Transcriptional regulation of the HIV-1 promoter by NF- κ B in vitro

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NF- κ B, purified from HeLa cell cytosol, and a recombinant p50 subunit of NF- κ B alone (expressed in and purified from bacteria) both stimulated transcription from the HIV-1 promoter in vitro (at least up to 15-fold). A deletion analysis of the p50 subunit revealed that transcriptional activation was mediated by the conserved c-rel-related domain. I κ B- β (or a related protein), which binds to the p65 but not the p50 subunit of NF- κ B, inhibited stimulation by natural NF- κ B but not by recombinant p50. Experiments employing a purified transcription system revealed that efficient induction of transcription by both natural NF- κ B or recombinant p50 required a cofactor fraction in addition to the general initiation factors. Combined with DNA-binding experiments, these studies suggest a role of p50 homodimers in transcriptional activation of certain promoters, with a possible preference for those carrying symmetric NF- κ B recognition sites, and a potential role of I κ B- β in direct transcriptional regulation within the nucleus.

[Key Words: NF- κ B; I κ B; cofactor USA; HIV-1 promoter; in vitro transcription]

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The transcription factor NF- κ B belongs to a family of c-Rel-related proteins that activate certain viral promoters and cellular genes involved in inflammation, immune and acute phase responses, and possibly the control of cell growth and differentiation (Lenardo et al. 1987, 1989; Nabel and Baltimore 1987; Boehnlein et al. 1988; Leung and Nabel 1988; Duyao et al. 1990; Liberman and Baltimore, 1990; for review, see Baeuerle and Baltimore 1991).

In many cell types, including T lymphocytes and HeLa cells, NF- κ B was found only in an inducible form in the cytoplasm, whereas in B lymphocytes NF- κ B DNA-binding activity is constitutively present in the nucleus (Sen and Baltimore 1986b). The inducible NF- κ B is part of a signal transduction pathway, and its activity seems to be regulated primarily at the post-translational level (Baeuerle and Baltimore 1989). According to the original model, active NF- κ B consists of two different subunits with molecular masses of ~50 kD (p50) and ~65 kD (p65) (Kawakami et al. 1988; Baeuerle and Baltimore 1989). Preceding induction, the p50/p65 heterodimer is located in the cytoplasm, presumably bound to an additional inhibitory subunit termed I κ B (Ghosh and Baltimore 1990; Zabel and Baeuerle 1990). Induction of this cytoplasmic heterotrimer may involve phosphorylation of I κ B by protein kinase C or heme-regulated eIF-2 kinase (HRI) (Shirakawa and Mizel 1989; Ghosh and Baltimore 1990). This causes dissociation of the protein complex and the subsequent translocation of the NF- κ B heterodimer (p50/p65) into the nucleus (Baeuerle and Baltimore 1988a,b).

Several other cellular and viral proteins have been reported to bind specifically to the decameric NF- κ B recognition motifs. These include a 49-kD protein (p49) (Schmid et al. 1991), the viral v-rel oncogene product, and its cellular counterpart, the c-Rel protein, which is identical to the HIVEN 86A protein (Ballard et al. 1990). These proteins are highly homologous in their amino-terminal regions to the p50 and p65 subunits of NF- κ B, as well as to the *Drosophila* maternal effect protein dorsal (Ghosh et al. 1990; Nolan et al. 1991; Ruben et al. 1991; Schmid et al. 1991). When present in the same cell, the different Rel-related proteins can presumably complex with each other (Kieran et al. 1990; Logeat et al. 1991; Schmid et al. 1991) via the conserved domain, which is necessary and sufficient for DNA binding and dimerization (Ghosh et al. 1990; Kieran et al. 1990; Ip et al. 1991; Nolan et al. 1991). Furthermore, the cellular proteins H2TF1 (Baldwin and Sharp 1988), EBP-1 (Clark et al. 1988), MBP-1 / PRDIIIBF1 (Baldwin et al. 1990; Fan and Maniatis 1990), and TCIB (Macchi et al. 1989) bind to NF- κ B-related sites. Taken together, depending on the status of a cell, various protein species may compete for NF- κ B target sequences in cellular or viral promoters with currently unknown consequences for the activation of specific genes.

Activation of transcription through NF- κ B sites was demonstrated in transfection experiments for a number of genes including the β -interferon gene (Lenardo et al. 1989), the interleukin-2 receptor α -chain gene (Boehnlein et al. 1988; Leung and Nabel 1988), the immunoglobulin κ light-chain gene (Lenardo et al. 1987),

and the HIV-1 promoter (Nabel and Baltimore 1987; Boehnlein et al. 1988). Cotransfection experiments using appropriate reporter constructs and expression vectors encoding Gal4/c-Rel fusion proteins p49, p50, or p65 suggested that transcriptional activity resides within c-*rel*, p65, and p49 but failed to show such activity for the p50 subunit of NF- κ B (Bull et al. 1990; Schmid et al. 1991). However, the activity of the different subunits may be regulated within the cell by modifications and/or binding of regulatory factors [i.e., specific inhibitors such as I κ B- α , I κ B- β , pp40, or related proteins (Zabel and Baeuerle 1990; Davis et al. 1991; Kerr et al. 1991)]. In addition, distinct homo- or heterodimers comprised of different members of the family recognize certain NF- κ B recognition sites with different affinities (Urban and Baeuerle 1990; Rattner et al. 1991; Urban et al. 1991; Zabel et al. 1991). Therefore, in vitro DNA-binding and transcription experiments will be crucial for further understanding of the activities of each family member. Although the DNA-binding properties of the NF- κ B subunits p50 and p65 have been studied extensively (Urban et al. 1991; Zabel et al. 1991 and references therein), very little is known about their transcriptional activities in vitro. Only one study analyzed a relatively defined purified NF- κ B (isolated from B-cell nuclei, where NF- κ B is present constitutively) for its ability to activate the HIV-1 promoter using a crude HeLa cell nuclear extract as transcription system (Kawakami et al. 1988).

Here, we analyzed purified inducible cytoplasmic NF κ B as well as the recombinant p50 subunit in DNA-binding and in vitro transcription experiments. To avoid interference with other Rel-related factors we employed for several crucial experiments a purified transcription system that included the general transcription factors IIA, IIB, IID, IIE, IIF, and RNA polymerase II. Our results indicate that both inducible heteromeric NF- κ B and the p50 subunit alone are potent activators of transcription. Stimulation by both activators was dependent on a recently defined cofactor fraction, designated USA (upstream factor stimulatory activity; Meisterernst et al. 1991). Activation by heteromeric natural NF- κ B, but not by recombinant p50 homodimers, was completely abolished by addition of purified I κ B- β , which confirmed that recombinant p50 alone (i.e., in the absence of p65) activates transcription. These experiments and DNA-binding studies suggest novel roles for the p50 subunit and the I κ B protein within the cell.

Results

Purification of NF- κ B and I κ B- β from HeLa cell cytosol and expression in and purification from bacteria of the p50 subunit of NF- κ B

NF- κ B and I κ B were isolated following ammonium sulfate precipitation of S-100 cytosolic extracts from HeLa cells (Fig. 1A). In the next purification step, NF- κ B and I κ B coeluted at 0.1 M KCl from a phosphocellulose (P11) column as an NF- κ B/I κ B complex (assayed by the dissociation-dependent formation of a NF- κ B/DNA complex;

see below). NF- κ B was then dissociated from I κ B by treatment with deoxycholate and formamide (Baeuerle and Baltimore 1988b) and separated from I κ B on a second phosphocellulose (P11) column, from which it eluted at 0.3 M KCl. Subsequently, NF- κ B was concentrated with DEAE-Sephadex and purified further by specific DNA affinity chromatography. Purified NF- κ B formed a single complex with synthetic DNA fragments containing the NF- κ B recognition motif of the Ig κ enhancer (Fig. 1B, lane 1). To analyze the peptide composition of this protein-DNA complex, a sample of the DE52 0.15 M KCl fraction was subjected to SDS-PAGE and the proteins were subsequently eluted and renatured from different gel slices. When fractions containing the proteins with molecular masses of ~50 and ~65 kD, respectively, were co-renatured, they formed a specific DNA-protein complex with an electrophoretic mobility identical to that of the complex formed with affinity-purified NF- κ B (Fig. 1B, cf. lanes 1 and 3). When renatured separately, the fraction containing the 50-kD proteins formed a complex with a lower mobility, whereas the 65-kD fraction alone failed to give rise to any detectable complex under the same conditions (data not shown). Together with an analysis on a silver-stained SDS-polyacrylamide gel (Fig. 1C), these results confirmed that the inducible NF- κ B complex consists of two subunits with molecular masses of ~50 kD (p50) and ~65 kD (p65). This is in accordance with previous determinations of the peptide composition of NF- κ B purified from Namalwa B-cell nuclear extract (Kawakami et al. 1988) and from HeLa cell cytosol (Baeuerle and Baltimore 1989). Western blot analyses using specific antibodies directed against p49, p50, or a carboxy-terminal region of c-Rel failed to reveal any of the related c-Rel protein (Fig. 1D, lane 4) or p49 in the purified NF- κ B fraction, but proved that p50 was a component of this complex (data not shown).

After dissociation from NF- κ B, I κ B was further purified and enriched on DEAE-Sephadex and Mono Q (FPLC) columns. I κ B activity was monitored in gel-shift assays as a reversible inhibition of NF- κ B binding to DNA. Treatment of I κ B with deoxycholate and formamide (as used in the purification procedure) prevented inhibition of NF- κ B. To determine the molecular weight of I κ B, a sample of the purified I κ B activity (Mono Q fraction) was separated on an SDS-gel and the polypeptides were renatured from the gel as described for NF- κ B. Subsequent gel-shift experiments revealed that the inhibitory activity was included in the 43- to 47-kD fraction (Fig. 1B, lane 2) but not in fractions containing polypeptides with molecular masses of 33–43 kD (data not shown). This indicated purification of I κ B- β (or a related protein of similar size), a 43- to 45-kD protein, but not of the smaller I κ B- α , which has a molecular mass of ~37 kD (Zabel and Baeuerle 1990; Davis et al. 1991; Haskill et al. 1991). A further control showed that co-renatured NF- κ B also was efficiently inhibited in DNA binding by renatured I κ B- β (Fig. 1B, lane 4).

Recently, several groups reported the cloning of a cDNA encoding the smaller subunit of NF- κ B, which has a molecular mass of ~50 kD (Bours et al. 1990; Ghosh et

Figure 1. Purification and characterization of natural NF- κ B and I κ B. (A) Scheme for the purification of the transcription factor NF- κ B and the inhibitor I κ B from HeLa cell cytosolic extracts. (B) Analysis of the peptide composition of purified NF- κ B and I κ B. Purified NF- κ B (DE52 fraction) and I κ B (Mono Q fraction) were subjected to SDS-PAGE, and proteins with different molecular weights, eluted and renatured from the gel, were tested in DNA-binding or I κ B assays using a probe [KK-1/2] that carried the NF- κ B-binding site of the mouse Ig κ enhancer. Proteins with molecular masses of ~50 and ~65 kD, derived from the NF- κ B fraction, were either renatured separately or corenated. Shown are DNA binding of purified NF- κ B (DE52 fraction) (lane 1), inhibition of NF- κ B-binding by renatured proteins with molecular masses of 43–47 kD derived from purified I κ B (lane 2), DNA binding of corenated proteins with molecular masses of 48–55 kD and 63–70 kD derived from purified NF- κ B (lane 3), and inhibition of the corenated NF- κ B by renatured I κ B (lane 4). (C) SDS-PAGE analysis of the purity of the DNA affinity-purified NF- κ B. DNA affinity-purified NF- κ B (lane 2) was analyzed by gel electrophoresis through a 15% SDS-polyacrylamide gel and subsequent silver staining. (Lane 1) Size markers with their molecular masses indicated. The arrows indicate protein bands that presumably correspond to the p50 and p65 subunits of NF- κ B, respectively. (D) Western blot analysis of the presence of c-Rel in DNA affinity-purified natural NF- κ B and various other fractions. Two microliters of TFIIA (DEAE 0.3 fraction) (lane 2), 1 μ l of TFIIA (Mono Q fraction) (lane 3), 5 μ l of NF- κ B (DNA affinity fraction) (lane 4), and 1 μ l of TFIIA (P11 0.1 fraction) (lane 5) were subjected to SDS-PAGE and subsequent Western blotting utilizing an antiserum directed against a nonconserved carboxy-terminal peptide of c-Rel (Brownell et al. 1989). Prestained protein size markers were used as control (lane 1). In lanes 2–4 the same fractions and amounts were loaded as were used in the transcription experiments shown in Figs. 4–6, with the exception of the experiments shown in Figs. 4A and 6C, in which only 0.25 and 0.5 μ l of TFIIA (Mono Q) were used, respectively.

al. 1990; Kieran et al. 1990; Meyer et al. 1991). The primary protein product of the cDNA clone is a 105-kD precursor molecule (p105), which is thought to be processed in the cell into the p50 subunit. To study the role of the p50 subunit in transcriptional regulation, we expressed various amino-terminal fragments of the human

p105 precursor in bacteria. Three different deletion fragments of the p105 precursor cDNA (Meyer et al. 1991) were subcloned into pET expression vectors, which were subsequently introduced into *Escherichia coli* strains developed for the T7 RNA polymerase expression system (Studier et al. 1990). The structures and designations of

the p105 precursor and the overexpressed p105 derivatives are illustrated schematically in Figure 2A. The derivatives were comprised of amino-terminal residues 18–368 (41.5 kD), 18–443 (48.6 kD), and 18–503 (55 kD) of the p105 precursor protein. All three recombinant p50 proteins were purified to near homogeneity by ammonium sulfate precipitation of the cell extracts and subsequent heparin–Sepharose chromatography. As one example, the expression and purification of p50(443) is demonstrated in Figure 2B. The procedure worked equally well for all three recombinant p50 proteins, which were estimated to be >95% pure after heparin–Sepharose chromatography (data not shown).

Bacterially expressed p50 binds in a sequence-specific manner to the Ig κ enhancer, but with considerably lower affinity than natural NF- κ B, and binding is not inhibited by I κ B- β

Natural cytoplasmic NF- κ B and the recombinant p50 proteins were subsequently compared with respect to their DNA-binding properties and their interactions with I κ B. As shown previously for renatured or in vitro-translated p50 (Kieran et al. 1990; Urban and Baeuerle 1990), the bacterially expressed p50 derivatives [exemplified here by p50(503)] bound specifically to the NF- κ B recognition site (Fig. 3A, lanes 7,8). In contrast to natural

NF- κ B, they were not inhibited by I κ B- β (Fig. 3A, cf. lanes 1–3 with lanes 4–6), in good agreement with a selective interaction of I κ B- β with the p65 subunit of NF- κ B (Urban and Baeuerle 1990; Zabel and Baeuerle 1990).

To analyze the potential role of p50 homodimers (as compared with heteromeric NF- κ B) in transcriptional activation of the HIV-1 promoter, we initially compared the binding affinities of purified natural NF- κ B, recombinant p50(503), and recombinant p50(443), which appears to correspond closely in size to the physiological p50 subunit, for the NF- κ B recognition site of the Ig κ enhancer. This site is identical in its core sequence to both HIV-1-binding sites. The equilibrium-binding constants of these proteins, determined by a method described previously (Meisterernst et al. 1988), were 7.8×10^{11} /mole for DNA affinity-purified natural NF- κ B, 3.8×10^{10} /mole for p50(443), and 12.1×10^{10} /mole for p50(503) [see Table 1]. Thus, natural heteromeric NF- κ B bound to the Ig κ enhancer site with a 10- to 20-fold higher affinity than did recombinant p50 homodimers.

Because p50 forms homodimers in solution (Baeuerle and Baltimore 1989), it appeared likely that p50 might recognize completely palindromic NF- κ B sequences with higher affinity than nonpalindromic sites such as those of the Ig κ and HIV-1 enhancers. To test this hypothesis we competed binding of the p50 subunit to a radioactively labeled Ig κ enhancer fragment with 4- or

A

Figure 2. Expression in and purification from bacteria of several fragments of the p50 precursor (p105). (A) Schematic illustration of the p50 subunit precursor (p105) and the bacterially expressed deletion proteins. All three deletion proteins contain 11 amino acids encoded by the expression vector at their amino-terminal end, whereas only p50(503) contains in addition a 22-amino-acid vector-encoded tail at its carboxy-terminal end (illustrated as checkered regions). (B) Coomassie-stained SDS–polyacrylamide gel showing the expression and purification of p50(443). Fifty-microliter samples of culture were taken just before (lane 1) and 4 hr after induction with IPTG (lane 2), washed, lysed in loading buffer, and subjected to SDS-PAGE. In addition, 5 μ g total protein of lysed culture (4 hr postinduction) (lane 3), of the redissolved 32% AS-pellet (lane 4), and of three protein peak fractions from heparin–Sepharose chromatography (lanes 5–8) were loaded on the same gel. (Lane 8) Size markers, whose molecular masses are indicated. The arrow indicates the position of the expressed protein, which was estimated to be >95% pure after heparin–Sepharose chromatography.

B

Figure 3. Analysis of the DNA-binding properties of recombinant p50 proteins. (A) Bacterially expressed p50(503) binds to the Ig κ enhancer NF- κ B-recognition site in a sequence-specific manner and is not inhibited by I κ B- β . Decreasing amounts of p50(503) (heparin-Sepharose fractions) were incubated with the probe (KK-1/2) carrying the Ig κ enhancer site either in the absence (lanes 1–3) or in the presence (lanes 3–6) of I κ B- β (Mono Q fraction). Equal amounts of I κ B- β were able to completely inhibit a similarly strong gel mobility shift of purified heteromeric NF- κ B (data not shown). Binding of p50(503) to the labeled probe was competed by a 40-fold molar excess of either unlabeled KK-1/2 oligonucleotide (lane 8) or an unlabeled oligonucleotide identical to KK-1/2 except for two point mutations in the NF- κ B-binding site (KKM-1/2) (lane 7). (B) Comparison of the binding affinities of p50(443) and of natural cytoplasmic NF- κ B to various DNA fragments. DNA affinity-purified p50(443) or natural DNA affinity-purified NF- κ B was incubated with the probe (KK-1/2) in the absence (lane 1) or in the presence (lanes 2–11) of 4- or 24-fold molar

excesses of various competitor DNAs. These DNA fragments carried either the NF- κ B-binding site of the Ig κ enhancer (KK-1/2) (lanes 2,3), the proximal or distal NF- κ B-binding site, respectively, of the HIV-1 promoter (KM-1/2 and KM-5/6) (lanes 4,5 and lanes 6,7), both HIV-1-binding sites [K-1/2] (lanes 8,9), or two completely palindromic binding sites [MP-1/2] (lanes 10,11). Proteins were added last to the reaction mixtures.

24-fold molar excesses of unlabeled DNA fragments containing either two completely palindromic sites, one of which is present in the promoter of the proencephalin gene (Rattner et al. 1991), or the two sites of the HIV-1 enhancer region. As exemplified here by p50(443), p50 showed at least a fivefold higher affinity for the palindromic sites than for the nonpalindromic sites (Fig. 3B, bottom, lanes 8 and 9 vs. 10 and 11). Under exactly the same conditions, natural heteromeric NF- κ B bound with about equal affinity to the palindromic and nonpalindromic sites (Fig. 3B, top, lanes 8 and 9 vs. 10 and 11). In-

terestingly, analysis of the HIV-1 enhancer for possible cooperative interactions between the two HIV-1-binding sequences revealed that neither p50 homodimers nor natural NF- κ B bind in a cooperative manner to the enhancer (Fig. 3B, lanes 6–9). For natural NF- κ B an HIV-1 enhancer fragment containing only an intact distal binding site showed a competitive strength equal to that of equimolar amounts of an enhancer fragment with both sites intact (Fig. 3B, top, lanes 6 and 7 vs. 8 and 9). Comparison of the competitions with the two individual HIV-1 and the single Ig κ enhancer-binding sequences revealed that neither recombinant p50 nor natural NF- κ B has a significant preference for any of these sites, although the distal binding site of the HIV-1 enhancer was recognized with slightly higher affinity than the proximal site (Fig. 3B, lanes 2–7). Because the decameric core sequences of all three binding sites are identical, these results indicated that the flanking sequences of the cores, at least in these cases, only marginally influence the binding affinities of NF- κ B or p50.

In summary, these DNA-binding studies suggest that homodimeric p50, if present in certain cells, might bind to NF- κ B target promoters, with some preference for those containing symmetric NF- κ B recognition sequences (e.g., the proencephalin gene promoter).

Table 1. Binding constants (K_B) of p50(443), p50(503), and purified NF- κ B to a synthetic DNA fragment containing the Ig κ enhancer site

p50(443) (per mole)	p50(503) (per mole)	NF- κ B (per mole)
3.1×10^{10}	4.0×10^{10}	4.6×10^{11}
3.7×10^{10}	13.8×10^{10}	5.5×10^{11}
3.9×10^{10}	14.7×10^{10}	5.9×10^{11}
4.5×10^{10}	15.9×10^{10}	15.2×10^{11}
Average values: 3.8×10^{10}	12.1×10^{10}	7.8×10^{11}

Activation of transcription by natural NF- κ B and recombinant p50

Natural NF- κ B and recombinant p50 were then analyzed in both crude and partially purified *in vitro* transcription systems for their potential effects on regulation of the HIV-1 promoter. The crude system was reconstituted with the 0.5 and 0.85 M KCl fractions obtained from the fractionation of HeLa nuclear extracts on a phosphocellulose (P11) column (Dignam et al. 1983) and a TFIIA fraction purified by two chromatographic steps (Meisterernst et al. 1990). The P11 0.5 M fraction provided the general transcription factors TFIIB, TFIIE, TFIIIF, and RNA polymerase II, whereas the P11 0.85 M fraction contained TFIID. The partially purified system consisted of these general transcription factors, which were all purified by at least three to four chromatographic steps (Meisterernst et al. 1991). Whereas the crude P11-derived system was used to mimic more closely the physiological conditions, the purified system was essential for more detailed analysis of the mechanism of activation based on its more clearly defined polypeptide composition.

Natural NF- κ B activated transcription from the HIV-1 promoter in the crude system efficiently (data not shown). However, upon further fractionation of the gen-

eral transcription factors this activation either was lost completely (under limiting TFIID concentrations; Fig. 4A, lanes 9,10) or was less than two- to threefold (under saturating TFIID concentrations; Fig. 4B, lanes 1,4). Activation levels were restored when transcription was performed in the presence of a previously described cofactor fraction, USA (Meisterernst et al. 1991) (Fig. 4A, lanes 4–6 vs. 9 and 10). Partially purified and DNA affinity-purified NF- κ B activated transcription equally well (Fig. 4A, lanes 5,6). As expected, transcriptional stimulation by NF- κ B required intact NF- κ B recognition sites (Fig. 4A, lanes 2 and 3 vs. 5 and 6). The level of induction by NF- κ B was >20-fold when increased amounts of USA were added to the reactions (Fig. 4B, lane 3 vs. 6). Under these conditions, basal levels of transcription were selectively repressed (lane 1 vs. 3), which may have been caused by an excess of the inhibitory component of USA, designated NC1, as suggested previously (Meisterernst et al. 1991).

The bacterially expressed p50 derivatives were passed through a DNA affinity column before analysis in transcription assays to select for molecules that were active in binding. As exemplified here for p50(443) and p50(503), the HIV-1 promoter was efficiently induced by the p50 subunit of NF- κ B in the crude system (Fig. 5A). To obtain full levels of transcriptional activation in the

Figure 4. Site-specific transcriptional activation of the HIV-1 promoter by NF- κ B is dependent on the cofactor fraction USA.

(A) Transcriptional activation by NF- κ B *in vitro* is dependent on the cofactor fraction USA under limiting TFIID concentrations. Equal amounts (as estimated from gel-shift assays) of affinity-purified or DE52-derived NF- κ B were incubated for 30 min at 25°C with the template DNA, 0.5 μ l of TFIID (Mono S fraction), and either 0.3 μ l of buffer or 0.3 μ l of USA (heparin-Sepharose fraction) before addition of the other components of the highly purified transcription system. As template DNA, either 5 ng of pMHIVMT (lanes 1–3, 7–8) or 5 ng of pMHIVWT (lanes 4–6, 9–10) were employed. Each lane contained 5 ng of pML Δ 53 as internal control. (B) Activation by heteromeric NF- κ B *in vitro* is strongly potentiated by the cofactor fraction USA. NF- κ B (DE52) (lanes 4–6) or equivalent amounts of buffer (lanes 1–3) were incubated for 30 min at 25°C with 40 ng of template DNA (pMHIVWT), 1 μ l of TFIID (Mono S fraction), and increasing amounts of USA (heparin-Sepharose fraction) before addition of the other components of the highly purified transcription system. The amounts of USA used are given in units (1 unit corresponds to 0.1 μ l of the heparin-Sepharose fraction) (Meisterernst et al. 1991). Each reaction contained 40 ng of pML Δ 53 as internal control.

A

B

Figure 5. Transcriptional activation of the HIV-1 promoter by p50 homodimers. (A) Both recombinant p50(443) and p50(503) stimulate transcription in a crude system to a similar extent. Increasing amounts of DNA affinity-purified p50(503) (lanes 1–5) or p50(443) (lanes 6–9) were incubated for 30 min at 25°C with 10 ng of linearized template DNA (pMHIVWT) before addition of the P11-derived transcription system. Each lane contained 10 ng of linearized pMLΔ53 as internal control. One unit of p50 protein corresponds to 40–80 fmoles of DNA-binding active protein. (B) Repression of transcription by high amounts of recombinant p50(503). Increasing amounts of p50(503) were incubated for 30 min (25°C) with 50 ng of linearized template DNA (pMHIVWT) before addition of the P11-derived transcription system. Each lane contained 50 ng of linearized pMLΔ53 as internal control. One unit of p50(503) corresponds to 100–200 fmoles of protein active in DNA binding. In lanes 4 and 5 the levels of activation could not be determined owing to the decreased levels of basal transcription from the internal controls (pMLΔ53). (C) Transcriptional activation by NF- κ B, p50(503), and p50(368) is dependent on the cofactor fraction USA. Purified natural NF- κ B (DE52 fraction), DNA affinity-purified p50(368), DNA affinity-purified p50(503), or equivalent amounts of buffer were mixed with 50 ng of template DNA (pMHIVWT) and either 0.5 μ l of USA (heparin-Sepharose fraction) or equivalent amounts of buffer followed by the addition of the other components of the highly purified transcription system. The reactions were started immediately without preincubation. Here, bacterially expressed recombinant TFIIIB (Malik et al. 1991) was used instead of natural purified TFIIIB. Each reaction included 50 ng of pMLΔ53 as internal control.

crude system we generally used 10- to 50-fold molar excesses of expressed p50 protein relative to the number of recognition sites on the templates. Higher concentrations repressed transcription from both the HIV-1 and the major late core promoter (Fig. 5B, lanes 4,5). To rule out the possibility that activation by p50 might have been mediated through other proteins present in the transcription system, which may be able to interact with p50 but unable to stimulate transcription by themselves (e.g., c-Rel or p65), we probed the transcription system with an antibody directed against a nonconserved carboxy-terminal peptide of c-Rel. c-Rel was found to partially coelute with TFIIA on the P11 and DEAE-Sepharose columns (Fig. 1C, lanes 2,5). Therefore, to be sure

that the activation was caused directly by p50 homodimers it was essential to use a more purified transcription system.

c-Rel was almost completely removed from TFIIA after two additional chromatographic steps (Fig. 1C, lane 3). Furthermore, no significant amounts of proteins that specifically recognized the NF- κ B sequences were detected in the purified transcription system (including the cofactor fraction) in gel-shift assays, and the components of the system were shown to be free of p49 and p50 by Western blot analyses (data not shown). Finally, when mixed and incubated with p50, none of the general transcription factor fractions or the cofactor fraction USA changed the mobility of the p50–DNA complex as as-

sayed with p50(368) and p50(503) (data not shown). This indicated that the transcription system was free of any NF- κ B-like activity, which alone or after heteromerization with p50 could recognize the NF- κ B sites in the HIV-1 promoter. Nevertheless, recombinant p50 stimulated transcription in this purified system severalfold (Fig. 5C, lanes 7,8), although the level of activation under saturating conditions was approximately threefold lower than that achieved with heteromeric NF- κ B (Fig. 5C, cf. lanes 3 and 4 with 7 and 8). An activation potential of p50 homodimers at least threefold lower was also observed when a crude transcription system was used (data not shown). The cofactor fraction USA was absolutely essential for activation (Fig. 5C, lanes 5 and 6 vs. 7 and 8), and this activation showed the same dependence on the concentration of USA as did stimulation by natural NF- κ B (data not shown). Surprisingly, the p50(368) protein, which is deleted almost precisely at the carboxy-terminal end of the *rel* domain (see Fig. 2A), retained full transcriptional activity as compared to the full-length p50(503) (Fig. 5C, lanes 9–12). This indicated that the stimulatory effect was mediated by the amino-terminal 368 amino acids, which essentially comprise the *rel* do-

main. In summary, both p50 and natural NF- κ B were potent activators in either a crude or a purified transcription system. Stimulation by these activators was abolished by removal of a cofactor and completely restored after addition of the cofactor fraction USA to the purified system.

Activation by cytoplasmic NF- κ B but not by recombinant p50 is inhibited by I κ B- β even after formation of a stable preinitiation complex

Partially purified I κ B- β was analyzed in in vitro transcription reactions for its ability to inhibit activation by NF- κ B. When added simultaneously with either cytoplasmic NF- κ B or recombinant p50(443), I κ B- β reduced significantly cytoplasmic NF- κ B but not p50(443)-induced levels of transcription (Fig. 6A, lanes 2 and 3 vs. 5 and 6). I κ B- β is thought to interact with the p65 subunit as well as with the c-Rel protein (Zabel and Bauele 1990; Kerr et al. 1991). Because we demonstrated earlier (Fig. 1C) that the affinity-purified NF- κ B does not contain significant amounts of c-Rel, this specific inhibition of natural cytoplasmic NF- κ B again confirmed that this

Figure 6. I κ B- β acts as a transcriptional inhibitor of heteromeric NF- κ B even after formation of a stable preinitiation complex. (A) I κ B- β inhibits transcriptional activation by heteromeric NF- κ B but not by p50 homodimers. NF- κ B (DE52 fraction) (lanes 2,3) or p50(443) (DNA affinity-purified) (lanes 5,6) was mixed with the P11-derived transcription system and the template DNA (10 ng of linearized pMHIVWT), followed by addition of either 4.0 μ l of buffer (lanes 2,5) or 4.0 μ l of I κ B (Mono Q fraction) (lanes 3,6). The transcription reactions were started immediately without preincubation. Controls were carried out in the same way but without NF- κ B, p50, or I κ B (lane 1) or with I κ B alone (lane 4). All reactions included 10 ng of linearized pML Δ 53 as internal controls. (B) I κ B- β inhibits transcriptional activation by NF- κ B even after formation of a stable preinitiation complex including all general factors, USA, and the upstream activator. As indicated, NF- κ B (DE52 fraction) (lanes 1,2) or an equivalent amount of buffer (lane 3) was preincubated with the template DNA (10 ng linearized pMHIVWT) for 15 min at room temperature before addition of the P11 0.85 fraction that provided USA and TFIID activities. After incubation for 10 min at 30°C, the other components of the P11-derived transcription system and ATP nucleotides were added, followed by another incubation period of 10 min at 30°C. Finally, 3.5 μ l of I κ B- β (Mono Q fraction) (lane 2) or equivalent amounts of buffer (lanes 1,3) were added and the transcription reactions were started 30 min thereafter. All reactions included 10 ng of linearized pML Δ 53 as internal controls. (C) I κ B- β eluted and renatured from an SDS-polyacrylamide gel inhibits NF- κ B activation in a highly purified transcription system. NF- κ B (DNA affinity-purified) (lanes 2,3) or an equivalent amount of buffer (lane 1) was mixed with the components of the highly purified transcription system, the template DNA (2 ng of pMHIVWT), and either 12 μ l of renatured I κ B- β fraction (molecular mass range: 43–47 kD) (lane 3) or equivalent amounts of buffer (lanes 1,2). The transcription reactions were started immediately without preincubation. Here, bacterially expressed recombinant TFIIB (Malik et al. 1991) was used instead of natural purified TFIIB. All reactions included 2 ng of pML Δ 53 as internal controls.

factor includes the p65 subunit. Most likely, the transcriptional inhibition by I κ B- β is brought about by removing the heterodimeric NF- κ B (p50/p65) from the template. Importantly, I κ B- β can perform this function in the presence of all the general factors and the necessary cofactors which may form a stable preinitiation complex including NF- κ B. Even when NF- κ B and all of the general factors and cofactors were preincubated with the template DNA, thus allowing the formation of such a stable preinitiation complex before the addition of I κ B- β , the inhibitor efficiently blocked transcriptional stimulation (Fig. 6B). In the experiment shown in Figure 6C, the highly purified transcription system, DNA affinity-purified NF- κ B, and I κ B- β , which had been eluted and renatured from a SDS-polyacrylamide gel, were used to demonstrate that this inhibition was a direct effect of I κ B- β and was not caused by another contaminating protein in the I κ B fraction.

In conclusion, these results show that I κ B- β could potentially play an important role in active regulation of transcription within the nucleus.

Discussion

Apart from one in vitro analysis with a constitutively activated NF- κ B from B cells, most studies of the activation of potential target genes by NF- κ B have involved transient transfection assays in cultured cells. To gain more insight into the function of NF- κ B and its component or associated polypeptides, we have analyzed the function of in vitro-activated heteromeric NF- κ B, the p50 subunit, and I κ B in in vitro transcription systems. The involvement of cofactors in the activation by NF- κ B or p50 has also been addressed.

Because several proteins are known to bind specifically to NF- κ B recognition sequences and to form heteromeric complexes with each other, it was important to utilize clearly defined purified or recombinant proteins and reconstituted in vitro transcription systems. Toward this end, both natural NF- κ B and recombinant p50 proteins were highly purified, enriched for molecules active in DNA binding, and, in the case of NF- κ B, subjected to polypeptide composition analysis by several methods. A crude transcription system was used to mimic more closely the physiological conditions, whereas an advanced system consisting of the general transcription factors IIA, IIB, IID, IIE, IIF, and RNA polymerase II, all highly purified from HeLa nuclear extracts, was employed for more detailed studies of the mechanism of transcriptional activation. As exemplified by the c-Rel analysis, potentially interfering proteins were shown to be absent in the highly purified transcription system.

Both inducible cytoplasmic NF- κ B (p50/p65) and p50 homodimers are potent transcriptional activators

Our analysis demonstrated that inducible cytoplasmic NF- κ B, here purified from HeLa cell cytosol, and the recombinant p50 subunit alone are both potent transcrip-

tional activators of the HIV-1 promoter in vitro. Surprisingly, deletion analysis revealed that the *rel* domain of p50 itself possesses full transcriptional activity as compared to the full-length p50 protein. This was unexpected, as this region was already shown to contain DNA-binding and dimerization functions (Ghosh et al. 1990; Kieran et al. 1990; Logeat et al. 1991). Furthermore, studies with Gal4-c-Rel fusion constructs functionally separated a *trans*-activation domain from the *rel* domain of the c-Rel protein (Bull et al. 1990). In contrast, our results indicated that p50 may not correspond to the typical structure of transcriptional activators, which have clearly distinct domains responsible for DNA binding and transcriptional activation. Only detailed mutational analysis of the *rel* domain of p50 will reveal whether the structural separation of these two functions is possible.

Because it is known that p50 can dimerize with p65 or c-Rel (Kawakami et al. 1988; Baeuerle and Baltimore 1989; Logeat et al. 1991), it was crucial for the interpretation of the results to exclude the possibility that recombinant p50 activated transcription only after heteromerization with one of the other Rel-related proteins, which might have been present in the transcription systems. Western blot analyses provided evidence that the purified system was basically free of p49, p50, and c-Rel, whereas the fact that I κ B did not inhibit activation by p50 also ruled out a participation of p65 in this activation process the recombinant protein. In addition, several other lines of evidence argue against a stoichiometric involvement of any Rel-related protein in this process. First, gel-shift assays revealed no significant amount of specific DNA-binding activity in the components of the purified system and absolutely no change in the mobility of the p50-DNA complex after incubation of p50 with these components (including the cofactor fraction USA) (data not shown). These experiments demonstrated that the purified system did not contain any components that could bind specifically to NF- κ B sites on their own or that could form stable complexes with p50. Second, absolutely no *cis*-activation of the HIV-1 promoter was observed in the purified system (Fig. 4A, cf. lanes 1 and 4), providing evidence that neither NF- κ B nor other transcriptionally active NF- κ B-related proteins were present.

Heteromeric NF- κ B (p50/p65) and p50 homodimers have different DNA-binding properties

Determinations of the equilibrium binding constants of recombinant p50s and natural NF- κ B using the Ig κ enhancer oligonucleotide as probe revealed that p50 homodimers bind to the Ig κ enhancer with ~ 10 – 20 times lower affinity than does natural purified NF- κ B. However, competition experiments showed that the affinity of p50 for completely palindromic recognition sites is at least fivefold higher than that for nonpalindromic sites such as those of the Ig κ or HIV-1 enhancers. Variations in the flanking regions of the decameric core consensus sequences did not influence significantly the binding af-

finities of NF- κ B or p50. Together, these results demonstrated that p50 homodimers recognize different NF- κ B motifs with distinct affinities and that their DNA-binding properties are different from those of inducible heteromeric NF- κ B.

Activation by both factors depends on the cofactor fraction USA

Initial experiments employing the purified transcription system did not show any activation of the HIV-1 promoter by NF- κ B or p50. Only upon complementation of the system with the cofactor fraction USA (Meisterernst et al. 1991) could full levels of induced transcription be restored. The activities of both NF- κ B and p50 were absolutely dependent on this cofactor. These results extend the range of transcriptional activators that require USA to yet another class of regulatory factors, as USA has been shown to be required for activation of the HIV-1 promoter by SP1 and of the major late promoter by USF (Meisterernst et al. 1991). Whereas SP1 is a zinc finger-containing protein belonging to the class of glutamine-rich activators (Courey and Tjian 1988), upstream stimulatory factor (USF) is a helix-loop-helix protein with an activation domain so far undefined (Gregor et al. 1990). p50 neither contains any of the known DNA-binding motifs nor shows any obvious sequence similarities to any of the characteristic activation domains of other *trans*-activators. This indicates that USA might play a very general role in the activation mechanism of various classes of *trans*-activators.

I κ B- β may potentially play an active role in transcriptional regulation within the nucleus

In vitro functional assays revealed that I κ B- β can efficiently inhibit activation by heteromeric NF- κ B but not by p50 homodimers. This is consistent with the observation that I κ B can actively sequester NF- κ B from DNA under gel-shift conditions (Zabel and Baeuerle 1990) and that it inhibits DNA binding of NF- κ B but not p50 under these conditions (Urban and Baeuerle 1990). The results of our functional assays are important because they demonstrate that I κ B also can elicit its inhibitory function under transcription conditions, where it must compete with the transcription machinery (including the cofactors) and possibly other proteins for protein-protein interactions with NF- κ B. Even after preincubation of NF- κ B with all components of the transcription system (including the cofactor USA) and the template DNA, I κ B- β inhibited stimulation of transcription by NF- κ B completely. In this preincubation protocol, the formation of a stable preinitiation complex, which included NF- κ B, was allowed before the addition of I κ B to the reaction. These results support strongly the idea that I κ B- β could potentially play an active role in transcriptional regulation within the nucleus. Such a regulatory role of I κ B- β could account for the observation that activation of genes by NF- κ B following stimulation of cells with TPA

is only transient (Sen and Baltimore 1986b; Baeuerle and Baltimore 1989). NF- κ B-binding activity reaches a maximum between 30 min and 2 hr after TPA treatment and then declines to an undetectable level by 8 hr. Future studies must clarify whether I κ B- β actually enters the nucleus or not.

Potential in vivo functions of p50 homodimers

The combined results from in vitro transcription and binding studies suggest that p50 homodimers might be involved in vivo in the regulation of genes with NF- κ B recognition sequences in their promoters or enhancers, especially those that contain completely palindromic binding sites [e.g., the proencephalin gene (Rattner et al. 1991)]. In addition, p50 homodimers, which are identical to the factor KBF-1 (Kieran et al. 1990), were implicated in the constitutive basal expression of murine major histocompatibility complex (MHC) class I genes in HeLa cells (Israel et al. 1989) and of the tumor necrosis factor α (TNF- α) gene in murine macrophages (Collart et al. 1990). Whether p50 homodimers can also be induced by reagents such as phorbol myristate acetate and concanavalin A (as suggested by Rattner et al. 1991) or whether they are constitutively present in the nuclei of all cell types remains to be clarified. However, considering the lower binding affinity of p50 homodimers to several non-palindromic recognition sites and its (approximately) threefold lower activation potential compared to inducible heteromeric NF- κ B in vitro, it seems likely that p50 may account for the low constitutive expression of certain NF- κ B target genes in uninduced cells.

Interestingly, the identification of p50 as a transcriptional activator in vitro appears to contradict "in vivo" studies reported recently (Schmid et al. 1991) in which cotransfection of a reporter plasmid and a p50 expression vector in Jurkat T cells did not lead to stimulation of transcription from a reporter gene. However, even cotransfection of both p50 and p65 expression vectors caused only weak induction (two- to threefold) of the reporter construct, and no induction at all of a reporter gene containing the HIV-1 promoter. The reasons for the differences between the in vitro and "in vivo" results are not yet clearly understood, but it is possible that cells contain either specific inhibitors of p50 homodimers (similar to the I κ B proteins) or proteins that bind to the promoters of the target genes and thereby block the access of activators to their binding sites. Recent results have shown that the Bcl-3 carboxy-terminal cleavage product of the p50 precursor as well as the proto-oncogene product Bcl-3 can inhibit DNA-binding of p50 homodimers (Hatada et al. 1992). Whereas the repression by specific I κ B-like inhibitors might be overcome by induction of certain signal transduction pathways, very high binding affinities may be necessary for the activators to compete with DNA-binding inhibitors. The 10- to 20-fold lower binding affinity of p50 compared with heteromeric NF- κ B for the Ig κ enhancer thus may account at least partially for the failure of p50 alone to activate transcription of the reporter gene in transfected cells.

This explanation is also consistent with our observation that full levels of transcription in the crude system require at least 5–10 times higher amounts of p50 than are required in the partially purified system, as the DNA-binding inhibitors are likely to be mostly removed (or titrated out) from the purified system but to a lesser extent from the crude system. In fact, we identified an abundant protein in the crude system, which bound with low specificity to the Ig κ enhancer and also to different regions of the HIV-1 or adenovirus major late promoters (M. Kretzschmar, M. Meisterernst, and R.G. Roeder, unpubl.). The fraction containing this protein was able to repress basal transcription in vitro from both the HIV-1 promoter and the major late core promoter, and the DNA-binding protein was mostly removed in the partially purified transcription system. Depending on the relative affinities of such inhibitors for distinct promoters, p50 could have varying effects on transcription of the corresponding target genes in vivo.

Another possible explanation for the failure of p50 to activate in transfected cells may be that activation by expressed p50 is obscured by the presence of endogenous p50 homodimers. These could cause a constitutively induced level of transcription, which cannot be significantly increased by additionally expressed p50 protein alone, but only by the coexpression of p50/p65 or p49/p65 heterodimers, both of which may have a higher activation potential in the cell than p50 homodimers. In support of this explanation it has been suggested previously that after induction of cells with TNF- α the low constitutive level of transcription of MHC class I genes is increased by the replacement of p50 homodimers, bound to the NF- κ B recognition sites, by an inducible NF- κ B-like factor (Israel et al. 1989). In addition, in transfection experiments using an HIV-LTR reporter construct, the ubiquitous activator SP1 and possibly other cellular factors, which bind to the HIV-1 promoter/enhancer region, might interfere with transcriptional activation by transfected p50 protein. In agreement with this idea we have observed that in vitro transcription from the HIV-1 promoter was induced only about twofold by p50 homodimers in the presence of saturating amounts of SP1 (M. Kretzschmar, M. Meisterernst, and R.G. Roeder, unpubl.). This suggests the exciting possibility that some regulatory transcription factors may activate basal transcription up to a similar maximal level by a common mechanism, whereas this transcriptional level may be exceeded by alternate mechanisms involving specific activators that carry additional functionally distinct activation domains (e.g., p65). These alternate activation domains may also account for the synergistic effects between activators that were observed in vivo.

Materials and methods

Purification of inducible cytosolic NF- κ B and I κ B- β

A 950-ml volume of cytosolic extract prepared from HcLa cells (Dignam et al. 1983) was adjusted to 0.1 M KCl and loaded onto

a phosphocellulose (P11) column. The flowthrough fraction, which contained the NF- κ B/I κ B complex, was treated with deoxycholate [0.8% (wt/vol)] and formamide [16% (vol/vol)] followed by addition of the nonionic detergent NP-40 [1.2% (vol/vol)] to dissociate the protein complex (Baeuerle and Baltimore 1988b). Subsequently, the fraction was rechromatographed on a phosphocellulose column (P11), from which free NF- κ B was eluted by a 0.3 M KCl step, whereas free I κ B was found in the flowthrough. To concentrate NF- κ B the 0.3 M KCl fraction was adjusted to 0.04 M KCl by dialysis and loaded on a DEAE-cellulose (DE52; Whatman) column. NF- κ B was step eluted with 0.15 M KCl and finally purified by specific DNA affinity chromatography (GIBCO BRL). Prior to loading, the 0.15 M KCl fraction was adjusted to 0.2 M KCl by addition of 0.4 M KCl containing buffer, and poly[d(A-T)]/[d(A-T)] and poly[d(I-C)]/[d(I-C)] (Pharmacia) were added as nonspecific competitors. The flowthrough was cycled three times over the DNA affinity column before bound NF- κ B was eluted with 0.4 M KCl.

For further purification of I κ B, the flowthrough fraction of the second P11 column was adjusted to 0.2 M KCl by addition of 1.0 M KCl containing buffer and loaded on a DEAE-Sephacel column (Pharmacia). I κ B was step eluted with 0.3 M KCl, subsequently dialyzed to 0.2 M KCl, and chromatographed on a Mono Q column (FPLC; Pharmacia). Application of a linear gradient from 0.2 to 1.0 M KCl resulted in the elution of the I κ B activity between 0.3 and 0.45 M KCl. All chromatographic steps were performed in buffer C containing 25 mM HEPES-KOH (pH 7.9), 20% glycerol (vol/vol), 0.25 mM EDTA, 5 mM dithiothreitol (DTT), 0.5 mM PMSF, 0.01–0.03% NP-40 (vol/vol), and the indicated concentrations of KCl.

Expression and purification of recombinant p50 proteins

The sequences coding for the recombinant p50 proteins were subcloned into the pET-3b or pET-5b expression vectors (Novagen) and introduced into the *E. coli* expression strain BL21 (DE3) pLysS (Novagen). Protein expression in this T7 RNA polymerase system (Studier et al. 1990) was carried out essentially as described elsewhere (Pognonec et al. 1991). Bacterial cultures were grown at 30°C up to an OD₆₀₀ of 0.7–0.9, induced with IPTG (0.4 mM), and again grown at 30°C for another 4–6 hr. Cells were spun down and resuspended in 20 ml ice-cold lysis buffer containing 20 mM Tris-Cl (pH 7.4 at 25°C) 10% glycerol (vol/vol), 0.5 M NaCl, 1 mM EDTA, 1 mM PMSF, 0.1% NP-40 (vol/vol), 1% aprotinin (vol/vol) (Sigma), and 5 μ g/ml of leupeptin (Boehringer). Cells were lysed by ultrasonication and centrifuged for 15 min at 4°C and 10,000g. The supernatant was diluted by an equal volume of water followed by slow addition of saturated ammonium sulfate solution (ICN; ultrapure) up to a final concentration of 32% ammonium sulfate. The precipitate was resuspended in buffer F containing 20 mM Tris-Cl (pH 7.3 at 25°C) 20% glycerol (vol/vol), 1 mM EDTA, 1 mM PMSF, 5 mM DTT, and 5 μ g/ml each of leupeptin, pepstatin (Sigma), and trypsin inhibitor (Boehringer), diluted to 0.1 M ammonium sulfate by further addition of buffer F and loaded on a heparin-Sepharose column (Pharmacia). The column was then washed extensively with buffer H containing 25 mM Hepes-KOH (pH 7.9), 10% glycerol (vol/vol), 0.1 M KCl, 2 mM EDTA, 5 mM DTT, 0.5 mM PMSF, and 0.01% NP-40 (vol/vol), and developed with a linear gradient from 0.1 to 1.0 M KCl in buffer H. The recombinant p50 proteins eluted between 0.3 and 0.5 M KCl. For specific DNA affinity purification one of the peak fractions was adjusted to 0.2 M KCl by dilution in buffer H without salt and chromatographed through a DNA affinity column (GIBCO BRL) as described for natural NF- κ B.

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DNA-binding assays

NF- κ B, which was not complexed with I κ B, or recombinant p50 proteins were incubated with a DNA probe in a 20- μ l reaction mixture for 20–30 min at 25°C before electrophoresis on a 5% native polyacrylamide gel (AA/BA, 50 : 1). The probe was a double-stranded synthetic oligonucleotide (KK-1/2) that carried the NF- κ B-binding site of the mouse Ig κ enhancer (Sen and Baltimore 1986a):

5'-dGAT CCT CAA CAG AGG GGA CTT TCC GAG GCC GAA TTC A-3'
3'-dGA GTT GTC TCC CCT GAA AGG CTC CGG CTT AAG TCT AG-5'

The oligonucleotide was end-labeled with [α -³²P]dATP (Amersham) by filling the overlapping ends with Klenow enzyme (Boehringer), and 12.5 fmoles of this probe were used in regular DNA-binding assays. The incubation buffer contained 10 mM Tris-Cl (pH 7.7 at 25°C), 10% glycerol (vol/vol), 0.1 M KCl, 0.2 mM EDTA, 3 mM DTT, 0.3 mM PMSF, 0.02% NP-40 (vol/vol), 400 ng/ μ l of BSA, and 0.2–1.0 μ g poly[d(A-T)] as nonspecific competitor. The gel contained 5% polyacrylamide (vol/vol), 5% glycerol (vol/vol), and 0.5 $\times$ TBE buffer and was developed with 9 V/cm at room temperature. Gels were dried and autoradiographed with a screen at –80°C. For assays of NF- κ B, which was complexed with I κ B, the protein fraction was incubated in a reaction mixture that contained 0.8% deoxycholate (vol/vol) and 10% formamide (vol/vol), followed by the addition of 1.2% NP-40 (vol/vol). In addition to KK-1/2 the following DNA fragments were used in competition experiments:

KKM-1/2

5'-dGAT CCT CAA CAG AGG CCA CTT TCC GAG GCC GAA TTC A-3'
3'-dGA GTT GTC TCC GGT GAA AGG CTC CGG CTT AAG TCT AG-5'

KM-1/2

5'-dAAT TCC AAG CTT CTT TCC GCT GGGGAC TTT CCA GGA-3'
3'-dTAA AGG TTC GAA GAA AGG CGA CCC CTG AAA GGT CCC T-5'

KM-5/6

5'-dAAT TCC AAG GGA CTT TCC GCT GCA GAC TTT CCA GGA-3'
3'-dTAA AGG TTC CCT GAA AGG CGA CGT CTG AAA GGT CCC T-5'

K-1/2

5'-dAAT TCC AAG GGA CTT TCC GCT GGGGAC TTT CCA GGA-3'
3'-dTAA AGG TTC CCT GAA AGG CGA CCC CTG AAA GGT CCC T-5'

MP-1/2

5'-dCC AGG GGA CGT CCC CGT GGGGAA TTC CCC AGG GGA TC-3'
3'-dCAT GGG TCC CCT GCA GGG GCA CCC CTT AAG GGG TCC CCT AG-5'

The decameric NF- κ B-binding motifs are underlined.

Assay for I κ B

I κ B assays were performed by preincubating the I κ B-containing fractions with purified NF- κ B for 10 min at 25°C before addition of the DNA probe. Subsequently, the assays were carried out exactly as the DNA-binding assays described for uncomplexed NF- κ B.

Renaturation of NF- κ B and I κ B from SDS-polyacrylamide gels

Renaturations from SDS-polyacrylamide gels were carried out essentially as described in Hager and Burgess (1980), with the variations reported by Baeuerle and Baltimore (1988b). Elution of the proteins from the gel fragments was allowed for 24 hr, whereas renaturation proceeded for 24–48 hr. After acetone precipitation, dried protein pellets were dissolved in 5 μ l of saturated solution of urea (BRL, ultrapure/enzyme grade) and diluted with 250 μ l of renaturation buffer. I κ B fractions that were to be used in *in vitro* transcription assays were subsequently dialyzed against buffer B containing 20 mM Tris-Cl (pH 7.3) at 25°C, 50 mM KCl, 10% glycerol (vol/vol), 0.25 mM EDTA, 5 mM DTT, and 0.5 mM PMSF.

Determination of equilibrium binding constants

The equilibrium-binding constants (K_B) of natural NF- κ B and recombinant p50 proteins were determined by gel-shift assays according to the method described in Meisterernst et al. 1988. The probe was a double-stranded oligonucleotide that carried the NF- κ B-binding site of the mouse Ig κ enhancer (Sen and Baltimore 1986a) flanked by 10 and 17 bp, respectively, of the *in situ* sequence. The probe was produced by annealing a primer (5'-dCTC AAC AGA GGG-3') to the 3' end of a single-stranded oligonucleotide with the sequence 5'-dGAT CTG AAT TCG GCC TCG GAA AGT CCC CTC TGT TGA G-3' and subsequently labeling the probe by primer extension using the Klenow enzyme (Boehringer) and the radionucleotides [α -³²P]dATP and [α -³²P]dCTP in addition to dGTP and dTTP (Amersham). A maximum of 13 labeled nucleotides were incorporated into the probe by this means. DNA-binding assays were performed as described previously but without addition of nonspecific competitor. The total number of DNA-binding protein units and the percentage of "active" DNA in the assays were determined as described in Meisterernst et al. (1988). For each protein, four K_B values were determined independently from four different gel lanes, and average values were calculated.

Western blotting

Western blot analysis was performed according to standard protocols using a 1 : 1000 dilution of a *c-rel*-specific antiserum (Brownell et al. 1989).

Preparation of nuclear extracts, transcription factors, and transcription templates

Preparation of nuclear extracts and transcription factors, as well as the construction of the transcription templates pMHIVWT and pML Δ 53, has been described previously [Meisterernst et al. 1991 and references therein]. pMHIVWT carries HIV-1 promoter sequences from position –109 to –8 followed by the adenovirus major late initiator element from –7 to +9 and a 379-bp G-free cassette [Sawadogo and Roeder 1985]. pML Δ 53 contains the major late core promoter from position –53 to +9 followed by a shortened G-free cassette. pMHIVMT was constructed identically to pMHIVWT except that oligonucleotides containing mutated HIV-1 NF- κ B-binding sites were used. The distal binding sequence was changed to 5'-dGCTTCTTTCC-3', whereas the proximal site was mutated to 5'-dCAGACTTTCC-3'. Both mutated sequences were inactive in binding to NF- κ B.

Transcription reactions

Transcription reactions were performed essentially as described in Meisterernst et al. (1991). When using the partially purified

transcription system, reactions included 0.25–1.0 μ l TFIIA [Mono Q fraction (2.70 mg/ml)], 0.5–1 μ l TFIIB [single-stranded DNA–agarose fraction (0.30 mg/ml) or Mono Q fraction (0.23 mg/ml)], 0.5–1.0 μ l TFIID [Mono S fraction (0.36 mg/ml)], 0.5–1 μ l TFIIE/F (A25 fraction, 0.6 mg/ml), and 0.5–2.0 μ l RNA polymerase II [DE52 fraction (0.30 mg/ml)], all in buffer A [Nakajima et al. 1988] with DTT (5 mM) replacing β -mercaptoethanol. The cofactor fraction USA [heparin–Sephacrose fraction (2.0 mg/ml)] was present in amounts (1 unit corresponds to 0.1 μ l) indicated in the figure legends. Transcription reactions using the crude P11-derived system contained 2 μ l of TFIIA (DEAE–cellulose fraction), 6 μ l of P11 0.5 M KCl fraction (providing TFIIB, TFIIE, TFIIF, and RNA polymerase II), and 5 μ l of P11 0.85 M KCl fraction (providing TFIID and USA). All transcription factors were added simultaneously to the reactions if not noted otherwise in the figure legends.

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