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Evidence that a Cell Cycle Regulator, $E2F_1$, Down-Regulates Transcriptional Activity of the Human Immunodeficiency Virus Type 1 Promoter

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Proliferation of eukaryotic cells is orchestrated by a series of cellular proteins which participate in various stages of the cell cycle to guide the cell through mitosis. Some of these proteins, including $E2F_1$, play a critical role in G_1 and S phases by coordinately regulating expression of several important cell cycle-associated genes. On the basis of recent observations indicating a block in human immunodeficiency virus type 1 (HIV-1) replication in cells arrested in G_1/S phase of the cell cycle, we sought to evaluate the regulatory action of $E2F_1$ on transcription from the HIV-1 long terminal repeat (LTR). Results from transient transfection of cells with an $E2F_1$ expression plasmid indicated that $E2F_1$ has the ability to suppress basal transcriptional activity of the LTR and to diminish the extent of the Tat-induced activation of the viral promoter. Deletion analysis of the HIV-1 LTR in transfection studies revealed the presence of two major elements responsive to $E2F_1$ repression located distally (−454 to −381) and proximally (−117 to −80) with respect to the +1 transcription start site. $E2F_1$-mediated suppression of LTR activity was observed in a wide range of human cell lines. Expression of $E2F_1$ by a transgene showed an inhibitory effect on the levels of reverse transcriptase activity obtained upon introduction of the proviral genome into cells. The data presented in this study suggest that cellular regulatory proteins involved in the progression of cells through the mitotic cycle could play crucial roles in determining the efficiency of HIV-1 replication during the various stages of infection. The possible roles of these factors in viral latency and activation are discussed.

Human immunodeficiency virus type 1 (HIV-1) is the etiological agent of AIDS (5, 18, 32). Following acute infection by HIV-1, the progression of the disease includes an asymptomatic period which varies in length from a few months to many years. To some extent this clinical latency correlates with microbiological latency, in which viral replication proceeds very slowly, if at all (14, 47). This period of latency may provide a biologic advantage to the virus and allow evasion of the host immune system. Although the mechanism which induces latency in viral replication is poorly understood, it is believed that multiple steps in the viral life cycle may participate in this event. These processes include reverse transcription, integration and methylation of viral cDNA, and transcription and nuclear export of viral mRNA (for a review, see reference 6).

After integration of HIV-1 DNA into the host genome, the collaborative action of viral and cellular factors on the long terminal repeat (LTR) determines the extent of HIV-1 gene transcription and the level of viral replication in the infected cells. In this respect, the status of several parameters, including the rate of cell proliferation, degree of differentiation, extent of activation, and phase in the mitotic cycle, becomes important, as these parameters influence the repertoire of regulatory proteins which can impact upon viral gene transcription. In support of this concept, earlier studies have indicated that resting peripheral blood lymphocytes sustain very low levels of viral expression, if any at all, but that high levels of viral replication can be induced through antigenic or mitogenic stimulation of these cells (16, 37). Also, differentiation of peripheral blood monocytes and promonocytic cell lines enhances productive HIV-1 infection in vitro (27, 45). Furthermore, HIV-1 replication in monocytes involves a proliferation-dependent step which occurs following stimulation by interleukin 4 (45). Progression through the cell cycle is a feature of activation and differentiation in lymphoid and monocytic cells and correlates with the level of HIV-1 replication. Commitment of a cell to enter the cell cycle involves modification of preexisting proteins and de novo expression of genes. It seems reasonable to speculate that cellular factors involved in cell proliferation simultaneously affect HIV-1 expression at the level of transcription.

Basal levels of viral transcription are mediated by a core promoter consisting of the TATAA element and the GC-rich motif, which constitutes three Sp1-binding sites (19, 24). Located immediately upstream of the core promoter is the HIV-1 enhancer region, which contains two binding sites for the transcription factor nuclear factor kappa B (NF-κB) (26, 39). This enhancer functions as the final common target for a wide variety of stimuli, including cytokines such as tumor necrosis factor alpha and mitogens which simultaneously activate T lymphocytes and up-regulate HIV-1 transcription (40, 46). The HIV-1 LTR also contains binding sites for numerous other cellular factors, including YY1, TCF-1, HIP, LBP, HIV-TF1, USF, NFAT, c-*myb*, and AP-1 (for a review, see reference 20). In general, the role of these transcription factors in HIV-1 expression in vivo is not well established. In addition to the influence of cellular factors, viral transcription is largely influenced by Tat (3). This viral regulatory protein is believed to

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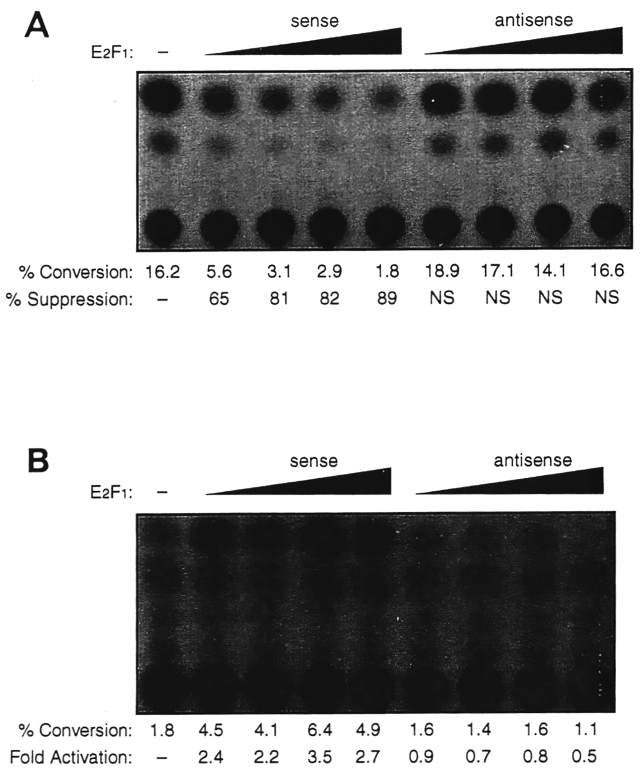


FIG. 1. Repression of basal HIV-1 LTR activity by E2F$_1$. (A) U-87MG cells were incubated in serum-free media for 24 h before transfection by the calcium phosphate method (21). Cells were cotransfected with 1.0 μg of pCD15-CAT and 0.0, 1.0, 2.5, 5.0, and 10.0 μg of pE2F$_{1S}$ or pE2F$_{1AS}$. The concentration of DNA in all the experiments were kept constant (10 μg) by addition of pCSMA$_1$, which contains only the promoter sequences of the expression vector. Whole-cell extracts were prepared after 36 h and analyzed for CAT enzyme activity. The percent acetylation and percent suppression indicated are the averages for three independent experiments. NS, no suppression was detected. The CAT assay whose results are shown was a representative experiment. (B) The c-*myc* promoter was used as a positive control to verify the specificity of E2F$_1$ activity in this study. U-87MG cells were treated as described above and transfected with 0.5 μg of p650-CAT containing the P2 promoter and 0 to 10.0 μg of pE2F$_{1S}$ or pE2F$_{1AS}$ expression plasmid. During these studies the trypan blue exclusion assay was performed to monitor cell viability in the transfected cells.

enhance the efficiency of elongation of transcripts initiated by RNA polymerase II (36).

E2F$_1$ is among the numerous proteins involved in regulating the progression of mammalian cells through the cell proliferation cycle. The promoters of several genes which are activated in mid to late G$_1$ contain putative E2F-binding sites. These genes include those for dihydrofolate reductase, thymidine kinase, and DNA polymerase alpha; c-*myb*, c-*myc*, and *cdc2*; and the gene for E2F$_1$ itself (for a review, see references 15 and 41). Presumably, E2F$_1$ influences the cell cycle, at least in part, by affecting the level of transcription of the above-mentioned genes, which are important for the G$_1$-to-S phase transition. The activity, as well as the function, of E2F is determined by the level of free E2F and by the formation of complexes with a variety of cofactors, including RB, p107, p130, cyclin A, cyclin E, and cdk2 at various points in the cell cycle (4, 8, 9, 11, 30). Although the functional differences between the various forms of E2F have not yet been determined, the multifunctional potential of E2F and its complexes is suggested by observations that the E2F-binding sites in the c-*myc* and B-myb promoters are involved in both the repression of these genes during G$_0$ and activation of these genes during G$_1$ and S phases (22, 29).

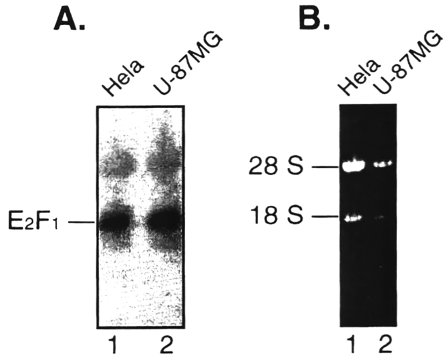


FIG. 2. Endogenous levels of E2F$_1$ RNA in U-87MG and HeLa cells. Approximately 15 μg of total RNA from HeLa and U-87MG cells was resolved by agarose-formaldehyde gel electrophoresis and after transfer to nitrocellulose was hybridized to ^{32}P-labeled E2F$_1$ probe. (A) Northern blot of the total RNA with E2F probe. (B) Ethidium bromide-stained agarose gel.

The data presented in this study suggest that E2F$_1$ can also function as a repressor of transcription from a promoter which does not contain consensus E2F$_1$-binding sites. We report that overexpression of E2F$_1$ results in suppression of basal transcriptional activity and Tat-mediated transactivation of the HIV-1 LTR and decreases the efficiency of viral replication in transfected cells.

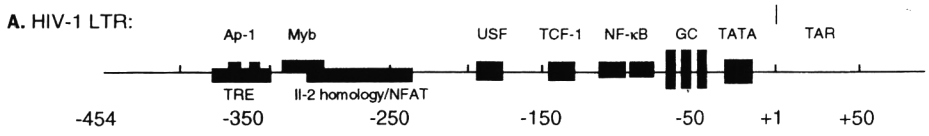
MATERIALS AND METHODS

Cells and tissue culture conditions. The following were obtained from the American Type Culture Collection: U-87MG cells, derived from a human glioblastoma; HeLa cells, derived from a human cervical epithelioid carcinoma; SW40 cells, derived from a human adenocarcinoma of the colon; RD cells, derived from a human rhabdomyosarcoma; and U-937 cells, derived from a human histiocytic lymphoma. The SupT$_1$ cell line was derived from a human pediatric T-cell lymphoma (kindly provided by D. Kozbor, Thomas Jefferson University). U-87MG, HeLa, SW40, and RD cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM), while U-937 and SupT$_1$ cells were grown in suspension in RPMI 1640 medium at 37°C in 5% CO_2. DMEM and RPMI 1640 medium were supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum and 1% penicillin-streptomycin.

Plasmid constructions. The proviral DNA construct pZ6Neo was originated from cells infected with HIV$_{Zr6}$ (48). Plasmid pCD15-CAT contains the entire U3 region and 75 nucleotides of the R region of the HIV-1 LTR inserted 5′ to the chloramphenicol acetyltransferase (CAT) gene in a pBR322-derived vector (3). The construction of deletion plasmids p(−381/+71), p(−321/+71), p(−273/+71), p(−219/+71), p(−117/+80), p(−117/+3), and p(−80/+3) has been reported previously (13, 50). The p650 *Ava*I construct contains 263 bp upstream and approximately 300 bp downstream of the murine c-*myc* promoter P2 initiation site (+1) and includes the E2F$_1$-binding site (43).

The expression plasmids ptat and pact-tat contain the *tat* coding region of HIV-1 under the control of the HIV-1 LTR (28) and β-actin promoters, respectively. To generate plasmids pE2F$_1$ sense (pE2F$_{1S}$) and pE2F$_1$ antisense (pE2F$_{1AS}$), an 804-bp *Hin*dIII-*Bam*HI DNA fragment of the cytomegalovirus immediate-early (CMVIE) promoter from pSSH (gift from Jay Nelson), containing DNA sequence from nucleotide position −750 to +54 relative to the transcription start site (+1), was placed upstream of the Moloney murine sarcoma virus poly(A) site in a pBR322 background and named pCSMA$_1$. The *Hin*dIII-*Bam*HI fragment of pCMV-β-globin E2F$_1$ (22a) containing 200 bp of the 5′ untranslated region of the β-globin gene fused to the full-length E2F$_1$ cDNA (23) was then cloned into the *Sma*I site of pCSMA$_1$ in the sense and antisense orientations, yielding pE2F$_{1S}$ and pE2F$_{1AS}$, respectively. The integrity of these clones was examined by direct DNA sequencing.

Transfections. U-87MG, HeLa, SW40, and RD cells were transfected by the calcium phosphate coprecipitation method (21), whereas SupT$_1$ and U-937 cells were transfected by the DEAE-dextran method (12). For CAT assays, 0.5 × 10^6 cells were plated in 60-mm-diameter dishes 24 h prior to transfection by the calcium phosphate method. Each plate of cells was transfected with 10 to 15 μg of DNA, depending on the experiment. A total of 10^7 cells were transfected with 20 μg of DNA by the DEAE-dextran method. For reverse transcriptase (RT) analysis, 10^6 cells were plated in 100-mm-diameter dishes and transfected with 30 μg of DNA. The amount of plasmid DNA containing the promoter sequence which drives the E2F$_1$ cDNA was kept constant by the addition of pCSMA$_1$. The



B. Deletion Constructs:

	% Conversion		
	-E2F1	+E2F1	% Suppression
-454 to +80	7.3±0.6	1.8±0.5	75
-381 to +71	19.0±1.0	8.8±1.3	54
-321 to +71	8.0±1.5	4.9±1.5	39
-273 to +71	12.4±3.6	9.2±3.6	26
-219 to +71	34.6±5.0	14.7±4.4	58
-117 to +80	28.5±8.7	10.6±1.7	63
-117 to +3	18.8±2.8	7.6±2.7	60
-80 to +3	3.8±0.2	3.0±0.0	21

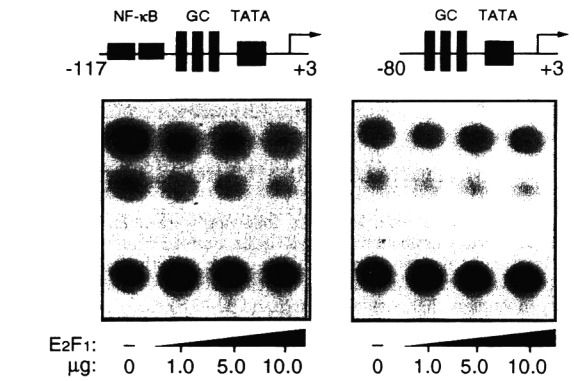


FIG. 3. Deletion analysis of the HIV-1 promoter. (A) Schematic representation of full-length HIV-1 LTR-CAT and the positions of the various *cis*-acting regulatory elements (20). (B) Schematic representation of the promoter deletions in each construct followed by the activity (indicated as percent conversion) of each promoter in the presence and absence of $E2F_1$. Also indicated is the percent suppression of CAT activity mediated by $E2F_1$. U-87MG cells were incubated in serum-free media for 24 h prior to transfection. The cells were cotransfected with 1.0 μg of pCD15-CAT (−454 to +80), p(−381/+71), p(−321/+71), p(−273/+71), or p(−219/+71); 0.4 μg of p(−117/+80) or p(−117/+3); 1.5 μg of p(−80/+3); and 2.5 μg of $pE2F_{1S}$. The values are averages for three independent experiments. (C) Representative CAT assay demonstrating the presence of an E2F-responsive element in the enhancer region (NF-κB motif) of the HIV-1 LTR. U-87MG cells were transfected with 0.4 μg of p(−117/+3) or 1.5 μg of p(−80/+3) and 0, 1.0, 5.0, and 10.0 μg of $pE2F_{1S}$.

viability of cells throughout these studies was monitored by the trypan blue exclusion assay.

CAT assay and RT analysis. Cells were prepared for the CAT assay by harvesting them 36 to 48 h after transfection and resuspending the cell pellet in 250 mM Tris (pH 7.8). Whole-cell extracts were prepared by five repeated freeze-thaw cycles followed by centrifugation at 14,000 rpm to remove cell debris. CAT enzymatic reactions were performed with 75 μg of protein extract, as described previously (21). RT analysis was performed as described previously (1).

Tat loading. Bacterially expressed recombinant Tat protein was kindly donated by J. Rappaport (Mt. Sinai Medical Center, New York, N.Y.) and used in a protein loading assay to supplement the media by the procedure described previously (17). Briefly, 0 to 10 μg of purified Tat in 1 ml of 75 μM chloroquine-containing DMEM was added to U-87MG cells 24 h after transfection. The cells were incubated for 1 h, following which the volume was adjusted to 5 ml with 75 μM chloroquine-DMEM. The cells were incubated for an additional 24 h before being harvested for the CAT assay.

RNA extraction and Northern (RNA) blot analysis. RNA was extracted from HeLa and U-87MG cells by the guanidinium thiocyanate method described previously (10). Briefly, 10^6 cells were washed with phosphate-buffered saline (PBS) and resuspended in buffer D containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M β-mercaptoethanol. Phenol-chloroform extraction was performed twice prior to precipitation with isopropanol. RNA pellets were washed with 70% ethanol and resuspended in water treated with diethylpyrocarbonate. Equal amounts (15 μg) of RNAs were fractionated by denaturing agarose gel electrophoresis and then transferred to a Hybond-N^+ membrane (Amersham) and hybridized with a ^{32}P-labeled $E2F_1$ cDNA probe.

RESULTS

Repression of HIV-1 LTR transcription by $E2F_1$. $E2F_1$ is a transcription factor which, by binding to its target sequence located in the promoter regions of a variety of cellular genes involved in cell proliferation, coordinately regulates their transcription throughout the cell cycle (for a review, see reference 15). In order to gain insight into the regulatory proteins which participate in replication of HIV-1 during cell proliferation, and to examine the importance of $E2F_1$ in regulation of HIV-1 LTR transcription, we transfected human glial cells, U-87MG cells, with reporter plasmid pCD15-CAT and an $E2F_1$ expression plasmid, $pE2F_{1S}$. As shown in Fig. 1A, expression of $E2F_1$ in the transfected cells caused a substantial decrease in the level of CAT activity, whereas expression of $E2F_1$ cDNA in the antisense orientation had negligible influence on the HIV-1 LTR-derived CAT activity. Overexpression of $E2F_1$ in the glial cells showed no significant effect on the expression of the CAT gene by the β-actin promoter (data not shown). Furthermore, under similar conditions the levels of $E2F_1$ were able to elevate the basal transcriptional activity of the control c-*myc* promoter (Fig. 1B). Expression of $E2F_1$ in the antisense orientation had a modest inhibitory effect on c-*myc* transcription in glial cells (Fig. 1B). These observations, taken together, suggest that $E2F_1$ could down-regulate expression of the HIV-1 LTR in a specific manner. As an additional control, viability of the transfected cells was monitored by the trypan blue exclusion assay in order to exclude the possibility that the observed repression of CAT activity is due to the death of the $E2F_1$-transfected cells (28a). These observations clearly indicate that $E2F_1$ has the ability to suppress transcription of the HIV-1 LTR in glial cells.

In a separate study, we examined the levels of endogenous $E2F_1$ gene expression in glial cells by a Northern blot assay. As a comparison, we utilized RNA from human cervical epithe-

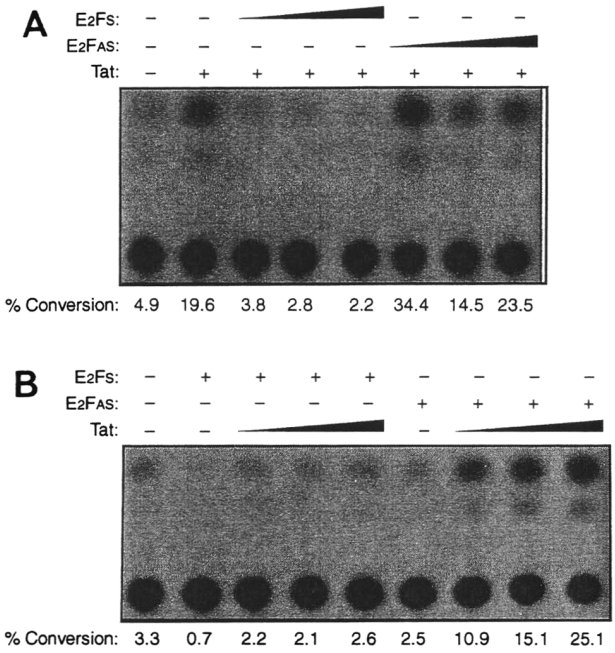


FIG. 4. Effect of exogenous Tat on E2F$_1$-mediated repression of the HIV-1 LTR. (A) U-87MG cells were incubated in serum-free media for 24 h prior to transfection. The cells were cotransfected with 1.0 μg of pCD15-CAT and 0.0 μg (lanes 1 and 2), 1.0 μg (lane 3), 5.0 μg (lane 4), or 10.0 μg (lane 5) of pE2F$_S$ and 1.0, 5.0, and 10.0 μg of pE2F$_{1AS}$. The following day, the cells were incubated in 75 μM chloroquine-DMEM containing 0.0 (lane 1) or 5.0 (lanes 2 to 8) μg of Tat. (B) U-87MG cells were transfected and samples were analyzed as for panel A. The cells were cotransfected with 0.5 μg of pCD15 and 5.0 μg of the control vector pCSMA$_1$ (lanes 1 and 6), 5.0 μg of pE2F$_{1S}$ (lanes 2 to 5), or 5.0 μg of pE2F$_{1AS}$ (lanes 6 to 9). After 24 h, the cells were washed with PBS and incubated in 75 μM chloroquine-DMEM containing 0.0 μg (lanes 1, 2, and 6), 2.5 μg (lanes 3 and 7), 5 μg (lanes 4 and 8), and 10.0 μg (lanes 5 and 9) of bacterially expressed recombinant Tat for 24 h. CAT enzyme activity was analyzed and is indicated in terms of percent acetylation.

lioid carcinoma HeLa cells, since the level of its E2F$_1$ expression has been well documented. Figure 2 indicates that E2F$_1$ is expressed in glial cells and that its level is slightly higher in U-87MG than in HeLa cells.

Characterization of E2F$_1$-responsive regions within the HIV-1 LTR. The presence of various regulatory motifs within the HIV-1 LTR, as schematized in Fig. 3A, confers a remarkable ability for the cellular proteins, including AP-1, Myb, USF, TCF-1, NF-κB, GC-rich, and TATA box-binding proteins, to regulate viral gene transcription under various conditions (for a review, see reference 20). Close inspection of the LTR sequence revealed no perfect consensus sequences for E2F$_1$ binding which may participate in modulation of viral transcription. Therefore, we utilized a collection of deletion mutants derived from the HIV-1 regulatory sequence in cotransfection assays to determine the potential E2F$_1$-responsive element(s). Towards this end, U-87MG cells were cotransfected with deletion constructs in the presence of the optimum amount of E2F$_1$ expression plasmid. As shown in Fig. 3B and C, removal of two regions, located distally (−454 to −381) and proximally (−117 to −80) with respect to the RNA start site at +1, exhibited a marked effect on the E2F$_1$-mediated suppression of HIV-1 LTR activity. The distal responsive element encompasses sequences whose importance in HIV-1 replication is largely uncharacterized. The proximal region spans the NF-κB enhancer motif and plays an important role in the induction of viral gene transcription by cytokines and other extracellular stimulators. The results of this study also indicated that the transacting responsive element (TAR) is dispensable for the E2F$_1$-mediated suppression of HIV-1 LTR activity (Fig. 3B; compare the activities of −117 to +80 and −117 to +3).

E2F$_1$ inhibits Tat-mediated transactivation of the HIV-1 LTR. Tat is a virally encoded 86-amino-acid protein produced at the early phase of infection which enhances the basal transcriptional activity of HIV-1 and plays a vital role in the productive phase of infection (3). Although interaction of Tat and TAR is important for activation of the viral LTR in a variety of cells, it is evident that the upstream promoter sequences and their binding proteins are critical for the observed activity (25, 34). In order to investigate the effect of E2F$_1$ on the activation of LTR by Tat, transfected cells containing the reporter construct pCD15-CAT and increasing amounts of pE2F$_{1S}$ or pE2F$_{1AS}$ were maintained in media containing 5 μg of Tat protein. Addition of Tat protein to the media enhanced the transcriptional activity of LTR in these cells (Fig. 4A, lanes 1 and 2), which is consistent with previous studies (17). Expression of E2F$_1$ in these cells drastically reduced the level of viral transcription mediated by Tat (Fig. 4A; compare lane 2 and lanes 3 to 5). Inclusion of the control plasmid pE2F$_{1AS}$ in the transfection mixture showed no inhibitory effect on viral promoter activation. In fact, expression of E2F$_1$ in the antisense orientation caused a modest enhancement in the Tat-mediated transcription of LTR (Fig. 4A; compare lane 2 with lanes 6 to 8), suggesting that endogenous E2F$_1$ may be involved in restraining Tat activation.

Next, we performed a series of transfection studies to evaluate the ability of Tat to liberate HIV-1 promoter activity from the repression imposed upon it by overexpression of E2F$_1$ in glial cells. Towards this end, cells were cotransfected with pCD15-CAT plus the optimum concentration of E2F$_1$ plasmid and were subsequently incubated in the presence of increasing concentrations of Tat protein which were added directly to the culture media. As shown in Fig. 4B, in the absence of Tat, expression of E2F$_1$ in transiently transfected cells caused a fourfold reduction of CAT activity (lanes 1 and 2). Addition of Tat protein to these cells augmented (three- to fourfold) the levels of CAT activity in these cells (Fig. 4B, lanes 2 to 5). In contrast, addition of Tat to cells transfected with the antisense E2F$_1$ resulted in a 4- to 10-fold increase in CAT activity. Results from the cotransfection studies indicated that E2F$_1$ effectively interferes with the transcriptional activation of the HIV-1 LTR by Tat which is produced by an expression plasmid, pact-tat, in glial cells (unpublished data). Altogether, these results strongly suggest that Tat is unable to liberate HIV-1 LTR activity from the repression imposed by E2F$_1$ and that although Tat is able to enhance transcription from the HIV-1 LTR in the presence of E2F$_1$, its ability to function as a potent activator is diminished in the presence of E2F$_1$. We also examined the inhibitory action of E2F$_1$ on transcriptional activation of the viral promoter by phorbol myristate acid. Our results indicated that overexpression of E2F$_1$ in glial cells negatively affects the levels of HIV-1 transcription in phorbol myristate acid-treated glial cells (data not shown).

The ability of E2F$_1$ to suppress HIV-1 LTR-directed expression is not glial cell specific. Transcription of the HIV-1 promoter has a broad range of specificities and is virtually active in all eukaryotic cell types (for a review, see reference 31). In order to examine the observed inhibitory function of E2F$_1$ on LTR activity in different cell types, we used HeLa, SW40, RD, and SupT$_1$ cell lines in transfection assays with pC15-CAT, ptat, and an E2F$_1$ expression plasmid. As shown in Fig. 5,

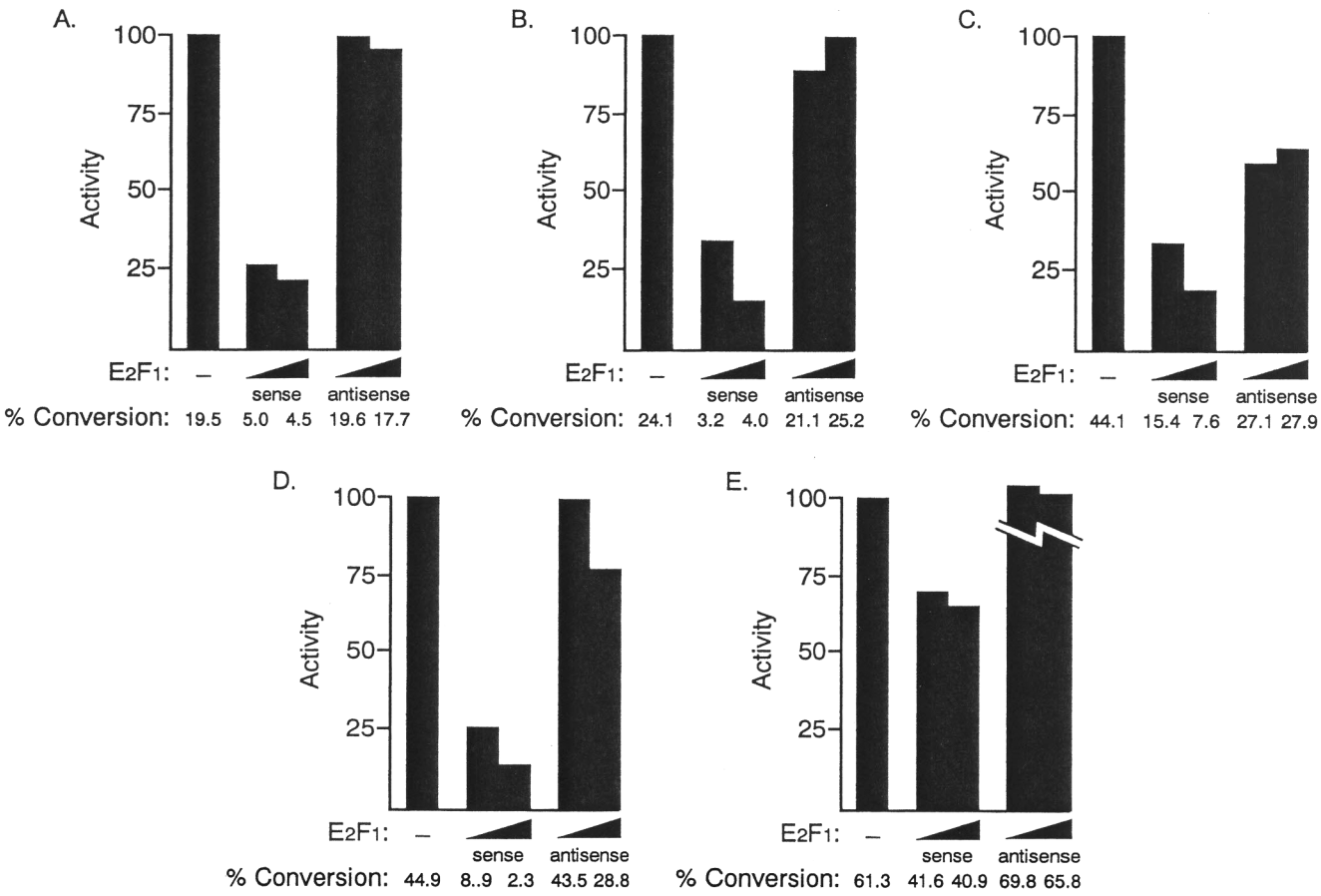


FIG. 5. Effect of E2F on Tat-mediated transactivation of the HIV-1 LTR in different cell lines. U-87MG (A), HeLa (B), SupT$_1$ (C), SW40 (D), and RD (E) cells were cotransfected with 1.0 and 5.0 μg of plasmids expressing pE2F$_1$ in the sense or antisense orientation plus optimal amounts of the pCD15-CAT and ptat constructs. The amount of DNA containing the promoter sequence was kept constant (10 μg) by the addition of pCSMA$_1$ in all experiments. U-87MG, HeLa, SW40, and RD cells were transfected by the calcium phosphate method (21), while SupT$_1$ cells were transfected by the DEAE-dextran method (12). Whole-cell extracts were prepared after 36 to 48 h and analyzed for CAT activity. Studies were performed in duplicate. Activity shown is CAT enzymatic activity normalized to levels obtained in the absence of E2F$_1$ plasmids rated on an arbitrary scale of 0 to 100. Percent acelytation values are the averages for two independent experiments.

expression of E2F$_1$ caused a decrease in the transcriptional activity of the viral promoter in the above-mentioned cell lines. Our recent studies have suggested that expression of E2F$_1$ may not significantly alter the levels of HIV-1 gene transcription in U-937 cell lines (unpublished observations). Therefore, it appears that with the exception of U-937 cells, a variety of cells contain the machinery required to mediate E2F$_1$ repression of the HIV-1 LTR.

Inhibition of HIV-1 replication by E2F$_1$. In order to determine whether E2F$_1$ has the potential to repress HIV-1 expression in the context of the whole viral genome, proviral DNA was introduced into RD cells alone or in combination with 5 μg of pE2F$_{1S}$ or pE2F$_{1AS}$. Relative levels of viral replication were determined by analysis of the RT activity obtained from the supernatant of each transfectant. As shown in Fig. 6, a >50% reduction in replication of the viral genome was detected upon expression of E2F$_{1S}$ in these cells. Transfection of cells with proviral DNA plus pE2F$_{1AS}$ or the vector plasmid pCSMA$_1$, containing only the promoter sequence, resulted in a slight increase in viral replication as examined by RT activity. These results clearly indicate that E2F$_1$ is involved in suppression of viral replication in cultured cells.

DISCUSSION

The results of this study strongly suggest that overexpression of E2F$_1$ can suppress replication of HIV-1 by down-regulating basal levels of LTR-directed transcription and inhibiting activation by Tat. This effect, which could be indirect, seems to be mediated, at least in part, by sequences encompassing the NF-κB-binding domain of the HIV-1 LTR and a region located upstream of nucleotide −381 relative to the start site of transcription. It is possible that E2F$_1$ exerts its effect by one or more of the following mechanisms: (i) direct interaction with the HIV-1 LTR through a novel binding motif, (ii) interaction with proteins which bind directly to the E2F$_1$-responsive sequence, or (iii) regulation of the expression and/or function of other cell cycle proteins which may then directly or indirectly influence transcription from the viral promoter. Also, it is likely that overexpression of E2F$_1$ may result in the formation of nonproductive complexes with important regulatory proteins involved in HIV-1 transcription of the viral genome. Perhaps we should mention that results from our most recent DNA binding studies have indicated that E2F$_1$ can interact with sequences overlapping the NF-κB binding elements (28b). This intriguing observation implies that E2F$_1$ may indirectly

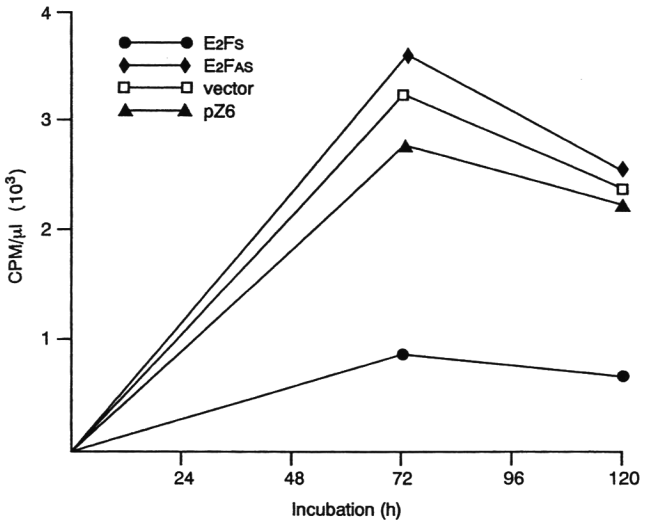


FIG. 6. Effect of $E2F_1$ on HIV-1 replication. RD cells were transfected with proviral DNA plasmid pZ6Neo, alone or with the promoter control vector ($pCSMA_1$), $pE2F_S$, or $pE2F_{AS}$. Supernatants from each sample were collected after 72 and 120 h and analyzed for RT activity. Experiments were performed twice, in duplicate each time.

inhibit transcriptional activity of the HIV-1 LTR by interfering with NF-κB function and/or directly through its participation in a repressive complex with the retinoblastoma protein p105. Studies to determine the mechanism by which $E2F_1$ influences HIV-1 LTR-directed transcription in lytically infected cells are in progress.

The role of $E2F_1$ may change through its association with a variety of proteins throughout the cell cycle. For example, it interacts with an underphosphorylated form of the retinoblastoma susceptibility protein Rb during G_0/G_1 (9, 11). This complex functions as a transcriptional repressor in certain promoters containing $E2F_1$-binding sites (22, 29). Subsequent phosphorylation of Rb is thought to release free $E2F_1$, allowing it to promote DNA synthesis by functioning as a transcriptional activator of genes involved in G_1 and S phases of the cell cycle (for a review, see reference 15). During the G_1 and S phases, E2F may associate with a phosphorylated form of Rb and the cyclin E-cdk2 protein kinase complex; late in G_1 phase and during S phase, $E2F_1$ may also interact with the cyclin A-cdk2 protein kinase complex (30). The picture is further complicated by the existence of other E2F family members, $E2F_2$ to $E2F_5$, which bind to E2F-binding sites (for a review, see reference 38), and of Rb-related proteins p107 and p130, which associate with the E2F family members (8, 11). Further elucidation of the nature of the E2F activity involved in regulation of HIV-1 expression will aid in assessing the relevance of these observations to AIDS pathogenesis and to the role of E2F as a negative regulator of transcription.

The observation that overexpression of $E2F_1$ can suppress HIV-1 replication leads to the hypothesis that cell cycle-regulatory proteins may be involved in inducing proviral latency. Several studies have suggested that viral infection can proceed to the stage of nuclear translocation of the HIV-1 preintegration complex in quiescent T lymphocytes and peripheral blood monocytes (7, 49). Other studies involving growth-arrested T-lymphocyte-derived cell lines and other transformed cells suggest that host cell activation, but not DNA synthesis, is involved in promoting subsequent steps of the viral life cycle (2, 33). Those studies do not exclude the possibility that crucial processes involved in modulating viral replication may occur during the transition from G_0/G_1 to S phase, following activation. A more recent study demonstrates that growth arrest at G_1/S phase of proliferating monocyte-derived macrophages by gamma irradiation prevents viral replication in these cells (44). This observation suggests that the state of the cell during G_1 and S phases is relevant to viral replication. Since gamma irradiation induces G_1 arrest through accumulation of tumor suppressor protein p53 (35), and since this protein has been shown to suppress HIV-1 transcription and replication (13), it is possible that p53 is, in part, responsible for the lack of viral replication in G_1-arrested cells. Another possibility is that G_1 arrest causes accumulation of cellular factors, such as $E2F_1$, which are normally involved in regulating G_1/S phase and that these factors may also be involved in suppressing viral replication. Thus, inhibition of HIV-1 transcription and replication by cellular factors involved in stimulating cells during the early phases of the cell cycle may provide another mechanism for inducing proviral latency.

The observations presented in this paper suggest that a regulatory protein involved in the cell cycle could influence HIV-1 gene transcription and replication. This interaction between viral gene expression and host cell proliferation is demonstrated by viral proteins such as Vpr, which arrests cells at G_2 phase of the cell cycle (42). Thus, it appears that interactions between virus and host via cell cycle modulators could be an important factor in the physiology of viral infection.

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