CCAAT/Enhancer Binding Proteins Are Not Required for HIV-1 Entry but Regulate Proviral Transcription by Recruiting Coactivators to the Long-Terminal Repeat in Monocytic Cells

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CCAAT/enhancer binding proteins (C/EBP) have been shown to be required for HIV-1 transcription and replication in macrophages. However, whether these transcription factors influence the ability of virus to establish infection by altering cytokine or receptor expression or primarily regulate HIV-1 transcription has not been determined. By inhibiting endogenous C/EBP activity with a dominant-negative protein, we demonstrate that functional C/EBPs are not required for HIV-1 infection and that these factors influence replication by a transcriptional mechanism. C/EBPβ recruits coactivators to the HIV-1 long-terminal repeat (LTR) and physically interacts with histone acetyltransferase (HAT) complexes, suggesting that C/EBPs participate in remodeling the chromatin organization of the HIV-1 provirus. Furthermore, overexpression of a C/EBP dominant-negative inhibits displacement of nucleosomes located at the HIV-1 transcriptional start site. These results provide insight into the general mechanisms by which C/EBPs regulate macrophage-restricted HIV-1 transcription.

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INTRODUCTION

HIV-1 transcription is controlled by cellular and viral factors that bind the HIV-1 long-terminal repeat (LTR) (Gaynor, 1992). NF-κB is one cellular factor that has been shown to be important in regulating HIV-1 expression. Recent experiments demonstrated that CCAAT/enhancer binding proteins (C/EBP) are also necessary for HIV-1 replication in macrophages and suggested that C/EBPβ is a macrophage-specific requirement for virus transcription (Henderson et al., 1996; Henderson and Calame, 1997; Tesmer et al., 1993). C/EBP family members are expressed in several tissues but have distinct expression patterns. For example, C/EBPβ is induced during monocytic cell differentiation and coordinates the expression of several genes during macrophage activation (Akira and Kishimoto, 1992; Poli, 1998). When infected macrophages are activated, HIV-1 transcription increases, partly due to induction of NF-κB and C/EBPβ activities (Griffin et al., 1989; Henderson et al., 1996; Henderson and Calame, 1997; Jacque et al., 1996). Whether the induction of transcription factors such as C/EBPβ solely regulate transcription or indirectly influence HIV-1 replication by modifying the expression of receptors or cytokines and thus the susceptibility of monocytes/macrophages to HIV-1 infection has not been examined.

The general mechanism by which C/EBPs regulate HIV-1 transcription is also not known. Although C/EBPs are necessary for HIV-1 expression and replication in macrophages (Henderson et al., 1996; Henderson and Calame, 1997), overexpression of C/EBPβ is not sufficient to activate latent virus expression, indicating that posttranslation events are necessary for this factor to induce HIV-1 transcription (Henderson et al., 1996). One explanation for this observation is that the ability of C/EBPβ to activate HIV-1 transcription is dependent on functional interactions with other factors. C/EBPβ has been shown to interact with several classes of transcription factors including transcriptional coactivators Swi/Snf and CBP/p300. Furthermore, it has been suggested that by interacting with these coactivators C/EBPβ potentially influences chromatin organization (Kowenz-Leutz and Leutz, 1999; Mink et al., 1997; Oelgeschlager et al., 1996).

HIV-1 expression is associated with displacement of nucleosomes and establishment of an open chromatin structure (El Kharroubi et al., 1998; Sheridan et al., 1997; Van Lint et al., 1996; Verdin, 1991; Verdin et al., 1993). CBP/p300 has been shown to functionally and physically interact with multiple factors that bind the LTR including NF-κB, Sp1, and Tat (Benkirane et al., 1998; Hottiger et al., 1998; Kiernan et al., 1999; Sheridan et al., 1997; Vanden Berghe et al., 1999). Chromatin-dependent in vitro tran-
scription systems have also demonstrated a critical role for T cell-restricted factors TCF-1/LEF-1 and Ets-1 in remodeling the HIV-1 LTR (Pazin et al., 1996; Steger and Workman, 1997). Factors that recruit chromatin remodeling complexes to the LTR in cells other than T cells have not been examined.

In this study we were interested in characterizing in greater detail the mechanisms by which C/EBPs regulate HIV-1 replication. We utilize monocytic cell lines that ectopically express a dominate-negative protein to inhibit endogenous functional C/EBPs. Our data show that C/EBP factors are not necessary for establishing HIV-1 infection in monocytic cells but primarily influence HIV-1 replication at a transcriptional level in part by reorganizing the chromatin structure of the HIV-1 proviral LTR.

RESULTS

C/EBP factors do not influence HIV entry but are necessary for efficient HIV-1 transcription in monocytic cells

Previous studies have shown that HIV-1 fails to productively infect monocytic cells overexpressing the C/EBP dominant-negative protein LIP (Henderson et al., 1996; Henderson and Calame, 1997). LIP, which lacks a strong transcriptional activation domain, inhibits C/EBPβ by forming antagonistic homodimers or nonfunctional C/EBPβ-LIP heterodimers (Descombes and Schibler, 1990). To assess the importance of functional C/EBP factors during HIV-1 replication in monocytic cells, we infected U937 monocyctic cells and U937 cells overexpressing LIP (U937-LIP) with a replication-competent GFP-tagged NL43 virus (HIV-GFP) to quantify the number of infected cells. Infection was monitored by flow cytometry and RT assays. The difference in the number of U937 and U937-LIP cells infected is not significant at the earlier times postinfection but the effects of LIP overexpression on HIV-1 infection become increasingly evident with time (Fig. 1). Twenty-four hours postinfection, equivalent numbers of U937 and U937-LIP cells were infected with HIV-GFP and by 2–3 days postinfection, twofold less U937-LIP cells were infected with HIV-GFP compared to U937 cells (Fig. 1A). Three weeks postinfection, U937 cells produced a significant amount of virus as indicated by RT activity (Fig. 1B) and 15–20% of the cells were HIV-1 positive (Figs. 1C). These data sug-
gest that functional C/EBP factors are required for productive replication of HIV-1 in monocytic cells.

The inability of the virus to replicate in cells overexpressing LIP could be due to a block in virus entry, possibly at the level of coreceptor usage. HIV-1 requires CD4 and a chemokine receptor, in particular CXCR4 or CCR5, to infect the host cell. Changes in the levels of these receptors have been shown to alter susceptibility to HIV-1 infection (Basta et al., 1999; Berger et al., 1999; Franchin et al., 2000; Moriuchi et al., 1998; Tuttle et al., 1998). To assure that C/EBP factors were not adversely influencing HIV-1 infection by altering the expression of CD4 or chemokine receptors, we used flow cytometry and semiquantitative RT-PCR to analyze U937 and U937-LIP cells for the expression of these receptors. When U937 and U937-LIP cells were analyzed for CD4, no significant difference in mRNA levels or surface expression was observed (Figs. 2A and 2B). Similarly, CXCR4 expression was unchanged between U937 and U937-LIP cells (Figs. 2C and 2D). Although there are slight shifts in the fluorescence intensity in CD4 and CXCR4 between U937 and U937-LIP cells, these changes represent less than a twofold difference in mean fluorescence intensity (mean fluorescence intensity for CD4, U937 = 128, U937-LIP = 72; mean fluorescence intensity for CXCR4, U937 = 15.1, U937-LIP = 27.7). Both cell types downregulated CXCR4 expression in response to PMA treatment, which is consistent with previous reports (Fig. 2C) (Moriuchi et al., 1998). Neither unstimulated nor stimulated U937 and U937-LIP cells expressed detectable levels of CCR5 as determined by RT-PCR and flow cytometry (data not shown). Therefore, LIP overexpression does not affect CD4 and CXCR4 expression, suggesting that endogenous C/EBPs are not required for HIV-1 infection of monocytic cells.

To confirm that U937 and U937-LIP cells were equally susceptible to HIV-1 infection, cells were infected with a replication-competent virus in which the envelope gene (Env) was replaced with a luciferase reporter gene (HIV-luc). Genomic DNA was prepared 48 h postinfection and assayed for proviral sequences using PCR. As shown in Fig. 3A, U937 and U937-LIP cells had similar levels of HIV-1 provirus, demonstrating that LIP did not block viral entry and that functional C/EBP proteins are not required for establishing HIV-1 infection.
The lack of a role for C/EBP factors in establishing infection suggests that the inhibition of HIV-1 replication in monocytic cells overexpressing LIP is through a direct effect on virus transcription. To investigate this possibility, HIV-luc and genomic DNA was prepared 48 h postinfection. Measures of 1, 0.1, or 0.01 μg genomic DNA were analyzed by PCR for proviral sequences. U937-LIP3 and U937-LIP4 refer to different clonal populations of cells. (B) Forty-eight hours postinfection with HIV-luc, U937 and U937-LIP cells were lysed and assayed for luciferase activity. These results are from a single experiment performed in triplicate and represent three independent experiments. Error bars show one standard deviation.

C/EBPβ requires coactivators to transactivate the HIV-1 LTR

Functional C/EBPs are necessary but not sufficient for HIV-1 transcription in macrophages (Henderson et al., 1996; Henderson and Calame, 1997) and may require interactions with other transcription factors or coactivators. To determine whether functional interactions between coactivators and C/EBPβ were relevant for HIV-1 LTR activity, we performed cotransfection experiments using a HIV-1 LTR-luciferase reporter and different combinations of expression constructs encoding C/EBPβ and CBP. Consistent with previous studies C/EBPβ was able to strongly transactivate the HIV-1 LTR (Henderson et al., 1995; Tesmer et al., 1993). Ectopic expression of CBP was also able to activate the HIV-1 LTR in a dose-dependent manner. However, cotransfecting with both C/EBPβ and CBP led to synergistic activation of the HIV-1 LTR. As shown in Fig. 4 cotransfections with both CBP and C/EBPβ resulted in a 56-fold increase in HIV-1 LTR activity, whereas approximately 10-fold increases in transcriptional activity were observed with C/EBPβ or CBP alone (Fig. 4A). These results are not due to CBP increasing C/EBPβ expression since immunoblots demonstrate that cells have approximately equivalent levels of C/EBPβ in the absence or presence of exogenous CBP (data not shown). Furthermore, C/EBPβ-binding activity is unaffected by CBP overexpression (Fig. 4D). These data would suggest that C/EBPβ functionally interacts with CBP and that CBP is a limiting factor for this activity.

Although CBP has histone acetyltransferase (HAT) activity, this coactivator functions in part by recruiting other HAT coactivators including PCAF to the promoter (Yang et al., 1996). Therefore, additional cotransfection experiments were performed to determine whether C/EBPβ and PCAF were able to cooperatively activate the HIV-1 LTR. Similar to the previous experiment, PCAF enhanced the ability of C/EBPβ to transactivate the HIV-1 LTR (Fig. 4B). This enhancement is not due to PCAF altering C/EBPβ binding activity, which remains unchanged in the absence or presence of PCAF (Fig. 4E). These data indicate that coactivators can interact with C/EBPβ to influence HIV-1 expression and are limiting the ability of C/EBPβ to fully induce HIV-1 transcription. Furthermore, ectopic expression of E1a, which binds directly to CBP/p300 and PCAF thereby inhibiting their activity (Chakravarti et al., 1999; Hamamori et al., 1999), decreases C/EBPβ-dependent HIV-1 LTR activity. The ability of C/EBPβ to transactivate the HIV-1 LTR was reduced by greater than 80% and this inhibition could be overcome by overexpression of either CBP or PCAF (Fig. 4C and data not shown), indicating that E1a inhibits the HIV-1 LTR by competing with C/EBPβ for coactivators. These data demonstrate that C/EBPβ activates HIV-1 transcription by recruiting HAT coactivators to the promoter.

C/EBPβ interacts with chromatin remodeling complexes

PCAF participates in large multi-subunit HAT complexes that are analogous to SAGA and Ada in yeast.
Native HATs purified from yeast have been well characterized and demonstrated to interact with transcription factors from higher eukaryotes (Grant et al., 1997; Howe et al., 1999; Sterner and Berger, 2000; Wallberg et al., 1999). Furthermore, yeast HATs have been shown to reorganize preassembled HIV-1 chromatin templates and stimulate transcription in vitro (Steger et al., 1998). Since the yeast HATs are structurally and functionally similar to the mammalian complexes (Howe et al., 1999; Ogryzko et al., 1998; Sterner and Berger, 2000; Vassilev et al., 1998), we examined the ability of C/EBPβ to bind SAGA using a GST pull-down assay. After incubating purified SAGA with glutathione-Sepharose beads and GST-C/EBPβ fusion protein, both beads and supernatant were assayed for HAT activity. Figure 5A shows HAT activity was retained on the GST-C/EBPβ-bound beads while no significant HAT activity was observed with beads alone. These data indicate that C/EBPβ directly interacts with HAT complexes and suggest that C/EBPβ in part regulates HIV-1 transcription by recruiting nucleosome-acetylating native complexes to the LTR.

To further explore the significance of the above interaction, we tested whether PCAF, the mammalian homolog of the GCN5 subunit of yeast SAGA, specifically interacts with C/EBPβ in a cellular system. 293T cells were transiently transfected with PCAF and C/EBPβ and nuclear extracts were analyzed for protein interactions by coimmunoprecipitation. As shown in Fig. 5B, immunoprecipitation with anti-C/EBPβ antibody pulled-down PCAF, demonstrating a physical interaction between
PCAF and C/EBPβ. However, we were unable to coimmunoprecipitate PCAF from cell extracts with transfected LIP, which lacks the N-terminal transactivation domain (Fig. 5B). These results demonstrate that C/EBPβ physically interacts with components of SAGA in a cellular context and that the transactivation domain of C/EBPβ mediates this interaction.

Inhibiting endogenous C/EBPs prevents chromatin remodeling of the HIV-1 LTR

We have used transient transfections to suggest a functional interaction between C/EBPβ and HATs; however, the majority of the transfected DNA is unintegrated and, although associated with histones, would be expected to have a simpler chromatin structure. Therefore, it is critical that we employ a relevant cell type containing endogenous C/EBPs. Furthermore, inhibition of C/EBP activity in monocytic cells does not adversely affect HIV-1 infection.

C/EBPβ has been shown to control the expression of a number of genes during macrophage activation including CD14, ICAM-1, Mac-1, and IL-6, which are expressed at significantly lower levels in cell lines that ectopically express the C/EBP dominant-negative protein, LIP (Henderson et al., 1996). Transcriptional induction is associated with an open chromatin structure, which can be detected by increased sensitivity to restriction enzymes (El Kharroubi et al., 1998; Verdin et al., 1993). Upon activation with PMA, which induces U1 HIV-1 transcription, the nucleosome positioned at −2 to +142 is displaced, making this region susceptible to HindIII and PstI digestion (Fig. 6B and data not shown) (Verdin et al., 1993). Other restriction sites that are adjacent to the positioned nucleosome, such as PvuII, generate fragments regardless of whether U1 cells have been activated (Fig. 6B) (Verdin et al., 1993). When U1-LIP cells were treated with PMA, consistent with previous studies, virus replication was significantly reduced (Fig. 6A) (Henderson et al., 1996). More importantly, despite detecting a PvuII-specific restriction fragment, HindIII did not efficiently digest DNA from nuclei obtained from activated U1-LIP cells (Figs. 6C and 6D). Therefore, inhibiting endogenous C/EBPs prevents the displacement of the nucleosome at the HIV-1 transcriptional start site. Since C/EBPβ is the primary C/EBP family member induced following activation of U1 cells (Natsuka et al., 1992), these results suggest that by recruiting chromatin remodeling complexes to the LTR, C/EBPβ regulates HIV-1 transcription.

DISCUSSION

Previous studies have shown that the transcriptional activator C/EBPβ activates the HIV-1 LTR and is necessary for HIV-1 replication in monocytes (Henderson et al., 1996; Henderson and Calame, 1997; Tesmer et al., 1993). However, the role of C/EBPβ in establishing HIV-1 infection has not been determined. We demonstrate that while C/EBP factors are not necessary for establishing HIV-1 infection of monocytes they influence HIV-1 replication by regulating the chromatin structure of the LTR and thus virus transcription.

FIG. 5. C/EBPβ interacts with chromatin remodeling complex SAGA. (A) GST pull-down assays were performed with either GST-C/EBPβ, mock-transformed bacterial extracts, or GST bound to glutathione-Sepharose beads. SAGA purified from yeast was incubated with the protein–bead complexes as described under Materials and Methods. Supernatants and beads were assayed for their ability to acetylate core protein–bead complexes as described under Materials and Methods. (B) 293T cells were transfected with 0 or 10 ng of PCAF and 0 or 5 ng of C/EBPβ or LIP. Nuclear extracts were immunoprecipitated with anti-C/EBPβ antibody and Western blotting was performed to detect PCAF and C/EBP proteins.

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C/EBPβ has been shown to control the expression of a number of genes during macrophage activation including CD14, ICAM-1, Mac-1, and IL-6, which are expressed at significantly lower levels in cell lines that ectopically express the C/EBP dominant-negative protein LIP (Henderson et al., 1996; Henderson, A. J., Lee, E. S., and Zhou, H., unpublished data). However, as shown in this study, the expression of receptors essential for HIV-1 infection, CD4 and CXCR4, does not require functional endogenous C/EBPs. Furthermore, inhibition of C/EBP activity in monocytic cells does not adversely affect HIV-1 infection. We were unable to detect CCR5 expression in U937 cells and therefore could not evaluate whether this chemokine receptor is potentially regulated by C/EBPs in monocytes/macrophages, although the promoter for CCR5 has been recently characterized and does have potential C/EBP binding sites (Moriuchi et al., 1997; Rosati et al., 2001). While these data suggest that C/EBP factors do
not directly affect HIV-1 infection in monocytes, C/EBPs may indirectly influence disease progression by contributing to aberrant macrophage function, inappropriate expression of inflammatory cytokines, and a more permissive microenvironment for disease progression (Akira and Kishimoto, 1992; Honda et al., 1998; Lee et al., 2001).

Although C/EBPβ does not influence HIV-1 infection, this factor impacts HIV-1 replication by regulating virus transcription in part through the recruitment of coactivators to the LTR. It has been demonstrated that activation of latent HIV-1 proviral expression in monocytic and T cell lines is associated with specific chromatin changes within the 5' LTR, including displacement of a nucleosome positioned at the transcriptional start site (El Kharroubi et al., 1998; Verdin, 1991; Verdin et al., 1993). Furthermore, treatment of cell lines harboring latent HIV-1 provirus with trichostatin A, trapoxin, or sodium butyrate, specific inhibitors of histone deacetylases, induces virus expression indicating that HIV-1 transcription is dependent on histone acetylation (Laughlin et al., 1993; Van Lint et al., 1996). The importance of histone acetylation and chromatin remodeling by HATs has been confirmed using nucleosome-associated HIV-1 templates with in vitro transcription systems (Pazin et al., 1996; Sheridan et al., 1995, 1997; Steger et al., 1997, 1998; Widak et al., 1997).

Transcription factors such as NF-κB, SP1, Ets-1, and LEF-1, which bind the proviral enhancer/promoter located in the site upstream of the nucleosome core particle, participate in reorganizing chromatin by facilitating the recruitment of HAT coactivators to the LTR (Pazin et al., 1996; Sheridan et al., 1995, 1997; Steger et al., 1997, 1998; Widak et al., 1997). However, the transcription factors Ets-1 and LEF-1 are restricted to T cells and it would be expected that macrophage-restricted factors may also interact with chromatin-remodeling complexes. Our results show that C/EBPβ functionally interacts with HAT coactivators and physically interacts with chromatin remodeling complexes in monocytic cells, demonstrating that coactivators are critical for the ability of C/EBPβ to induce HIV-1 transcription. However, it is unclear the exact role chromatin remodeling plays during de novo infection since the number of cells with latent provirus is estimated to be low, although reactivation of virus expression in these cells may be a major source of HIV-1 at late stages of infection or upon removal of anti-retroviral treatments (McCune, 1995).

Previous work by Mink et al. demonstrates that the amino-terminal transcriptional activation domain of C/EBPβ binds regions of CBP/p300 and PCAF through the E1a binding domain (Mink et al., 1997). E1a com-
pletely inhibits the ability of C/EBPβ to transactivate the HIV-1 LTR, suggesting that E1a is competing with C/EBPβ for binding to CBP/p300 or PCAF (Chakravarti et al., 1999; Hamamori et al., 1999). Furthermore, the LIP dominant-negative protein, which lacks this domain (Mink et al., 1997), does not physically interact with PCAF, fails to activate HIV-1 transcription, and is unable to mediate the displacement of nucleosomes that repress HIV-1 transcriptional initiation. Therefore, the HIV-1 LTR is similar to other myeloid-specific promoters requiring C/EBPβ and coactivators for differential activity (Kowenz-Leutz and Leutz, 1999; Mink et al., 1997; Oelgeschlager et al., 1996).

C/EBPβ has also been demonstrated to interact with components of the basal transcriptional machinery such as TBP and TFIIB (Nerlov and Ziff, 1995) and other transcription factors, including NF-kB and CREB (Hohaus et al., 1995; LeClair et al., 1992; Ross et al., 2001; Stein et al., 1993; Westendorf et al., 1998; Zhang et al., 1996). These proteins may cooperate in the recruitment of coactivators to the HIV-1 LTR in monocyctic cells. Furthermore, the recruitment of coactivators by transcription factors, including C/EBPβ, may functionally impact Tat, which physically interacts with HATs including CBP/p300, PCAF, TAF,250, and Tip60 (Benkirane et al., 1996; Kamine et al., 1996; Kiernan et al., 1999; Marzio et al., 1998; Weissman et al., 1998). In addition to recruiting HATs, C/EBPβ physically interacts with the ATP-dependent chromatin remodeling complex Swi-Snf (Kowenz-Leutz and Leutz, 1999) and therefore may coordinate multiple factors that participate in establishing a transcriptionally accessible HIV-1 LTR.

**MATERIALS AND METHODS**

**Cell lines and expression constructs**

U937, U937-LIP, U1, and U1-LIP pro-monocytic cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. U937 and U1 cells overexpressing LIP have been previously described (Henderson et al., 1996; Henderson and Calame, 1997). 293T human embryonic kidney cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The LTR-luciferase reporter and the GST-C/EBPβ constructs have been previously described (Artandi and Calame, 1993; Henderson et al., 1996). The C/EBPβ expression construct was generated by subcloning C/EBPβ cDNA from pSCT-LAP (Descombes and Schibler, 1991) into the EcoRI site of the MSCV-IRES-GFP retroviral expression construct (generously provided by G. Nolan, Stanford University) (Anderson et al., 1996). The CMV-LIP construct has been previously described (Henderson et al., 1996). The CBP expression construct was previously described (Kwok et al., 1996) and was provided by Dr. R. Goodman (Oregon Health Sciences University). Dr. D. Livingston (Dana-Farber Cancer Institute, Harvard University) generously provided the 12s E1a expression construct (White et al., 1991), and PCAF expression construct (Yang et al., 1996) was obtained from Dr. Y. Nakatani (National Institute of Child Health and Human Development, Bethesda, MD).

**Transient transfections and luciferase assays**

293T cells were transfected with different DNA constructs as indicated in the figure legends by calcium phosphate. Forty-eight hours posttransfection cells were harvested, lysed, and assayed for luciferase activity using the Promega Reporter Assay System (Madison, WI). Transfection efficiencies were determined by monitoring GFP expression from either the MCSV-C/EBPβ-IRES-GFP or the cotransfected MCSV-IRES-GFP. Protein expression was monitored by immunoblot as previously described (data not shown) (Cooper et al., 1995).

**Generation of HIV-1 infectious titers and infections**

Replication-competent virus was generated by transfecting 293T cells with 15 μg T-tropic pHIV-EGFP R5/E2 DNA and 3 μg RSV-Rev DNA by CaPO4 transfection (Pear et al., 1993). Production of viral particles was monitored by RT activity 48 h posttransfection. Infectious virus stocks were added to 1.0 × 10^6 cells/ml U937 and U937-LIP cells for 24 h. The infection medium was then removed and replaced with fresh RPMI supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. At various times postinfection, infected cells were fixed with 2% paraformaldehyde and quantitated using a Coulter flow cytometer at the Penn State flow cytometry core facility.

Replication-incompetent virus was also generated by transient transfection using 10 μg of pNL43-Luc(+)Env(-) (HIV-luc) DNA (Connor et al., 1995), 2 μg HXB2-Env DNA, and 2 μg RSV-Rev DNA. Transfection efficiency was assessed by luciferase activity. Two hundred microliters of undiluted viral stocks was added to 1.0 × 10^6 cells/ml U937 and U937-LIP cells for 24 h. Cells were harvested 48 h postinfection and assayed for viral transcription by luciferase assays. 1.0 × 10^5 cells were lysed in 1× Reporter Lysis Buffer (Promega) and supernatants were collected. Twenty microliters of cell extract was added to 100 μl luciferase substrate (Promega) and activity was measured using a luminometer.

**Reverse transcriptase (RT) assays**

Virus replication was measured by RT assays at various times postinfection (Henderson et al., 1996). Briefly, 10 μl of supernatant was added to a mixture containing 60 mM Tris, 24 mM DTT, 7 mM MgCl2, 75 mM NaCl, 6 μg/ml poly(dG), 12 μg/ml poly(dC), 0.06% NP-40, and 10
μCi [α-32P]dGTP in a final volume of 50 μl and incubated at 37°C for 1 h. Ten microliters of this reaction mixture was then transferred to DEAE paper and washed twice in 2× SSC (0.3 M NaCl, 0.03 M Na citrate) for 15 min at 25°C. RT activity was quantitated using a phosphorimager.

Immunofluorescent staining

To detect cell-surface receptors CD4 or CXCR4, 1.0 × 10^6 U937 or U937-LIP cells were incubated in staining media (1% FCS in PBS) with 1 μg IgG, isotype FITC-conjugated mouse anti-human CD4 mAb (Pharmingen, San Diego, CA) or 1 μg IgG, isotype PE-conjugated mouse anti-human CXCR4 mAb (Pharmingen) for 30 min at 4°C. Cells were washed three times in staining media and fixed with 2% paraformaldehyde. Fluorescence was measured using a Coulter flow cytometer at the Penn State flow cytometry core facility.

RNA extraction and RT-PCR

Total RNA was prepared by lysing cells with 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarkosyl, and 0.1 M 2-mercaptoethanol followed by guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 1 mM EDTA, 1% SDS) and treated with 0.5 mg/ml proteinase K at 55°C for 5 h. Measures of 1, 0.1, or 0.01 μl genomic DNA were amplified by a 30-cycle PCR of 94°C for 1 min, 72°C for 2 min using the upstream primer 5'-ATGGTAGTAGCCCCTCAGTGC-3' and downstream primer 5'-CTTGGTAGTAGCCCCTCAGTGC-3'. CXCR4 cDNA was amplified using the upstream primer 5'-TTCCTGCCCACCATCTACTC-3' and downstream primer 5'-CACCACAGGTTCACCTTCC-3'. cDNA was prepared from 2 μg of RNA using murine leukemia virus reverse transcriptase and random primers. To amplify the cDNA, a 30-cycle polymerase chain reaction of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min was performed with 0.2 μg of cDNA using various primers. CD4 cDNA was amplified using the upstream primer 5'-CTTGGTAGTAGCCCCTCAGTGC-3' and downstream primer 5'-AGCTCCTGGAAAGTCCCAGGGAAATCCCT-3'.

PCR for proviral sequences

To detect proviral sequences in genomic DNA, U937 and U937-LIP cells were lysed 48 h postinfection with TES buffer (10 mM Tris, pH 8.0, 400 mM NaCl, 2 mM EDTA, 1% SDS) and treated with 0.5 ml/ml proteinase K at 55°C for 5 h. Measures of 1, 0.1, or 0.01 μg genomic DNA were amplified by a 30-cycle PCR of 94°C for 1 min, 55°C for 2 min, 72°C for 2 min using the upstream primer 5'-CCATGTATGGCTGAAACTGG-3' and downstream primer 5'-CTTGCATGCTAGAGATTTTCCAC-3'.

Nuclear extract preparations and electromobility shift assays (EMSA)

Nuclear extracts from 2.0 × 10^6 293T cells were prepared as described previously (Schreiber et al., 1989) by lysing cells with 10% Nonidet P-40 in buffer A [10 mM HEPES (pH 7.5), 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF]. Extracts were recovered in buffer C [20 mM HEPES (pH 7.5), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF]. Fifty nanograms of annealed C/EBP-binding site DNA (5'-GATCGCCTAAGCATTTCTACACGT-3' and 5'-AGCTCCTGGAAAGTCCCGAGGAATCCCTT-3') was end-filled with [α-32P]-dCTP using bacterial Klenow fragment (Promega). Four micrograms of nuclear extract was incubated in a reaction mixture containing the DNA probe, 3 μg of poly(dIdC) (Amershams Pharmacia Biotech, Arlington Heights, IL), 0.25 M HEPES (pH 7.5), 0.6 M KCl, 50 mM MgCl2, 1 mM EDTA, 7.5 mM DTT, and 9% glycerol for 20 min at 25°C. Fiftyfold excess of unlabeled C/EBP or NF-kB binding site DNA was used as specific and nonspecific competitors, respectively. The samples were run on a 6% polyacrylamide gel and visualized by autoradiography.

Coimmunoprecipitation and Western blotting

Nuclear extracts from 293T cells transfected with 0 or 1 μg PCAF and 0 or 5 μg C/EBPβ or LIP were immunoprecipitated with 1 μg rabbit-anti-C/EBPβ polyclonal antibody and 10 μg A/G-agarose beads (Santa Cruz). Complexes were washed four times with PBS and resuspended in 50 μl 1× SDS buffer [50 mM Tris–HCl (pH 8.0), 2% SDS, 0.1% bromphenol blue, 10% glycerol, 100 mM DTT]. Ten microliters of each sample was run on a 10% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). Membrane was probed for PCAF using mouse-anti-Flag monoclonal antibody (Sigma) for 1 h at 25°C and horseradish peroxidase conjugated sheep anti-mouse antibody (Chemicon, Temecula, CA) for 1 h at 25°C. For detection of C/EBP proteins, membrane was stripped of PCAF and 0 or 5 μg C/EBPα or L1 rabbit-anti-C/EBPα fusion products were purified from bacterial extracts by binding to glutathione-Sepharose 4B beads (Pharmacia) as described (Barlev et al., 1995). SAGA complexes were obtained from Dr. J. Warkman’s laboratory (Penn State) (Grant et al., 1997). Equivalent amounts of SAGA, precleared with glutathione-Sepharose beads, were incubated with immobilized GST fusion proteins for 2 h at 4°C in binding buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 0.1% Tween 20, 0.5 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin A]. The supernatants were removed and the

GST pull-down assay

GST and GST-C/EBPβ fusion products were purified from bacterial extracts by binding to glutathione-Sepharose 4B beads (Pharmacia) as described (Barlev et al., 1995). SAGA complexes were obtained from Dr. J. Warkman’s laboratory (Penn State) (Grant et al., 1997). Equivalent amounts of SAGA, precleared with glutathione-Sepharose beads, were incubated with immobilized GST fusion proteins for 2 h at 4°C in binding buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 0.1% Tween 20, 0.5 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin A]. The supernatants were removed and the
beads were washed three times with binding buffer. Input SAGA, supervenants, and beads were directly assayed for HAT activity on histones (Grant et al., 1997). Briefly, 2 \( \mu \)g of core histones was incubated with 10 \( \mu \)l of sample to be tested and \(^3\)H-labeled acetyl-CoA (0.125 \( \mu \)Ci) in HAT buffer [50 mM Tris–HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl\(_2\), and 0.3 M sucrose] for 5 min and then lysed in 0.3% NP-40 for 30 s. Nuclei were collected by centrifugation and resuspended in Buffer B [10 mM Tris–HCl (pH 7.9), 10 mM MgCl\(_2\), 50 mM NaCl, 1 mM DTT, 100 \( \mu \)g/ml BSA, and 0.1 M PMSF]. Nuclei were digested with 400 U of HindIII, PvuI, PsiI, and Hinfl (New England Biolabs) for 8–16 h. Genomic DNA was prepared by lysing nuclei with TES buffer [10 mM Tris (pH 8.0), 400 mM NaCl, 5% glycerol, 0.1 mM EDTA, 1 mM DTT, 10 mM sodium butyrate] at 30°C for 30 min. The reactions were then run on 18% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Grant et al., 1997). Gels were Coomassie stained to determine the amounts of histone loaded in each lane and destained before determining acetylation by fluorography using EN\(^3\)HANCE autoradiography enhancer (NEN, Boston, MA).

**Restriction enzyme accessibility assay and ligation-mediated PCR**

U1 and U1-LIP cells were stimulated with phorbol myristate acetate (PMA) for 48 h and HIV-1 transcription was monitored by RT activity in the cell culture supernatant. Cells were harvested and resuspended at 2.5 \( \times \) 10\(^7\) cells/ml in cold buffer A [10 mM Tris–HCl (pH 8.0), 10 mM NaCl, 3 mM MgCl\(_2\), and 0.3 M sucrose] and treated with lysing nuclei with TES buffer [10 mM Tris–HCl (pH 8.0), 50 mM HCl, 5% glycerol, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate] at 30°C for 30 min. The reactions were then run on 18% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Grant et al., 1997). Gels were Coomassie stained to determine the amounts of histone loaded in each lane and destained before determining acetylation by fluorography using EN\(^3\)HANCE autoradiography enhancer (NEN, Boston, MA).

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