binding sites in their promoters. For this reason, the use of low concentrations of G+C-rich-selective DNA binding drugs could be a reasonable approach if their activity is potentiated by the simultaneous administration of promoter-specific TFOs.

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