We report the characterization of a CAAT enhancer-binding protein (C/EBP) (NF-IL6) element encompassing the region from −174 to −166 of the U3 long terminal repeat (LTR) region of HIV-1. This C/EBP cis sequence was found to bind to C/EBPβ and C/EBPδ factors in DNA band shift assay. Transfection of Ntera-2 cells with a HIV-1-LTR CAT construct (pC15CAT), together with C/EBPβ or C/EBPδ expression plasmids showed that C/EBP proteins strongly activated the HIV-1 promoter. Deletions encompassing the C/EBP-binding site resulted in the enhancement of the LTR activation mediated by C/EBP proteins, suggesting that other sequences located 3′ to −170 were indeed the target for C/EBP factors. This possibility was confirmed by using the pCD54E9CAT plasmid, in which the NF-κB enhancer was inserted 5′ to the HIV-1 LTR TATA box. A NF-κB1(p50) expression plasmid was also utilized to test for functional co-operation between NF-κB and C/EBP factors. We observed that p50-C/EBPβ and p50-C/EBPδ complexes were generated in tested cells and strongly activated the HIV-1 LTR by binding to the NF-κB sequences. The physical association of NF-κB1(p50) with C/EBP factors was assayed by direct interaction of in vitro translated p50 proteins with C/EBPβ or C/EBPδ produced as glutathione S-transferase fusion proteins. Moreover, p50-C/EBPβ complexes were observed in vivo by using DNA affinity studies with biotinylated NF-κB oligonucleotides. By using mutant forms of p50 or C/EBPβ proteins we found that the transactivation of HIV-1 LTR by p50-C/EBPβ complexes required the DNA-binding domain of p50 and the transcription activation domain of C/EBPβ.

Despite the intensive investigation on the immunopathogenesis of AIDS, many questions concerning the molecular mechanisms of HIV-1 primary infection and progression remain unanswered (1, 2). Recently, the identification of cohorts of HIV-exposed individuals who remain free of infection over a long period of viral exposure (3) as well as the existence of a small subgroup of HIV-1-infected subjects who are long-term non-progressors, were described (4, 5). Together with recent reports on viral life cycle (6, 7), the above evidence argue that HIV infection and disease progression may ultimately result from the levels of viral gene expression. The regulation of HIV-1 gene transcription depends on the recognition of cis regulatory regions in the 5′-long terminal repeat (LTR) by a set of transcription factors which interact with the basal transcriptional complex. These include a TATA box, three Sp1 sites, and a strong enhancer composed of two NF-κB sites (8, 9). In addition, a number of binding sites for transcriptional regulatory proteins have been identified 5′ to the NF-κB enhancer, in the so-called negative regulatory element (NRE) of HIV-1 LTR. The NRE includes binding sites for USF, AP1, NF-AT, and ETS transcription complex, whose activity on HIV-1 gene expression is uncertain (10, 11). Inducible activation of the viral LTR appears to depend principally on the generation of functional NF-κB complex and requires a trans-activating protein, Tat, that interacts with a trans-activating responsive element (12–15). NF-κB defines a family of transcription factors composed by members of the NF-κB/Rel family, namely NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, v-Rel, and c-Rel, which share a sequence homology over a 300 amino acids “rel homology domain” (16). NF-κB proteins form homo- or heterodimers that bind with different affinities to the NF-κB enhancer of HIV-1 (17). NF-κB activation by different stimuli results from proteolytic degradation of IκBα (18), IκBβ (19), and from processing of p105 and p100 precursors to p50 and p52, respectively (19–21), followed by nuclear translocation and DNA binding of NF-κB complexes (17).

Stimuli, such as LPS, UV light, and the cytokines interleukin (IL)-1 (IL-1), IL-6, and tumor necrosis factor-α, that activate NF-κB, are also potent inducers of C/EBP proteins (22–25). The C/EBP family of transcription factors belongs to a class of DNA binding factors named bZIP proteins, which include C/EBPα, C/EBPβ (also termed LAP, NF-IL6α, IL6-DDB, AGBP/EFBP), C/EBPγ (previously defined Ig/EBP-1), and C/EBPδ (NF-IL6β) (22). C/EBPα is mainly involved in the transcription activation of adipose-specific genes of 3T3-L1 preadipocytes (23). C/EBPβ and C/EBPδ are induced in response to inflammatory stimuli.
such as lipopolysaccharide (LPS), IL-1, IL-6, and tumor necrosis factor-α (24, 25). These proteins are all characterized by a leucine zipper domain and by a DNA-binding basic region located in the C-terminal half of the proteins. Members of the C/EBP family can all associate through the leucine zipper domain, and in the case of C/EBPβ and C/EBPδ are phosphorylated (26, 27). In addition, C/EBP factors can associate with members of the NF-xB/Rel family, generating C/EBP-NF-xB complexes which efficiently activate transcription of cellular genes (28, 29). Accordingly, both the C/EBP and NF-xB cis sequences have been identified in the regulatory regions of many genes involved in inflammation and immune regulation (16). In fact, adjacent or overlapping binding sites for NF-xB and C/EBP factors have been identified in the promoter regions of IL-6, IL-8, and angiotensinogen genes (30–32). In these cases, NF-xB and C/EBP factors cooperate in modulating gene expression by binding to the respective cis sequences (33–35). This suggests that formation of C/EBP-NF-xB complexes may represent a common response to selected stimuli, and may also regulate the transcription of viral genes. In support of this possibility, we show in this study that C/EBP-NF-xB heterodimers are generated both in vitro and in vivo, and are potent activators of HIV-1 LTR. A C/EBP cis region was identified in the viral LTR 5′-upstream to the NF-xB enhancer, and functioned as a negative regulatory element, while the NF-xB enhancer was bound and activated by C/EBP/NF-xB complexes. By using mutant forms of C/EBPβ and p50, we identified the domains involved in DNA-binding and in transcription activation.

MATERIALS AND METHODS

Plasmids—The plasmid pC15CAT contains the HIV-1 LTR cloned in the HindIII site of plasmid pSVOCAT (Pharmacia, Uppsala, Sweden) and thus lacking the NRE (negative regulatory element) cis region located in front of the cat gene, and thus lacking the NRE (negative regulatory element) cis region located in front of the cat gene. The plasmid pC15CAT contains the HIV-1 LTR cloned in the HindIII site of plasmid pSVOCAT (Pharmacia, Uppsala, Sweden) and thus lacking the NRE (negative regulatory element) cis region located in front of the cat gene. The pC15CAT plasmid was digested with HindIII and XbaI, and blunt-end ligated to generate the pC15CAT plasmid. The pC15CAT plasmid was digested with HindIII and XbaI, and blunt-end ligated to generate the pC15CAT plasmid.

CAT Assay—Cell extracts were prepared by three cycles of freezing-thawing in 0.25 M Tris, pH 7.8, and CAT assays were performed as described previously (39, 41). Proteins were measured in each cell extract by a protein assay (Bio-Rad) and equal amounts were analyzed for each sample. Each CAT assay contained 20–50 μg of proteins, 20 μl of 4 μM acetyl-coenzyme A (Boehringer Mannheim, Germany), 1 μl (0.5 μCi) of [3H]-chloramphenicol (DuPont NEN) in a final volume of 150 μl of 0.25 M Tris, pH 7.8. Reactions were incubated for 3 h at 37 °C, extracted with ethyl acetate, dried, and spotted on silica gel plates (Polyscrim Sil G; Macherey-Nagel, Duren, Germany). Plates were run in a thin-layer chromatography (TLC) tank containing chloroform:methanol (95:5). After 20 h of autoradiography, the TLC plates were cut and samples were counted in a scintillation counter (LS5000TD; Beckman Instruments, Inc., Palo Alto, CA).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed as described previously (39) for 0.2 μg of proteins, the plasmids pGEX-C/EBPβ and pGEX-C/EBPδ were kindly donated by S. Akira (33). These plasmids express the C/EBPβ wild-type gene, the C/EBPβ gene lacking the DNA binding domain (S288A), or the transcriptional activation domain (ΔSp1). pMT2Tp65, pMT2Tp50, and pMT2Tp50 (59–69), hereafter referred to as p65, p50, and p50 (59–60), carrying the wild-type p65 and p50 DNA or p50 DNA with single base substitutions of the DNA-binding domain, respectively, were previously described (38). The pCD23/XC/EBPβ/CAT plasmids, harboring three copies of the HIV-1 C/EBP binding site identified at −174 to −166 of HIV-1 LTR (5′-AGCATTTGCTCA-3′) in a sense and antisense orientation, were generated by cloning a single copy of a 3X HIV-1 C/EBP oligonucleotide at −65 of pCD52CAT, which contains the HIV-1 LTR sequences from −65 to +80 in front of the cat gene (shown in Table II). The correct sequence was analyzed by direct sequencing (37). To generate the pGEX-C/EBPβ plasmid, the coding region of C/EBPβ was excised from pCD23/XC/EBPβ/CAT plasmids and inserted into compatible sites (SmaI-BamHI) of pGEX-4T3 (Pharmacia, Sweden) using standard techniques (37). The pGEX-C/EBPβ was obtained by inserting the C/EBPβ DNA, excised from pBlue610 (obtained from S. Akira) by SmaI digestion, in compatible sites of pGEX-4T3.

Cells and Transfection—NTERa-2 cells, a human teratocarcinoma cell line, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and were harvested after incubation of 10 min in ice, then subjected to a double electrical pulse (0.2 kV, 960 microfarads) using a Bio-Rad apparatus (Bio-Rad), recovered, and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. After incubation, cells were harvested, washed once with phosphate-buffered saline and collected for CAT assay. The transient expression experiments were performed at least 5 times with different plasmid preparations. Transfection efficiency was monitored by co-transfecting the cells with 5 μl of pNls-LacZ plasmid, β-Galactosidase activity was assayed by using 50 μg of protein extracts as described (38). To obtain nuclear extracts from NTERa-2 cells enriched in NF-xB or C/EBP proteins, cells were transfected with the expression plasmids coding for NF-xB1 (p50) or C/EBP genes. 24 h after transfection the cells were harvested and used for nuclear extracts preparation, as reported (39). Peripheral blood mononuclear cells were isolated by centrifugation over a Ficoll-Hipaque (Sigma, Milan, Italy) density gradient at 400 × g for 30 min, washed twice with phosphate-buffered saline, and resuspended in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Monocytes were isolated from peripheral blood mononuclear cells by centrifugation through a 46% Percoll (Pharmacia, Uppsala, Sweden) density gradient at 2000 × g for 30 min. For isolation of monocytes in Dulbecco's modified Eagle's medium supplemented with 10% FCS containing 1 μl/ml LPS (Sigma) for 24 h.

To produce GST-C/EBPβ and mutants HIV-1/C/EBP M1, 5′-AGCATTTGCTCA-3′; HIV-1/C/EBP M2, 5′-AGCATATTGCTCA-3′; HIV-1/C/EBP M3, 5′-AGCATATTGCTCA-3′; and HIV-1/C/EBP M4, 5′-AGCATTTGCTCA-3′; C/EBP from HIV-1 LTR (from −177 to −164) 5′-AGCATTTGCTCA-3′ and mutants HIV-1/C/EBP M1, 5′-AGCATTTGCTCA-3′; HIV-1/C/EBP M2, 5′-AGCATATTGCTCA-3′; HIV-1/C/EBP M3, 5′-AGCATATTGCTCA-3′; and HIV-1/C/EBP M4, 5′-AGCATTTGCTCA-3′; C/EBP from IL-6 promoter (hereafter referred to as IL-6/C/EBP M5) 5′-GATCGGACGTCACATTGCA-3′, each oligonucleotide was annealed to its complementary strand and end-labeled with [γ-32P]ATP (Amersham Corp.) using polynucleotide kinase (New England Biolabs, Beverly, MA). Equal amounts (5 μg) of cell extracts were incubated in a reaction mixture containing 20 μl of buffer containing 20% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 2 μg of polyclonal antisera (Boehringer Mannheim) for 10 min in ice. Each reaction contained 0.2 ng, 5–8 × 104 cpm) was then added with or without a 25- and 50-fold molar excess of competitor wild-type or mutant oligonucleotides. The reactions were incubated at room temperature for 30 min and run on a 6% acrylamide/bisacrylamide (30:1) gel in 22.5 mM Tris borate, 0.5 mM EDTA. Gels were dried and autoradiographed. To identify the individual proteins present in the complexes, polyclonal antisera against p50, p55, p65, C/EBPβ, and C/EBPδ (Santa Cruz Biotechnology, Santa Cruz, CA) were used in combination with the EMSA. Antisera (5 μg) were incubated with nuclear extracts (5 μg) for 30 min at 4 °C prior to the addition of poly(dI-C)- and 32P-labeled probe as described for the EMSA.

In Vitro C/EBPβ protein interaction—To produce GST-C/EBPβ and GST-C/EBPδ proteins, the plasmids pGEX-C/EBPβ and pGEX-C/EBPδ were kindly donated by S. Akira.
Identification and Characterization of a C/EBP cis Sequence in the HIV-1 LTR—HIV-1 LTR are activated by a variety of stimuli, including LPS, IL-1, IL-6, tumor necrosis factor-α, and UV light (22, 25). These stimuli induce NF-κB, previously bound to streptavidin-conjugated Dynabeads (Dynal, Oslo, Norway) and incubated with 200 μg of nuclear extracts from monocytes stimulated with LPS (1 μg/ml), and 20 μg of poly(dI−dC), in 200 μl of EMSA buffer at room temperature for 90 min with slow agitation. The DNA-protein complexes were washed with EMSA buffer plus 0.5% bovine serum albumin and 0.1% Nonidet P-40, using a magnetic particle concentrator, and were solubilized in SDS-gel sample buffer. The eluted proteins were analyzed on 10% SDS-polyacrylamide gel. Gels were stained with Coomassie blue, dried, and exposed.

DNA Affinity Purification and Immunoblot Analysis—25 ng of biotinylated oligonucleotide corresponding to HIV-1 NF-κB, previously bound to streptavidin-conjugated Dynabeads (Dynal, Oslo, Norway) and incubated with 200 μg of nuclear extracts from monocytes stimulated with LPS (1 μg/ml), and 20 μg of poly(dI−dC), in 200 μl of EMSA buffer at room temperature for 90 min with slow agitation. The DNA-protein complexes were washed three times with EMSA buffer plus 0.5% bovine serum albumin and 0.1% Nonidet P-40, using a magnetic particle concentrator, and were solubilized in SDS-gel sample buffer. The eluted proteins were analyzed on 10% SDS-polyacrylamide gel. Gels were stained with Coomassie blue, dried, and exposed.

Expression of C/EBPβ and C/EBPβ expression plasmids along with a HIV-1 LTR CAT plasmid (pC15-CAT, shown in Fig. 1). As shown in Table I, both C/EBPβ and C/EBPβ activated the LTR-driven expression of CAT and acted cooperatively in activating the HIV-1 LTR. The above results suggested the existence of a C/EBP-responsive sequence in the HIV-1 LTR. A computer assisted analysis of the U3 and R regions of HIV-1 identified a region that matched the C/EBP(NF-IL6) consensus 5′-AC/TTNC-NN(A/C)A-3′. This sequence, ATTTCCGCTCA, located at −174/−166 upstream of the tandem NF-κB cis sequence, was defined as HIV-1 C/EBP (shown in Fig. 1). An oligonucleotide representative of this sequence was tested for C/EBP DNA binding activity in EMSA. As shown in Fig. 2A, nuclear extracts from NTera-2 cells transfected with C/EBPβ expression plasmids strongly bound to HIV-1 C/EBP. The binding was competitively displaced by unlabeled HIV-1 C/EBP oligonucleotide, but not by unlabeled oligonucleotide corresponding to the C/EBP(NF-IL6) cis element of the IL-6 promoter (31). The specificity of the binding was further demonstrated by using mutant oligonucleotides of HIV-1 C/EBP (shown in Fig. 2B). In fact, single base substitutions of the C/EBP oligonucleotides abolished the capability of mutant oligonucleotides to compete with wild-type C/EBP (Fig. 2C). Similar results were seen with extracts obtained from C/EBPβ-transfected NTera-2 cells (not shown). In other experiments, 32P-labeled mutant oligonucleotides were unable to bind to nuclear factors from C/EBP-transfected cells (not shown).
Co-expression of NF-κB1(p50) and C/EBPβ Results in the Increase in HIV-1 C/EBP Binding Activity—C/EBP and NF-κB regulatory sequences are often contiguous in certain promoters, such as the IL-8 promoter (33). The HIV-1 C/EBP elements lie about 60 base pairs from the NF-κB enhancer, thus raising the question of whether C/EBP-NF-κB complexes bind simultaneously to both C/EBP and κB DNA sequences, or whether they alternatively bind to a single regulatory element. These possibilities were tested by transfecting Ntera-2 cells, which express very low levels of endogeneous NF-κB or C/EBP factors, with plasmids expressing NF-κB1 (p50) and C/EBPβ alone or in combination. The results shown in Fig. 3 indicate that the binding to HIV-1 C/EBP was strongly increased by co-expressing C/EBPβ and p50. Moreover, the complexes were supershifted by antibodies to C/EBPβ or to p50, while an antibody to p65 (relA) was ineffective, suggesting that C/EBPβ:p50 complexes were generated in vivo, and bound strongly to HIV-1 C/EBP.

Next, we tested nuclear extracts of Ntera-2 cells transfected with C/EBPβ and p50 for binding to a HIV-1 NF-κB oligonucleotide. These experiments showed that co-expression of p50 together with increasing amounts of C/EBPβ led to a parallel increase in the binding activity to the HIV-1 κB site, suggesting the in vivo generation of increasing amounts of C/EBPβ:p50 complexes (Fig. 4). Altogether, the above results indicate that either C/EBPβ homodimers or C/EBPβ:p50 complexes were able to bind to both HIV-1 C/EBP or NF-κB site (Figs. 3 and 4). Similar results were obtained in the case of C/EBPβ (not shown).

Functional Cooperation of C/EBPβ and NF-κB1 (p50) in Inducing the Transcriptional Activation of HIV-1 LTR—To test whether the C/EBPβ:p50 complexes were functional on HIV-1 LTR, we co-transfected C/EBPβ and/or p50 expressing plasmids together with LTR-CAT plasmids carrying the region of −572/+80 (pC15-CAT), or a truncated region (−117/+80) 5’ to the CAT gene (pCD23, shown in Fig. 1). The plasmid pCD23-CAT lacks the C/EBP cis sequence, while retaining the two κB enhancers and the Sp1 sites. We found that C/EBPβ activated the pC15-CAT plasmid, and significantly cooperated with p50 (Fig. 5). Moreover, a stronger activation was observed when the pCD23-CAT plasmid was used, suggesting that C/EBP homodimers and C/EBPβ:p50 complexes acted on a region located at −117/+80 of HIV-1 LTR (Fig. 5). The data also indicated that the C/EBP region located upstream to the NF-κB enhancer acted as a negative regulator of C/EBP and C/EBP:p50 heterodimers.

To test whether the negative function of HIV-1 C/EBP was depending on its location upstream of the κB enhancer, we generated the pCD52(3XC/EBP)CAT plasmid, where three copies of the HIV-1 C/EBP were positioned at −65 of the viral LTR. As shown in Table II, the pCD52(3XC/EBP)CAT plasmid was responsive to C/EBP factors, indicating that HIV-1 C/EBP is intrinsically functional and behaves as a negative regulatory element only in the context of the HIV-1 LTR.

To test whether C/EBPβ:p50 complexes activated HIV-1 LTR through the κB enhancer, we used the pCD54E9-CAT and pCD54E8-CAT plasmids, in which the two κB sites of the viral LTR were placed 5’ to the TATA box in forward and backward orientation, respectively (shown in Fig. 1). Co-expression of C/EBPβ or p50 showed that both C/EBPβ and C/EBPβ:p50 activated pCD54E9-CAT (Table III). In these experiments, a strong synergism of C/EBPβ and p50 was observed. Moreover, we found that the activation induced by C/EBPβ:p50 was stronger than the one induced by co-transfecting p50- and κB expression plasmids, indicating that C/EBPβ:p50 complexes were efficient activators of κB sites (Table III). Similar results were obtained by using the pCD54E8-CAT plasmid (not shown).

C/EBP Factors Associate In Vitro and Bind to HIV-1 NF-κB—The above results suggested that C/EBP and p50 proteins...
could physically associate to generate transcriptional complexes binding to the HIV-1 \(k_B\) element. To verify this possibility, \textit{in vitro} translated and \([35S]\)Met-labeled p50 proteins were tested for binding to GST, GST-C/EBP \(b\), or GST-C/EBP \(d\) proteins. As shown in Fig. 6A, p50 was selectively eluted from GST-C/EBP \(b\) or GST-C/EBP \(d\) fusion proteins, indicating an \textit{in vivo} physical association of p50 and C/EBP factors.

Next, we tested whether C/EBP \(z\) complexes could also form \textit{in vivo} upon stimulation, and could bind to the HIV-1 \(k_B\) element. Human monocytes were stimulated with LPS, a known inducer of C/EBP and NF-\(\kappa\)B factors (24, 25, 31, 43). After 24 h, nuclear extracts were incubated with biotinylated HIV-1 \(k_B\) oligonucleotides. Proteins binding to the oligonucleotides were eluted and subjected to immunoblotting with antibodies to C/EBP\(b\) or to p50. As shown in Fig. 6B, both C/EBP\(b\) and p50 were recovered from the HIV-1 \(k_B\) oligonucleotide, suggesting the \textit{in vivo} formation of C/EBP\(b\)-p50 complexes. In this experiment we were unable to detect any base-line or LPS-induced C/EBP\(d\) proteins (not shown).

Identification of C/EBP and p50 Domains Involved in Functional Co-operation—To identify the domains of C/EBP and p50 involved in the transcriptional activity of C/EBP:p50 heterodimers, we took advantage of expression plasmids carrying single base pair substitutions or deletions of functional domains in p50 and C/EBP\(b\) factors.

Next, we tested whether C/EBP-p50 complexes could also form \textit{in vivo} upon stimulation, and could bind to the HIV-1 \(k_B\) element. Human monocytes were stimulated with LPS, a known inducer of C/EBP and NF-\(\kappa\)B factors (24, 25, 31, 43). After 24 h, nuclear extracts were incubated with biotinylated HIV-1 \(k_B\) oligonucleotides. Proteins binding to the oligonucleotides were eluted and subjected to immunoblotting with antibodies to C/EBP\(b\) or to p50. As shown in Fig. 6B, both C/EBP\(b\) and p50 were recovered from the HIV-1 \(k_B\) oligonucleotide, suggesting the \textit{in vivo} formation of C/EBP\(b\)-p50 complexes. In this experiment we were unable to detect any base-line or LPS-induced C/EBP\(b\) proteins (not shown).

Identification of C/EBP and p50 Domains Involved in Functional Co-operation—To identify the domains of C/EBP and p50 involved in the transcriptional activity of C/EBP:p50 heterodimers, we took advantage of expression plasmids carrying single base pair substitutions or deletions of functional domains in p50 and C/EBP\(b\). These plasmids were cotransfected with pC15-CAT or pCD23-CAT plasmids (shown in Fig. 1). CAT activities were determined at 48 h post-transfection by using 50 \(\mu\)g of cell extracts.
Regulation of HIV-1 Expression by NF-κB/C/EBP Complexes

**Table II**
Responsiveness of pCD52CAT and pCD52(3XC)/EBP/CAT plasmids to C/EBP and to NF-κB factors

| Target plasmids<sup>a</sup> | Transactivating plasmids<sup>b</sup> | CAT activity<sup>c</sup> | Induction<sup>d</sup> |
|---------------------------|------------------------------------|--------------------------|----------------------|
| pCD52-CAT                | None                               | 0.2                      | 1.0                  |
| pCD52CAT                 | p50                                | 0.2                      | 1.0                  |
| pCD52CAT                 | pC/EBPβ                            | 0.3                      | 1.5                  |
| pCD52CAT                 | pC/EBPβ                            | 0.3                      | 1.5                  |
| pCD52CAT                 | pC/EBPβ + pC/EBPβ                   | 0.3                      | 1.5                  |
| pCD52CAT                 | pC/EBPβ + p50                       | 0.2                      | 1.0                  |
| pCD52(3XC)/EBP/CAT       | None                               | 0.5                      | 1.0                  |
| pCD52(3XC)/EBP/CAT       | p50                                | 0.6                      | 1.2                  |
| pCD52(3XC)/EBP/CAT       | pC/EBPβ                            | 2.5                      | 5.0                  |
| pCD52(3XC)/EBP/CAT       | pC/EBPβ                            | 4.0                      | 8.0                  |
| pCD52(3XC)/EBP/CAT       | pC/EBPβ + p50                       | 6.7                      | 13.4                 |
| pCD52(3XC)/EBP/CAT       | pC/EBPβ + p50                       | 7.2                      | 14.4                 |
| pCD52(3XC)/EBP/CAT       | pC/EBPβ + p50                       | 10.7                     | 21.4                 |

<sup>a</sup> Ntera-2 cells were transfected with 5 μg of the target plasmids alone or together with 10 μg of p50 and 2 μg of C/EBPβ and C/EBPβ expressing plasmids. Target plasmids are derivatives of the wild type HIV-1 LTR (pC15CAT). pCD52CAT carries the region of –65 to +80 bp of HIV-1 LTR inserted upstream of the cat gene. In the pCD52(3XC)/EBP/CAT plasmid a double strand oligonucleotide corresponding to three copies of the HIV-1 C/EBP is positioned upstream of the HIV-1 LTR sequence of pCD52CAT.

<sup>b</sup> Determined by scintillation counting of unacylated and acetylated spots.

<sup>c</sup> Fold induction of chloramphenicol acetylation induced by the transactivating plasmids is expressed as the ratio of percentages acetylated. The data are representative of four independent experiments.

**Table III**
Responsiveness of pC15CAT, pCD54CAT, and pCD54E9CAT plasmids to C/EBP and to NF-κB factors

| Target plasmids<sup>a</sup> | Transactivating plasmids<sup>b</sup> | CAT activity<sup>c</sup> | Induction<sup>d</sup> |
|---------------------------|------------------------------------|--------------------------|----------------------|
| pC15CAT                   | None                               | 0.4                      | 1.0                  |
| pC15CAT                   | p50                                | 0.5                      | 1.25                 |
| pC15CAT                   | pC/EBPβ                            | 4.0                      | 10.0                 |
| pC15CAT                   | pC/EBPβ                            | 9.0                      | 22.5                 |
| pC15CAT                   | pC/EBPβ + p50                       | 10.0                     | 25.0                 |
| pC15CAT                   | pC/EBPβ + p50                       | 16.8                     | 42.0                 |
| pC15CAT                   | p50 + p65                           | 5.2                      | 13.0                 |
| pCD4E9CAT                 | None                               | 0.4                      | 1.0                  |
| pCD4E9CAT                 | p50                                | 0.3                      | 0.7                  |
| pCD4E9CAT                 | pC/EBPβ                            | 1.5                      | 3.7                  |
| pCD4E9CAT                 | pC/EBPβ                            | 1.6                      | 4.0                  |
| pCD4E9CAT                 | pC/EBPβ + p50                       | 3.8                      | 9.5                  |
| pCD4E9CAT                 | pC/EBPβ + p50                       | 4.6                      | 16.5                 |
| pCD4E9CAT                 | p50 + p65                           | 2.5                      | 6.2                  |
| pC54CAT                   | None                               | 0.4                      | 1.0                  |
| pC54CAT                   | p50                                | 0.2                      | 0.5                  |
| pC54CAT                   | pC/EBPβ                            | 0.6                      | 1.5                  |
| pC54CAT                   | pC/EBPβ                            | 0.6                      | 1.5                  |
| pC54CAT                   | pC/EBPβ + p50                       | 0.8                      | 2.0                  |
| pC54CAT                   | pC/EBPβ + p65                       | 1.2                      | 1.2                  |

<sup>a</sup> Ntera-2 cells were transfected with 5 μg of the target plasmids alone or together with 2 μg of p50 and p65 and 10 μg of C/EBPβ and C/EBPβ. Target plasmids are derivatives of the wild type HIV-1 LTR (pC15CAT). pCD54CAT carries the TATA box and the trans-activating responsive element region of HIV-1 LTR inserted upstream of the cat gene. In the pCD54E9CAT plasmid the HIV-1 NF-κB enhancer is positioned upstream of the TATA in place of the Sp1 sites (shown in Fig. 1).

<sup>b</sup> Determined from scintillation counting of unacetylated and acetylated spots.

<sup>c</sup> Fold induction of chloramphenicol acetylation induced by the transactivating plasmids is expressed as the ratio of percentages acetylated. The data are representative of four independent experiments.

Driven transcription of CAT. To verify this possibility, a plasmid carrying mutations at codons 59–60 of p50 (p50(59–60)), and therefore expressing a mutant form of p50 lacking a functional DNA-binding domain (36), was used in combination with pC/EBPβ plasmid in transient expression experiments. As shown in Fig. 7B, p50(59–60) did not act as an activator of the HIV-1 LTR when co-expressed with pC/EBPβ (lane c). Moreover, increasing amounts of p50(59–60) down-regulated the transcriptional activity of C/EBPβ/p50 complexes (lanes f–h), suggesting the in vivo generation of inactive C/EBPβ/p50(59–60) complexes.

**Discussion**

HIV-1 is the etiologic agent for AIDS and causes various clinical and immunological abnormalities, including activation of polyclonal B cells that manifests as hypergammaglobulinemia and auto-antibody production, lymphadenopathy, Kaposi's sarcoma, and lymphoma of the B-cell phenotype (46–48). Studies on small cohorts of subjects exposed to HIV-1 and who do not develop HIV-1 infection, and individuals who harbor HIV-1 but remain disease free for long periods (49, 50), strongly suggest that the development of AIDS may depend on a dynamic interplay between viral and host gene products, such as EBV-EBNA2 and LPS, which can activate the HIV-1 LTR (39, 40), while inflammatory stimuli, including the cytokines IL-1, IL-6, and tumor necrosis factor-α, enhance the expression of HIV-1 genes by inducing active NF-κB complexes (16, 43). These stimuli can also activate C/EBP proteins binding to cognate cis sequences found in the regulatory regions of a variety of cellular and viral genes (34–36). Recent evidence indicate that members of the NF-κB and C/EBP families of transcription factors can physically associate, generating active transcriptional complexes (28, 29). In fact, both NF-κB and C/EBP sites are present in the promoters of cellular genes such as IL-6, IL-8, serum amyloid A, and angiotensinogen (31–33, 35, 44, 45), suggesting that cooperative activation by C/EBP-NF-κB complexes could represent a substantial way of achieving high gene expression. In support of this possibility, we have shown here that C/EBPβ/p50 and C/EBPβ/p65 complexes are generated in LPS-stimulated monocytes and in p50- and C/EBP-transfected Ntera-2 cells, and act as potent activators of HIV-1 LTR driven gene expression. This activity was consistently stronger than the one induced by p50/p65 complexes. We found that C/EBPβ/p50 complexes acted on the NF-κB enhancer by utilizing the DNA-binding domain of p50 and the transcription activation domain of C/EBPβ. In these experiments, the mutant form of p50 functioned as negative trans-dominant of the activity of C/EBPβ/p50, suggesting that it could down-regulate the expression of HIV-1 genes. The sequence matching the C/EBP consensus was found at position −174/−166, upstream of the κB enhancer and immediately downstream of the so-called NRE of HIV-1 LTR. Functional characterization of this HIV-1 C/EBP revealed that, although it efficiently bound C/EBP homodimers and C/EBP/p50 complexes, its deletion led to an up-regulation of the transcription activity exerted by C/EBP and C/EBP/p50 complexes. This indicates that the HIV-1 C/EBP element functions as a negative regulatory region by possibly squelching C/EBP and C/EBP/p50 transcription complexes. This activity may be due to the relative
distance of C/EBP site to the TATA box and to the NF-κB enhancer, since positioning of the C/EBP site 5' to the HIV-1 LTR basal promoter restored its enhancer function (Table II). It is noteworthy that C/EBP factors distort DNA upon binding, but they do not introduce a large DNA bending (51). This could make the bound complexes incapable of interacting with the transcription machinery.

The HIV-1 C/EBP overlaps with a consensus (E box) which is a binding site for proteins of the B class of basic-helix-loop-helix-leucine zipper (b-HLH-Zip) family (52). Members of this family include c-Myc, Max, Mad, TFE3, TFE6, and Mxi1 (53–58), which form homo- and hetero-multimers, and are potentially able to associate with C/EBP or NF-κB factors. The role of these factors in the regulation of HIV-1 LTR is, however, uncertain. Recently, the b-HLH-Zip USF protein has been shown to function as a positive regulator of LTR-driven transcription (59). The USF consensus is located at –173 to –157, and overlaps with HIV-1 C/EBP site. As USF is an efficient DNA-bending factor, the positive effects of USF on LTR function could be due to a different DNA bending which could bring USF close to the TATA box of HIV-1 LTR. The NRE of LTR also harbors a variety of cis sequence, such as Ets, AP1, NFAT1, LEF/TCF1a, and nuclear receptor responsive elements (10, 11). The role of these factors in HIV-1 gene expression is, however, unclear in cell culture systems, while they may play a role in the transcription of integrated proviruses, since their binding sequences are conserved in HIV-1 isolates of AIDS patients.
Regulation of HIV-1 Expression by NF-κB/C/EBP Complexes

ACKNOWLEDGMENTS—We thank G. Ciliberto and S. Akira for providing the pH2-CEBPz and pEF-NF-kB6a plasmids, respectively. We are grateful to U. Siebenlist for critical discussions and A. Wilchoks for editorial work.

REFERENCES

1. Pantaleo, G., Graziosi, C., and Fauci, A. S. (1993) N. Engl. J. Med. 328, 327–335
2. Paul, W. E. (1995) Cell 82, 177–182
3. Salk, J., Bretscher, P. A., Salk, P. M., Clerici, M., and Shearer, G. M. (1993) Nature 360, 1270–1272
4. Pantaleo, G., Menzo, S., Vaccarezza, M., Graziosi, C., Cohen, O. J., Demarest, W. A., (1990) Science 247, 223–232
5. Cao, Y., Qin, L., Zhang, L., Safrit, J., and Ho, D. D. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 21128–21133
6. Wey, X., Ghosh, S. K., Taylor, M. E., Johnson, V. A., Emini, E. A., Deutsch, P., Friedman, A. D., Nakabeppu, Y., Kelly, T. J., and Lane, M. D. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 288–293
7. Sambrook, J., Frolich, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
8. Scalia, G., Quinto, I., Ruocco, M. R., Arcucci, A., Mallardo, M., Caretto, F., Perico, G., and Venuta, S. (1990) J. Exp. Med. 172, 61–68
9. Scalia, G., Quinto, I., Ruocco, M. R., Mallardo, M., Ambrosio, C., Squitiere, B., Tassone, P., and Venuta, S. (1993) J. Virol. 67, 2583–2586
10. Pzhakcius, K. J., Ferg, M. B., Trono, D., and Baltimore, D. (1990) J. Exp. Med. 172, 253–261
11. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051
12. Scalia, G., Ruocco, M. R., Ambrosio, M., Mallardo, M., Giordano, V., Baldassarre, F., Dragonetti, E., Quinto, I., and Venuta, S. (1994) J. Exp. Med. 179, 961–971
13. Oserbin, L., Kunkel, S., and Nabel, G. J. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 8202–8206
14. Li, X., and Liao, W. S.-L. (1991) J. Biol. Chem. 266, 15192–15202
15. Ray, A., Hannink, M., and Ray, B. K. (1985) J. Biol. Chem. 260, 7365–7374
16. Fauci, A. S., Mach, A. M., Longo, D. L., Lane, H. C., Rock, A. H., Masur, H., and Gelman, E. P. (1984) Ann. Int. Med. 100, 92–103
17. Beral, V., Peterman, A., Berrkelman, R. L., and Jaffe, H. W. (1990) Lancet 335, 123–128
18. Levine, A. M. (1992) Blood 80, 8–15
19. Nowak, M. A., Anderson, R. M., McLean, A. R., Wolfs, T. F., Goudsmit, J., and May, R. M. (1991) Science 254, 963–969
20. Biggar, R. J. (1990) AIDS 4, 1059–1065
21. Avital, N., and Calame, K. (1994) J. Biol. Chem. 269, 23553–23562
22. Dang, C. V., Dolce, C., Gillison, M. C., and Kato, G. J. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 599–602
23. Murre, C., McCaw, P. S., Vaessen, H., Caudy, M., Jan, Y. N., Baldassarre, F., Dragonetti, E., Quinto, I., and Venuta, S. (1994) J. Exp. Med. 179, 961–971
Regulation of HIV-1 Long Terminal Repeats by Interaction of C/EBP(NF-IL6) and NF-κB/Rel Transcription Factors

Maria Rosaria Ruocco, Xueni Chen, Concetta Ambrosino, Emila Dragonetti, Weimin Liu, Massimo Mallardo, Giulia De Falco, Camillo Palmieri, Guido Franzoso, Ileana Quinto, Salvatore Venuta and Giuseppe Scala

J. Biol. Chem. 1996, 271:22479-22486.
doi: 10.1074/jbc.271.37.22479

Access the most updated version of this article at http://www.jbc.org/content/271/37/22479

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 62 references, 35 of which can be accessed free at http://www.jbc.org/content/271/37/22479.full.html#ref-list-1