

# Independent modes of transcriptional activation by the p50 and p65 subunits of NF- $\kappa$ B

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**Recombinant subunits of the transcription factor NF- $\kappa$ B, p50 and p65, were analyzed both for binding to various  $\kappa$ B motifs and in vitro activation. The subunits preferentially form a heterodimer that activates transcription. Although p50 and p65 bind DNA individually as homodimers and are structurally related, their activation mechanisms are distinct. p65 activates transcription by its unique carboxy-terminal activation domain. (p50)<sub>2</sub> displays higher affinity DNA binding than (p65)<sub>2</sub> for many distinct  $\kappa$ B motifs and provides strong transcriptional activation only when adopting a chymotrypsin-resistant conformation induced by certain  $\kappa$ B motifs but not others. Thus, (p50)<sub>2</sub> acts as a positive regulator in vitro, consistent with its isolation as a putative constitutive regulator of MHC class I genes. Both subunits of NF- $\kappa$ B, therefore, contribute independently to provide regulation at given  $\kappa$ B motifs.**

[*Key Words*: NF- $\kappa$ B; p50; p65; *c-rel*; transcription; protein conformation]

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Nuclear factor NF- $\kappa$ B was originally described as an immunoglobulin  $\kappa$  light-chain enhancer-binding protein, constitutively present in the nuclei of B lymphocytes (Sen and Baltimore 1986b). Later studies showed that NF- $\kappa$ B binding sites are involved in the regulation of many other genes such as major histocompatibility complex (MHC) class I, cytokines, and viruses (Lenardo and Baltimore 1989; Baeuerle and Baltimore 1991). In cells other than mature B cells and perhaps macrophages (Griffin et al. 1989), NF- $\kappa$ B activity is inducible by cellular-activating stimuli such as antigens for lymphocytes (Sen and Baltimore 1986a), cytokine stimulation (Osborn et al. 1989), or virus infection (Fujita et al. 1989; Lenardo et al. 1989; Visvanathan and Goodbourn 1989). In unstimulated cells, NF- $\kappa$ B is complexed with an inhibitory protein, termed I $\kappa$ B, in a non-DNA-binding form that is localized to the cytosolic fraction (Baeuerle and Baltimore 1988). Cellular activation results in the release of NF- $\kappa$ B from I $\kappa$ B, at least partially as a consequence of phosphorylation of I $\kappa$ B (Ghosh and Baltimore 1990). Thus, NF- $\kappa$ B acts as a transducer of cytoplasmic signals to the nucleus by a translocation mechanism.

Biochemical analysis has shown that the major form of NF- $\kappa$ B consists of two distinct polypeptides, 50 and 65 kD, termed p50 and p65. Purified p50 exhibits DNA-binding activity as a homodimer, with specificity for  $\kappa$ B motifs (Baeuerle and Baltimore 1989). Similarly purified p65, tested under these same conditions, failed to bind to

the  $\kappa$ B site (Baeuerle and Baltimore 1989). Moreover, it has been demonstrated that p65, but not p50, is capable of complexing with I $\kappa$ B (Baeuerle and Baltimore 1989; Ghosh and Baltimore 1990; Urban and Baeuerle 1990).

cDNA cloning studies suggested that p50 is equivalent to the previously identified KBF-1 (an MHC class I enhancer-binding protein) and that p50 is a processed product from a p105 precursor (Ghosh et al. 1990; Kieran et al. 1990). Both p50 and p65 share homology with the viral oncogene product *v-rel*, its cellular homolog *c-rel*, and the *Drosophila* morphogen *dorsal* (Ghosh et al. 1990; Kieran et al. 1990; Nolan et al. 1991; Ruben et al. 1991). More recently, it has been shown that *c-rel* can be detected as a species of  $\kappa$ B motif-binding protein in activated T lymphocytes (Lee et al. 1991; Urban et al. 1991). These results suggest that various *rel*-related proteins, in homo- or hetero-interaction complexes, might differentially regulate the expression of many genes.

To understand the roles of these various polypeptides in gene regulation will require both in vitro and in vivo analyses. Here, we report initial in vitro studies using NF- $\kappa$ B proteins made in a baculovirus expression system. p50 and p65 produced by this system specifically bound to  $\kappa$ B motif DNAs. We reconstituted NF- $\kappa$ B activity by preincubation of the p50 and p65 preparations in vitro to selectively convert them into a heterocomplex with binding characteristics similar to the NF- $\kappa$ B purified from cells. In vitro transcription assays with these proteins showed that p50/p65 could activate transcription from all  $\kappa$ B motifs tested. Some motifs sup-

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ported activation by (p65)<sub>2</sub>, and the activation was dependent on the p65 non-*rel*-related, carboxy-terminal region. Surprisingly, (p50)<sub>2</sub> activated transcription from certain  $\kappa$ B motifs, particularly the KBF-1 site, and this activity resides at least partially in the *rel* region. Activation by (p50)<sub>2</sub> correlated with a chymotrypsin-resistant p50 DNA-binding structure. These results suggest that when it is bound to certain DNA motifs, p50 adopts a particular structure that makes it competent for transcriptional activation. Our functional analyses have revealed that each subunit functions independently in gene regulation.

## Results

### Recombinant NF- $\kappa$ B subunits p50 and p65

To obtain relatively large amounts of pure NF- $\kappa$ B subunits, we adopted the baculovirus expression system, using the previously cloned cDNAs for p50 and p65 (Ghosh et al. 1990; Nolan et al. 1991). This system was likely to be advantageous over a prokaryotic expression system because many proteins produced in this manner are soluble and the insect cells can carry out certain eukaryotic post-translational modifications such as phosphorylation (Miyamoto et al. 1985; Ollo and Maniatis 1987). In addition, several reports demonstrate that recombinant transcriptional regulators produced by this system are active in vitro (Patel and Jones 1990; Watson and Hay 1990).

cDNA cloning studies of both mouse and human p50 have suggested that it is primarily synthesized as a larger precursor protein, p105, and may be matured by proteolytic removal of its carboxyl region. To produce the mature form of p50 without processing, we introduced a

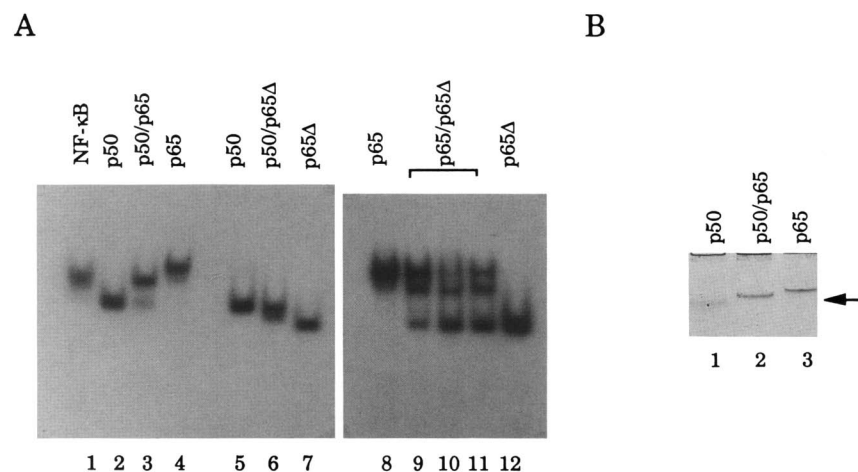
translation terminator at the approximate position of cleavage as deduced in cDNA truncation experiments (Ghosh et al. 1990; see Materials and methods). To produce p65, the full-length cDNA was used. A derivative of p65 known to possess efficient  $\kappa$ B-specific DNA binding, p65 $\Delta$ 314–549 (equivalent to the *Bsp*HI truncation of the cDNA in Nolan et al. 1991), was produced lacking the carboxyl 250 amino acids of p65 by introducing a translation terminator into the cDNA (hereafter referred to as p65 $\Delta$ ).

Seventy-two hours after infection of insect cells with the recombinant viruses, but not after infection with wild-type virus,  $\kappa$ B-specific DNA-binding activity was detectable in the whole-cell extract of SF9 cells. Detection of DNA-binding activity of the full-length p65 was unexpected, because previous reports had failed to detect efficient DNA-binding activity (see Discussion; Baeuerle and Baltimore 1989; Nolan et al. 1991). Each of the proteins was purified by ion exchange and  $\kappa$ B-motif DNA affinity chromatography. Purified preparations were analyzed by SDS-PAGE, and their apparent molecular masses were as predicted: 50, 65, and 35 kD for p50, p65, and p65 $\Delta$ , respectively. The purity determined by stained electrophoretic gels of each subunit was 30%, 95%, and 95% for p50, p65, and p65 $\Delta$ , respectively.

### DNA-binding activity of recombinant NF- $\kappa$ B subunits

Purified subunit proteins were analyzed by the electrophoretic mobility-shift assay (EMSA), either singly or after preincubation with other subunits (Fig. 1). The probes used were <sup>32</sup>P-labeled oligonucleotides with partially self-complementary sequences that form a stem-loop structure and provide a 16-bp double-stranded region

**Figure 1.** Heterodimer formation between NF- $\kappa$ B subunits p50 and p65. **(A)** Recombinant NF- $\kappa$ B subunits, either alone or after preincubation (37°C for 60 min) with the other subunit, were subjected to EMSA using <sup>32</sup>P-labeled probe containing the Ig $\kappa$   $\kappa$ B motif (10 fmoles). (Lane 1) Partially purified NF- $\kappa$ B from rabbit lung; (lanes 2,5) p50 (10 fmoles); (lane 3) p50 (5 fmoles) + p65 (5 fmoles); (lanes 4,8) p65 (10 fmoles); (lane 6) p50 (5 fmoles) + p65 $\Delta$  (5 fmoles); (lanes 7,12) p65 $\Delta$  (10 fmoles); (lane 9) p65 (7.5 fmoles) + p65 $\Delta$  (2.5 fmoles); (lane 10) p65 (5 fmoles) + p65 $\Delta$  (5 fmoles); (lane 11) p65 (2.5 fmoles) + p65 $\Delta$  (7.5 fmoles). The p65/p65 $\Delta$  heterocomplex was reproducibly observed as a single band (T. Fujita et al., unpubl.). **(B)** Recombinant subunits were analyzed by native PAGE in the absence of  $\kappa$ B motif DNA. (Lane 1): p50 (2 pmoles); (lane 2) p50 (2 pmoles) + p65 (2 pmoles), preincubated (37°C, 60 min); (lane 3) p65 (2 pmoles). Protein bands were visualized by staining with Coomassie brilliant blue.



containing 10 bp of a  $\kappa$ B motif (Materials and methods). The control fractions prepared from wild-type virus-infected cells did not show any detectable DNA-binding activity (data not shown).

It was shown previously that p50 exists as a homodimer in solution, and it was suggested to specifically recognize  $\kappa$ B motifs as a homodimer (Baeuerle and Baltimore 1989). To assess how many subunits are included in a p65- $\kappa$ B motif DNA complex, we measured the number of complexes formed between full-length p65 and truncated p65 $\Delta$  (Hope and Struhl 1987). p65 $\Delta$  (35 kD) contains only the *rel* homology region and has been shown previously to bind to DNA (Nolan et al. 1991). In EMSA,  $\kappa$ B motif DNA complexed with p65 migrated more slowly than the same DNA bound to p65 $\Delta$  (lanes 8,12). Preincubation of various ratios of p65 and p65 $\Delta$  resulted in the appearance of single additional complex with intermediate mobility (lanes 9–11). This result is best explained by assuming that p65, like p50, binds to the  $\kappa$ B motif as homodimer, and the new complex is a p65/p65 $\Delta$  heterodimer. Other possibilities, such as a tetrameric structure, are unlikely because three or more heteromers should be detected.

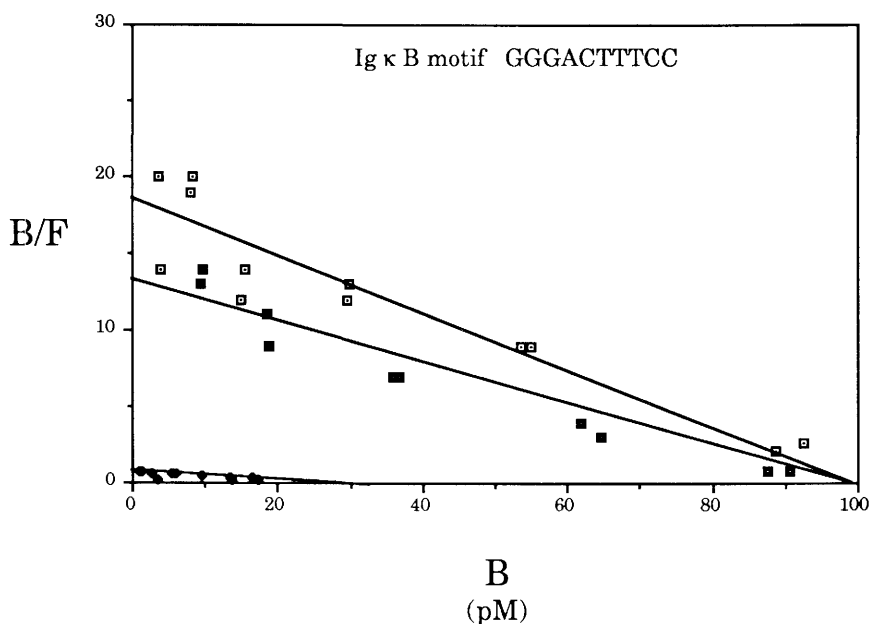
p50/p65 heterocomplexes were made by mixing equimolar amounts of p50 and p65, followed by incubation at 37°C for 60 min. This preincubation resulted in both the formation of a single, new complex with intermediate mobility by EMSA and the virtual disappearance of the homodimer complexes [Fig. 1A (lanes 3,6), Fig. 1B]. Mixing of wild-type p50 and p65 resulted in a complex with mobility indistinguishable from that of purified native NF- $\kappa$ B (cf. lanes 1 and 3). Because both p50 and p65 appear to bind to DNA as homodimers (above), this result is in apparent conflict with the previous heterotetramer-binding model of NF- $\kappa$ B (Baeuerle and Baltimore 1989). The fact that p65 itself is capable of binding to  $\kappa$ B motifs

and that p65 is involved in direct contact with DNA (see Discussion) leads us to believe that NF- $\kappa$ B actually binds to DNA as a p50/p65 heterodimer; and, for convenience, hereafter we refer to the p50/p65 complex as a heterodimer. Taking advantage of the availability of a relatively large amount of purified recombinant subunits, we analyzed them by native gel electrophoresis in the absence of the  $\kappa$ B motif DNA (Hope and Struhl 1987). The result (Fig. 1B) was very similar to that obtained by EMSA and indicated that mixing p50 and p65 selectively formed a heterocomplex species in solution.

#### DNA-binding affinity of NF- $\kappa$ B to various $\kappa$ B motif DNA sequence

The  $\kappa$ B motifs in different genes have slightly different sequences (Lenardo and Baltimore 1989; Baeuerle and Baltimore 1991). Using EMSA with purified NF- $\kappa$ B components, we measured the DNA-binding affinity to representative  $\kappa$ B motifs: 5'-GGGACTTTCC-3' (mouse immunoglobulin  $\kappa$  chain); 5'-GGGAAATTCC-3' [interferon- $\beta$  (IFN- $\beta$ )]; 5'-GGGATTCCCC-3' (H-2K gene); and 5'-GGGGAATCCC-3' (mutated IFN- $\beta$ ). Scatchard analysis was performed by the use of varying concentrations of  $^{32}$ P-labeled oligonucleotide probe containing a single copy of the individual  $\kappa$ B motifs and the various p50/p65 complexes. A representative plotting of the values obtained for binding to the Ig $\kappa$   $\kappa$ B motif is shown in Figure 2 and calculated  $K_d$  values for all complexes are presented in Table 1. To test whether the protein made in insect cells differs from that made in mammalian cells, a small amount of p50 was produced by transient transfection of 293 human kidney cells and was shown to have the same binding affinity as the insect protein for the  $\kappa$ B sites from the H-2 and IFN- $\beta$  site.

The p50 homodimer bound to the various  $\kappa$ B motifs



**Figure 2.** Scatchard plot analysis of the recombinant NF- $\kappa$ B subunits. Recombinant NF- $\kappa$ B subunits: p50 homodimer (■,  $K_d = 6.7$  pM); p65 homodimer (◆,  $K_d = 32.2$  pM); and p50/p65 heterodimer (□,  $K_d = 5.7$  pM) were analyzed for Ig $\kappa$   $\kappa$ B motif-binding affinity. Quantitative EMSA results obtained from various probe concentrations and a constant amount of recombinant protein subunits were plotted (Materials and methods).

**Table 1.**  $\kappa$ B Motif-binding affinity of NF- $\kappa$ B subunit homo- and heterodimers

	$K_d$ (pM)				
	p50/p50	p65/p65	p50/p65	p65 $\Delta$ /p65 $\Delta$	p50/p65 $\Delta$
Ig $\kappa$ (G)GGGACTTTCC(G)	6.7	32.2	5.7	27.0	ND
H-2 (G)GGGATTCCCC(G)	6.2	77.5	10.9	ND	7.0
IFN- $\beta$ (G)GGGAAATTCC(G)	5.9	24.8	8.9	15.0	7.7
mutant IFN- $\beta$ (G)GGGGAATCCC(G)	7.3	57.2	13.5	ND	ND

Values were obtained by quantitative EMSA and Scatchard plot analysis as described in Fig. 2. Nucleotides in parenthesis are residues surrounding the  $\kappa$ B motifs within the probes used. (ND) Not determined.

with similarly high affinities ( $K_d = 5.9$ – $7.3$  pM). For example, by sequence, the Ig $\kappa$   $\kappa$ B motif (5'-GGGACTT-TCC-3') and H-2  $\kappa$ B motif (5'-GGGATTCCCC-3') are significantly diverged, but their affinities for p50 were indistinguishable ( $K_d = 6.7$  and  $6.2$  pM, respectively). In contrast, the p65 homodimer recognized these  $\kappa$ B motifs with very different affinities. The IFN- $\beta$   $\kappa$ B motif appeared to be the highest affinity site for p65 ( $K_d = 24.8$  pM). This value is still three- to fourfold lower than the affinity of this site for p50. Changing the central portion of the sequence of the IFN- $\beta$   $\kappa$ B motif GGGAAATTCC to GGGGAATCCC (mutant IFN- $\beta$ ) significantly lowered the binding affinity for p65. Removal of the carboxy-terminal region of p65 (p65 $\Delta$ ) did not significantly alter the DNA-binding affinity by this assay; however, evidence exists that this truncation alters the specificity of DNA sequence recognition (G. Nolan, unpubl.).

The heterodimer p50/p65 recognized these  $\kappa$ B motifs with a uniformly high affinity ( $K_d = 5.7$ – $13.5$  pM; Table 1). The mouse Ig $\kappa$   $\kappa$ B motif exhibited the highest affinity ( $K_d = 5.7$  pM), which is comparable to published DNA-binding affinity of purified natural NF- $\kappa$ B ( $K_d = 2.7$  pM; Zabel et al. 1991).

#### *In vitro* transcriptional activity of NF- $\kappa$ B subunits

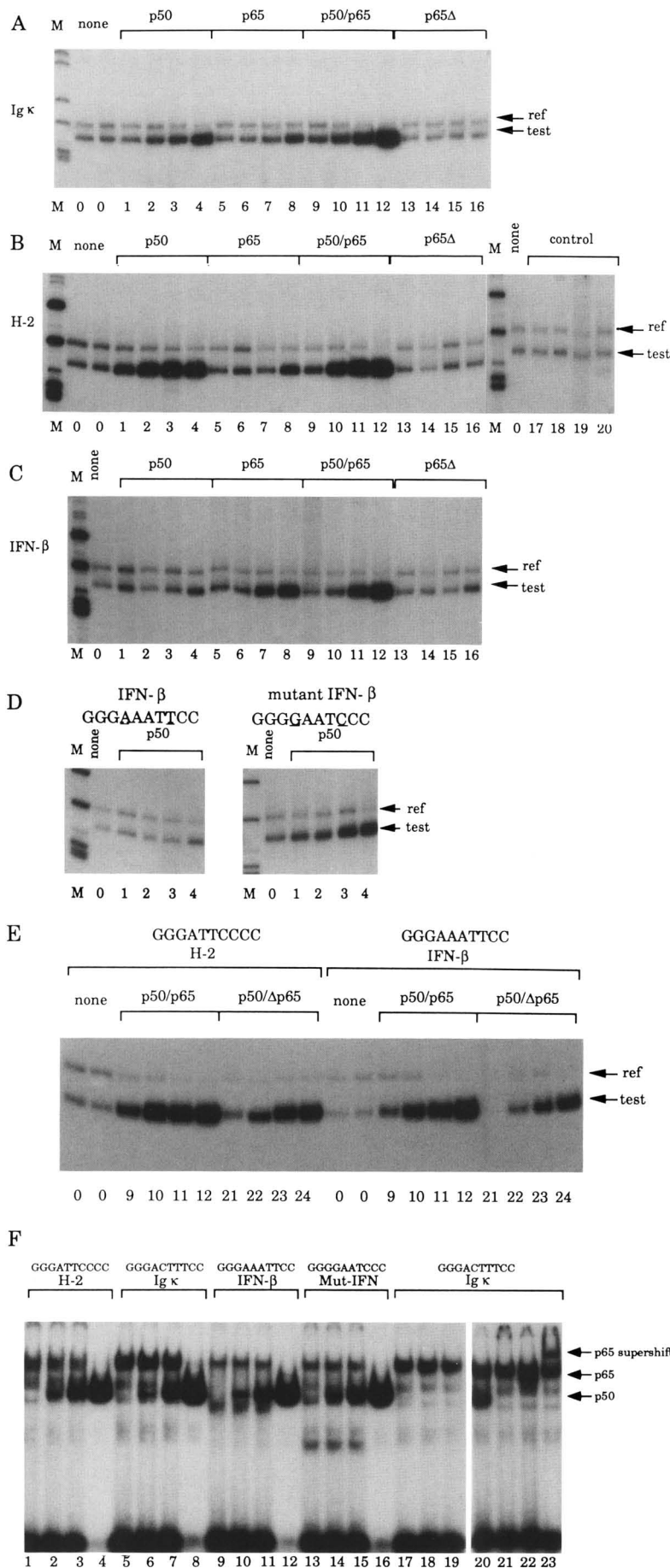
The various homo- and heterodimers of NF- $\kappa$ B subunits were tested for transcriptional activation *in vitro* by the use of HeLa cell nuclear extracts (Dignam et al. 1983). The test templates used contained three copies of a  $\kappa$ B motif upstream of an IFN- $\beta$  TATA box (Fujita et al. 1989). Two of the templates containing either IFN- $\beta$  or H-2  $\kappa$ B motifs were shown previously to be transcriptionally active in an induction-dependent manner when transfected into mouse L929 cells (Fujita et al. 1989). The control template (denoted ref in Fig. 3) contained only the IFN- $\beta$  TATA box, which is not detectably expressed when transfected into mouse L929 cells. After *in vitro* transcription, total RNA was extracted from the mixture and specific transcripts were quantitated by primer extension.

Under the conditions for *in vitro* transcription, the

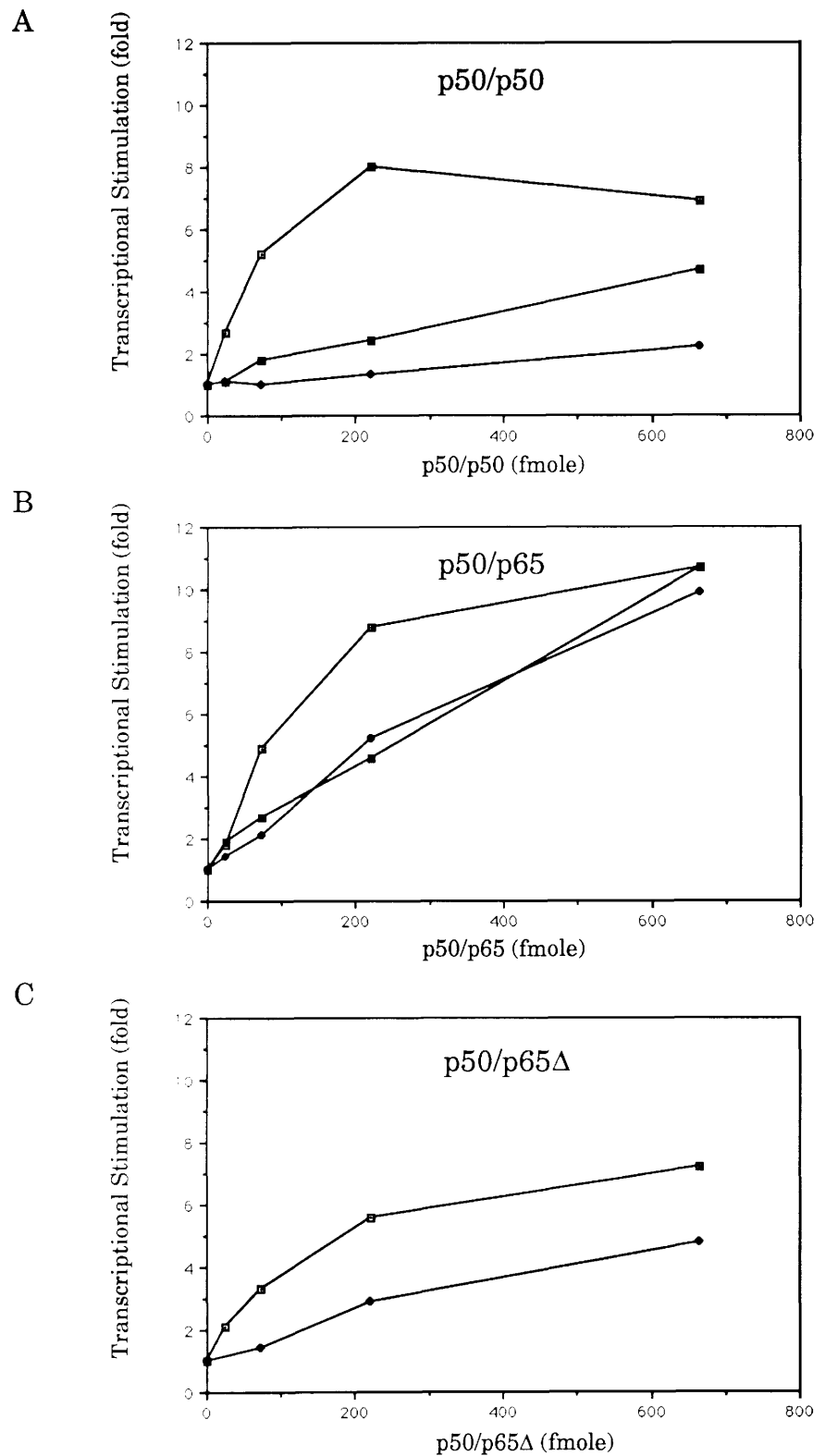
HeLa nuclear extract showed no NF- $\kappa$ B-specific binding to  $\kappa$ B motif by EMSA (Fig. 3F): The complexes formed did not coincide with those formed by p50 or p65; none of the complexes showed any reactivity to specific antibody prepared against p50 and p65; and addition of exogenous p50 or p65 subunits did not generate any new complexes. These results indicate that there is no detectable subunit exchange with endogenous proteins. Moreover, the extract showed a similar basal level of transcription on both the reference and test templates (Fig. 3, lanes 0, bands ref and test). Also, the addition of excess amounts of  $\kappa$ B motif-containing oligonucleotides did not influence these basal level of transcription (data not shown). We conclude, therefore, that this nuclear extract does not contain a significant level of NF- $\kappa$ B or related subunit activity but does utilize the constructs to provide a low level of transcriptional initiation.

The p50/p65 heterodimer, which bound to all tested  $\kappa$ B motifs with similarly high affinity (Table 1), showed extensive activation on all of the templates containing a  $\kappa$ B motif [Figs. 3A (lanes 9–12) and 4B,C]. A quantitation of the result (Fig. 4B) showed clearly that the H-2  $\kappa$ B motif is activated most efficiently among these three  $\kappa$ B motifs. This was unexpected because the affinity of p50/p65 to H-2  $\kappa$ B motif ( $K_d = 10.9$  pM) is lower than that to other two  $\kappa$ B motifs ( $K_d = 5.7$  and  $8.9$  pM, for Ig $\kappa$  and IFN- $\beta$ , respectively).

Interestingly, significant transcriptional stimulation was observed by the addition of p50 alone to transcription mixtures containing the H-2 or Ig $\kappa$   $\kappa$ B motif templates [Figs. 3A (lanes 1–4) and 4B]. This activation was dependent on the presence of the  $\kappa$ B motif in the template DNA because the reference template that contained no  $\kappa$ B motif was not stimulated by p50. Because the p50 preparation contained several copurified polypeptides, we prepared a similarly purified protein fraction from wild-type virus-infected cells. This control fraction contained an indistinguishable set of proteins from those contaminating the authentic p50 fraction (data not shown) but showed no transcriptional stimulation with the H-2  $\kappa$ B motif (Fig. 3B, lanes 17–20). This suggests that p50 is responsible for the transcriptional



**Figure 3.** Transcriptional activity of recombinant NF- $\kappa$ B subunits in vitro. The transcriptional activity of recombinant NF- $\kappa$ B subunits was tested using HeLa cell nuclear extracts in vitro. Templates used contained three tandem copies of Ig $\kappa$   $\kappa$ B motif (A; p-55Ig $\kappa$ ); H-2  $\kappa$ B motif (B,E; p-55A4); IFN- $\beta$   $\kappa$ B motif (C-E; p-55A2); mutated IFN- $\beta$   $\kappa$ B motif (D; p-55mutA2). Bands corresponding to the primer extension product for reference transcript (ref) and test template (test) are indicated. (Lanes M) Size markers (pUC18 digested by *Sau*3A); (lane O) buffer control; (lanes 1–4) p50; (lanes 5–8) p65; (lanes 9–12) p50/p65; (lanes 13–16) p65 $\Delta$ ; (lanes 17–20) control fractions from wild-type baculovirus-infected cells; (lanes 21–24) p50/ $\Delta$ p65. Amount of recombinant subunits added: (lanes 1,5,9,13,21) 25 fmoles; (lanes 2,6,10,14,22) 74 fmoles; (lanes 3,7,11,15,23) 220 fmoles; (lanes 4,8,12,16,24) 670 fmoles. Control lanes 17–20 contained equivalent amounts of the protein containing p50 (isolated from cells infected with a nonrecombinant baculovirus) as in lanes 1–4, respectively. In lanes 11 and 12, a reduction of transcription from the reference template was observed. We attribute this decrease to competition for general transcription factors/polymerase between reference and test templates at high overall transcription levels. (F) Detection of  $\kappa$ B DNA-binding activity in the transcription mixture. A  $^{32}$ P-labeled oligonucleotide probes, indicated at the top of figure, were mixed with a HeLa cell nuclear extract under the conditions for transcription (Materials and methods) in the presence or absence of recombinant p50. The mixture was analyzed by EMSA. (Lanes 1,5,9,13,17,18,19) Without recombinant NF- $\kappa$ B; (lanes 2,6,10,14) 25 fmoles of p50; (lanes 3,7,11,15,20,21) 74 fmoles of p50; (lanes 4,8,12,16) 220 fmoles of p50; (lanes 22,23) 220 fmoles of p65; (lanes 17,20,22) with preimmune rabbit serum; (lanes 18,21) with anti-p50 serum; (lanes 19,23) with anti-p65 serum.



**Figure 4.** Different  $\kappa$ B motifs are regulated differentially in vitro by NF- $\kappa$ B subunits. Results of in vitro transcription using  $\kappa$ B motif-containing templates [legend to Fig. 3] and p50 homodimer (A), p50/p65 (B), or p50/p65 $\Delta$  (C) was quantitated by Betascope. ( $\square$ ) H-2; ( $\diamond$ ) IFN- $\beta$ ; ( $\blacksquare$ ) Ig $\kappa$ . Calculated fold stimulation over the basal transcription (buffer control) was plotted.

activity in our purified fractions. Also heat treatment (68°C, 10 min) completely abolished the activation functions of the p50 and p65 subunits (data not shown).

Transcription activated by the H-2  $\kappa$ B motif was stim-

ulated most efficiently by p50, whereas that directed by the IFN- $\beta$   $\kappa$ B motif was barely stimulated [Figs. 3C (lanes 1–4) and 4A]. Although the templates contained three copies of either  $\kappa$ B motif, DNA-binding analysis by

EMSA showed no detectable difference in binding affinity or change in the number of p50 molecules bound. This difference in transcriptional activation appears to be primarily the result of recognition by p50 of the fine structure of the nucleotide sequence within the  $\kappa$ B motif and not simply binding affinity. This is suggested by the observation that mutation of 2 nucleotides in the IFN- $\beta$   $\kappa$ B motif (GGGAAATTCC to GGGGAATCCC) conferred susceptibility to activation by p50 (Fig. 3D), although the binding affinity of p50 to these motifs (Table 1) and actual occupancy of these sites by p50 (Fig. 3F) was practically indistinguishable. Interestingly, we observed eightfold higher constitutive gene expression by mutated IFN- $\beta$   $\kappa$ B site than wild-type IFN- $\beta$   $\kappa$ B site in mouse L cells (Table 2) in which KBF-1 factor is constitutive (Shirayoshi et al. 1987), suggesting an effect of  $\kappa$ B site in vivo.

The p65 homodimer also activated transcription from the  $\kappa$ B motifs tested [Figs. 3A (lanes 5–8) and 4B,C]. This activation was most prominent on the IFN- $\beta$   $\kappa$ B motif as expected from the relatively high-affinity binding to this  $\kappa$ B motif (Table 1). Note, however, that activation by p65 on the IFN- $\beta$  motif was greater than the activation by p50. This is the opposite of the activation potential of these same proteins upon the I $\kappa$ g  $\kappa$ B motif.

#### Mapping of the transcriptional activation domains of p65

A derivative of p65, p65 $\Delta$ , which lacks the carboxyl acidic region, failed to activate any  $\kappa$ B motif, including that of IFN- $\beta$  (lanes 13–16), although p65 $\Delta$  exhibited quite a high affinity for the IFN- $\beta$   $\kappa$ B motif (Table 1). This suggests that the carboxyl region of p65 is responsible for transcriptional activation by the p65 homodimer and that this region probably contains an activation domain that is functional in NF- $\kappa$ B. In this regard, heterodimers of p50/p65 $\Delta$  exhibited a reduced transcriptional activity from the H-2 and IFN- $\beta$   $\kappa$ B motifs when compared with p50/p65, without a notable reduction in DNA-binding affinity (Table 1; Fig. 4C). Thus, it is likely that a primary role of the carboxyl region of p65 in the NF- $\kappa$ B complex is to activate transcription.

It is worth noting that although p50/p65 $\Delta$  bound to the H-2 and IFN- $\beta$   $\kappa$ B motifs with indistinguishable affinity ( $K_d = 7.0$  and  $7.7$   $\mu$ M, respectively), its transcriptional activity on these motifs was significantly different (Figs. 3E and 4C). p50/p65 $\Delta$  is slightly more active than p50/

p50 on IFN- $\beta$   $\kappa$ B motif. Presumably, this is because the activation domain of p50 is not completely masked in the context of p50/p65 $\Delta$  heterodimer (Fig. 6A,C, below).

#### DNA binding-induced conformational change of NF- $\kappa$ B subunits

In the preceding sections we showed that the DNA-binding affinity and transcriptional activity of NF- $\kappa$ B subunits were not necessarily correlated. Particularly, p50 homodimer activated transcription from an H-2  $\kappa$ B motif but not from an IFN- $\beta$   $\kappa$ B motif, although actual occupancy of these sites by p50 was indistinguishable (Fig. 3F). To investigate the mechanism of this apparent discrepancy, we examined the gross structure of p50 bound to these motifs using a chymotrypsin-sensitivity assay. After binding of p50 to  $^{32}$ P-labeled probe oligonucleotides containing different  $\kappa$ B motifs for 20 min (binding was completed in  $<5$  min; data not shown), the complex was treated with chymotrypsin for various times. The reaction was terminated by adding chymostatin, and the complex containing labeled probe was analyzed by EMSA (Fig. 5A). Chymotrypsin treatment rapidly converted (within 1 min) the complex containing either of the  $\kappa$ B motifs into a faster migrating species. Thereafter, however, p50 bound to different  $\kappa$ B motifs was digested with different kinetics (Fig. 5C). The chymotrypsin insensitivity of p50 bound to different  $\kappa$ B sites correlated positively with its transcriptional activating potential (Fig. 3). In the absence of DNA, p50 was very sensitive to chymotrypsin: Its DNA-binding activity was reduced to 50% after a 1-min treatment under the same conditions (data not shown). p65 however, did not have an altered chymotrypsin sensitivity dependent on the binding site sequence (Fig. 5A).

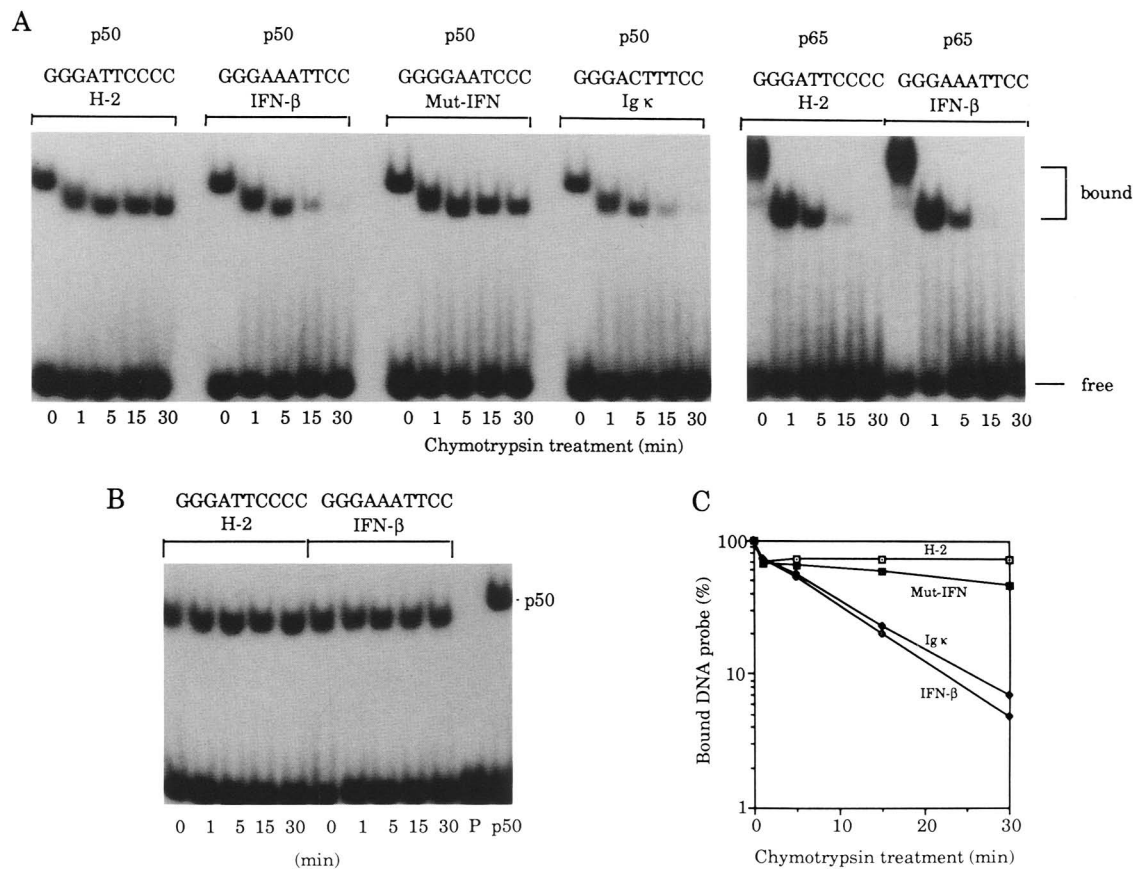
This difference of chymotrypsin sensitivity in p50 might be caused by an altered p50 conformation and/or differential dissociation rates of the DNA–protein complexes. We determined the dissociation rates of partially digested (1 min) p50 from both the H-2 and the IFN- $\beta$   $\kappa$ B motif DNAs (Fig. 5B). After chymotrypsin digestion of p50–probe complex for 1 min, the reaction was terminated by chymostatin. A large excess of unlabeled oligonucleotide containing the H-2  $\kappa$ B motif was then added (2000-fold excess over probe oligonucleotide; 1000-fold excess over p50 homodimer), and the mixture was incubated further. The remaining labeled DNA complex was quantitated by EMSA. The p50– $\kappa$ B motif complexes were extremely stable. Most important, the half-lives of p50 bound to the H-2 and IFN- $\beta$   $\kappa$ B motifs were both  $>30$  min. Because chymotrypsin digested the p50–IFN- $\beta$   $\kappa$ B motif complex much faster than it dissociates (half-lives of 5 min and 30 min, respectively), the rapid digestion of the p50–IFN- $\beta$   $\kappa$ B complex cannot be solely the result of digestion of dissociated, unbound p50 and must be due primarily to digestion of bound p50. We conclude that p50 adopts a conformation upon binding to the H-2  $\kappa$ B motif that is resistant to digestion by chymotrypsin and that is different from the structure of p50 bound to other  $\kappa$ B motifs. The chymotrypsin-resistant p50– $\kappa$ B motif

**Table 2.** Activity of wild-type and mutated IFN- $\beta$   $\kappa$ B motif in cells

	Relative CAT activity <sup>a</sup>
TATA–CAT	0.0
(IFN- $\beta$ $\kappa$ B) <sub>3</sub> –CAT	1.0
( $\mu$ IFN- $\beta$ $\kappa$ B) <sub>3</sub> –CAT	8.3

<sup>a</sup>Values are normalized by reference to a cotransfected CRE–luciferase construct, and background is subtracted using no DNA transfected as control.

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**Figure 5.** p50 binds to H-2 and IFN- $\beta$   $\kappa$ B motifs with distinct conformations. (A) p50 or p65 bound to  $^{32}$ P-labeled probe containing various  $\kappa$ B motif was digested with chymotrypsin for the indicated length of time. (0 min) No enzyme digestion. After terminating the digestion by adding chymostatin, the protein-DNA complex was analyzed by EMSA. (bound) Probe DNA bound to p50; (free) free probe. (B) Measurement of dissociation rate of p50 from H-2 or IFN- $\beta$   $\kappa$ B motif DNA. p50 was mixed with  $^{32}$ P-labeled probe containing either H-2 (left) or IFN- $\beta$  (right)  $\kappa$ B motif for 20 min. Chymotrypsin (400 mg/ml) was added and allowed to incubate for 1 min. The reaction was stopped by the addition of chymostatin. A 1000-fold molar excess of unlabeled oligonucleotide containing the H-2  $\kappa$ B motif was added for the indicated length of time. The remaining complex was separated from free probe by EMSA, followed by Betascope quantitation. (C) Quantitation of chymotrypsin sensitivity. p50 results in A were quantitated by Betascope and plotted.

complexes are those that gave the highest activation in *in vitro* transcription. These results suggest that transcriptional activity of the p50 homodimer is dependent on its conformation as determined by interaction of p50 with the specific sequence constituting a given  $\kappa$ B motif.

## Discussion

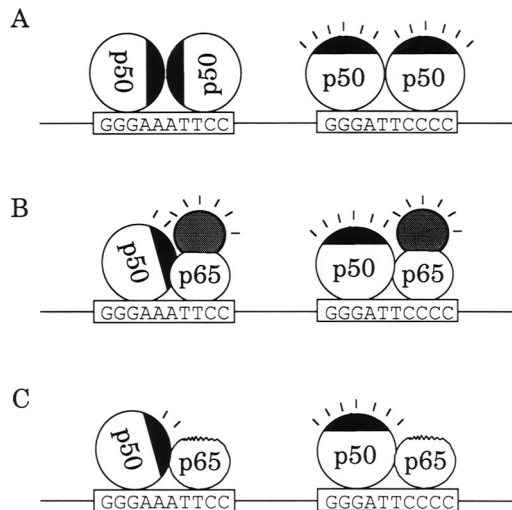
By analyzing both the DNA-binding activity and the *in vitro*  $\kappa$ B transcriptional activation ability of the two NF- $\kappa$ B subunits, an unexpected complexity of behavior has emerged as schematically represented in Figure 6. First, each subunit forms a homodimer, but when mixed, preferentially forms a heterodimer. Second, the homo- and heterodimers all bind to DNA, but (p50) $_2$  binds ~10-fold better than (p65) $_2$ , and the heterodimer binds with an intermediate efficiency. Third, both p50/p65 and (p65) $_2$  activate transcription from all binding sites, albeit with somewhat varying efficiency, whereas (p50) $_2$  activation is highly dependent on the sequence of the site to which

it binds. Fourth, virtually all of the activating ability of the p65 subunit is in its carboxy-terminal non-*rel* extension, a region with no homology to p50. Fifth, the activating ability of p50 is correlated to a chymotrypsin-resistant structure that it adopts when bound to an activating sequence. Sixth, in the heterodimer, each component contributes to the activation potential individually. This complexity of biochemical behavior makes two biological predictions: that the specific sequence of each  $\kappa$ B site has evolved to provide a particular level of activation determined by the combined effects of p50 and p65 and that the (p50) $_2$  found as KBF-1 in the nuclei of many cells probably is a functional activator with a high site specificity. Whether (p65) $_2$  is ever found in cells as a functional entity needs to be determined.

### DNA-binding activity

Previous evidence had led to the suggestion that p65 could not bind to DNA; thus, a heterotetramer model for





**Figure 6.** Model for transcriptional activation by NF- $\kappa$ B subunits. A totally schematic representation of the behavior of p50 and p65 in various combinations is provided. The solid region of p50 is its activation domain, which is shown as being partially or completely revealed, depending on the DNA sequence to which it is bound. The activation domain of p65 is shown as a knob that is active whenever p65 is bound to a site. In p65 $\Delta$ , the knob [carboxy-terminal region of the protein] is deleted. (A–C) The activities of (p50)<sub>2</sub>, p50/p65, and p50/p65 $\Delta$ , respectively, when bound to either the IFN- $\beta$   $\kappa$ B site (left) or the H-2  $\kappa$ B site (right).

p50/p65 was proposed (Baeuerle and Baltimore 1989). It is now evident that p65 binds well to DNA. Previously, binding had been done with poly[d(I-C)] · poly[d(I-C)]. In the present experiments, by using recombinant protein and omitting the competing polymer, the binding potential of p65 was revealed. Urban et al. (1991) also noted this effect and found that renatured p65 could bind to DNA. Our mixing experiments are consistent with p65 forming a dimer, and previously, p50 had been shown to dimerize; thus, we now believe that p50/p65 is a dimer, a conclusion also reached by Urban et al. (1991). An equimolar mixture of p50 and p65 forms almost exclusively p50/p65, indicating that the heterodimerization constant is lower than at least one of the homodimerization constants. We tested four  $\kappa$ B motifs for their ability to bind (p50)<sub>2</sub>, (p65)<sub>2</sub>, and p50/p65. Although binding constants for any one dimer varied up to threefold, depending on the DNA sequence, the major effect was that (p50)<sub>2</sub> bound with the highest affinity ( $K_d \sim 6.5$   $\mu$ M), (p65)<sub>2</sub> with the lowest ( $K_d = 25$ – $75$   $\mu$ M), and p50/p65 with a lower intermediate affinity ( $K_d = 6$ – $14$   $\mu$ M). Thus, it is the specificity of heterodimerization that determines the form of NF- $\kappa$ B bound to DNA, and not the affinity for DNA. It is significant that the highest DNA-binding affinity is that of p50/p65 for the immunoglobulin  $\kappa$ B site, the site also found twice in the human immunodeficiency virus long terminal repeat (HIV LTR). In the immunoglobulin  $\kappa$  enhancer, NF- $\kappa$ B plays the central role in activating transcription (Lenardo et al. 1987), and

for HIV, NF- $\kappa$ B is probably the crucial regulator of its replication rate (Nabel and Baltimore 1987; M. Feinberg and D. Baltimore, unpubl.).

#### Transcriptional activation

Previous data had indicated that p50/p65 purified from cells can activate transcription up to threefold in a HeLa cell-free system (Kawakami et al. 1988). By using recombinant subunits of unlimited quantity, we could show 10-fold activation dependent on  $\kappa$ B motifs and could reveal the activation potential of the individual subunits. (p50)<sub>2</sub> and (p65)<sub>2</sub> both activate, but activation is dependent on very different parameters. Activation by p65 is dependent on its carboxy-terminal region—as found previously for the *c-rel* product (Kamens et al. 1990)—and is correlated to its  $\kappa$ B motif-binding affinity: The IFN- $\beta$   $\kappa$ B motif is the highest affinity site and is activated most efficiently by (p65)<sub>2</sub>. Activation by (p50)<sub>2</sub> is dependent on its conformation as detected by the chymotrypsin probe: Although the H-2 and IFN- $\beta$   $\kappa$ B motifs bind to (p50)<sub>2</sub> with approximately equal high affinity, only the former, which induces a chymotrypsin-resistant structure in the bound (p50)<sub>2</sub>, activates transcription (Fig. 6A).

Transcriptional regulation dependent on a DNA-induced conformation of a transcription factor has been suggested previously in two cases. The activation functions of the thyroid hormone and glucocorticoid receptors were found to be modulated by the specific site that they are bound in transfection experiments in cells (Glass et al. 1988; Sakai et al. 1988). Yeast pheromone/receptor transcription factor (PRTF) was shown to adopt a specific conformation only when bound to the *a*-specific upstream activating sequences, and this was correlated to transcriptional activity in vivo (Tan and Richmond 1990). In neither case, however, was it shown by direct in vitro analysis that transcription factor conformation when bound to specific DNA sites determines activation potential. Our demonstration of an in vitro effect, however, makes it likely that the other cases do involve DNA site-dependent conformational effects and suggests that this may be a widespread phenomenon.

Although we have found that it is difficult to demonstrate any  $\kappa$ B-dependent stimulation of in vivo transcription by cotransfection with p50-producing constructs, we were able to derive in vivo results that correlate with the in vitro phenomenology without added p50, presumably because of endogenous (p50)<sub>2</sub> (KBF-1). There was eightfold more activity of a mutated IFN- $\beta$   $\kappa$ B site than a wild-type IFN- $\beta$   $\kappa$ B site in mouse L cells (Table 2) in which KBF-1 factor is constitutive (Shirayoshi et al. 1987), suggesting an effect of specific  $\kappa$ B site sequence in vivo.

The segment of p50 that provides activation activity has yet to be identified. Comparing the sequence of p50 with that of p65, however, suggests potential activation domains. p65 has a *rel*-related region of  $\sim 35$  kD while the *rel*-related region of p50 is spread over  $\sim 50$  kD. The difference is the result of a number of “inserts” in the p50 *rel* homologous region. One or more of these inserts

could well provide the activation activity (T. Fujita and D. Baltimore, unpubl.). How the structure of the p50 molecule is altered by DNA binding to release its activation potential remains to be discovered.

#### Dual elements in p50/p65

In the heterodimer, each component plays a separate activating role. We come to this conclusion from two types of evidence. First, deletion of the carboxyl terminus of p65, which almost completely inactivates the transcriptional activity of (p65)<sub>2</sub> in our assay, leaves more activation function in the context of the heterodimer. Second, the activity of the p50/p65Δ heterodimer is dependent on the sequence of the site to which it is bound, showing that the configuration-dependent property of p50 is evident in the heterodimer (Fig. 6C). Third, the activity of the p50/p65 heterodimer is a function of the site, with the H-2 κB site giving the highest activity at low NF-κB input (Figs. 4B and 6B). The H-2 κB site binds (p65)<sub>2</sub> poorly and activates by (p65)<sub>2</sub> poorly (Table 1; Fig. 3), so it must be the combination of the high affinity for p50 and the activating role of p50 bound to the H-2 motif that makes the H-2 site so active.

#### Biological significance

These biochemical studies have revealed a complexity to NF-κB that has clear biological implications. The NF-κB constituents p50 and p65 and the κB site are both seen to provide previously unsuspected elements of specificity. The p50 and p65 polypeptides, although homologous in sequence, are quite different in function. (p50)<sub>2</sub> which was purified previously as a constitutive nuclear protein (KBF-1), has a DNA-induced transcriptional activation potential that identifies it as a probable constitutive transcription factor. Although it binds to all tested κB sequences, only a subset provides the conformation to the molecule that makes it a strong activator.

Although the full range of active sequences remains to be defined, the site upstream of H-2 genes is highly active, supporting the notion that (p50)<sub>2</sub> provides a basal level of H-2 gene transcription by interaction with this site. No other site with this exact sequence and activity is yet known, but it will be interesting to see whether other genes have such a site or whether (p50)<sub>2</sub> is dedicated to providing a basal level of H-2 class I gene products on cells. Almost all cells have class I proteins on their surface because such proteins are important to the recognition of cells as self by the immune system. In this regard, it is significant that the identical κB motif is preserved in H-2K<sup>b</sup> (Israël et al. 1987), H-2D<sup>d</sup> (Korber et al. 1988), HLA-A3, HLA-A2, HLA-A11, HLA-B7, HLA-B27, and HLA-B51 genes (Hakem et al. 1989). (p50)<sub>2</sub> has partial in vitro activity on other sites, such as that in the immunoglobulin κ gene. This κB motif is also found upstream of the β<sub>2</sub>-microglobulin gene, which encodes a non-polymorphic class I polypeptide chain, suggesting an involvement of (p50)<sub>2</sub> in its universal expression. In contrast, class II genes are known to be regulated by dif-

ferent mechanisms (Lee 1988; Liou et al. 1990). Synthesis of the p50 protein is complicated because it is the amino-terminal region of the p105 protein. How a pool of (p50)<sub>2</sub> is produced is not yet understood.

(p65)<sub>2</sub> is also a transcriptional activator, but no pool of such a protein is known. Given that p50 and p65 heterodimerize so efficiently it is unlikely that cells could have pools of both (p65)<sub>2</sub> and (p50)<sub>2</sub>. We assume that p50 is maintained in excess and that (p65)<sub>2</sub> does not exist, but until a direct study of the question has been provided, it is possible that a secondary modification could inhibit heterodimerization in a fraction of the p50 or p65 molecules and allow for coexistence of all forms of the polypeptides.

κB sites are found in the regulatory regions of many genes. In general, they are genes that are important to cells in conditions of attack by pathogens, immunologic responses, or cellular stress (Lenardo and Baltimore 1989; Baeuerle and Baltimore 1991). In particular, many cytokine genes rely on κB sites for their transcription. The IFN-β site, studied here, is a good example in that it shows no (p50)<sub>2</sub> stimulation but responds to the p50/p65 that is released from IκB by induction stimuli. These sites are generally highly asymmetric but now must be investigated separately to ascertain their individual activities. They are often coupled to sites for other transcription factors, and their particular structures could facilitate or even demand such interaction.

## Materials and methods

### Plasmid construction

The *FspI* fragment of p105 cDNA (Ghosh et al. 1990) was subcloned into the *SmaI* site of pBluescript(SK+) (pBSK+) phagemid, and single-stranded DNA was obtained by superinfection with the helper M13 phage. Using this single-stranded DNA and the oligonucleotide 5'-GAAGCCCTGGCCAGCT-TAAGGCTACTCGAACTAC-3', site-directed mutagenesis was performed. The mutagenesis altered codons 401 and 402 [GGG (glycine) and TAT (tyrosine)] into GCT (alanine) and TAA (terminator), respectively, and also created a new restriction enzyme site, *AflII*. The mutagenized cDNA was excised by digestion of double-stranded plasmid DNA with *EcoRI* and *AflII*, ends were made flush with T4 DNA polymerase, and it was cloned into pVL1392. This plasmid is designated pVLp50.

The pBSK+ containing p65 cDNA (Nolan et al. 1991) was used for subcloning the whole p65-coding region (nucleotide 1–2208) into pVL1392. This plasmid is designated pVLp65.

To express the short p65, p65Δ, the cDNA was truncated by *BspHI* and subcloned into pVL1392 using the synthetic adaptor.

5'-CATGTGAGCGGCCGCC-3'  
3'-ACTCGCCGCGGG-5'

The adaptor provides a translational terminator at codon 314 and a *NotI* recognition site. This plasmid is designated pVLp65Δ.

In vitro transcription templates p-55A2 and p-55A4 were described previously (Fujita et al. 1989). New plasmids p-55 IκB

and p-55muA2 were generated by use of the following oligonucleotides, as described previously.

p-55I $\kappa$ :

5'-TCGACCCGGGACTTTCCGCGGGGACTTTCCGCGGGGACTTTCCGG-3'  
3'-GGGCCCTGAAAGGGCGCCCTGAAAGGGCGCCCTGAAAGGCCCTAG-5'

p-55muA2:

5'-TCGACCCGGGGAATCCGCGGGGGAATCCGCGGGGGAATCCCGG-3'  
3'-GGGCCCTTAGGGCGGCCCTTAGGGCGGCCCTTAGGGCCCTAG-5'

The reference template p-55Spe was made by inserting one copy of a *SpeI* linker (5'-GGACTAGTCC-3') into the *HindIII* site of p-55cat.

#### Production of recombinant NF- $\kappa$ B proteins and purification

Recombinant baculoviruses were prepared by the MAXIBAC baculovirus expression system (Invitrogen Corp.). SF9 cells ( $10^8$  cells/15-cm dish) were infected with recombinant baculoviruses at a m.o.i. of 5–10 and cultured for 3–4 days. Infected cells ( $10^9$  cells) were harvested with a rubber policeman, washed once with PBS, and lysed by vortexing in buffer D' [20 mM HEPES (pH 7.9), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.1 % NP-40, 10% glycerol] containing 100  $\mu$ g/ml of leupeptin. Nuclei and ribosomes were removed by centrifugation at 2000g for 10 min and 100,000g for 30 min, respectively. The supernatant was applied to a phosphocellulose column (10 ml) equilibrated with buffer D', and the column was washed with the same buffer and eluted with a 50 mM to 2 M NaCl gradient in buffer D'. The eluted fractions were tested for  $\kappa$ B motif-binding activity by EMSA. The active fractions were pooled, diluted sixfold with buffer D', and applied to an oligonucleotide column (Kadonaga and Tjian 1986) containing multimerized oligonucleotide

5'-GATCAGAGGGGACTTTCCGAGG-3'  
3'-TCTCCCCTGAAAGGCTCCCTAG-5'

The column was washed and eluted as for P-cellulose. The active fractions were pooled and dialyzed against buffer D'. The recovery of proteins by each of these chromatographies is >90%.

Heterodimers were formed by incubating equal amounts of p50 and p65 (or their derivatives) at 37°C for 60 min (final protein concentration, 0.2 pmole/ml).

#### EMSA

The following oligonucleotides containing self-complementary regions were used as probes: I $\kappa$   $\kappa$ , 5'-GAGAGGGGACTTTCCGATTAGCTTTCCGAAAGTCCCCTCT-3'; H-2, 5'-GAGAGGGGATTCCCGATTAGCTTTCCGGGGAATCCCCTCT-3'; IFN- $\beta$ , 5'-GAGAGGGGAAATCCCGATTAGCTTTCCGAAATTTCCCCTCT-3'; and mutant IFN- $\beta$ , 5'-GAGAGGGGGAATCCCGATTAGCTTTCCGGGATTCCCCTCT-3' (underlined sequences show self-complementary regions).

Probes were labeled with polynucleotide kinase and [ $\gamma$ - $^{32}$ P]ATP (7000 Ci/mmol). The labeled probe was separated

from unincorporated [ $\gamma$ - $^{32}$ P]ATP and diluted to 4 pmole/ml. This probe was heated (100°C for 3 min) and self-annealed at 68°C for 30 min.

Our EMSA mixture (10  $\mu$ l) contained 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 10 mg/ml of BSA, 1% NP-40, 5% glycerol, 0.5–1  $\mu$ l of protein fraction, and 2–4 fmoles of  $^{32}$ P-labeled probe. The mixture was incubated at 25°C for 5–10 min and applied to a 4% polyacrylamide gel (0.25 $\times$  TBE) and electrophoresed (10 V/cm) for 60 min at room temperature.

For detection of  $\kappa$ B motif-binding activity in transcription reaction, the following solutions were mixed: 1  $\mu$ l of  $^{32}$ P-labeled probe (320 fmoles) and 5  $\mu$ l of carrier poly[d(I-C)] $\cdot$ poly[d(I-C)]; 10  $\mu$ l of recombinant subunits, as indicated in figures (in buffer D'); 10  $\mu$ l of buffer and nucleotide mix (see in vitro transcription); and 10  $\mu$ l of nuclear extract. After incubation at 30°C for 30 min, a portion (6  $\mu$ l) was subjected to EMSA. Where indicated, 0.1  $\mu$ l of preimmune or immune rabbit serum was included.

#### Native gel electrophoresis

To analyze the NF- $\kappa$ B subunits under nondenaturing condition, sample fractions were electrophoresed in native polyacrylamide gel (8% acrylamide; 0.5 $\times$  TBE; 7% glycerol), 10 V/cm, for 9 hr. The gel was stained with Coomassie brilliant blue.

#### Scatchard analysis

For Scatchard analysis, a constant amount (1 fmoles) of recombinant subunit was incubated with serially diluted oligonucleotide probe (<2 fmoles) in 10  $\mu$ l of EMSA mixture. The bound and free probes were separated by gel electrophoresis and quantitated by Beta Scope (Betagen) or PhosphorImager (Molecular Dynamics). Reactions were performed in duplicate.

#### Preparation of HeLa cell nuclear extract and in vitro transcription

HeLa cell nuclear extract was prepared by the published procedure (Dignam et al. 1983), with the following modifications: Nuclei were extracted with 0.6 M KCl; to remove KCl, the extract was dialyzed against buffer without KCl and dialysis was stopped when the salt concentration reached 100 mM (monitored by conductivity).

The in vitro transcription reactions were performed by adding the following components: 1  $\mu$ l of template mixture (test and reference plasmid, each 400 ng); 10  $\mu$ l of recombinant subunit (in buffer D'); 10  $\mu$ l of buffer and nucleotide mixture; and 10  $\mu$ l of nuclear extract. The final concentration of the mixture was 13 mM HEPES (pH 7.9), 0.5 mM MgCl<sub>2</sub>, 60 mM KCl, 66  $\mu$ M EDTA, 0.5 mM DTT, 8.3 % glycerol, and 0.6 mM NTP. The incubation was performed at 30°C for 60 min.

The total RNA was extracted from the mixture with phenol-chloroform and precipitated with ethanol. The transcripts were quantitated by primer extension, using a  $^{32}$ P-labeled oligonucleotide primer complementary to the chloramphenicol acetyltransferase (CAT) structural gene, 5'-CAACGGTGGTATATC-CAGTG-3', and Maloney murine leukemia virus (M-MLV) reverse transcriptase (BRL). The product sizes were 95 and 105 nucleotides for the test and reference transcripts, respectively. The products were quantitated by counting the radioactivity as described in Scatchard analysis.

#### Limited proteolysis of p50- $\kappa$ B motif DNA complex

p50- $\kappa$ B motif DNA complex formed in the EMSA mixture was treated with chymotrypsin (400  $\mu$ g/ml) for the indicated time,

and the reaction was terminated by adding chymostatin (final 500  $\mu\text{g/ml}$ ) and immediately applied to the gel for EMSA.

#### DNA transfection and assay for CAT activity

CAT constructs (p-55cat, p-55A2, and p-55muA2; 2  $\mu\text{g}$ ) were each cotransfected with a plasmid containing the reference gene pCRE-LUC [a construct containing two copies of CRE (TGACGTCA) in front of the *fos* TATA box, followed by luciferase; 1  $\mu\text{g}$ ] to L cells ( $2 \times 10^6$  cells), and CAT activity was determined as described [Fujita et al. 1989]. Luciferase activity was determined by Promega Luciferase Assay System.

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