JOURNAL OF VIROLOGY, Jan. 1992, p. 244–250
0022-538X/92/010244-07$02.00/0

Vol. 66, No. 1

Regulation of Human Immunodeficiency Virus Enhancer Function by PRDII-BF1 and c-*rel* Gene Products

CHRISTIAN MUCHARDT, JACOB-S. SEELER, AJAY NIRULA, DIXIE-LEE SHURLAND, AND RICHARD B. GAYNOR*

Division of Molecular Virology, Departments of Medicine and Microbiology, Southwestern Medical School, Dallas, Texas 75235

Received 19 June 1991/Accepted 4 October 1991

The human immunodeficiency virus (HIV) enhancer element is important in the regulation of HIV gene expression. A number of cellular proteins have been demonstrated to bind to the NF-κB motifs in this element. The genes encoding several of these proteins, including members of the *rel* family and PRDII-BF1, have been cloned. We characterized the binding of proteins encoded by the human c-*rel* and PRDII-BF1 genes to HIV NF-κB motifs and related enhancer elements. Both the human c-*rel* protein and two proteins derived from the PRDII-BF1 gene by alternative splicing bound specifically to the HIV NF-κB motif and related enhancer elements found in the immunoglobulin kappa, class I major histocompatibility complex, and interleukin-2 receptor genes. To determine the role of these factors in regulating HIV gene expression, we fused the cDNAs encoding either of the two proteins derived by alternative splicing of the PRDII-BF1 gene or the c-*rel* gene to the DNA binding region of the yeast transcription factor GAL4. GAL4 binding sites were inserted in place of the native HIV enhancer sequences in an HIV long terminal repeat chloramphenicol acetyltransferase construct. Cotransfection of these constructs revealed that c-*rel* was a strong activator of basal HIV gene expression but did not result in synergistic effects in the presence of *tat*. PRDII-BF1-derived cDNAs did not result in stimulation of either basal or *tat*-induced activated gene expression. These results indicate that multiple enhancer binding proteins may potentially regulate HIV in both a positive and negative manner.

Human immunodeficiency virus (HIV) gene expression is regulated by a variety of different regulatory regions in the long terminal repeat (LTR), including the negative regulatory, enhancer, SP1, TATA, and TAR elements. In addition, the transactivator tat protein is critical for high levels of HIV gene expression (summarized in reference 24). A number of different cellular DNA- and RNA-binding proteins have been demonstrated to bind to these regions and regulate HIV gene expression in both a positive and negative manner (14). One of these regions, the HIV enhancer, has been studied in great detail. This region contains two consensus NF-κB binding motifs which are critical for HIV gene expression when assayed in transfection assays (14, 15, 29). The role of these sites in viral growth seems less critical (28), but they appear to be important for high-level HIV gene expression in activated T lymphocytes (20). A number of different cellular factors have been demonstrated to bind to these motifs, including NF-κB, EBP-1, KBF-1, HIVen86, H_2TF1, and PRDII-BF1 (4, 6, 7, 11, 12, 13, 22, 25, 34, 37, 42). However, which of these proteins serve as positive activators of HIV gene expression has not been elucidated.

Recently, the NF-κB proteins have been extensively characterized (1–3, 7, 16, 17, 26, 30). The NF-κB protein family comprises a number of proteins with different kinetics of nuclear localization following T-cell activation. These proteins have in common amino-terminal DNA binding and dimerization domains which are highly homologous to similar domains found in products of the v-*rel*, c-*rel*, and *Drosophila* dorsal genes (7, 9, 10, 17, 19, 26, 30, 40). NF-κB-related proteins of 50, 55, 65, 75, and 85 kDa which bind to NF-κB motifs in the interleukin-2 receptor (IL-2R) promoter have been characterized (7). The p50 subunit of NF-κB is capable of forming homodimers. In addition, different members of the NF-κB family form heterodimers or heterotetramers (3, 7, 26), and one member, p65, complexes with a cytoplasmic inhibitory protein, IκB (1, 2, 16, 30, 33, 43). It is not clear which members of the *rel* family are critical in the regulation of HIV enhancer function.

In addition to the *rel* family, the genes of another family of proteins which bind to the HIV enhancer motif have been characterized (4, 12, 32, 34). One member of this group, known as MBP-1 or PRDII-BF1, encodes a 300-kDa cellular protein with two widely spaced zinc fingers (4, 12). Each of these zinc finger domains is capable of binding to DNA recognition sequences with homology to NF-κB motifs (4, 12). Though the expression of PRDII-BF1/MBP-1 RNA is inducible by phorbol ester and mitogen stimulation of cells, no functional role of these proteins in regulating gene expression or cellular growth properties has been reported (4, 12).

In an attempt to determine the role of the PRDII-BF1 and human c-*rel* genes in regulating HIV gene expression, cDNAs encoding the binding domains of each of the proteins were expressed in bacteria and their binding specificities were determined. These unrelated proteins both bind specifically to the HIV NF-κB motifs and related motifs found in the immunoglobulin kappa (Igκ), major histocompatibility complex (MHC) class I, and IL-2R enhancers (5, 8, 22, 37). To determine their role in regulation of HIV gene expression, cDNAs encoding either c-*rel*, which encodes an 85-kDa protein (9), or genes encoding each of the spliced gene products of PRDII-BF1 (200- and 70-kDa proteins, respectively) were each fused to the DNA binding domain of the yeast protein GAL4 (35, 36). These constructs were assayed in cotransfection assays with HIV LTR chloramphenicol acetyltransferase (CAT) constructs containing GAL4 sites inserted in place of the enhancer element. These results indicate that c-*rel* is capable of activating basal HIV gene

* Corresponding author.

expression, while proteins derived from alternative splicing of the PRDII-BF1 gene do not activate HIV gene expression.

MATERIALS AND METHODS

Cloning of PRDII-BF1 and c-*rel*. (i) PRDII-BF1. Oligo(dT) and random-primed HeLa cell lambda gt11 cDNA expression libraries purchased from Clontech Laboratories Inc. were screened with ligated, nick-translated oligonucleotides corresponding to the wild-type or mutated HIV enhancer regions (38). Inserts from positive phage were excised with *Eco*RI, cloned into pUC19, and characterized by restriction mapping and dideoxy sequencing. This resulted in the cloning of a partial cDNA encompassing the second binding domain of PRDII-BF1 (12). A second round of screening using this partial cDNA as a probe led to the isolation of a fragment covering nucleotides 5641 to 9010. The same probe was also used to screen a lambda GEM 11 genomic library made from leukocytes. This screening resulted in the isolation of a clone covering nucleotides 417 to 6530. This portion of the coding region was not interrupted by introns. The remaining portion of the open reading frame of PRDII-BF1 (nucleotides 280 to 759) was amplified from HeLa cDNA by the polymerase chain reaction (PCR).

(ii) c-*rel*. PCR was used to amplify two fragments from the c-*rel* cDNA, and the fragments were fused at the unique *Eco*RV site at position 1010 in the c-*rel* cDNA sequence (10).

PCR. cDNA was synthesized by hexamer priming 2 μg of poly(A)-selected HeLa RNA, using a Promega RiboClone kit under conditions recommended by manufacturer. PCRs were performed in a DNA Thermal Cycler, using reagents from a GeneAmp kit (Perkin Elmer-Cetus). For each reaction, 1/10 of the cDNA was used along with 50 pmol of primer in a total volume of 100 μl. Mixes were heated for 5 min at 94°C and then incubated for 40 cycles at 55°C for 1 min, at 72°C for 1 min, and at 94°C for 1 min; 2 μl from each reaction mix was then reamplified with at least one nesting primer. Products were analyzed on 5% polyacrylamide gels. Products were usually visible only after the second round of amplification.

PCR primers. Sequences of primers are shown in Fig. 1.

Bacterial expression. The first binding domain (amino acids 342 to 551) and the second binding domain (amino acids 2045 to 2406) of PRDII-BF1 were cloned in frame in pGEX-2T (Pharmacia). A *Bam*HI-*Dra*I (nucleotide 1539) fragment from the c-*rel* cDNA was cloned in frame into *Bam*HI-*Sma*I-cut pGEX-3X. Constructs and a control pGEX-2T without insert were transformed into *Escherichia coli* JM109. Cultures (500 ml) were grown to an optical density at 600 nm of 0.7 to 0.9 and induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Cells were pelleted and resuspended in 10 ml of phosphate-buffered saline (PBS) plus 1% Triton X-100 (Sigma). Cells were mildly sonicated, debris was pelleted, and the supernatant was incubated with 1 ml of glutathione-Sepharose for 90 min at 4°C (39). The beads were then washed five times with 10 ml of PBS. Fusion protein was eluted in 3 ml of Tris-HCl (pH 8.0)–10 mM glutathione for 90 min at 4°C and dialyzed against protein storage buffer (10 mM Tris-HCl [pH 8.0], 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride); 1 μl of this extract was used in gel retardation assays.

In vitro transcription and translation. For in vitro translation of PRDII-BF1 Δ1, a *Pst*I fragment from nucleotides 282 to 733 (obtained by PCR) was fused to a genomic fragment containing nucleotides 733 to 6392 (first stop codon is the seventh codon after the end of the exon). The resulting construct was cloned in pGEM7 downstream of the T7 polymerase promoter and was linearized with either *Sca*I (site in pGEM7), *Xho*I (amino acid 1409), or *Bst*XI (amino acid 926). The PRDII-BF1 Δ2 cDNA was cloned in pGEM2 downstream the SP6 polymerase promoter. This construct was linearized with *Hin*dIII. The full-length c-*rel* cDNA was cloned into the *Sma*I site of pGEM4, linearized with *Hin*dIII, and transcribed with SP6 polymerase. The DNA templates were transcribed in vitro, using a commercial kit for RNA synthesis (Promega). RNA was in vitro translated with micrococcal nuclease-treated rabbit reticulocyte lysate in a 50-μl reaction volume as directed by the supplier (Promega). For analysis of the translation products, proteins were labeled with [^{35}S]methionine and a 5-μl aliquot was run on sodium dodecyl sulfate–8% polyacrylamide gels. After electrophoresis, the gels were dried and subject to autoradiography.

Eucaryotic expression and reporter constructs. To construct fusions of the DNA binding domain of GAL4 (35, 36) and PRDII-BF1 Δ1, an end-filled *Eco* 109I (nucleotide 306)-*Xba*I (in the pGEM7 polylinker) fragment from the pGEM7 expression construct was joined to a *Hin*dIII-*Sma*I fragment containing the first 147 amino acids of GAL4 and inserted into the pDP18 expression vector. To construct fusions of GAL4 and PRDII-BF1 Δ2, an *Eco*RI (nucleotides 6433 to 9010) fragment was fused to the first 147 amino acids of GAL4 (*Hin*dIII-*Eco*RI) and inserted into pDP18 (20). To construct fusions of GAL4 and c-*rel*, an *Nco*I-*Sma*I extending from the *rel* initiating methionine at nucleotide 126 to the end of the cDNA clone was isolated, fused to the first 147 amino acids of GAL4 (*Hin*dIII-*Nco*I), and inserted into

For PRDII-BF1 Δ1:

-CACTATACTGTCAAGCAATA (nucleotide 6313 to 6333)

-TAAGAGGAAGTCAGTTCATTTGATGA (nucleotide 7447 to 7422)

-CGCTGCTGTCTGGGGGCTGTGCTCA (nucleotide 7359 to 7335)

For PRDII-BF1 Δ2:

-ATCTGCAGTTTTTAAGAAGA (nucleotide 280 to 299)

-TTCATTTTCATTGTCATCCTCATCT (nucleotide 6894 to 6869)

-TCCTGTTCATCTATTAAACCTACTG (nucleotide 6794 to 6768)

Spliced forms: (last nucleotide of acceptor and first nucleotide of donor)

	Last	First	Sequence of junction
PRDII-BF1 Δ1	6409	7254	agcattaggtΔATTCTGAGA
PRDII-BF1 Δ2	416	6395	tttcaaaaaaΔAGAGAGCATT

c-*rel*:

Fragment 1:

- GGGAAGGTGCGGGGAGCGGAGCCAT (nucleotide 155 to 180)

-CGTGATCCTGGCACAGTTTCTGGAA (nucleotide 1098 to 1025)

-CCGTAAGTATCTTTTTCATCTGGCA (nucleotide 1040 to 1016)

Fragment 2:

-CAGTAAAAATGCAGTTGCGGAGACC (nucleotide 950 to 974)

-GAAGTTAGTGAATCTATGGATTTTA (nucleotide 985 to 1009)

-GGGTTATACTTGAAAAAATTCATATGG (nucleotide 2038 to 2014)

FIG. 1. Oligonucleotides used in PCR for characterizing regions of the full-length PRDII-BF1 cDNA (12) which were removed by alternative splicing.

pDP18. The HIV LTR CAT construct containing GAL4 binding sites (35, 36) in place of enhancer sequences was constructed by inserting a *Dra*I site at −81 in the HIV LTR and inserting five GAL4 binding sites as a *Pst*I-*Hin*cII fragment from a GAL4/E1B plasmid (28).

DNA binding assays. DNA binding assays were performed with four sets of double-stranded oligonucleotides. These oligonucleotides correspond to enhancer sequences found in HIV (29), Igκ (39), MHC class I (4, 22), and IL-2Rα (8) and were end labelled with [^{32}P]ATP and T4 polynucleotide kinase. The sequences are as follows: HIV enhancer, 5′-CTTTCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3′; Igκ enhancer, 5′-TCGACAACAGAGGGGACTTTCCGAGAGGCCATCC-3′; MHC class I (H-2K^b), 5′-TCGACAAGCCCAGGGCTGGGGATTCCCCATCTCC-3′; and IL-2Rα, 5′-GACAACGGCAGGGGAATCTCCCTCTCCTTCCC-3′.

A second set of mutated oligonucleotides was used to test the specificity of the binding in competition assays. In these oligonucleotides, the underlined sequences were mutated A to G, G to A, C to T, and T to C.

Protein extract was incubated with 1 μg of co-poly(dI-dC) (Pharmacia) in binding buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 50 mM NaCl) for 10 min. When competition assays were performed, the proteins were incubated for a further 10 min with 50 ng of unlabeled oligonucleotide. The labeled probe (50,000 cpm) was added to the mixture, and after 20 min of incubation at room temperature, the samples were loaded on a 4% polyacrylamide gel and electrophoresed in 0.25× TBE (1× TBE is 89 mM Tris, 89 mM borate [pH 8.3], and 1 mM EDTA).

Transfection assays. An HIV LTR CAT plasmid containing five GAL4 binding sites in place of the HIV enhancer region was transfected into HepG2 cells in the presence of constructs containing the DNA binding domain of GAL4 fused to either PRDII-BF1 Δ1, PRDII-BF1 Δ2, c-*rel*, or VP-16. The HepG2 cells were transfected at 40 to 50% confluence and maintained in 10% fetal bovine serum with penicillin and streptomycin. The cells were harvested at 48 h posttransfection, and CAT assays were performed as described previously (18).

RESULTS

PRDII-BF1 yields multiple enhancer-binding proteins by alternative splicing. To characterize the role of PRDII-BF1 on HIV gene expression, we wanted to further characterize and isolate cDNAs encoding this protein. Southern analysis revealed that in addition to the major species hybridizing with the PRDII-BF1 DNA, a number of fainter species were detected (Fig. 2A). This finding is consistent with recent studies including chromosomal mapping of PRDII-BF1, which indicated the likelihood of related gene products (15a, 34). Northern (RNA) analysis of HeLa cytoplasmic mRNA using a portion of the carboxyl terminus of PRDII-BF1 revealed that in addition to the previously described 9.1-kb full-length PRDII-BF1 RNA (4, 12), several smaller mRNA species were routinely detected (Fig. 2). To determine whether these different species were due to the presence of alternative splicing or due to degradation, PCR with a variety of different primers corresponding to different regions of PRDII-BF1 cDNA was performed.

Two cDNAs isolated by PCR analysis of HeLa cytoplasmic RNA revealed that they contained only the first or second zinc finger DNA binding domains of the PRDII-BF1 gene. A schematic of the PRDII-BF1 cDNA and two PCR

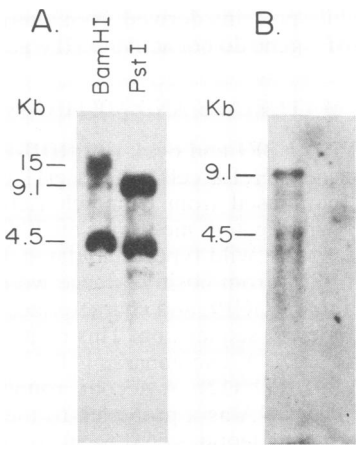

FIG. 2. Northern and Southern analysis of PRDII-BF1. (A) HeLa DNA was restricted with either *Bam*HI or *Pst*I, and Southern analysis was performed with a portion of the PRDII-BF1 cDNA extending from nucleotides 5641 to 9010 (12). (B) HeLa poly(A) RNA was electrophoresed on agarose formamide gels and probed with the same portion of the PRDII-BF1 cDNA used in Southern analysis. The sizes of the hybridizing species are indicated.

products known as Δ1 and Δ2, which contained the first and second binding domains, respectively, are shown (Fig. 3). The nucleotides indicated refer to those in the complete PRDII-BF1 cDNA (12). The Δ1 construct contained the same initiating methionine as PRDII-BF1, and removal of the second DNA binding domain by splicing resulted in a change in the reading frame with introduction of a stop codon at position 7339. The Δ2 construct contained a second initiating methionine in an alternative reading frame; the first DNA binding domain was removed by splicing, and the previously described PRDII-BF1 reading frame was preserved from position 6394 to a stop codon at 8497. Northern analysis using either the amino or carboxy portion of PRDII-BF1 revealed that in addition to hybridizing to the 9.1-kb full-length PRDII-BF1 RNA, these probes hybridized selectively to different smaller RNA species, further indicating their specificity (data not shown). Sequence analysis of these cDNAs revealed potential open reading frames encoding proteins of 200 kDa (Δ1) and 70 kDa (Δ2). To further characterize these cDNAs, each was cloned downstream of either the SP6 or T7 promoter in pGEM-based vectors, in

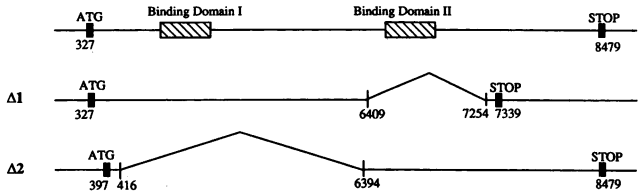

FIG. 3. Schematic diagram of the PRDII-BF1 cDNA and spliced forms. The structure of the PRDII-BF1 cDNA, including the positions of the initiating methionine, two zinc finger DNA binding domains, and the stop codon, is indicated. The structures of two alternatively spliced PRDII-BF1 cDNAs encoding proteins Δ1 and Δ2 are shown, and the positions of the initiating methionines, stop codons, and region of each protein removed by alternative splicing of the full-length PRDII-BF1 cDNA are indicated. Nucleotide numbers shown for the PRDII-BF1 cDNA are those described for the original PRDII-BF1 cDNA (12).

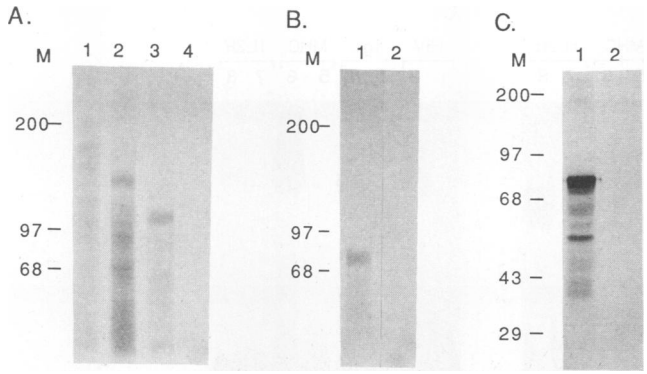

FIG. 4. In vitro translation of PRDII-BF1 cDNAs and c-*rel*. (A) The PRDII-BF1 Δ1 clone in pGEM containing the first zinc finger binding domain was cut at the *Sca*I site in the vector (lane 1) or at either the *Xho*I (lane 2) or *Bst*XI (lane 3) site in the protein coding sequence, in vitro transcribed, and translated. Reticulocyte lysate in the absence of added RNA (lane 4) is also shown. (B) The PRDII-BF1 Δ2 clone in pGEM2 which contains the second zinc finger binding domain was cut at the *Hin*dIII site in the vector and in vitro translation was performed as for lane 1 in panel A. Reticulocyte lysate in the absence of RNA is also shown (lane 2). (C) The c-*rel* cDNA in pGEM4 was cut at the *Hin*dIII site in the vector, in vitro transcribed, and translated (lane 1); reticulocyte lysate in the absence of RNA is also shown (lane 2). Sizes of markers (lanes M) are shown in kilodaltons.

vitro transcribed, and translated with rabbit reticulocyte lysate. As predicted, these cDNAs yielded specific translation products of 200 kDa (Fig. 4A, lane 1) and 70 kDa (Fig. 4B, lane 1), respectively. In addition, a human c-*rel* cDNA was isolated by PCR analysis and then in vitro transcribed and translated (9). This c-*rel* cDNA yielded an 80-kDa translation product, in agreement with published reports (Fig. 4C, lane 1).

PRDII-BF1 and c-*rel* bind specifically to NF-κB motifs in HIV and related enhancers. Previous data indicated that both the *rel* family (7, 17, 21, 26) and PRDII-BF1 (4, 12) bound to recognition elements with homology to the NF-κB binding motifs. To compare the binding of these proteins to related enhancer elements, gel retardation was performed with oligonucleotides corresponding to the HIV enhancer and related motifs in the Igκ, MHC, and IL-2R genes (5, 8, 22, 29, 37) (Fig. 5). Proteins containing either the first or second zinc finger binding domains of PRDII-BF1 or the amino end of c-*rel* were expressed as glutathione-*S*-transferase fusions in bacteria and purified by glutathione-agarose chromatography (39). Gel retardations with these proteins were then performed (Fig. 6).

Protein containing the first binding domain of the PRDII-BF1 gene bound with high affinity to both the HIV and MHC

HIV-1 (prox.)	GGGGACTTTCC
HIV-1 (dist.)	AGGGACTTTCC
Human Ig K enhancer	GGGGATTTCC
MHC class I (H-2K^b)	GGGATTCCCC
Human IL-2Rα	GGGAATCTCC

FIG. 5. NF-κB and related sequences found in HIV (29) (prox., proximal; dist., distal), Igκ (37), MHC class I (5, 22), and IL-2Rα (8) enhancers are shown.

enhancer sequences (Fig. 6A, lanes 2 and 6) but with lower affinity to the Igκ and IL-2R sequences (Fig. 6A, lanes 4 and 8). Protein containing the second PRDII-BF1 binding domain bound with high affinity to all four of the enhancer sequences (Fig. 6B, lanes 2, 4, 6, and 8). This finding indicated that these two zinc finger binding domains in PRDII-BF1 have different affinities for related enhancer sequences. Next we determined the ability of c-*rel* to bind to these same motifs. The c-*rel* protein bound with high affinity to the HIV and MHC enhancer sequences and with somewhat lesser affinity to the Igκ and IL-2R sequences (Fig. 6C, lanes 4 and 8). These results indicate that both PRDII-BF1 and c-*rel* were capable of binding to related enhancer motifs.

To determine the specificity of binding by PRDII-BF1 and c-*rel*, gel retardation was performed with labeled MHC oligonucleotides and competition analysis was performed with unlabeled wild-type and mutated MHC oligonucleotides (Fig. 7). Both the first (Fig. 7A, lane 2) and second (Fig. 7B, lane 2) DNA binding domains of PRDII-BF1 and c-*rel* (Fig. 7C, lane 2) bound specifically to the MHC probe. This binding was specifically competed for by unlabeled MHC oligonucleotides (lanes 3) but not by mutated MHC oligonucleotides (lanes 4). Specific binding to the HIV, Igκ, and IL-2R enhancers was also demonstrated (data not shown).

c-*rel* activates HIV gene expression. Next we determined whether either of the PRDII-BF1 proteins or c-*rel* was capable of activating HIV gene expression. This type of analysis was complicated by the fact that the authentic PRDII-BF1 and c-*rel* proteins may have different binding affinities for the HIV enhancer. Also, *rel* proteins have been demonstrated to bind to the cytoplasmic inhibitor protein IκB, which inhibits the transport of *rel* proteins into the nucleus (1–3, 16, 30, 43). Finally, both PRDII-BF1 and c-*rel* proteins were constitutively expressed in a wide variety of cells, and transfected gene products may not result in effective competition with endogenous factors.

To circumvent these difficulties, we fused the cDNAs encoding the full-length proteins derived from alternative splicing of the PRDII-BF1 gene or the full-length human c-*rel* protein to the DNA binding domain of the yeast protein GAL4 (35, 36). These gene fusions were then cloned into a Rous sarcoma virus (RSV) expression vector (20). Multiple copies of GAL4 binding sites were inserted in place of the enhancer sequences in an HIV LTR CAT plasmid for use as a reporter plasmid in cotransfection assays in both the presence and absence of *tat* (Fig. 8). The HepG2 cell line was used in transfection assays because it contains low levels of several enhancer-binding proteins, including AP-2 (41). Similar results were also seen in the Jurkat cells (data not shown). Neither GAL4/PRDII-BF1 cDNA fusions containing the Δ1 protein (Fig. 8A, lanes 1 to 3) or the Δ2 protein (Fig. 8A, lanes 4 to 6) were capable of activating basal HIV gene expression. In fact, constructs containing the Δ1 protein reproducibly gave lower levels of HIV gene expression with increasing amounts of transfected plasmid (Fig. 8A, lane 3). A GAL4/c-*rel* fusion reproducibly gave 10- to 15-fold stimulation of basal HIV gene expression (Fig. 8A, lanes 7 to 9). The level of activation of the HIV LTR CAT construct by c-*rel* was nearly as high as seen with GAL4/VP-16 fusions (Fig. 8A, lanes 10 to 12), which have been demonstrated to activate a variety of viral and cellular promoters (35, 36).

These constructs were also tested in both the presence and absence of *tat*. Again GAL4/c-*rel* (Fig. 8B, lane 7) significantly activated HIV LTR basal gene expression compared with GAL4 (Fig. 8B, lane 1), GAL4/Δ1 (Fig. 8B, lanes 3), and GAL4/Δ2 (Fig. 8B, lane 5). There was no significant

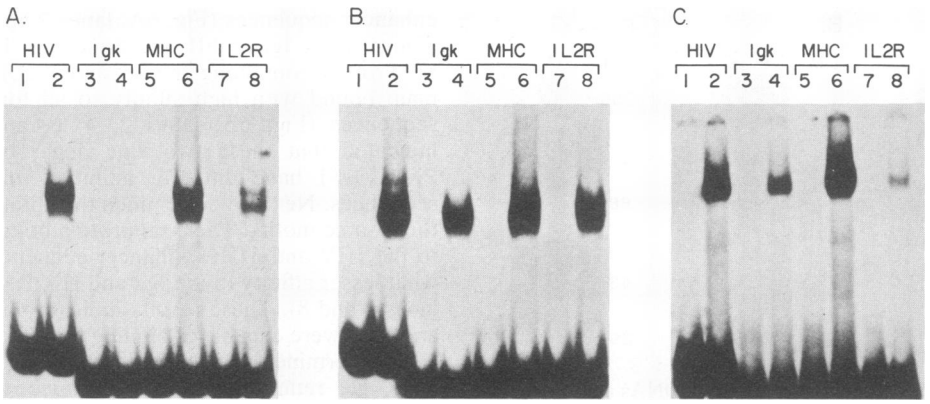

FIG. 6. Binding of bacterially produced PRDII-BF1 and c-*rel* to related NF-κB-related motifs. The first binding domain of PRDII-BF1 (A), the second binding domain of PRDII-BF1 (B), and c-*rel* (C) were expressed as glutathione-*S*-transferase fusions and purified by glutathione-agarose column chromatography. Gel retardation was performed with glutathione-*S*-transferase protein alone (lanes 1, 3, 5, and 7) or each of the bacterially synthesized DNA-binding proteins (lanes 2, 4, 6, and 8) and labeled oligonucleotides corresponding to the indicated enhancers.

difference in the level of activation with use of each of these constructs in the presence of *tat* (Fig. 8B, lanes 2, 4, 6, and 8). These results demonstrated that c-*rel*- but not PRDII-BF1-derived proteins was a potent activator of basal HIV gene expression, though no synergy with *tat* activation was detected. Moreover, the specificity of c-*rel* activation of HIV was confirmed by the fact that GAL4/c-*rel* did not activate other promoter constructs containing multimerized GAL4 sites, including the well-characterized construct containing the E1B TATA box (28) (data not shown).

DISCUSSION

The cloning of genes encoding cellular factors which bind to important regulatory motifs in the HIV LTR is critical for understanding the mechanisms governing HIV gene expression. One regulatory region, the enhancer, has been the subject of intense investigation. This region extending between −108 and −80 in the HIV LTR contains two NF-κB motifs which are also found in a number of other viral and cellular enhancer elements, including those of the cytomegalovirus, simian virus 40, Igκ, and IL-2R (summarized in reference 24). Mutagenesis of the HIV enhancer region results in decreases in both basal and *tat*-induced gene expression from the HIV LTR in a variety of different cell lines (15, 29). NF-κB motif mutations inserted into proviral constructs do not result in defects in viral growth (27), but a role for these motifs in HIV gene expression is seen in activated T cells (20). This finding further substantiates the role of the enhancer element in the regulation of HIV gene expression.

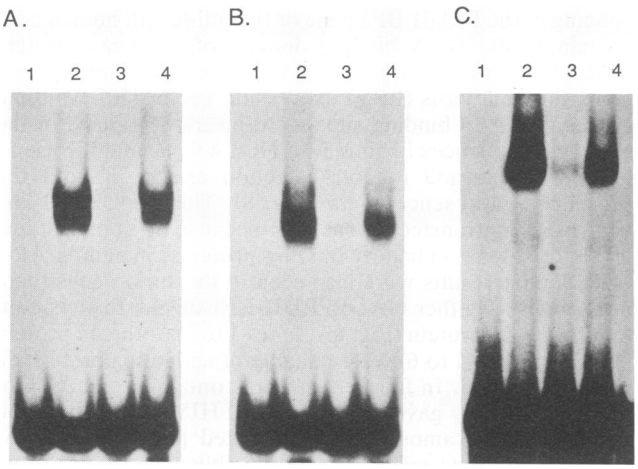

FIG. 7. Gel retardation and competition analysis of bacterially produced PRDII-BF1 and c-*rel* with the MHC enhancer. The first (A) and second (B) binding domains of PRDII-BF1 and c-*rel* protein (C) expressed as glutathione-*S*-transferase fusions were assayed by gel retardation with labeled oligonucleotides corresponding to the MHC enhancer. Lanes: 1, glutathione-*S*-transferase protein alone; 2, protein with either the first (A) or second (B) binding domain of PRDII-BF1 or c-*rel* protein (C); 3, the same proteins with a 20-fold molar excess of unlabeled wild-type MHC oligonucleotides; lane 4, the proteins with a 20-fold molar excess of unlabeled mutated MHC oligonucleotides.

A number of different cellular factors, including NF-κB (37), EBP-1 (11, 42), H_2TF1 (5, 6), KBF-1 (22), and HIVen86 (13), have been demonstrated to bind to NF-κB motifs. Recent studies indicate that NF-κB proteins, including HIVen86, comprise a family of proteins related to the proto-oncogene c-*rel* (1–3, 7, 16, 26, 30). This family of proteins is evolutionarily conserved in that a *rel*-related protein known as dorsal is also found in *Drosophila* cells (40). The genes encoding several of these *rel*-related proteins, including p50, p65, and p80, have been cloned and shown to be highly conserved in their amino-terminal DNA binding and dimerization domains (7, 9, 10, 17, 26, 30, 33). Another cellular factor known as PRDII-BF1/MBP-1 also binds to NF-κB motifs (4, 12). This 300-kDa protein contains two widely separated zinc finger binding domains, each of which bind to NF-κB motifs (4, 12). Like the *rel* family, factors related to PRDII-BF1 have been identified in both human and rat tissues (32, 34). These studies define two families of enhancer-binding proteins which bind to NF-κB and related enhancer motifs.

Since we noted that several mRNAs were detected on Northern blots with a PRDII-BF1 cDNA as a probe, we used PCR analysis of HeLa cytoplasmic RNA to determine whether alternative splicing of PRDII-BF1 gives rise to different enhancer-binding factors. We isolated two cDNAs encoding cellular proteins of 200 and 70 kDa which contain either of two zinc finger DNA binding domains. The binding of bacterially produced PRDII-BF1 and c-*rel* proteins were

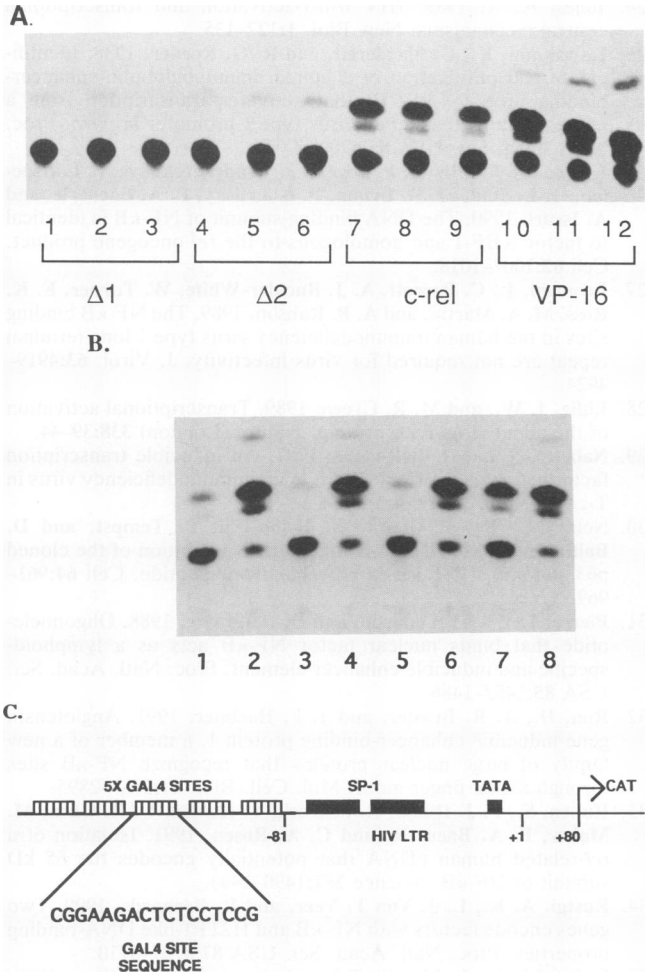

FIG. 8. Activation of HIV gene expression by GAL4/c-*rel* fusions. (A) An HIV LTR CAT plasmid containing five GAL4 binding sites in place of the NF-κB enhancer sequences was cotransfected into HepG2 cells with either 1, 5, or 10 μg of eucaryotic expression vector plasmids containing GAL4/Δ1 (lanes 1 to 3), GAL4/Δ2 (lanes 4 to 6), GAL4/c-*rel* (lanes 7 to 9), or GAL4/VP-16 (lanes 10 to 12), and CAT activity determined 48 h posttransfection. In each transfection, the total concentration of the Rous sarcoma virus expression vector was kept constant. (B) The HIV LTR CAT reporter construct was transfected with 5 μg of either GAL4 (lanes 1 and 2), GAL4/Δ1 (lanes 3 and 4), GAL4/Δ2 (lanes 5 and 6), or GAL4/c-*rel* (lanes 7 and 8) in either the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of *tat*. (C) Diagram of the HIV LTR CAT construct with five GAL4 binding sites replacing the enhancer and a part of the third SP-1 binding site.

tested in gel retardation assays, using oligonucleotides corresponding to enhancers found in HIV, Igκ, MHC class I, and IL-2R genes (5, 8, 22, 37). Our results indicate that both of the PRDII-BF1-derived gene products are able to bind specifically to related motifs. Likewise, c-*rel* is able to bind specifically to these same regulatory motifs. However, we note that the PRDII-BF1 and c-*rel* bind differently to several NF-κB-related binding sequences. These studies indicate that at least two different genes may potentially be involved in the regulation of HIV enhancer function.

To determine the role of these factors in the regulation of HIV gene expression, we constructed fusions of these different enhancer-binding proteins with the DNA binding domain of the yeast transcription factor GAL4 (35, 36). These fusions allow a direct comparison of the activation phenotypes of different factors that bind to the same recognition motif. Furthermore, both the c-*rel* and PRDII-BF1 gene products are expressed constitutively in most cell lines, complicating the assay of these transfected genes. Our results demonstrate that c-*rel* but not cDNAs derived from PRDII-BF1 by alternative splicing are capable of activating HIV LTR CAT gene expression via GAL4 sites inserted in place of the enhancer element. The specificity of this c-*rel* activation of HIV was suggested by the failure of GAL4/c-*rel* to activate gene expression from other reporter constructs containing multimerized GAL4 sites.

In our binding and transfection experiments, we assayed cDNAs derived from the PRDII-BF1 gene but did not assay the full-length PRDII-BF1 cDNA, which encodes a 300-kDa protein. Further studies using full-length PRDII-BF1 and c-*rel* proteins to assay their role on both basal and *tat*-induced HIV gene expression are under way. However, potential competition of the PRDII-BF1 and *rel* family of proteins on the regulation of HIV gene expression remains possible. Since NF-κB proteins mediated by the p65 subunit complex with IκB in the cytoplasm of cells (1–3, 16, 30, 43), the concentration of *rel* proteins in the nucleus of resting cells is likely quite low. Under these conditions, PRDII-BF1, which is a nuclear protein (4, 12), likely binds to the HIV LTR, resulting in low levels of HIV gene expression. However, upon activation of cells by various mitogens, *rel* proteins are released by phosphorylation of IκB and localize to the nucleus (16). When *rel* proteins are translocated to the nucleus, they are likely capable of competing with PRDII-BF1-derived proteins for binding to the HIV enhancer region, with subsequent activation of HIV gene expression. Similar competition between inducible and constitutive enhancer-binding factors in the MHC class I gene have been described (23). Thus, a study of different cellular factors which bind to NF-κB motifs will be important in determining the mechanism by which HIV enhancer function is regulated.

ACKNOWLEDGMENTS

We thank Tamyra Comeaux and Chuck Leavitt for the preparation of the manuscript.

This work was supported by grants from the National Institutes of Health, Veterans Administration, and UC AIDS Task Force. R.G. was supported by a grant from an American Cancer Society Faculty Research Award, and C.M. was supported by a NATO grant.

REFERENCES

1. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB: a specific inhibitor of the NF-κB transcription factor. Science **242**:540–546.
2. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53**:211–217.
3. **Baeuerle, P. A., and D. Baltimore.** 1989. A 65 kD subunit of active NF-κB is required for inhibition of NF-κB by IκB. Genes Dev. **3**:1689–1698.
4. **Baldwin, A. S., K. P. LeClair, S. Harinder, and P. A. Sharp.** 1990. A large protein containing zinc finger domains binds to related sequence elements in the enhancers of the class I major histocompatibility complex and kappa immunoglobulin genes. Mol. Cell. Biol. **10**:1406–1414.
5. **Baldwin, A. S., and P. A. Sharp.** 1987. Binding of a nuclear factor to a regulatory sequence in the promoter of the mouse H-2K^b class I major histocompatibility gene. Mol. Cell. Biol. **7**:305–313.
6. **Baldwin, A. S., and P. A. Sharp.** 1988. Two transcription factors, NF-κB and H2TF1, interact with a single regulatory

sequence in the class I major histocompatibility complex promoter. Proc. Natl. Acad. Sci. USA **85**:723–727.
7. **Ballard, D. W., W. H. Walker, S. Doerre, P. Sista, J. A. Molitor, E. P. Dixon, N. J. Peffer, M. Hannink, and W. C. Greene.** 1990. The v-*rel* oncogene encodes a κB enhancer binding protein that inhibits NF-κB function. Cell **63**:803–814.
8. **Bohnlein, E., J. W. Lowenthal, M. Siekevitz, D. W. Ballard, B. R. Franza, and W. C. Greene.** 1988. The same inducible nuclear protein(s) regulates mitogen activation of both the interleukin-2-receptor-alpha gene and type 1 HIV. Cell **53**:827–836.
9. **Brownell, E., N. Mittereder, and N. R. Rice.** 1989. A human *rel* proto-oncogene cDNA containing an Alu fragment as a potential coding exon. Oncogene **4**:935–942.
10. **Bull, P., K. L. Morley, M. F. D. Hoekstra, T. Hunter, and I. M. Verma.** 1990. The mouse c-*rel* protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain. Mol. Cell. Biol. **10**:5473–5485.
11. **Clark, L., R. M. Pollock, and R. T. Hay.** 1988. Identification and purification of EBP1: a HeLa cell protein that binds to a region overlapping the 'core' of the SV40 enhancer. Genes Dev. **2**:991–1002.
12. **Fan, C. M., and T. Maniatis.** 1990. A DNA-binding protein containing two widely separated zinc finger motifs that recognize the same DNA sequence. Genes Dev. **4**:29–42.
13. **Franza, B. R., S. F. Josephs, M. Z. Gillman, W. Ryan, and B. Clarkson.** 1987. Characterization of cellular proteins recognizing the HIV enhancer using a microscale DNA-affinity precipitation assay. Nature (London) **330**:391–395.
14. **Garcia, J. A., D. Harrich, E. Soultanakis, F. Wu, R. Mitsuyasu, and R. B. Gaynor.** 1987. Interactions of cellular proteins involved in the transcriptional regulation of the human immunodeficiency virus. EMBO J. **6**:3761–3770.
15. **Gaynor, R. B., M. D. Kuwabara, F. K. Wu, J. A. Garcia, D. Harrich, M. Briskin, R. Well, and D. S. Sigman.** 1988. Repeated B motifs in the human immunodeficiency virus long terminal repeat enhancer region do not exhibit cooperative factor binding. Proc. Natl. Acad. Sci. USA **85**:9406–9410.
15a. **Gaynor, R. B., C. Muchardt, A. Diep, T. K. Mohands, R. S. Sparkes, and A. J. Lusis.** 1991. Localization of the zinc finger DNA-binding protein HIV-EA1/MBP-1/PRDII-BF1 to human chromosome 6p223-p24. Genomics **9**:758–761.
16. **Ghosh, S., and D. Baltimore.** 1990. Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. Nature (London) **334**: 678–682.
17. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D. Baltimore.** 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. Cell **62**:1019–1029.
18. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
19. **Hannink, M., and H. M. Temin.** 1989. Transactivation of gene expression by nuclear and cytoplasmic *rel* proteins. Mol. Cell. Biol. **9**:4323–4336.
20. **Harrich, D., J. A. Garcia, R. Mitsuyasu, and R. B. Gaynor.** 1990. TAR-independent activation of the human immunodeficiency virus in phorbol ester stimulated T-lymphocytes. EMBO J. **9**:4417–4423.
21. **Inoue, J. I., L. D. Kerr, L. J. Ransone, E. Bengel, T. Hunter, and I. M. Verma.** 1991. c-*rel* activates but v-*rel* suppresses transcription from κB sites. Proc. Natl. Acad. Sci. USA **88**:3715–3719.
22. **Israel, A., A. Kimura, M. Kieran, D. Yano, J. Kanellopoulos, O. Le Bail, and P. Kourilsky.** 1987. A common positive trans-acting factor binds to enhancer sequences in the promoters of mouse H-2 and β2-microglobulin genes. Proc. Natl. Acad. Sci. USA **84**:2653–2657.
23. **Israel, A., O. LeBail, D. Hatat, J. Dietta, M. Kieran, F. Logeat, D. Wallach, M. Fellows, and P. Kourilsky.** 1989. TNF stimulates expression of mouse MHC class I genes by inducing an NF-κB-like enhancer binding activity which displaces constitutive factors. EMBO J. **8**:3793–3800.
24. **Jones, K. A.** 1989. HIV *trans*-activation and transcriptional control mechanisms. New Biol. **1**:127–135.
25. **Kawakami, K., C. Sheidereit, and R. G. Roeder.** 1988. Identification and purification of a human immunoglobulin-enhancer-binding protein (NF-κB) that activates transcription from a human immunodeficiency virus type I promoter *in vitro*. Proc. Natl. Acad. Sci. USA **85**:4700–4704.
26. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israel.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF-1 and homologous to the *rel* oncogene product. Cell **62**:1007–1018.
27. **Leonard, J., C. Parrott, A. J. Buckler-White, W. Turner, E. K. Ross, M. A. Martin, and A. B. Rabson.** 1989. The NF-κB binding sites in the human immunodeficiency virus type 1 long terminal repeat are not required for virus infectivity. J. Virol. **63**:4919–4924.
28. **Lillie, J. W., and M. R. Green.** 1989. Transcriptional activation of the adenovirus E1A protein. Nature (London) **338**:39–44.
29. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T-cells. Nature (London) **326**:711–713.
30. **Nolan, G. P., S. Ghosh, L. Hsiou-Chi, P. Tempst, and D. Baltimore.** 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a *rel*-related polypeptide. Cell **64**:961–969.
31. **Pierce, J. W., M. Lenardo, and D. Baltimore.** 1988. Oligonucleotide that binds nuclear factor NF-κB acts as a lymphoid-specific and inducible enhancer element. Proc. Natl. Acad. Sci. USA **85**:1482–1486.
32. **Ron, D., A. R. Brasier, and J. F. Haebner.** 1991. Angiotensin gene-inducible enhancer-binding protein 1, a member of a new family of large nuclear proteins that recognize NF-κB sites through a zinc finger motif. Mol. Cell. Biol. **11**:2887–2895.
33. **Ruben, S., P. J. Dillon, R. Schreck, T. Henkel, C. H. Chen, M. Maher, P. A. Baeuerle, and C. A. Rosen.** 1991. Isolation of a *rel*-related human cDNA that potentially encodes the 65 kD subunit of NF-κB. Science **251**:1490–1493.
34. **Rustgi, A. K., L. J. Van T. Veer, and R. Bernards.** 1990. Two genes encode factors with NF-κB and H2TF1-like DNA-binding properties. Proc. Natl. Acad. Sci. USA **87**:8707–8710.
35. **Sadowski, I., J. Ma, S. Triezenberg, and M. Ptashne.** 1988. GAL4-VP16 is an unusually potent transcriptional activator. Nature (London) **335**:563–564.
36. **Sadowski, I., and M. Ptashne.** 1989. A vector for expressing GAL4 (1-147) fusion in mammalian cells. Nucleic Acids Res. **17**:7539.
37. **Sen, R., and D. Baltimore.** 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a post-translational mechanism. Cell **47**:921–928.
38. **Singh, H., J. H. LeBowitz, A. S. Baldwin, and P. A. Sharp.** 1988. Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA. Cell **52**:415–423.
39. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene **67**:31–40.
40. **Steward, R.** 1987. *Dorsal*, an embryonic polarity gene in *Drosophila* is homologous to the vertebrate proto-oncogene c-*rel*. Science **238**:692–694.
41. **Williams, T., A. Admon, B. Luscher, and R. Tjian.** 1988. Cloning and expression of AP-2, a cell-type specific transcription factor that activates inducible enhancer elements. Genes Dev. **2**:1567–1569.
42. **Wu, F. K., J. A. Garcia, D. Harrich, and R. B. Gaynor.** 1988. Purification of the human immunodeficiency virus type 1 enhancer and TAR binding proteins EBP-1 and UBP-1. EMBO J. **7**:2117–2129.
43. **Zabel, U., and P. A. Baeuerle.** 1990. Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor and its cognate DNA. Cell **61**:255–265.