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# Physical and Functional Interaction of HIV-1 Tat with E2F-4, a Transcriptional Regulator of Mammalian Cell Cycle*

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**Tat protein of the human immunodeficiency virus type-1 (HIV-1) plays a critical role in the regulation of viral transcription and replication. In addition, Tat regulates the expression of a variety of cellular genes and could account for AIDS-associated diseases including Kaposi's Sarcoma and non-Hodgkin's lymphoma by interfering with cellular processes such as proliferation, differentiation, and apoptosis. The molecular mechanisms underlying the pleiotropic activities of Tat may include the generation of functional heterodimers of Tat with cellular proteins. By screening a human B-lymphoblastoid cDNA library in the yeast two-hybrid system, we identified E2F-4, a member of E2F family of transcription factors, as a Tat-binding protein. The interaction between Tat and E2F-4 was confirmed by GST pull-down experiments performed with cellular extracts as well as with *in vitro* translated E2F-4. The physical association of Tat and E2F-4 was confirmed by *in vivo* binding experiments where Tat·E2F-4 heterodimers were recovered from Jurkat cells by immunoprecipitation and immunoblotting. By using plasmids expressing mutant forms of Tat and E2F-4, the domains involved in Tat·E2F-4 interaction were identified as the regions encompassing amino acids 1–49 of Tat and amino acids 1–184 of E2F-4. Tat·E2F-4 complexes were shown to bind to E2F *cis*-regions with increased efficiency compared with E2F-4 alone and to mediate the activity of E2F-dependent promoters including HIV-1 long terminal repeat and cyclin A. The data point to Tat as an adaptor protein that recruits cellular factors such as E2F-4 to exert its multiple biological activities.**

The human immunodeficiency virus type-1 (HIV-1)[1] is the etiologic agent of the AIDS, a progressive and degenerative disease of the immune system (1). Although the central defect in AIDS is the depletion of $CD4^+$ lymphocytes, the infected subjects show a complex immunological dysfunction that is often associated with the development of tumors including Kaposi's Sarcoma and non-Hodgkin's lymphoma and involvement of the central nervous system (2, 3) Among the HIV-1 regulatory proteins, Tat plays a critical role in the regulation of viral transcription and replication (4, 5). Tat is a small nuclear protein (86 or 101 amino acid residues according to viral strains) that acts through a *cis*-acting element termed the transactivation response region (TAR) located within the long terminal repeat (LTR) and encompasses nucleotides +1 to +44 from the transcription start site (6, 7). Tat binds directly to TAR-RNA (8–11) and promotes the full activation of viral gene transcription by enhancing the processivity and the transcription rate of RNA polymerase II (12, 13). The mutational analysis of the protein revealed two functional domains coded for by the first exon of *tat* and required for the optimal activation of viral gene transcription: the activation domain extending from the N terminus to residue 48 and the arginine-rich RNA binding motif from residues 49 to 58, which also encompasses a nuclear localization signal (14–18). The transcription activation domain functions as a typical eukaryotic domain as demonstrated by swapping experiments where the Tat-(1–49) region was fused to the DNA-binding domain of heterologous proteins (19, 20). Three functional domains can be identified in the Tat-(1–49) region: the acidic activation domain (residues 1–21) and the cysteine-rich region (residues 22–31), which together with the conserved core region are involved in the *in vitro* formation of Tat-linked metal dimers and Tat protein interactions *in vivo* (21, 22). The Tat functional interaction with cellular proteins plays a key role in the regulation of Tat transcriptional activity (23, 24). Tat-mediated regulation of cellular gene expression is strongly related to its physical and functional interaction with proteins directly involved in the basal transcriptional process including TFIID, TFIIB (25–27), and eukaryotic transcription factors such as Sp1 (28), and CAAT enhancer-binding protein ((29). Moreover, Tat binds to cyclin T and recruits CDK9 to increase the processivity of RNA polymerase II (12, 30–32). The second exon of *tat* codes for the C terminus of the protein and appears to mediate a large array of cellular activities by interacting with several cell surface receptors including integrin receptors (33), vascular endothelial growth factor, and chemokine receptors (34, 35). Tat protein may be directly involved in the development of some AIDS-related diseases by interfering with cellular processes such as proliferation, differentiation, and apoptosis (36). In fact, Tat deregulates the expression of several genes including proto-oncogenes and genes encoding for metabolic enzymes, cytokines, and cytokine receptors (36–38). The above evidence points to Tat as an adaptor protein that affects the expression of viral and cellular genes by associating with cellular proteins.

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[1] The abbreviation used are: HIV-1, human immunodeficiency virus, type 1; TAR, transactivation response region; LTR, long terminal repeat; DTT, dithiothreitol; CAT, chloramphenicol acetyltransferase; E2, ubiquitin carrier protein.

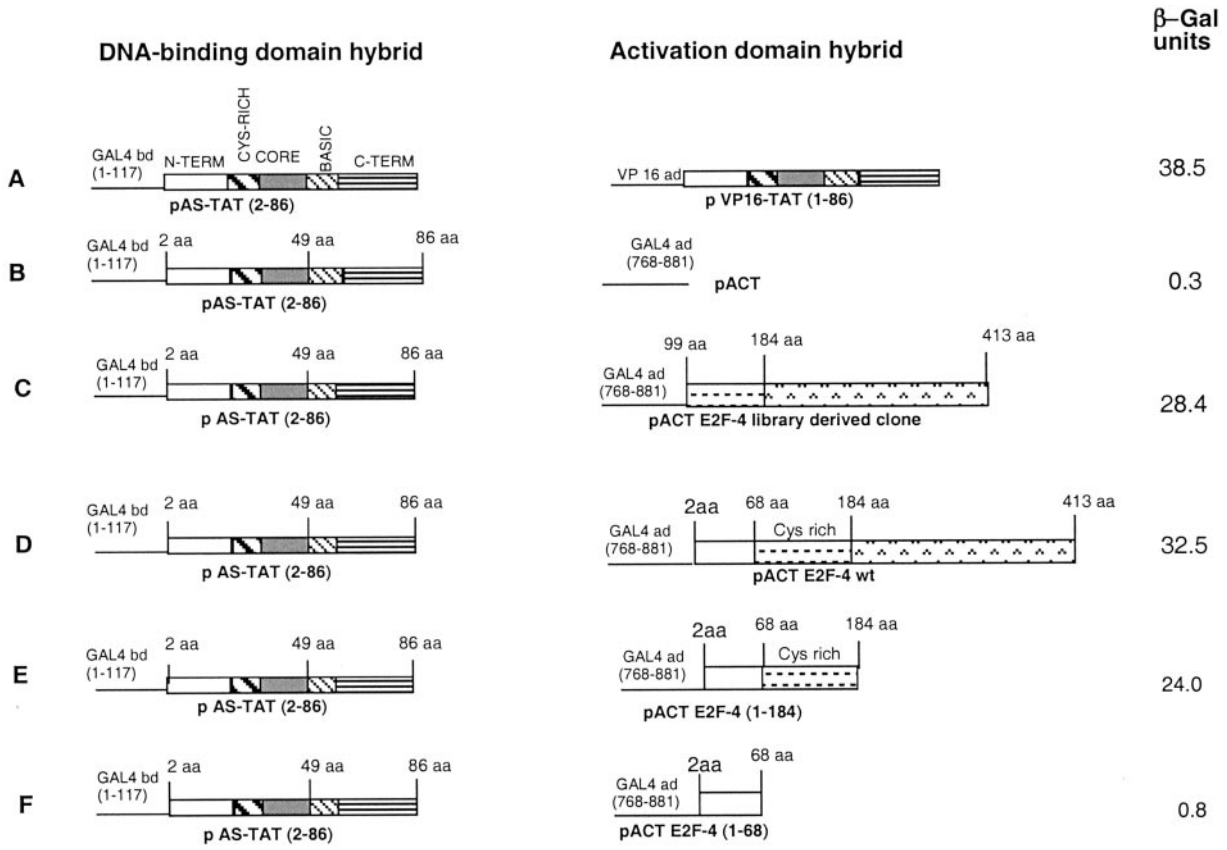


FIG. 1. **Functional interaction between full-length Tat and discrete regions of E2F-4 in yeast cells**. *A*, the strong Tat-Tat interaction (29) as a positive control. *B*, the absence of interaction of Tat with Gal4 activation domain. pAS-Tat-(2–86) plasmid carrying a cDNA coding for the full-length of Tat fused to the Gal4 DNA-binding domain was co-transfected in yeast Y190 together with pACT-(99–413) plasmid expressing the region of E2F-4 encompassing amino acids 99–413 and identified by screening a human B-cell library as detailed under "Experimental Procedures" (*C*). *D–F*, in parallel experiments, pACT plasmid expressing either the full-length or discrete regions of E2F-4 were tested. Results are expressed as the level of β-galactosidase of the yeast colonies selected as detailed under "Experimental Procedures."

In this regard, the complex role of Tat in the establishment and progression of HIV-1 infection indicates that the yet unknown cellular partners of Tat remain to be identified. To this end, we took advantage of the yeast two-hybrid system to identify cellular proteins interacting with HIV-1 Tat. Among the identified Tat-binding proteins, E2F-4, a member of the E2F transcription factor family, was selected for further studies.

The family of E2F transcription factors forms heterodimers with pRb and DP (differentiation regulated transcription factor 1 protein) family proteins resulting in DNA-binding complexes (39, 40). E2F proteins share a conserved DNA-binding domain and an acidic transcriptional activation domain, which includes the "pocket proteins" binding site. E2F proteins share a similar structural organization with minor modifications: a N terminus region, which includes the DNA-binding domain, followed by the dimerization domain and the transcriptional activation domain located at the C terminus (41). Functional E2F binding sites have been detected in the promoters of genes controlling cell cycle progression such as dihydrofolate reductase (42), thymidine kinase, cyclin A (43, 44), cyclin E (45), E2F-1, E2F-2, pRb107, and some cellular proto-oncogenes (43). The E2F DNA-binding complexes can be composed of DP-E2F heterodimers or by larger complexes containing the pRB pocket proteins (46–49) or cyclin A-CDK2 and cyclin E-CDK2 (49–51) with E2F-4 as a major component of E2F complexes in every stage of the cell cycle. E2F-4 presents some structural and functional peculiarities, because it lacks a nuclear localization signal (52–54) and the cyclin A binding site (located at the N terminus of the other E2F proteins) (41). E2F-4 transcriptional activities are regulated by modifications of its phosphorylation status (41, 46, 49) by association with other cellular proteins (55) and by its subcellular localization (56, 57). E2F-4 can bind all "pocket proteins" (pRb, p107, and p130), although a preferential association with p107 (48, 58) and p130 has been documented (59, 60). The resulting E2F-4 heterodimers exert different biological roles. In fact, E2F-4·p130 complex is more abundant in the $G_0$ phase of the cell cycle, whereas E2F-4·p107 complex is mainly found in the S phase. In addition, the investigation of E2F·pRb complexes is made difficult by the capacity of Rb proteins to substitute for each other (56–61). The above evidence points to E2F-4 as a major mediator E2F cellular activity.

In this work, we provide evidence that Tat physically associates to E2F-4 *in vitro* as well as *in vivo*. The resulting Tat·E2F-4 complexes enhance the transcriptional activity of E2F-driven promoters, indicating that Tat recruits E2F-4 to exert multiple biological activities.

## EXPERIMENTAL PROCEDURES

*Plasmids and Cloning Strategies*—The yeast plasmids expressing the wild-type and mutated HIV-1 Tat proteins fused to GAL4 DNA-binding domain (amino acids 1–117) were constructed by cloning the *tat* cDNA fragments into the *Nde*I site (Klenow-filled) of pAS2 plasmid. The DNA fragments coding for either the wild-type Tat protein (amino acids 2–86) or the activation domain of Tat (amino acids 2–49) or the basic region of Tat (amino acids 49–86) were recovered from pAS-Tat plasmids (a kind gift of B. Cullen) by *Eco*R1/*Xho*I digestion and were inserted into pAS2. pACTE2F-4-(1–481) was constructed by cloning the E2F-4 wild type cDNA downstream of the GAL4 activation domain in pACT vector. The E2F-4 cDNA was obtained by PCR using the pCDNA3-E2F-4 plasmid (provided by A. Giordano) as a template for the following primers: 5′-CGCGGATCCCGCGGAGGCCGGG-3′ (5′ primer)

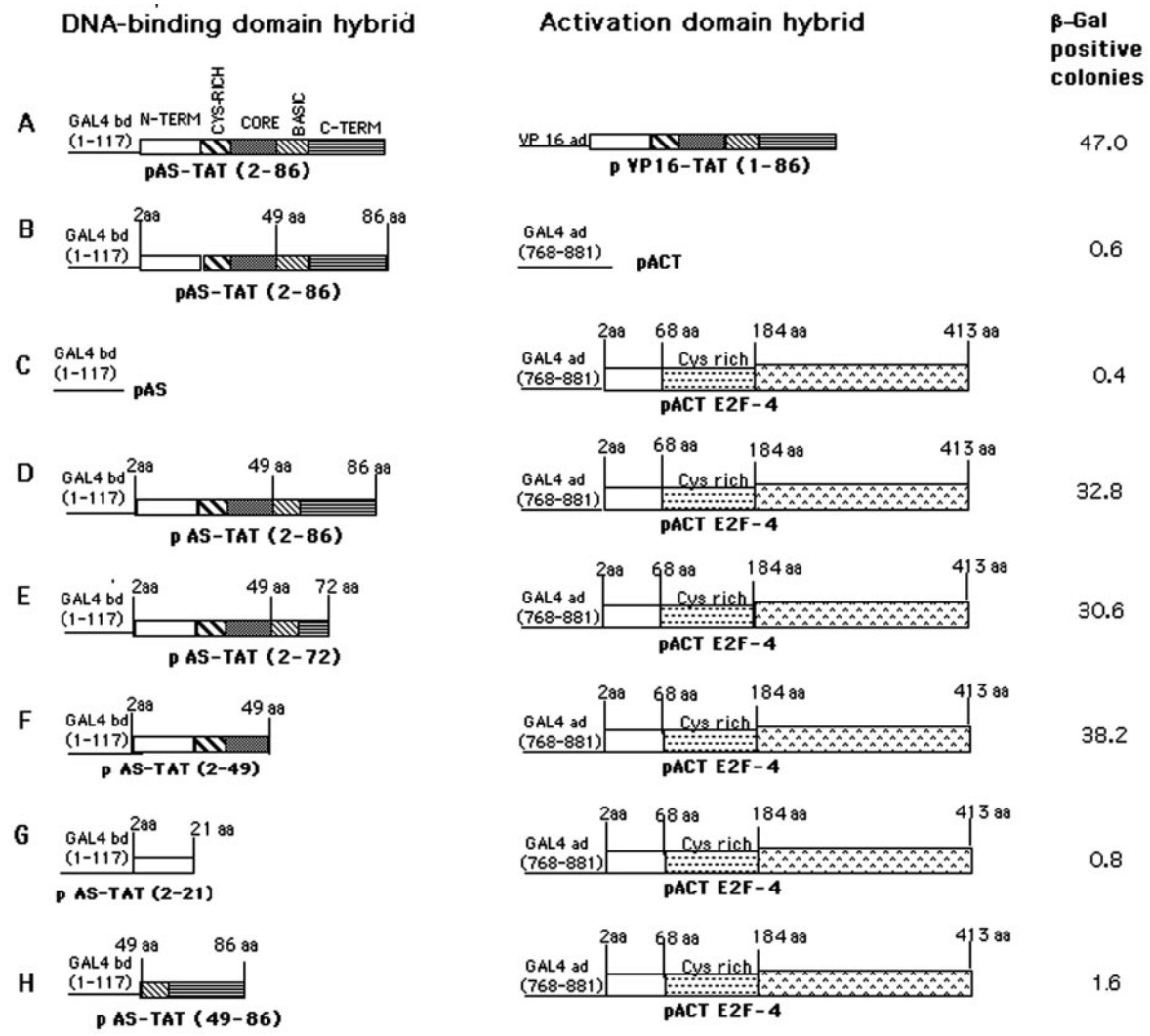


FIG. 2. **Functional interaction between full-length E2F-4 and discrete regions of Tat in yeast cells.** pAS-TAT plasmids carrying discrete regions of Tat fused to the Gal4 DNA-binding domain were co-transfected in Y190 yeast cells with pACTE2F-4 plasmid expressing E2F-4 full-length. *A* and *B*, positive and negative controls of Tat interaction as detailed in legend to Fig. 1.

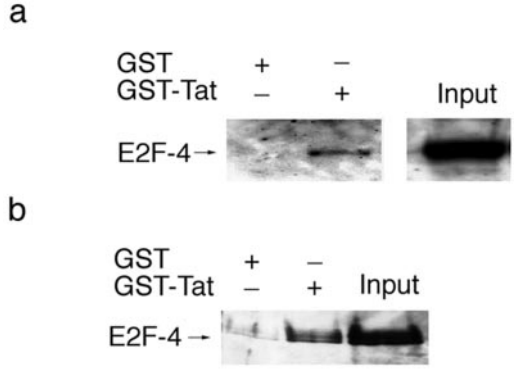


FIG. 3. ***In vitro* interaction of Tat and E2F-4.** *a*, Tat was produced and purified *in vitro* as a GST fusion protein as described previously (29). 5 μg of purified GST or GST-Tat proteins were incubated with whole cellular extracts prepared from Jurkat cells. The cellular proteins complexed either with GST or with GST-Tat were recovered by adding a glutathione affinity matrix and subjected to immunoblotting with a E2F-4-specific antibody. *b*, GST or GST-Tat proteins were incubated with *in vitro* translated E2F-4. E2F-4 proteins binding to GST or to GST-Tat were detected by a anti-E2F-4 antibody.

and 5′-CGCGGATCCTGAGAGGTTGAGAACAGGCAG-3′ (3′ primer). The PCR fragment was digested with *Bam*H1 and cloned in the *Bam*H1 site of pACT. The DNA fragments coding for the 3′-truncated forms of E2F-4 were obtained by PCR using the following 3′ primers: 5′-CGGCCTCGAGTCACAGGTGAATCTGGTACTTC-3′ in the case of E2F-4-(1–184) and 5′-CGGCTCGAGTCACCAAAACATTGGTAATGTCGTAAATCCG-3′ for E2F-4-(1–68). The PCR fragments were digested with *Bam*H1/*Xho*I and cloned in the compatible sites of pACT. The correct sequence of the cDNA fragments was analyzed by sequencing (Sequenase Version 2, Amersham Biosciences). The expression of the fusion proteins was verified by Western blot by using antibodies specific for either the GAL4 DNA-binding domain or the GAL4 activation domain (Santa Cruz Biotechnology). pGEX-Tat plasmid was a gift of M. Giacca. Mammalian plasmids expressing either the wild type Tat, pCMVTat-(1–86), or the mutant forms of the viral protein, namely pCMVTat-(1–21) and pCMVTat-(1–49), were constructed by cloning the respective PCR fragments in pRC-CMV vector. The PCR was performed with the following oligonucleotides: 5′-CGGGGTACCATGGAGCCAGTAGATCCTAG-3′ as 5′ primer; 5′-GGAATTCCATATGGCCTTAGGCATCTCC-3′ as 3′ primer in the case of Tat-(1–86); 5′-CTA**TCCCTGTCTCCGCTTCTTCCT**AGCAGTTTTAGGCAGACAACC-3′ for Tat-(1–21); and 5′-CTA**TCCCTGTCTCCGCTTCTTCCT**CCTGCCATAGGAGATCG-3′ as 3′ primer for Tat-(1–49). The above 3′ primers included the nuclear localization signals (bold nucleotides) and the stop codon. The PCR fragments were subcloned in BKS (Stratagene) and recovered by *Kpn*I/*Bam*H1 digestion. The purified fragments were cloned in pRC-CMV digested with the same enzymes. pCMV-5′-FLAG-Tat-(1–86) was obtained by cloning the FLAG-Tat fragment previously digested with *Kpn*I/*Bam*H1 in the compatible sites of pRC-CMV. The FLAG epitope (bold sequence) recognized by the M2 monoclonal antibody (Sigma) was inserted in-frame upstream of the Tat coding sequence by PCR using the following primers: 5′-CGGGGTACC**ATGGACTACAAAGACGATGACGACAAG**GAGCCAGTAGATCCTAGACTA-3′

(5′ primer) and the above reported 3′ primer for Tat-(1–86). To obtain pCMVTat-(1–49), the cDNA coding for residues 1–49 of Tat was amplified by using the primers 5′-CCGGAATTCAGAGCCAGTAGATC-TTAGACTA-3′ and 5′-GCTCTAGACTAGCCATAGGAGATGCCTAA-G-3′ followed by *Eco*RI-*Xba* digestion and insertion into the corresponding sites of p3XFLAG-CMV-7.1 (Sigma). The HIV-1 Tat expression vectors pSVT8 and pSVT10 carrying the *tat* gene cloned downstream of the SV40 promoter in sense or antisense orientation, respectively, were provided by A. Caputo. The reporter plasmids pWTcat carrying the HIV-1 promoter from −644 to +78 bp cloned upstream to the *cat* gene, pTARcat carrying mutations abolishing the Tar structure, and pNFA-cat carrying mutations of the two NFκB sites were obtained by A. Rabson (62). pCD23 plasmid harboring the HIV-1 promoter sequence from −117 to +80 bp and pCD52 lacking the two NFκB sites were obtained from the AIDS Reference and Reagents Program. In pCD23ΔTar, the Tar region was functionally deleted as described previously (63). pBLTK/E2F-cat and pBLTK/E2FM-cat were prepared by inserting a double-stranded oligonucleotide corresponding to two copies of either wild type or mutant E2F binding site upstream of the TK promoter in the *Sal*I site of pBLCAT2. The following oligonucleotides corresponding to the E2F binding site of the promoter of the adenovirus *E2* gene were utilized: 5′-GATCCACTAGTTTCGCGCGTT-TCTACACTATTTCGCGCGC-3′ (wild type sequence) and 5′-GATCCA-CTAGTTT**ACTCA**GATAA**C**TACACTATTT**ACTCA**GATAA**C**TATCG-A-3′ (mutant oligonucleotides, mutated basis are in *bold*).

*Library Screening by Using the Yeast Two-hybrid System*—Y190 and Y187 yeast strains were described previously (64). For the library screening, *Saccharomyces cerevisiae* Y190 was grown in selective minimal medium containing 6.7 g/liter yeast nitrogen base without amino acids (20 g/liter glucose, 1× amino acid mixture lacking uracil and lysine, 5 μg/ml cycloheximide, and 20 g/liter Bacto-agar (Difco). For yeast transformation, a yeast culture from a single colony was diluted and grown overnight at 30 °C. The day after, the yeast culture (300 ml) was diluted at $A_{600}$ = 0.2 and grown to $A_{600}$ = 0.5. Yeast cells were harvested by centrifugation at 6000 × *g* and resuspended in 1.5 ml of lithium acetate-TE buffer (100 mM lithium acetate, pH 7.5, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) and incubated at 30 °C for 1 h in agitation. For each transformation, 20 μg of library DNA were mixed with 5 μg of pASTAT-(2–86) and 175 μg of carrier DNA previously boiled and chilled on ice. 200 μl of yeast suspension were added to each DNA mix in the presence of 1.3 ml of polyethylene glycol solution (40% polyethylene glycol 3350, 100 mM lithium acetate, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) freshly prepared. After incubation at 30 °C for 45 min, the mixture of yeast and DNA was heat-shocked at 42 °C for 15 min, resuspended in 5 ml of synthetic complete medium, and grown for 5 h at 30 °C. The yeast cultures then were harvested and plated on a larger plate containing selective medium lacking tryptophan, leucine, and histidine plus 25 mM 3-amino-triazole (Sigma). The plates were incubated at 30 °C, and the colonies grown after 3–5 days were tested for β-galactosidase activity by filter assay. To this end, 45-nm nitrocellulose filters (Schleicher & Schuell) were laid onto the plates that were incubated at 30 °C overnight. The day after, the filters were lifted and placed at −80 °C for 1 h and laid onto 3-mm chromatography paper soaked with buffer Z containing 1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, INALCO). Blue colonies were picked and grown on selective medium lacking leucine plus 5 μg/ml cycloheximide to induce the pAS-Tat-(2–86) plasmid expulsion. A single colony for each yeast clone was grown in liquid synthetic medium lacking leucine plus cycloheximide, and 200 μl of this culture were mixed with 40 μl of Y187 stably carrying pACT-laminin or pACT-SNF1. The mixed yeast cultures were incubated at 30 °C onto a nitrocellulose filter laid on a complete medium plate. After a 4-h incubation, the filters were recovered, and diploid cells were identified by plating on synthetic medium lacking tryptophan and leucine. The grown colonies were screened for β-galactosidase expression by filter assay. To recover the library plasmids from yeast, positive yeast clones were grown in synthetic medium minus leucine until saturation, harvested, washed, and lysed in breaking buffer (0.2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) with glass beads (Sigma) and an equal volume of phenol/chloroform/isoamyl alcohol. The DNA was purified and introduced in *Escherichia coli* DH5α by electroporation (Bio-Rad apparatus). Two-hybrid assays were performed with purified plasmids by using a similar transformation procedure using 5 μg of each of the tested plasmids.

*Cell Cultures and Transfection Experiments*—Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics as reported previously (65). For transfection experiments, 4 × $10^6$ cells were resuspended in 0.3 ml of RPMI 1640 medium and 20% fetal calf serum and subjected to a double electrical pulse at 200 V, 960 microfarads by a Bio-Rad apparatus (66). CAT activity was determined 36 h post-transfection as described previously (29). Each assay contains 15 μg of cell extracts, 30 μl of 4 mM acetyl-coenzyme A (Sigma), 0.5 μCi of D-threo-1,2-[$^{14}$C]chloramphenicol (Amersham Biosciences) in a final volume of 150 μl of 0.25 M Tris-HCl, pH 7.8. The reactions were incubated at 37 °C for 3 h, extracted with ethyl acetate, dried, and spotted on Polygram Sil G silica gel plates (Macherey-Nagel). The plates then were run in a thin layer chromatography tank containing a mixture of chloroform:methanol (95:5). Following autoradiography, the thin layer chromatography spots were counted in a Beckman LS5000TD scintillation counter.

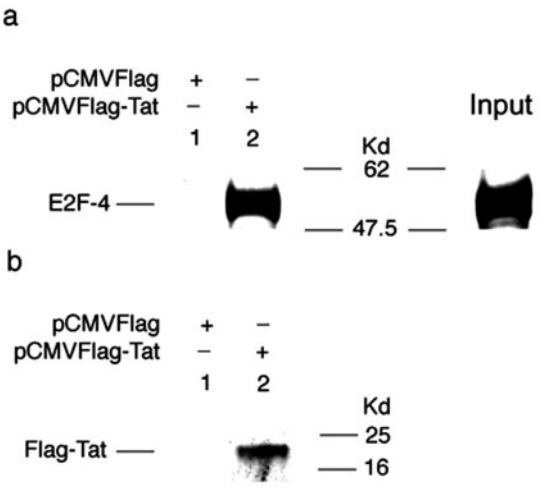


FIG. 4. ***In vivo* interaction of Tat with cellular E2F-4.** *a*, pCMV-FLAG and pCMV-FLAG-Tat were used to transfect Jurkat cells. At 36 h post-transfection, nuclear cell extracts (*lanes 1* and *2*) were subjected to immunoprecipitation by using an anti-FLAG affinity matrix for 4 h at 4 °C followed by immunoblotting with an anti-E2F-4 antibody. *Lane 3* shows the endogenous levels of E2F-4 in nuclear cell extracts. *b*, Tat expression in the tested cell extracts was analyzed by immunoblotting with an anti-FLAG antibody.

*Protein-Protein Interactions*—For *in vitro* interaction studies, GST and GST-Tat proteins were produced and purified as described previously (29, 65). For protein interaction, 5 μg of purified GST and GST-Tat proteins were incubated with 400 μg of whole cellular extracts in binding buffer (20 mM Hepes, pH 7.9, 0.4 M KCl, 25% glycerol, 1 mM EDTA, 2 mM $MgCl_2$, and 5 mM DTT). After a 2-h incubation at 4 °C on a rocking platform, glutathione-Sepharose beads (Amersham Biosciences) previously equilibrated in binding buffer containing 1 mg/ml bovine serum albumin were added to the samples and left under agitation in a cold room for 2 h. The beads were collected by centrifugation at 2000 × *g* for 30 s, washed several times in binding buffer containing 60 mM KCl, and resuspended in modified Laemmli buffer containing 7 M urea and 10% β-mercaptoethanol. The proteins were resolved on SDS-polyacrylamide gel, blotted onto nitrocellulose filters, and probed with E2F-4-specific antibody (C-20, Santa Cruz Biotechnology). A similar protocol was used for the *in vitro* interaction between GST proteins and E2F-4 *in vitro* translated by using the TNT™ coupled reticulocyte system (Promega) according to the instruction of the manufacturer.

Co-immunoprecipitation experiments were performed as reported previously (65). 250 μg of whole cellular extracts or 100 μg of nuclear proteins were precleared in a final volume of 300 μl in binding buffer with protein G-Sepharose for 2 h at 4 °C on a rocking platform. Unbound complexes were recovered by centrifugation at 500 × *g* for 10 min at 4 °C and incubated with 20 μl of M2-anti-FLAG affinity matrix (Sigma) for 3 h in a cold room under agitation. Bound proteins were collected by centrifugation at 500 × *g* for 10 min, washed several times in binding buffer containing 60 mM KCl, and resuspended in Laemmli buffer. The samples were boiled for 2 min and centrifuged at 13,5000 × *g* for 2 min. The recovered supernatants were run on SDS-polyacrylamide gel and subjected to immunoblotting. The filters were probed with E2F-4-specific antibody (C-20).

For immunoblotting assay, the samples were resuspended in modified 2× Laemmli buffer (0.25 M Tris-HCl, pH 6.8, 30% glycerol, 10 mM EDTA, 4% SDS, 0.1% bromphenol blue, 7 M urea, and 10% β-mercaptoethanol) and boiled before loading on SDS-polyacrylamide gel. The gel was run in running buffer (0.025 M Tris, 0.190 M glycine, and 0.1% SDS)

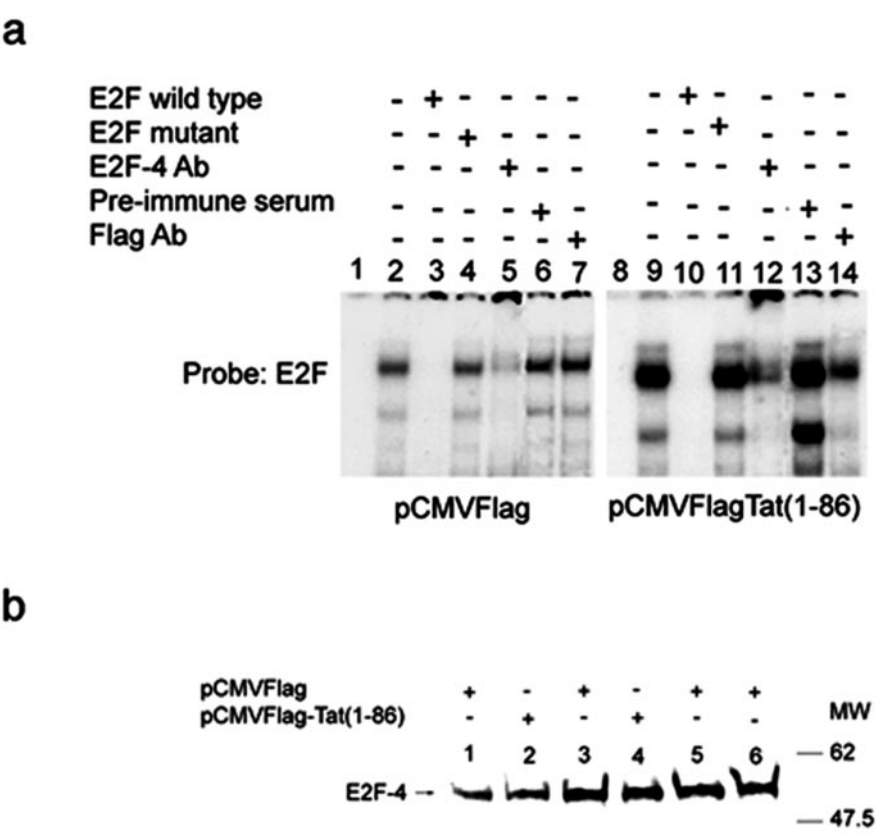


FIG. 5. **Induction of E2F binding activity by Tat**. *a*, nuclear extracts prepared from Jurkat cells transiently transfected with either pCMV-FLAG or pCMV-FLAG-Tat were analyzed for binding to an oligonucleotide corresponding to the E2F binding site located in the adenovirus E2 promoter. Anti-FLAG or anti-E2F-4 antibodies were added to the reaction mixture as detailed under "Experimental Procedures." *b*, E2F-4 content of nuclear (*lanes 1* and *2*), cytosolic (*lanes 3* and *4*), and whole cell extracts (*lanes 5* and *6*) prepared from pCMV-FLAG-transfected or pCMV-FLAG-Tat-transfected Jurkat cells. Cell extracts obtained from the same cell number were subjected to immunoblotting by using an E2F-4 antibody.

and blotted onto nitrocellulose filter (Schleicher & Schuell). The filters were blocked in phosphate-buffered saline, 5% nonfat dry milk, and incubated with the reported antibodies for 2 h at room temperature. The filters were then washed several times and incubated with a peroxidase-conjugated goat-anti rabbit/mouse IgG (Roche Molecular Biochemicals). After a 1 h incubation, the filters were washed and subjected to enhanced chemiluminescence system (ECL, Amersham Biosciences). Anti-E2F-4 (C-20) antibody was purchased from Santa Cruz Biotechnology.

*Electrophoretic Mobility Shift Assay and Northern Blot Analysis*—Whole cellular extracts were prepared as reported previously (37). The cells were resuspended in lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 10% glycerol, 10 mM NaF, 1 mM $Na_2VO_3$, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml aprotinin, and 10 $\mu$g/ml leupeptin). Cells lysates were frozen in dry ice, thawed on ice, and pelleted at 13,5000 $\times$ *g* for 30 min at 4 °C. The cell extracts were aliquoted and stored at −80 °C. To isolate nuclear and cytosolic protein fractions, cells were harvested, washed with cold phosphate-buffered saline, and transferred to a 1.5-ml tube for a second wash at 4 °C. The pellets were resuspended in NP buffer (10 mM Hepes, pH 7.9, 1 mM EDTA, 60 mM KCl, 5 mM DTT, and 0.2% Nonidet P-40 plus protease and phosphatase inhibitors). After a 3-min incubation on ice, cells were checked by microscopy, and nuclei were collected by centrifugation for 500 $\times$ *g* for 5 min at 4 °C. The supernatants (cytosolic fraction) were recovered, diluted in nuclear extraction buffer, and centrifuged at 13,5000 $\times$ *g* for 30 min. The proteins then were aliquoted and stored at −80 °C. The nuclei were washed twice in lysing buffer without Nonidet P-40 and incubated for 1 h in nuclear extraction buffer (lysis buffer plus 450 mM KCl) on ice. After a centrifugation at 13,5000 $\times$ *g* for 30 min at 4 °C, the proteins were aliquoted and stored at −80 °C. Protein concentration was determined by Bio-Rad protein assay kit.

For electrophoretic mobility shift assay, the following double-stranded oligonucleotides obtained by annealing the reported oligonucleotide with the complementary strand were used as probe and competitors: E2F binding site, 5′-GATCCACTAGTTTCGCGCGCTTTCTA-3′; mutant E2F binding site, 5′-GATCCACTAGTTT**ACTCA**G**A**T**AA**C-TA-3′ (bold nucleotides represent the introduced mutations); and Sp1, 5′-GGGAGGTGTGGCCTGGGCGGGACTGGGGAGTGGCG-3′. The oligonucleotides were end-labeled with [$\gamma$-$^{32}$P]ATP by using polynucleotide kinase (Roche Molecular Biochemicals). 5 $\mu$g of nuclear proteins were incubated in a reaction mixture (final volume 10 $\mu$l) containing 10% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 2 $\mu$g of poly(dI·dC) (Roche Molecular Biochemicals) for 5 min on ice, and 1 $\mu$l of [$\gamma$-$^{32}$P]-ATP-labeled double-stranded probe (0.2 ng, 4−6 $\times$ $10^4$ cpm for 5 min on ice). After a 5-min incubation on ice, the cold competitors (200 times) were added, and the samples were left on ice for 5 min and supplemented with the labeled probes. The samples were incubated at room temperature for 20 min followed by the addition of 0.5 $\mu$g of the monoclonal antibodies against E2F-4 (RK-13, Santa Cruz Biotechnology), anti-M2-FLAG epitope (Eastman Kodak Co.), or mouse preimmune serum. The samples were incubated for 1 h on ice and loaded on 4% native acrylamide gel and run in 0.3× Tris borate EDTA for 5 h at 4 °C. A similar protocol was followed in the case of Sp1 where the reaction buffer consisted of 10% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM DTT, and 2 $\mu$g of poly dI·dC. The gels were dried and analyzed by autoradiography. Northern blots were performed on total RNA as reported previously (65).

## RESULTS

*Identification of E2F-4 as a Tat-binding Protein in the Yeast Two-hybrid System*—To screen for human proteins interacting with HIV-1 Tat protein, the Y190 yeast strain (64) was co-transfected with a pACT-based human B-lymphocyte cDNA library fused to the GAL4 acidic activation domain together with pAS-Tat plasmid-expressing HIV-1 Tat-(2–86) fused to the GAL4 DNA-binding domain. The characteristics of the yeast expression vectors and of the library used in the screening are described elsewhere (67). A total of 2.5 $\times$ $10^6$ transformants were placed under selection by spreading on synthetic medium lacking tryptophan, leucine, and histidine and containing 25 mM 3-amino-1,2,4-triazole (3-AT). The 3-AT is a competitive inhibitor of histamine biosynthesis and increases the stringency of the selection of His3 expression. After selection for *his3* gene expression, the transformants were screened for their ability to produce $\beta$-galactosidase using a filter lift assay (68). 279 $His^+$ blue colonies were screened for interaction with Tat-unrelated proteins by mating the type assay. The colonies were grown in synthetic medium minus leucine and plus cycloheximide to eliminate the bait plasmid pAS2-Tat, which confers cycloheximide sensitivity to the Y190 strain.

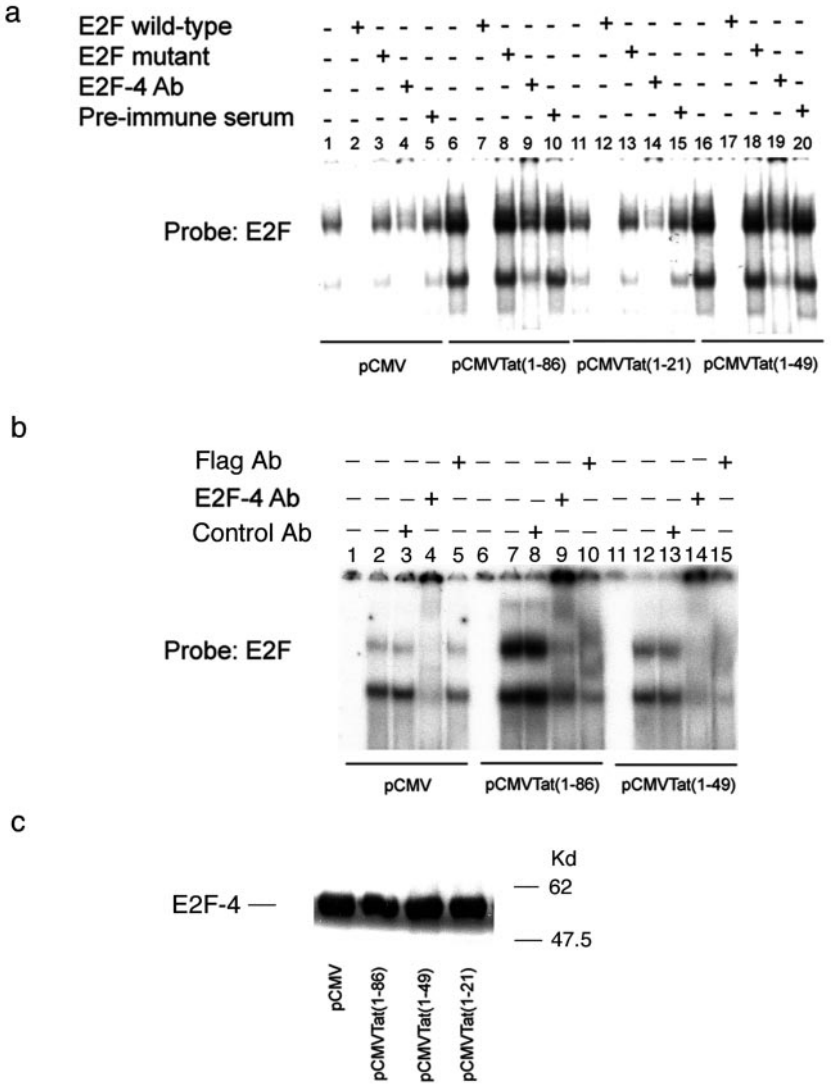


FIG. 6. **Identification of the Tat region required for Tat-induction of E2F-binding activity.** *a*, Bandshift assays were performed on nuclear extracts prepared from Jurkat cells transiently transfected with pCMV, pCMVTat-(1–86), pCMVTat-(1–21), and pCMVTat-(1–49) as detailed under "Experimental Procedures." Oligonucleotides corresponding to an E2F *cis*-region were end-labeled with [$\gamma$-$^{32}$P]ATP and incubated with 5 $\mu$g of nuclear extracts. *b*, protein extracts from Jurkat cells expressing either FLAG-Tat-(1–86) or FLAG-Tat-(1–49) were tested for E2F DNA binding activity. Supershift experiments were performed by using either an anti-E2F-4 or an anti-FLAG antibody. *Lanes 1*, *6*, and *11* denote the free probe. *c*, E2F-4 proteins were detected in transfected cells by immunoblotting with an anti-E2F-4 antibody.

Yeast cells from these liquid cultures were mated with yeast strain Y187 stably expressing pAS-laminin or pAS-SNFI plasmids (64). The resulting diploid cells were selected and assayed for $\beta$-galactosidase activity. The yeast colonies that tested positive in this assay were considered to be not actually positive and were discarded. A sequence analysis from one of the positive clones revealed that the cDNA insert encoded a protein identical to E2F-4 from amino acids 99 to 413. To verify the ability of wild-type E2F-4 to interact with HIV-1 Tat protein, the full-length E2F-4 cDNA coding for amino acids 2–413 was cloned downstream to the GAL-4 activation domain in the pACT yeast expression vector. As shown in Fig. 1, *C* and *D*, the full-length E2F-4 protein interacts with HIV-1 Tat in a manner similar to the library-derived clone (compare *panel C* with *D*). The specificity of the interaction was verified by two-hybrid experiments performed with yeast vectors expressing mutant forms of GAL4-E2F-4. We constructed the pACTE2F-4-(2–184) and pACTE2F-4-(2–68) plasmids expressing 3′-truncated forms of E2F-4 lacking either the transcriptional activation domain (amino acids 185–413) or the transcription activation domain together with the cysteine-rich region (amino acids 68–184), respectively. As shown in Fig. 1, *E* and *F*, the deletion of E2F-4 transcriptional activation domain (amino acids 185–413) resulted in a reduced interaction with Tat, whereas the deletion of E2F-4 cysteine-rich region (amino acids 69–184) completely abolished it.

To identify the Tat domain required for the interaction with E2F-4, we generated the mutant plasmids pAS-Tat-(2–49) and pAS-Tat-(49–86). The above plasmids were then co-transfected in Y190 together with pACTE2F-4-(2–413). The deletion of a Tat region encompassing the basic and the C terminus domains (amino acids 49–86) did not affect the Tat interaction with E2F-4 (Fig. 2*F*), whereas the tat domain encompassing amino acids 2–21 did not interact with E2F-4 (*panel G*). Consistently, the Tat C terminus region (amino acids 49–86) failed to sustain a physical interaction between HIV-1 Tat protein and E2F-4 (*panel H*). Considered collectively, the results shown in Figs. 1 and 2 indicate that Tat interacts with the cellular transcription factor E2F-4. In addition, this finding demonstrates that the interaction is mediated by the Tat activation domain (amino acids 2–49) and requires the Cys-rich domain of E2F-4.

*HIV-1 Tat Protein Binds to E2F-4 Both in Vitro and in Vivo*—Tat was next produced and purified *in vitro* as a GST fusion protein as described previously (65). 5 $\mu$g of purified GST or GST-Tat proteins were incubated with whole cellular extracts prepared from Jurkat cells. The cellular proteins complexed either to GST or to GST-Tat were recovered by adding a glutathione affinity matrix and subjected to immunoblotting with a E2F-4-specific antibody. As shown in Fig. 3*a*, GST-Tat proteins specifically associated with E2F-4. Similar results were obtained when GST or GST-Tat proteins were added to *in*

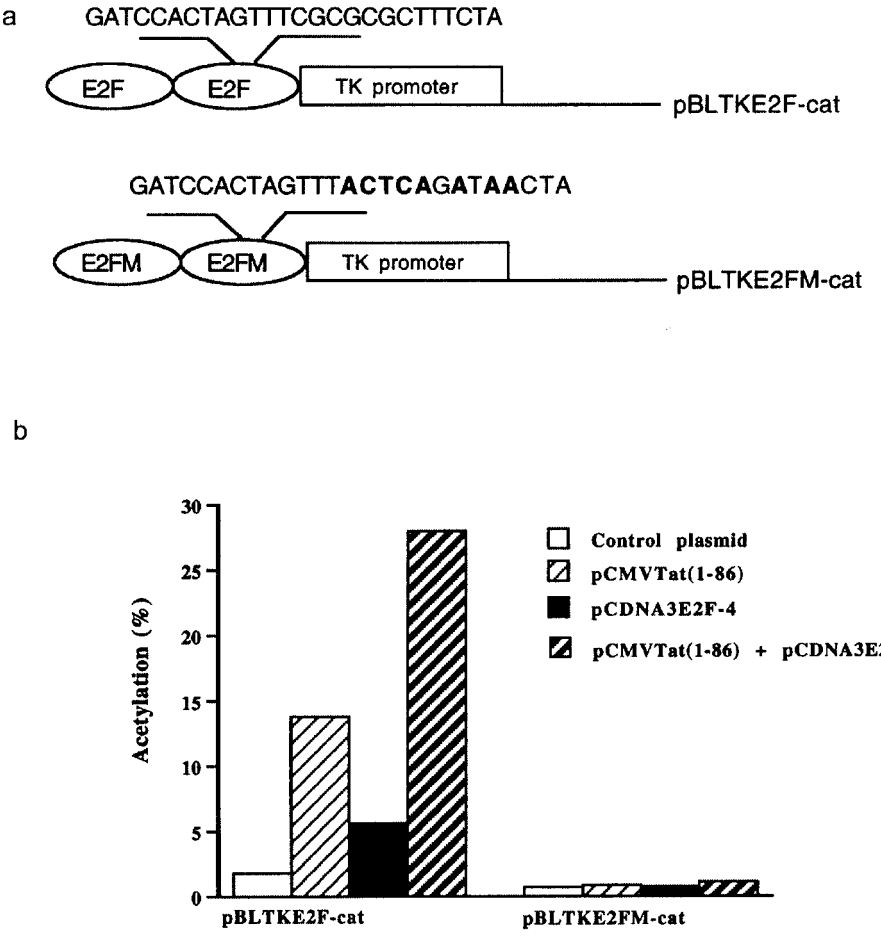


FIG. 7. **Tat up-regulates the transcriptional activity of a E2F-driven promoter**. *a,* an oligonucleotide reproducing two copies of either wild type or a mutant E2F binding site was cloned upstream to the thymidine kinase promoter in pBL-CAT2. The resulting pBLTK-E2F-cat and pBLTK-E2FM-cat were transiently co-transfected in Jurkat cells together with pCMVTat-(1–86) alone or in combination with pCMV-E2F-4. Cell extracts prepared 36 h post-transfection were tested for CAT activity as detailed under "Experimental Procedures." *b*, results are expressed as percentages of acetylated [1,2-$^{14}$C]chloramphenicol. The above experiments were repeated three times with similar results.

*vitro* translated E2F-4 (as shown in Fig. 3*b*), suggesting that the post-translational modifications of E2F-4 such as phosphorylation do not play a role in the observed interaction.

To further characterize the Tat·E2F-4 interaction, we examined whether Tat could associate with endogenous E2F-4 proteins. To this end, Jurkat cells were transfected with pCMV-FLAG and pCMV-FLAG-Tat. 36-h post-transfection nuclear cell extracts were subjected to immunoprecipitation by using an anti-FLAG affinity matrix for 2 h at 4 °C followed by immunoblotting with an anti-E2F-4 antibody. As shown in Fig. 4*a*, *lane 2*, E2F-4 was selectively immunoprecipitated from the Tat-transfected cells, indicating the *in vivo* presence of Tat·E2F-4 complexes. Tat expression in the cell extracts was analyzed by immunoblotting with an anti-FLAG antibody (Fig. 4*b*).

*Tat·E2F-4 Complexes Bind to a E2F cis-Sequence*—The Tat·E2F-4 complexes may bind to E2F *cis*-sequences and regulate E2F-dependent promoters. To test this possibility, the nuclear extracts prepared from Jurkat cells transiently transfected with either pCMV-FLAG or pCMV-FLAG-Tat were analyzed for binding to an oligonucleotide corresponding to the E2F binding site located in the adenovirus E2 promoter. In these experiments, Tat-transfected cells expressed a nuclear E2F DNA binding activity higher than the control cells (Fig. 5*a*, *lanes 2* and *9*). The increased DNA binding activity was mediated by E2F-4 as demonstrated by using a E2F-4-specific antibody (Fig. 5*a*, *lanes 5* and *12*) and by the lack of any activity by a control pre-immune serum (Fig. 5*a*, *lanes 6* and *13*). The addition of an anti-Flag monoclonal antibody reduced the E2F DNA binding specifically in the Tat-expressing cells (Fig. 5*a*, *lanes 7* and *14*), indicating that Tat was present in the DNA-binding complex. The increased level of E2F-4 nuclear DNA binding activity of the Tat-transfected cells was not the result of a higher amount of nuclear E2F-4. In fact, immunoblotting experiments performed with nuclear, cytosolic, and whole cell extracts prepared from the same number of pCMV-FLAG or pCMV-FLAG-Tat-transfected cells showed similar levels of cellular E2F-4 (Fig. 5*b*). The above results indicate that Tat binds to E2F-4 transcription factors and increases their affinity for the cognate *cis*-sequence.

Bandshift assays performed on nuclear extracts prepared from Jurkat cells transiently transfected with pCMV, pCMVTat-(1–86), pCMVTat-(1–49), and pCMVTat-(1–21) demonstrated that the Tat activation domain (Tat-(1–49)) was as efficient as the full-length Tat in enhancing the E2F DNA binding activity, whereas the N terminus of the protein (Tat-(1–21)) as well as the control pCMV was ineffective (as shown in Fig. 6*a*). Supershift experiments performed in the presence of an anti-E2F-4 antibody confirmed the specificity of the Tat induction of the E2F DNA binding activity (Fig. 6*a*, *lanes 4*, *9*, *14*, and *19*). Thus, consistent with the interaction results shown in Figs. 1–4, the Tat region encompassing amino acids 22–49 is required for the generation of Tat·E2F-4 DNA-binding complexes. In other experiments, protein extracts from Jurkat cells expressing either FLAG-Tat-(1–86) or FLAG-Tat-(1–49) were tested for E2F DNA binding activity (Fig. 6*b*). We observed increased levels of E2F binding activity in FLAG-Tat-expressing cells (Fig. 6*b*, *lanes 7*, *8* and *12*, *13*). The DNA-protein complexes were supershifted by an anti-E2F-4 and reduced by an anti-FLAG antibody, indicating that both E2F-4 and Tat contributed to the E2F-binding complex (Fig. 6*b*, *lanes 9*, *14* and *10*, *15*).

*Functional Cooperation between Tat Protein and E2F-4 in the Regulation of Gene Transcription*—E2F *cis*-sequences have been identified in the promoter regions of several viral and

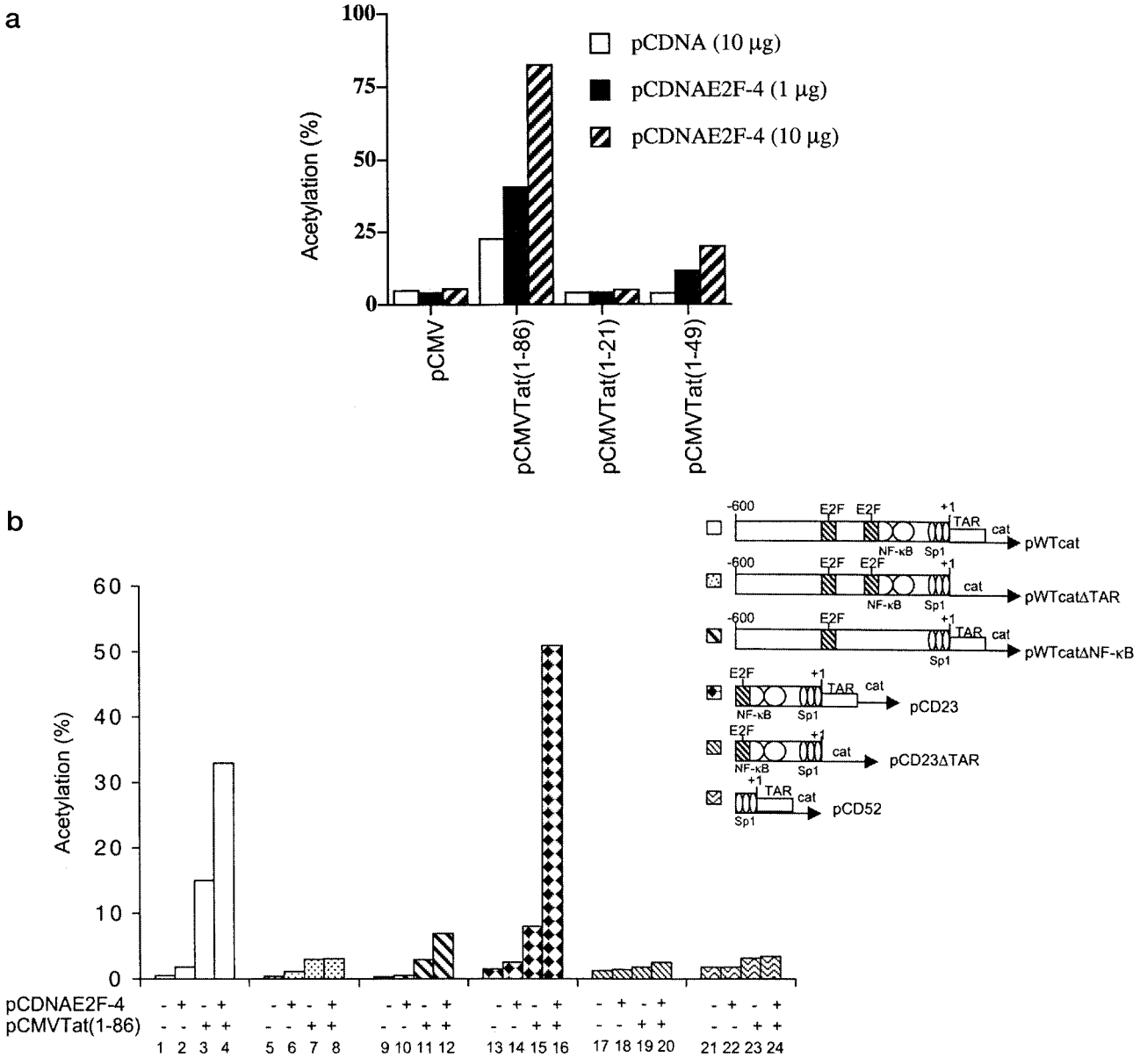


FIG. 8. **Tat·E2F-4 complexes regulate the HIV-1 LTR promoter activity.** *a*, Jurkat cells were co-transfected with pWT-cat, an LTR-driven reporter construct together with the indicated *tat*-expressing plasmids (0.5 μg) in combination with the indicated amounts of pCDNA-E2F-4 plasmid. CAT activity was assayed on cell extracts at 36 h post-transfection as detailed under "Experimental Procedures." Results are expressed as percentages of acetylated [1,2-$^{14}$C]chloramphenicol. *b*, pCMVTat-(1–86) (0.5 μg) was co-transfected with pCDNA-E2F-4 plasmid (10 μg) together with the indicated LTR-CAT constructs carrying either the wild-type sequence or deletions of discrete *cis*-sequences. CAT activity was assayed on cell extracts at 36 h post-transfection as detailed under "Experimental Procedures." Results are expressed as percentages of acetylated [1,2-$^{14}$C]chloramphenicol. The results are representative of four independent experiments.

cellular genes (41). To test whether HIV-1 Tat protein could modify the activity of a E2F-regulated promoter, an oligonucleotide consisting of two tandem copies of either wild type or mutant E2F binding site was cloned upstream of the thymidine kinase promoter in pBL-CAT2, a *cat* reporter plasmid (Fig. 7*a*). The resulting pBLTK-E2F-cat and pBLTK-E2F-4-cat were transiently co-transfected in Jurkat cells together with pCMVTat-(1–86) alone or in combination with pCMV-E2F-4 (Fig. 7*b*). In these experiments, Tat induced a substantial amount of CAT activity only in cells co-transfected with pCMV-E2F-4 plasmid, indicating that Tat cooperates with E2F-4 in regulating the E2F-dependent gene expression.

Two functional E2F binding sites at positions −454 to −381 and −117 to −80 bp have been identified in the LTR region of HIV-1 and regulate HIV-1 promoter activity (69). Based on these observations, we tested whether Tat could cooperate with E2F-4 in the regulation of gene transcription driven by the HIV-1 LTR. To this end, Jurkat cells were co-transfected with pWTcat either alone or in the presence of pCMVTat and pCDNA3-E2F-4. We found that E2F-4 cooperates with suboptimal doses of Tat (0.5 μg) to induce the HIV-1 promoter activity (Fig. 8*a*). In these experiments, a synergistic increase in CAT activity induced by E2F-4 and full-length Tat was observed. To define the Tat domains involved in the observed increase in CAT activity, plasmids encoding deletion mutants of Tat protein, namely pCMVTat-(1–21) and pCMVTat-(1–49), were transfected in Jurkat cells. We observed that the co-expression of pCDNA3-E2F-4 resulted in a substantial increase in HIV-1 promoter activity only when pCMVTat-(1–86) or pCMVTat-(1–49) were co-transfected, whereas mutant Tat-(1–21) was ineffective. These results indicate that Tat activation domain (amino acids 1–49) was the minimal region required

for a functional interaction with E2F-4 in the context of the HIV-1 LTR (Fig. 8*a*).

We next addressed the role of E2F-4 and Tat-binding *cis*-regions in the regulation of HIV-1 LTR-driven transcription. The expression vectors pCMVTat and pCMV-E2F-4 were co-transfected in Jurkat cells together with *cat* reporter plasmids carrying the deletions of discrete regions of the viral LTR. As reported in Fig. 8*b*, the deletion of the TAR region in pWTCatΔTAR strongly reduced the Tat induction of LTR-driven transcription (*bars* 3 and *7*) and abolished the TAT-E2F-4 cooperation (*bars 4* and *8*). The deletion of the NFκB binding sites and of the overlapping E2F *cis*-region in pWTcatΔNFκB reduced the induction of viral transcription by Tat (*bars 3* and *11*), but it did not abolish the functional cooperation between Tat and E2F-4 (*bars 4* and *12*), indicating that the upstream E2F binding site was used in the Tat-E2F-4 regulation of the transcription (*bar 12*). When pCD23 plasmid carrying a promoter region encompassing the two NFκB sites and the contiguous E2F site was used in the assay, a strong cooperation between Tat and E2F-4 was observed, indicating that both the E2F-4 and NFκB *cis*-sequences are required for an optimal function of Tat·E2F-4 heterodimers (*bars 15* and *16*). The deletion of either the TAR region (pCD23ΔTAR) or of the NFκB binding site overlapping the proximal E2F site (pCD52) completely abolished the Tat·E2F-4 cooperation (*bars 17–24*).

*Induction of Cyclin A Gene Expression by Tat*—We next examined whether Tat could cooperate with E2F-4 in regulating the transcription of endogenous genes. In fact, the functional E2F *cis*-regions are present in the promoter regions of several genes whose expression is regulated during cell cycle (19). These include cyclin A, a key regulator of $G_1$-S transition (44). The results shown in Fig. 5 suggest that Tat may modulate cyclin A gene expression by forming E2F-4·Tat complexes acting on the endogenous cyclin A promoter. To test this possibility, Jurkat cells were transfected with a *tat*-expressing plasmid and tested for the expression of the endogenous cyclin A gene. As shown in Fig. 9, an increase in cyclin A mRNA was observed in Tat-positive cells at 36 h post-transfection, suggesting that Tat may deregulate the cell cycle of lymphoid cells by increasing the levels of cyclin A-CDK2 complexes.

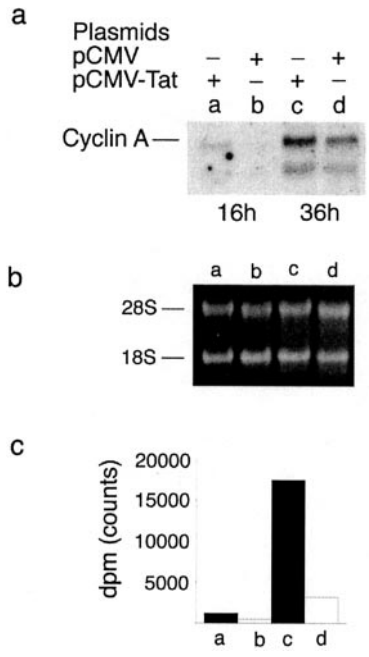


FIG. 9. **Tat activates the transcription of the endogenous cyclin A gene in Jurkat cells.** *a* and *b*, Jurkat cells were transfected with 10 μg of pcDNA-Tat-(1–86) as reported previously (37). At the indicated time points, total RNA was isolated and analyzed by Northern blot by using a $^{32}$P-labeled probe consisting of the cDNA of human cyclin A gene. *b*, the panel shows the amounts of RNA subjected to Northern blot. *c*, the relative increase in cyclin A gene expression was assessed by a PhosphorImager-assisted comparison of the disintegrations/min counts. Results representative of three independent experiments are shown.

## DISCUSSION

A wealth of studies have recently shed light on the pleiotropic activity of HIV-1 Tat on the viral life cycle and host cell physiology (5). In particular, the molecular mechanisms underlying the transcriptional function of Tat have been clarified by showing that Tat interacts with the p-TEFb complex, which includes the Cdk9 cyclin-dependent kinase physically associated to members of cyclin T such as T1, T2a, and T2b (12, 31, 32, 70). The resulting Tat·p-TEFb complex binds TAR RNA with high affinity and promotes an efficient transcription from the viral LTR. In addition, Tat binds directly to several eukaryotic transcription factors including TFIID (27), TFIIB (25), TFIIH (71), Sp1 (28), NF-interleukin-6-CAAT enhancer-binding protein (29), and RNA polymerase II (10). However, the above studies could not account for the large array of biological activities ascribed to Tat and may imply that Tat could functionally interact with additional cell factors. To address this issue, we used the yeast two-hybrid system to identify Tat-interacting proteins and identified a cDNA clone encoding for E2F-4, a transcription factor that plays a key role in the regulation of the cell cycle progression (42–45). We verified in yeast that the full-length E2F-4 protein (amino acids 1–413) could specifically interact with HIV-1 Tat in a similar manner as the library clone (amino acids 99–413) (as shown in Figs. 1 and 2). The interaction requires the E2F-4 residues from 1 to 184, a region encompassing the DNA-binding and dimerization domains of the protein and the HIV-1 Tat activation domain (amino acids 1–49). The interaction was confirmed by GST pull-down experiments performed with cellular extracts as well as with *in vitro* translated E2F-4. In both cases, Tat interacted with E2F-4 (Fig. 3, *a* and *b*). This interaction was verified *in vivo* by co-immunoprecipitation experiments. (Fig. 4). Subsequent DNA-protein interaction experiments showed that Tat increases the E2F-4 DNA binding activity and is a component of the DNA-binding complexes (Fig. 5*a*). Moreover, the enhanced E2F binding activity occurred in the absence of the increased nuclear level of E2F-4 (Fig. 5*c*), indicating that Tat increases the affinity of E2F-4 for its cognate *cis*-elements. A similar mechanism accounts for human T-cell lymphotrophic virus, type I Tax activity on transcription mediated by bZip proteins (72, 73), suggesting that the two viral transactivators have evolved to regulate the function of crucial cell transcription factors. In fact, the transcriptional activity of Tat·E2F-4 complexes was verified by a using a E2F-regulated reporter plasmid. In these experiments, Tat activation domain (amino acids 1–49) promoted a transcriptional activity similar to the one contributed by wild type Tat (shown in Fig. 5*a*). The biological relevance of the Tat·E2F-4 complex was analyzed in gene expression experiments where the two proteins were tested for the capacity to regulate the LTR-driven transcription of a *cat* reporter gene. We found that E2F-4 could cooperate with Tat to activate the transcription of HIV-1 promoter (Fig. 8*a*). The analysis of Tat mutants showed that the Tat activation domain strongly increased the activity of E2F-4. The experiments with HIV-1 promoter mutants revealed that the deletion of TAR region strongly undermines the E2F-4-Tat cooperation (Fig. 8*b*) and points to an indispensable role of TAR for Tat·E2F-4 cooperation in the context of HIV-1 LTR. Consistent with this possibility, similar results were obtained in

the case of Tat-CAAT enhancer-binding protein complexes (29). The deletion of one E2F binding site (−454 to −381 bp) does not abolish the E2F-4 induction and Tat cooperation, whereas a deletion of both sites of E2F *cis*-regions results in the complete lost of E2F-4 cooperation with Tat. The synergistic cooperation of Tat and E2F-4 can be explained in the context of the capacity of Tat to increase the DNA binding activity of E2F-4 as shown by DNA bandshift assay (Fig. 5*a*).

The capacity of Tat to promote the transcription of E2F-regulated endogenous genes was tested by analyzing the amounts of cyclin A mRNA in Jurkat cell transfected with *tat*. As shown in Fig. 9, Tat expression resulted in an increase in cyclin A mRNA at 36 h post-transfection. In this regard, cell cycle regulation in non-transformed cells is characterized by a repression of cyclin A gene expression during $G_1$ phase followed by an induction at S-phase entry (44). In addition to the $G_1$-S transition, cyclin A is required throughout S and M phases (74, 75). In fact, cyclin A may be a component of the DNA replication complex (14, 44). Accordingly, constitutive expression of cyclin A is associated with a tumorigenic phenotype, and its repression results in cell growth arrest (28, 75). The above evidence together with the functional interaction results reported in this work points to Tat as a major regulator of cell cycle. Consistent with this possibility, Tat binds to p300 (76–78), which in turn associates to cyclin E-Cdk2 to activate NFκB/rel transcription factors (79). Additional evidence for a role of Tat in cell cycle regulation comes from the evidence that Tat modulates cell cycle $G_1$ phase of glial cells (80) and induces apoptosis by interfering with a proper cyclin E-CDK2 complex (81). Moreover, Tat transactivation occurs distinctly during mid-to-late $G_1$ and at $G_2$ phases of the cell cycle (82). Thus, Tat appears to have evolved as an adaptor protein that binds to cellular factors such as E2F-4 to promote transcriptional events required for cell cycle progression.

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