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# Different members of the Sp1 multigene family exert opposite transcriptional regulation of the long terminal repeat of HIV-1

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## ABSTRACT

**Recently, a family of transcription factors structurally related to Sp1 has been described; thus, more than one activator may bind to the GC boxes present in a number of viral and cellular promoters. We have compared the transactivation potentials of Sp1, Sp3 and Sp4 proteins on the human immunodeficiency virus type 1 (HIV-1) promoter. The long terminal repeat (LTR) of HIV-1 contains three binding sites for the transcription factor Sp1 (GC boxes) which are involved in both basal and Tat-mediated transcriptional activation. Moreover, a cooperative interaction between NF-κB and Sp1 is required for HIV enhancer activation. We now demonstrate that Sp4 is an activator, while the Sp3 protein represses basal expression of HIV promoter. Remarkably, we found that over-expression of the transcription factor Sp3 was able to suppress Tat-mediated transactivation. These inhibitory effects of Sp3 correlate with its DNA binding activity, suggesting that Sp3 inhibition involves competition with Sp1 for occupancy of the GC boxes. Next, we have analyzed the role of different Sp1-related proteins in the stimulation of HIV-1 promoter in response to mitogens. We found that the binding of NF-κB is not by itself sufficient to induce HIV gene expression. Instead, an interaction between NF-κB and the *trans*-acting domain (A domain) of Sp1 bound to an adjacent site must occur. We found that the cooperative interaction between NF-κB and Sp1 is highly specific, since neither Sp3 nor Sp4 is capable of cooperating with NF-κB.**

## INTRODUCTION

Regulation of RNA polymerase II-dependent promoters is largely governed by specific DNA binding proteins that are thought to help assemble and/or engage the transcription complex. Extensive studies on the DNA binding properties of the transcription factors revealed that most of the target sequences are recognized by multiple DNA binding proteins. Presence of a set of factors that interact with common binding sites raised the cogent question of how a promoter is regulated by different transcription factors with a similar DNA binding specificity. The human transcription factor Sp1 has long been thought to be a unique GC box binding protein; the GC box sequence is one of the most widely distributed promoter elements in cellular and viral genes. Recently, two groups have independently reported the isolation of cDNAs encoding Sp1-related proteins, indicating the presence of a novel Sp1 multigene family. To date, three Sp1-related proteins, which have been called Sp2, Sp3, Sp4 have been described (1,2). Like Sp1, all three proteins are expressed ubiquitously, they contain zinc fingers and glutamine- and serine/threonine-rich amino acid stretches similar to those of Sp1. The DNA binding domains of the Sp1, Sp3 and Sp4 proteins are highly conserved and they recognize GC boxes with specificity and affinity closely similar to that of Sp1 (1,2). Clearly, the existence of proteins similar to Sp1 indicates that gene regulation by Sp1 is more complicated that previously assumed.

In this paper we began to explore the transcriptional properties of different members of the Sp1 gene family in the regulation of the HIV-1 promoter. Transcription initiation of the HIV-1 promoter is a complex process involving interplay between *cis*-acting regulatory sequences present in the viral LTR and *trans*-acting cellular transcription factors as well as a viral transactivator, the Tat protein (3–9). Mutational analysis of the contributory role of the various elements of the HIV-1 promoter indicated that Tat-mediated activation requires cooperation with promoter-bound cellular transcription factors (10,11). A number of studies have shown that transcriptional activation by Tat from both TAR and GAL4 binding sites displays a marked synergy with the transcription factor Sp1 (12,13). Moreover, it has been recently reported that a cooperative interaction between NF-κB and Sp1 is required for HIV-1 enhancer activation (14). Finally, mutation analyses of LTR in the context of full-length proviral DNA have indicated the contributory role of the Sp1 binding motifs in the replicative capacity of HIV-1 (15–17). From the above-mentioned considerations, the GC sites present in HIV-1 LTR appear to be involved in several aspects of the HIV-1 cell

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cycle: (a) regulation of basal transcription; (b) functional interaction with NF-κB in enhancer activation; (c) a synergistic role in Tat-mediated transactivation; (d) a contributory role in retroviral replication.

In this report we demonstrated that the Sp4 protein is a transcriptional activator of HIV-1 LTR promoter, and it is able to support Tat function. Conversely, the Sp3 protein markedly represses the HIV-1 promoter activity. Notably, we found that over-expression of the transcription factor Sp3 suppresses Tat-mediated transactivation. These inhibitory effects of Sp3 correlate with its DNA binding activity, suggesting that Sp3 inhibition involves competition with Sp1 for occupancy of the GC boxes. From these results it appears that both Sp1 and Sp4 are activators, whereas Sp3 is a dominant suppressor of both basal and Tat-mediated activation of HIV gene expression. Next, we have analyzed the role of Sp1-like proteins in the stimulation of HIV-1 promoter in Jurkat T leukemia cells in response to mitogens. We found that an interaction between NF-κB and Sp1 is required for inducible HIV-1 gene expression. The cooperative interaction between NF-κB and Sp1 is highly specific, since neither Sp3 nor Sp4 is capable of cooperating with NF-κB. Together, these data suggest that competition between different members of the Sp1-related proteins at the GC sites may play an important role in the regulation of HIV-1 LTR expression.

## MATERIALS AND METHODS

### Plasmids

The Sp1-related cDNAs were inserted into pRc-CMV (Invitrogen) downstream of the human cytomegalovirus (CMV) immediate early promoter. The expression plasmid Sp3DBD is a fingerless Sp3 mutant, in which the 169 C-terminal codons of SP3 have been removed. A full description of these plasmids has been published elsewhere (18). The Tat expression plasmid (pSV-Tat) and the reporter pC15-CAT were kindly provided by Dr Martini (19). GAL4−SP1 containing the activating region A of SP1 inserted into pSG424 GAL4 (1−147) has been previously described (12). The GAL4−SP3 derivative was constructed by inserting a *Sma*I−*Kpn*I fragment from plasmid pBS A31416, containing the Sp3 sequences (aa 1−527), into the *Eco*RI site of pSG424 (20) via blunt ligation. In a similar manner the *Sau*I−*Sfu*I fragment from the SP4 cDNA (aa 98−621) was inserted into the *Bam*HI−*Xba*I site of pSG424 to generate GAL4−SP4. The reporter plasmids G5−83-HIV−CAT and G5−38-HIV−CAT were constructed by PCR amplification of the HIV-1 sequences from −83 to +83 and −38 to +83 with primers containing the *Xba*I site at the 5′ and the *Kpn*I site at the 3′ ends respectively and subsequent insertion of the PCR products between the *Xba*I and *Kpn*I sites of the G5E1bCAT reporter (12). G5LTR−HIV contains 5 GAL4 DNA binding sites (*Xba*I−*Hin*dIII fragment of G5E1B) cloned in both orientations (G5′LTR−HIV in opposite orientation) via blunt ligation into the filled *Xho*I site (−700) of pC15-CAT. LTR−HIVG5 was constructed by blunt ligation of the *Xba*I−*Hin*dIII fragment of G5E1B containing 5 GAL4 DNA binding sites into the filled *Bam*HI site (+1600) of pC15-CAT. The κB−G5-HIV reporter was constructed by inserting an oligonucleodide containing the κB sequences (from nt −106 to −78), flanked by *Xho*I (5′) and *Pst*I (3′) sites, in the corresponding sites of the G5−38-HIV−CAT, immediately upstream of the GAL4 sites. The mutant 5′ κB−G5-HIV contains a mutation of the 5′ κB sites by changing, AGGGACTTTCC to AGATCTCTTTCC; the 3′ κB-G5-HIV plasmid contains a mutation of the 3′ B site, GGGGACTTTCC to GAGATCTTTCC. ΔκB−G5-HIV contains both κB mutations. pPacSp1 (21), a Sp1 expression plasmid was kindly provided by Dr R.Tjian. pPacSp3 and the pPacSp3DBD, a fingerless Sp3 mutant in which the 169 C-terminal codons of SP3 have been removed, have been described elsewhere (18). All plasmids were analyzed by DNA sequencing to confirm correct construction. Full details of each construction are available upon request.

### Transfection and CAT assays

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Transfections were performed by calcium phosphate precipitation using subconfluent cell cultures. For normalization of transfection efficiencies a β-Gal expression plasmid was included in the co-transfections (pSV-β-Gal expression plasmid; Promega). Each set of transfections was repeated at least 3−5 times with different plasmid preparations. Transfections using DEAE dextran with Jurkat T leukemia cells were performed as described (22). Twenty hours after transfection the cells were stimulated with 10 ng/ml PMA and 2 mg/ml PHA (Sigma Chemical, St Louis, MO). Sixteen hours later the cells were harvested for CAT assays. *Drosophila melanogaster* SL2 cells were grown in Schneider's medium supplemented with 10% heat-inactivated fetal calf serum, and transfected by calcium phosphate precipitation as previously described (23). Cells were harvested 48 h after addition of the DNA, and extracts were assayed for CAT activity. For normalization of transfection efficiencies a β-Gal expression plasmid (pB97, kindly provided by P.P.Di Nocera) was included in the co-transfections. The CAT activity was quantified by counting the amount of [$^{14}$C]chloramphenicol converted to the mono-acetylated form using the Molecular Dynamics PhosphorImager™ system.

### Electrophoretic mobility shift assays

Gel shift assays were performed with radiolabeled GC binding sites of the HIV-1 LTR (nt −77 to −45). The oligonucletides were synthesized on an Applied Biosystems automated DNA synthesizer, deprotected and gel purified. Oligonucletides were labeled with kinase. Jurkat and HeLa nuclear extracts and DNA binding assays were performed as described (18,24). For DNA binding assays in which antibodies were included, antibodies were added to the binding assay mixtures and incubated for 30 min in ice before addition of radiolabeled oligonucleotide. Polyclonal rabbit antisera against Sp1 and Sp3 proteins were generated using bacterially expressed Sp1 and Sp3 proteins as previously described (1,18). EMSA experiments and Western blot analysis reported elsewhere indicated that antibodies against Sp3 did not cross-react with bacterially expressed Sp1 and vice versa (1,18).

### *In vitro* transcription

*In vitro* transcription experiments were performed using the HeLa cell extract transcription kit from Promega. Transcription products were analyzed by primer extension using a CAT primer as previously described (25). Reaction products were resolved on a 6% polyacrylamide−8 M urea gels. Sequencing ladders were generated by the dideoxy chain termination method. For *in vitro* transcription assays in which antibodies were included, antibodies were added to the nuclear extracts and incubated for 40 min in ice before addition of DNA template. Immunodepletion of nuclear extracts was monitored by EMSA using radiolabeled

oligonucleotide (nt −77 to −45) containing the GC binding sites of the HIV-1 LTR. The Molecular Dynamics PhosphorImager™ system was used to evaluate the extended products.

## RESULTS

### Various members of the Sp1 family exhibit different transcriptional properties on HIV-1 LTR

To directly compare the transactivation potentials of Sp1, Sp3 and Sp4 on HIV-1 promoter, CMV expression plasmids encoding each of these proteins were transfected in HeLa cells together with a CAT reporter plasmid containing the full-length HIV-1 LTR (pC15LTR-CAT). Consistent with prior reports we found that Sp1 induced transcription of HIV-1 LTR. Sp4 was also able to increase LTR transcription, whereas Sp3 was not able to activate the HIV-1 promoter and even slightly repressed this reporter (Table 1). To confirm the specificity of these biological effects, and since the transfection experiments were performed in mammalian cells, which contain endogenous Sp1, we sought to analyze the role of these transcription factors in a heterologous system. The *Drosophila melanogaster* Schneider cell line SL2 is devoid of endogenous Sp1-like activity and so serves as a useful cell to examine Sp1-mediated activation (18,21,26,27). SL2 cells were transfected with expression vectors (pPac) for Sp1 and Sp3 together with the HIV-1 LTR reporter plasmid, respectively (Table 1). In these cells Sp1 enhanced the CAT activity; in contrast, Sp3 did not activate the HIV-1 LTR reporter plasmid.

### Sp3 represses Sp1-mediated transcription of HIV promoter

The distinct transactivation potential of Sp1 versus Sp3 prompted investigation of whether Sp3 might negatively regulate Sp1-mediated transcriptional activation of HIV-1 LTR. To explore the potential functional consequences of the over-expression of Sp1 and Sp3 proteins, HeLa cells were co-transfected with the pC15LTR-CAT reporter and a mixture of Sp1 and Sp3 expression vectors. We found that co-expression of Sp3 markedly repressed the Sp1-mediated stimulatory effect in a dose-dependent manner (Figure 1A). No antagonistic effect was observed with Sp3DBD, a deletion mutant of Sp3 lacking the C-terminal region containing the zinc finger region required for DNA binding activity (18). A similar experiment was also performed in *Drosophila* SL2 cells. As reported in Figure 1B, co-expression of Sp3 in these cells markedly repressed the Sp1-mediated stimulatory effect in a dose-dependent manner. No repression was observed with a deletion mutant of Sp3 (pPacSp3DBD) lacking the C-terminal region containing the zinc finger region required for DNA binding activity. The results obtained in both HeLa and *Drosophila* cells indicated that Sp3 represses Sp1-mediated transcriptional activation of HIV-1 LTR promoter, and the Sp3-mediated repression is dependent on the presence of the zinc finger DNA binding motifs.

**Table 1.** Effects of Sp1-related proteins on the expression of CAT activity driven by the HIV-1 promoter

| Effector | HeLa cells | | *Drosophila* cells | |
|---|---|---|---|---|
| | CAT activity | Fold activation | CAT activity | Fold activation |
| Sp1 | 18 | 12 | 125 | 125 |
| Sp3 | 0.4 | 0.2 | 0.6 | 0.6 |
| Sp4 | 17 | 11 | ND | ND |
| None | 1.5 | 1 | 1 | 1 |

2 μg of pC15HIV-CAT reporter were co-transfected into HeLa cells with 8 μg of the indicated Sp-related cDNAs inserted downstream of the CMV immediate early promoter. Transfections in *Drosophila* cells were performed with 2 μg of pC15HIV-CAT together with of the indicated Sp-related cDNAs inserted downstream of the pPac vector (2 μg). Transfections were performed by calcium phosphate precipitation in a total of 20 μg adjusted with the parental vector without insert. The results are presented as the mean of six independent transfection experiments with a standard deviation < 10%.

### Both Sp1 and Sp3 bind to the HIV-1 GC sites

Prior studies have indicated that the bacterially produced Sp1 and Sp3 proteins recognize GT and GC boxes with identical affinities (1,2). Accordingly, we did not detect any significant differences in binding affinities of bacterially produced Sp1 and Sp3 on the three GC boxes present in the HIV-1 LTR. It has recently been shown that Sp3 is abundant in many mammalian cell lines, and both Sp1 and Sp3 proteins bind to the GT and GC motif with similar if not identical affinities (18). Since it is well documented that HeLa cells contain Sp1 protein which is able to bind the HIV-1 GC boxes (6,9), we sought to determine whether Sp3 protein binds to the HIV GC motif. EMSA experiments were performed using HeLa and Jurkat T cell nuclear extracts following incubation with a labeled oligonucleotide containing the three GC boxes present in the HIV-1 LTR, in the presence or absence of specific Sp1 and Sp3 antibodies as previously described (18). As reported in Figure 2A, two specific complexes were observed (lane 1). Complex I appears to be composed of two very closely retarded bands. In fact, addition of specific anti-Sp1 antibody in the binding reaction results in reduction of intensity of this complex, while complex II was not altered (lane 2). Addition of Sp3-specific antibody in the binding reaction resulted in the absence of complex II (lane 3). Inclusion of both Sp1 and Sp3 antibodies in the binding reaction resulted in the absence of both complexes (lane 4). From the results reported in Figure 2A, it appears that complex II is due to Sp3 binding, while complex I may be resolved into two distinct DNA−protein complexes due to specific interaction with Sp1 and Sp3, respectively. In addition, both complexes were competed with similar efficiencies by unlabeled oligonucleotide (Fig. 2B). Next,

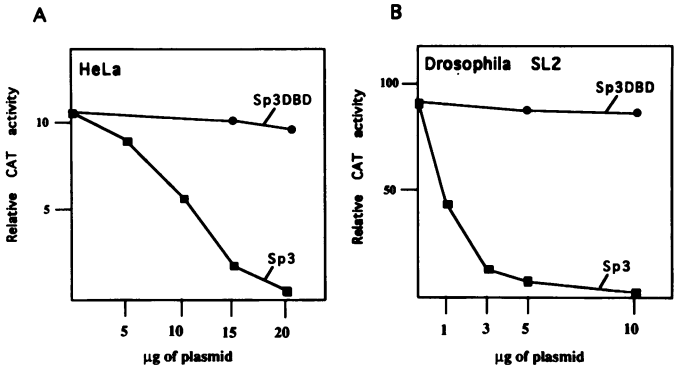


**Figure 1.** Sp3 represses Sp1-mediated activation of HIV-1 promoter in HeLa and *Drosophila* cells. (**A**) The reporter plasmid pC15HIV-CAT (2 μg) was transfected into HeLa cells with constant amount of pCMV-Sp1 (8 μg) in the presence of increasing amounts of pCMV-Sp3 or pCMV-Sp3DBD plasmid DNAs as indicated. (**B**) *Drosophila* Schneider SL2 cells were transfected with pC15HIV-CAT (4 μg) reporter plasmid in the presence of a constant amount (0.2 μg) of pPac-Sp1 together with increasing amounts of the effector plasmids pPac-Sp3 or pPac-Sp3DBD as indicated in a total of 20 μg adjusted with the parental pPac expression vector without insert.

we sought to determine the relative amount of Sp1 and Sp3 in Jurkat T cells using nuclear extracts prepared in the presence and absence of PMA stimulation. A similar pattern of DNA binding specificity was observed using both extracts (Figure 2C). These data indicate that both Sp1 and Sp3 proteins are expressed in HeLa and Jurkat cells, and both proteins bind specifically and with similar affinities to the HIV-1 LTR GC motifs; in addition, the relative amount of Sp1 and Sp3 proteins did not change after PMA stimulation.

### *In vitro* transcription of HIV promoter using immunodepleted extracts

To further substantiate the notion that Sp3 causes a dominant negative effect on Sp1-stimulated expression of HIV-1 promoter, we sought to analyze the *in vitro* transcription of HIV-1 promoter using immunodepleted nuclear extracts. HeLa nuclear extracts were pre-incubated with: (i) pre-immune serum; (ii) anti-Sp1 serum; and (iii) anti-Sp3 serum. Immunodepletion of Sp1 and Sp3 proteins from nuclear extracts was monitored by EMSA experiments, as those described in Fig. 2 (data not shown). Two different templates were used. The −83HIV-CAT, which contains the LTR sequence from −83 to +83, and the −38-HIV−CAT, in which the CAT gene is under the control of the HIV-1 promoter sequence from −38 to +83. The latter plasmid does not contain the GC boxes. The *in vitro* transcripts were analyzed by primer extension from a primer complementary to the CAT mRNA sequences as previously described (25). *In vitro* transcription assays were repeated several times using two different batches of HeLa nuclear extracts. An example of the transcription pattern observed is reported in Fig. 3. Immunodepletion of Sp1 depresses basal transcription of the G5−83-HIV−CAT (Fig. 3, compare lanes 2 and 3), whereas immunodepletion of Sp3 resulted in a modest but reproducible increased transcription (2- to 3-fold) (compare lanes 1 and 2), No significative effects were observed with pre-immune serum (lane 4). As expected, the low basal level of transcription of −38-HIV−CAT was unaffected by the presence of the Sp antiserum (lanes 5−7).

### Sp3 represses Tat-mediated activation

The Sp1 sites have been shown to most effectively augment Tat function and it has been suggested that Tat functions in concert

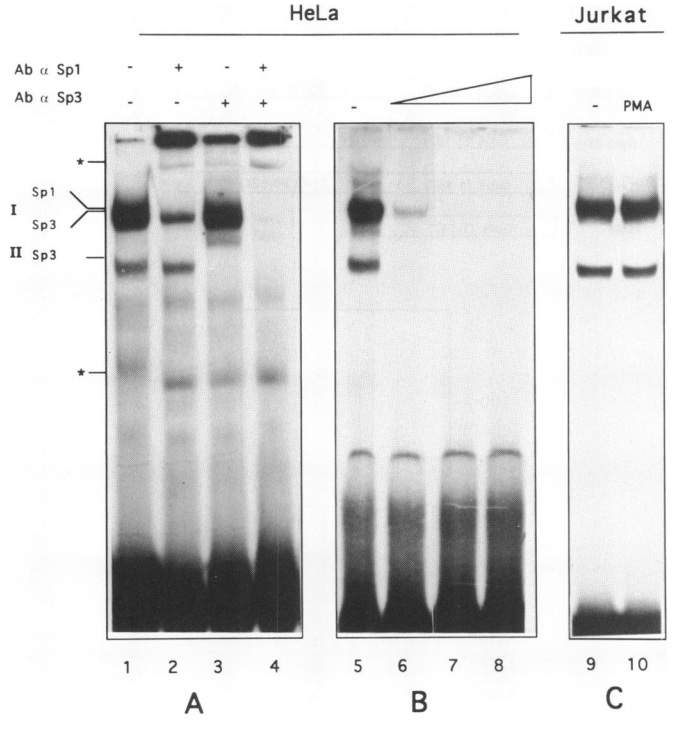


**Figure 2.** Sp1 and Sp3 bind to HIV GC boxes. (**A**) Gel mobility shift analysis was performed by using a labeled oligonucleotide spanning the HIV 1 LTR (nt −77 to −45) and nuclear extracts from HeLa cells. The presence of specific antibody in the binding reaction is indicated above each lane. Specific complexes for Sp1 and Sp3 are indicated on the left. Non-specific complexes which are also seen with the pre-immune serum (data not shown) are indicated by asterisks. (**B**). HeLa cell extract (3 μg) was incubated with labeled oligonucleotide in the absence (lane 5) or in the presence of various amounts (10-, 50- and 100-fold molar excess) of unlabeled oligonucleotide (lanes 6−8). (**C**) Cell extract (3 mg) from Jurkat T cells prepared from unstimulated and PMA-stimulated cells (lanes 9 and 10) were incubated with the HIV GC site-containing oligonucleotide.

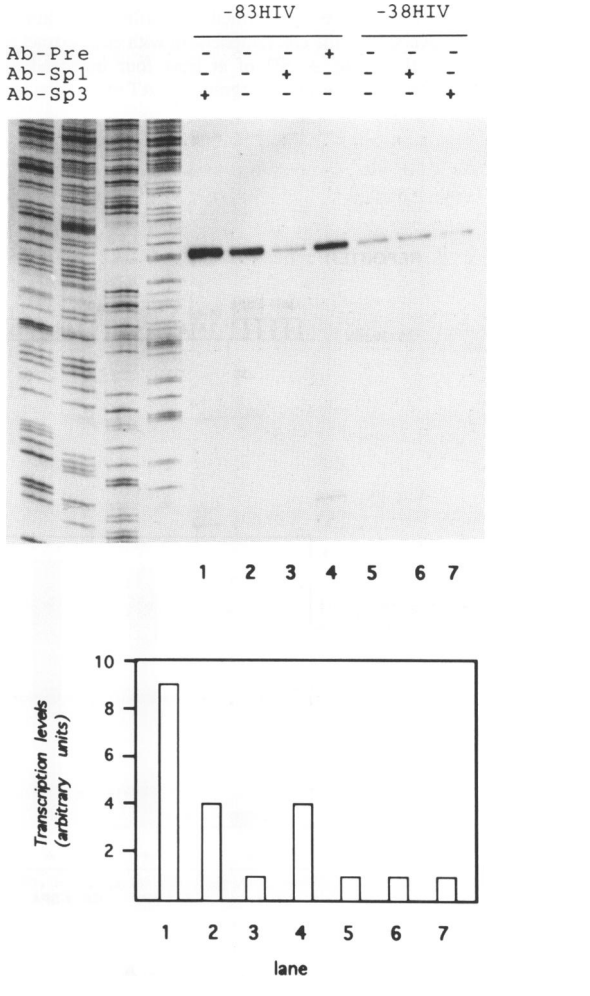


**Figure 3.** Transcriptional activity of HIV-1 promoter in immunodepleted HeLa nuclear extracts. RNA synthesized *in vitro* was detected by primer extension analysis using a primer complementary to the CAT coding region. The presence of specific antibody in the nuclear extract is indicated above each lane. Immunodepleted extracts were analyzed for the presence of Sp1/Sp3 proteins by EMSA as described in Fig. 2. Transcription levels were quantitated using a phosphorimager and are presented below in bar graph form. Data collected over multiple independent experiments (3−5) using different preparations of nuclear extracts showed similar results.

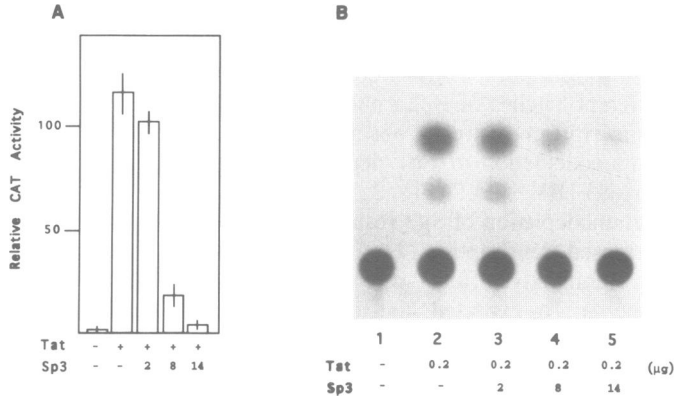


**Figure 4.** Repression by Sp3 of Tat activation. (**A**) HeLa cells were transfected with the reporter plasmid pC15HIV-CAT (2 μg) alone and together with 0.2 μg of Tat expression plasmid DNA in the presence of an increasing amount of pCMV-Sp3 plasmid. CAT assays were performed with different amounts of extracts to ensure linear conversion of the chloramphenicol with each extract and the results are presented as the mean ± SD of at least four independent transfection experiments. A representative autoradiogram of CAT expression is shown in (**B**).

with the Sp1 transcription factor (10–13,28). Since Sp3 represses Sp1, we wished to determine whether Sp3 influences Tat activation. HeLa cells were transfected with the LTR–CAT reporter together with a Tat expression plasmid in the presence of an increased amount of CMV-driven Sp3 expression plasmid. As expected, the presence of Tat expression plasmid alone produced a strong stimulation of HIV-1 promoter activity. However, co-expression of Sp3 in these cells markedly repressed the Tat-mediated stimulatory effect in a dose-dependent manner (Fig. 4). In addition, Tat response was unaffected by the presence of the Sp3 deletion mutant lacking the zinc finger region required for DNA binding activity (data not shown), suggesting that Sp3-mediated repression is strictly dependent on the presence of the zinc finger DNA binding motifs.

## Transcriptional properties of Sp–Gal4 derivatives

Since the expression of both endogenous Sp1 and Sp3 proteins in HeLa cells might interfere with the effect of exogenous Sp3, we further addressed the Sp3 role in Tat transactivation using the well-defined GAL4-based fusion proteins system. For this experiment, we constructed two plasmids that express Sp3 amino acids 1–527 and Sp4 amino acids 98–621 fused to Gal4 DNA

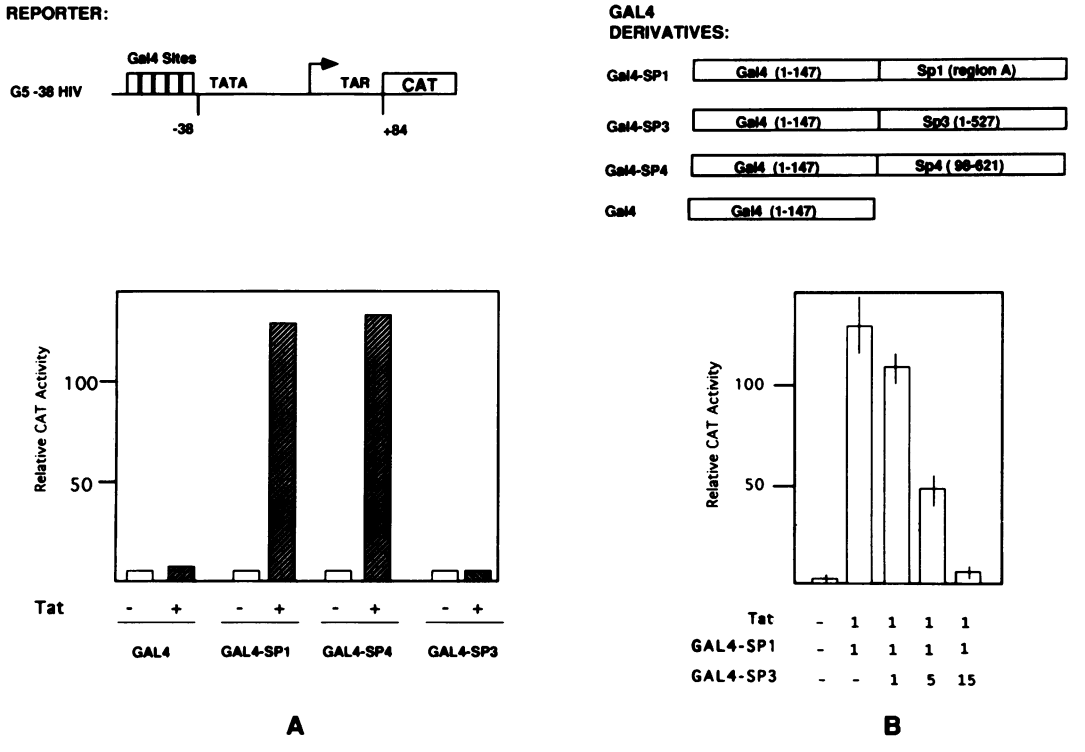


**Figure 5.** Effect of GAL4 derivatives on Tat activation. Five GAL4 DNA binding sites were cloned upstream of the TATA box in the (–38) HIV LTR to generate G5–38 HIV–CAT. The GAL4 (1–147) and the GAL4–SP1 expression plasmids have been described elsewhere. (**A**) The N-terminal half of Sp3 (1–527) and Sp4 (98–621) of the coding region of SP3 and SP4 cDNAs were fused in frame to the DNA binding domain of GAL4, respectively. G5–38-HIV–CAT (1 μg) was co-transfected into HeLa cells together with a GAL4 derivative expression plasmid (4 μg) in the presence or absence of pSV-Tat (1 μg), and CAT activity was quantified. (**B**) G5–38-HIV–CAT (1 μg) was co-transfected into HeLa cells together with pSV-Tat (1 μg) and GAL4–SP1 (1 μg) in the presence of increasing amounts of GAL4–SP3 expression plasmid. For all the experiments reported the CAT activity was quantified using appropriate amount of extract to ensure linear conversion of the chloramphenicol with each extract. The values represent the average of at least four independent transfections after normalization for the internal control β-galactosidase activity.

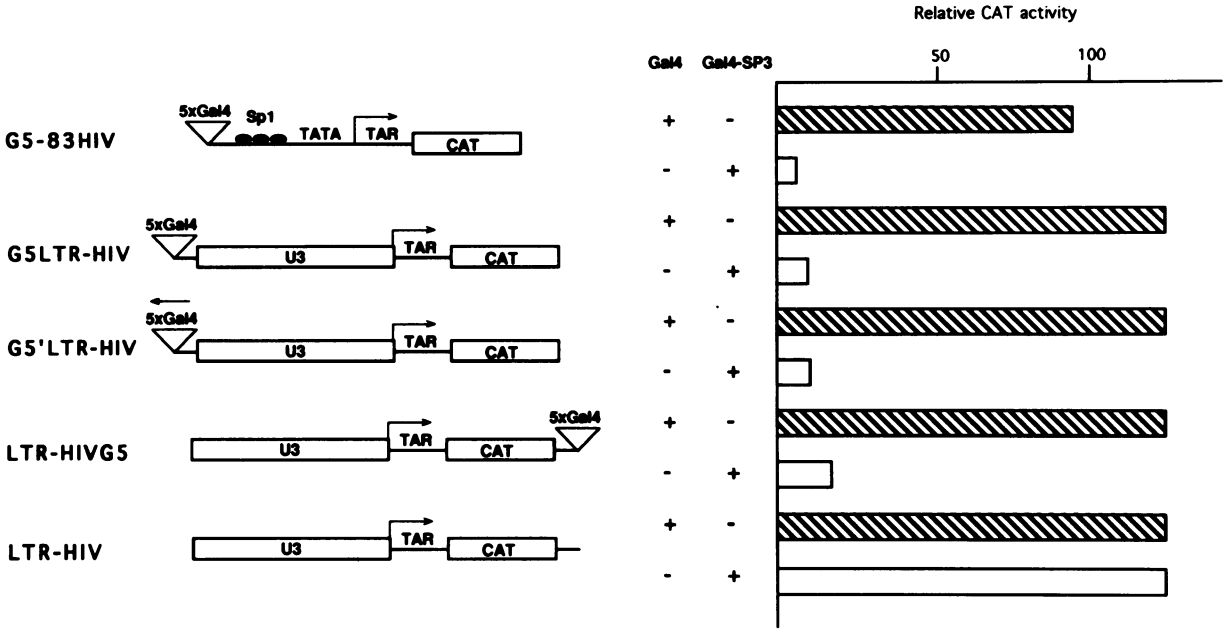


**Figure 6.** Sp3 represses transcription in a distance-independent manner. The HIV reporters (1 μg) were co-transfected into HeLa cells with pSV-Tat (1 μg) in the presence of control Gal4 (1–147) (open bars) or GAL4–SP3 (10 μg) (dashed bars). The relative CAT activities in the absence and presence of the GAL4–SP3 plasmid are reported. These results were representative of four independent transfections. Standard deviations were <10%.

binding domain, respectively. The GAL4–SP1 plasmid expresses the glutamine-rich A domain of Sp1 (12). The fusion proteins encoded by these plasmids are dependent upon the GAL4 DNA binding domain for DNA binding, since the zinc finger DNA binding domain of each Sp1-like protein has been deleted. We tested the ability of the Gal4–Sp1, GAl4–Sp3 and GAl4–Sp4 fusion proteins to support a Tat response using as reporter a plasmid containing five GAL4 DNA binding sites inserted immediately upstream of the HIV-1 LTR TATA box (G5–38-HIV–CAT). Each of the GAL4 derivatives was co-transfected with the reporter plasmid into HeLa cells in the absence or presence of the Tat expression plasmid. We found that the activating domains of Sp1 and Sp4 were able to support a Tat response, whereas Gal4–Sp3 failed to cooperate with Tat (Fig. 5A). Furthermore, co-expression of both Gal4–Sp1 And Gal4–Sp3 in the presence of Tat resulted in repression of Tat function (Fig. 4B), suggesting that Gal4–Sp3 inhibition of the Tat response involved competition with Gal4–Sp1 for occupancy of the Gal4 DNA binding sites. The experiments reported in Figure 5 did not prove that Sp3 possesses intrinsic repressing activity. In fact, a similar dominant negative effect was also observed using as effector the GAL4 (1–147) control vector (data not shown). Therefore, Sp3-mediated repression of both basal and Tat-mediated activation of HIV-1 promoter may occur by mechanisms involving either steric hindrance or inhibitory protein–protein interactions; the latter mechanism suggests that Sp3 possesses an intrinsic repressing activity. We sought to distinguish these two possibilities by determining whether Sp3 might repress transcription in a distance-independent manner. To this end the effector construct GAL4–SP3 was co-transfected with different reporter plasmids containing 5 GAL4 DNA binding sites at various distances from the start site of transcription. The GAL4 DNA binding sites were inserted at −83 (G5–83-HIV), at −700 from the HIV-1 start site in both orientations (G5LTR–HIV and G5'LTR–HIV) and at the 3′ of the CAT gene (LTR–HIV-G5). Each of the GAL4 derivatives was co-transfected with the reporter plasmid into HeLa cells in the presence of the Tat expression plasmid, together with the GAL4–SP3 effector or the control GAL4(1–147). Tat-mediated activation of each construct was strongly reduced in the presence of the Gal4–Sp3 protein, whereas no significant effects were observed using the GAL4(1–147) control effector (Fig. 6). Thus, the non-finger domain of Sp3 protein must be tethered to the DNA template in order to function as a repressor.

Together, these findings suggest that both Sp1 and Sp4 support Tat activation, whereas Sp3 transcription factor may represent a dominant suppressor of Tat-mediated activation of HIV-1 promoter.

### Interaction of NF-κB with Sp1-like proteins

The HIV-1 LTR contains two binding sites for NF-κB which are the targets of the inducible transcription factor NF-κB (29). These κB enhancer elements are recognized by a structurally related family of interactive proteins that includes p50, p65 and the product of the c-*rel* proto-oncogene (c-Rel). In most cell types NF-κB is found as an inactive form, bound to the inhibitor I-κB. Activating agents, such as phorbol esters and tumor necrosis factor alpha, promote phosphorylation and dissociation of I-κB, subsequent migration of NF-κB into the nucleus, and consequent transcriptional activation (reviewed in 30,31).

Perkins *et al.* (14) have recently reported that a cooperative interaction between NF-κB and Sp1 is required for HIV-1 enhancer activation in Jurkat T cells. Since Sp1-related proteins are expressed in Jurkat cells (Fig. 2), we sought to test the

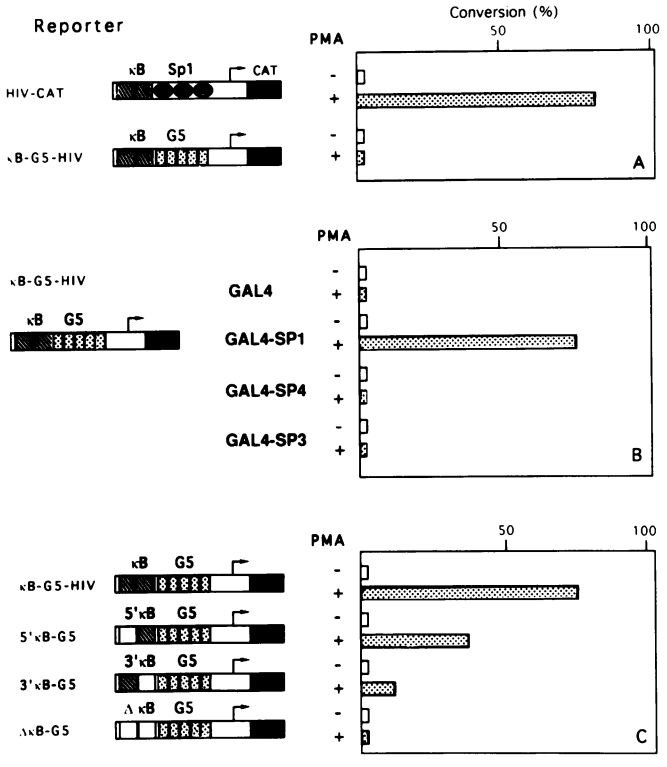


**Figure 7.** Functional interaction between NF-$\varkappa$B and Sp1 is required for HIV enhancer activation. (**A**) Jurkat T leukemia cells were transfected using DEAE dextran with the indicated reporter plasmid (4 mg) Twenty hours after transfection, cells were incubated with PMA (40 ng/ml) and PHA (2 $\mu$g/ml) for an additional 16 h. (**B**) The $\varkappa$B−G5-HIV plasmid was co-transfected with the indicated GAL4 plasmid (4 $\mu$g) and cells stimulated by PMA. (**C**) Jurkat T cells were co-transfected with the indicated plasmid (4 $\mu$g) together with the GAL4−SP1 expression plasmid (4 $\mu$g). These results were representative of at least three independent transfections.

potential transcriptional activity of Sp1, Sp3 and Sp4 in stimulation of HIV promoter. Jurkat T leukemia cells were transfected with reporter plasmids containing wild-type (HIV−CAT) and a mutant form of HIV-1 promoter ($\varkappa$B−G5-HIV) in which the three GC motifs were substituted by five GAL4 DNA binding sites. In accordance with previous results, we found that the absence of Sp1 binding motifs rendered the enhancer completely unresponsive to PMA stimulation (Fig. 7A). Next, we co-transfected the mutant $\varkappa$B−G5-HIV with different GAL4 derivative expression plasmids. We found that the presence of Gal4−Sp1 restored PMA inducibility. Conversely, the presence of Gal4−Sp3 and Gal4−Sp4 fusion proteins did not restore PMA inducibility (Fig. 7B). These results suggest that the interaction between NF-$\varkappa$B and Sp1 is highly specific. Notably, Sp4, which is able to support Tat activation, fails to cooperate with NF-$\varkappa$B. In addition, the Tat-mediated expression of $\varkappa$B−G5-HIV reporter in PMA-stimulated cells was only observed in the presence of GAL4−SP1 effector (data not shown). To further characterize the functional relationship between $\varkappa$B and the A domain present in the Gal4−Sp1 fusion protein, the GAL4-SP1 expression plasmid was co-transfected in Jurkat cells with plasmids containing different mutations of the NF-$\varkappa$B binding sites. Mutation of the 3′ $\varkappa$B element caused a 7-fold reduction in PMA stimulation, whereas mutation of the 5′ $\varkappa$B element resulted in a 2-fold reduction. Mutations of both NF-$\varkappa$B elements ($\Delta\varkappa$B−G5-HIV) abolished PMA stimulation (Fig. 7C). These data strongly suggested that $\varkappa$B-dependent inducible activation involves the specific interaction of NF-$\varkappa$B and Sp1.

## DISCUSSION

### Differential regulation of HIV-1 promoter by Sp1, Sp3 and Sp4 proteins

The results reported in this paper indicate that Sp1 and Sp4 are activators of both basal and Tat-mediated transcription of HIV-1 LTR, while Sp3 fails to activate the HIV-1 promoter and it does not support Tat activation. Further, when both Sp1 and Sp3 transcription factors are co-expressed, Sp3 markedly represses the Sp1 activating effects. Since the inhibitory effect of Sp3 is dependent on the DNA binding domain, it appears that Sp3 represses Sp1-dependent transcription of HIV-1 through interference with Sp1 binding to the GC motifs. In addition, the GAL4 system shows that Sp3 contains a repression domain which can function independently from the zinc finger DNA binding domain. Using EMSA assays we have shown that the GC motifs present in HIV-1 promoter interact specifically with both proteins in HeLa and Jurkat cells. We found that both Sp1 and Sp4 transcription factors support Tat activation. Conversely, enforced expression of Sp3 results in a marked inhibition of Tat-mediated transactivation of HIV-1 LTR. Together, these findings suggest that Sp3 transcription factor may represent a dominant suppressor of Tat. Thus, one might predict that the relative presence of Sp3 transcription factor in different cell types will influence Tat transactivation. The role Sp3 may play in regulating both basal and Tat-mediated transcription of HIV-1 promoter *in vivo* by binding to the GC binding sites is not yet clear. Since mutations of the GC binding sites are known to decrease both basal and Tat-mediated transcription (6,7,11−13), activators as such Sp1 and Sp4 appear to compete successfully with Sp3 repressor for binding to the GC sites *in vivo*. However, binding by the repressor Sp3 may modulate the degree of activation conferred by the GC binding sites.

### Repression of Tat stimulation of HIV-1 promoter at a distance by Sp3

Sp3-mediated transcriptional repression can result from competition for the same DNA binding sites or steric hindrance between repressors and positively acting transcription factors, such as Sp1. Because this type of repression results from displacement of transcription factors from the DNA, Sp3 might not necessarily possess an active repression function. However, we found that Tat-mediated activation of several HIV−LTR reporters containing five GAL4 DNA binding sites inserted at different distances from the transcription start site was abrogated by co-expression of the chimeric Gal4−Sp3 protein. Distance-independent repression observed with the Gal4−Sp3 fusion protein rules out the possibility that Sp3 is simply sterically blocking the promoter. In addition, it is unlikely that Sp3 when bound at great distances from the promoter could precisely position a nucleosome over the promoter and occlude its access to transcription factors. Repression at distance supports a mechanism requiring protein−protein interactions between distantly bound Sp3 protein and proximal factors, such as TAFs, looping out intervening DNA.

### A specific interaction between NF-κB and Sp1 is required for HIV-1 enhancer activation

The LTR of HIV-1 contains two κB enhancer elements involved in the regulation of inducible viral gene expression upon T cell activation. Regulation of transcription by NF-κB is mainly exerted by phosphoryation of I-κB gene product which allows the release of the active NF-κB binding protein into the nucleus and consequent transcriptional activation (1,9).

We have shown that an HIV promoter mutant lacking the Sp1 binding sites does not respond to PMA stimulation of Jurkat T leukemia cells. However, PMA inducibility was restored by the presence of the Gal4−Sp1 fusion protein, suggesting that a cooperative interaction between NF-κB and Sp1 is strictly required for HIV enhancer activation. Neither Sp3 nor Sp4 was able to cooperate with NF-κB for enhancer activation. We found that the mutant plasmid with an intact 5′ κB site responds poorly to PMA stimulation, whereas the mutant plasmid containing a proper 3′ κB site retains a high level of inducibility. Together these data suggest that a specific interaction between NF-κB and Sp1 is required for inducible HIV-1 gene expression, and these factors must be correctly positioned for optimal enhancer activation. Such cooperative interaction has not been observed with the Sp4 protein, although both Sp1 and Sp4 share many common structural and functional properties, i.e. both proteins contain glutamine- and serine/threonine-rich amino acid stretches, both proteins activate basal HIV transcription and they are both able to support Tat-mediated activation. Thus, the cooperation with NF-κB appears to represent a functional assay to distinguish between two very similar Sp-related transcription factors.

Altogether, our results allow depiction of a complex picture of the involvement of different Sp1-like transcription factors in HIV-1 enhancer activation, and the potential cross-talk between these factors and the Tat protein. In the uninduced state, the low basal level of transcription might be maintained by a dynamic mechanism of positive and negative regulation exerted by different members of the Sp1 gene family, with Sp3 being a potent repressor. Upon PMA stimulation, active NF-κB form becomes available in the nucleus and binds to the κB sites in close proximity to three Sp1 binding sites. However, this binding alone is not sufficient to activate transcription. Instead, a protein−protein interaction must occur between NF-κB and Sp1 protein bound to the adjacent site. We suggest that the function of NF-κB is to mediate the assembly of a functional transcription multiprotein complex on HIV promoter, and it does so by facilitating the binding of the positive transcription factor Sp1. The concomitant presence of NF-κB and Sp1 could be sufficient for the induction of a sustained rate of transcription, which will be further triggered by the synergistic action of the newly synthesized Tat protein.

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