Induction of Monocytic Differentiation and NF-κB-like Activities by Human Immunodeficiency Virus 1 Infection of Myelomonoblastic Cells

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Summary

The effects of human immunodeficiency virus 1 (HIV-1) infection on cellular differentiation and NF-κB DNA binding activity have been investigated in a new model of myeloid differentiation. PLB-985 cells represent a bipotential myelomonoblastic cell population capable of either granulocytic or monocytic differentiation after induction with appropriate inducers. By virtue of the presence of CD4 on the cell surface, PLB-985 cells were chronically infected with HIV-1 strain IIIB. PLB-IIIB cells clearly possessed a more monocytic phenotype than the parental myeloblasts, as determined by differential staining, increased expression of the myeloid-specific surface markers, and transcription of the c-fms proto-oncogene. NF-κB binding activity was inducible by tumor necrosis factor and phorbol myristate acetate in PLB-985. However in PLB-IIIB cells, constitutive expression of a novel NF-κB complex was detected, composed of proteins ranging between 70 and 110 kD. These proteins interacted specifically with the symmetric NF-κB site from the interferon β (IFN-β) promoter. Mutations affecting the 5' guanine residues of the κB site were unable to compete for these NF-κB-related proteins. Inducibility of endogenous IFN-β and IFN-α RNA was also increased in PLB-IIIB cells. These studies indicate that HIV-1 infection of myelomonoblastic cells may select for a more mature monocytic phenotype and that unique subunit associations of NF-κB DNA binding proteins may contribute to differential NF-κB-mediated gene expression.

The host range of the HIV-1 includes, in addition to CD4^{+} Th lymphocyte, cells of the monocyte/macrophage lineage, and hematopoietic progenitor cells (1–3). Whereas Th cells are ultimately destroyed by HIV infection, infection of monocytic cells is not cytolytic, and these cells may act as reservoirs for viruses, thus contributing to virus spread to peripheral tissues including the lungs, brain, skin, and lymph nodes (3). Furthermore, HIV infection of myeloid cells results in the impairment of crucial functions involved in host defense, such as antigen presentation, chemotaxis, cell killing, and cytokine release (4–6).

HIV-1 infection of PBMC and monocytes/macrophages may have a dramatic impact on cellular gene expression and in particular on cytokine production (7–17). HIV infection does not generally lead to constitutive cytokine gene transcription and secretion (9, 12, 13); however, the cytokine response of HIV-infected cells to subsequent antigenic challenge by viruses, lipopolysaccharides, or poly(I:C) can be affected in HIV-infected cultures, often leading to increased expression of TNF and IL-1 (9, 12, 15–17). Cytokine release by HIV-infected cells may, in turn, alter the course of virus pathogenesis in vitro. For example, treatment of cells with TNF-α, IL-1, or GM-CSF has been shown to increase gene expression driven by the HIV-1 LTR in transfection studies, and to stimulate virus multiplication in vitro (10, 11, 18–21).

Cytokines, phorbol esters, virus infections, double-stranded RNA, and other mitogenic agents potentiate HIV replication, in part via activation of NF-κB, a family of mitogen- and phorbol-ester–inducible DNA binding transcription factors which bind to and activate the HIV enhancer (22–24). NF-κB proteins bind to a decameric recognition sequence (consensus 5'-GGGRNNYYCC-3') present in the promoter regions of many cellular genes encoding immunoregulatory molecules (IL-2 receptor-α, MHC class I antigens, and TNF receptor) and cytokines (IL-2, IL-6, IL-8, IFN-β, TNF, and GM-CSF), as well as the enhancer domains of several viruses (22). To date, NF-κB induction has been characterized predominantly as a posttranslational event. Latent NF-κB exists in the cytoplasm as a heterotrimeric protein; induction in-

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volves the dissociation of a p65-p50 heterodimer from a regulatory protein (inhibitor of κB or IκB), and nuclear translocation of the DNA-binding NF-κB complex (22, 25, 26). Phosphorylation of IκB appears to be a necessary requirement for dissociation of the heterotrimeric form of NF-κB (22). Recent cloning of the p65 and p50 subunits has revealed that the NH₂-terminal DNA binding region of both proteins shares homology with the c-rel proto-oncogene, (27–32). Furthermore these studies indicate that NF-κB induction may also be controlled at the level of transcription.

As a step toward understanding the consequences of HIV-1 infection on myeloid differentiation and cellular gene expression, we have investigated the induction of monocytic differentiation and NF-κB/rel activities in a new myelomonoblastic model of HIV-1 infection. PLB-985 cells represent an immature blast population, capable of both monocytic and granulocytic differentiation (33). By virtue of CD4 expression on the cell surface, these cells were infected by HIV-1 strain IIIB and a chronically infected line (PLB-IIIB) was established. Characterization of PLB-IIIB cells and NF-κB-related activity has demonstrated that HIV-1 infection of myelomonoblastic cells may select for a more mature monocytic phenotype, and that distinct subunit associations of NF-κB DNA binding proteins may contribute to developmental and inducible NF-κB-mediated gene expression.

Materials and Methods

Cell Culture

Myelomonoblastic PLB-985 and PLB-IIIB cells were maintained at 37°C, 5% CO₂, in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS, 1% glutamine, 1,000 IU/ml penicillin, 20 μg/ml streptomycin, and 1 μg/ml gentamycin. Induction of exponentially growing cells was performed as described for the individual experiments. Cells were treated with PMA (Sigma Chemical Co., St. Louis, MO) to a final concentration of 25 ng/ml, DMSO (Aldrich Chemical Co.) to a final concentration of 1.25%, 2'-dibutyryl adenosine-3', 5'-cyclic monophosphate (Bt2cAMP; Sigma Chemical Co.) to a final concentration of 750 μM, cis-retinoic acid (RA; Sigma Chemical Co.) to a final concentration of 1 μM, and TNF-α to a final concentration of 100 U/ml.

Infection of PLB-985

Cells were infected using supernatant HTLV-IIIB virus obtained from U9-IIIB cells as previously described (34).

P24 Antigen Determination

Immunofluorescence. Production of HIV-1–specific p24 antigen was monitored over a period of 60 d by indirect immunofluorescence. Cells were fixed on multibeveled slides with methanol/acetone (1:1). An aliquot of mouse anti-p24 antibody (a gift from Dr. R. Gallo, National Institutes of Health, Bethesda, MD) diluted 1:1,000 in PBS was added to each well for 30 min. After washing with PBS, goat anti-mouse-conjugated FITC antibody (diluted 1:1,000 in PBS) (Caltag Laboratories, San Francisco, CA) was added for 30 min. Slides were washed with PBS plus 0.25% Triton X-100, air-dried, and mounted with glycerol.

ELISA. An ELISA (Abbott Labs, North Chicago, IL) was used to detect HIV-1 p24 in culture fluids, as per manufacturer's instructions.

Reverse Transcriptase Analysis

At different time points after infection 1 ml of culture medium was centrifuged at 1,200 rpm for 10 min. Reverse transcriptase activity was evaluated without further virus concentration using ³H TTP incorporation into acid-precipitable nucleic acid (34).

Analysis of RNA

Total cellular RNA was isolated from untreated and PMA-treated PLB-985 and PLB-IIIB cells at specific times using a modified guanidinium isothiocyanate procedure (35). Total RNA (20 μg) was electrophoresed in a 1% denaturing formaldehyde gel, transferred to a nylon membrane, and hybridized with α-[³²P]ATP nick translated probes at 42°C. The probes include a 1.23-kb c-fms fragment generated by EcoRI cleavage of clone pc-fms 104 (American Type Culture Collection, Rockville, MD), a 1.1-kb β-actin fragment, a 4.5-kb c-myc fragment containing exons 2 and 3 produced by cleavage of plasmid pHSR-1 (American Type Culture Collection) with XbaI, EcoRI, and HB10, a 12.5-kb HIV-IIIB probe (a gift from Dr. F. Wong-Staal, University of California at San Diego, La Jolla, CA). The blots were washed at 55–60°C, air-dried, and exposed to X-OMAT film (Kodak) at -70°C. The relative amounts of each signal were quantified by laser densitometry (Ultrascan XL; Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

Analysis of Surface Markers

PLB-985 and PLB-IIIB cells treated with PMA or retinoic acid were analyzed for expression of myeloid specific surface markers using mAbs directed against CD13, CD14, CD33, CD34, and CD67 and analyzed in an Epics Profile II cytofluorograph; (Coulter Electronics, Hialeah, FL).

Whole Cell Extract Preparations

Whole cell extracts (WCE)[2] were prepared from untreated PLB-985 and PLB-IIIB cells or from cells treated for 6 or 15 h with PMA (25 ng/ml) or TNF-α (100 U/ml) as previously described (24).

Gel Retardation Assays

Cell protein in WCE buffer (0.1 M KCl), was preincubated with 5 μg of poly(dI:dC) as nonspecific competitor DNA for 10 min on ice. ³²P-labeled probe (0.2 ng) consisting of the P2 oligonucleotide (5'-GGGAAATTCCGGGAAATTCC-3') or the HIV enhancer (5'-AGGGACTTTCCGCTGGGGACTTTCC-3') was added to the protein, and incubated for 30 min at room temperature. Competition assays were performed by incubating 125-fold excess of unlabeled competitor (25 ng) with the protein for 10 min on ice, before the addition of radiolabeled probe.

UV Crosslinking Analysis

For UV crosslinking analysis, assays were performed using double-stranded BUdR-substituted ³²P-labeled P2 or HIV oligonucleotide probes. The crosslinking procedure was performed as previously described (36).

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[2] *Abbreviation used in this paper:* WCE, whole cell extract(s).

Analysis of IFN RNA

Total RNA was isolated from uninduced or Sendai virus–induced PLB-985 or PLB-IIIB cells. Cells were IFN-α primed (250 U/ml), then treated with Sendai virus (2,000 HAU/ml) for 6 h before RNA isolation. IFN-α– and IFN-β–specific RNA was analyzed by PCR-mediated RNA phenotyping as previously described (9) using the IFN-α (sense: 5'-CAATATCTACGATGGCTTCGC-3' and antisense 5'CAGAAGGCTCCAGCCATCTCT-3') and IFN-β primers described previously (12).

Results

Isolation and Characterization of PLB-IIIB Cells. PLB-985 cells were originally derived from a patient with acute non-lymphocytic leukemia and characterized as a diploid my-elomonoblastic cell line (33). PLB-985 cells are capable of differentiation along the monocytic lineage after treatment with phorbol esters or along the granulocytic lineage after treatment with agents such as DMSO, dibutyryl cAMP, or retinoic acid (33). By virtue of the expression of CD4 on the surface of >99% of the cells, PLB-985 cells were infected with HIV-IIIB using culture fluids of HIV-1–infected U937 cells (34). At various times after infection, the cells and/or culture fluids were assayed for RT activity, p24 core antigen, and accumulation of viral RNA (Fig. 1). After a lag period of about 10 d, the number of p24-positive cells increased rapidly together with levels of RT. By day 18, more than 50% of the population was infected with HIV-1 (Fig. 1 A). Spliced and unspliced viral RNA species were easily detectable by Northern blot analysis using the HB10 probe (Fig. 1 B). During the next several weeks, the infected cell population underwent considerable death, presumably because of rapid virus multiplication. Within 2 mo, a chronically infected cell population (>95% positive for p24 antigen and <4% CD4$^+$) emerged that possessed a more mature monocytic phenotype than the immature parental blast cells (compare Fig. 2, A and C). Phorbol ester treatment of PLB-985 produced an adherent, monocyte-like phenotype (Fig. 2 B). In contrast, PLB-IIIB without induction displayed several characteristics of monocyte maturation, including less basophilic cytoplasm, decreased nuclear/cytoplasmic ratio, production of vacuoles, less visible nucleoli, ruffling at the cytoplasmic membrane, and lobularization of the nucleus (Fig. 2 C). PMA treatment of the PLB-IIIB cells further accentuated these features of the monocytic phenotype (Fig. 2 D).

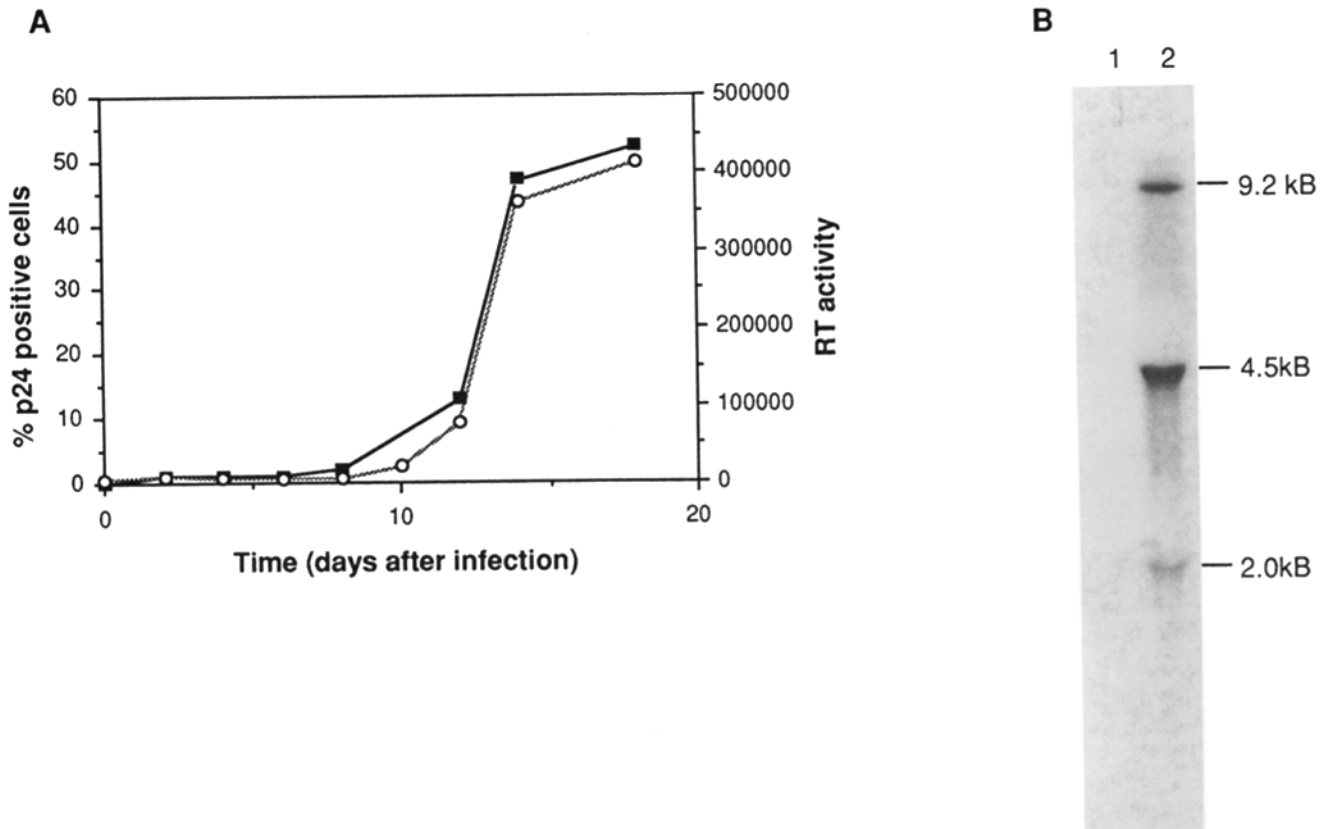*Expression of Myeloid Specific Surface Markers.* To deter-

Figure 1. Kinetics of infection of PLB-985 cells by HIV-1. (*A*) The kinetics of infection were assessed by p24 core antigen immunofluorescence (■) and by analysis of reverse transcriptase activity (○) in culture supernatants at various times after initial infection. (*B*) Detection of HIV-1 viral RNA species in chronically infected PLB-IIIB cells. Total RNA (20 μg) from PLB-985 (lane *1*) and/or PLB-IIIB (lane *2*) was electrophoresed in a 1% formaldehyde gel, transferred to a nylon filter (Amersham Corp.), and hybridized with the HB10 probe.
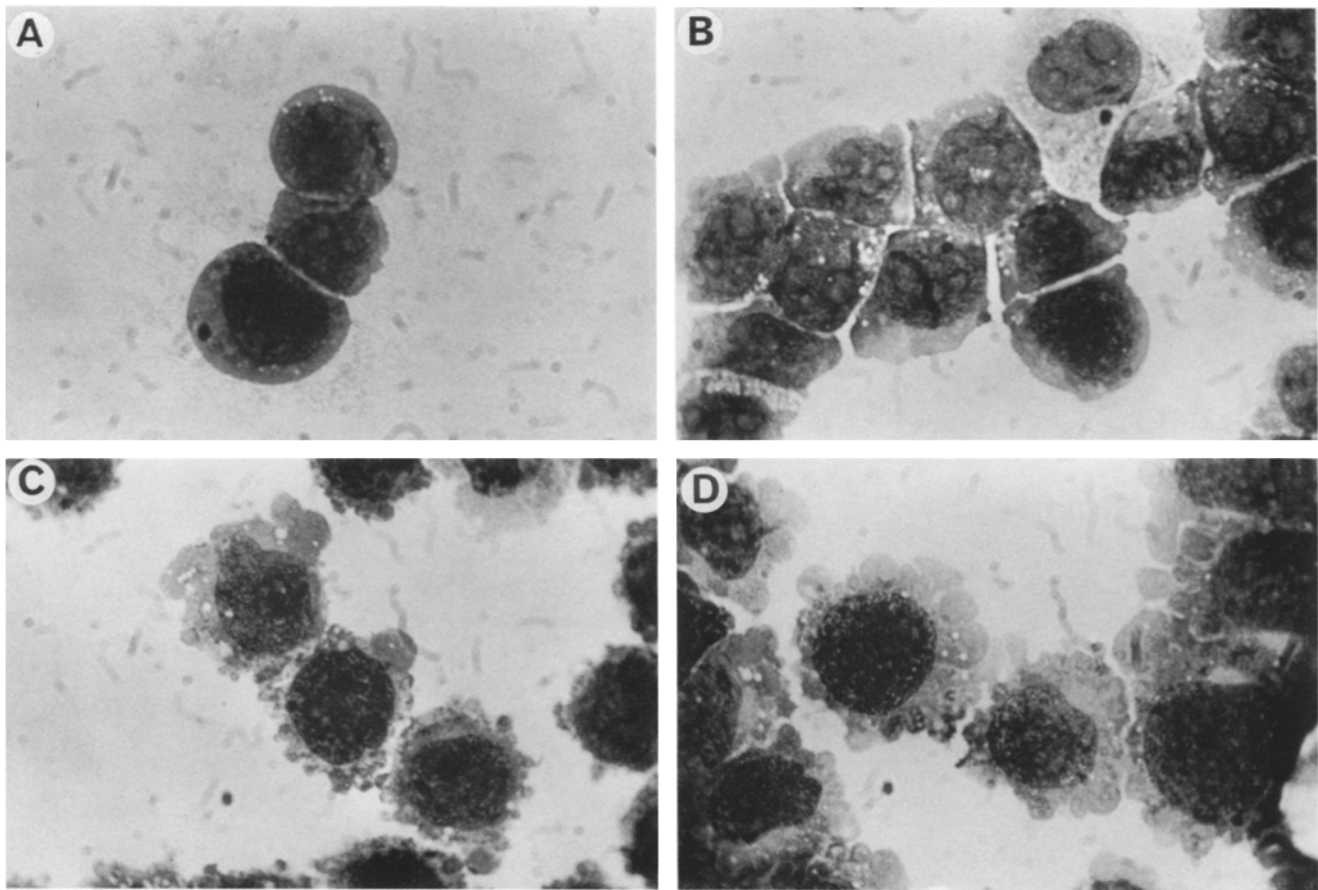
Figure 2. Morphological characteristics of PLB-985 and PLB-IIIB cells. PLB-985 cells (*A* and *B*) and PLB-IIIB (*C* and *D*) were fixed and differentially stained with Giemsa and examined by phase contrast microscopy. Cells were either untreated (*A* and *C*) or PMA treated for 6 h (*B* and *D*) before staining.

mine whether the morphological alterations were accompanied by other changes in the PLB-IIIB phenotype, expression of myeloid-specific markers was determined by flow cytometry. PLB-985 cells were <5% positive for the monocyte-specific marker CD14, whereas the PLB-IIIB cells were about 20% positive for the monocyte-specific CD14 surface antigen. After PMA treatment, PLB-IIIB cells became >60% CD14 positive within 24 h (Fig. 3 *A*). The levels of other myeloid markers CD13, CD33, and CD67 remained at >90%. Similarly 5–15% of the cells were CD34 positive both before and after infection (Fig. 3 *B*).

Expression of c-fms and c-myc Proto-oncogenes. Alteration of proto-oncogene expression is a recognized consequence of induction of differentiation in a variety of hematopoietic cell types (37, 38). To examine the effects of HIV-1 infection on PMA-induced proto-oncogene expression, the steady state mRNA levels for c-*myc* and c-*fms* were measured at different times after PMA treatment of PLB-985 and PLB-IIIB cells (Fig. 4). In both cell types, c-*myc* RNA levels decreased about 2–3 fold by 24 h after PMA treatment (Fig. 4, *upper*). In contrast, c-*fms* RNA was only weakly detectable by Northern blot analysis in PLB-985 cells before or after PMA induction (Fig. 4, *middle*, lanes 1–5). In untreated PLB-IIIB cells, c-*fms*

RNA was present in low quantities. This level of c-*fms* was induced more than 20-fold by PMA treatment (Fig. 4, *middle*, lanes 6–10). The level of β-actin RNA did not fluctuate significantly with induction or differentiation (Fig. 4, *bottom*). After granulocyte induction of PLB-985 and PLB-IIIB cells with dibutyryl cAMP, no increase in c-*fms* was observed (data not shown). Finally, morphological and histochemical studies demonstrated that PLB-IIIB cells were nonresponsive to inducers of granulocytic differentiation, indicating that the cell population had irreversibly differentiated along the monocytic lineage (data not shown).

Induction of NF-κB Binding Activity in PLB-IIIB Cells. Previous studies have demonstrated a correlation between monocyte maturation and the expression of NF-κB DNA binding activity (39–41). More recently, NF-κB has been shown to represent a family of *rel*-related DNA binding proteins involved in both transcriptional control and oncogenesis (reviewed in references 22, 27–32). We therefore sought to examine the relationship between HIV infection, monocytic differentiation, and activation of the NF-κB/*rel* transcription complex in this new myelomonoblastic model of HIV-1 infection. Induction of NF-κB DNA binding activity was examined after treatment with PMA or TNF-α, using
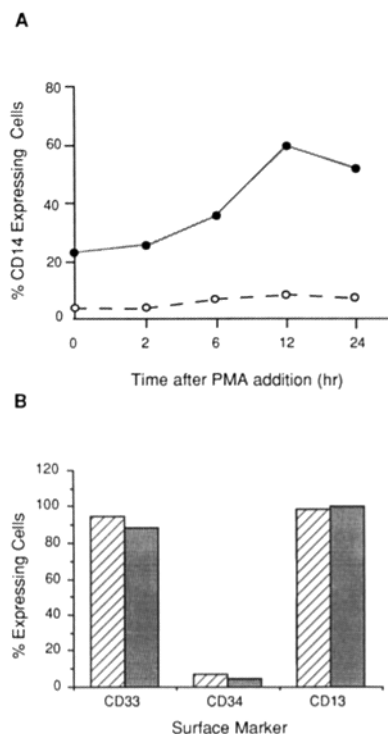
A

B

Figure 3. Myeloid-specific surface marker expression. PLB-985 and PLB-IIIB cells were either untreated or PMA treated for various times before surface marker analysis using the EPICS cytofluorograph (Coulter Electronics Inc.). (*A*) The percentage of CD14 positive cells is plotted as a function of time after PMA treatment. (●) PLB-IIIB; (○) PLB-985. (*B*) The levels of CD13-, CD33-, and CD34-positive PLB-985 (▨) and PLB-IIIB (■) cells.

sociation rate analysis, complex A dissociated from the radiolabeled probe with a $T_{1/2}$ of <4 min in the presence of excess P2 oligonucleotide, whereas addition of excess HIV enhancer failed to dissociate the A complex ($T_{1/2}$ >30 min) (Fig. 7). Thus, the proteins involved complex A bound preferentially to the 5'-GGGAAATTCC-3' site. These proteins had a >20-fold higher affinity for P2 sequences than for HIV enhancer sequences.

To evaluate the sequence specificity of complex A binding, mutational analysis of the NF-κB sites was performed. Mutations that altered the 5' guanine residues of the HIV or P2 oligonucleotides completely inhibited NF-κB competition in PLB-985 extracts (Fig. 8 *A*, lanes *3* and *5*), whereas mutation of the AA dinucleotide to CG partially inhibited >60% of the NF-κB binding (Fig. 8 *A*, lane *6*). With extracts from PLB-IIIB cells, only the homologous P2 oligonucleotide competed effectively for complex A (Fig. 8 *A*, lane *10*). Mutation of either the 5' guanine residues or the AA dinucleotide of the NF-κB site blocked competition (Fig. 8 *A*, lanes *11* and *12*). These results suggest that complex A is composed of NF-κB related proteins that require the entire P2 site for efficient DNA binding.

The proteins involved in protein-DNA complex formation were evaluated by in situ UV crosslinking analysis using BUdR-substituted P2 and HIV enhancer probes together with extracts from the TNF-treated PLB-985 or PLB-IIIB cells (Fig. 9). The regions of the mobility shift assay corresponding to NF-κB and complex A were cut from the gel (Fig. 9 *A*), crosslinked in situ for 30 min and resolved on an SDS polyacrylamide gel (Fig. 9 *B*). Both P2 and HIV probes bound proteins of 50, 40, and 20 kD from PLB-985 cell extracts (Fig. 9 *B*, lanes *1* and *2*). In addition, proteins of 75 and 60 kD interacted weakly with the HIV enhancer. Differences in protein migration were attributable to the size difference between the two probes. It is striking that the HIV probe did not efficiently bind proteins from the PLB-IIIB extracts (Fig. 9 *B*, lane *4*), whereas multiple proteins ranging in size from 50-105 kD were detected using the P2 probe (Fig. 9 *B*, lane *3*). Three prominent proteins of 100, 90, and 70 kD were identified, as well as several other proteins. Comparison of uninduced and TNF induced extracts by UV crosslinking also demonstrated that only the 70-kD protein was inducible by TNF (data not shown). Overall, the profiles of NF-κB DNA binding proteins in PLB-985 and PLB-IIIB cells differ dramatically, indicating that the nature of NF-κB-like interactions is altered in the HIV-infected cells.

Transcription of IFN-α and IFN-β Genes. To examine if changes in the state of differentiation or NF-κB-related activities affected gene expression, the transcription of IFN-α and IFN-β genes was investigated in PLB-985 and PLB-IIIB cells (Fig. 10). In PLB-985 cells, IFN-β RNA was induced by Sendai virus infection 6 h after infection. In PLB-IIIB cells, a low level of constitutive IFN-β mRNA was detected and coinfection by Sendai virus resulted in three- to fourfold more IFN-β RNA than similarly induced PLB-985 cells. In contrast to these results, IFN-α transcription was only weakly induced in PLB-985 cells, but 50-fold higher levels of IFN-α RNA were detected in PLB-IIIB cells. Together, these results
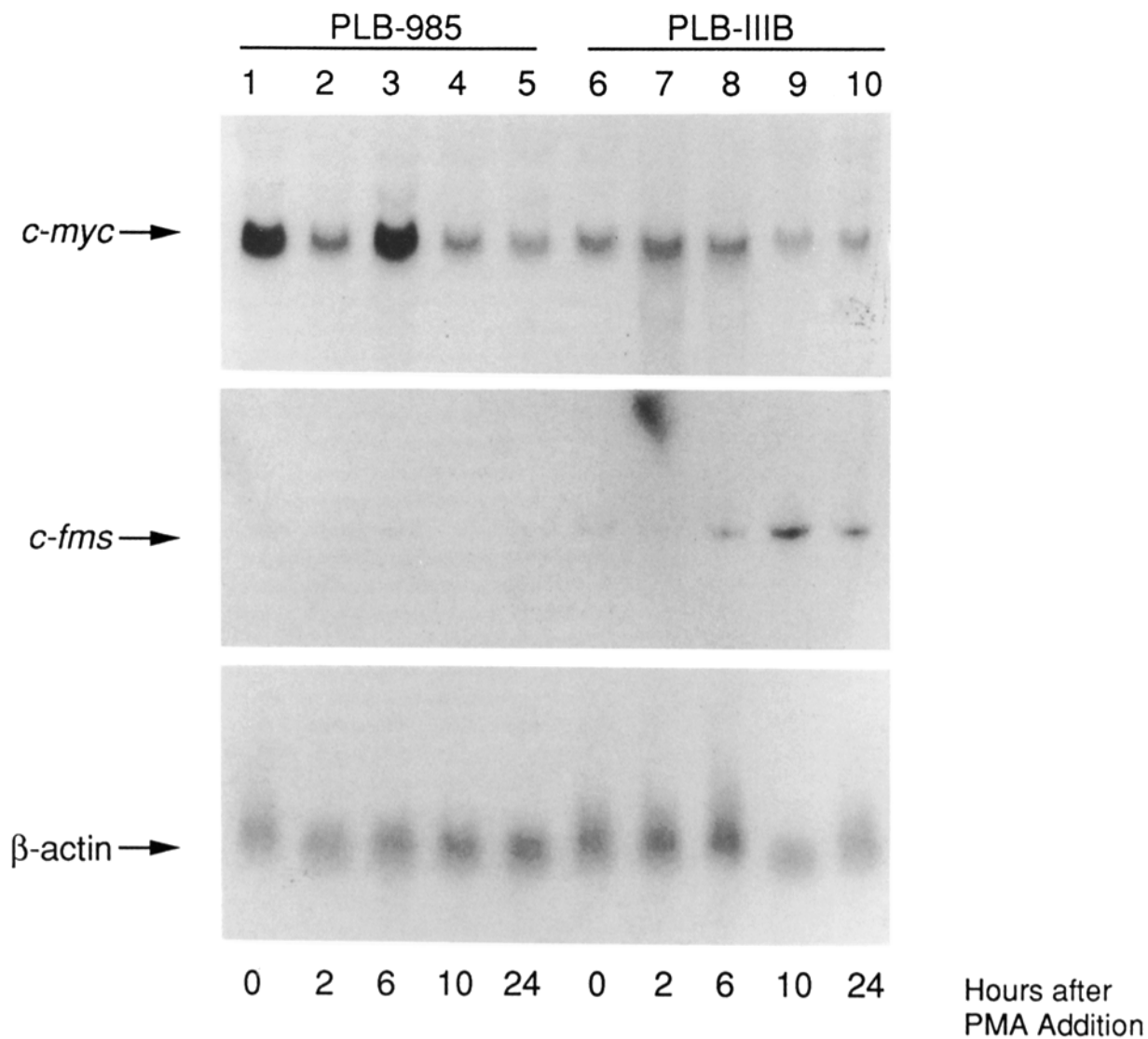
the P2 oligonucleotide which consists of two copies of the NF-κB site found in IFN-β (PRDII site, −64 to −55) and human c-rel (−28 to −18) or the HIV enhancer oligonucleotide which contains two NF-κB–binding sites found in the HIV-1 LTR (−105 to −80). In PLB-985 cells, multiple protein-DNA complexes were induced by 5 or 16 h of PMA or TNF treatment. Both inducers appeared to be equally effective in stimulating NF-κB–DNA binding activities (Fig. 5 *A*, lanes *1-3* and *7-9*). In PLB-IIIB cells, a distinct pattern of protein-DNA complex formation (complex A) was detected consisting of strong constitutive P2 DNA binding activity; formation of slowly migrating complexes; and weak inducibility of this DNA binding activity by either PMA or TNF (Fig. 5 *A*, lanes *4-6* and *10-12*). A similar profile was observed using the HIV enhancer sequence as probe although about fivefold less complex A was detected with this probe (Fig. 5 *B*). In competition experiments, both the P2 oligonucleotide and the HIV enhancer oligonucleotide were able to compete effectively for the TNF-induced NF-κB binding activities in PLB-985 cells (Fig. 6 *A*). However, in PLB-IIIB cells only the P2 probe (Fig. 6 *B*, lanes *2*, *5*, and *8*) competed efficiently for the slower migrating constitutive A complex. The HIV enhancer competed weakly for this DNA binding activity (Fig. 6 *B*, lanes *3*, *6*, and *9*). In dis-

Figure 4. Modulation of c-*myc* and c-*fms* RNA levels in PMA-treated PLB-985 and PLB-IIIB cells. Total RNA (20 μg) from PMA-treated PLB-985 cells (lanes 1–5) or PLB-IIIB cells (lanes 6–10) was electrophoresed in a 1% formaldehyde gel, transferred to a nylon membrane, and hybridized sequentially with c-*myc*, c-*fms*, and β-actin probes as described in Materials and Methods. RNA was isolated at 0, 2, 6, 10, and 24 h after treatment (shown below the autoradiogram). The arrows indicate the positions of the correct RNA signal.

indicate that HIV infection of myelomonoblastic cells results in selection of a cell population with a more monocytic phenotype by several criteria, changes in NF-κB–like protein-DNA interactions and modulation of cytokine gene expression.

Discussion

HIV-1 infection of PLB-985 blast cells resulted in the generation of a chronically infected cell population (PLB-IIIB) possessing morphological, immunological, histochemical, and molecular characteristics of monocytes. Concomitant with HIV infection and myeloid differentiation, dramatic alterations in the protein-DNA interactions occurring at the NF-κB recognition sequence 5'-GGGAAATTCC-3' were observed. Distinct proteins of 100, 90, and 70 kD were identified

in PLB-IIIB cells that possessed a higher affinity for the more symmetric PRDII binding site than for the HIV enhancer sites. These two sequences differ only by the -AA- and -CT dinucleotides in the middle of the binding site, resulting in a more palindromic recognition sequence.

NF-κB represents a family of *rel*-related DNA binding polypeptides that include p45, p50, p55, p65, p75, and p85 (c-*rel*). The activities of these proteins are controlled at several regulatory levels. Multiple DNA binding NF-κB heterodimers with distinct affinities for DNA can form in vitro (22, 42, L. Cohen and J. Hiscott, manuscript submitted for publication), single base changes in the NF-κB site can significantly alter the binding affinity of these heterodimers. Biphasic kinetics of NF-κB induction has been described, characterized by rapid appearance of p55 and p75 forms of NF-κB within
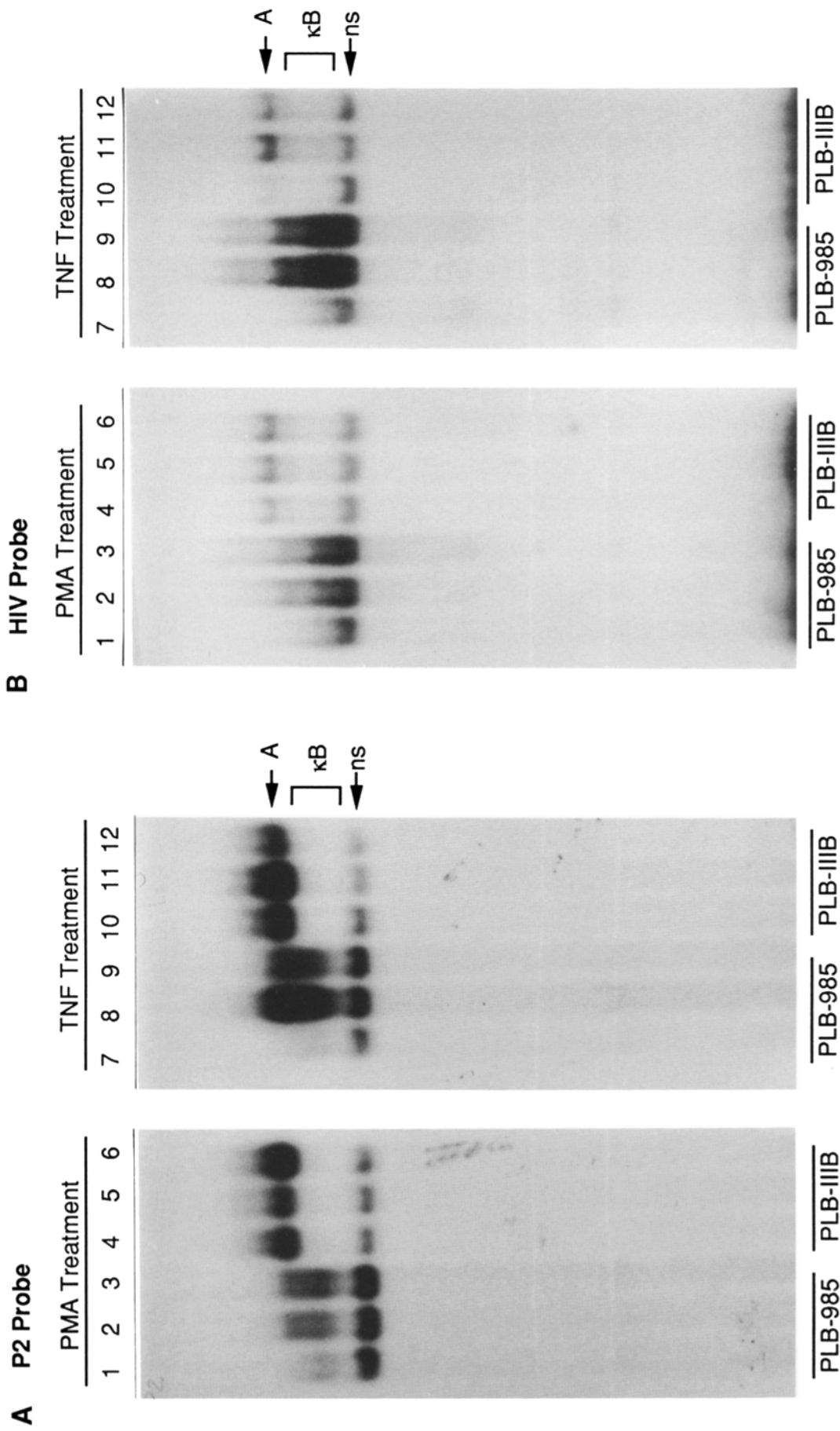
Figure 5. Mobility shift analysis of NF-κB-binding proteins in PMA and TNF treated PLB-985 and PLB-IIIB cells. (*A*) Whole cell extracts (10 μg) from PLB-985 (lanes *1–3*, and *7–9*) and PLB-IIIB (lanes *4–6* and *10–12*) were analyzed for NF-κB-binding proteins using the P2 oligonucleotide as described in Materials and Methods. Extracts were prepared from cells without treatment with inducers (lanes *1, 4, 7*, and *10*), after PMA treatment for 4 h (lanes *2* and *5*), PMA treatment for 16 h (lanes *3* and *6*), TNF-α treatment for 4 h (lanes *8* and *11*), or TNF-α treatment for 16 h (lanes *9* and *12*). Complex A and a nonspecific (ns) complex are indicated by arrows; NF-κB is indicated by the bracket. (*B*) The HIV enhancer oligonucleotide was used as probe with the same complex.

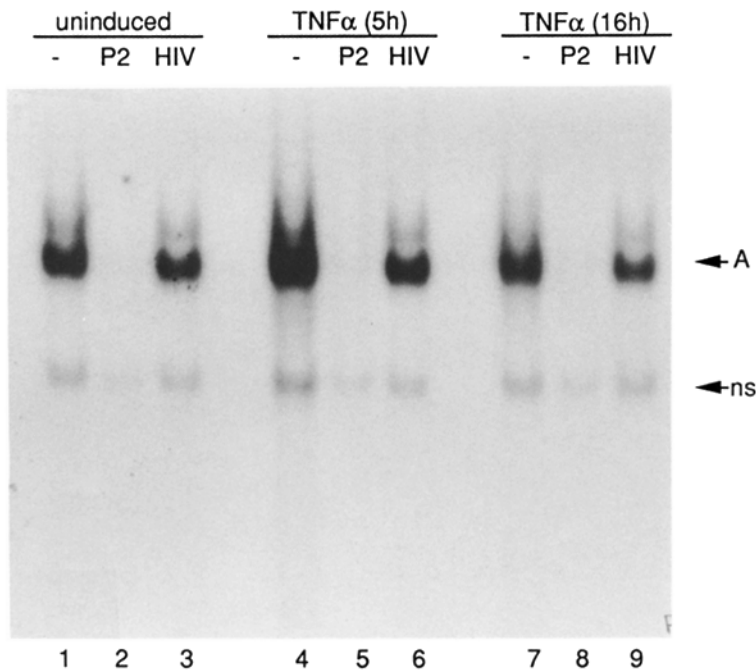## A PLB-985
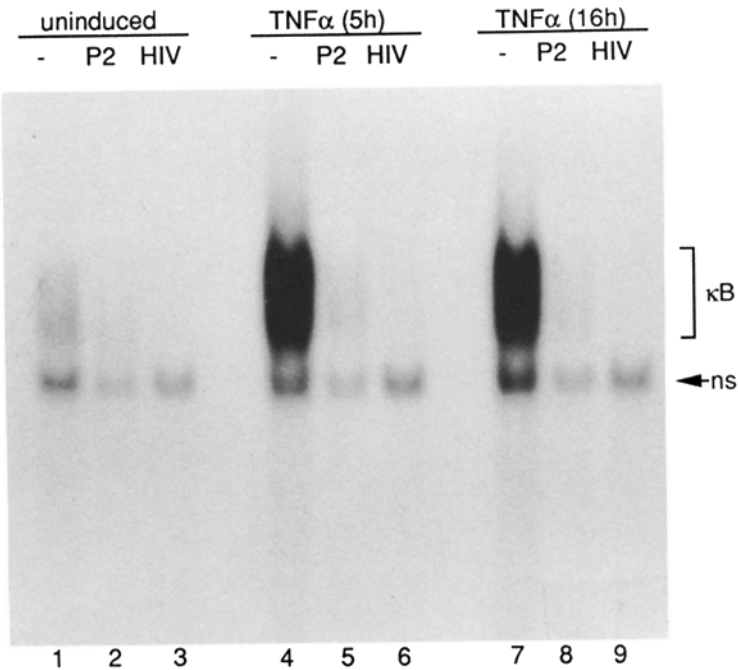
B PLB-IIIB

Figure 6. (*A*) Competition of NF-κB-binding proteins in PLB-985 cells. The protein-DNA complexes present in uninduced (lane *1*), 5-h TNF-treated (lane *4*) or 16-h TNF-treated (lane *7*) extracts were competed using a 125-fold excess of unlabeled P2 oligonucleotide (lanes *2*, *5*, and *8*) or unlabeled HIV enhancer oligonucleotide (lanes *3*, *6*, and *9*). The bracket indicates the position of NF-κB complexes; and the arrow indicates the nonspecific (ns) complex. (*B*) Competition NF-κB-binding proteins in PLB-IIIB cells. The protein-DNA complexes present in uninduced (lane *1*), 5-h TNF-treated (lane *4*), or 16-h TNF-treated (lane *7*) extracts were competed using a 125-fold excess of unlabeled P2 oligonucleotide (lanes *2*, *5*, and *8*) or unlabeled HIV enhancer oligonucleotide (lanes *3*, *6*, and *9*). The arrows indicate the position of the slowly migrating A complex and the nonspecific complex.

minutes, and by the delayed induction of p50 and p85 after several hours of PMA treatment (32). These observations indicate that variations in DNA affinity, heterodimer formation, and induction kinetics may contribute to both positive and negative transcriptional control.

With the availability of cloned NF-κB subunits, it is now clear that transcription of NF-κB-related genes represents an-

other important level of regulation. NF-κB p50, IκB, and c-*rel* gene transcription is rapidly induced by phorbol esters and mitogens (29, 43–45). Furthermore, a new NF-κB 100-kD precursor gene was described recently that is regulated by both alternate splicing and proteolytic cleavage to yield two forms of a distinct p50 subunit (52). HIV-1 infection may have an impact on posttranslational processing of NF-κB
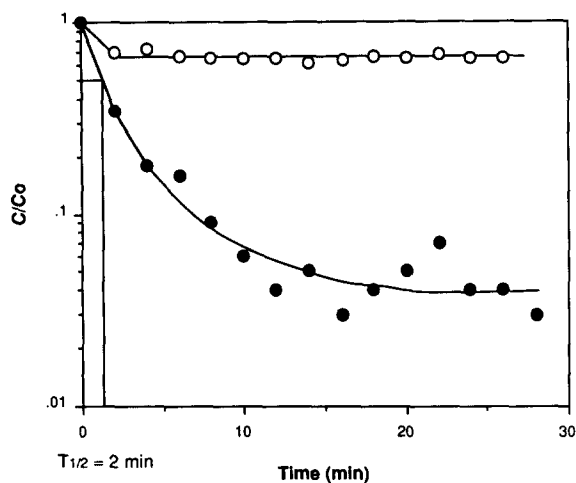
Figure 7. Dissociation rate analysis of the P2 complex. The P2 probe (0.2 ng) was mixed with protein from TNF-induced PLB-IIIB cells (10 μg) in the presence of 5 μg of poly(dI:dC) as nonspecific competitor; at T_0, the amount of complex A was determined. A 125-fold molar excess of P2 oligonucleotide or HIV enhancer oligonucleotide was added to the reaction, and at 2-min intervals the amount of complex A remaining was measured. The relative intensity of the bands at different times was quantified by laser densitometry and plotted as a function of the amount of complex at T_0. (●) P2; (○) HIV.

since the p105 precursor may be cleaved by the HIV-1 protease in vitro (46).

Several possibilities may account for the dramatic differences in DNA binding activity between PLB-985 and PLB-IIIB cells. The induction of distinct or NF-κB–related proteins in PLB-IIIB cells may occur as a result of monocytic differentiation. Recent studies indicate that c-rel may be transcriptionally induced in PLB-IIIB cells (data not shown). Altered stoichiometry of NF-κB subunits because of changes in precursor processing may result in binding of larger, incompletely processed subunits. The 100- and 105-kD p50 precursors may not be processed efficiently in PLB-IIIB cells, resulting in the formation of new combinations of DNA-binding heterodimers, possibly via interactions of the ankyrin repeat. Overexpression of chromatin-associated proteins may stabilize the binding of proteins that would otherwise possess an affinity too low to be detected by mobility shift analysis.

Recently a protein capable of interacting with the PRDII site but not other NF-κB sites has been described that may act as such an accessory protein and, in part, explain the unique pattern of NF-κB binding observed only in the PLB-IIIB cells (47). The HMG I/Y proteins are chromatin-associated proteins that bind in the minor groove of DNA to sequences that are A-T rich or that possess Oct-1 like recognition sites
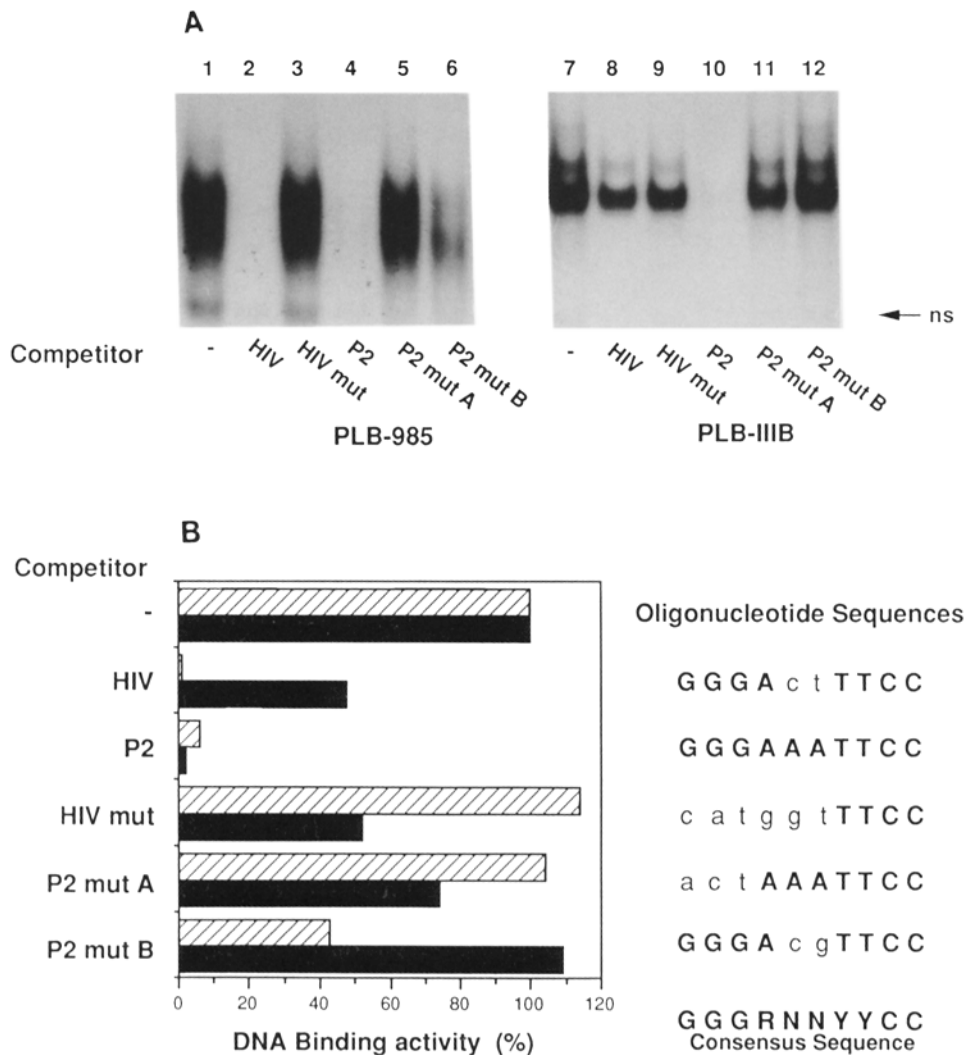

Figure 8. Mutational analysis of the NF-κB-binding sites. (*A*) The protein-DNA complexes present in PLB-985 cells (lane *1*) and PLB-IIIB cells (lane *7*) were competed using a 125-fold excess of unlabeled oligonucleotide corresponding to the HIV enhancer (lanes *2* and *8*), the HIV mutated enhancer (lanes *3* and *9*), the P2 oligonucleotide (lanes *4* and *10*), the P2 mutant A (lanes *5* and *11*), and the P2 mutant B (lanes *6* and *12*). The sequence of these oligonucleotides is shown adjacent to the quantification data. Altered nucleotides relative to the sequence of wild-type P2 are indicated by small letters. (*B*) Quantification of mutant competition. The amount of DNA binding activity remaining after competition was measured by laser densitometry and quantified relative to the amount of binding obtained without competitor. The results represent the average of duplicate competition experiments. (▨) amount of complex remaining from PLB-985 extracts; (■) amount of complex remaining from PLB-IIIB extracts.
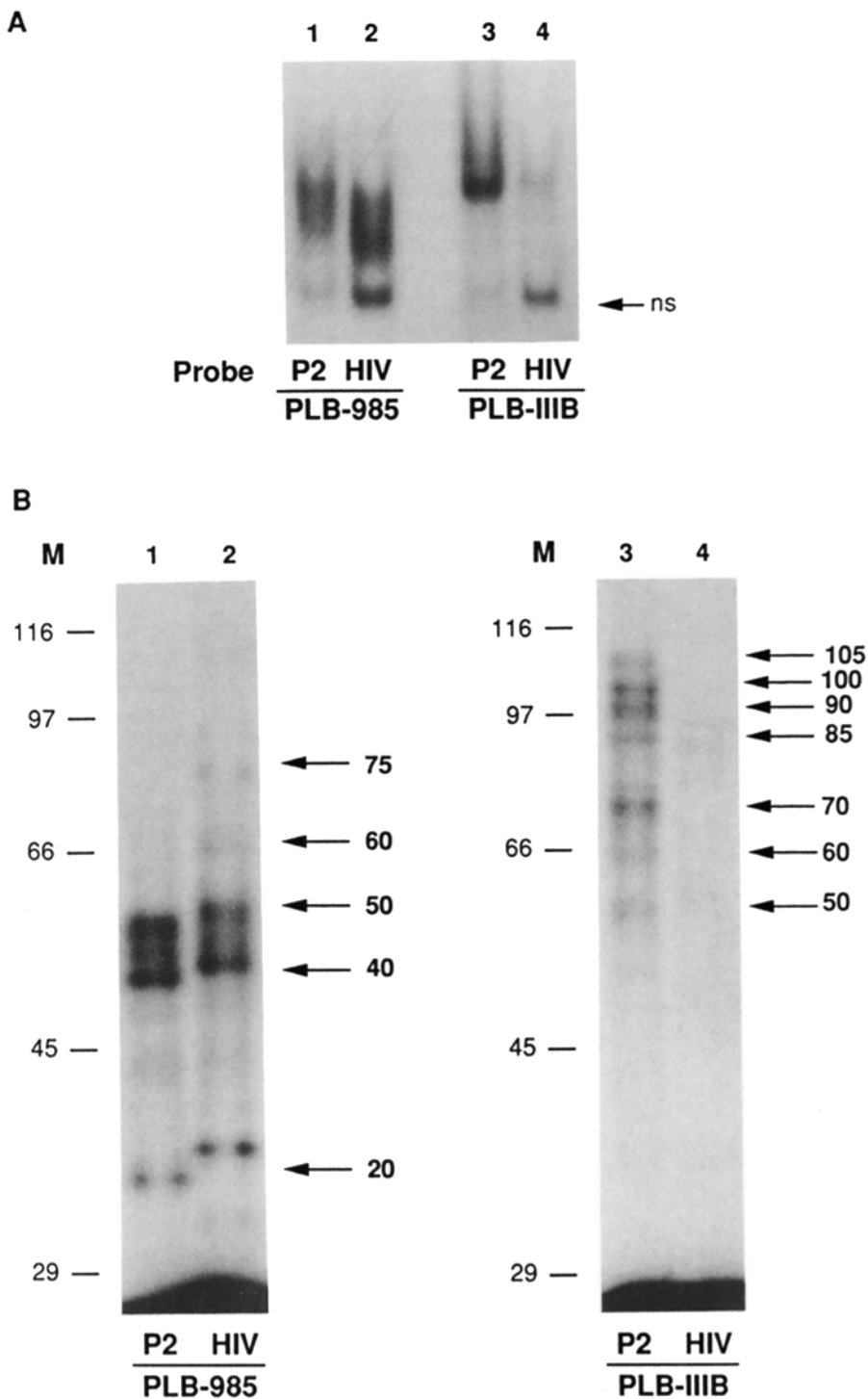
Oligonucleotide Sequences

GGGA c t TTCC

GGGAAATTCC

c a t g g t TTCC

a c t AAATTCC

GGGA c g TTCC

GGGRNNYYCC
Consensus Sequence

A

1 2   3 4

← ns

Probe P2 HIV   P2 HIV
       ───────   ───────
       PLB-985   PLB-IIIB

B

M   1   2

116 —

97 —

← 75

66 —   ← 60

← 50

← 40

45 —

← 20

29 —

P2   HIV
───────
PLB-985

M   3   4

116 —   ← 105
         ← 100
         ← 90
97 —   ← 85

← 70

66 —   ← 60

← 50

45 —

29 —

P2   HIV
───────
PLB-IIIB

Figure 9. UV crosslinking analysis of proteins binding to HIV and P2 oligonucleotides. (A) Mobility shift analysis of NF-κB binding activities in PLB-985 (lanes 1 and 2) and PLB-IIIB (lanes 3 and 4) after treatment with TNF for 5 h using BUdR substituted, ^{32}P-labeled P2 (lanes 1 and 3) or HIV (lanes 2 and 4) probes. The nonspecific complex (ns) was not analyzed. (B) The region of the gel containing NF-κB complex A was cut from the native gel, exposed to UV (302 nm) for 30 min, soaked in SDS sample buffer, and analyzed on a 10% SDS polyacrylamide gel. The positions of molecular mass standards (M) are indicated; the molecular masses of crosslinked proteins are indicated with arrows.

(48, 49). Increased HMG I/Y protein levels have been detected in undifferentiated, rapidly growing and transformed cells (50, 51). HIV-1 infection may be sufficient to increase HMG I/Y levels in PLB-IIIB cells.

In parental PLB-985 cells, PMA treatment resulted in the induction of NF-κB binding activity and monocytic differentiation. These results are in agreement with previous studies that showed that NF-κB binding activity, expression of an HIV-LTR reporter gene, and monocytic differentiation were coordinately inducible in immature myeloid cells (HL-60 and U937). In the more mature P388, THP-1, and P45-1.8 cells, and in primary macrophages, binding activity and basal level expression of the HIV-LTR reporter gene were constitutive and not further inducible, suggesting that NF-κB binding activity is induced during the promyelocytic to monocytic transition (39). However, in other studies, NF-κB binding
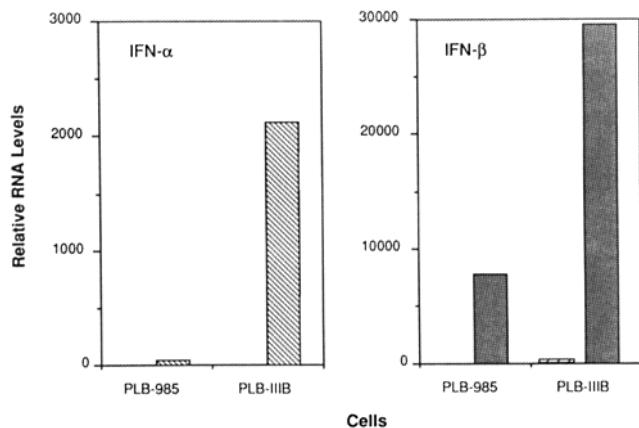
Figure 10. Transcription of IFN α/β genes. Total RNA was isolated from PLB-985 and PLB-IIIB cells after Sendai virus induction for 6 h. Reverse transcription and PCR-mediated amplification was carried out using 1 μg of RNA and ^{32}P-labeled IFN-α and IFN-β consensus primers (12). The products of amplification were resolved by denaturing polyacrylamide gel electrophoresis. The relative RNA levels were quantified by laser densitometry of autoradiographs and presented as a bar graph.

activity was not induced by HIV infection of U937 cells. It is interesting that these experiments identified but did not further characterize a virus induced, slowly migrating protein-DNA complex associated with the HIV enhancer (40). In primary monocytes, both NF-κB and slowly migrating complexes were induced in response to HIV infection (40). The PLB cell line represents a relevant new myeloid model to investigate the relationship between HIV infection, monocytic differentiation, and the induction of these novel NF-κB related activities. It will nonetheless be important to evaluate the results obtained with the cell model in light of findings with primary monocytic cells. Given the abundant regulatory levels of NF-κB activity, it appears that whether developmentally regulated or induced by HIV infection, NF-κB provides a range of potent signals for stimulation of HIV gene expression and cytokine activation.

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