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Interaction of Enhancer-Binding Protein EBP1 (NF-κB) with the Human Immunodeficiency Virus Type 1 Enhancer

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Human EBP1, isolated from HeLa cells, binds to a 10-base-pair (bp) sequence in cellular and viral enhancers that is also recognized by the inducible transcription factor NF-κB. Here we describe the interaction of purified EBP1 with the 10-bp repeated sequence that is responsive to signals which activate T cells and which form part of the human immunodeficiency virus type 1 (HIV-1) enhancer. DNase I footprinting indicates that both 10-bp sites on the same molecule, located between −80 and −105 on the HIV-1 long terminal repeat, can be occupied by EBP1, while dimethyl sulfate protection and methylation interference experiments indicate which purine bases are in contact with the protein. The presence of bases which exhibit increased rates of dimethyl sulfate-induced methylation in the presence of EBP1 indicate that interaction of EBP1 with its recognition site is accompanied by distortion of the DNA double helix. Supporting this conclusion is the observation that the polyamine spermidine dramatically increases EBP1 binding to its cognate site on the DNA. Studies with human T cells (Jurkat) and nucleotide stimulation data suggest that EBP1 is the activated form of NF-κB in these cells.

Human immunodeficiency virus type 1 (HIV-1) is the etiological agent of acquired immune deficiency syndrome (4, 15, 30). HIV-1 and HIV-2 infect T lymphocytes, macrophages, and cells of the central nervous system that display the CD4 glycoprotein on their surfaces (13, 26, 31, 33). Infected individuals may remain asymptomatic for months or years, and it is thought that viral latency is maintained, at least in part, by low transcriptional activity of the integrated provirus in resting cells. However, when T cells are activated (for example, after encountering antigen-presenting cells), viral transcription is induced by cellular factors and gene expression is further amplified by the action of viral transactivators (36). The DNA sequence within the HIV-1 long terminal repeat (LTR) that responds to T-cell activation signals has been identified and exhibits the properties of a transcriptional enhancer (14, 23, 34, 41, 42, 44). T-cell activation of transcription directed by the HIV-1 LTR correlates with activation of the transcription factor NF-κB, which binds to two repeated 10-base-pair (bp) sequences located in the region previously identified as responsive to T-cell mitogenic signals (34). NF-κB is induced by a post-translational mechanism (39) in which the inactive precursor is held in the cytoplasm by an inhibitor molecule that inactivates the DNA-binding activity. Upon activation, the inhibitor dissociates from NF-κB, activating the DNA-binding domain and allowing the protein to migrate to the nucleus (2). Dissociation of the inhibitor molecule to expose the DNA-binding form of NF-κB can also be accomplished in vitro by the detergent deoxycholate (1). The 10-bp sequence recognized by NF-κB which is repeated in the HIV-1 LTR is also present in a large number of viral and cellular gene regulatory regions, including the enhancers of simian virus 40 (SV40) (47), cytomegalovirus (7), the immunoglobulin κ light-chain gene (37, 38), the interleukin 2 receptor α-chain gene (5), the major histocompatibility complex class I *H-2K*^{b} gene (3, 20), and the beta interferon regulatory element (17). In many cases the 10-bp element that is recognized by NF-κB is required for the inducible expression of linked genes, but it is also clear that in some circumstances the same element is required for constitutive transcription. Although NF-κB may be involved in the constitutive expression of these genes, additional transcription factors, such as κBFI (46), H2TFI (3), and EBP1 (12), which, along with NF-κB and HIVen86 (5), appear to recognize similar DNA sequences, have been identified. The relationships among these sequence-specific DNA-binding proteins have not been clarified but are important in establishing the role of the proteins in the activation of HIV-1 transcription.

We recently purified from human HeLa cells a protein (EBP1) which binds to the SV40 enhancer (11, 12) and a variety of other viral and cellular enhancers, including the PRDII region of the human beta interferon gene regulatory element (10). Here we demonstrate that EBP1 also binds to the repeated 10-bp sequence located between −80 and −105 on the HIV-1 LTR. DNase I footprinting indicates that both sites in the HIV-1 LTR can be occupied by EBP1 simultaneously, while dimethyl sulfate (DMS) protection and methylation interference experiments indicate which purine bases are in contact with the protein. The presence of bases which exhibit increased rates of DMS-induced methylation in the presence of EBP1 indicates that interaction of EBP1 with its recognition site is accompanied by distortion of the DNA double helix. Studies carried out with the human T-cell line Jurkat suggest that EBP1 may in fact represent the activated form of NF-κB in these cells.

MATERIALS AND METHODS

Cell culture. HeLa cells were grown in suspension at 37°C in Earle minimal essential medium containing 5% newborn calf serum. Jurkat cells in 75-cm^2 flasks were grown in RPMI 1640 medium containing 10% fetal calf serum and 50 μM β-mercaptoethanol in an atmosphere of 5% CO_2 at 37°C. When necessary, Jurkat cells were incubated in growth medium containing 1 μg of phytohemagglutinin (PHA) per ml and 50 nM phorbol ester myristate (PMA).

Preparation of cell extracts and purification of EBP1. Jurkat cells were collected from the growth medium by centrifuga-

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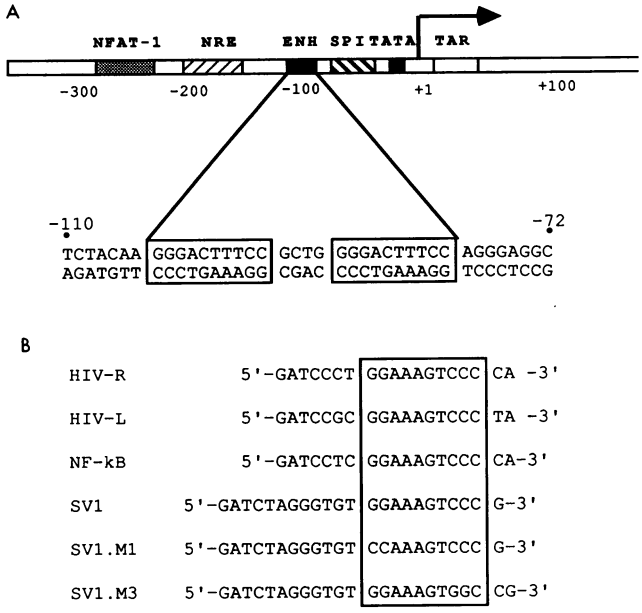

FIG. 1. (A) DNA sequence elements in the HIV-1 LTR involved in viral gene expression. Indicated are the *trans*-acting responsive element (TAR) located between +19 and +42 (21), the TATA box located between −21 and −24, three tandem binding sites for transcription factor SPI (22), the negative regulatory element (NRE) (35), and the binding site for the inducible transcription factor NFAT-1 (40). The DNA sequence of the enhancer (ENH) region containing the two repeated 10-bp sequences is also shown. (B) DNA sequence of the top strand of the double-stranded oligonucleotides used in this study. The boxed region shows the nucleotide sequences related to the 10-bp repeats in the HIV-1 enhancer.

tion, washed once in phosphate-buffered saline, and fractionated into nuclei and cytosol (9). Nuclei were extracted in a buffer containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), NaOH (pH 7.5), 0.5 mM dithiothreitol, 0.5 mM $MgCl_2$, 0.35 M NaCl, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 2 μg each of antipain, pepstatin, and leupepsin per ml) for 30 min on ice. The extract was clarified by centrifugation, divided into equal portions, and stored at −70°C. EBP1 was purified from nuclear extracts of HeLa cells by ion-exchange chromatography followed by three successive rounds of sequence-specific, recognition site affinity chromatography as described previously (12).

Plasmids, oligonucleotides, and labeled fragments. Plasmid pHIVen contains DNA sequences from the HIV-1 enhancer from positions −79 to −108 inserted into the *Bam*HI site of the pUC13 polylinker. Plasmids pHIV-L and pHIV-R contain one copy of either the HIV-L or HIV-R double-stranded oligonucleotide (HIV with EBP1 binding site at distal or proximal end, respectively) (Fig. 1B) inserted into the *Bam*HI site of the pUC13 polylinker. ^{32}P-labeled DNA was prepared by digestion of pHIVen, pHIV-L, and pHIV-R with *Eco*RI and *Pst*I or *Hin*dIII and *Sac*I followed by 3' end labeling at the *Eco*RI or *Hin*dIII site with [α-^{32}P]dATP (Amersham Corp.; specific activity, 3,000 Ci/mmol); unlabeled dCTP, dTTP, dGTP; and the large Klenow fragment of *Escherichia coli* DNA polymerase I. Labeled fragments containing HIV-1 enhancer sequences were purified on 8% polyacrylamide gels and electroeluted (19).

Oligonucleotides were synthesized on a model 381A DNA synthesizer (Applied Biosystems, Inc.). Nucleotide sequences of one strand of the various double-stranded oligonucleotides used in this study are shown in Fig. 1. Complementary single-stranded, synthetic oligonucleotides were annealed by first heating them to 100°C in 0.1 M NaCl–10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA and then cooling them slowly to 16°C. The resultant double-stranded oligonucleotides, which all contained 5'-GATC overhangs, were 3' end labeled with [α-^{32}P]dATP and isolated as described above.

Chloramphenicol acetyltransferase (CAT) expression vectors are derivatives of plasmid pKTMLP, which contains the adenovirus type 2 major late promoter and the bacterial CAT gene inserted into the pUC18 polylinker. Plasmid pKTMLP was a generous gift from C. Goding, Marie Curie Research Institute, Oxted, Surrey, United Kingdom, and was modified by the insertion of *Bgl*II linkers into the *Sma*I site. Double-stranded SV1 oligonucleotides were then inserted into this engineered *Bgl*II site, generating plasmids which contained one, two, or three tandem copies of the EBP1 binding site.

In vivo transient expression assays. HeLa S1 monolayer cells were grown to ~30 to 40% confluency (approximately 8 × 10^5 cells per 50-mm plate) in Glasgow modified minimal essential medium containing 10% newborn calf serum. Calcium phosphate-DNA coprecipitates were prepared and added dropwise to the medium of the tissue culture cells, which were incubated at 37°C for 48 h. The preparation of cell extracts and assay of CAT activity were performed as described previously (18).

Gel electrophoresis DNA-binding assay. Gel electrophoresis DNA-binding assays were performed as described previously (12) with the following modifications: reactions with affinity-purified EBP1 did not contain unlabeled carrier DNA, and with crude Jurkat cell extracts, reaction mixtures were preincubated in the presence of unlabeled carrier DNA for 5 min prior to the addition of the specific, labeled DNA fragment.

DNase I protection. Binding reactions contained 0.5 to 1.0 ng of ^{32}P-labeled probe (10,000 cpm), 25 mM HEPES, NaOH (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.05% Nonidet P-40, 10% glycerol, and various amounts of affinity-purified EBP1 protein fractions in a final reaction volume of 100 μl. Binding was allowed to proceed for 20 min at 20°C, after which the products were treated with DNase I and prepared for electrophoresis in 8% polyacrylamide gels as described previously (12).

Methylation protection. Binding reactions were set up as described for DNase I protection. After 20 min at 20°C, the products were treated with 0.5 μl of DMS for 2 min at 20°C. The reaction was stopped by the addition of 2 volumes of 0.3 M sodium acetate–1.5 M β-mercaptoethanol–50 μg of yeast tRNA per ml. The DNA was extracted once with phenol and once with chloroform, precipitated twice with ethanol, washed with 70% ethanol, dried, and treated with piperidine according to the "G greater than A" specific chemical cleavage method of Maxam and Gilbert (32). Cleavage products were fractionated and visualized as described previously (11). Autoradiographs were analyzed and band densities were quantitated with a Shimadzu flying spot densitometer; only those autoradiograms exposed within the linear response of the film were utilized. Ratios of peak areas were determined for each base in the absence and presence of EBP1 and normalized by comparison with bases known to lie outside the recognition site for EBP1.

Methylation interference. DMS (1 μl) was added to ~10 ng of end-labeled probe DNA and 0.5 μg of unlabeled poly d(A · T)-poly d(G · C) in 200 μl of 50 mM sodium cacody-

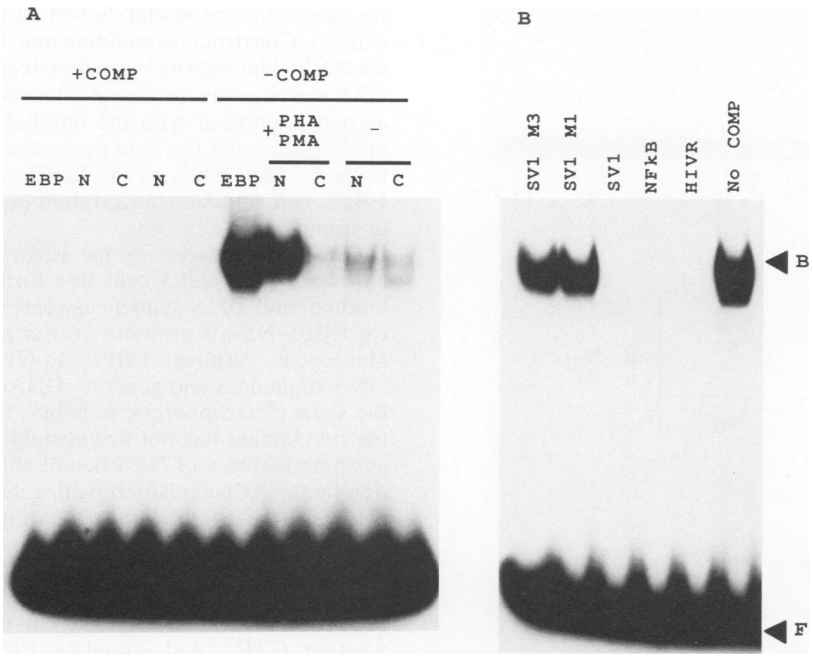

FIG. 2. Gel electrophoresis DNA-binding assay of purified EBP1 and the inducible transcription factor NF-κB. (A) ^{32}P-labeled HIV-L double-stranded oligonucleotide was incubated with purified EBP1 or with nuclear (N) or cytoplasmic (C) extracts (10 μg) of Jurkat cells either unstimulated (−) or treated with PHA and PMA for 3.5 h. Samples were incubated in either the absence (−COMP) or presence (+COMP) of 100 ng of unlabeled HIV-L double-stranded oligonucleotide prior to electrophoresis in a 6% native polyacrylamide gel. (B) Nuclear extract (10 μg) from PHA- and PMA-stimulated Jurkat cells was incubated with ^{32}P-labeled HIV-L double-stranded oligonucleotide in the absence of competitor (No COMP) or the presence of 100 ng of the indicated double-stranded oligonucleotides (for sequences, see Fig. 1).

late. Incubation was continued for 10 min at 20°C, after which the reaction was terminated by the addition of 50 μl of 1.5 M sodium acetate, 1.0 M β-mercaptoethanol containing 4 μg of unlabeled poly d(A · T)-poly d(G · C), and the DNA isolated by ethanol precipitation. Methylated DNA was suspended in binding buffer, and binding reactions in the presence of affinity-purified EBP1 were carried out as described for DNase I protection. Specific DNA-protein complexes were separated from free DNA in 6% polyacrylamide gels, and bound and free DNA were isolated. DNA was solubilized and treated with NaOH, and the cleavage products were fractionated as described previously (10).

RESULTS

Relationship of EBP1 to the inducible transcription factor NF-κB. Within the HIV-1 enhancer region are two direct repeats of a DNA sequence, -GGAAAGTCCC-, similar to that present in the core region of the SV40 enhancer (Fig. 1). We have previously demonstrated by competition analysis that EBP1 binds to both of these sequences present in the HIV-1 enhancer (10), and others have shown that the inducible transcription factor NF-κB binds to these same sequences already described for EBP1 (34). EBP1 can be purified from actively growing HeLa suspension cells, whereas NF-κB binding activity is not detected in pre-B cells but is detected in mature B-cell lines. In addition, NF-κB binding activity is detected in the T-cell line Jurkat only after treatment of the cells with mitogenic agents such as phorbol esters and PHA. In view of this, we sought to establish the relationship between purified HeLa cell EBP1 and the NF-κB present in extracts from activated human T cells. Jurkat cells were therefore incubated in the presence or absence of a combination of PMA and PHA. After 3.5 h of exposure to these mitogens, cells were collected and nuclear and cytoplasmic extracts were prepared essentially as described previously (12). T-cell extracts and purified EBP1 were incubated with ^{32}P-labeled HIV-L double-stranded oligonucleotide (Fig. 1B), and DNA protein complexes were resolved from free DNA by native polyacrylamide gel electrophoresis (Fig. 2). It is clear that in the presence of PMA and PHA, a nuclear protein which forms a DNA protein complex with HIV-L is induced. This complex comigrates with the EBP1 DNA complex, and an excess of unlabeled HIV-L competes for the formation of both DNA protein complexes. To establish the specificity of the induced factor, the nuclear extract from PMA- and PHA-stimulated T cells was incubated with a series of unlabeled double-stranded oligonucleotides (Fig. 1) which had previously been used to define the binding site for purified EBP1 (10). As with purified EBP1, competition for the induced factor was observed with the SV1, NF-κB, and HIV-R oligonucleotides but not with the oligonucleotides SV1 M1 and SV1 M3, which contain mutations in the EBP1 binding site (Fig. 2). It is therefore apparent that under the conditions of the assays employed, EBP1 and NF-κB have indistinguishable DNA-binding specificities and form DNA protein complexes with similar electrophoretic mobilities.

HIV-1 enhancer is constitutively active in HeLa cells. As demonstrated above, it appears that EBP1, which may represent the activated form of NF-κB, is active (in DNA binding) in HeLa cells. Since the tandem binding sites for EBP1 and NF-κB constitute the HIV-1 enhancer, these sequences were tested for their ability to activate transcription in HeLa cells. Copies of the EBP1–NF-κB binding site present in the enhancer region of the HIV-1 LTR were inserted upstream from a truncated version of the adenovi-

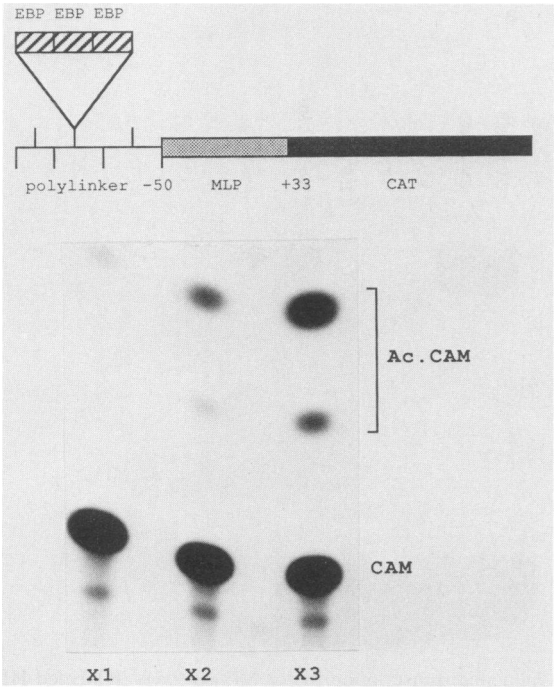

FIG. 3. Activity of the HIV-1 enhancer in vivo. HeLa cells were transfected by calcium phosphate coprecipitation with plasmids containing one, two, or three copies of the EPB1 binding site inserted upstream from a CAT gene linked to the adenovirus type 2 major late promoter. CAT activity was assayed 48 h after transfection by determining the conversion of [^{14}C]chloramphenicol (CAM) into its acetylated forms (Ac. CAM) by thin-layer chromatography.

rus major late promoter linked to the bacterial CAT gene (Fig. 3). Constructs containing one, two, and three copies of the EBP1 binding site were transfected into HeLa cells, and CAT activity was measured after 48 h. The level of enzyme activity increased with the number of copies of the EBP1 binding site, and the data presented in Fig. 3 suggest that a protein which binds to the EBP1 binding site, presumably EBP1, can activate transcription of a linked gene in vivo in unstimulated HeLa cells.

Cofactor requirements for EBP1 binding. EBP1 can be extracted from HeLa cells in a form that is active in DNA binding, and DNA sequences representing the binding sites for EBP1–NF-κB promote transcription of a linked gene in HeLa cells. Although EBP1 and NF-κB recognize the same DNA sequences and generate DNA-protein complexes with the same electrophoretic mobility, the relationship between the two factors has not been established. One of the distinguishing features of NF-κB, not shared by other transcription factors which also recognize its binding site (H2TFI or κBFI), is the stimulation of binding activity by nucleotide triphosphates (29). To establish the optimal conditions for EBP1 binding and as a means of comparison with NF-κB, the cofactor requirements for binding of EBP1 to its recognition site were investigated. In addition to determining whether GTP could stimulate EBP1 binding, two other components already known to be required for efficient in vitro transcription, $MgCl_2$ and spermidine, were also examined for their effects on the binding of EBP1 to DNA in vitro. As with NF-κB, GTP stimulation of EBP1 binding activity was maximal at a concentration of 3 mM (Fig. 4). However, this effect was not as dramatic as that observed with spermidine, which considerably increased the binding of EBP1 to its recognition site. Since the magnitude of the stimulation with spermidine was greater than that obtained with GTP, it was difficult to determine whether the stimulatory effect of

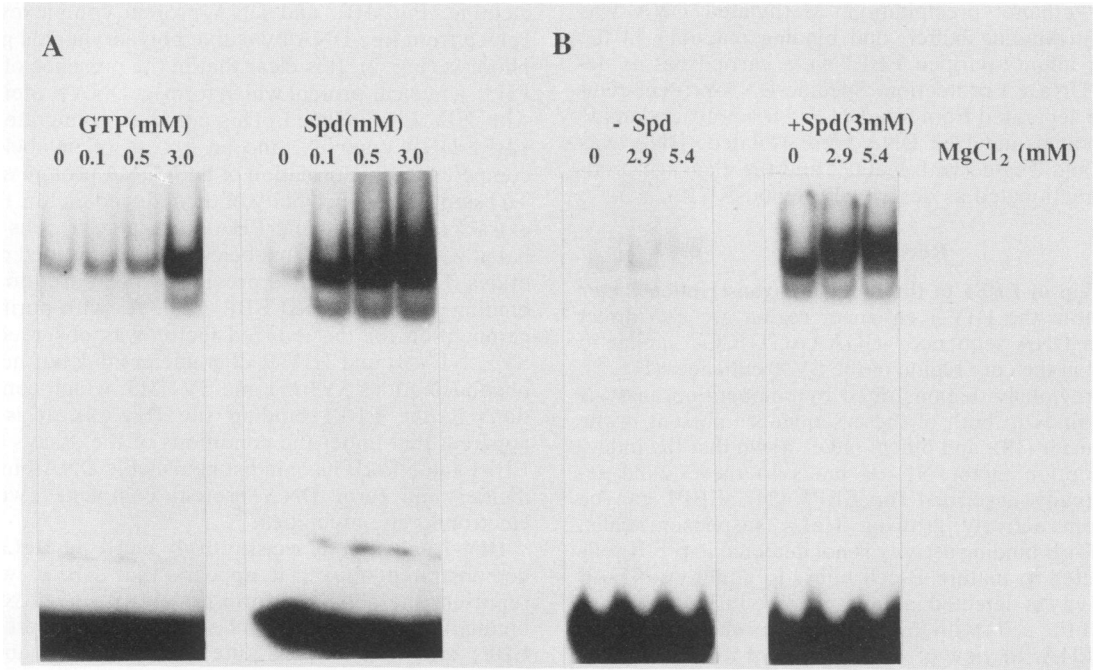

FIG. 4. Cofactor requirements for EBP1 binding. Affinity-purified EBP1 was incubated with ^{32}P-labeled HIV-L double-stranded oligonucleotide in a standard gel electrophoresis DNA-binding assay containing in addition the indicated amounts of GTP or spermidine (Spd) (A) or the indicated amount of $MgCl_2$ and spermidine (B). DNA-protein complexes were resolved from free DNA by electrophoresis in a native 6% polyacrylamide gel.

the two factors was additive, although we believe that this may be the case. Although $MgCl_2$ had no quantitative effect on the binding of EBP1 to its recognition site, its combination with spermidine resulted in a decreased mobility of the DNA-protein complex (Fig. 4). While these experiments were performed with highly purified EBP1, the stimulatory effect of spermidine on binding was also apparent in crude extracts of PHA- and PMA-induced Jurkat cells (data not shown).

DNA protected by EBP1 from DNase I cleavage. To determine whether both of the recognition sites in the HIV-1 enhancer could be occupied simultaneously by EBP1, the protein was incubated in the absence of a carrier with a restriction enzyme fragment, ^{32}P labeled on either the top or bottom strand of the HIV-1 enhancer, and digested with DNase I. The cleavage products were fractionated on a denaturing polyacrylamide gel. In the presence of EBP1, a 34-bp region of the HIV-1 enhancer on both the top and bottom strands was protected from DNase I digestion (Fig. 5). This region contains both -GGGACTTTCC- direct repeats and indicates that EBP1 can occupy both of these sites on the same DNA molecule, although it appears that the promoter-proximal site may be occupied at a lower concentration of EBP1 than the distal site is. On the bottom strand, the region of protection extends from the phosphate bond 3′ of base −75 to the phosphate bond 3′ of base −108, and on the top strand, the region extends from the phosphate bond 3′ of position −74 to the bond 3′ of position −107. From these and other experiments utilizing DNA fragments with two binding sites in gel electrophoresis DNA-binding assays (data not shown), we find no evidence for cooperativity in the binding of EBP1 to the tandem binding sites.

Base-specific contacts of EBP1 on the HIV-1 enhancer. To ascertain which purine bases in the EBP1 recognition sites of the HIV-1 enhancer are in close contact with EBP1, methylation protection and interference experiments were carried out. DNA-protein complexes formed between EBP1 and the HIV-1 enhancer were treated with DMS, and guanine residues methylated at the N-7 position were identified by treating with piperidine to cleave the DNA backbone adjacent to the base modified by DMS. Cleavage products were fractionated by electrophoresis in denaturing polyacrylamide gels and visualized by autoradiography. X-ray film was exposed for 15 or 48 h to allow a linear autoradiographic response, and scanning densitometry of autoradiograms was used to quantitate the extent of DMS methylation in the presence and absence of EBP1. Bases at positions −88G, −89G, −102G, and −103G on the top strand and −80G, −81G, −85G, −94G, −95G, and −99G on the bottom strand were protected from DMS modification in the presence of EBP1 (Fig. 6). In addition, −86A, −104A, −87G, −90G, and −85C on the top strand (Fig. 6) along with −82A, −96A, and −92G on the bottom strand (Fig. 6) exhibited increased rates of DMS methylation in the presence of EBP1.

A complementary series of experiments was carried out to determine which methylated purine bases interfered with the binding of EBP1. In these experiments, DNA previously methylated at purine bases in the absence of protein was incubated with purified EBP1 and DNA-protein complexes resolved from free DNA by electrophoresis in a native polyacrylamide gel. Since there are two binding sites for EBP1 on the HIV-1 enhancer, interference in the binding of EBP1 to one of these sites would still result in the formation of a DNA-protein complex at the other site. Thus it would be difficult to observe interference at specific residues. We therefore used individual copies of each repeated element

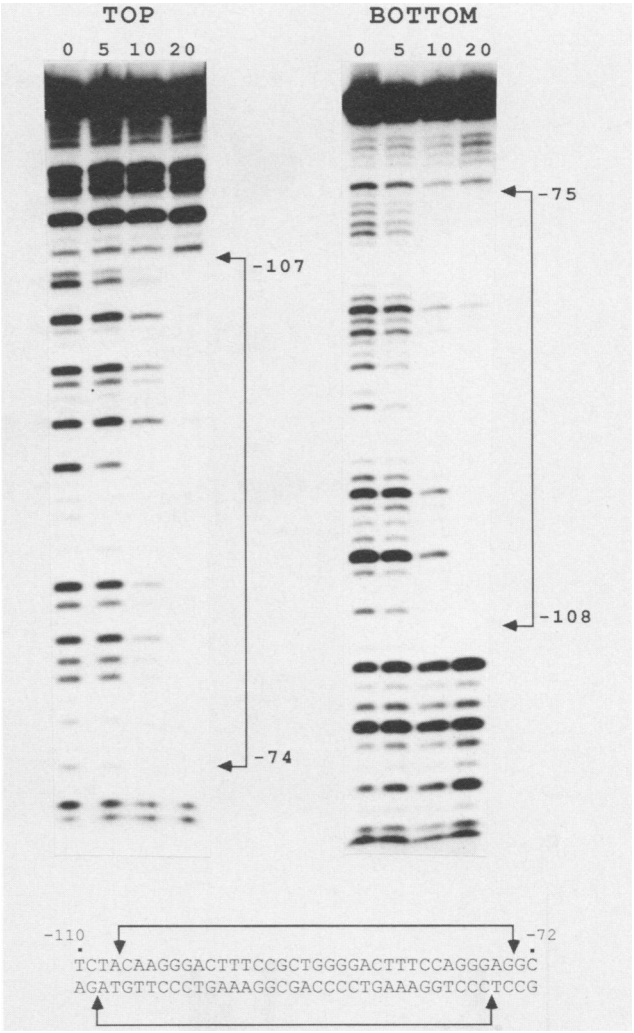

FIG. 5. DNase I protection of the HIV-1 enhancer in the presence of EBP1. A *Hin*dIII-to-*Sac*I fragment or *Eco*RI-to-*Pst*I fragment from pHIVen was 3′ end labeled at the *Hin*dIII (BOTTOM) or *Eco*RI (TOP) site; incubated in the absence (0) or presence of 5, 10, or 20 μl of affinity-purified EBP1; and digested with DNase I. DNA was isolated and fractionated by electrophoresis in 8% denaturing polyacrylamide gels, and the cleavage products were visualized by autoradiography. Phosphate bonds within the bracketed regions were protected from DNase I cleavage in the presence of EBP1. Nucleotide positions are given relative to the HIV-1 mRNA start site.

and performed interference experiments with restriction fragments containing the proximal (right) or distal (left) EBP1 binding sites on the HIV-1 enhancer. DNA fragments ^{32}P labeled on either the top or bottom strands were treated with DMS to generate, on average, less than one methylated base per labeled fragment and were incubated with EBP1, and DNA-protein complexes were separated from free DNA as indicated above. DNA present in the bound and free fractions was eluted from the gel and treated with NaOH to cleave the DNA backbone adjacent to *N*-7-methylated guanines and *N*-3-methylated adenines. Comparing bound and free DNA on a denaturing polyacrylamide gel revealed which purine bases interfered with EBP1 binding when methylated. On the top strand of the distal site, four consecutive purine bases (−100A, −101G, −102G, and −103G) all

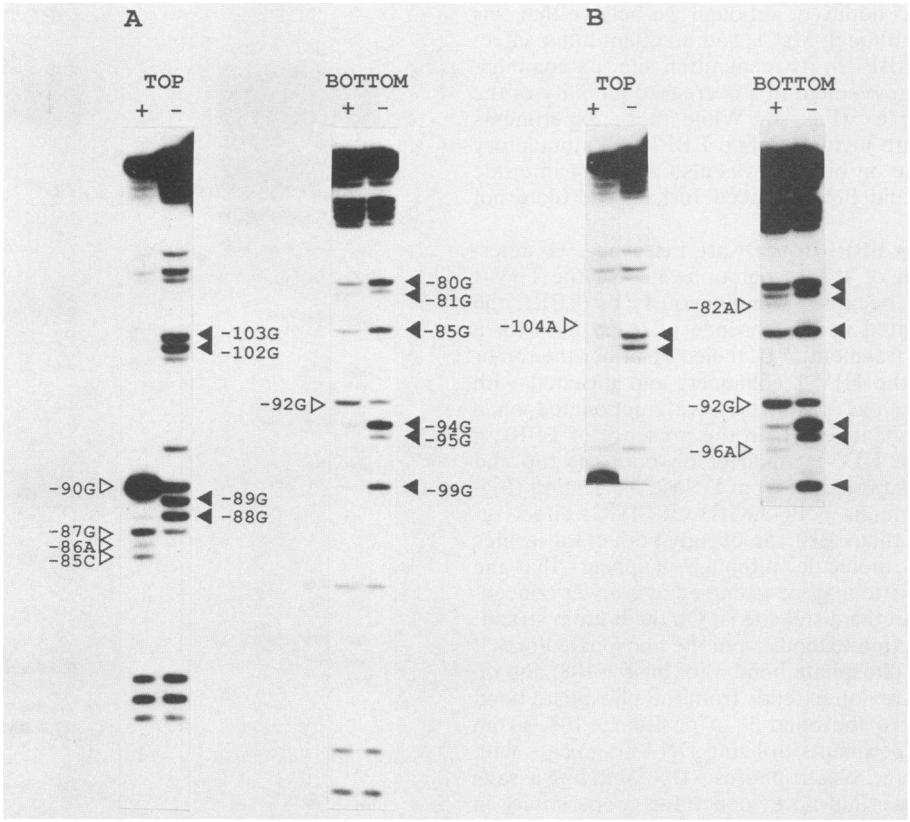

FIG. 6. Methylation protection of the EBP1 binding sites by EBP1. A *Hin*dIII-to-*Sac*I fragment or *Eco*RI-to-*Pst*I fragment from pHIVen was 3′ end labeled at the *Eco*RI (TOP) or *Hin*dIII (BOTTOM) site, incubated in the absence (−) or presence (+) of 20 μl of EBP1, and treated with DMS as described in Materials and Methods. DNA was cleaved at modified bases by treatment with piperidine, and the reaction products were fractionated by electrophoresis in 8% denaturing polyacrylamide gels. (A) Cleavage products were visualized by autoradiography after 15 h of exposure to film. Bases which show either increased (▷) or decreased (◀) rates of methylation by DMS in the presence of EBP1 are indicated. (B) As a result of the increased rate of methylation of −90G, 70% of all DNA molecules, labeled on the top strand, were cleaved by piperidine at this point. Thus, labeled molecules longer than that produced by cleavage at −90G were underrepresented in the population. For this region of the top strand, we compared a shorter exposure in the absence of EBP1 with a longer exposure in the presence of EBP1 such that the intensity of bands representing cleavages outside the binding site are similar. For the bottom strand, a 48-h exposure is shown to illustrate cleavage at bases with increased rates of DMS modification in the presence of EBP1. (C) Quantitation of DMS protection data. Autoradiographs were analyzed by scanning densitometry, and the logarithm of the bound-to-free (B/F) ratio was computed for each base.

interfered with EBP1 binding when they were methylated. A similar situation was evident on the bottom strand, where six consecutive purines (−94G, −95G, −96A, −97A, −98A, and −99G) all interfered with EBP1 binding when they were methylated (Fig. 7). An identical pattern of interfering bases was found for the proximal EBP1 binding site, where four consecutive purine bases (−86A, −87G, −88G, and −89G) on the top strand all interfered with EBP1 binding when they were methylated and six consecutive purine bases on the bottom strand (−80G, −81G, −82A, −83A, −84A, and −85G) interfered with binding (Fig. 7). Thus all purine bases in the 10-bp stretches from −80 to −89 and −94 to −103 interfered with EBP1 binding when they were methylated. The guanine nucleotides that were protected by EBP1 from methylation all interfered with binding when they were methylated, although −90G and −104A, which both demonstrated a very pronounced increase in reactivity toward DMS in the presence of EBP1, did not interfere with EBP1 binding when they were methylated. These nucleotides lie outside the two 10-bp stretches of interfering purine bases.

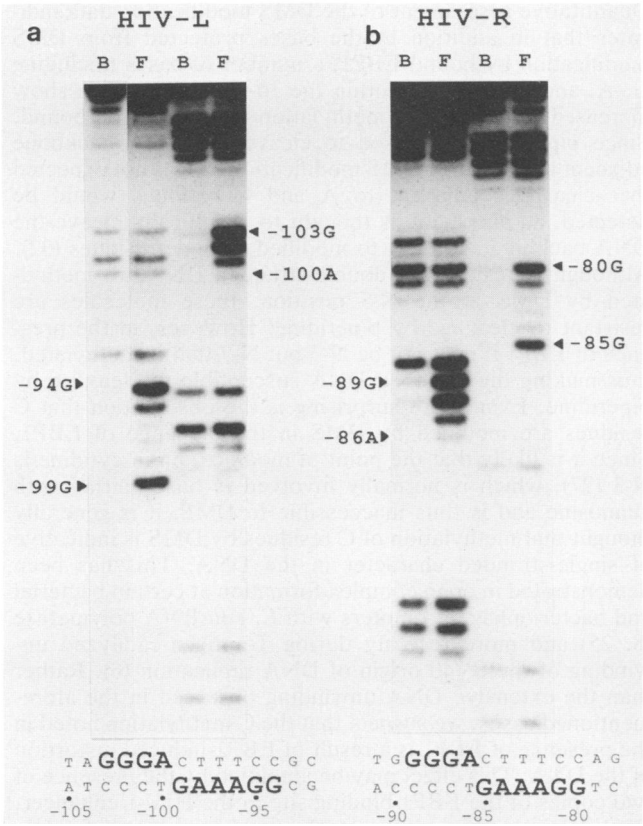

FIG. 7. Methylated bases on the HIV-1 enhancer that interfere with EBP1 binding. A *Hin*dIII-to-*Sac*I fragment or *Eco*RI-to-*Pst*I fragment from pHIV-R (b) was 3′ end labeled at the *Eco*RI (top) or *Hin*dIII (bottom) site and treated with DMS, and the methylated DNA was incubated with 20 μl of affinity-purified EBP1 as described in Materials and Methods. Free DNA (F) and EBP1-DNA complexes (B) were separated by electrophoresis in a native polyacrylamide gel. DNA was eluted from the gel and cleaved at modified purine bases by treatment with NaOH. Cleavage products were fractionated by electrophoresis in an 8% denaturing polyacrylamide gel. A *Hin*dIII-to-*Sac*I fragment or *Eco*RI-to-*Pst*I fragment from pHIV-L (a), was 3′ end labeled at the *Eco*RI (top) or *Hin*dIII (bottom) site, treated with DMS, incubated with EBP1, and processed as for HIV-R. Nucleotide positions of the outermost interfering bases are indicated (▶). The DNA sequences of HIV-R, containing the proximal EBP1 binding site, and HIV-L, containing the distal EBP1 binding site, are presented. Bases that interfere with EBP1 binding are shown in boldface type.

Summary of EBP1 base contacts on the HIV-1 LTR and comparison with binding to other viral enhancers. The pattern of bases which interfere with EBP1 binding when they are methylated or which are protected from methylation by DMS in the presence of EBP1 is strikingly similar on all of the DNA sequence elements which we have analyzed to date (Fig. 8). The binding sites for EBP1 in the SV40 enhancer (11), the human beta interferon gene regulatory element (10), and the HIV-1 enhancer each contain six purine residues on the top strand followed by four purine residues on the bottom strand. In each case, all of the purine bases within this 10-bp region interfere with binding when they are modified by DMS. This 10-bp region is identical in the SV40 enhancer and both of the repeats in the HIV-1 enhancer. Although the pattern of DMS protection is identical in all three cases, subtle differences can be observed in the pattern

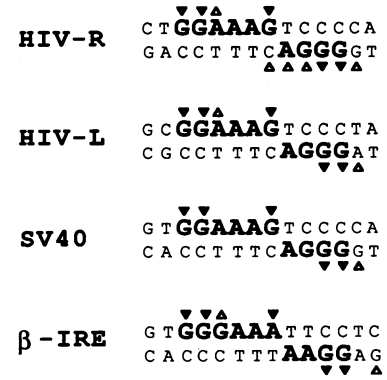

FIG. 8. Comparison of binding of EBP1 to HIV-R and HIV-L with that previously determined for binding to the SV40 enhancer (11) and the beta interferon gene regulatory element (β-IRE) (10). Bases that interfere with EBP1 binding when methylated are shown in boldface type. Bases showing increased (△) or decreased (▼) rates of methylation by DMS in the presence of EBP1 are indicated.

of bases which exhibit an increased rate of methylation by DMS in the presence of EBP1. In the SV40 and HIV-R sequences, a G residue flanking the binding site showed a 10-fold increase in reactivity toward DMS in the presence of EBP1. However, differences in the reactivities of bases within the binding site were apparent. Although not observed in previous studies of the single element in the SV40 enhancer, it is clear that −82A, −85C, −86A, and −87G in HIV-R and −96A in HIV-L have increased rates of modification with DMS in the presence of EBP1.

DISCUSSION

In the experiments reported here, we have shown that the transcription factor EBP1 binds to the HIV-1 LTR at two sites located between 80 and 103 bp upstream from the mRNA cap site. This region of the genome has previously been shown to represent the binding site for the inducible transcription factor NF-κB (34) in T cells and to be required for transcriptional activation induced by proliferative stimuli. Consequently, this raises the question of the relationship between EBP1 and NF-κB. By several criteria, the activated DNA-binding form of NF-κB detected in human T cells appears to be very similar and may in fact be identical to the constitutively active EBP1 isolated from HeLa cells. The DNA-protein complex formed between NF-κB inducible in Jurkat cells and DNA containing its recognition site has the same electrophoretic mobility as the DNA-protein complex containing purified EBP1 (Fig. 2). NF-κB and EBP1 also appear to have indistinguishable DNA-binding specificities, in that double-stranded oligonucleotides which compete for the binding of NF-κB (Fig. 2) also compete for the binding of EBP1 (10). Likewise, oligonucleotides which fail to compete for the binding of NF-κB (Fig. 2) also fail to compete for the binding of EBP1 (10). Data from other studies also support this conclusion. Induction of the human beta interferon gene by virus or double-stranded RNA appears to involve activation of NF-κB and subsequent binding to the PRDII element of the beta interferon response element (28, 43). Moreover, single base-pair changes in the NF-κB binding site present in PRDII which alter the binding of NF-κB in crude extracts (43) similarly alter the binding of purified EBP1 (10). Purified EBP1 has a molecular weight of 57,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

and bromodeoxyuridine cross-linking (12). This is similar to that determined for NF-κB by gel elution and renaturation (2) but somewhat larger than the 45,000 reported by other workers (24, 29). According to methylation interference data, EBP1 makes contacts identical (Fig. 7) to that shown for NF-κB in crude extracts (3, 28). Thus all purine residues in the conserved 10-bp sequence interfere with binding when they are methylated, but purine residues immediately flanking the 10-bp sequence do not interfere with binding when they are methylated. This distinguishes EBP1 and NF-κB from the similar constitutively active proteins H2TFI and κBFI, in which G residues at each end of an 11-bp region interfere with binding when they are methylated (3, 20). Nucleotide triphosphates have been shown to stimulate the binding of NF-κB to its recognition site but have no effect on the binding of H2TFI and κBFI. EBP1 is also stimulated by GTP, although to a lesser extent than that reported for NF-κB. Thus, as far as can be determined, the protein we have designated EBP1 appears to represent the activated form of NF-κB. Definitive proof of this conclusion will, however, require the isolation of cDNAs corresponding to each protein and comparison of the predicted protein sequence. Although we have purified EBP1 from HeLa cells (12), it has been reported that NF-κB binding activity is absent from HeLa cells (39). We do not believe that EBP1 was artifactually activated (by proteolysis or detergents, for example) during preparation of our HeLa extracts, since the binding activity, in these circumstances, should appear in the cytoplasmic extracts, where inactive NF-κB is known to be partitioned (1). This is not the case, as EBP1 is located in the nuclear fraction of hypotonically lysed HeLa cells (12). Transfection experiments with plasmids containing multiple copies of the EBP1 binding site, inserted upstream from the adenovirus major late promoter linked to the CAT gene, support the contention that EBP1 is constitutively active in the HeLa cell lines we have utilized (Fig. 3). In this respect, it is also worth noting that HeLa cells bearing the CD4 receptor support the growth of HIV-1 (31). It is possible that the different levels of DNA-binding activity detected can be explained by the conditions under which the cells are grown (there may be differences in the growth factor content of different batches of newborn calf serum) or that the lines of HeLa cells used have diverged substantially from their common ancestor. Since the labile inhibitor molecule which controls NF-κB binding activity appears to be inactivated by many diverse signals, it would not be surprising if its level in some tissue culture cells fluctuated under different growth conditions. Another protein, HIVen86, is inducible in T cells and appears to have the same DNA-binding specificity as EBP1 and NF-κB (5), but since it has a molecular weight of 86,000, its relationship to EBP1 and NF-κB remains to be clarified. Unfortunately, another protein that binds to the HIV-1 LTR has also been named EBP1 (45), but this protein is quite distinct from the subject of this report in its DNA-binding specificity and chromatographic behavior.

Availability of the purified protein has enabled a detailed analysis to be performed on the binding of EBP1 to the recognition sites present in the HIV-1 LTR. Data presented in previous studies have indicated that human NF-κB can bind to either of the 10-bp repeated sequences, but occupation of both sites on the same DNA molecule was not observed (16, 24). This is in contrast to the bovine analog of NF-κB, which was shown to bind to both sites on the same molecule (29). Using saturating levels of EBP1, we have demonstrated that both binding sites on the same molecule are protected from DNase I cleavage and DMS modification. Quantitative assessment of the DMS modification data indicates that in addition to the bases protected from DMS modification by bound EBP1, a number of bases (including G, A, and C residues) within the 10-bp binding sites show increased rates of DMS methylation when EBP1 is bound. Since piperidine was used to cleave the DNA backbone adjacent to the site of DMS modification, it was not expected that cleavages adjacent to A and C residues would be detected, as piperidine is thought to specifically cleave the DNA backbone adjacent to modified guanine residues (32). Although A residues in double-stranded DNA are methylated by DMS at the N-3 position, these molecules are resistant to cleavage by piperidine. However, in the presence of EBP1 it may not be N-3 but N-7 that is methylated, thus making the modified DNA susceptible to cleavage by piperidine. Even more surprising is the observation that C residues are modified by DMS in the presence of EBP1. Since it is likely that the point of modification in cytidine is N-3 (27), which is normally involved in base pairing with guanosine and is thus inaccessible to DMS, it is generally thought that methylation of C residues by DMS is indicative of single-stranded character in the DNA. This has been demonstrated in open complex formation at certain bacterial and bacteriophage promoters with *E. coli* RNA polymerase (8, 25) and more recently during T-antigen-catalyzed unwinding of the SV40 origin of DNA replication (6). Rather than the extensive DNA unwinding observed in the aforementioned cases, we suspect that the C methylation noted in the presence of EBP1 is a result of EBP1-induced distortion of the DNA. This effect may be amplified by the presence of two copies of the EBP1 binding site in the HIV-1 enhancer, since C methylation was not observed in studies of single copies of the EBP1 binding site (10, 11). Alteration in the structure of the DNA accompanying EBP1 binding may explain the stimulatory effect of spermidine on DNA binding, since this polyamine is known to induce changes in the conformation of DNA molecules. Thus spermidine may allow DNA in the binding site to adopt a conformation that is optimal for the interaction with EBP1.

Although we have demonstrated that the HeLa cell protein EBP1 is probably the activated form of NF-κB present in Jurkat cells and that purified EBP1 binds to and activates transcription from the HIV-1 enhancer, it is not clear how these proteins are activated in vivo. However, since stimuli such as antigen presentation to T cells and beta interferon induction activate EBP1–NF-κB, it is likely that viral infection of individuals harboring HIV-1 induces transcription from the provirus, breaking latency and resulting in progression of the disease.

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