JOURNAL OF VIROLOGY, Dec. 1997, p. 9375–9382 Vol. 71, No. 12
0022-538X/97/$04.00+0


# Repression of Human Immunodeficiency Virus Type 1 through the Novel Cooperation of Human Factors YY1 and LSF

FABIO ROMERIO, MATTHEW N. GABRIEL, AND DAVID M. MARGOLIS*

*Institute of Human Virology, Medical Biotechnology Center, University of Maryland Biotechnology Institute, Baltimore, Maryland 21201*

Received 7 April 1997/Accepted 20 August 1997

**A subpopulation of stably infected CD4$^+$ cells capable of producing virus upon stimulation has been identified in human immunodeficiency virus (HIV)-positive individuals (T.-W. Chun, D. Finzi, J. Margolick, K. Chadwick, D. Schwartz, and R. F. Siliciano, Nat. Med. 1:1284–1290, 1995). Few host factors that directly limit HIV-1 transcription and could support this state of nonproductive HIV-1 infection have been described. YY1, a widely distributed human transcription factor, is known to inhibit HIV-1 long terminal repeat (LTR) transcription and virus production. LSF (also known as LBP-1, UBP, and CP-2) has been shown to repress LTR transcription in vitro, but transient expression of LSF has no effect on LTR activity in vivo. We report that both YY1 and LSF participate in the formation of a complex that recognizes the initiation region of the HIV-1 LTR. Further, we have found that these factors cooperate in the repression of LTR expression and viral replication. This cooperative function may account for the divergent effects of LSF previously observed in vitro and in vivo. Thus, the cooperation of two general cellular transcription factors may allow for the selective downregulation of HIV transcription. Through this mechanism of gene regulation, YY1 and LSF could contribute to the establishment and maintenance of a population of cells stably but nonproductively infected with HIV-1.**

The paradigm of human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) transcriptional activation and virion production following cellular stimulation has been long established. The nuclear level of several cellular factors, most notably NF-κB, is upregulated following antigen- or lectin-induced lymphocyte activation, allowing HIV-1 mRNA expression and successful production of virions. As few infected, activated CD4$^+$ cells appear to return to the resting state (6), little attention has been given to mechanisms that repress HIV transcription and possibly allow some cells to support stable, unproductive infection. However, the regulation of proviral expression within this reservoir of infected CD4$^+$ cells may assume greater relevance as potent combination antiretroviral therapies deplete HIV from productively infected cell populations.

YY1 is a widely distributed 68-kDa multifunctional transcription factor that directly interacts with many viral and cellular nuclear factors (5, 15, 24, 30). Previously, we demonstrated by electrophoretic mobility shift assay (EMSA) that a protein complex present in HeLa nuclear extract binds the HIV-1 LTR initiation region (−17 to +27) and that this complex is specifically depleted by anti-YY1 monoclonal antibodies. Moreover, we found that expression of YY1 inhibited HIV-1 LTR transcription and virion production (18). Further studies of the YY1-containing LTR-binding complex suggested that this might be a multiprotein complex. Biochemical studies revealed the presence of a second factor that proved to cooperate in function with YY1.

Three other factors, USF (8), LSF (also known as LBP-1, UBP-1, and CP-2 [9, 12, 14, 29]), and TDP-43 (19), have been shown to bind the HIV-1 LTR within the −17 to +27 region that is also recognized by YY1. LSF (LBP-1/UBP/CP-2) is a ubiquitous factor demonstrated to directly inhibit HIV transcription (9, 11, 12, 14, 16, 28). Paradoxically, while LSF can repress HIV transcription in vitro (14), transient expression of LSF has no effect on the in vivo activity of the HIV-1 LTR (16, 28). The function of TDP-43 is unknown, while USF acts as an activator of the HIV-1 LTR (8, 19).

We find that the protein complex that recognizes the HIV-1 LTR initiation region contains a second factor in addition to YY1. Further, YY1 cooperates with this factor in inhibition of HIV-1 LTR expression and virus production. This unique interaction may allow for selective repression of HIV gene expression and could allow some cells to establish stable, nonproductive infection. The discovery of a selective mechanism of transcriptional repression may lead to targeted approaches to inhibiting HIV gene expression.

## MATERIALS AND METHODS

**Nuclear extracts.** Large-scale preparation of nuclear extracts from CEM cells for chromatographic purification of the repressor complex sequence (RCS) was carried out as described previously (7) with the following minor modifications: buffers A and C were supplemented with 1 mM NaF, 1 mM $Na_3VO_4$, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, and 1 μg of pepstatin A per ml. Chymostatin (1 μg/ml) was also added to buffer A, and 50 mM β-glycerolphosphate was added to buffer C.

**EMSA.** The double stranded oligonucleotides used in this study were LTR (corresponding to the −17 to +27 region of the HIV-1 LTR); RCS (−10 to +27 of the HIV-1 LTR); mRCS (−10 to +27 of the HIV-1 LTR), carrying three mutations at positions −1/+1 (5′-GG-3′→5′-CC-3′), +10/+11 (5′-GG-3′→5′-TC-3′), and +17/+18 (5′-CC-3′→5′-GG-3′); and P5, carrying a canonical binding site for YY1 from the −60 region of the adeno-associated virus (AAV) P5 promoter (24). All oligonucleotides were end labeled with polynucleotide kinase (New England Biolabs, Beverly, Mass.) and [γ-$^{32}$P]ATP. Purified oligonucleotide probe ($2 \times 10^4$ cpm) and 4 μg of nuclear extract or 0.2 μl of affinity chromatography fraction were incubated for 30 min on ice in a buffer containing 12% glycerol, 12 mM HEPES (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 4 mM Tris-HCl (pH 7.9), 0.6 mM EDTA, 0.6 mM dithiothreitol (DTT), and 10 μM zinc acetate (final volume, 20 μl). Two micrograms of poly(dI-dC) was added to reaction mixtures containing nuclear extract; 100 ng of poly(dI-dC) and 50 μg of bovine serum albumin were added to binding reaction mixtures with purified fractions. Binding reactions were resolved on a 4% nondenaturing polyacrylamide gel

* Corresponding author. Mailing address: Institute of Human Virology, MBRF N552, 725 W. Lombard St., Baltimore, MD 21201. Phone: (410) 706-4607. Fax: (410) 706-1992. E-mail: margolis@umbi.umd.edu.

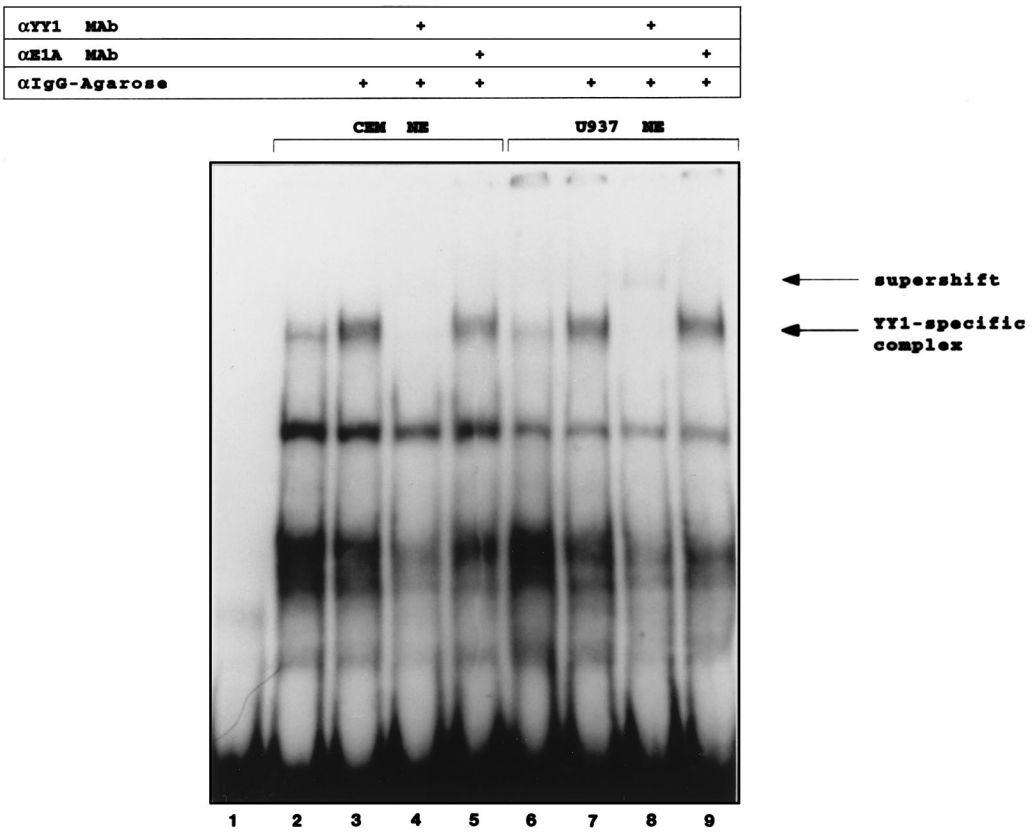


FIG. 1. YY1 LTR-binding activity in CEM lymphocytes and U937 monocytoid cells. The LTR (−17 to +27) probe was incubated with CEM or U937 nuclear extract. The YY1-specific DNA-protein complex was depleted by the addition of anti-YY1 monoclonal antibody (αYY1 MAb) and anti-IgG agarose (lanes 4 and 8). The addition of control antibody (anti-E1A) and anti-IgG agarose (lanes 5 and 9) or anti-IgG agarose alone (lanes 3 and 7) had no effect.

(20:1) in a buffer containing 12.5 mM Tris-borate and 0.05 mM EDTA. Competition EMSA was performed with unlabeled RCS, mRCS, or P5 oligonucleotide in the indicated amount. For experiments using crude nuclear extract, anti-YY1 monoclonal antibody or control monoclonal antibody (anti-E1A, a gift of Y. Shi) was added to the EMSA reaction mixture and incubated for 1 h at 4°C. The antibody-protein-DNA complexes were depleted by the addition of 3 μl of anti-immunoglobulin G (IgG) agarose (Sigma, St. Louis, Mo.), incubation for 30 min at 4°C, and centrifugation at 2,500 × *g* for 5 minutes. The supernatant was recovered and complexes were resolved on a 4% nondenaturing gel. For antibody interference EMSA with nuclear extract fractions, 3 μg of purified anti-YY1 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.), an equivalent amount of rabbit IgG (Sigma), 1 μl of anti-LSF rabbit antiserum, or 1 μl of preimmune rabbit serum was added and the EMSA reaction mixtures were incubated for an additional 30 min at 4°C.

**Phosphatase experiments.** For phosphatase experiments, EMSA was performed as described above, but nuclear extracts were preincubated at room temperature for 30 min in the presence or absence of 1.0 U of calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Where indicated, 10 mM NaF was added to inhibit phosphatase activity. The reaction mixtures were then incubated on ice for 30 min with DNA probe and poly(dI-dC) and resolved on a 4% acrylamide gel as described above.

**Ion-exchange chromatography.** Activated P11 phosphocellulose (Whatman, Clifton, N.J.) was equilibrated with a solution containing 50 mM NaCl, 50 mM HEPES (pH 7.9), 10% glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM DTT. CEM cell nuclear extract was loaded at 0.4 ml/min, washed, and eluted in a linear gradient of 50 mM to 1 M NaCl. Fractions shown by Western blotting with anti-YY1 antibody (Santa Cruz Biotechnology) to contain YY1 and by EMSA to contain RCS-binding activity were pooled and dialyzed against a solution containing 20 mM Tris-HCl (pH 7.9), 10% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 50 mM NaCl before DEAE-cellulose chromatography. A DEAE-cellulose DE52 column (Whatman) was loaded with pooled fractions at 0.2 ml/min. The column was washed and eluted, and the fractions were analyzed as above. Fractions positive by both Western blot and gel shift analysis were subjected to further purification by DNA affinity chromatography.

**DNA affinity chromatography.** A double-stranded oligonucleotide spanning the region −10 to +27 of the HIV-1 LTR was ligated and coupled to CNBr-activated Sepharose CL-4B (Pharmacia, Piscataway, N.J.) as previously described (13). Active fractions from DEAE-cellulose chromatography were equilibrated in buffer Z (25 mM HEPES [pH 7.6], 0.1 M NaCl, 20% glycerol, 12.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, and 0.1% Nonidet P-40). Affinity resin was washed extensively with buffer Z without glycerol and Nonidet P-40. Fractions were incubated for 10 min at 4°C with 10 μg of poly(dI-dC), loaded by gravity, washed, and eluted with a step gradient of 0.1 to 1 M NaCl. Western blot analysis was performed as described previously (2).

**Cell lines, transfections, and assays.** CEM and U937 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum. HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and transfected as follows. Twenty micrograms of plasmid DNA was prepared in 430 μl of distilled water; 60 μl of a 1 M $CaCl_2$ solution was added to the DNA. Five hundred microliters of 2× HBS solution (1% HEPES, 1.6% NaCl [pH 7.08]) and 10 μl of 100× $PO_4$ solution (483 mg of $Na_2HPO_4$ and 497 mg of $NaH_2PO_4$ in 100 ml of distilled water) were premixed and then added to the DNA-$CaCl_2$ mixture. After 30 min at room temperature, the solution was added to the cells ($2.5 \times 10^5$ to $4 \times 10^5$ cells/plate). Twelve hours after transfection, the cells were washed with phosphate-buffered saline and fed fresh medium. Forty-eight hours later the cells were harvested, cellular extracts were prepared, and chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were performed as previously described (2, 17). Luciferase assays were performed as suggested by the manufacturer (Promega, Madison, Wis.), but cells were resuspended in 200 μl of lysis buffer and three freeze-thaw steps were performed. Five micrograms of cellular extract in a final volume of 10 μl was used for luciferase reactions.

To measure transfection efficiency and effects on other promoters, cellular extract was tested for β-galactosidase or luciferase activity. CAT assays were then normalized for β-galactosidase or luciferase activity. The control reporter constructs pCH110, expressing β-galactosidase (Pharmacia Biotech), and pHβ-actin-luciferase (26), were used in cotransfection. Where indicated, aliquots of culture medium were sampled for detection of HIV-1 $p24^{gag}$ protein by antigen capture enzyme-linked immunosorbent assay according to the manufacturer's instructions (Coulter Corporation, Hialeah, Fla.).

**ms(ABC)′-CAT construction.** For CAT reporter assays of HIV-1 LTR unresponsive to YY1 and LSF, a CAT gene was cloned into plasmid ms(ABC)′. This construct contains mutations that abolish LSF binding to the HIV-1 promoter (12), as well as six compensatory mutations that maintain the folding of the TAR

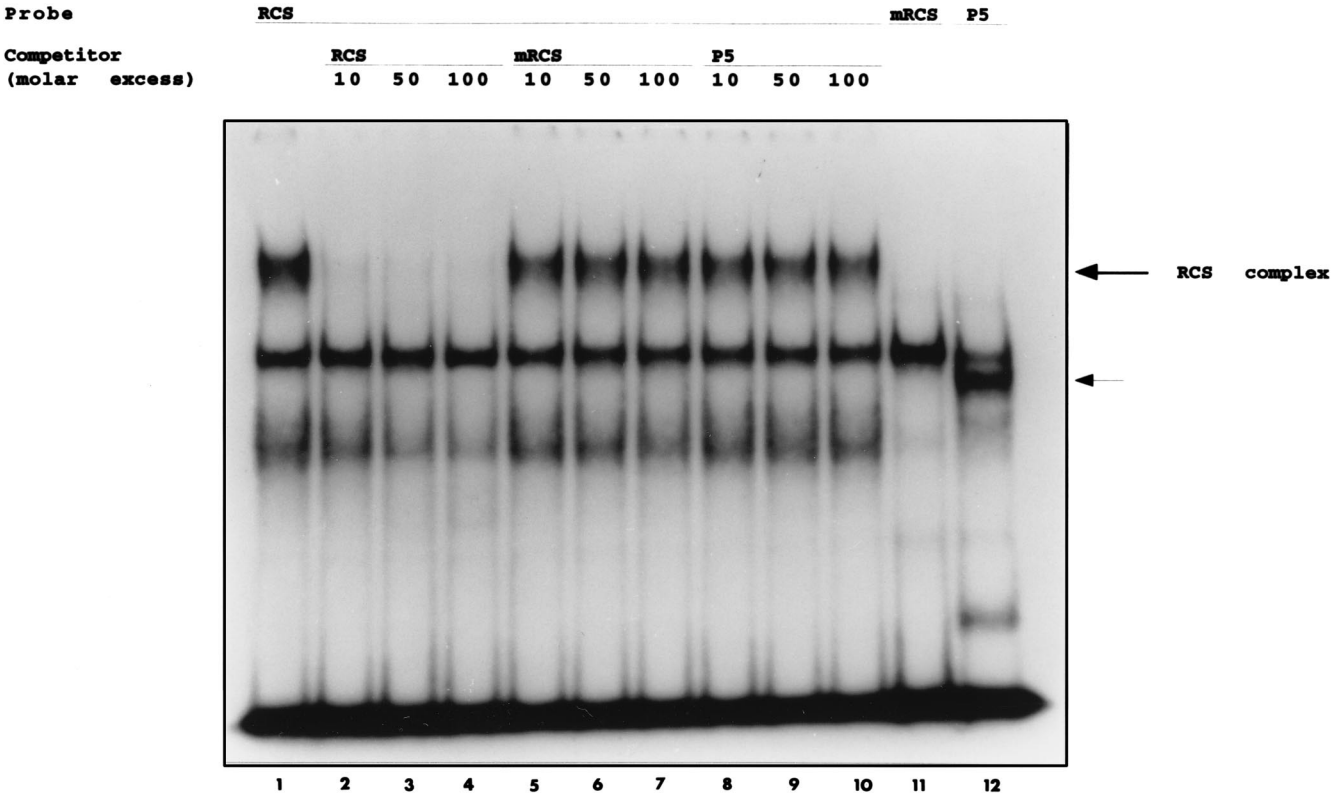


FIG. 2. A mutated RCS probe and a high-affinity YY1 binding sequence do not compete for formation of the YY1-specific complex on the HIV-1 LTR. When used in EMSA, an mRCS probe carrying three mutations described to prevent the binding of LSF to the HIV-1 LTR (12) was unable to induce formation of the YY1-specific complex (lane 11). In competition experiments, this oligonucleotide failed to compete with the RCS probe for formation of the RCS complex. The P5 oligonucleotide encoding the canonical YY1 binding site present at position −60 of the AAV P5 promoter (24) was able to induce the formation of a YY1-specific complex, as indicated by the smaller arrow (lane 12). Nevertheless, when used as a competitor this oligonucleotide was unable to inhibit formation of the YY1-specific complex with the RCS probe (lanes 8 to 10).

stem-loop structure and response to Tat activation (23). *Bam*HI sites were created flanking a CAT gene by PCR amplification. The CAT gene was then inserted into the *Bam*HI site (+82) of ms(ABC)′, and the promoter and reporter sequences were confirmed.

## RESULTS

**YY1-specific LTR-binding activity in lymphocyte and monocyte cell lines.** EMSAs using a −17 to +27 oligonucleotide and nuclear extract from the $CD4^+$ lymphocyte cell line CEM or the monocytoid cell line U937 revealed several DNA-protein complexes (Fig. 1, lanes 2 and 6). Treatment with the anti-YY1 monoclonal antibody 1G3 (a gift of Y. Shi) specifically depleted the lowest-mobility complex (Fig. 1, lanes 4 and 8). A control antibody (anti-E1A, a gift of Y. Shi) or an agarose-conjugated anti-IgG antibody had no effect (Fig. 1, lanes 3, 5, 7, and 9). For these studies we modified the protocol used to prepare nuclear extracts to minimize protein degradation and dephosphorylation (see Materials and Methods). Previous studies using HeLa nuclear extract prepared from large batches of cultured cells detected two YY1-specific complexes (18). We detected only one YY1-specific complex in nuclear extracts prepared by a modified protocol from smaller culture volumes of the lymphoid cell lines CEM, Jurkat, and A3.01, the monocytoid cell line U937, and primary lymphocyte cell populations (Fig. 1 and data not shown).

We tried to further delimit the sequence necessary for the formation of the LTR-binding complex that contains YY1. EMSAs using a −10 to +27 oligonucleotide and crude or

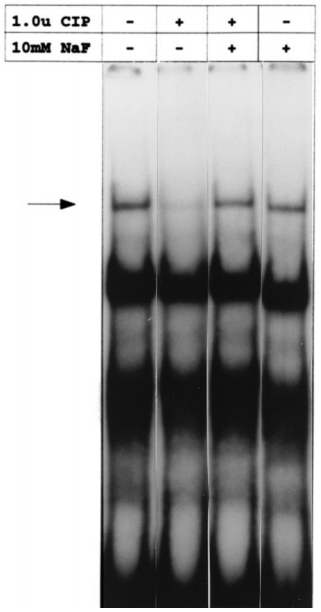


FIG. 3. YY1-LSF complex formation is inhibited by phosphatase. CEM nuclear extracts were treated with calf intestinal phosphatase (CIP), the phosphatase inhibitor NaF, or both and then used in EMSA with the LTR probe. Treatment with CIP abolished formation of the YY1-LTR complex, indicated by the arrow; this effect was inhibited in the presence of NaF.

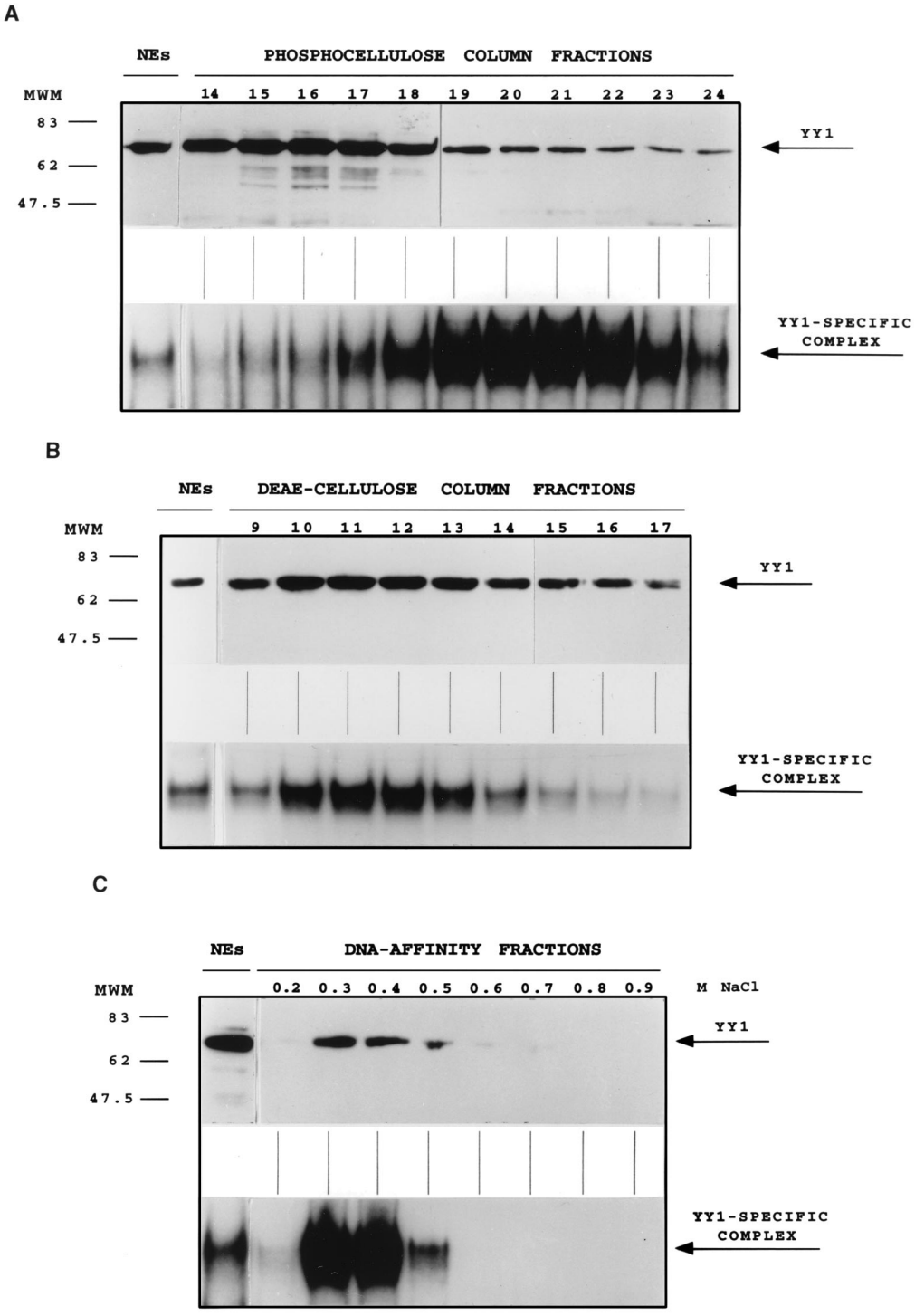


FIG. 4. Copurification of RCS-binding activity and YY1. Western blotting (upper panels) used rabbit polyclonal anti-YY1 C-20 (Santa Cruz Biotechnology), and EMSA (lower panels) used the RCS probe. Western blotting was performed with 20 μg of nuclear extract and 5 μg of phosphocellulose (A), 2.5 μg of DEAE-cellulose (B), or 200 ng of DNA affinity column eluate (C). EMSA was performed with 4 μg of nuclear extract and 1 μg of phosphocellulose (A), 500 ng of DEAE-cellulose (B), or 20 ng of DNA affinity column eluate (C). Arrows indicate YY1-specific complexes as validated by anti-YY1 interference in EMSA (data not shown). Molecular weight markers (MWM) are indicated, in thousands. Values at the top of each panel are molar concentrations of NaCl. NEs, unfractionated nuclear extract.

partially purified nuclear extract fractions from the $CD4^+$ T-lymphocyte cell line CEM (see below) revealed only one retarded band, which contains YY1 as evidenced by the ability of anti-YY1 antibodies to specifically abrogate its formation. Further deletion of residues from either the 5′ or 3′ end of this oligonucleotide greatly reduced the formation of this EMSA complex (data not shown). Hereafter we refer to this sequence as RCS (for repressor complex sequence).

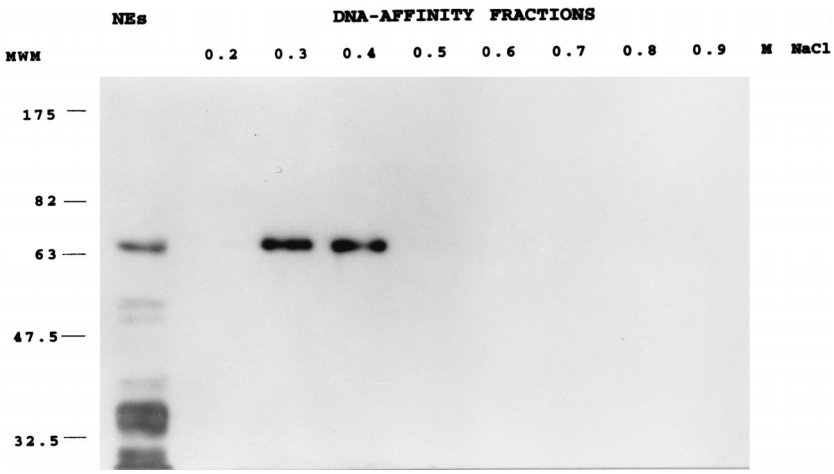


FIG. 5. LSF copurifies with YY1 and the RCS-binding activity. Equal amounts of the DNA affinity chromatography fractions were subjected to Western blot analysis with an anti-CP-2 (LSFc/LBP-1c; a gift of M. Sheffery) antiserum. The 0.3 and 0.4 M NaCl fractions were found to contain LSF and to copurify with YY1 and the RCS-binding activity. Molecular weight markers (MWM) are indicated, in thousands. NEs, unfractionated nuclear extract.

**Another factor participates in LTR binding with YY1.** While the LTR-binding complex we have identified contains YY1, several observations suggested that this complex might contain additional components and/or a uniquely modified form of YY1.

First, the lymphoid transcription factor LSF (LBP-1/UBP/CP-2) has been shown to recognize the same LTR sequence as YY1 (9, 11, 12, 14, 16, 28). We found that a triple mutant of the −10/+27 RCS probe very similar to a triple mutant that disrupts LSF binding at this site (12) was not able to form YY1-specific complexes or compete for their formation (Fig. 2, lanes 5 to 7 and 11). Also, an oligonucleotide encoding the binding site for YY1 from the AAV P5 promoter, a sequence that efficiently binds YY1 (24), did not compete for formation of the YY1-specific complex with the RCS probe (Fig. 2, lanes 8 to 10). These results suggest that YY1 binds the HIV-1 LTR with cofactors or is modified in a way that makes it unavailable for binding to the P5 probe.

Further, while purified LSF can repress LTR transcription when tested in vitro (14), transfection of LSF expression constructs failed to inhibit transcription in vivo (28). Phosphorylation of LSF has been shown to modulate its DNA binding activity (25). We found that phosphatase treatment of nuclear extract disrupted the formation of the YY1-specific LTR-binding complex (Fig. 3).

Finally, purified YY1 expressed in *E. coli* efficiently bound the P5 probe but did not bind the LTR probe in EMSA (data not shown). This could be due to the absence of a cofactor or proper posttranslational modification of YY1 expressed in *Escherichia coli*. These observations led us to hypothesize that LSF and YY1 bind the HIV-1 LTR and function as part of a multiprotein complex.

**LSF participates in RCS complex formation.** We sought to identify other components of the YY1-specific LTR-binding complex with a DNA affinity purification strategy. To delineate components of the RCS-binding complex, CEM nuclear extract was serially fractionated by P11 phosphocellulose (Fig. 4A), DEAE-cellulose (Fig. 4B), and DNA affinity chromatography with a double-stranded oligonucleotide encoding the RCS (Fig. 4C). At each step, fractions enriched for RCS-binding activity were identified by EMSA, and the presence of YY1 was assayed by Western blotting. YY1 and RCS-binding activity copurified in the 0.3 and 0.4 M NaCl fractions of the final DNA affinity chromatography step. RCS-binding activity was enriched approximately 10,000-fold by this procedure. Western blot analysis showed that LSF copurified with YY1 and the RCS-binding activity in the 0.3 and 0.4 M NaCl fractions (Fig. 5). TDP-43, another nuclear protein reported to bind near the RCS site (19), was not detected (data not shown).

Since anti-YY1 antibodies (Fig. 6A) as well as anti-LSF antisera (Fig. 6B) disrupted the formation of the RCS complex, we concluded that both YY1 and LSF participate in RCS complex formation. Total rabbit IgG, preimmune rabbit serum, or irrelevant antibodies such as anti-E1A had no effect on RCS complex formation (Fig. 6A and B and data not shown). Further, anti-YY1 or anti-LSF had no effect on EMSA complexes formed on a Sp1 binding site (data not shown). This suggests that both YY1 and LSF bind the HIV-1 LTR as part of a multiprotein complex.

**YY1 and LSF synergize in repression of HIV-1 LTR expression.** Cooperation in repression of HIV-1 gene expression by YY1 and LSF was demonstrated by cotransfection of HeLa cells with the infectious molecular clone pNL4-3 (1) and vectors expressing YY1 (24) and/or LSF (a gift of Q. Zhu and U. Hansen). As these cells support HIV replication but cannot be infected, a measurement of the effects of YY1 and LSF on a single round of viral replication can be made. The effects of YY1 and LSF on viral production were measured by testing the culture supernatant for the presence of the viral protein $p24^{gag}$. In conditions in which transfection of a vector expressing YY1 or LSF produced little inhibition of virion production, cotransfection of both factors synergistically inhibited HIV production (Fig. 7A). The data shown are representative of three independent experiments.

In a second set of experiments, we tested the effects of YY1 and LSF on the expression of a CAT reporter gene driven by the HIV-1 LTR in the presence of the viral protein Tat. As previously reported (18), YY1 inhibited the basal level of HIV-1 LTR-driven CAT expression. We found little inhibition of the basal level of LTR expression following cotransfection of LSF (data not shown). Similarly, Tat-activated, LTR-directed CAT expression was inhibited by YY1 alone and not significantly affected by LSF alone. However, cotransfection of YY1 and LSF strongly inhibited CAT expression (Fig. 7B).

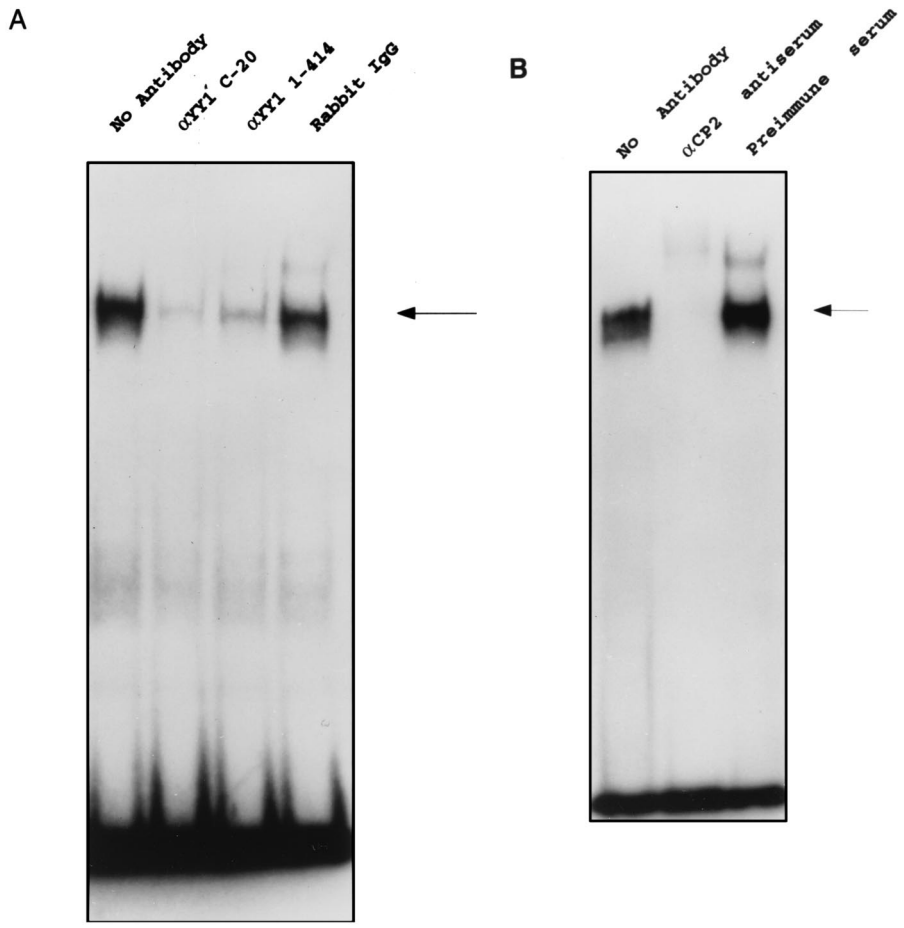


FIG. 6. The RCS-binding complex contains YY1 and LSF. (A) EMSA binding reaction with the RCS probe (−10/+27) and 5 ng of DNA affinity chromatography eluate. Complexes were disrupted by a rabbit polyclonal antibody that recognizes the carboxyl-terminal domain of YY1 (anti-YY1 [αYY1] C-20) or a polyclonal antibody directed against the entire molecule (anti-YY1 [αYY1] 1-414) but not by an equal amount of total rabbit IgG. (B) EMSA binding reaction with the RCS probe and 5 ng of DNA affinity chromatography eluate. Complexes were disrupted by a rabbit antiserum raised against LSF-c (anti-CP-2 [αCP2]) but not by preimmune rabbit serum.

The results shown are representative of four independent experiments.

In all of these experiments, cell growth, expression of a cotransfected simian virus 40 (SV40) early-driven β-galactosidase reporter and expression of a β-actin promoter-driven luciferase reporter were unaffected by YY1 and LSF (data not shown).

The level of inhibition of LTR-directed CAT expression by YY1 and LSF was similar when CAT assay reaction mixtures were normalized for either SV40 early-driven β-galactosidase activity or β-actin promoter-driven luciferase activity (data not shown). While LSF is known to activate the SV40 late promoter, it has little effect on the early promoter (22). As YY1 and LSF do not modulate the expression of a broadly active viral promoter or a constitutive cellular promoter, the cooperative repressive effect of these factors appears somewhat selective for the HIV-1 LTR. The effects of LSF and YY1 cannot be attributed to general repression of transcription or nonspecific cell toxicity. Taken together, these data indicate that YY1 and LSF participate in the formation of the RCS-binding complex and cooperatively inhibit virion production by repressing HIV-1 transcription.

**Mutations which abolish YY1 and LSF binding abrogate the abilities of YY1 and LSF to repress HIV-1 LTR expression.** To confirm that the negative effects of YY1 and LSF on HIV-1 expression correlated with the abilities of these proteins to recognize the −10/+27 region of the LTR, a CAT reporter gene was inserted downstream of the *Bam*HI site (+82) of plasmid ms(ABC)′. This construct contains mutations that abolish LSF binding to the HIV-1 promoter (12) as well as RCS complex formation (Fig. 2). ms(ABC)′ contains six compensatory mutations that maintain the folding of the TAR stem-loop structure and is defective in the production of short transcripts, but responds to Tat (23). We found that the expression of ms(ABC)′-CAT in the presence of Tat was unaffected by the presence of YY1, LSF, or both factors (Fig. 8).

## DISCUSSION

We have described a nuclear protein complex isolated from CEM lymphocytes that binds the initiator region of the HIV-1 LTR. We have designated the binding site RCS (for repressor complex sequence). We have shown that two transcription factors, YY1 and LSF, are required for formation of the RCS-binding complex. Further, YY1 and LSF cooperate in repression, as their coexpression synergistically inhibits LTR-directed gene expression and HIV-1 virion production. The ability of

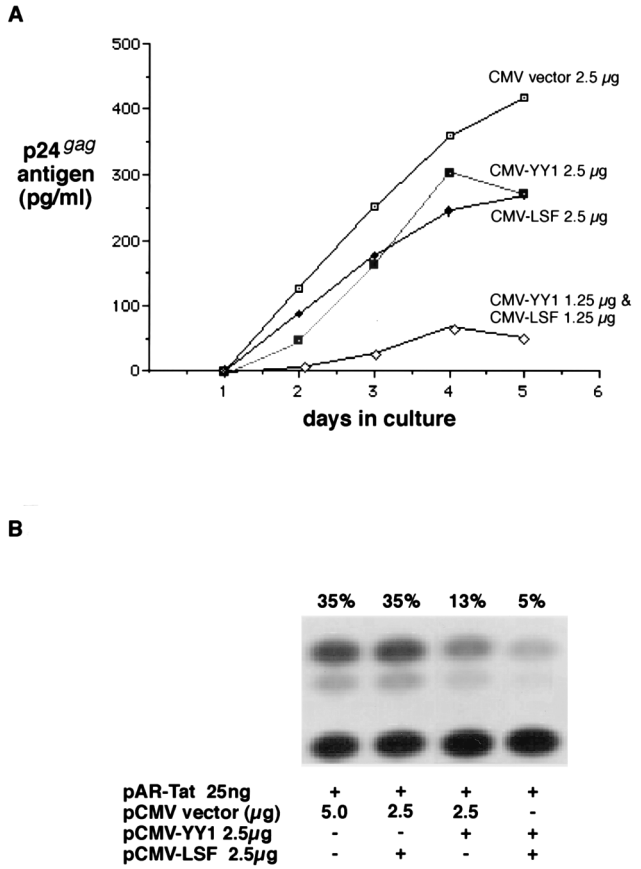


FIG. 7. Cooperative repression by YY1 and LSF. (A) HeLa cells were transfected with 1.25 μg of HIV-1 molecular clone pNL4-3 and the indicated amounts of expression vector. Repression mediated by YY1 and LSF was synergistic. (B) HeLa cells were transfected with 20 ng of HIV-1 LTR-CAT reporter (1), 25 ng of Tat expression vector (10), and empty cytomegalovirus (CMV) expression vector, CMV-YY1, or CMV-LSF, as indicated. As shown, the repression of CAT expression by YY1 and LSF was more than additive. Percent acetylation is indicated above each lane.

YY1 and LSF to cooperate in function has not been previously described.

Our findings suggest that LSF allows YY1 to recognize a site on the LTR that YY1 cannot bind by itself. Anti-YY1 antibodies completely and specifically disrupt RCS complex formation. However, bacterially expressed YY1 binds weakly to the RCS, suggesting that YY1 of bacterial origin is unable to bind the RCS or that YY1 requires a cofactor for binding. Previous studies have shown that bacterially expressed LSF as well as LSF purified from nuclear extract can bind an oligonucleotide encompassing the RCS in EMSAs (14, 28, 29). Further, anti-LSF antibodies completely and specifically disrupt RCS complex formation.

These observations are consistent with a model in which YY1 participates in RCS complex formation through both direct contact with LSF and a DNA site(s) within the RCS. This model has precedent in YY1 function in other promoters. The human papillomavirus type 18 URR promoter is regulated by YY1, but YY1 requires C/EBP-β to cooperatively bind a site in the human papillomavirus promoter not recognized by YY1 alone (3). YY1 can directly repress transcription; it binds and represses the AAV P5 promoter (24). We suggest that YY1 requires a second protein, LSF, to bind the RCS within the HIV-1 LTR and that binding of the HIV-1 promoter by YY1 and LSF results in repression of transcription. However, we have not yet demonstrated a direct physical interaction between YY1 and LSF or a biochemical cooperation in binding the RCS. Therefore, we cannot yet exclude other models, such as those involving a third factor. Studies to explore the protein-protein and protein-DNA interactions of YY1 and LSF are underway.

Three transcription factors, USF, LSF (LBP-1c/UBP-1/CP-2), and TDP-43, have been shown to bind the HIV-1 LTR within the −17 to +27 region that is also recognized by YY1 (8, 9, 12, 14, 19, 29). USF has been shown to activate transcription (8). We were unable to detect TDP-43 in the column fractions containing the RCS-binding activity. As inhibition of LTR expression is not seen upon the overexpression of LSF in

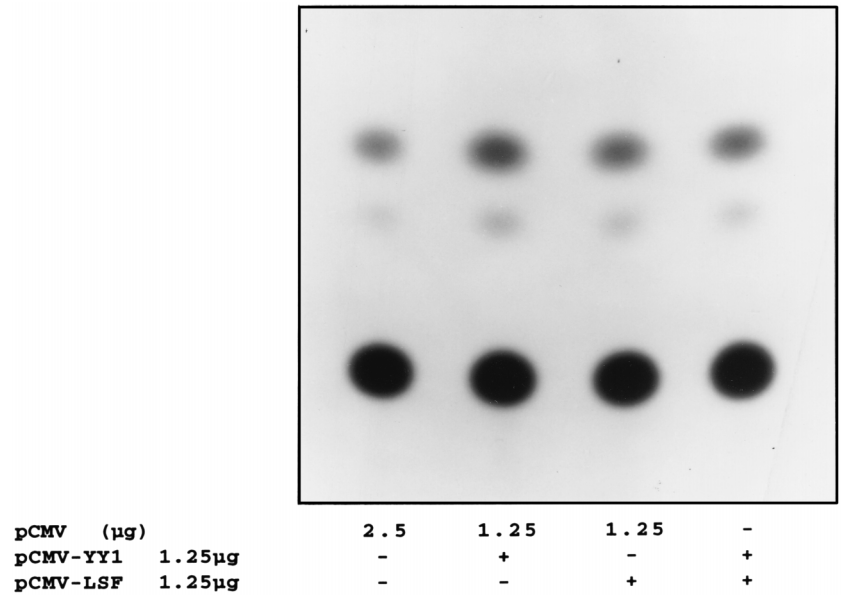


FIG. 8. Mutations which abolish YY1 and LSF binding abrogate the ability of YY1 and LSF to repress HIV-1 LTR expression. HeLa cells were transfected with 20 ng of ms(ABC)′-CAT reporter, 25 ng of Tat expression vector (10), and empty cytomegalovirus (CMV) expression vector, CMV-YY1, or CMV-LSF, as indicated. As shown, YY1 and LSF had no significant effect on CAT expression.

cells, these observations lead us to speculate that YY1 directly mediates transcriptional repression following its recruitment and/or stabilization within the RCS complex by LSF.

Previously, we reported that YY1 was able to repress the basal level of LTR expression and inhibit HIV-1 virion production (18). We now find that coexpression of YY1 and LSF synergistically inhibits Tat-activated LTR expression and virion production. Promoter mutations which block RCS complex formation abrogate repression by YY1 and LSF. These mutations also abolish the function of the inducer of short transcripts (23). However Pessler et al. have recently reported that neither YY1 nor LSF regulates the function of the inducer of short transcripts, and they attribute that function to a novel factor, FBI-1 (21).

The mechanism through which YY1 and LSF repress HIV-1 transcription remains to be studied. YY1 interacts directly with cellular enhancer factors such as Sp1 (15) and with other general transcription factors (5). Such interactions may mediate repression by blocking the assembly of a preinitiation transcription complex or by inhibiting the processivity of an assembled complex. Alternatively or additionally, as the viral activator protein Tat can interact with the TATA-binding protein and can induce phosphorylation of RNA polymerase II, YY1 and LSF may inhibit these putative activating functions of Tat. Finally, several groups have recently reported that histone acetylation may result in transcriptional repression (20). YY1 may act by bringing a histone acetylase to the promoter and inducing an inhibitory modulation of chromatin structure (27).

Repression by YY1 and LSF appears to some degree to be selective for the HIV-1 promoter. Under conditions that significantly inhibited LTR expression, coexpression of YY1 and LSF did not significantly affect the activity of a promiscuous viral promoter (SV40 early) or of a constitutive cellular promoter (β-actin). While other cellular processes are likely to be affected by YY1 and LSF, the unique cooperation of these factors may allow specificity in the negative regulation of HIV-1 LTR expression. It is attractive to speculate that YY1 and LSF participate in cellular pathways opposing those signals that activate lymphocytes, induce IL-2 expression, upregulate NF-κB, and promote HIV-1 replication.

## ACKNOWLEDGMENTS

We thank Q. Zhu and U. Hansen for the gift of the LSF expression vector prior to publication, S. Johnston for β-actin luciferase, R. Gaynor for anti-TDP-43, M. Sheffery for anti-CP-2, and both Y. Shi and U. Hansen for helpful discussions and the communication of unpublished results. We also thank R. C. Gallo, G. K. Lewis, R. R. Redfield, and E. C. Tramont for their support.

## REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1995. Current protocols in molecular biology. John Wiley and Sons, Inc., New York, N.Y.
3. **Bauknecht, T., F. Jundt, I. Herr, T. Oehler, H. Delius, Y. Shi, P. Angel, and H. zur Hausen.** 1995. A switch region determines the cell-type-specific positive or negative action of YY1 on the activity of the human papillomavirus type 18 promoter. J. Virol. **69:**1–12.
4. **Bauknecht, T., R. See, and Y. Shi.** 1996. A novel C/EBP β-YY1 complex controls the cell-type-specific activity of the human papillomavirus type 18 upstream regulatory region. J. Virol. **70:**7695–7705.
5. **Chiang, C.-M., and R. G. Roeder.** 1995. Cloning of an intrinsic human TFIID subunit that interacts with multiple transcriptional activators. Science **267:** 531–536.
6. **Chun, T.-W., D. Finzi, J. Margolick, K. Chadwick, D. Schwartz, and R. F. Siliciano.** 1995. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. Nat. Med. **1:**1284–1290.
7. **Dignam, J. D.** 1990. Preparation of extracts from higher eukaryotes. Methods Enzymol. **182:**194–203.
8. **Du, H., A. L. Roy, and R. G. Roeder.** 1993. Human transcription factor USF stimulates transcription through the initiator elements of the HIV-1 and the Ad-ML promoters. EMBO J. **12:**501–511.
9. **Garcia, J. A., F. K. Wu, R. Mitsuyasu, and R. B. Gaynor.** 1987. Interactions of cellular proteins involved in the transcriptional regulation of the human immunodeficiency virus. EMBO J. **6:**3761–3770.
10. **Gendelman, H. E., W. Phelps, L. Feigenbaum, J. M. Ostrove, A. Adachi, P. M. Howley, G. Khoury, H. S. Ginsberg, and M. A. Martin.** 1986. Transactivation of the human immunodeficiency virus long terminal repeat sequence by DNA viruses. Proc. Natl. Acad. Sci. USA **83:**9759–9763.
11. **Huang, H.-C., R. Sundseth, and U. Hansen.** 1990. Transcription factor LSF binds two variant bipartite sites within the SV40 late promoter. Genes Dev. **4:**287–298.
12. **Jones, K. A., P. A. Luciw, and N. Duchange.** 1988. Structural arrangements of transcription control domains within the 5′-untranslated leader regions of the HIV-1 and HIV-2 promoters. Genes Dev. **2:**1101–1114.
13. **Kadonaga, J. T.** 1991. Purification of sequence-specific binding proteins by DNA affinity chromatography. Methods Enzymol. **208:**10–23.
14. **Kato, H., M. Horikoshi, and R. G. Roeder.** 1991. Repression of HIV-1 transcription by a cellular protein. Science **251:**1476–1479.
15. **Lee, J.-S., K. M. Galvin, and Y. Shi.** 1993. Evidence for physical interaction between the zinc-finger transcription factors YY1 and Sp1. Proc. Natl. Acad. Sci. USA **90:**6145–6149.
16. **Lim, L. C., S. L. Swendeman, and M. Sheffery.** 1992. Molecular cloning of the α-globin transcription factor CP2. Mol. Cell. Biol. **12:**828–835.
17. **Liu, F., M. A. Thompson, S. Wagner, M. E. Greenberg, and M. R. Green.** 1993. Activating transcription factor-1 can mediate $Ca^{2+}$- and cAMP-inducible transcriptional activation. J. Biol. Chem. **268:**6714–6720.
18. **Margolis, D. M., M. Somasundaran, and M. R. Green.** 1994. Human transcription factor YY1 represses human immunodeficiency virus type 1 transcription and virion production. J. Virol. **68:**905–910.
19. **Ou, S.-H. I., F. Wu, D. Harrich, L. F. Garcia-Martinez, and R. B. Gaynor.** 1995. Cloning and characterization of a novel cellular protein, TDP-43, that binds to the human immunodeficiency virus type 1 TAR DNA sequence motifs. J. Virol. **69:**3584–3596.
20. **Pazin, M. J., and J. T. Kadonaga.** 1997. What's up and down with histone deacetylation and transcription? Cell **89:**325–328.
21. **Pessler, F., P. S. Pendergrast, and N. Hernandez.** 1997. Purification and characterization of FBI-1, a cellular factor that binds to the human immunodeficiency virus type 1 inducer of short transcripts. Mol. Cell. Biol. **17:** 3786–3798.
22. **Rio, D., A. Robbins, R. Myers, and R. Tjian.** 1980. Regulation of simian virus 40 early transcription in vitro by a purified tumor antigen. Proc. Natl. Acad. Sci. USA **77:**5706–5710.
23. **Sheldon, M., R. Ratnasabapathy, and N. Hernandez.** 1993. Characterization of the inducer of short transcripts, a human immunodeficiency virus type 1 transcriptional element that activates the synthesis of short RNAs. Mol. Cell. Biol. **13:**1251–1263.
24. **Shi, Y., E. Seto, L.-S. Chang, and T. Shenk.** 1991. Transcriptional repression by YY1, a human GLI-Krüppel-related protein, and relief of repression by adenovirus E1A protein. Cell **67:**377–388.
25. **Volker, J. L., L. E. Rameh, J. DeCaprio, and U. Hansen.** 1997. Mitogenic stimulation of resting T cells causes rapid phosphorylation of the transcription factor LSF and increased DNA-binding activity. Genes Dev. **11:**1–12.
26. **Williams, R. S., S. A. Johnston, M. Riedy, M. J. DeVit, S. G. McElligott, and J. C. Sanford.** 1991. Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. Proc. Natl. Acad. Sci. USA **88:**2726–2730.
27. **Yang, W.-M., C. Inouye, Y. Zeng, D. Bearss, and E. Seto.** 1996. Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. Proc. Natl. Acad. Sci. USA **93:**12845–12850.
28. **Yoon, J.-B., G. Li, and R. C. Roeder.** 1994. Characterization of a family of related cellular transcription factors which can modulate human immunodeficiency virus type 1 transcription in vitro. Mol. Cell. Biol. **14:**1776–1785.
29. **Zhong, F., S. L. Swendeman, W. Popik, P. M. Pitha, and M. Sheffery.** 1994. Evidence that levels of the dimeric cellular transcription factor CP-2 play little role in the activation of the HIV-1 long terminal repeat in vivo or following superinfection with herpes simplex virus type 1. J. Biol. Chem. **269:**21269–21276.
30. **Zhou, Q., R. W. Gedrich, and D. A. Engel.** 1995. Transcriptional repression of the c-*fos* gene by YY1 is mediated by a direct interaction with ATF/CREB. J. Virol. **69:**4323–4330.