

# Genetic analysis of NF- $\kappa$ B/Rel transcription factors defines functional specificities

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**The NF- $\kappa$ B transcription factors consist of dimeric proteins of the Rel homology family. They activate many promoters containing highly divergent  $\kappa$ B-site sequences. We have generated cell lines lacking individual and multiple NF- $\kappa$ B proteins and used them to establish interactions between components of the NF- $\kappa$ B-I $\kappa$ B signaling system. Functional compensation within the family of dimers was evident in knockout cell lines. Analysis of transiently transfected genes gave an impression of promiscuity that was not borne out by analysis of endogenous genes. Using TNF $\alpha$  as an inducer, a panel of endogenous genes showed a wide range of subunit specificities as well as highly variable kinetics of induction. Comparing the function and subunit specificity of genes with the sequence of the  $\kappa$ B DNA-binding site we found little correlation, indicating that NF- $\kappa$ B family member specificity for endogenous promoters is not solely encoded by the  $\kappa$ B site sequence itself.**

**Keywords:** compensation/NF- $\kappa$ B/Rel/specificity/transcription

## Introduction

Cell signaling molecules such as transcription factors are encoded in gene families whose members have distinct yet overlapping functions. The fact that increasingly complex genomes contain a greater number of gene family members rather than novel families (Lander *et al.*, 2001) suggests that diversity within a gene family may provide for specificity and versatility in the regulation of cellular responses. Characterizing the specific functions of the members of transcription factor gene families contributes to our understanding of gene regulatory networks and to the development of specific therapeutic strategies. For example, biochemical studies of the nuclear hormone receptor family have shown that the spacing between two tandem cognate DNA half-sites determines which family members bind and function (Rastinejad, 2001). Similarly within the large zinc finger transcription factor family, biochemical and biophysical characterization of DNA-protein interactions are the basis for synthetic transcription factor engineering (Wolfe *et al.*, 2000). However, for many other transcription factor families, such as the C/EBP

(Ramji and Foka, 2002), E2F (Trimarchi and Lees, 2002), AP-1 (Chinenov and Kerppola, 2001; Mehta-Grigoriou *et al.*, 2001) and NF- $\kappa$ B families, marked DNA-binding specificity rules have not been identified. Indeed, the *raison d'être* of multiple members within signal transduction protein families may not be molecular interaction specificities but could be found in their own differential regulation. Considering the immediate regulators of NF- $\kappa$ B activity, the I $\kappa$ B protein family, for example, the difference between family members that is of primary physiological importance appears to be regulation of synthesis, as demonstrated by the fact that I $\kappa$ B $\alpha$  knockout lethality was rescued by placing I $\kappa$ B $\beta$  under the control of the I $\kappa$ B $\alpha$  promoter (Cheng *et al.*, 1998). A quantitative analysis of the signaling characteristics of I $\kappa$ B family members has supported that conclusion (Hoffmann *et al.*, 2002).

The NF- $\kappa$ B (also called NF- $\kappa$ B/Rel) family of dimeric transcription factors mediates cellular responses to a wide variety of different stimuli by regulating the expression of a large number of genes of highly diverse functions (Pahl, 1999). Mouse knockout studies have revealed this pleiotropic signal transducer to be involved in inflammatory and stress responses, the control of the cell cycle, apoptosis, growth and proliferation, cell-cell communication, lymphocyte development and maturation, and neuronal learning (Gerondakis *et al.*, 1999; Meffert *et al.*, 2003). The functional transcription factors consist of pairs taken from the NF- $\kappa$ B protein family (p50, p52, p65, cRel, RelB), proteins that utilize the Rel homology domain (RHD) for DNA binding and dimerization. Their cognate DNA-binding element, the  $\kappa$ B-site, is found in the promoters of NF- $\kappa$ B responsive genes but displays a remarkably loose consensus, often described as G<sub>-5</sub>G<sub>-4</sub>G<sub>-3</sub>R<sub>-2</sub>N<sub>-1</sub>N<sub>0</sub>Y<sub>+1</sub>Y<sub>+1</sub>C<sub>+2</sub>C<sub>+3</sub> (Ghosh *et al.*, 1998). The heterogeneity of  $\kappa$ B-sites has been thought to confer specificity of regulation mediated by differential affinities of NF- $\kappa$ B isoforms. Purified recombinant RHD protein dimers could indeed be shown to select differential optimal DNA-binding motifs from a pool of random oligonucleotides (Kunsch *et al.*, 1992). Similarly, one study with transfected promoter-reporter constructs showed that multimerized  $\kappa$ B-sites from one of three different promoters (I $\kappa$  $\kappa$ , MHC H2 and IFN- $\beta$ ) exhibited differential responses when co-transfected with p50 and/or p65 expression plasmids (Fujita *et al.*, 1992).

However, more recent structural studies did not reveal evidence for highly sequence-specific DNA-NF- $\kappa$ B interactions, with base-specific contacts within the p50 homodimer structure involving primarily 5'-G<sub>-5</sub>G<sub>-4</sub>G<sub>-3</sub> (Ghosh *et al.*, 1995; Muller *et al.*, 1995) and contacts within the p65 homodimer structure involving primarily 5'-G<sub>-4</sub>G<sub>-3</sub> (Chen *et al.*, 1998). In fact, these studies have not revealed sufficient DNA base contacts to rationalize

$\kappa$ B-site sequence-specific binding. Though homodimers of p65 or cRel require only a 9 bp sequence for binding, this may be contained within certain 10 bp consensus-conforming  $\kappa$ B-sites. X-ray structures of the p50:p65 heterodimer bound to different  $\kappa$ B-sites, such as those derived from the immunoglobulin and HIV enhancers G<sub>-5</sub>G<sub>-4</sub>G<sub>-3</sub>A<sub>-2</sub>C<sub>-1</sub>T<sub>0</sub>T<sub>+1</sub>T<sub>+2</sub>C<sub>+3</sub>C<sub>+4</sub> (Chen *et al.*, 1998), the IFN- $\beta$  enhancer G<sub>-5</sub>G<sub>-4</sub>G<sub>-3</sub>A<sub>-2</sub>A<sub>-1</sub>A<sub>0</sub>T<sub>+1</sub>T<sub>+2</sub>C<sub>+3</sub>C<sub>+4</sub> (Berkowitz *et al.*, 2002; Escalante *et al.*, 2002), and the urokinase plasminogen activator gene promoter G<sub>-5</sub>G<sub>-4</sub>G<sub>-3</sub>A<sub>-2</sub>A<sub>-1</sub>A<sub>0</sub>G<sub>+1</sub>T<sub>+2</sub>A<sub>+3</sub>C<sub>+4</sub> (Chen-Park *et al.*, 2002) demonstrate that NF- $\kappa$ B binds a variety of sequences. Furthermore, the two RHDs contained in each dimer constituent relate to each other differently in different structures and lead to different degrees of DNA bending (Chen and Ghosh, 1999), emphasizing the remarkable permissiveness of NF- $\kappa$ B-DNA interactions.

Despite the difficulty in discerning specificity at the level of DNA-protein interactions, mice deficient in a single RHD protein show specific phenotypes. *rela*<sup>-/-</sup> animals are embryonic lethal with massive hepatocyte apoptosis (Beg *et al.*, 1995), while *cret*<sup>-/-</sup> mice develop normally but have defects in lymphocyte proliferation (Kontgen *et al.*, 1995). In part, family member-specific functions can be inferred by tissue-specific expression. While p65 appears to be ubiquitous, cRel is expressed constitutively primarily in mature monocytic and lymphocytic lineages (Liou *et al.*, 1994), while in others, such as fibroblasts, only following stimulation (Grumont and Gerondakis, 1990). In addition, there are differences in the molecular characteristics of RHD family members: transfection studies have identified activation domains in p65 and cRel, but not in p50 and p52 (Ghosh *et al.*, 1998). DNA-binding assays were used to identify dimerization rules: p50 (and presumably p52) can partner with all RHD family members including themselves, while RelB and cRel do not appear to engage in homotypic interactions. RelB, furthermore, does not appear to dimerize with p65 or cRel. Little is known about how the results from *in vitro* biochemical characterizations of RHD proteins can serve to explain physiologically relevant functional specificity in gene regulation, because selectivity of endogenous  $\kappa$ B-site containing promoters remains largely unexplored. In fact, in the case of the IL-12 promoter, results from cell-free or extra-chromosomal templates are shown not to recapitulate endogenous promoter specificity (Sanjabi *et al.*, 2000).

Genetic approaches are suitable to address questions of *in vivo* mechanistic specificity, but must take into account the possibility of interdependent regulation of factors in mammalian signaling networks; resulting compensatory mechanisms among functionally related molecules may lend increased robustness to signaling systems and affect the phenotype of genetic deletions. Here, we have undertaken a genetic analysis of RHD proteins with respect to NF- $\kappa$ B-dependent gene activation by creating a panel of single and double knockout cell lines. We have biochemically characterized NF- $\kappa$ B activation within them, and measured the TNF $\alpha$  responsiveness of a diverse set of NF- $\kappa$ B target genes. Our results reveal that RHD protein family members exhibit differential target gene specificities and demonstrate that a combination of genetic and biochemical analyses can be applied to decode

functional specificity rules of gene promoters in mammalian cells.

## Results

### **Cross-regulation and compensation within the NF- $\kappa$ B-I $\kappa$ B regulatory system**

The dominant  $\kappa$ B-binding activity induced by TNF $\alpha$  in fibroblasts is composed of p50 and p65, the mature gene products of the *nf $\kappa$ b1* and *rela* genes. Microarray studies with *nf $\kappa$ b1*<sup>-/-</sup>*rela*<sup>-/-</sup> doubly deficient cells have confirmed their key role in activating most TNF $\alpha$ -responsive genes; fibroblasts lacking both factors show no activation of genes controlled by NF- $\kappa$ B (A.Hoffmann and D.Baltimore, in preparation). To address the specific roles of p50 and p65 in NF- $\kappa$ B-dependent gene expression, we generated fibroblast cell lines from *nf $\kappa$ b1*<sup>-/-</sup> (Sha *et al.*, 1995), *rela*<sup>-/-</sup> (Beg *et al.*, 1995) and *nf $\kappa$ b1*<sup>-/-</sup>*rela*<sup>-/-</sup> embryos (Horwitz *et al.*, 1997). Western blots confirmed that p50 and p65 proteins were absent in respective knockout cell lines (Figure 1A), while other RHD proteins remained, though at somewhat altered levels. In particular, p52 levels were enhanced in *nf $\kappa$ b1*<sup>-/-</sup> cells, cRel levels were increased in *rela*<sup>-/-</sup> cells, and cRel as well as RelB levels were somewhat reduced in doubly deficient cells. Strikingly, *rela*<sup>-/-</sup> cells contained markedly reduced levels of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  proteins, while all three I $\kappa$ B proteins were reduced to almost undetectable levels in doubly deficient cells (Figure 1B).

To investigate the result of genetic knockouts within the RHD family on the activated NF- $\kappa$ B transcription factor, we examined the induction of nuclear  $\kappa$ B-binding activity in response to TNF $\alpha$  stimulation in wild-type and mutant cell lines (Figure 2). Gel shift and western blot analyses of the nuclear and cytoplasmic compartments of wild-type cells revealed a characteristic biphasic induction of nuclear NF- $\kappa$ B (Figure 2A) that results from the coordinated degradation and synthesis of I $\kappa$ B family members (Figure 2B). Cells lacking p50 showed only a marginal reduction in  $\kappa$ B-binding activity (Figure 2C) with no alteration in the temporal regulation. In p65-deficient cells, however, induction produced two distinct  $\kappa$ B-binding activities that did not undergo post-induction repression (Figure 2E), correlating with defective I $\kappa$ B $\alpha$  protein synthesis (Figure 2F). Finally, no significant  $\kappa$ B-binding activity was induced at any time point within a 6 h TNF $\alpha$  time course in doubly deficient cells (Figure 2G). These results confirm that p50 and p65 make up the major constituents of NF- $\kappa$ B in fibroblasts. However, neither p50 nor p65 single knockout cells were deficient in  $\kappa$ B-binding activity. In addition, the temporal regulation of nuclear NF- $\kappa$ B is altered in *rela*<sup>-/-</sup> cells.

### **Compensation and specificity on non-chromosomal promoter DNA**

To characterize the molecular composition of induced  $\kappa$ B-binding complexes, we employed antibodies specific for each Rel protein in gel mobility supershift analyses of nuclear extracts made after 30 min of TNF $\alpha$  stimulation. As expected, nuclear  $\kappa$ B-binding activity in wild-type cells (Figure 3A) was sensitive to antibodies specific to p50 and p65; indeed distinct complexes comprising dimers of p65 (upper arrow), p50:p65 (lower arrow) and p50 (data

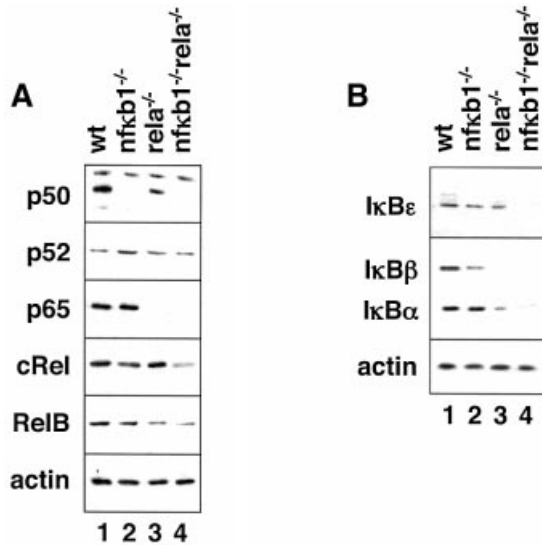
not shown) were identified. The lower complex (indicated by an asterisk) appeared to be non-specific as revealed by competition analysis with wild-type and mutant double-stranded oligonucleotides (lanes 2 and 3 in each panel). Not surprisingly, complexes apparent at long exposure in extracts from *nfkb1<sup>-/-</sup>rela<sup>-/-</sup>* double knockout cells (Figure 3D) were not recognized by any of the NF- $\kappa$ B family antibodies and were not specifically competed by double-stranded oligonucleotides.

Turning to single knockouts, anti-p50 antibodies, as expected, did not recognize  $\kappa$ B-binding complexes in extracts derived from *nfkb1<sup>-/-</sup>* cells (Figure 3B), nor did anti-p65 antibodies recognize  $\kappa$ B-binding complexes derived from *rela<sup>-/-</sup>* cells (Figure 3C). Instead, antibodies directed against p52 ablated the *nfkb1<sup>-/-</sup>* complex of the same mobility as the p50:p65 complex found in wild-type

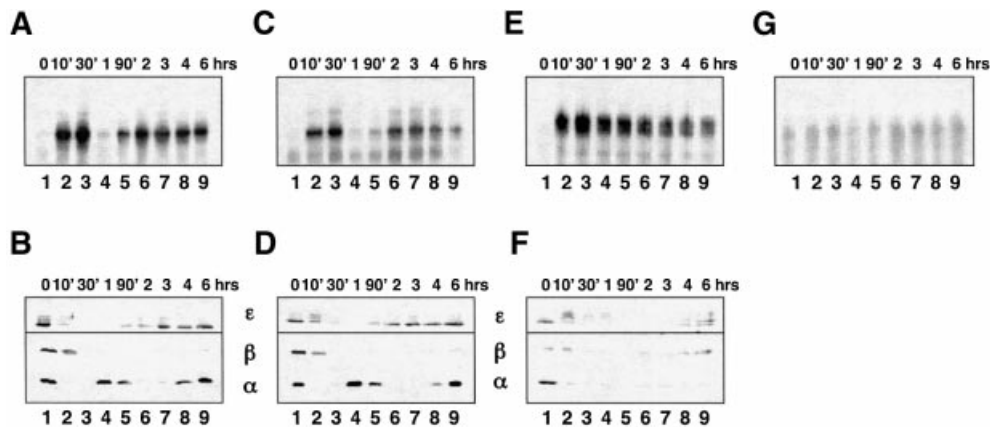
cells. Similarly, antibodies directed against cRel supershifted the analogous *rela<sup>-/-</sup>*  $\kappa$ B-binding complex, as well as a much weaker, slower migrating complex that may correspond to a cRel homodimer. These results strikingly revealed molecular compensation within the NF- $\kappa$ B/Rel family; however, not every family member functioned interchangeably with one another. Molecular compensation rules appear to reflect previously noted homology relationships, and the results from molecular characterization studies that inferred structural and functional similarities between p50 and p52, and p65 and cRel proteins (Ghosh *et al.*, 1998).

These results suggest multiple mechanisms of cross-regulation between the RHD and I $\kappa$ B protein family members. One known mechanism is transcriptional feedback: cRel (Grumont *et al.*, 1993), RelB (Bren *et al.*, 2001) and I $\kappa$ B $\alpha$  (Scott *et al.*, 1993) are known NF- $\kappa$ B target genes, which presumably explains observed decreases in protein levels in p50 and p65 doubly deficient cells, as well as the absence of post-induction repression of nuclear NF- $\kappa$ B in *rela<sup>-/-</sup>* cells. Another cross-regulation mechanism may be based on the protein stability differential between uncomplexed and complexed polypeptides; indeed reduced I $\kappa$ B protein levels in mutant cells may be the result of the loss of NF- $\kappa$ B dimers available for complex formation. Similar reasoning might explain the increased levels of p52 and cRel-containing complexes in *nfkb1<sup>-/-</sup>* and *rela<sup>-/-</sup>* cells by respective substitution in dimer formation.

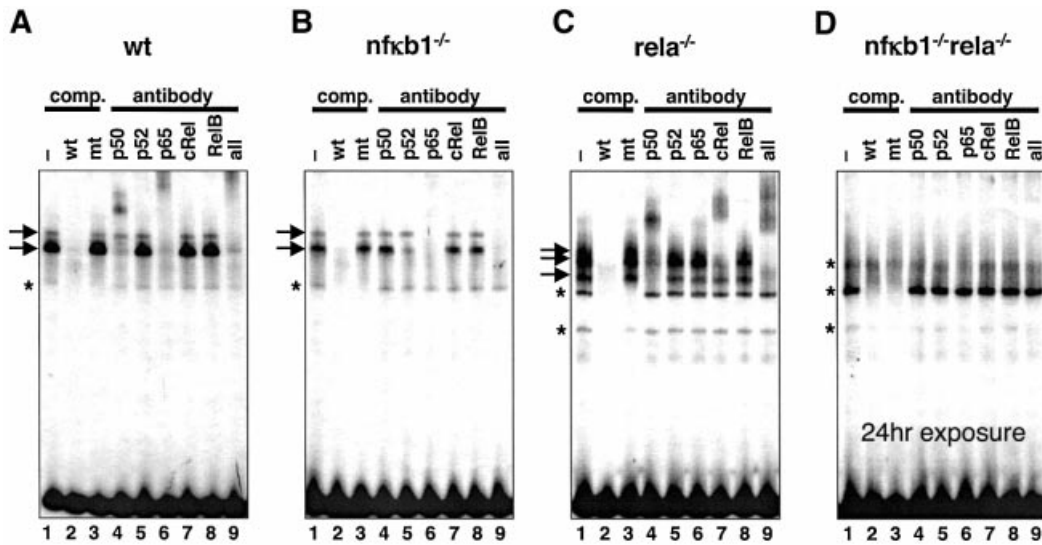
To address the transcriptional activity of p50- or p65-deficient NF- $\kappa$ B complexes, we utilized transient transfections with promoter-reporter constructs driven by multimerized  $\kappa$ B-sites. Each construct contained  $\kappa$ B-binding site sequences taken from one of three different promoters: I $\kappa$ g, MHC H2 or IFN- $\beta$ . These constructs had previously been shown to have differential responses to co-transfected p50 and p65 expression plasmids (Fujita *et al.*, 1992). We observed significant transcriptional activation of  $\kappa$ B-site-containing reporters in wild-type fibroblasts following TNF $\alpha$  stimulation (Figure 4A, white bars). Such activation was dependent on the presence of  $\kappa$ B-sites (lower right panel) and was minimal in cells deficient in



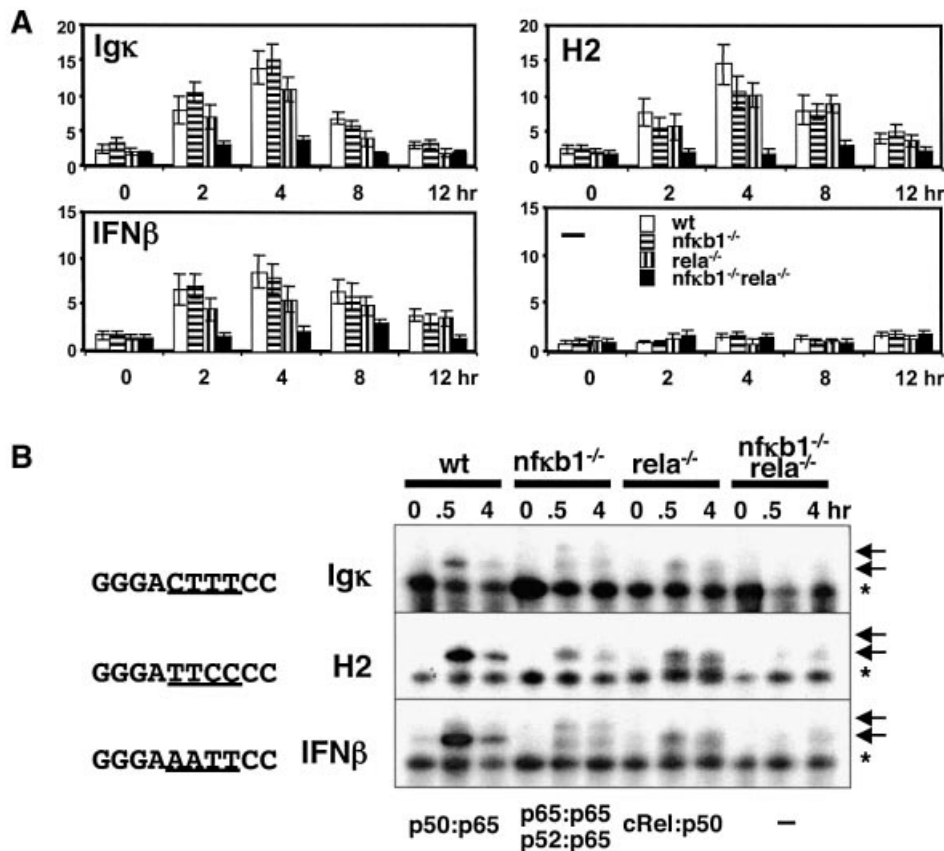
**Fig. 1.** RHD proteins in NF- $\kappa$ B knockout cells. Western blots of cells derived from wild-type, *nfkb1<sup>-/-</sup>*, *rela<sup>-/-</sup>* and *nfkb1<sup>-/-</sup>rela<sup>-/-</sup>* mouse embryos with antibodies directed against p50, p52, p65, cRel, RelB and actin (A), and I $\kappa$ B $\epsilon$ , - $\beta$ , - $\alpha$  and actin (B) as indicated. Specific bands are shown, but additional bands were detectable with some antibodies.



**Fig. 2.** TNF $\alpha$  induction of  $\kappa$ B-binding activity in NF- $\kappa$ B knockout cells. EMSA was used to monitor nuclear NF- $\kappa$ B in wild-type (A), *nfkb1<sup>-/-</sup>* (C), *rela<sup>-/-</sup>* (E) and *nfkb1<sup>-/-</sup>rela<sup>-/-</sup>* (G) cells in the indicated (in min and h) time course following the onset of TNF $\alpha$  stimulation. The cytoplasmic portion from wild-type (B), *nfkb1<sup>-/-</sup>* (D), *rela<sup>-/-</sup>* (F) was probed in western blots to monitor concurrent degradation and synthesis of I $\kappa$ B proteins, I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  (bottom panels) and I $\kappa$ B $\epsilon$  (top panels).



**Fig. 3.** Molecular composition of  $\kappa$ B-binding activity in NF- $\kappa$ B knockout cells. Immediate early protein-DNA complexes induced by TNF $\alpha$  in wild-type (A), *nf $\kappa$ b1*<sup>-/-</sup> (B), *rela*<sup>-/-</sup> (C) and *nf $\kappa$ b1*<sup>-/-</sup>*rela*<sup>-/-</sup> (D) cells and detected by EMSA in Figure 2 are indicated by arrows in lanes 1, while constitutive complexes are indicated by asterisks. They are specific for  $\kappa$ B-sites as shown by competition with double-stranded wild-type and mutant oligonucleotides (lanes 2 and 3) and are probed with antibodies directed against the indicated RHD proteins (lanes 4–9). This results in ‘supershift’ or ablation of the characteristic protein-DNA complex. We conclude that the  $\kappa$ B-binding activity in wild-type cells consists of p50:p65 heterodimer and p65 homodimer, in *nf $\kappa$ b1*<sup>-/-</sup> cells p52:p65 heterodimer and p65 homodimer, in *rela*<sup>-/-</sup> cells p50 homodimer, cRel:p50 heterodimer, and most likely, but not unambiguously cRel homodimer. While experiments shown in panels A, B and C were exposed to film for 6 h, panel D shows a 24 h exposure.



**Fig. 4.** Compensation on different  $\kappa$ B-site sequences in extra-chromosomal plasmids. Reporter plasmids driven by the *c-fos* core promoter alone or fused to two  $\kappa$ B-sites derived from the Ig $\kappa$  the MHC H2 and IFN $\beta$  promoter, as indicated in (A), were assayed in response to TNF $\alpha$  stimulation for the indicated time in wild-type (empty bars), *nf $\kappa$ b1*<sup>-/-</sup> (horizontally striped bars), *rela*<sup>-/-</sup> (vertically striped bars) and *nf $\kappa$ b1*<sup>-/-</sup>*rela*<sup>-/-</sup> (black bars). EMSA (B) was used to monitor  $\kappa$ B-binding activity in TNF $\alpha$ -stimulated NF- $\kappa$ B knockout cells with indicated  $\kappa$ B-site containing double-stranded oligonucleotide probes. Induced, specific bands are indicated by arrows, a prominent constitutive protein-DNA complex is indicated by an asterisk.

**Table I.** RHD proteins induced by TNF $\alpha$  stimulation in NF- $\kappa$ B knockout cells

MEFs genotype	$\kappa$ B-binding proteins				$\kappa$ B-binding complexes in EMSA		
	p50	p52	p65	cRel			
wt	+	–	+	–	p50:p50 <sup>a</sup>	p50:p65	p65:p65 <sup>a</sup>
<i>nfkbl</i> <sup>-/-</sup>	–	+	+	–		p52:p65	p65:p65 <sup>a</sup>
<i>nfkbl</i> <sup>-/-</sup> <i>nfknb2</i> <sup>-/-</sup>	–	–	+	–			p65:p65
<i>nfknb2</i> <sup>-/-</sup>	+	–	+	–	p50:p50 <sup>a</sup>	p50:p65	p65:p65 <sup>a</sup>
<i>rela</i> <sup>-/-</sup>	+	–	–	+	p50:p50	p50:cRel	cRel:cRel
<i>rela</i> <sup>-/-</sup> <i>crel</i> <sup>-/-</sup>	+	–	–	–	p50:p50		
<i>crel</i> <sup>-/-</sup>	+	–	+	–	p50:p50 <sup>a</sup>	p50:p65	p65:p65 <sup>a</sup>
<i>nfkbl</i> <sup>-/-</sup> <i>rela</i> <sup>-/-</sup>	–	–	–	–		–	

Fibroblast cell lines derived from embryos of indicated genotypes (column 1) exhibit TNF $\alpha$ -inducible  $\kappa$ B-binding activity that consists of indicated RHD proteins (column 2). These proteins form  $\kappa$ B-binding complexes indicated in column 3. All data are based on EMSA with  $\kappa$ B-site-containing probes and antibodies directed against specific RHD proteins.

<sup>a</sup>Complexes detected with only a subset of  $\kappa$ B-site-containing probes.

both p50 and p65 (black bars). However, fibroblasts lacking only one family member, either p50 (horizontally striped bars) or p65 (vertically striped bars), still showed significant levels of activation, with some reduction observed (about half) in *rela*<sup>-/-</sup> cells. Surprisingly, the results were similar for all three promoter constructs. Those  $\kappa$ B-sites also revealed little difference in NF- $\kappa$ B complexes when employed in a gel shift assay with nuclear extracts from the above-described TNF $\alpha$ -stimulated mutant cell lines. Each probe revealed p50:p65 complexes in wild-type cells, p52:p65 complexes in *nfkbl*<sup>-/-</sup> cells, and p50:cRel complexes in *rela*<sup>-/-</sup> cells (Figure 4B; data not shown). However, we note that the H2  $\kappa$ B-probe does not show p65 homodimer complexes in *nfkbl*<sup>-/-</sup> cells and appears to impart somewhat lower transcriptional activity than the other  $\kappa$ B-sites.

The above-described analyses indicate that molecular compensation occurs within the RHD protein family and results in functional compensation on extra-chromosomal templates; the 10 bp  $\kappa$ B-sites did not reveal much specificity for NF- $\kappa$ B isoforms, although certain sequences may certainly provide a restriction with respect to the entire panel of possible dimers.

We next turned to the regulation of *endogenous* genes to determine whether they exhibit similar promiscuity. However, compensation evident in single knockouts required the inclusion of fibroblast cell lines that are *nfkbl*<sup>-/-</sup>*nfknb2*<sup>-/-</sup> and *rela*<sup>-/-</sup>*crel*<sup>-/-</sup> doubly deficient, as well as single knockout controls derived from *nfknb2*<sup>-/-</sup> and *crel*<sup>-/-</sup> embryos. For example, to address the function of p65 in wild-type cells, *rela*<sup>-/-</sup>*crel*<sup>-/-</sup> cells are an informative genotype because the results will not be affected by cRel compensation for the loss of p65. In turn, we can address the question of molecular specificity of RHD proteins in transcriptional activation by determining whether molecular compensation by cRel results in functional compensation on endogenous promoters.

### Systematic genetic analysis of a transcription factor family

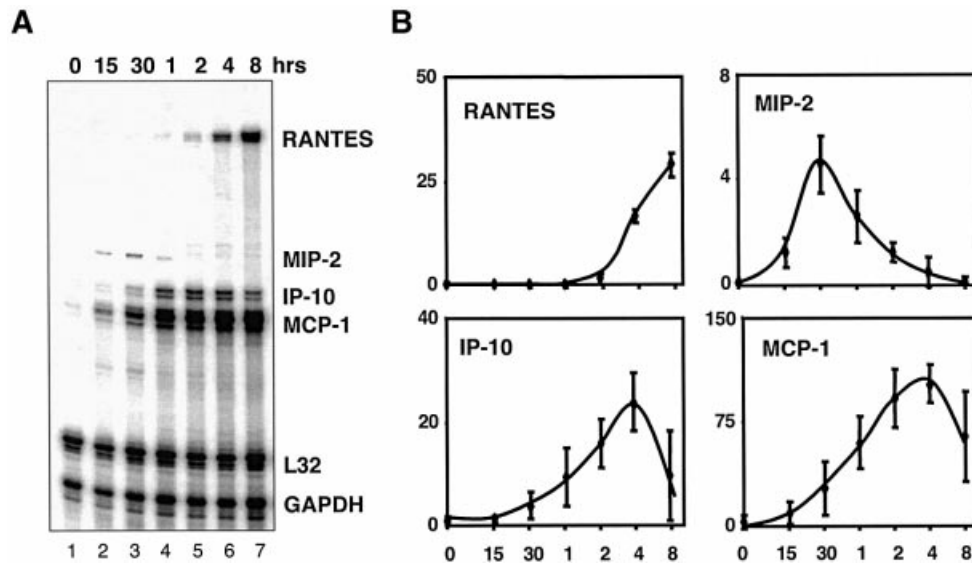
To undertake such a study, we interbred NF- $\kappa$ B knockout mouse strains and derived 3T3 fibroblast lines that were doubly deficient in p50 and p52 or p65 and cRel. We also derived control lines from *nfknb2*<sup>-/-</sup> (Caamano *et al.*, 1998) and *crel*<sup>-/-</sup> (Kontgen *et al.*, 1995) embryos. Gel mobility

shift assays were then used to detect  $\kappa$ B-binding complexes in extracts made from each of these cell lines following TNF $\alpha$  stimulation (data not shown); their molecular composition was determined by employing the previously used panel of antibodies. As summarized in Table I, each mutant cell line showed a characteristic set of TNF $\alpha$ -inducible RHD proteins that gave rise to particular  $\kappa$ B-binding activities. Therefore, the systematic panel of mutants allowed us to distinguish between NF- $\kappa$ B-dependent promoters that strictly required a particular RHD protein for activation and those promoters that had a broader requirement for one or the other RHD protein subclass. Specifically, we could distinguish genes that require p50 for TNF-induced activation, those on which either p50 or p52 must function, and those that do not require either protein for NF- $\kappa$ B-dependent activation. Similarly, those same genes could be further classified according to their requirement for p65 or cRel.

For a quantitative, yet high throughput analysis of endogenous gene expression, we used a multiplex RNase protection assay (RPA) that allows independent monitoring of up to 10 different messenger RNA transcripts (Figure 5A). Two housekeeping genes, GAPDH and L32, allowed for normalization such that consistent expression units can be used to compare different experiments. Multiple data sets from four different wild-type cell lines demonstrated reproducibility, which therefore allows comparison of datasets from different cell lines bearing different mutations. Interestingly, a detailed time course revealed that different genes have widely different kinetics of activation. For example, our panel of genes included several chemokine genes (Figure 5B). After TNF $\alpha$  stimulation, MIP-2 mRNA levels increase as early as 15 min, reaching a peak at 1 h and disappearing by 2 h. On the hand, RANTES transcripts are not detectable until 2 h post-stimulation and continue to rise beyond 8 h. Finally, MCP-1 and IP-10 display an intermediary profile. The dynamic nature of NF- $\kappa$ B activation emphasizes the importance of conducting gene expression assays in detailed time courses.

### Differential NF- $\kappa$ B/Rel protein requirements

We focused our attention on nine genes that represent diverse classes of NF- $\kappa$ B responsive genes, are induced in fibroblasts by TNF $\alpha$  stimulation, and the mRNA levels of



**Fig. 5.** Quantitative monitoring of gene expression by multiplex RPA. Multiple double-stranded RNA products indicative of mRNA transcripts derived from indicated NF- $\kappa$ B-dependent and housekeeping genes following TNF $\alpha$  stimulation of wild-type fibroblasts resolved by electrophoresis (A). Three independent experiments with independently derived wild-type 3T3 cell lines were quantitated by PhosphorImager and mRNA abundance was graphed for the indicated chemokine genes in arbitrary units relative to housekeeping genes L32 and GAPDH (B).

which could be monitored reliably by RPA. These genes were: I $\kappa$ B $\alpha$ ; aforementioned chemokine genes RANTES, IP10, MCP-1 and MIP-2; the immune regulatory factor LIF; the growth factor M-CSF; transcription factor junB; and apoptosis-inducing TNF receptor family member Fas. Quantitative RPA results for each gene are summarized in three graphs which group related genotypes (Figure 6). Graphs in the left column demonstrate NF- $\kappa$ B dependence because cells lacking both p50 and p65 have no NF- $\kappa$ B-mediated activity; those in the center column address dependence on p50 and/or p52 proteins, and those in the right column address dependence on p65 and/or cRel proteins. An eight point time course extending to 8 h following the onset of TNF $\alpha$  stimulation revealed diverse kinetics in gene activation. In each case, transcriptional induction proved to be protein synthesis-independent but cycloheximide did affect the transcript levels of some genes at time points following initial activation (A.Hoffmann, unpublished results). TNF $\alpha$  activation of all genes proved to be NF- $\kappa$ B-dependent as determined with cells lacking both p50 and p65 (left column). Two genes, M-CSF and junB, showed residual induction in the absence of NF- $\kappa$ B, but the NF- $\kappa$ B contribution to their activation was found to be reproducible and analyzable with respect to NF- $\kappa$ B protein requirement (see below). Interestingly, junB transcription was found to be highly induced in NF- $\kappa$ B knockouts at the time when these cells undergo widespread TNF $\alpha$ -induced apoptosis, indicating that this gene is subject to additional transcriptional regulation mechanisms independent of NF- $\kappa$ B.

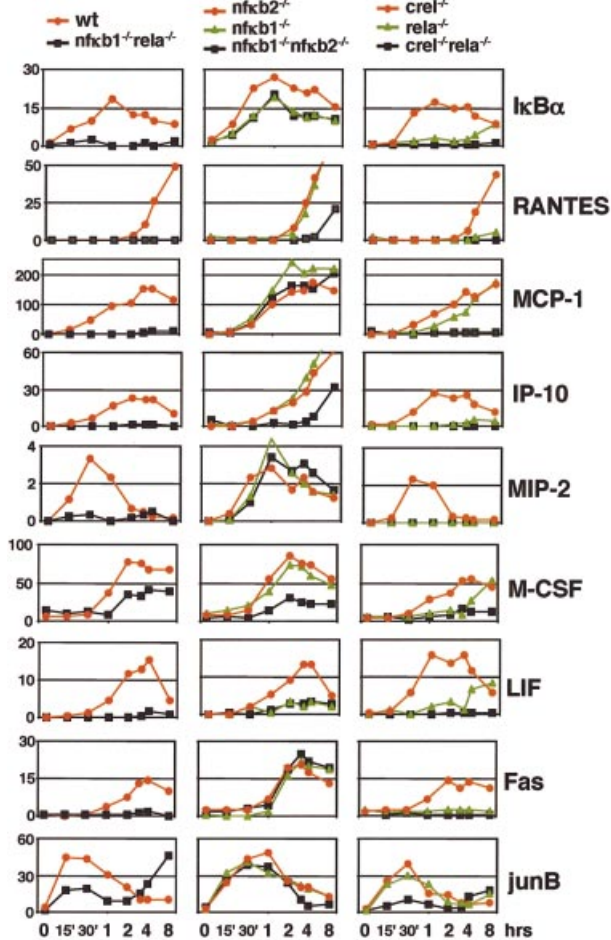
Turning to p50 and p52 NF- $\kappa$ B family members, we expected no activation defects in *nfkb2*<sup>-/-</sup> fibroblasts, because our supershift analysis did not reveal any p52 protein in  $\kappa$ B-binding complexes in TNF $\alpha$ -stimulated wild-type cells. As shown in the center column graphs, that is in fact the case; however, some genes, notably IP-10, appear to be super-induced at later time points in *nfkb2*<sup>-/-</sup> cells. Surprisingly, TNF $\alpha$ -induced gene expression is

largely unaffected by p50 deficiency (green line) as well. In fact, only one gene in our collection, LIF, cannot be activated by TNF $\alpha$  stimulation in *nfkb1*<sup>-/-</sup> cells. The major reason for such a mild gene expression phenotype appears to be efficient functional compensation by p52, because the activation of several genes, such as RANTES, IP-10 and M-CSF, is defective in cells lacking both p50 and p52. Thus, NF- $\kappa$ B-dependent genes appear to fall into three categories: those requiring p50 for induction in TNF $\alpha$ -stimulated fibroblasts, those on whose promoters either p50 or p52 can efficiently function, and those whose TNF $\alpha$  induction can proceed without either of these two RHD proteins.

Transcript quantitation for these genes in TNF $\alpha$ -induced cells deficient in cRel, p65 or both is shown in the right column of Figure 6. Because cRel was not found in  $\kappa$ B-binding complexes in wild-type fibroblasts, it is not surprising that none of the nine genes are significantly attenuated in *crel*<sup>-/-</sup> cells (red lines). In fact, most genes appear to be dramatically dependent on the p65 protein (green lines), although in some cases (e.g. I $\kappa$ B $\alpha$ , LIF, M-CSF) we saw significant transcript levels at the latest time points that are probably the cumulative result of residual transcriptional initiation activity. However, the chemokine MCP-1 and the transcription factor junB did not exhibit such a strict requirement for p65 because cRel could also function on these promoters (compare green and black lines). Importantly, we find that no NF- $\kappa$ B-dependent promoter can be activated in the absence of both p65 and cRel. This observation confirms that these two proteins contain the principal activation functions within  $\kappa$ B-binding complexes, while p50 and p52 can be considered binding partners that have essential functions in a subset of gene induction events.

Our panel of knockout cell lines revealed that endogenous genes have remarkably different requirements for RHD proteins when induced by TNF $\alpha$  in fibroblasts. While none could be induced in cells lacking both p50 and





**Fig. 6.** Transcriptional induction by TNF $\alpha$  of NF- $\kappa$ B target genes in NF- $\kappa$ B knockout cells. Results from a representative RPA experiment are graphed for the indicated genes in three panels. The left panel shows data from wild-type (red line) and *nfκb1*<sup>-/-</sup>*rela*<sup>-/-</sup> cells (black line), the center panel from *nfκb2*<sup>-/-</sup> (red), *nfκb1*<sup>-/-</sup> (green) and *nfκb1*<sup>-/-</sup>*nfκb2*<sup>-/-</sup> (black), and the right panel from *crel*<sup>-/-</sup> (red), *rela*<sup>-/-</sup> (green), and *rela*<sup>-/-</sup>*crel*<sup>-/-</sup> (black) cells.

p65, or p50 and cRel, the ability to be induced in *nfκb1*<sup>-/-</sup>, *nfκb1*<sup>-/-</sup>*nfκb2*<sup>-/-</sup> or *rela*<sup>-/-</sup> cells was gene-specific (Table II, left columns). Therefore, specific RHD protein requirements are determinable for every TNF $\alpha$ -induced gene in fibroblasts, as summarized for the group of nine NF- $\kappa$ B-dependent genes that were part of this study (Table II, right columns). Within this small sample of genes, the pattern of RHD protein requirement does not appear to correlate with the temporal control of gene expression (indicated in the second column) or the known or presumed function of the gene product; the six secreted immune response regulatory proteins represented here (RANTES, MCP-1, IP-10, MIP-2, M-CSF and LIF) cover diverse temporal kinetics as well as diverse combinations of RHD protein requirements.

**Discussion**

We have presented genetic evidence that mammalian gene activation requires not only specific transcription factors, but specific members of the RHD transcription factor family for the activation of endogenous NF- $\kappa$ B-responsive

**Table II.** Distinct RHD protein requirements for NF- $\kappa$ B-dependent genes

	wt time	RHD protein required		
		p50	p52	p65 cRel
IκBα	E	-	-	+
RANTES	L	-	+	+
MCP-1	M	-	-	+
IP-10	M	-	+	+
MIP-2	E	-	-	+
M-CSF	M	-	+	+
LIF	L	+	-	+
Fas	M	-	-	+
JunB	E	-	-	+

Transcriptional regulation results are summarized for each of the ten genes (column 1) monitored: temporal regulation (column 2), expression in indicated knockout cells (columns 3-5) and deduced RHD protein requirement (columns 6 and 7).

genes. However, *in vitro* binding assays with extracts from cells harboring deletions of specific family members reveal DNA binding characteristics that are sufficiently overlapping that functional specificity of family members cannot be reproduced on naked templates in transient transfections. In fact, in the absence of exogenously expressed family members, transiently transfected promoters appear to be remarkably promiscuous with regard to family member-specific requirements, although some  $\kappa$ B-site sequences may be more specialized, restricting access to a subset of NF- $\kappa$ B dimers.

Specificity was revealed when taking a genetic approach to a mechanistic question. The specific RHD protein requirement for the activation of an endogenous promoter implies that a particular subset of all available RHD protein dimers (Table I) is capable of functioning on the promoter in question. Thus the RHD protein requirement (Table II) can be translated into the subset of RHD protein dimers that appear capable of mediating the induction of each gene in our study (Table III). For example, LIF induction by TNF $\alpha$  is abolished in both *nfκb1*<sup>-/-</sup> as well as *rela*<sup>-/-</sup> single knockout cells leading to the conclusion that of all possible RHD protein dimers only the p50:p65 heterodimer is functional on this promoter. In contrast, IP-10 (as well as M-CSF and RANTES) can be induced by dimers containing either p50 or p52, with p65 being a required partner. A representative of a third group is MIP-2; this chemokine (as well as IκB $\alpha$  and Fas) is induced by p65-containing complexes that may not only be heterodimers with p50 or p52, but can also be p65:p65 homodimers. MCP-1 and junB exhibited the least stringent RHD protein requirement with NF- $\kappa$ B/Rel complexes consisting of either p65 or cRel capable of functioning on these promoters. While cRel protein can partner with p50, p65 can function as a homodimer or as a heterodimer with p50 or p52 to activate transcription of these two genes.

**Determinants of NF- $\kappa$ B protein requirements**

Because individual NF- $\kappa$ B dimers have been shown to have particular binding sequence preferences *in vitro* (Kunsch *et al.*, 1992), we examined whether RHD protein requirements can be correlated with the sequence of the

**Table III.**  $\kappa$ B-site sequences do not correlate with RHD dimer specificity

Gene	Functioning RHD protein dimers				$\kappa$ B-site sequence	Reference
MCP-1	p50:p65	p52:p65	p50:cRel	p65:p65	<sup>-2811</sup> GGGAATTTCC <sup>-2640</sup> GGGAATTC	Ping <i>et al.</i> (1999)
JunB	p50:p65	p52:p65	p50:cRel	p65:p65	<sup>+2057</sup> GGGCTTTCC	Phinney <i>et al.</i> (1995)
MIP-2	p50:p65	p52:p65		p65:p65	<sup>-66</sup> GGGAATTTCC	Widmer <i>et al.</i> (1993)
I $\kappa$ B $\alpha$	p50:p65	p52:p65		p65:p65	<sup>-260</sup> GGGAAGTCC <sup>-70</sup> GGAAATTC	Chiao <i>et al.</i> (1994)
Fas	p50:p65	p52:p65		p65:p65	<sup>-59</sup> GGAATGCCCA	Zheng <i>et al.</i> (2001)
RANTES	p50:p65	p52:p65			<sup>-87</sup> GGGAGTTCC	Lee <i>et al.</i> (2000)
IP-10	p50:p65	p52:p65			<sup>-169</sup> GGGAAATTC <sup>-113</sup> GGGACTTC	Ohmori <i>et al.</i> (1992)
M-CSF	p50:p65	p52:p65			<sup>-378</sup> GGAAAGTCC	Harrington <i>et al.</i> (1991)
LIF	p50:p65				<sup>-77</sup> GGGGATCCCG	Willson <i>et al.</i> (1992)

NF- $\kappa$ B-dependent genes (column 1) are listed in order of increasingly restrictive RHD protein dimer requirement (column 2). Corresponding gene promoters contain conserved  $\kappa$ B-sites of indicated sequences (column 3) that were shown to be functional in previously published studies (column 4).

$\kappa$ B-site. Such a correlation would indicate that DNA-protein interaction affinity, or more precisely  $K_{on}$  and/or  $k_{off}$  rates, of RHD protein dimers to specific  $\kappa$ B-site sequences determine which RHD protein dimers are functional on a particular promoter. The promoters for most of the genes in this study have been previously investigated by standard transiently transfected reporter assays that led to the identification of the apparent functional transcription factor-binding sites. For LIF, we used human and mouse genomic databases and previous promoter studies to identify a novel  $\kappa$ B-binding element. Grouping the NF- $\kappa$ B-dependent genes in our study according to their specific RHD protein requirement in Table III failed to reveal a correlation with  $\kappa$ B-site sequences. In fact, genes whose TNF $\alpha$  induction can be mediated by the same set of RHD protein dimers, such as RANTES, IP-10 and M-CSF, contain different  $\kappa$ B-site sequences that are responsible for this induction. Conversely, promoters containing the same  $\kappa$ B-site sequence (GGGAATTTCC), such as MIP-2 and MCP-1, exhibit different RHD protein requirements. Furthermore, focusing on genes that can be activated by p65 homodimers (MCP-1, JunB, MIP-2, I $\kappa$ B $\alpha$  and Fas) we cannot discern any common features in the relevant  $\kappa$ B-site sequences. In each case, the  $\kappa$ B element is embedded in a promoter that is bound by many proteins, such as neighboring transcription factors, co-activators and chromatin components that may interact with NF- $\kappa$ B/Rel dimers bound at the  $\kappa$ B-site. The results of our genetic analysis suggest that the ability of RHD protein dimers to function on a particular promoter is not solely determined by DNA-protein interactions at the  $\kappa$ B-site. While the regulatory control regions of the genes in this study have previously been characterized, we cannot rule out that NF- $\kappa$ B may function via unrecognized binding sites, whose sequence may not be conserved between mouse and human. Mutations of endogenous sequences via knock-in technology or methodologies that allow faithful expression of transgenic constructs will be needed to confirm and extend our conclusions.

Interactions with DNA is likely to restrict the subset of possible transcription factors that may function at a particular promoter element, and within the family of  $\kappa$ B-sites it is known that p65 or cRel homodimers bind better to 9 bp than 10 bp sites. However, our results (based on the highly divergent set of 10 bp  $\kappa$ B-sites) point to the importance of protein-protein interactions within

promoter-DNA assemblies in determining a specific family member requirement and generating transcriptional specificity *in vivo*. Transcriptional synergy (Lin *et al.*, 1990) between adjacent transcription factors and within enhanceosomes (Thanos and Maniatis, 1995), as well as specific co-activator requirements (Merika *et al.*, 1998), have been observed in non-chromosomal experimental systems and have long been thought to play a role in generating specificity through combinatorial control. Differential specificity of NF- $\kappa$ B protein family members in interactions with contextual transcriptional factors may thus account for family member-specific requirements for gene activation. Chromatin has also been shown to control NF- $\kappa$ B/Rel accessibility *in vivo* (Saccani *et al.*, 2001) and may in fact do so in a manner that is specific for a subset of RHD protein dimers. Furthermore, some genes require chromatin re-organization for gene activation (Lomvardas and Thanos, 2002), and this may be dependent on protein-protein interactions specific to a particular family member.

Other groups have taken complementary approaches to studying transcription factor specificity. In particular, Farnham and colleagues used chromatin immunoprecipitation in combination with microarray technology (ChIP-Chip) to examine the role of a single member of the E2F family across a wide spectrum of genes (Weinmann *et al.*, 2002), while Young and colleagues have begun to characterize extensive networks of transcriptional control in budding yeast (Lee *et al.*, 2002). Recently, ChIP experiments have revealed differential NF- $\kappa$ B dimer recruitment to target promoters that may complement the present study (Saccani *et al.*, 2003). While NF- $\kappa$ B occupancy as assayed by ChIP does not indicate the functional activity of the bound dimer without the use of genetic tools, such assays indicate that transcriptional control may involve the dynamic interplay of different dimers to attenuate or sustain transcriptional activity (also A.Hoffmann and D.Baltimore, manuscript in preparation). Perfecting the ChIP-chip technology to allow for a comprehensive characterization of occupancy states of regulatory regions will shed light on promoter architecture. Complementing that approach with transcript measurements in a variety of relevant knockouts may illuminate the operative promoter code, and in particular the question of family member specificity.

Are distinct  $\kappa$ B-site sequences functionally equivalent? While our genetic analysis has failed to correlate functional requirement with  $\kappa$ B-site sequence, and NF- $\kappa$ B co-



crystal structure studies do not reveal extensive NF- $\kappa$ B dimer-specific DNA-binding contacts (Chen and Ghosh, 1999), this does not rule out that the  $\kappa$ B-site sequence plays a functional role. In fact, structural comparisons of the p50:p65 dimer bound to different  $\kappa$ B-site sequences have revealed significant differences in conformation (Chen and Ghosh, 1999). The particular conformations of DNA-bound NF- $\kappa$ B complexes (probed by protease sensitivity and X-ray crystallography) have indeed been correlated with their ability to transactivate on transfected promoters (Fujita *et al.*, 1992; Chen-Park *et al.*, 2002); however it is unknown whether the conformational state of NF- $\kappa$ B complexes may have similar importance on chromosomal promoters, and whether a particular conformation of an NF- $\kappa$ B dimer may be functional on one promoter but not another. In addition to examining binding affinities, investigating the conformations of NF- $\kappa$ B dimers bound to  $\kappa$ B elements and the functional consequence within the context of endogenous regulatory region is likely to bring further understanding to the promoter code.

Multiple protein–DNA complexes may be able to assemble on a given promoter depending on the cell-type and stimulus (Falvo *et al.*, 2000). Because the  $\kappa$ B-site sequence is not the sole determinant of the specific RHD dimer requirement, we may find that the specific RHD protein requirement for the activation of a particular gene is also specific to cell-type and stimulus. If combinatorial control by multiple promoter-bound transcription factors generates specific transcription factor requirements, it can form the basis for the inclusion or exclusion of a particular gene in specific gene expression programs in response to multiple activation pathways.

## Materials and methods

### Cell culture and transfections

Immortalized fibroblast cell lines were generated from E12.5–14.5 embryos according to the 3T3 protocol in 10% bovine calf serum (Aaronson and Todaro, 1968) and maintained in the same manner. Previously published NF- $\kappa$ B luciferase reporter plasmids (Fujita *et al.*, 1992) were transfected together with  $\beta$ -actin–lacZ control plasmid into subconfluent fibroblasts using Ca-PO<sub>4</sub> or fugene6 (Roche) methodologies. After 24 h, cells were starved for 24 h with 0.5% serum containing medium and then stimulated with 10 ng/ml TNF $\alpha$ .

### DNA-binding assays and western blot

For whole cell western blots, cells were lysed within six well plates using SDS–PAGE sample buffer, and probed with specific antibodies from Santa-Cruz Biotechnology, Inc. For electrophoretic mobility shift assays (EMSA), previously described methods and  $\kappa$ B-site containing probes were used (Fujita *et al.*, 1992; Hoffmann *et al.*, 2002). Supershift assays were done with antibody cocktails specific to indicated RHD proteins composed of rabbit antibodies available at Santa Cruz Biotechnology. The reactivity and specificity of these is demonstrated in the data shown in Figure 3A–D.

### RNase protection assays

Total RNA was made from confluent and starved fibroblasts using Tri-Reagent (Molecular Research Center, Inc). RPA was performed with 5  $\mu$ g RNA using Riboquant probe sets (PharMingen) according to the manufacturer's instructions. Data was quantitated using a Molecular Dynamics PhosphorImager. Following local background subtraction, data was normalized using GAPDH and L32 gene data, which allowed results to be compared across experiments. Every experiment was performed at least twice, many three to five times with high reproducibility.

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