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Association of p65 and C/EBPβ With HIV-1 LTR Modulates Transcription of the Viral Promoter

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Abstract In human immunodeficiency virus type 1 (HIV-1) latently infected cells, NF-kappaB (NF-κB) plays a critical role in the transcriptional induction of the HIV-1 promoter. The *trans*-activating ability of NF-κB can be modified by another nuclear factor C/EBPβ that can physically bind to NF-κB and regulate its activity. Because the HIV-1 promoter also contains a C/EBPβ site adjacent to the NF-κB site, the present study examined cooperative functional in vivo interaction of the p65 subunit of NF-κB and C/EBPβ, and the impact of Tat in this event. We demonstrated that ectopic expression of p65 along with Tat increases p65 binding to HIV-1 LTR, and that this increase correlates with enhanced HIV-1 promoter activity. Further, co-expression of C/EBPβ and Tat leads to a decrease in p65 binding, which allows C/EBPβ to bind more efficiently to the LTR. Inhibition of p65 expression by siRNA significantly decreases C/EBPβ-binding and LTR expression. Using ChIP assay, we confirmed the existence of an interchange between p65 and C/EBPβ and their abilities to bind to the LTR in vivo. These observations demonstrate that a delicate balance of interaction between p65, C/EBPβ, and Tat can dictate the level of HIV-1 LTR transcription. J. Cell. Biochem. 100: 1210–1216, 2007.

Key words: NF-κB; transcription; HIV-1; C/EBPβ

The human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) serves as a common point for regulation by many cellular and viral proteins such as NF-κB, Sp1, C/EBPβ [Pereira et al., 2000; Coyle-Rink et al., 2002]. NF-κB is an inducible cellular transcription factor that regulates expression of a wide variety of cellular and viral genes, including that of HIV [Nabel and Baltimore, 1987; West et al., 2001; Muthumani et al., 2003]. In cells, NF-κB, a hetero- or homodimer consisting of the Rel family proteins p65 (RelA), RelB, c-Rel, p50/p105, and p52/p100, resides in the cytoplasm and is complexed with an inhibitory molecule, IκB [Phelps et al., 2000; Ghosh and Karin, 2002]. Phosphorylation targets IκBα for its ubiquitination and degradation by the β-transducin repeat-containing protein ubiquitin ligase and 26S proteasome, respectively, thus allowing free NF-κB to translocate to the nucleus to activate gene expression [Karin and Ben-Neriah, 2000; Trubiani et al., 2000]. The role of NF-κB in activating HIV transcription has been extensively studied. HIV-1 replication is positively regulated by several cytokines, or T-cell activators, most of which act either completely or partially via NF-κB. NF-κB has been shown to regulate viral transcription via the two NF-κB sites located in the HIV-1 LTR enhancer region [West et al., 2001]. This effect has been shown to be further enhanced through synergism with Sp1 [Chun et al., 1998; Mischitelli et al., 2005]. In HIV-1 latently infected cells, activation of NF-κB could trigger the transcription of viral genes, including the transactivator Tat, resulting in an explosive increase in HIV replication [Demarchi et al., 1996]. Treatment with compounds that block

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NF-κB activation results in inhibition of HIV-1 gene expression and viral replication [Pande and Ramos, 2003].

The CAAT/enhancer binding protein (C/EBPs) family of transcription factors contains six members (α, β, γ, δ, ε, and, ζ) and is characterized by the presence of a COOH-terminal basic DNA binding domain and a leucine zipper domain that promotes homo- and heterodimerization of the different family members [Cassel and Nord, 2003; Barber et al., 2004]. These latter factors, which are induced by several cytokines, play key roles in numerous cellular responses, including immune and inflammatory processes, by regulating cytokine and acute phase response genes [Cassel and Nord, 2003]. Among their many functions, the C/EBP family of proteins is also involved in the regulation of cellular and viral gene expression (e.g., HIV-1 LTR) [Schwartz et al., 2000]. In this regard, C/EBPβ was shown to interact with HIV-1 LTR DNA through two binding sites known as C/EBP sites I and II [Tesmer et al., 1993].

Thus, the presence of putative binding sites for both NF-κB and C/EBPβ in the 5′-flanking region of the *HIV-1* gene prompted us to assess their role in the regulation of viral transcription, as well as their mutual effect on each other's binding capabilities in vivo.

MATERIALS AND METHODS

Plasmids

HIV-1 LTR-CAT reporter plasmid, CMV-Tat (86 aa), CMV-p65, and CMV-C/EBPβ plasmids were described previously [Amini et al., 2002; Abraham et al., 2005].

Cell Culture, Transfection, and CAT Assays

The HL3T1 HeLa-derived cell line contains stably integrated, silent copies of the HIV-1 LTR promoter linked to the *CAT* gene. Cells were transfected with expression vector using the lipofectamine transfection reagent according to the manufacturer's protocol (Roche, Indianapolis, IN). To control the concentration of the promoter DNA sequence in transfection, $pcDNA_3$ empty plasmid was added to the transfection mixture. Each transfection was repeated a minimum of three separate times with at least three different plasmid preparations. Cell extracts were prepared 48 h after transfection, and CAT assays were performed as previously described [Sawaya et al., 1998; Coyle-Rink et al., 2002].

RNA Interference

Transient knockdown of p65 was performed with p65-specific siRNA: 5′-GCCCUAUCCCUUUACGUCAdTdT (Dharmacon Research, Inc., Lafayette, CO). After 24 h of plating, cells were rinsed once with Optimem. siRNA was added at a final concentration of 50 nm by the method of Surabhi and Gaynor [2002]. Western blot analysis of protein extracts from untransfected cells or cells transfected with p65-specific siRNA using anti-p65 was performed. For loading controls, anti-Grb2 antibody was used. Control non-targeting siRNA was also obtained from Dharmacon.

Chromatin Immunoprecipitation (ChIP) Assay

HL3T1 cells were grown overnight in 100-mm dishes to 60%–70% confluency. Cells were then transfected with 1 μg of CMV-Tat, CMV-p65, and/or CMV-C/EBPβ expression plasmids using various combinations, using a lipofectamine transfection reagent (Roche Applied Sciences). Cells were incubated for 40–48 h and then cross-linked with formaldehyde, harvested, and ChIP was performed. For these studies, only 5×10^6 cells were used per immunoprecipitation reaction since the plasmid is present at a high copy number. The remainder of the procedure followed standard protocols for ChIP analysis, as has been described in Upstate protocol. The resulting DNA was analyzed by PCR reactions using the following HIV-LTR primers: (sense)(−120) 5′ aactggtaccatcgagcttgct 3′ and (anti-sense) (+66) 5′ ttgaggatccagcagtgggttc 3′. Antibody used in the ChIP procedure is p65 or C/EBP((from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as well as rabbit anti-mouse IgG.

The specificity of the bands obtained by PCR was analyzed using Southern blot hybridization. Briefly, PCR products were isolated from agarose gels and transferred to a nylon membrane (Amersham, Piscataway, NJ) using a Turbo-blotter system (Schleicher & Schuell, Keene, NH) according to the manufacturer′s instructions. The DNA probes were then labeled using a random primer labeling reaction with Klenow enzyme and α^{32}P dCTP as previously described [Sweet et al., 2003]. The primers used

for PCR are as follow: (−80) GGGAGGTACCA-GGGAGGCGTGG and (+3) CAGAGGATCC-CAGTACA GGCAA from LTR region.

RESULTS AND DISCUSSION

Earlier observations on cell free interaction of p65 and C/EBPβ with HIV-1 LTR DNA prompted us to examine whether this event can also occur in living cells where either protein is overexpressed. Further, to assess the possible cooperative or antagonistic effect of p65 and C/EBPβ on each other's DNA binding activity, we transfected HL3T1, a cell line that contains integrated DNA segment of the HIV-1 LTR fused to the *CAT* gene (Fig. 1A) with p65 and/or C/EBPβ expression plasmids. Forty-eight hours post-transfection, the cells were harvested and processed for chromatin immunoprecipitation (ChIP) assay. To examine the basal binding of p65 and/or C/EBPβ, untransfected cells were also used in the study. ChIP assay was performed using antibodies directed against p65 protein as well as rabbit anti-mouse serum as a negative control. As shown in Figure 1B, endogenous as well as ectopically expressed p65 bind to HIV-LTR DNA (lanes 3 and 6), and as expected, an increase in the level of p65–DNA complex was observed when p65 was ectopically expressed (compare lanes 3 and 6). The p65-DNA interaction was noticeably elevated in cells co-expressing p65 and C/EBPβ (lane 12) but not in cells with only C/EBPβ overexpression (lane 9). No DNA–protein complexes were observed when the control serum was used (lanes 2, 5, 8, and 11).

In a reciprocal study, we utilized antibody directed against C/EBPβ in ChIP assay. Again, rabbit anti-mouse serum was used as a negative control. As shown in Figure 1C, endogenous and over-expressed C/EBPβ proteins bind to HIV-LTR DNA with the same intensity, suggesting that C/EBPβ may occupy the promoter of the LTR (lanes 3 and 6). A slight decrease of C/EBPβ–DNA interaction was observed when the cells were co-transfected with C/EBPβ and p65 (compare lane 12 to lanes 3 and 6) but not with p65 alone (compare lanes 9 and 12). No interaction was observed when the non-specific control serum was used in the precipitation steps (lanes 2, 5, 8, and 11).

Next we sought to assess the effect of Tat on the binding of p65 or C/EBPβ to DNA. To that end, HL3T1 cells were transfected with Tat, p65, and C/EBPβ expression plasmids using various combinations. As shown in Figure 2A,

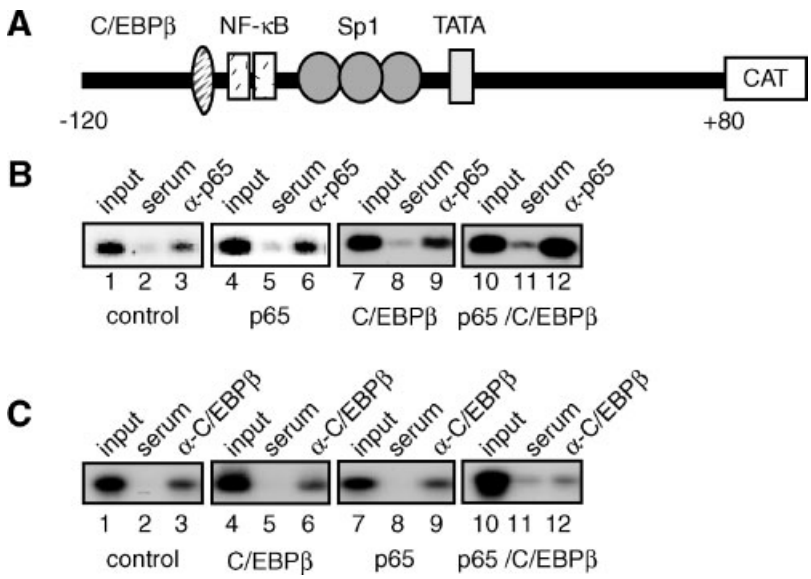

Fig. 1. Interaction of p65 and C/EBPβ with the LTR. **A**: Schematic representation of the HIV-1 LTR (−120/+80). The potential binding sites of several DNA-binding transcription factors are also indicated. **B** and **C**: HL3T1 cells were transfected with 1.0 μg of p65 (**Panel B**, **lanes 4–6** and 1**0–12**, and **Panel C**, **lanes 7–12**) or C/EBPβ (Panel C, lanes 4–6 and 10–12, and Panel B, lanes 7–12) expression plasmid as indicated. Interaction of p65 or C/EBPβ with HIV-LTR was demonstrated by ChIP assay. The primers used in these experiments are described in Materials and Methods. Anti-p65 (Panels B, **lanes 3**, 6, 9, and 12), anti-C/EBPβ (Panel C, lanes 3, 6, 9, and 12), pre-immune serum (Panels B and C, **lanes 2**, 5, 8, and 11), and no antibody (**lanes 1**, 4, 7, and 10) were used in these experiments.

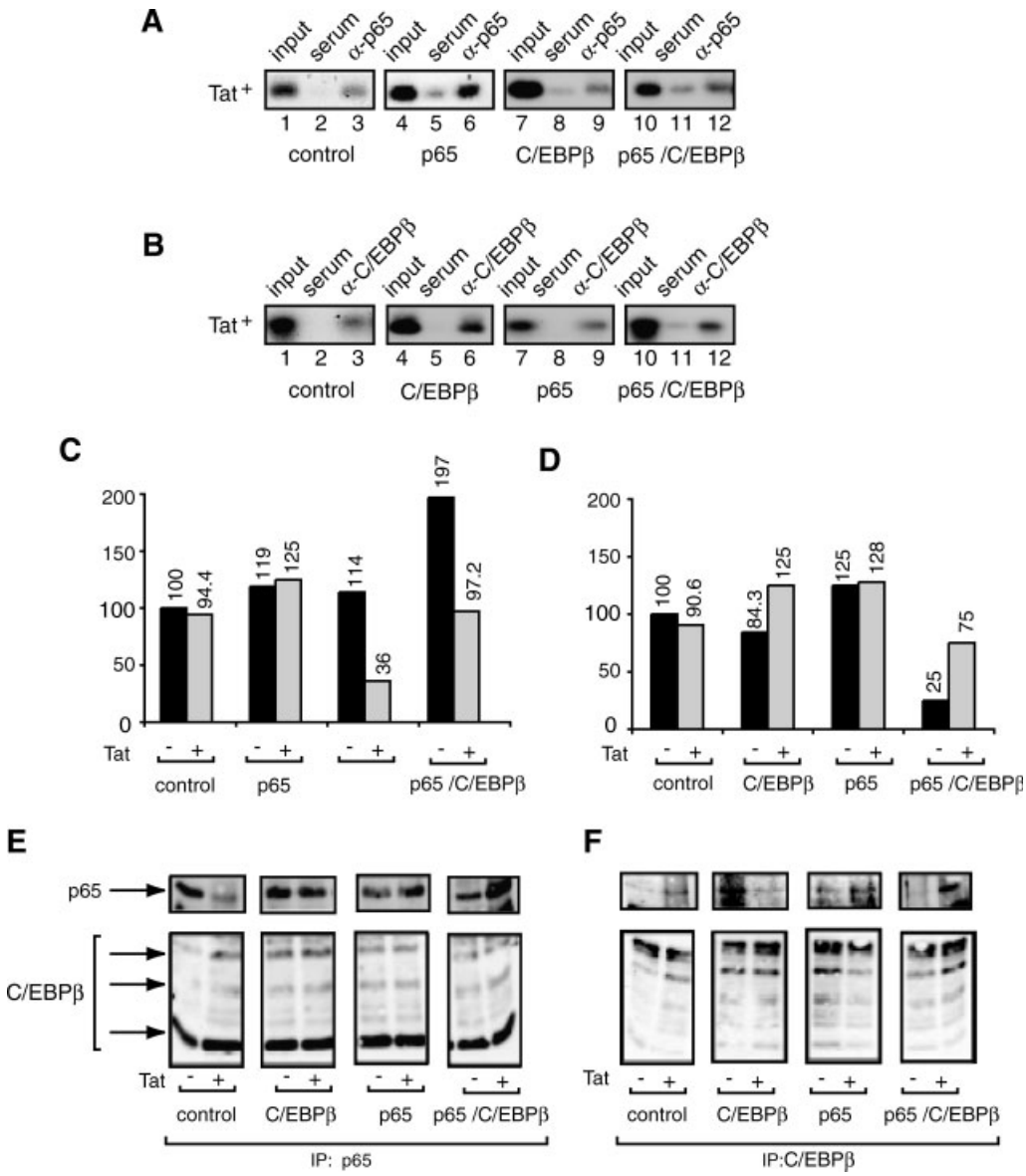

Fig. 2. Effect of Tat on in vivo association of p65 and C/EBPβ with LTR. **A** and **B**: HL3T1 cells were transfected with HIV-1 Tat, p65, or C/EBPβ expression plasmids as indicated. Interaction of p65 or C/EBPβ with HIV-LTR in the presence of Tat was demonstrated by ChIP assay. Anti-p65 or anti-C/EBPβ antibodies, pre-immune serum, and no antibody were used in these experiments. **C** and **D**: Histogram representing the values of each band obtained from ChIP using densitometry. The values are based on the arbitrary units of 1–200 and the baseline derived from control without Tat was set at 100. **E** and **F**: Western blot analysis using anti-p65 and anti-CEBPβ antibodies demonstrated the specificity of the antibodies. Arrows indicate the positions of p65 and the various C/EBPβ isoforms.

endogenous and ectopic p65 were able to associate with LTR DNA (lanes 3 and 6) in the presence of Tat with a similar efficiency as seen in the absence of Tat (compare Fig. 1B, lanes 3 and 6 to Fig. 2A, lanes 3 and 6). Co-expression of Tat and C/EBPβ slightly decreased p65–DNA interaction (compare lanes 3 and 9) (also compare Fig. 1B, lanes 3 and 9). Interestingly, in the presence of Tat, cells with ectopic expression of C/EBPβ and p65 showed a significant decrease in the binding of p65 to LTR (compare lanes 12 Fig. 2A) (also compare lanes 12 in Fig. 1B). In this regard, we should add that we previously demonstrated the ability of Tat to associate with C/EBPβ [Abraham et al., 2005]. Therefore, the observed decrease could be

explained by the formation of a Tat–C/EBPβ complex, which leads to an inhibition of p65–DNA complex formation and suppression of p65 transcriptional activity. No interaction was observed when the, non-specific control, serum was used (lanes 2, 5, 8, and 11).

When ChIP assay was performed using anti-C/EBPβ antibody, C/EBPβ-DNA association showed no change in cells expressing Tat or Tat plus p65 (Fig. 2B, compare lanes 3 and 9). Surprisingly, C/EBPβ–DNA interaction showed an elevation in cells expressing Tat and C/EBPβ (compare lanes 3 and 6). Differences in the levels of interaction of p65 or C/EBPβ to the DNA were further evaluated by densitometric analysis using Scion image software (Scion Image Corp) (Fig. 2, panels C and D). The values represent the percentage of variation in the intensity of p65-DNA or C/EBPβ–DNA complexes. Similar results were obtained with Jurkat cells, which led us to conclude that the events may also occur in T-cells (data not shown). It should be noted that the specificity of the antibodies was examined using Western blot analysis using anti-p65 and anti-CEBPβ as shown in Figure 2 (panels E and F).

Next, we sought to determine the functional interplay of p65 and C/EBPβ upon LTR transcription in Tat-producing HL3T1 cells. As anticipated, Tat-enhanced HIV-1 promoter activity in HL3T1 cells (Fig. 3, lane 2). Expression of p65 and C/EBPβ enhanced, albeit to various extents, Tat activation of the LTR, respectively (compare lane 2 to lanes 4 and 6). Interestingly, C/EBPβ decreased the level of p65 and Tat cooperativity as evidenced by the decrease in the level of LTR transcription (compare lane 4 to lane 8). Lanes 3, 5, and 7 represent the basal levels of p65 and/or C/EBPβ on HIV-1 LTR.

To further confirm the significance of p65-DNA association and p65-C/EBPβ functional interplay, we knocked down p65 expression using small interference RNA directed against p65 (siRNA-p65). As shown in Figure 4A, p65 was silenced in extracts prepared from siRNA-transfected cells (compare lanes 1 and 3). Note that control non-targeting siRNA was used as a negative control (lane 2). Results from ChIP assay showed that a decrease in the level of p65 can negatively impact on p65 as well as C/EBPβ association with LTR, and that this event can occur in the absence and presence of Tat.

In this report, we demonstrate the functional association of members of two important transcription factor families that include p65 subunit of NF-κB and C/EBPβ of C/EBP with each other and their impact on HIV-1 LTR transcription. The functional association is characterized by (i) the inhibition of p65-induced expression of HIV-1 LTR by C/EBPβ and (ii) the enhancement of p65 association with the DNA in the presence of C/EBPβ. This functional interplay is more evident in the presence of HIV-1 Tat.

The functional interaction between members of the two families is not without precedent. Earlier studies have shown existence of a

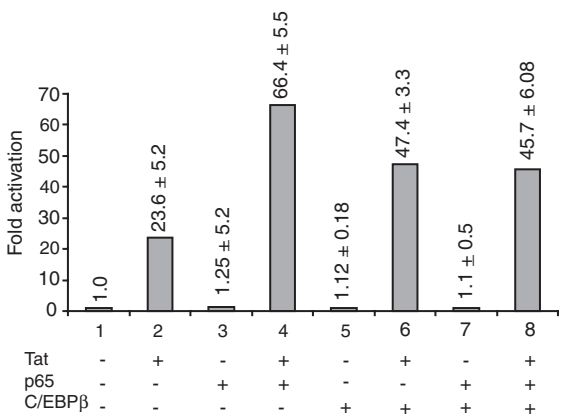

Fig. 3. Transcription regulation of HIV-1 promoter by p65 and C/EBPβ in the presence of Tat. HL3T1 cells were transfected with 1.0 μg of Tat, p65, or C/EBPβ expression plasmids, as indicated. Total amount of transfected DNA was kept constant by the addition of empty control vector. The data represent the mean value of at least three separate transfection experiments. Cell extracts were prepared 48 h after transfection and CAT activity was determined as described [Coyle-Rink et al., 2002].

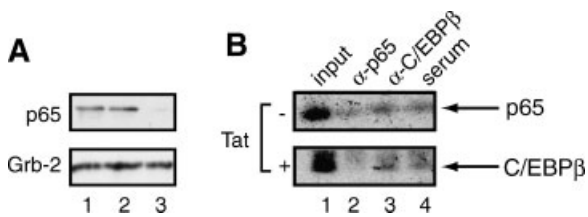

Fig. 4. Effect of p65 on interaction of C/EBPβ with the LTR in the absence and presence of Tat. **A**: HL3T1 cells were transfected with siRNA-p65 (5′-GCCCUAUCCCUUUACGUCAdTdT; Dharmacon Research, Inc., Lafayette, CO). Approximately, 50 μg of extract were utilized in Western blot assay utilizing anti-p65 or anti-Grb2 antibodies, respectively. Cells transfected only with pcDNA3 were utilized as negative controls. The arrows depict the positions of the different proteins. **B**: ChIP assay was performed to evaluate p65 and C/EBPβ association with the DNA after 48 h of siRNA-p65 transfection in the presence or absence of Tat.

physical interaction between the various members of NF-κB, (i.e., p50, p65, and c-Rel) through the Rel homology domain with the bZIP region of C/EBP (α, β, and δ) [Stein et al., 1993]. The nature of the molecular complex (i.e., domains of interaction, heterodimer formation) formed between p65 and C/EBPβ remains unknown. Previous studies have shown that a mutation in the putative p65 leucine zipper domain does not block the functional or physical interaction between p65 and C/EBPβ. On the contrary, an alternatively spliced form of p65 fails to interact with C/EBPβ subunits [Stein et al., 1993]. These observations suggest that the same motif that is necessary for dimerization of NF-κB may also be involved in its cross-interaction with C/EBPβ. Further, earlier reports suggest that a single C/EBP binding site may be sufficient to serve as a target for its cooperativity with NF-κB subunits [Cha-Molstad et al., 2000]. Based on this information, one may envision a model in which a dimer of a C/EBP family member interacts with the DNA motif and associates through its bZIP region with the Rel homology region of NF-κB family member. In this regard, and based on our results, the functional interplay between p65 and C/EBPβ is more obvious as shown in Figures 1 and 4, where we show that C/EBPβ interacts more efficiently with the DNA in the presence of p65 then in its absence. These results could be explained by the dependency of C/EBPβ in the presence of p65 in order to bind and activate the HIV-LTR. Further, these results may also point to a potential role for p65 in the stabilization of C/EBPβ, which may explain the observed data.

Functional interaction of NF-κB and C/EBP may also explain IL-1 and IL-6 cooperativity. IL-1 is known to induce NF-κB [Miyazawa et al., 1998], whereas IL-6 activates C/EBPβ [Xia et al., 1997]. The physical and functional interactions observed between p65 and C/EBPβ may explain the functional synergy observed between IL-1 and IL-6 in several physiological responses [Murakami et al., 2001]. Further, functional interactions between C/EBPβ and p65 subunits could have profound influences on cellular growth control. It has been demonstrated that C/EBPα is growth inhibitory and likely functions in the terminal differentiation of certain cells [Xia et al., 1997]. The expression of C/EBP isoforms could alter the ability of NF-κB subunits to control cellular proliferation.

Here we utilized ChIP assays to illustrate in vivo association of NF-κB and C/EBP factors to their respective DNA-binding sites on HIV-1 LTR. In co-transfection experiments, we demonstrated that p65 and C/EBPβ alone or co-expressed along with Tat modulate the HIV-1 promoter activity. These observations correlate with the previous reports on cooperativity of C/EBPβ and NF-κB upon transcription of Fas promoter [Darville and Eizirik, 2001]. In their study, the authors demonstrated that addition of p65 inhibits transactivation of Fas promoter by C/EBPβ. Recent studies also revealed cross-talk between C/EBP and NF-κB pathways in TNF-α signaling [Papin et al., 2003]. Cross-family interactions of specific transcription factors within a particular family were also demonstrated between the GR and the AP-1 family of proteins (i.e., Fos and Jun) [Ogata et al., 2003], or between the leucine zipper protein Jun and the helix-loop-helix protein MyoD [Finkel et al., 1993].

In summary, we conclude that the regulation of gene expression is not mediated solely by the presence or absence of a particular set of transcription factors. The function of any particular transcription factor can be inhibited by the presence of another factor or a specific inhibitor that blocks the ability of that particular transcription factor to bind to DNA. In addition, our data demonstrate that the important transcription factor families C/EBP and NF-κB functionally interact. This observation, along with those made previously, highlights a unique mechanism by which the levels of one transcription factor can strongly modulate the functional activity of another.

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