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USF-related transcription factor, HIV-TF1, stimulates transcription of human immunodeficiency virus-1

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ABSTRACT

The transcription factor HIV-TF1, which binds to a region about 60 bp upstream from the enhancer of the human immunodeficiency virus-1 (HIV-1), was purified from human B cells. HIV-TF1 had a molecular weight of 39,000. Binding of HIV-TF1 to the HIV long terminal repeat (LTR) activated transcription from the HIV promoter *in vitro*. The HIV-TF1-binding site in HIV LTR was similar to the site recognized by upstream stimulatory factor (USF) in the adenovirus major late promoter. DNA-binding properties of HIV-TF1 suggested that HIV-TF1 might be identical or related to USF. Interestingly, treatment of purified HIV-TF1 by phosphatase greatly reduced its DNA-binding activity, suggesting that phosphorylation of HIV-TF1 was essential for DNA binding. The disruption of HIV-TF1-binding site induced a 60% decrease in the level of transcription from the HIV promoter *in vivo*. These results suggest that HIV-TF1 is involved in transcriptional regulation of HIV-1.

INTRODUCTION

Human immunodeficiency virus (HIV) is a cytopathic retrovirus and is the etiologic agent of the acquired immunodeficiency syndrome (AIDS) (1–4). Transcription of HIV is regulated by viral proteins such as Tat (5–7) and also by cellular transcription factors (8–11). Two regulatory regions in the HIV long terminal repeat (LTR), the enhancer and TAR regions, are important for regulation by the enhancer-binding protein (EBP) and the viral *trans*-activator Tat.

The binding of regulatory protein(s) to the HIV enhancer is induced by compounds such as phytohemagglutinin (PHA) or phorbol ester (11, 12). This process is thought to be a key step for HIV production from latently infected T lymphocytes. Multiple proteins were identified as the HIV EBP: NF-κB consisting of a 50 kDa and 65 kDa polypeptide (13), 51-kDa NF-κB from Namalwa Burkit lymphoma cells (14), 48-kDa KBF-1 from mouse BW5147 thymoma cells (15), 36–42 kDa polypeptides from human BALL-1 B cells (16), H2TF1 found in many types of cells (17), 57–60 kDa EBP-1 from HeLa cells (18), and 86-kDa HIVEN 86 found in activated human T-cells (19). NF-κB localized in the cytoplasm in sequestered form is dissociated from its inhibitor IκB by phosphorylation of IκB (20–22). Differences between H2TF1 and NF-κB were noted in their contacts with DNA, their relative affinity with the related sequences, their inducibility, and cell type distribution (13, 14, 17).

Recently, cDNA clones encoding two types of HIV EBP were isolated. HIV-EP1/PRDII-BF1/MBP-1 has a relative molecular mass of 298 kDa, and contains two zine fingers in the DNA-binding domain (23–25). The related proteins were also identified by cDNA cloning (26, 27). The level of mRNAs encoding these proteins is induced by treatment of cells with mitogen and phorbol ester (25, 27). On the other hand, cDNA cloning of the DNA-binding subumit (p50) of NF-κB indicated that its DNA-biding domain had a homology with the *rel* oncoprotein and *Drosophila dorsal* gene product (28, 29). Therefore, two different types of proteins, the metal-finger protein and the *rel*-related protein, can bind to the HIV-1 enhancer, but the mechanism of activation by mitogen and phorbol ester is different between these two types of proteins.

Another putative regulatory region (nucleotides −159 to −173) upstream from the enhancer in the HIV-1 LTR was identified by DNase I footprinting with HeLa cell extracts (10). This region was also pointed out to contain a sequence similar to the site for upstream stimulatory factor (USF) in the adenovirus major late promoter (Ad MLP) (30). So far, however, little is known about the role of this site and the regulatory protein binding to this site. Here, we report the purification and characterization of the transcription factor HIV-TF1 binding to this site. HIV-TF1 also bound to the USF site in Ad MLP. Interestingly, phosphorylation of HIV-TF1 was required for its DNA binding.

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MATERIALS AND METHODS

Purification of HIV-TF1

HIV-TF1 was purified from whole cell extract (WCE) of human B cells, BALL-1, using heparin-Sepharose and sequence-specific affinity column chromatography as described (16). A oligonucleotide column was prepared as described by Kadonaga and Tjian (31) with the synthetic duplex oligonucleotides shown in Fig. 1B.

DNA binding assay and photoaffinity labeling

The gel retardation assay and DNase I footprinting were done as described (16). For the gel retardation assay, the synthetic duplex oligonucleotides shown in Fig. 1B were used as a probe. For the DNase I footprinting, the *Bgl*II-*Eco*RV fragment (nucleotides +23 to −338 in the HIV-1 recombinant, pCD12 (32)) was ^{32}P-labeled at the 5′ end of the upper strand. Photoaffinity labeling was done by the method of Wu *et al.* (33).

In vitro transcription

Transcription using HeLa WCE was done as described (16) except for that 0.5 μg of HIV-1 template DNA and 0.5 μg of control template DNA were used. The *Eco*RI-cut pCD12 plasmid DNA containing the HIV-1 promoter was used as a template. The *Eco*RI-cut pRSVCAT plasmid DNA in which the Rous sarcoma virus (RSV) LTR promoter is linked to the chloramphenicol acetyltransferase (CAT) gene was used as the control template. Run-off transcripts were analyzed on a 7 M urea/4% polyacrylamide gel. The amount of each transcript was measured by using a Bioimage Analyzer (Fuji Photo Film Co., Ltd.).

Mutant plasmid construction, DNA transfection, and CAT assay

The HIV-TF1-binding site in the HIV LTR was disrupted as follows. The *Hin*dIII fragment (nucleotides +82 to −190) containing the HIV promoter was generated from the plasmid pCD12 by the method of polymerase chain reaction (PCR), and then the *Hin*dIII fragment containing the whole region of the HIV LTR in pCD12 was replaced by this 272-bp *Hin*dIII fragment to generate the plasmid pCD-190. By using the primer corresponding to the mutant sequence of the HIV-TF1-binding site at nucleotide −166, the mutant plasmid pCD-190TF1m was constructed similarly. For the CAT assay, the plasmid pCD-190 and the mutant plasmid pCD-190TF1m harboring the disrupted HIV-TF1-binding site were used. Mixtures of each CAT plasmid DNA and 2 μg of pRSV-β-gal plasmid DNA were transfected into Jurkat T cells by electroporation (34). The plasmid pRSV-β-gal, which carries the *E. coli* β-galactosidase gene linked to the RSV LTR (35) was used as an internal control for differences in transfection efficiency between precipitates. CAT assays were done by the method of Gorman *et al.* (36). The amounts of cell extract used for CAT assays were normalized with respect to β-galactosidase activity. The degree of conversion was measured by the density of the spot corresponding to either unreacted [^{14}C]chloramphenicol or its accetylated forms using a Bioimage analyzer (Fuji Photo Film Co., Ltd.).

Phosphatase treatment

Purified HIV-TF1 or HIV EBP was treated with potato acid phosphatase (Boehringer Mannheim). Purified protein (2 ng) was incubated with 8×10^{-5} units of phosphatase in 15 μl of gel retardation-binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl. 1 mM dithiothreitol, 0.05% Nonidet P-40, 100 μg/ml bovine serum albumin, 5% (vol/vol) glycerol] for 20 min at 30°C. The ^{32}P-labeled probe was then added and incubated for 30 min at 25°C, and the material was analyzed by polyacrylamide gel electrophoresis in low ionic strength.

RESULTS

Purification of HIV-TF1

HIV-TF1 was purified by chromatography on (i) heparin-Sepharose and (ii) specific oligo-DNA Sepharose. The WCE from BALL-1 cells were fractionated with heparin-Sepharose column chromatography, and the fractions were used in the gel retardation assay. The synthetic duplex oligonucleotides corresponding to nucleotides −177 to −151 of the HIV LTR (see Fig. 1B) were

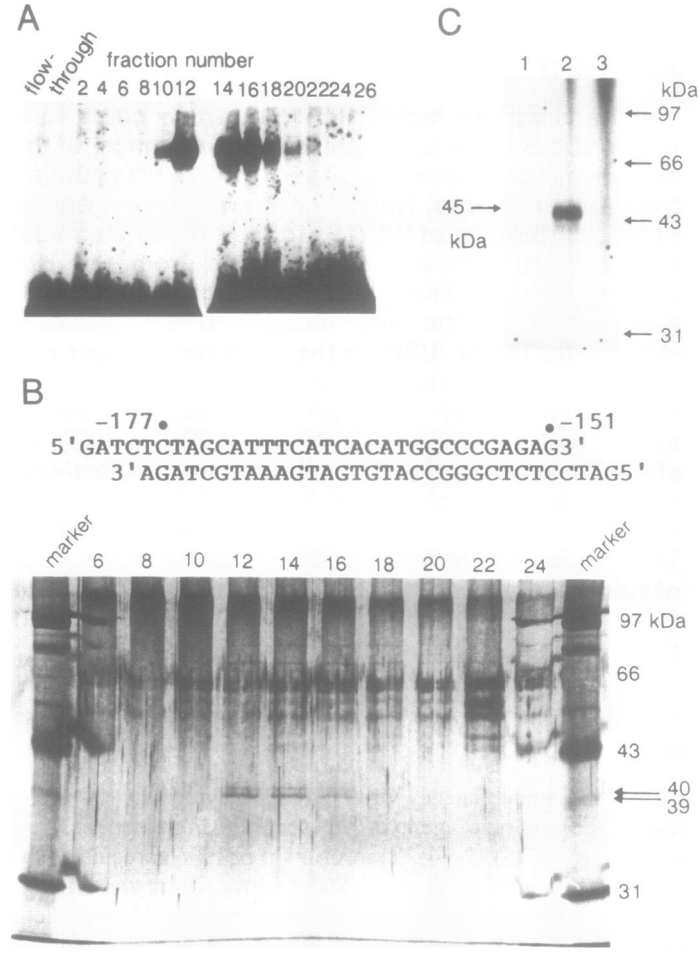

Figure 1. Purification of HIV-TF1. A, Fractions from the oligo-DNA Sepharose column were assayed by gel retardation with the probe shown in B. B, Fractions from the oligo-DNA Sepharose column were analyzed by SDS-polyacrylamide gel electrophoresis followed by silver staining. The sequence of oligonucleotides used as a ligand is shown above. C, Ten ng of the affinity-purified fraction was incubated with the 5-bromodeoxy-uridine-substituted specific oligonucleotide probe shown in B, UV cross-linked, digested with nuclease, and analyzed on an SDS-polyacrylamide gel (lane 2). Lane 1, without purified fraction. Lane 3, with 40 ng (100-fold molar excess) of specific oligonucleotide as a competitor. Molecular weights of markers and protein-DNA adducts are shown on the right and left, respectively.

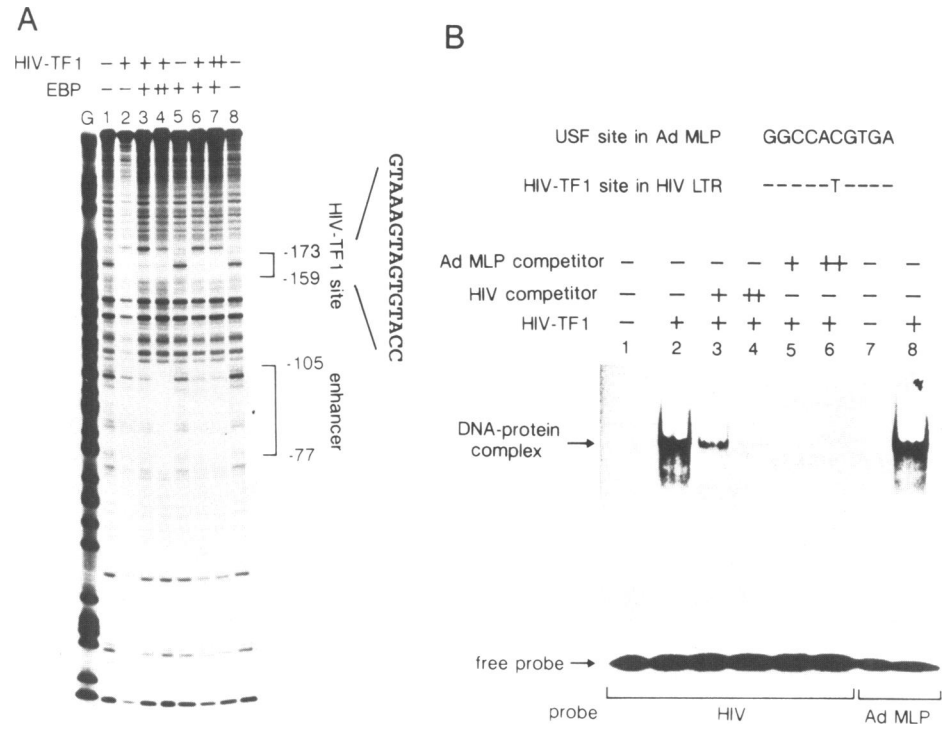

Figure 2. DNA-binding property of HIV-TF1. A, DNase I footprinting with purified HIV-TF1 and HIV EBP. End-labeled *Bgl*II-*Eco*RV DNA fragment from pCD12 plasmid was incubated with purified HIV-TF1 (4 ng/μl) and HIV EBP (5 ng/μl) (16). The amount of two factors used was indicated above each lane; +, 3 μl and ++, 6 μl. After treatment with DNase, the DNA was purified and analyzed on an 8% sequencing gel. The protected region is indicated by the brackets. B, Binding of HIV-TF1 to the USF site in the Ad MLP. At the top, DNA sequence of HIV-TF1-binding site in the HIV LTR and USF-binding site in the Ad MLP is indicated. Dash of HIV-TF1 site shows the same sequence as that of USF site. The purified HIV-TF1 was used for a gel retardation assay with the oligonucleotide probe containing the sequence of HIV LTR shown in Fig. 1B (lanes 1−6) or the USF-binding site of Ad MLP (5′GATCAGGTGTAGGCCACGTGACCGGGTGTTCC3′, 3′TCCACATCCGGTGCACTGGCCCACAAGGCTAG5′) (lanes 7 and 8). Lane 1, without purified HIV-TF1; lanes 3 and 4, with 20- and 100-fold molar excess of HIV oligonucleotide as a competitor, respectively; lanes 5 and 6, with 20- and 100-fold molar excess amount of Ad MLP oligonucleotide as a competitor, respectively.

used as a probe. The binding activity was detected in fractions corresponding to 0.4−0.5 M KCl (data not shown). This fraction was then put on an oligo-DNA Sepharose column, in which the same duplex oligonucleotides as those used in the gel retardation assay were used as a ligand, and the binding factor was eluted by increases in the KCl concentration. The binding factor was detected in fractions 10−18 corresponding to 0.5−0.6 M KCl, which included 39 and 40 kDa polypeptides as the major protein and other minor polypeptides of 70−80 kDa (Fig. 1A and B). The bands between 65 and 50 kDa corresponded to a silver staining artifact. About 10 μg of polypeptides of 39 and 40 kDa (HIV-TF1) was purified from 100 g of BALL-1 cells.

To examine which polypeptide binds specifically to the sequence shown in Fig. 1B, we used UV cross-linking. The photoaffinity labeling probe was prepared from the oligonucleotide shown in Fig. 1B. After incubating the DNA probe with the purified factor, the mixture was exposed to UV light, digested with nuclease, and analyzed on a SDS-polyacrylamide gel. We observed the protein-DNA adduct of about 45 kDa as a major band that was resistant to further increases in DNase digestion (Fig. 1C, lane 2). In a competition experiment an unlabeled specific oligonucleotide abolished the signal (lane 3). Omission of purified facror from the reaction also failed to produce any signal (lane 1). These results indicated that two polypeptides of 39 and/or 40 kDa had a binding activity.

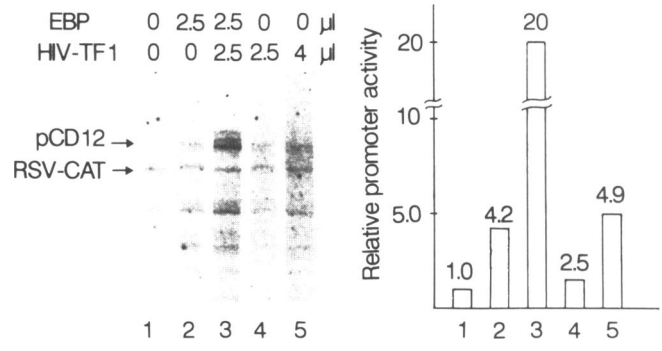

Figure 3. Stimulation of transcription from HIV promoter by purified HIV-TF1 *in vitro*. Transcription was done with *Eco*RI-cut pCD12 as a DNA template. The *Eco*RI-cut pRSVCAT DNA was used as the control template. Each reaction contained variable amouts of purified HIV-TF1 (4 ng/μl) and HIV EBP (5 ng/μl) as indicated above each lane. The locations of the run-off transcripts of 373 nucleotides from the HIV promoter and that of 291 nucleotides from the RSV promoter are indicated by arrows. The multiple transcripts in the lower part of gel were generated by degradation of the transcripts from the HIV promoter or premature termination of transcription from the HIV promoter. On the right, the results of *in vitro* transcription are indicated by a bar graph. The amounts of 373-nucleotides transcripts were normalized with respect to the amounts of 291-nucleotides transcripts. The activity of HIV-1 promoter in the presence of HIV-TF1 and/or HIV EBP is expressed relative to that in the absence of factors.

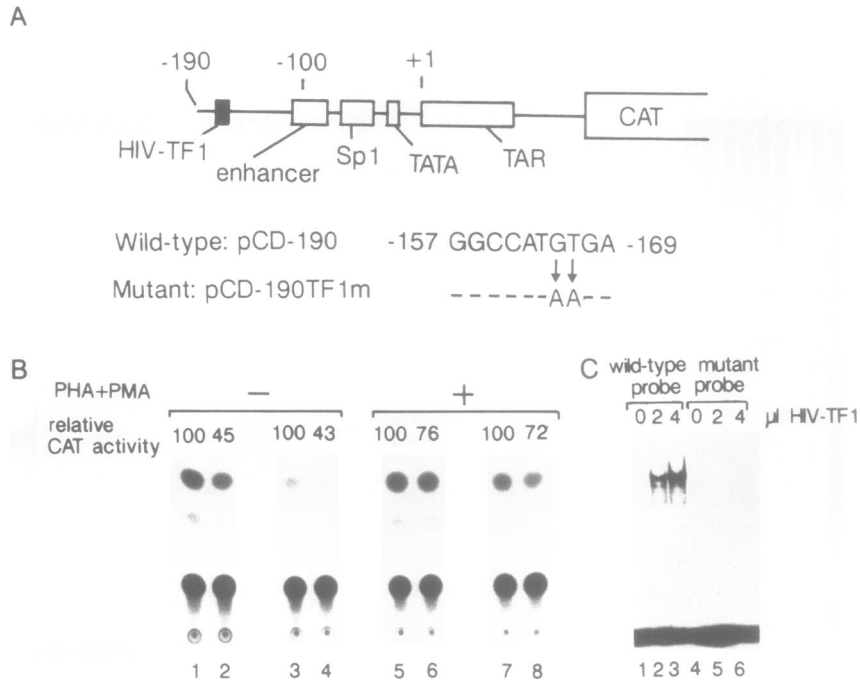

Figure 4. Effects of disruption of HIV-TF1 site on CAT activity directed by HIV promoter-CAT. A, Transcriptional regulatory elements in the pCD-190 plasmid, in which the HIV LTR was linked to the CAT gene, are indicated at the top. Two nucleotides of the HIV-TF1-binding site in pCD-190 were changed to generate the plasmid pCD-190TF1m. B, Mixtures of pCD-190 (lanes 1, 3, 5, and 7) or pCD-190TF1m (lanes 2, 4, 6, and 8) and pRSV-β-gal plasmid DNA were transfected into Jurkat cells. Amount of CAT plasmid DNA used was 15 μg (lanes 1 and 2) or 7.5 μg (lanes 3–8). Cells were treated with PHA (2 μg/ml) and PMA (10mM) for 20 h (lanes 5 and 6) or 8 h (lanes 7 and 8) before harvest. CAT activity was measured as described (36). Experiments were repeated three times, and typical results are shown. The differences between each set of experiments were within 10%. CAT activity of pCD-190TF1m was shown above each lane relative to that of pCD-190 under same assay conditions. C, Gel retardation analysis was carried out by using the wild-type probe shown in Fig. 1B or the mutant probe. The nucleotide sequence of mutant probe is the same as the wild-type probe except for 2 nucleotides as shown in A. The amounts of HIV-TF1 used are indicated above each lane.

We also did renaturation experiments after the SDS-polyacrylamide gel electrophoresis. After isolation and renaturation, only the protein samples containing the 39 and 40 kDa polypeptides generated the specific retarded band in a gel retardation assay (data not shown). The behavior of the 39 and 40 kDa polypeptides in various column chromatographies (data not shown) suggested that the two polypeptides had very similar biochemical properties, and the difference between them may be a modification such as phosphorylation or degradation.

DNA-binding properties of HIV-TF1

To demonstrate the specific binding of the purified HIV-TF1 with the HIV-1 LTR, a DNase I footprinting experiment was done. The purified HIV-TF1 protected a region corresponding to nucleotides −173 to −159 (Fig. 2A, lane 2). To examine the functional relationship between HIV-TF1 and HIV EBP, we used the purified HIV-TF1 and the purified EBP in a DNase I footprint experiment. The concentration of purified EBP required to observe a clear footprint on the HIV-1 LTR was lower in the presence of HIV-TF1 than in its absence (Fig. 2A, lanes 3–7). These results suggested that binding of HIV-TF1 increased binding of the factor to the enhancer region.

The DNA sequence of the HIV-TF1-binding site is similar to that of the USF site in the Ad MLP and there is only one base difference out of 10 nucleotides between them (Fig. 2B). To examine whether purified HIV-TF1 also binds to the USF site, a gel retardation analysis was done. Both probes containing the HIV-TF1-binding site of HIV LTR or the USF site of the Ad MLP bound to the purified HIV-TF1 and generated a specific retarded band (Fig. 2B, lanes 2 and 8). To examine the relative affinity of HIV-TF1 for the two related sequences, a competition analysis was done. Addition of a 20-fold molar excess of competitor containing the HIV-TF1-binding site of HIV LTR

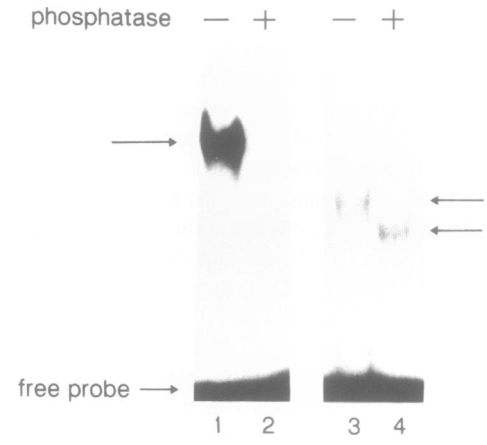

Figure 5. Phosphatase treatment of HIV-TF1. Purified HIV-TF1 was treated with (+) or without (−) potato acid phosphatase. After incubation, DNA-binding activity was measured in a gel retardation assay with a probe described in Fig. 1B. In a control experiment, DNA-binding activity of purified HIV EBP was assayed in a similar way by using the DNA probe containing the HIV enhancer (16). Arrows indicate the retarded band corresponding to specific DNA-protein complex.

partially competed for binding of HIV-TF1 to the HIV-TF1 probe, while a 20-fold molar excess of competitor containing the USF site abolished the binding of HIV-TF1 completely (Fig. 2B, lanes 3 and 5). Thus, HIV-TF1 has a higher affinity for the USF site than the HIV-TF1 site in the HIV-1 LTR.

Transcriptional activation by purified HIV-TF1

The effect of the purified HIV-TF1 in stimulating transcription was examined in the WCE of HeLa cells. When transcribed in HeLa WCE, the pCD12 plasmid DNA digested with *Eco*RI generated 373 nucleotide run-off transcripts corresponding to specific initiation at the *in vitro* CAP site (Fig. 3, lane 1). The *Eco*RI-cut pRSVCAT control template generated 291 nucleotide run-off transcripts. Addition of purified HIV-TF1 stimulated transcription from the HIV-1 promoter 2.5- to 4.9-fold, but did not stimulate transcription from the RSV LTR promoter (Fig. 3, lane 1, 4, and 5). As reported previously (16), addition of purified HIV EBP stimulated transcription from the HIV-1 promoter 4.2-fold (Fig. 3, lanes 1 and 2). Interesitingly, the effects of HIV-TF1 and EBP were synergistic, and simultaneous addition of both proteins stimulated transcription from the HIV-1 promoter 20-fold (Fig. 3, lane 3).

Disruption of HIV-TF1-binding site

To examine whether the HIV-TF1-binding site in the HIV LTR functions as a transcriptional control element, we introduced a mutation (Fig. 4A) By using a gel retardation analysis, it was confirmed that HIV-TF1 could not bind to this mutant promoter (Fig. 4C). The mutant HIV promoter was linked to the bacterial CAT gene to generate the plasmid pCD-190TF1m. Two CAT constructs containing the wild-type (pCD-190) or mutant promoter (pCD-190TF1m) were transfected into Jurkat T-cells and CAT activity was assayed. The level of CAT activity obtained by the mutant promoter was about 40% of that by wild-type promoter (Fig. 4B, lanes 1−4). Activation of Jurkat T cells with PHA and 4β-phorobol 12-myristate 13-acetate (PMA) induces the activation of HIV EBP (11). Therfore, treatment of cells by PHA and PMA stimulated CAT activity with pCD-190 about 10-fold (Fig. 4B, lanes 3, 5, and 7). In the presence of PHA and PMA, the level of activity with mutant promoter was about 76% of that with wild-type promoter (Fig. 4B, lanes 5−8). Thus, the HIV-TF1-binding site is a positive element for transcriptional control, although in the presence of an excess of activated EBP, the HIV-TF1 site appears to be not so important for transcription of HIV-TF1.

Phosphatase treatment of HIV-TF1

The HIV EBP, NF-$\varkappa$B, is activated by phosphorylation of its inhibitor I$\varkappa$B (22). To examine whether HIV-TF1 is also regulated by phosphorylation, we treated the purified HIV-TF1 with or without acid phosphatase and assayed them in parallel for DNA-binding activity. DNA-binding activity was greatly reduced by phosphatase treatment (Fig. 5, lanes 1 and 2). In a control experiment, the purified HIV EBP was treated with phosphatase and its DNA-binding activity was examined. Although the mobility of protein-DNA complex was changed, no change in the DNA-binding activity was detected after phosphatase treatment (Fig. 5, lanes 3 and 4). Thus, the phosphorylation of HIV-TF1 appeared to be essential for its DNA-binding activity.

DISCUSSION

We have purified the protein HIV-TF1, which binds to the specific site in the HIV-1 LTR and stimulates transcription from the HIV-1 promoter *in vitro*. The DNA sequence of the HIV-TF1-binding site is similar to that of USF site in the Ad MLP, and only one base out of 10 is different between the two sequences. Although a sequence homologous with USF site in the HIV LTR was noted previously (30), our report probably gave the first evidence showing that the factor binding to this site stimulates transcription from the HIV-1 promoter. The molecular weight of purified HIV-TF1 was 39,000 and 40,000 and is indistinguishable from that of purified USF from HeLa cells, 43,000 and 44,000 (37). Furthermore, purified HIV-TF1 bound to the USF site in the Ad MLP, and was perfectly stable at temperatures as high as 100°C (data not shown) like purified USF (37). These results strongly suggest that HIV-TF1 is identical or closely related to USF. Recently, cDNA clones encoding USF have been isolated (38). Interestingly, another protein, TEF3, which binds to the USF site in the Ad MLP was also identifited by cDNA cloning (39). Both proteins contain a helix-loop-helix and a leucine zipper structure, but the sequence of USF is totaly different from that of TEF3. Thus, multiple proteins which bind to the USF site appear to exist. Therefore, the possibility that HIV-TF1 is different from USF remains.

Using a transfection assay, we have shown that the HIV promoter containing a mutation in the HIV-TF1-binding site has a reduced activity, about 40% of that of wild-type promoter in Jurkat T-cells. In Jurkat cells activated by PHA and PMA, the effect of disruption of the HIV-TF1-binding site on the level of transcription was lower than that in the absence of PHA and PMA. Since treatment of cells with PHA and PMA increased the level of activated HIV EBP, HIV-TF1 may not dramatically affect HIV transcription in the presence of a saturating amount of EBP. Thus, our results suggest that HIV-TF1 may be a key regulator only when the level of EBP is low, such as in latently infected cells. Phosphatase treatment of the purified HIV-TF1 greatly reduced its DNA-binding activity. Therefore, it is possible that some extracellur stimuli, which leads to virus production from latently infected cells, may activate HIV transcription partly by phosphorylation of HIV-TF1.

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