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C/EBP Proteins Activate Transcription from the Human Immunodeficiency Virus Type 1 Long Terminal Repeat in Macrophages/Monocytes

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Three binding sites for C/EBP proteins are found in the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) (V. M. Tesmer, A. Rajadhyaksha, J. Babin, and M. Bina, Proc. Natl. Acad. Sci. USA 90:7298–7302, 1993). We have determined the functional role of C/EBP proteins and C/EBP sites in regulating transcription from the HIV-1 LTR in monocytes/macrophages. Inhibition of endogenous C/EBP proteins, using either an excess of C/EBP binding sites or a *trans*-dominant negative inhibitor, demonstrated that C/EBP proteins are required for basal and activated levels of HIV-1 LTR transcription in the promonocytic cell line U937. Northern (RNA) blots and binding assays showed that NF-IL6 is the only known C/EBP family member which is increased when U937 cells are activated. Mutational analyses of the HIV-1 LTR showed that one C/EBP site is required for normal LTR transcription both before and after cellular activation and that the two 3′ C/EBP sites are functionally equivalent. However, transcription from crippled HIV-1 LTRs lacking C/EBP sites can still be induced following activation of U937 cells. Several models are suggested for how elevated NF-IL6 may participate in an autostimulatory loop involving HIV infection, macrophage activation, cytokine expression, and HIV replication.

Monocytes/macrophages, in addition to $CD4^+$ T cells, are a primary target of human immunodeficiency virus (HIV). Unlike HIV-infected T cells, HIV-infected monocytes/macrophages appear to be resistant to the cytopathic effects of the virus and thus serve as a reservoir for persistent infection and virus dissemination (16, 18). HIV-infected monocytes/macrophages are also associated with the AIDS-related pathologies observed in the lungs, skin, lymph nodes, and central nervous system (9, 16, 18, 23, 24, 62). Despite the importance of these cells in AIDS, the regulation of HIV expression in monocytes/macrophages has not been extensively studied.

Inflammatory cytokines such as interleukin 1 (IL-1), IL-6, and tumor necrosis factor alpha are elevated in patients with AIDS, and these cytokines mediate activation and differentiation of monocytes/macrophages (5, 15, 25, 27, 43, 44). The exact relationship between HIV type 1 (HIV-1) infection, macrophage activation, induction of transcription factors, cytokine expression, and HIV-1 replication is not known, but there is evidence that macrophages, cytokines, and HIV-1 could be involved in an autostimulatory loop. Activation of macrophages induces an array of cellular transcription factors, including NF-κB/rel and NF-IL6 (2, 4). These proteins activate cytokine gene expression. NF-κB/rel proteins also activate HIV-1 transcription. However, the function of C/EBP proteins for regulation HIV-1 LTR transcription in monocytes/macrophages is not known.

NF-IL6 is a strong transcriptional activator that is induced during monocyte/macrophage differentiation (1, 2, 37). NF-IL6 appears to be important in the coordinate expression of several cellular genes which are expressed in activated monocytes, including genes encoding IL-1, IL-6, IL-8, granulocyte colony-stimulating factor, and tumor necrosis factor alpha (2). Targeted disruption of the NF-IL6 gene demonstrated that it is necessary for normal macrophage bactericidal and tumoricidal functions (57). Recently, NF-IL6 was shown to bind three sites on the HIV-1 long terminal repeat (LTR), suggesting that it could also be important for HIV-1 transcription in monocytes/macrophages (58).

NF-IL6 (C/EBPβ) is a member of the C/EBP family of transcription factors, which also includes activators C/EBP (C/EBPα), CRP3 (C/EBPδ), and CRP1 and negative regulators liver inhibitory protein (LIP), Ig/EBP (C/EBPγ), and CHOP-10 (2, 6, 13, 47, 48, 63). Family members share homology in their basic leucine zipper regions and as a result recognize the same DNA consensus sequence and can form heterodimers with one another (61). C/EBP sites occur in regulatory regions of many cytokine genes, liver-specific genes, including those encoding acute-phase proteins, immunoglobulin heavy- and light-chain genes, and several viruses in addition to HIV-1 (2).

In this study, we have addressed the functional role that C/EBP family proteins and C/EBP binding sites play in regulating transcription of the HIV-1 LTR in the human promonocytic cell line U937. U937 cells were chosen for this study because activation of U937 cells with mediators such as lipopolysaccharide (LPS) and phorbol myristate acetate (PMA) induces cellular differentiation (22, 37, 46). We show that C/EBP proteins are important activators of HIV-1 transcription both before and after activation and that at least one C/EBP site is necessary for normal HIV-1 LTR transcriptional activity. NF-IL6 appears to be a key C/EBP family activator for the HIV-1 LTR since it is the only family member which is induced upon LPS-PMA activation of U937 cells.

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MATERIALS AND METHODS

Plasmids. The 500-bp *Bgl*II fragment of the HIV-1 LTR from strain HXB2 (49) was cloned into the *Sma*I site located 5′ of the luciferase reporter gene (LUC) in the p19LUC vector (60). The −158ΔLTR construct was generated by

subcloning the 178-bp *Ava*I-*Bgl*II fragment of the HIV-1 LTR into the *Sma*I site of p19LUC. The mκB HIV LTR-LUC construct was constructed by subcloning the *Bgl*II fragment from mκB-CAT, generously provided by G. Nabel (University of Michigan) (35), into p19LUC as described above. For the −93ΔLTR construct, the 117-bp *Acc*I-*Bgl*II fragment from mκBLTRCAT was cloned into the *Sma*I site of p19LUC. The deletion mutations −205ΔLTR and −109ΔLTR and the site-directed mutations mC2LTR and mC3LTR were generated by PCR (28, 42) using oligonucleotides −205Δ (5′ AGAGAAGCTTTAGAGTGGAGG 3′), −109Δ (5′ GACATCGAAGCTTGGGACTTTCCGCT 3′), mC3 (5′GCTGA CATCGACAGCTGTACAAGGGAC 3′), mC2 (5′ GCCGCCTAGCTGCAG GGGCGTGGCCCGAGAG 3′), and 3′LTR (5′ CCACTGCTAGAGATTTTC CAC 3′). The PCR products were digested with *Hin*dIII and cloned into the *Hin*dIII site of p19LUC. The mC2,3 double mutation was also generated by PCR, but the mC2LTR construct rather than the wild-type LTR was used as the template in the PCRs. Mutations were confirmed by restriction analysis and sequencing.

The expression vectors for rat NF-IL6, LIP, and Ig/EBP have been previously described (11, 13). pUC-E4 was generated by cloning four tandem C/EBP binding sites into pUC19 (3).

Transfections. For transfection experiments, DNA was added to 0.3 ml of U937 cells resuspended in RPMI supplemented with 10% fetal calf serum at 1.0×10^7 to 2×10^7 cells per ml. The cell suspensions were electroporated by pulsing with 960 μF and 240 V from a Bio-Rad (Richmond, Calif.) Gene Pulser apparatus. Cells were resuspended in 5 ml of RPMI supplemented with 10% fetal calf serum in the absence or presence of 10 μg of LPS and 10 ng of PMA per ml. Cells were incubated at 37°C in 5% CO_2 in air for 24 h before harvesting for luciferase assays. At the time of harvesting, cells were counted and luciferase values were corrected on the basis of these counts. At least three independent plasmid preparations were tested for each construct, and all transfections were done in at least duplicate. Transfection efficiencies in some experiments were controlled by cotransfecting a human growth hormone expression vector, pXGH5 (Nichols Institute Diagnostics, San Juan Capistrano, Calif.).

For luciferase quantitation, cells were washed twice with phosphate-buffered saline and lysed with 0.5 ml of lysis buffer (1% Triton X-100, 25 mM glycylglycine [pH 7.8], 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol [DTT]). Lysates were cleared by centrifugation. To assay for luciferase activity, 500 μl of reaction buffer (15 mM KPO_4 buffer [pH 7.8], 2 mM ATP [pH 7.0], 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM DTT) and 100 μl of substrate buffer (0.4 mM luciferin, 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM DTT) were added to 100 μl of the supernatant. Samples were measured with a luminometer.

Northern (RNA) blots. Total RNA was prepared by lysing cells with 4 M guanidium isothiocyanate–25 mM sodium citrate–0.5% *n*-lauryl sarcosine–100 mM mercaptoethanol. Lysates were pelleted through a 5.7 M CsCl cushion by centrifugation for 12 h at 36,000 rpm in an SW50 rotor (10). Forty micrograms of total RNA was resuspended in 1× formaldehyde gel-buffer (0.1 M morpholinepropanesulfonic acid [MOPS; pH 7.0], 40 mM sodium acetate, 5 mM EDTA)–17.5% formaldehyde–50% formamide–1× gel-loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF) and separated on a formaldehyde-agarose gel (1% agarose, 1× formaldehyde gel buffer, 2.2 M formaldehyde) (32). RNA was transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, Ill.) by capillary action, using 20× SSC (3 M NaCl, 0.3 M sodium citrate) (32). RNA was fixed to the membrane by baking for 2 h at 80°C and hybridized with radiolabeled probes at 65°C in 1.5× SSPE (0.23 M NaCl, 1.5×10^{-2} M NaH_2PO_4, 1.5×10^{-3} M EDTA)–10% polyethelene glycol–7% sodium dodecyl sulfate (SDS)–0.1 μg of salmon sperm DNA per ml for at least 12 h. Filters were washed once at 22°C in 2× SSC–0.1% SDS for 15 min, once at 65°C in 1× SSC–0.1% SDS for 15 min, and once at 65°C in 0.3× SSC–0.1% SDS for 15 min. Filters were stripped by washing at 100°C in 0.1% SDS. Radiolabeled probes with a specific activity of $>2 \times 10^8$ were generated by random priming gel-isolated DNA fragments as specified by the manufacturer (United States Biochemical, Cleveland, Ohio).

EMSAs and DNase I footprinting. Nuclear extracts from U937 cells were prepared as previously described (53). Electrophoretic mobility shift assays (EMSAs) were performed with 2 to 4 μg of protein from nuclear extracts or 1 μg of protein from extracts of bacteria expressing recombinant rat NF-IL6, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 60 mM KCl, 7.5% glycerol, 0.1 mM EDTA, 0.75 mM DTT, and 5 mM $MgCl_2$. Reaction mixtures were preincubated with specific and nonspecific competitors and polyclonal antisera (12) for 15 min at room temperature before addition of 10^4 cpm of probe and incubation for an additional 20 min. Samples were loaded directly onto a 6% polyacrylamide gel and electrophoresed at 120 V in 1× Tris-borate-EDTA. Probes for EMSA were generated by using oligonucleotides representing the C/EBP site that spans −169 bp (5′ GATCGCCTAGCATTTCATCACA CGT 3′ and 5′ GATCACGTGTGATGAAATGCTAGGC 3′) and the HIV-1 LTR. Probes were generated by end filling with the Klenow fragment of *Escherichia coli* polymerase (32) or by PCR using a kinase-treated oligonucleotide (26).

For DNase I footprinting, binding reactions were performed with bacterially expressed rat NF-IL6, 1 μg of dI-dC, 0.5 μg of salmon sperm DNA, 25 mM HEPES (pH 7.5), 60 mM KCl, 7.5% glycerol, 0.1 mM EDTA, 0.75 mM DTT, 5 mM $MgCl_2$, and 10^4 cpm of probe. Binding reaction mixtures were incubated at 22°C for 20 min. Following binding, 0.1 U of RQ1 DNase I (Promega, Madison, Wis.) diluted in 25 mM $CaCl_2$ was added to each reaction mixture, and the mixtures were incubated for 1 min at 22°C (64). The reaction was stopped by adding 100 μl of stop buffer (1% SDS, 20 mM EDTA, 200 mM NaCl). Samples were extracted with phenol and 24:1 chloroform-isoamyl alcohol, precipitated, separated on a 6% sequencing gel with G and G+A ladders, and visualized by autoradiography. End-labeled probes for DNase I footprinting were generated by PCR using a kinase-labeled −205Δ oligonucleotide and either the wild-type LTR or the mC2,3LTR construct as a template (26). Following PCRs, probes were gel purified.

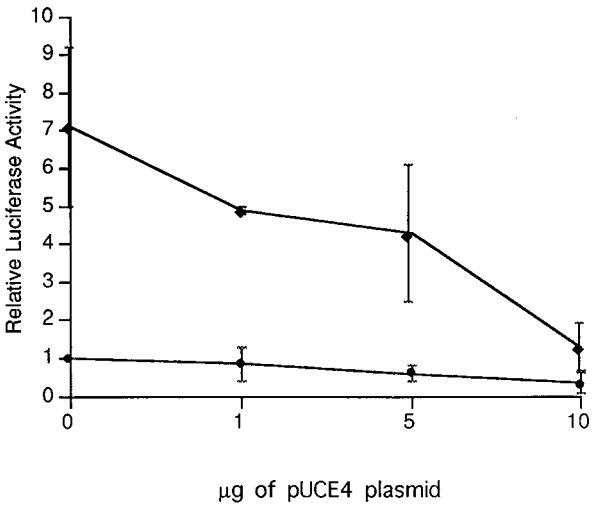

FIG. 1. Basal and activated HIV-1 LTR activity is decreased by an excess of C/EBP binding sites. U937 cells were cotransfected with 10 μg of LTR-LUC and increasing amounts of pUC-E4, a plasmid with four C/EBP binding sites. pUC19 was used to ensure that all transfection mixtures had a total of 20 μg of DNA. Following transfection, cells were cultured in the absence (●) or presence (◆) of 10 μg of LPS and 10 ng of PMA per ml for 24 hours. The data represent three independent experiments, and error bars show 1 standard deviation.

RESULTS

Endogenous C/EBP proteins are required for HIV-1 LTR transcription in U937 cells. NF-IL6 is involved in the coordinate regulation of several genes expressed in monocytes/macrophages. Previous studies, as well as our own experiments, have demonstrated that NF-IL6 can bind three sites in the HIV-1 LTR (reference 58 and data not shown). In addition, NF-IL6 can transactivate the HIV-1 LTR in the variety of cells, including monocytes/macrophages (reference 58 and data not shown). Although these studies indicate that NF-IL6 can activate the HIV-1 LTR in a cotransfection assay, they do not address the role that endogenous C/EBP proteins may play in HIV-1 expression in vivo.

To examine the role that endogenous C/EBP proteins play in regulating HIV-1 LTR activity in monocytes/macrophages, we used two different strategies, both of which depended on functional inhibition of endogenous C/EBP proteins. First, U937 cells were cotransfected with an HIV-1 LTR-LUC reporter and increasing amounts of control pUC19 vector or pUC-E4, a vector that contains four C/EBP binding sites (3) and would be expected to bind endogenous C/EBP proteins. Following transfection, the U937 cells were cultured in the absence or presence of 10 μg of LPS and 10 ng of PMA per ml for 24 h. Activation of U937 cells with LPS and PMA has been shown to induce differentiation of these cells (22, 37, 46). The results of these experiments are shown in Fig. 1. The presence of pUC-E4 diminished HIV-1 LTR activity by approximately 70% prior to LPS-PMA treatment. After U937 cell activation, HIV-1 LTR transcription was inhibited by 80% at the highest

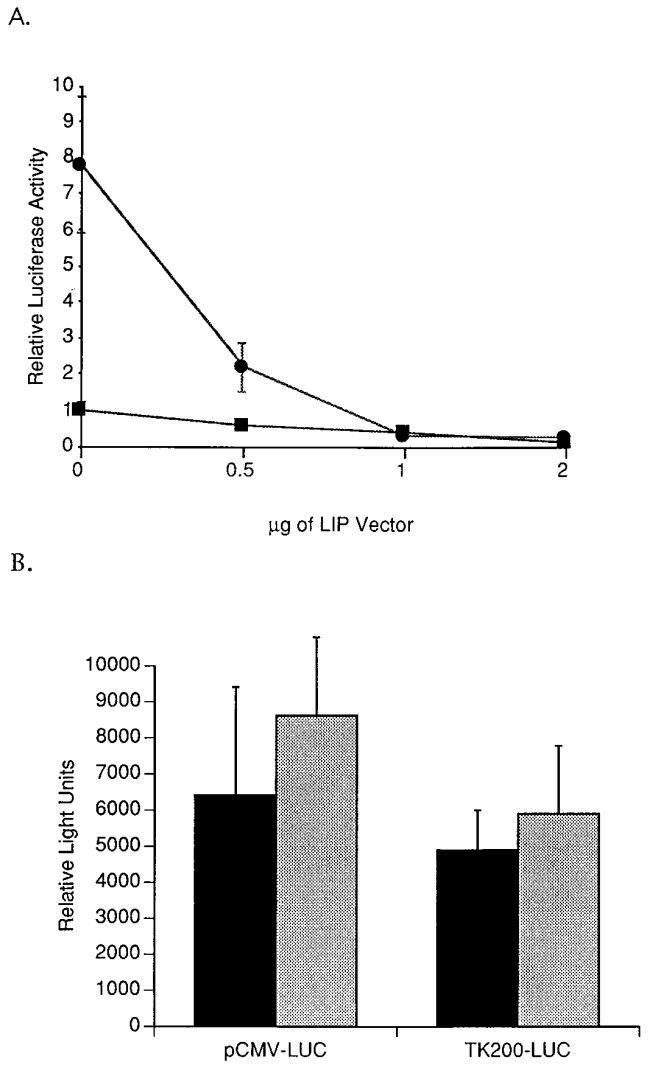

FIG. 2. Basal and activated HIV-1 LTR activity is inhibited by the *trans*-dominant negative protein LIP. (A) Untreated (■) and LPS-PMA-treated (●) U937 cells were cotransfected with LTR-LUC and increasing amounts of CMV-LIP expression vector. (B) U937 cells were cotransfected with 2 μg of pCMV-LUC or 5 μg of TK200-LUC and 2 μg of CMV-LIP expression (stippled bars) vector or pCMV (solid bars). pCMV was used to ensure that all transfections had a total of 10 μg of CMV promoter. The data are representative of three independent experiments, and error bars show 1 standard deviation.

concentration of pUC-E4. These data show that endogenous C/EBP-binding proteins are important for transcription from the HIV-1 LTR in U937 cells both before and after activation of the cells by LPS and PMA.

This conclusion was corroborated by experiments using an expression vector for LIP, a truncated form of NF-IL6 which acts as a *trans*-dominant negative regulator of C/EBP family activators, including C/EBP and NF-IL6 (13). LIP contains the conserved basic leucine zipper domain which permits DNA binding and dimerization but lacks a strong transcriptional activation domain; therefore, inactive LIP-containing heterodimers can form between LIP and C/EBP activators (13). Cotransfection of U937 cells with increasing amounts of a LIP expression vector inhibited HIV-1 LTR-dependent transcription both before and after LPS-PMA differentiation in a dose-dependent manner (Fig. 2A). The observed inhibition was not

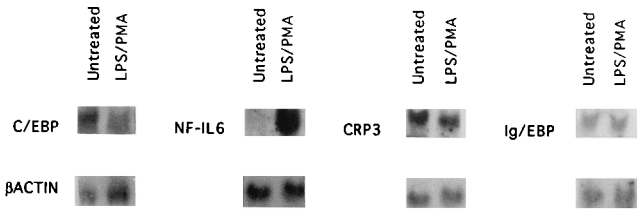

FIG. 3. Expression of C/EBP family members before and after U937 differentiation. U937 cells were cultured in the absence or presence of 10 μg of LPS and 10 ng of PMA per ml for 24 h. Total RNA was analyzed by Northern blotting with the indicated probes.

due to nonspecific inhibition of transcription by LIP because cotransfecting the cytomegalovirus (CMV)-LIP construct with luciferase reporters driven by the CMV promoter or the herpes simplex virus *tk* promoter did not reduce their activity (Fig. 2B). Furthermore, a second negative regulator of C/EBP activators, Ig/EBP (11), also inhibited LTR activity following U937 differentiation (data not shown). Taken together, these results show that endogenous C/EBP proteins are necessary for HIV-1 LTR transcription in U937 promonocytes both before and after activation.

NF-IL6 is the only C/EBP family member whose levels are increased upon activation of U937 cells. The experiments described above indicated that C/EBP family members are required for transcription from the HIV-1 LTR but did not distinguish which family members might be responsible for this transcriptional activation. Therefore, mRNA levels of four C/EBP family members, NF-IL6, C/EBP, CRP3, and Ig/EBP, were examined to gain insight into their regulation during monocyte differentiation. C/EBP, NF-IL6, and CRP3 are transcriptional activators, while Ig/EBP is a negative regulator of these activators (2, 6, 11, 13, 47, 63). mRNA for CRP3 and Ig/EBP was detected at modest levels in U937 cells, and these levels did not change following activation of the cells with LPS and PMA (Fig. 3). Consistent with previous studies, C/EBP mRNA levels were reduced 2-fold and NF-IL6 mRNA levels were increased 15-fold following U937 activation (Fig. 3) (37). These results show that mRNAs encoding three activators which recognize C/EBP sites—C/EBP, CRP3, and NF-IL6—are present in U937 cells. C/EBP and CRP3 are present before cell activation, and NF-IL6 is the only family member whose mRNA is induced by treatment with LPS and PMA. It is likely that either heterodimers or homodimers of C/EBP and CRP3 are responsible for activating the HIV LTR via the C/EBP sites prior to cell activation and that NF-IL6 plays an important role following activation.

EMSAs were performed to examine proteins in U937 nuclear extracts which could bind to the HIV-1 C/EBP sites. Nuclear extracts were prepared from unstimulated and LPS-PMA-stimulated U937 cells. Using an oligonucleotide probe representing the C/EBP site which spans the −169 bp site in the HIV-1 LTR, a weak complex, presumably due to C/EBP, CRP3, and Ig/EBP (Fig. 3), was observed in nuclear extracts from unstimulated U937 extracts (Fig. 4, lanes 1 and 2). The intensity of the complex increased approximately fivefold in extracts prepared from LPS-PMA-stimulated U937 cells (Fig. 4, lanes 4 and 5). This complex was competed for by C/EBP site competitors but not by heterologous competitors (Fig. 4; compare lanes 2 and 3 and lanes 5 and 6). The induced complex was ablated when an NF-IL6 polyclonal antiserum was included in the EMSA reaction mixture, demonstrating that NF-IL6 is a major component of the C/EBP site complex in activated U937 cells (Fig. 4, lane 8). Therefore, these binding data

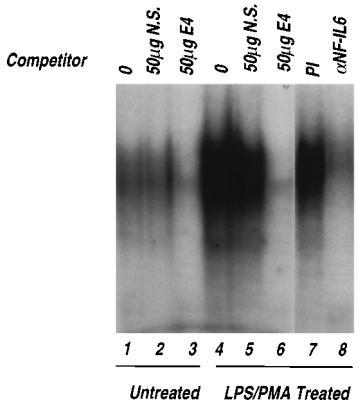

FIG. 4. LPS-PMA stimulation increases binding of NF-IL6 to the HIV-1 C/EBP binding sites. EMSA was performed with nuclear extracts prepared from unstimulated and LPS-PMA-stimulated U937 cells and an oligonucleotide probe which spans the −169 bp C/EBP binding site. Extracts were incubated with no competitor (lanes 1 and 4), 50 μg of unrelated (nonspecific [N.S.]) GATA competitors (lanes 2 and 5), 50 μg of specific C/EBP binding-site competitor (lane 3 and 6), preimmune (PI) serum (lane 7), and an NF-IL6 polyclonal antiserum (αNF-IL6) (lane 8).

are consistent with the mRNA steady-state levels and demonstrate that C/EBP proteins which bind the HIV-1 LTR increase following activation and that this change is due primarily to increased NF-IL6.

One C/EBP binding site is required for HIV LTR transcription in quiescent U937 cells. Tesmer et al. tested the function of an HIV-1 LTR containing a mutation in the highest-affinity C/EBP binding site and found that it did not affect the ability of NF-IL6 to transactivate (58). However, their mutant reporter still contained two wild-type C/EBP sites. We wished to determine the functional importance of the three C/EBP sites in monocytes/macrophages. Specifically, we wished to examine whether (i) one or more of the three C/EBP sites are required for the promoter activity of the HIV-1 LTR, (ii) the sites are functionally equivalent, and (iii) one or more C/EBP site is necessary for induction of HIV-1 LTR activity when U937 cells are activated by LPS and PMA.

To address these questions, we generated a series of 5′ truncation mutations in the HIV-1 LTR reporter (Fig. 5A) and tested their transcriptional activity by transfection into U937 cells. Deletion of the two most 5′ C/EBP sites did not decrease transcription relative to the wild-type construct in cells prior to LPS-PMA treatment. As shown in Fig. 5A, the −205ΔLTR construct, which contains two C/EBP binding sites, is as active as the full-length HIV-1 LTR, whereas the −158ΔLTR construct, which contains a single C/EBP site, is slightly more active than the wild-type LTR. However, deletion of all three C/EBP sites (the −109ΔLTR construct) reduced transcription approximately fivefold even though the two NF-κB sites and the three Sp1 sites remained. A similar decrease was observed with mκBLTR, a construct which lacks functional κB sites but contains C/EBP sites. These data suggest that at least one C/EBP binding site is required for normal levels of HIV-1 LTR transcription in U937 cells.

To confirm that loss of C/EBP binding sites, rather than loss of other sequences, caused the observed decrease of HIV-1 LTR transcription and to determine if the HIV-1 C/EBP binding sites were functionally equivalent, site-directed mutagenesis was performed in the context of the −205ΔLTR. The two 3′ C/EBP sites (C2 [−178 to −159 bp] and C3 [−120 to −109 bp]) were mutated by PCR; the sequences of the mutant sites are shown in Fig. 5B. DNase I footprinting confirmed that these mutations ablated the ability of recombinant NF-IL6 to bind the −178 to −159 and −120 to −109 bp sites (Fig. 5C). The site-directed mutations were then tested by transfection into U937 cells. Mutants containing single mutations at either site had activity similar to that of the wild-type LTR (Fig. 5A). However, the construct containing mutations in both C/EBP binding sites (mC2,3LTR) had activity which was decreased by 70% and was not significantly different from that of the truncated construct (−109ΔLTR) lacking all C/EBP sites (Fig. 5A). Thus, the results of the truncations and site-directed mutations are consistent and show that at least one C/EBP binding site is required for full transcriptional activity of the HIV-1 LTR in U937 cells. Furthermore, since both single mutations, mC2LTR and mC3LTR, show wild-type activity, the two 3′ C/EBP binding sites appear to be functionally equivalent.

Following activation of U937 cells, one C/EBP site is required for full transcriptional activity of the HIV-1 LTR but not for induction of HIV-1 LTR transcription. Transcription from the truncated and mutated HIV-1 LTR constructs was also determined after the transfected U937 cells were activated and induced to differentiate by treatment with LPS and PMA (Fig. 6). Following LPS-PMA stimulation, transcription from the −109ΔLTR and mC2,3LTR constructs was dramatically reduced; it was approximately 26% of the wild-type LTR activity. However, we note that all constructs, even −93ΔLTR, which lacks the three C/EBP sites as well as the two κB sites, and mκBLTR showed activation-dependent induction in the range of six- to eightfold after LPS-PMA treatment. One possible explanation for these results is that there exist undetected C/EBP sites within the HIV LTR. However, no C/EBP sites were found by EMSAs using probes that spanned the −109 to +78 region of the HIV-1 LTR to bind recombinant NF-IL6 (data not shown).

LPS and PMA act through different signal transduction pathways (2, 4, 59). To determine whether C/EBP sites were important in the induction of the HIV-1 LTR in response to activation by more specific stimuli, U937 cells were activated with IL-6, LPS, PMA, or PMA plus LPS (Table 1). The wild-type LTR, −205ΔLTR, −109ΔLTR, and mC2,3LTR constructs were induced by similar amounts in response to each treatment. Activating U937 cells with either IL-6 or LPS alone induced these constructs by three to fivefold, whereas treatments involving PMA resulted in at least a sevenfold increase in transcription (Table 1). However, constructs lacking NF-κB sites, mκBLTR and −93ΔLTR, were reduced in the ability to be activated by individual activators although not by PMA plus LPS (Table 1). No significant induction was observed with −93ΔLTR in U937 cells treated with IL-6 or LPS, and only a threefold activation of both −93ΔLTR and mκBLTR was observed following PMA treatment (Table 1). Thus, the data show that C/EBP sites are required for elevated levels of HIV-1 LTR transcription following activation but that transcription from crippled promoters lacking C/EBP sites is still inducible.

DISCUSSION

The studies reported here establish for the first time the functional importance of C/EBP proteins and C/EBP binding sites for basal and activated levels of HIV-1 LTR transcription in monocytes/macrophages. The data demonstrate that endogenous C/EBP proteins are required for normal HIV-1 LTR transcription in U937 cells both before and after cellular activation and differentiation. We also demonstrate a functional requirement for at least one C/EBP site in the HIV-1 LTR.

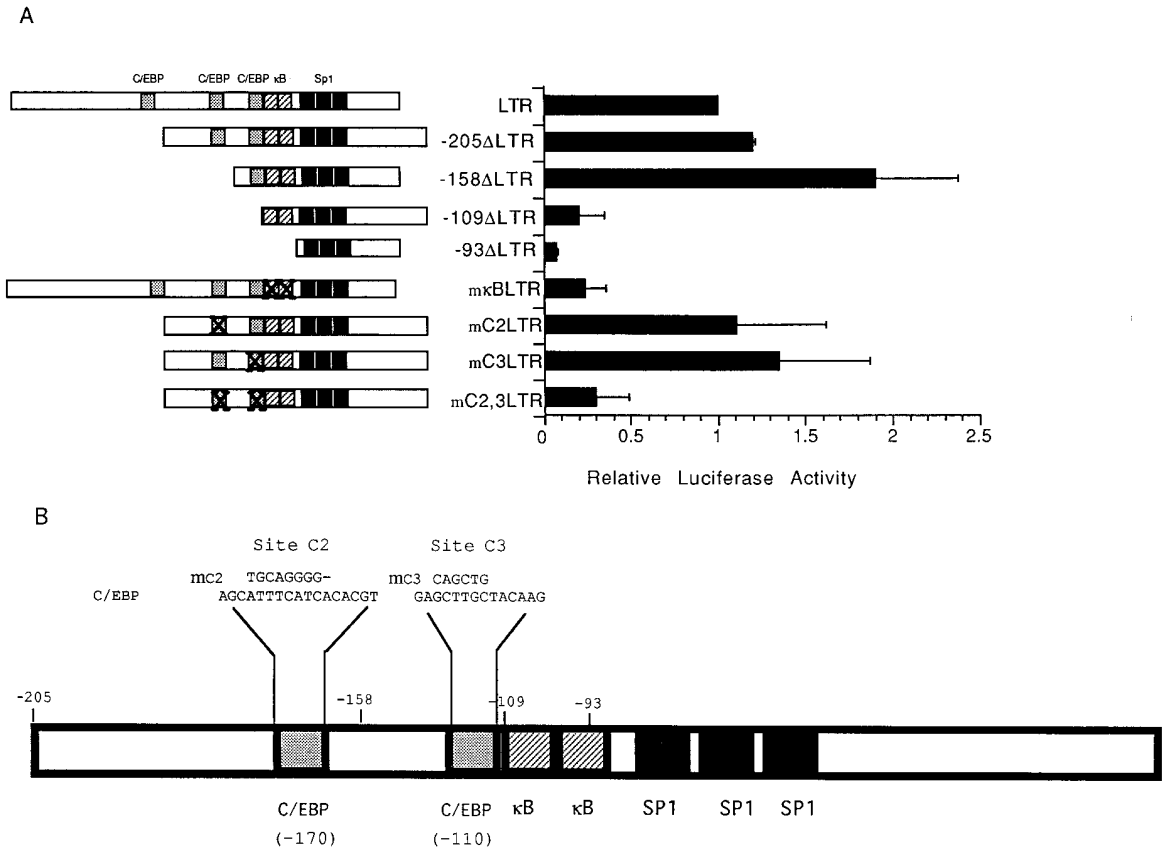

FIG. 5. C/EBP sites are required for basal HIV-1 LTR activity. (A) U937 cells were transfected with 10 μg of various truncated and mutated LTR constructs. Each bar represents an average of at least three experiments using three or more independent DNA preparations. Error bars represent 1 standard deviation. (B) Site-directed mutagenesis of the C/EBP sites. Altered bases are indicated above the wild-type HIV-1 LTR sequences. Numbers in parentheses represent the central base pairs in the recognition sequences. (C) Mutations disrupt sequence-specific binding of NF-IL6. Probes were generated with either the HIV LTR or mC2,3LTR and incubated with increasing amounts of bacterially expressed rat NF-IL6 (rNF-IL6). Following binding, the samples were treated with DNase I.

Therefore, at least three families of cellular transcription factors, C/EBP, NF-κB/rel, and Sp1, are required for HIV-1 LTR transcription in monocytes/macrophages, and members of two of these families, C/EBP and NF-κB/rel, are induced upon activation of monocytes/macrophages (22, 37, 40, 46, 51). The C/EBP protein NF-IL6 may have a key role in regulating the HIV-1 LTR in monocytes, since it can bind the HIV-1 LTR and is the only C/EBP family protein which is induced during monocyte differentiation (37, 50, 58) (Fig. 3 and 4).

Role of C/EBP binding sites in the HIV-1 LTR. A series of truncated and mutated HIV-1 LTR constructs was used to determine the functional importance of C/EBP binding sites. Our results obtained by using these constructs are generally consistent with results obtained by other groups who performed mutational analyses of the HIV-1 LTR in various cell types, including fibroblasts, T cells, monocytic cell lines, and undifferentiated and differentiated primary monocytes (8, 17, 19, 20, 30, 34, 36, 49, 52, 54, 65). However, our studies are the first to test directly and systematically the importance of C/EBP sites within the HIV-1 LTR. We have shown that at least one C/EBP site is required for optimum LTR transcription in undifferentiated U937 cells and for high levels of transcription following differentiation of U937 cells (Fig. 5A and 6). Spacing of the C/EBP site relative to the NF-κB and Sp1 sites does not appear to be critical, since LTRs retaining either the −169 bp C/EBP site or the −110 bp site showed similar activities (Fig. 5A and 6). It is interesting to note, however, that

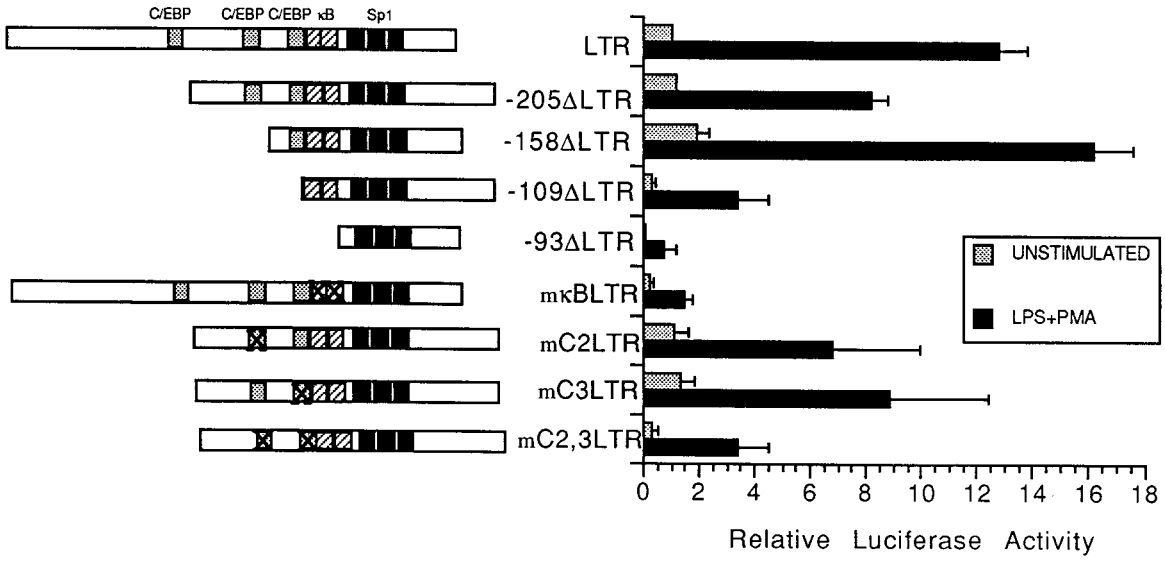

FIG. 6. C/EBP sites are required for efficient transcription of the HIV-1 LTR following LPS-PMA activation but are not necessary for transcriptional induction. U937 cells were transfected with 10 μg of various LTR constructs and were cultured in the absence or presence of 10 μg of LPS and 10 ng of PMA per ml for 24 h. Each bar represents an average of at least three experiments using three or more independent plasmid preparations.

when the sequences of different HIV-1 strains are compared, the −110 bp C/EBP site, which is adjacent to the NF-κB sites, is more highly conserved than the −169 bp site. Thus, it may be that although our transfection experiments did not reveal a functional difference between the two 3′ C/EBP sites in the HIV-1 LTR, in the context of overall viral growth, the −110 bp site is more important.

The region of the HIV-1 LTR from −340 to −158 bp, which contains the two 5′ C/EBP binding sites, has been referred to as the negative regulatory element (19, 21, 31, 49). Results of experiments in which sequences in this region have been deleted or mutated have suggested that this region negatively regulates the LTR in some cell lines (17, 31, 49). Deletion of this region has also been shown to result in more viral replication in T cells and monocytes/macrophages (30). Our data are in agreement with these reports, since deletion of this region produced a modest increase in HIV-1 LTR-driven transcription in U937 cells (Fig. 5A). The experiments of Tesmer et al. suggested that C/EBP sites are involved in this negative regulation in T-cell and hepatocyte lines (58). However, our data indicate that even though two C/EBP sites lie in this region, they are not responsible for negative regulation in the monocytic cell line U937. Inhibiting the function of C/EBP proteins or mutating the −169 bp C/EBP site did not increase HIV-1 LTR activity (Fig. 1, 2, and 5). It is possible that U937 cells, T cells, and hepatocytes have different strategies for both the positive and negative regulation of the HIV-1 LTR, and these different strategies may be reflected in the expression patterns of C/EBP family members (7, 13, 37, 45). We are currently investigating the importance of other sites for negative regulation of the HIV-1 LTR in monocytes.

TABLE 1. Fold induction following different treatments[a]

Construct	Fold induction after treatment with:			
	IL-6	LPS	PMA	LPS + PMA
LTR	5.5 ± 1.3	2.5 ± 0.5	10.3 ± 0.4	12.8 ± 1.2
−205ΔLTR	4.0 ± 1.0	3.2 ± 0.4	14.4 ± 3.0	6.8 ± 0.9
−109ΔLTR	ND	2.9 ± 0.6	7.8 ± 0.1	12.5 ± 4.2
−93ΔLTR	1.6 ± 0.8	0.7 ± 0.3	2.6 ± 0.5	10.4 ± 2.3
mκBLTR	ND	ND	3.0 ± 1.0	6.0 ± 0.5
mC2,3LTR	3.5 ± 0.8	3.0 ± 1.0	8.1 ± 3.2	11.3 ± 3.2

[a] U937 cells were transfected with 10 μg of HIV-1 LTR reporter constructs. Following transfection, cells were cultured in the absence or presence of IL-6 (80 U/ml), LPS (10 μg/ml), PMA (10 ng/ml), or LPS (10 μg/ml) plus PMA (10 ng/ml) for 24 h. The data represent means and standard deviations of at least three independent experiments. ND, not determined.

The basic helix-loop-helix leucine zipper (bHLHZIP) proteins TFE3 and USF have also been shown to bind a site which overlaps the −169 bp C/EBP site (14, 55). Data from our laboratory have shown that the bHLHZIP protein TFE3 and NF-IL6 cannot bind this region simultaneously and that the two proteins compete for binding (2a). However, unlike the expression of NF-IL6, the expression of TFE3 and USF is not induced following monocyte/macrophage differentiation (data not shown). While it is formally possible that changes in the amount of bHLHZIP proteins relative to C/EBP proteins play a role in regulating the HIV-1 LTR, our data suggest that this is unlikely, since mutations which ablate the −169 C/EBP site and the TFE3/USF site (mC2LTR) had no effect on HIV-1 LTR transcription when another C/EBP site was present (Fig. 5 and 6). Thus, our results suggest that bHLHZIP proteins may not be important regulators of the HIV-1 LTR in U937 cells.

Role of C/EBP proteins in the induction of HIV-1 LTR transcription. Our data show that C/EBP proteins and at least one C/EBP site are required for full transcription from the HIV-1 LTR in U937 cells after activation; however, transcription from mutant HIV-1 LTRs lacking C/EBP sites can be induced after treatment with PMA, LPS, IL-6, or LPS plus PMA. We also found that mutant LTRs lacking κB sites could be induced after treatment with LPS plus PMA, although they were poorly or only partially induced upon treatment with individual agents. These results are in agreement with previous studies which have demonstrated that NF-κB is required for full induction of the HIV-1 LTR transcription after cellular activation (22, 35). They are also in agreement with the findings of Sakaguchi et al. (52), who showed that induction of the HIV-1 LTR can occur in the absence of functional NF-κB

sites. The data of Sakaguchi et al. suggested that cellular activation may induce HIV-1 transcription through an alternative pathway by increasing activity of the basal transcriptional machinery (52).

While our data show that an HIV-1 LTR lacking C/EBP sites can be induced, they do not show whether the elevated levels of NF-IL6 which accompany U937 cell activation are important for induced HIV-1 LTR transcription in vivo. It is important to determine the role that elevated NF-IL6 plays in induction of HIV-1 transcription because elevation of NF-IL6 accompanies monocyte/macrophage activation (Fig. 3 and 4) and could be important in overcoming viral latency or increasing HIV-1 replication in macrophages. On the basis of our experiments, we envision three models for the role of NF-IL6 in HIV-1 LTR transcription which are consistent with all of the data.

The first model posits that although C/EBP proteins are required for HIV-1 LTR activity in monocytes/macrophages, basal levels of these proteins are sufficient for mediating induction following cellular activation. In this model, other inducible activators such as NF-κB/rel proteins would be limiting and thus required for the inductive response, whereas increases in the levels of NF-IL6 would have no effect on HIV-1 LTR induction following cellular activation.

A second model is that increased NF-IL6 does induce HIV-1 LTR transcription but can be functionally redundant with other mechanisms such as increases in NF-κB/rel proteins (22, 35) or basal transcription factors (52). Our cotransfection and cell activation assays may not be sufficiently sensitive to distinguish the apparent redundancy of these effects although in vivo each could be important in particular physiological situations, i.e., in response to different combinations of activation signals or different cellular microenvironments. It is interesting that macrophages from mice in which the NF-IL6 gene was disrupted by gene targeting display normal induction for most cytokines (57), consistent with functional redundancy of transcription activators present after cellular activation. In this model, elevated NF-IL6 would be important for HIV-1 induction in some but not all circumstances.

Finally, it is possible that increased concentrations of NF-IL6 are important for HIV-1 LTR induction in vivo but there is an apparent site independence in our assay because of protein-protein associations. These protein-protein associations are likely to be functionally important in vivo as well as in the transfection assay. In transiently transfected cells which have been activated, elevated levels of NF-IL6 and NF-κB/rel proteins may be sufficient to allow their association, even in the absence of their cognate DNA binding sites, with DNA-bound transcription proteins, such as Sp1 or components of the basal transcription machinery, leading to increased HIV-1 LTR transcription. This suggestion is consistent with previous studies showing functional association between NF-IL6 and other transcription activators. NF-IL6 interacts physically with several proteins, including the NF-κB subunits, p50 and p65 (29, 33, 38, 56). Moreover, p65 appears to increase the binding of NF-IL6 to C/EBP binding sites, suggesting that p65 can stabilize NF-IL6 complexes (56). C/EBP proteins or C/EBP–NF-κB complexes may also associate with DNA-bound Sp1. A physical and functional association between NF-κB and Sp1 has been recently reported (40, 41). Thus, in this model, increased levels of NF-IL6 would be important for increased HIV-1 LTR activity in vivo, but protein-protein associations with DNA-bound activators would confer binding site independence in our assay.

In summary, our work establishes a role for C/EBP proteins in HIV-1 LTR transcription in monocytes/macrophages. To determine which of the aforementioned models for how elevated NF-IL6 may act is valid, we are currently altering levels of endogenous NF-IL6 by means other than cellular activation to determine whether such changes affect transcription rates of latent HIV-1. We are also testing the ability of viruses lacking C/EBP sites to be induced.

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