

One Nucleotide in a κ B Site Can Determine Cofactor Specificity for NF- κ B Dimers

Thomas H. Leung,¹ Alexander Hoffmann,^{1,2}
and David Baltimore^{1,*}

¹Division of Biology
California Institute of Technology
Pasadena, California 91125

Summary

The transcription factor NF- κ B regulates a wide variety of genes involved in multiple processes. Although the apparent consensus sequence of DNA binding sites for NF- κ B (κ B sites) is very broad, the sites active in any one gene show remarkable evolutionary stability. Using a lentivirus-based methodology for implantation of gene regulatory sequences we show that for genes with two κ B sites, both are required for activity. Swapping sites between κ B-dependent genes altered NF- κ B dimer specificity of the promoters and revealed that two κ B sites can function together as a module to regulate gene activation. Further, although the sequence of the κ B site is important for determining κ B family member specificity, rather than determining the ability of a particular dimer to bind effectively, the sequence affects which coactivators will form productive interactions with the bound NF- κ B dimer. This suggests that binding sites may impart a specific configuration to bound transcription factors.

Introduction

Study of mammalian gene transcription is often confounded by the recognition of a single regulatory sequence by multiple members of a transcription factor family. Expansion of the number of members in a particular family may occur over evolutionary time suggesting that particular members evolve to serve restricted functions in a complex organism. However, outside of the nuclear hormone receptor superfamily (Rastinejad, 2001), little is known about how individual family members provide specificity.

NF- κ B is a dimeric protein that mediates cellular responses to a wide variety of stimuli including TNF α , LPS, IL-1, and ultraviolet light (Ghosh et al., 1998; Karin and Ben-Neriah, 2000; Karin et al., 2002). NF- κ B plays an integral role in many important and diverse processes, including inflammatory responses, immune system development, apoptosis, learning in the brain, and bone development. In resting cells, NF- κ B is held inactive via associations with inhibitory proteins called inhibitors of NF- κ B (I κ B). When the cell is stimulated, I κ B proteins are phosphorylated, ubiquitinated, and degraded, allowing NF- κ B to bind DNA and activate appropriate target genes.

In response to inflammatory stimuli, four members of

the NF- κ B family are involved in gene activation: p50, p52, p65, and cRel. They are the mature gene products from four genes: *nfkB1*, *nfkB2*, *relA*, and *rel*, respectively, and they homo- or heterodimerize with one another to bind DNA. It is noteworthy that mice deficient in a single NF- κ B/Rel family member exhibit unique, largely non-overlapping phenotypes. For instance, *relA*^{-/-} animals are embryonic lethal and develop massive liver apoptosis (Beg et al., 1995), while *nfkB1*^{-/-} mice develop normally but have deficits in immune function (Sha et al., 1995).

Past studies predominantly used transient-transfection reporter systems with overexpressed NF- κ B proteins to conclude that certain NF- κ B complexes bind particular NF- κ B binding sites (called κ B sites) preferentially (Fujita et al., 1992; Kunsch et al., 1992). When similar transient-transfection reporter assays were repeated in NF- κ B knockout cells, NF- κ B family members exhibited no preference for particular κ B sequences (Hoffmann et al., 2003). With increased knowledge about transcriptional regulation, questions about the recruitment of specific NF- κ B family members to a promoter should be addressed within a physiological context.

Our previous study utilized a genetic approach to study NF- κ B-dependent gene activation by creating a panel of single and double NF- κ B knockout cell lines (Hoffmann et al., 2003). We showed there that TNF α -stimulated, κ B-dependent genes may require specific κ B family members for activation. Some genes had stricter requirements than others. For example, any p65 or cRel-containing hetero- or homodimer could activate the MCP-1 gene (*scya2*), while the LIF gene (*lif*) specifically required a p50:p65 heterodimer for activation. We were unable to document a strict correlation between κ B family member requirements and their κ B site sequences.

Structural studies have also not revealed evidence for highly specific interactions between NF- κ B and its cognate DNA binding sites. κ B sites display a remarkably loose consensus sequence, often cited as G₋₅G₋₄G₋₃R₋₂N₋₁N₀Y₊₁Y₊₁C₊₂C₊₃ (Ghosh et al., 1998). Crystal structures have shown that p50 homodimers often interact specifically with 5'-G₋₅G₋₄G₋₃, while p65 homodimers interact specifically with 5'-G₋₅G₋₄. X-ray structures of p50:p65 heterodimers demonstrate that they can bind to a variety of κ B site sequences (Berkowitz et al., 2002; Chen and Ghosh, 1999; Chen-Park et al., 2002; Escalante et al., 2002). Taken together, structural studies have not identified enough dimer-specific DNA binding contacts to rationalize specific NF- κ B binding sites and underscore the remarkable permissiveness in NF- κ B-DNA interactions.

In this study, we demonstrate that the sequence of the κ B site does play an important role in determining κ B family member specificity. But rather than the site determining the ability of a particular dimer to bind effectively, we find that the sequence of the κ B site affects which coactivators will form productive interactions with the bound κ B dimer, suggesting that binding sites may affect the configuration of the bound dimer.

*Correspondence: baltimo@caltech.edu

²Present address: Signaling Systems Laboratory, Department of Chemistry and Biochemistry, UCSD, La Jolla, California 92093

A

Gene	Mouse	Human
MCP-1	GGGAAGTTCC GGGAATTTCC	GGGAAGTTCC GGGAATTTCC
IκBα	GGAAATTTCC GGGGAAGTCC	GGAAATTTCC GGGGAAGTCC
Fas	GGGAATGCCC	GGGAATGCCC
MIP2	GGGAATTTCC	GGGAATTTCC
M-CSF	GGAAAGTCCC	GGAAAGTCCC
IP-10	GGGAAATTCC GGGACTTTCC	GGGAAATTCC GGGACTTTCC
RANTES	GGGAGTTTCC	GGGAGTTTCC
LIF	GGGGATCCCG	GGGGATCCCG
IFNβ	GGGAAATTCC	GGGAAATTCC
IL-6	GGATTTTCCC	GGATTTTCCC
JunB	GGGGCTTTCC	GGGGCTTTCC

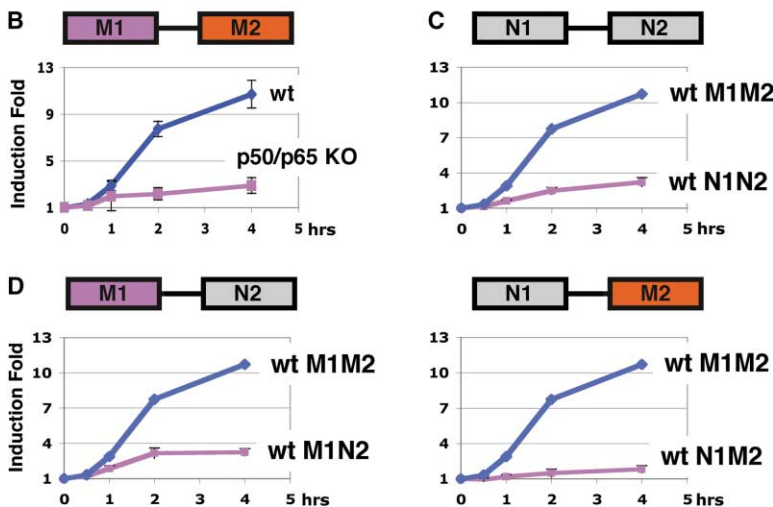


Figure 1. Functional κB Site Sequences Are Strictly Conserved and Activation of Lentiviral MCP-1 Transgene Is Responsive to Stimuli and Dependent on NF-κB

Validated mouse and human κB site sequences for eleven κB-dependent genes were compared using the Celera and Ensembl databases (A). The κB site sequences for all of the eleven genes studied were 100% conserved between mouse and human. Roughly 5 kb of the MCP-1 gene promoter and a luciferase reporter gene (ML M1M2) was implanted into wild-type and p50/p65 knockout 3T3 cells by lentiviral infection. Cells were stimulated with TNF α over a four hour time course. Four variations of the ML transgene are shown: a 5 kb wild-type version “ML M1M2” (B), a version with both κB binding sites mutated to a null sequence “ML N1N2” (C), a version where the distal κB binding site is intact and the proximal site is mutated to a null sequence “ML M1N2” (D, left image), and a version where the distal binding site is mutated to a null sequence and the proximal site is intact “ML N1M2” (D, right image).

Results

κB Site Sequences Are Strictly Conserved between Mouse and Human

To understand whether the sequences of κB sites play a significant role in determining functional specificity, validated mouse and human κB site sequences for eleven κB-dependent genes were compared using the Celera and Ensembl databases (Hoffmann et al., 2003; Libermann and Baltimore, 1990; Thanos and Maniatis, 1995b). The κB site sequences for all eleven genes studied were 100% conserved between mouse and human (Figure 1A). In contrast, a pairwise sequence comparison program (Family Relations) revealed that the regions surrounding the κB sites often displayed <85% conservation (data not shown) (Brown et al., 2002). If the sequence of the κB site did not play a significant role in determining functional specificity, we would have expected the sequence of some of the κB sites to mutate over time. We therefore decided to test whether the sequence of the κB site can alter the functional properties of bound dimers.

To address the role of κB site sequence, we wanted to

swap the sequences between two κB-dependent genes with different properties. The IP-10 (*cxcl10*) and MCP-1 genes were chosen for investigation. When IP-10 and MCP-1 respond to TNF α , they have different κB family member requirements. MCP-1 mRNA is induced in both wild-type (wt) and p50/p52-deficient cells, while IP-10 mRNA is induced in wt cells but not p50/p52-deficient cells (Hoffmann et al., 2003). p65 homodimers are the only detectable dimer in p50/p52-deficient 3T3 cells. Therefore, both hetero- and homodimers of NF-κB are able to function on the MCP-1 promoter while only heterodimers of NF-κB appear able to activate IP-10. The regulatory sequences of the genes have certain similarities but significant differences. Based on previous work as well as mouse/human sequence comparisons, MCP-1 and IP-10 each appear to have two functioning κB sites that are both required for gene activation (Ohmori and Hamilton, 1993, 1995; Ping et al., 1999). Both genes are highly expressed in fibroblasts and are responsive to multiple stimuli. However, the promoter architecture for the two genes is strikingly different. IP-10’s κB sites are less than 200 bp from the transcription start site while MCP-1’s functional κB sites are more

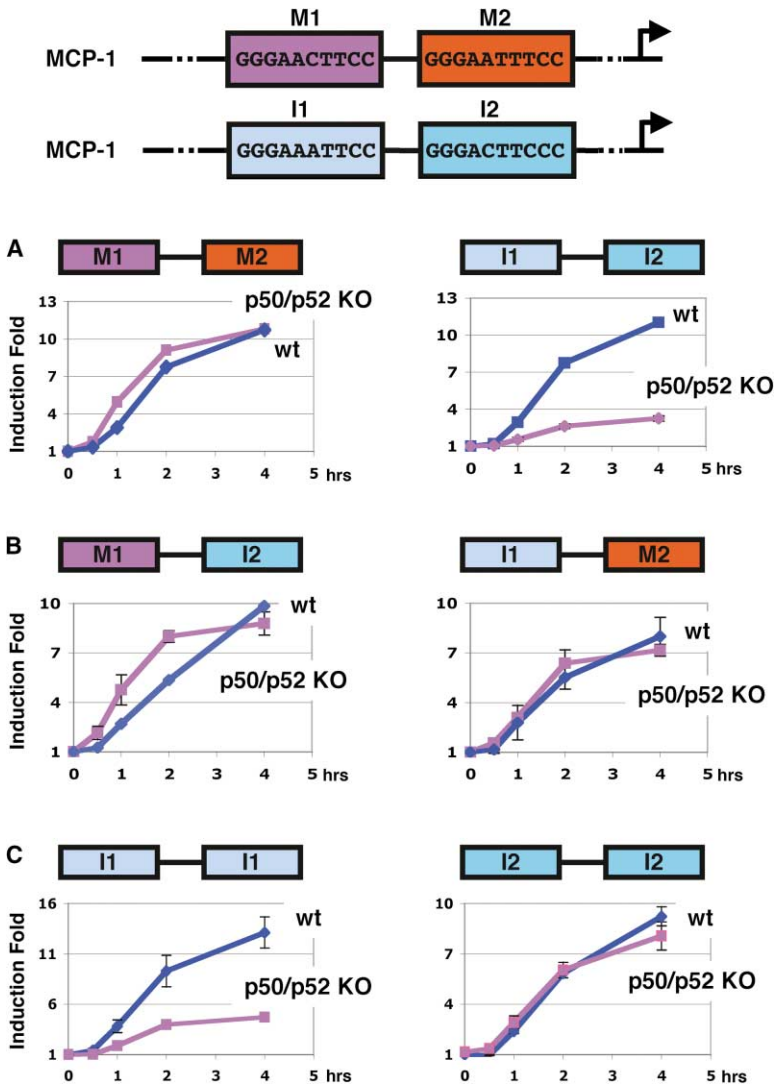


Figure 2. Swapping MCP-1 κ B Site Sequences for IP-10 κ B Site Sequences Imposes IP-10 κ B Family Member Requirements on the MCP-1 Promoter

IP-10 κ B site sequences (ML I1I2) were swapped for the MCP-1 κ B site sequences in the ML transgene by PCR mutagenesis. Lentivirus was produced and applied to wild-type and p50/p52 knockout 3T3 cells.

(A) compares the activity of the integrated ML M1M2 transgene to the ML I1I2 transgene.

(B) shows the activity of the transgene when one MCP-1 κ B binding site is swapped with its corresponding IP-10 κ B binding site sequence (ML M1I2 or ML I1M2).

(C) compares the activity of the transgene when either one of the IP-10 κ B sites are duplicated and inserted into the transgene (ML I1I1 and ML I2I2). All cells were stimulated with TNF α over a four hour time course.

than 2.3 kb away from the transcription start site. (MCP-1 has another κ B-like sequence near the promoter, which was shown to be nonfunctional and not conserved between mouse and human.) IRF-3 has been shown to be important in IP-10 gene regulation but not for MCP-1 (Sakaguchi et al., 2003). The κ B site sequences are not dramatically different between MCP-1 and IP-10. All sites are 10 bp long. The distal sites differ in one base pair, and the proximal sites differ in two base pairs (Figure 1A).

Lentiviral-Based Reporter System Recapitulates Endogenous Gene Regulation

To effect the sequence interchanges, we needed a system that would allow us easy access for nucleotide alteration but would retain the regulatory properties of the endogenous gene promoters. The use of a retrovirus vector allows insertion of up to 7 kb of regulatory information and permits sequence alteration at will. We chose a self-inactivating lentiviral vector system that infects both dividing and nondividing cells (Lois et al., 2002). The self-inactivating characteristic involves dele-

tion of the virus' own promoter, ensuring that the inserted regulatory sequence is the only such information in the vector. We used a luciferase reporter gene to monitor transcriptional activity.

To test the reporter system, we cloned 5 kb of the mouse MCP-1 gene promoter into the lentiviral construct. (We designate this ML M1M2, denoting its origin from the MCP-1 gene, its use of a luciferase reporter gene, and its two κ B sites containing MCP-1-derived sequence). Lentiviral stocks were prepared, concentrated by ultracentrifugation, and applied to either wt mouse 3T3 cells or 3T3 cells lacking both p50 and p65 subunits (effectively NF- κ B null cells (Hoffmann et al., 2003)). Routinely, multiple cell populations were created by infecting cells with serial dilutions of virus. Populations that expressed low basal luciferase activity (roughly 5-fold above background) were used for analysis to ensure a low number of integrated proviruses per cell.

wt and p50/p65-deficient cells with the integrated ML M1M2 transgene were stimulated with TNF α (Figure 1B) over a 4 hr time course. wt cells responded robustly,

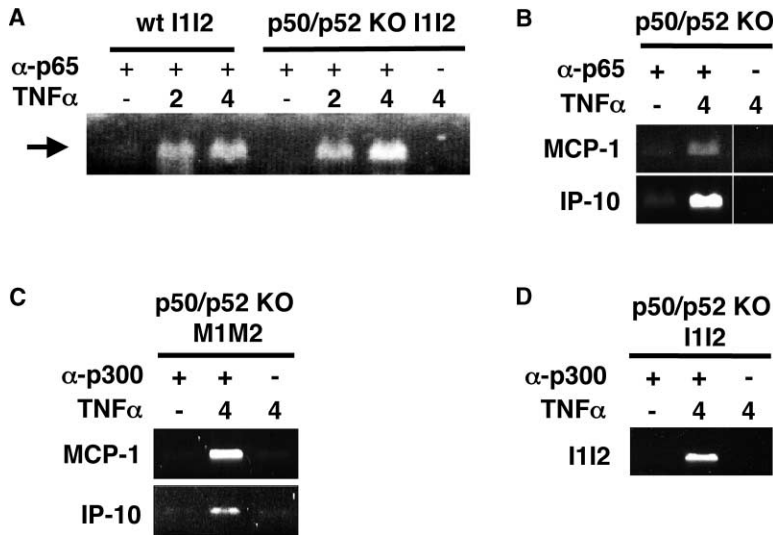


Figure 3. p65 Homodimer and p300/CBP Bind to ML I112

Chromatin immunoprecipitation assays with antibodies against p65 (A and B) and p300 (C and D) demonstrate that p65 and p300 binds the MCP-1 promoter, the IP-10 promoter, and ML I112 transgene in a TNF α -dependent manner. The white line indicates where lanes irrelevant to the figure have been cropped (B).

while p50/p65-deficient cells had minimal inducible luciferase activity, showing that the system recapitulates the NF- κ B dependence shown previously for the in situ gene. Next, the κ B sites within the ML transgene were mutated to null κ B site sequences (ML N1N2). wt cells carrying the integrated ML N1N2 transgene showed no inducible luciferase activity following TNF α treatment (Figure 1C). Finally, two different constructs were created with one κ B site left intact and the other κ B site mutated to a null sequence (ML M1N2 and ML N1M2) (Figure 1D). As previously shown (Ping et al., 1999), the mutation of either κ B site to a null sequence abrogated inducible luciferase activity in wt cells. These transgenic cells lines were also stimulated with LPS, and the results were similar to those with TNF α (data not shown). Thus, the ML M1M2 transgene is inducible by multiple stimuli in a κ B-dependent fashion, and both κ B sites must be intact for the promoter to be functional.

Swapping MCP-1 κ B Site Sequences for IP-10 κ B Site Sequences Imposes IP-10 κ B Family Member Requirements on the MCP-1 Promoter

The κ B site sequences from the IP-10 promoter were swapped into the ML M1M2 transgene by PCR to create ML I112. wt cells infected with ML I112 were responsive to TNF α stimulation, but TNF α -stimulated, ML I112-infected, p50/p52-deficient cells displayed no inducible luciferase activity (Figure 2A, right image). In contrast, p50/p52-deficient cells infected with ML M1M2 displayed inducible luciferase activity comparable to that of wt cells (Figure 2A, left image). We conclude that the ML I112 transgene requires NF- κ B heterodimers for activation.

To test whether the altered κ B family member requirements in ML I112 were dependent on one or both of the IP-10 κ B site sequences, two MCP-1 promoter constructs were created carrying one IP-10 κ B site and one MCP-1 κ B site (ML M1I2, ML I1M2, Figure 2B). Both wt and p50/p52-deficient cells carrying these constructs remained responsive to TNF α stimulation. Therefore, either MCP-1 κ B site sequence alone is sufficient to provide a response to the p65 homodimer.

To determine if one or both of the IP-10 κ B site se-

quences were unresponsive to the homodimer, two different MCP-1 promoter constructs containing duplicated IP-10 κ B site sequences were made (ML I1I1, ML I2I2, Figure 2C). wt cells infected with either ML transgene remained responsive to TNF α stimulation. However, p50/p52-deficient cells carrying the ML I1I1 transgene were not responsive to TNF α stimulation, while those carrying the ML I2I2 transgene displayed inducible luciferase activity. Thus, the I1 or distal κ B site of the IP-10 promoter determines the heterodimer requirement of the IP-10 promoter. The κ B site sequences in the ML I1I1 construct differ from those in the ML M1M2 construct only at the sixth nucleotide for each site. Therefore, we can identify this single nucleotide as responsible for the difference in κ B family member requirements.

To exclude the possibility that nearby factors bound to the MCP-1 promoter may affect κ B-dimer specificity, lentiviral constructs containing only the *c-fos* minimum promoter and the κ B sites from MCP-1 or IP-10 were tested. Upon TNF α treatment, the construct containing the MCP-1 κ B sites was responsive to hetero- and homodimers of NF- κ B, while the construct containing IP-10 κ B sites only responded to heterodimers of NF- κ B (data not shown).

p65 and CBP Are Bound to the Inactive ML I112 Transgene in TNF α -Stimulated p50/p52-Deficient Cells

One possible explanation of the inability of the IP-10 distal κ B site to respond to a homodimer of p65 would be an inability of the site to bind the homodimers. To address this possibility, we performed chromatin immunoprecipitation (ChIP) assays with an anti-p65 antibody in TNF α -stimulated wt and p50/p52-deficient cells containing the ML I112 transgene. wt cells with the ML I112 transgene showed robust p65 binding after TNF α stimulation (Figure 3A). Surprisingly, p50/p52-deficient cells with the ML I112 transgene also demonstrated p65 binding after TNF α stimulation. Therefore, even though the ML I112 transgene shows minimal activity in TNF α stimulated p50/p52-deficient cells, p65 homodimers are still bound to the promoter. Examination of the endogenous

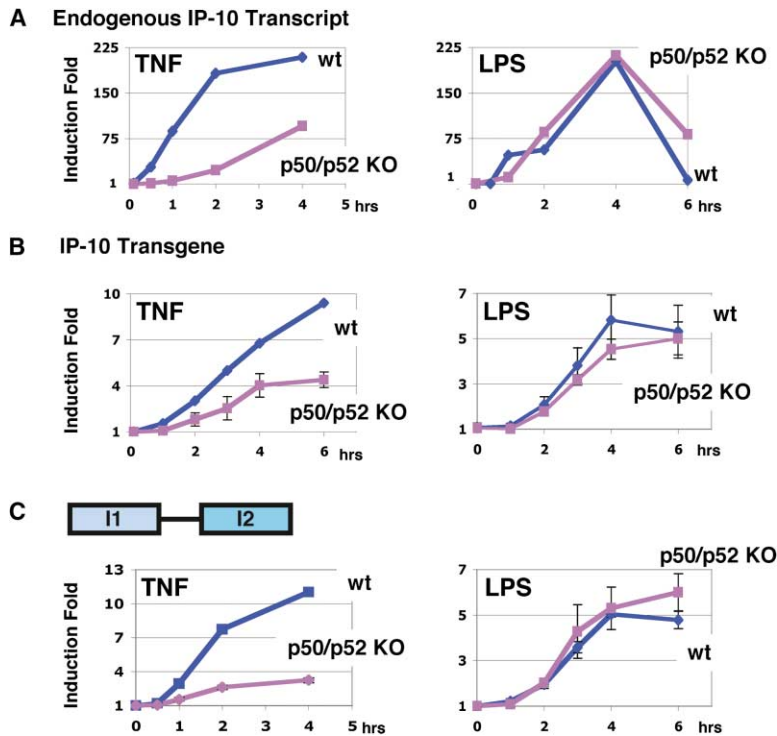


Figure 4. IP-10 κ B Binding Sites Display Stimulus-Specific κ B Family Member Requirements

Wild-type (wt) and p50/p52-deficient cells were stimulated with LPS. IP-10 RNA transcript was measured by quantitative PCR (A). 1 kb of the IP-10 promoter was cloned into the lentiviral transfer vector (IL). wt and p50/p52 knockout cell lines with integrated IL were stimulated with TNF α or LPS (B). wt and p50/p52 knockout cells integrated with the ML I1I2 transgene were stimulated with TNF α or LPS (C).

IP-10 promoter gave a congruent result. In TNF α -stimulated p50/p52-deficient cells, p65 was still bound to an inactive IP-10 promoter (Figure 3B).

If p65 homodimers are bound to an inactive ML I1I2 transgene, the κ B site sequence might not support transcription because the conformation of the p65 homodimers might not recruit the appropriate coactivators to the promoter. One type of coactivator known to interact with the transactivation domain of p65 is CBP/p300 (Perkins et al., 1997; Sheppard et al., 1999). We performed ChIP assays against p300 using TNF α -stimulated, ML M1M2-infected, p50/p52-deficient cells and discovered that p300 is recruited to the MCP-1 and IP-10 promoters in a stimulus-dependent manner (Figure 3C). Furthermore, we found that p300 was bound to the ML I1I2 transgene in TNF α -stimulated p50/p52-deficient cells (Figure 3D). Thus, this cofactor is recruited even though the promoter is not firing.

IP-10 Displays Stimulus-Specific Requirements for NF- κ B Family Members

To test whether the IP-10 gene shows the same heterodimer requirement to an inducer other than TNF α , wt and p50/p52-deficient cells were stimulated with TNF α or LPS over a 6 hr time course, and IP-10 mRNA induction was assayed by quantitative PCR (Q-PCR). The induction of IP-10 mRNA in TNF α -stimulated wt and p50/p52-deficient cells recapitulated our previously published ribonuclease protection assays showing the heterodimer requirement for activation (Figure 4A, left image). In contrast, LPS-stimulated wt and p50/p52-deficient cells induced IP-10 mRNA to similar levels (Figure 4A, right image). Thus, the heterodimer requirement is an inducer-specific phenomenon.

To determine if the lentiviral-based reporter system could recapitulate IP-10's stimulus-specificity, 1 kb of the

IP-10 promoter was cloned into our retrovirus vector (IL). Consistent with the endogenous gene data, TNF α -stimulated wt cells with the integrated IL transgene displayed induced luciferase activity, while p50/p52-deficient cells with the IL transgene showed significantly lower activity (Figure 4B, left image). In contrast, LPS-stimulated wt and p50/p52-deficient cells with the IL transgene demonstrated similar luciferase activity (Figure 4B, right image). Thus, the lentiviral system recapitulated the stimulus-specific behavior of the endogenous IP-10 gene.

To be certain that the stimulus-specificity was determined by the κ B site, we went back to the chimeric transgenes. In fact, as opposed to the data with TNF α -stimulated cells, LPS-treated wt and p50/p52-deficient cells containing the ML I1I2 transgene displayed similar levels of luciferase induction (Figure 4C, right image). Studies with the ML I1I1 transgene demonstrated similar results (data not shown) and supported the conclusion that p65 homodimers are bound to the I1 site. Taken together, these results confirmed that the stimulus-specificity was a consequence of the κ B site sequence and was consistent with the observation of p65 homodimers bound to the ML I1I2 transgene. It strongly suggested that the unresponsiveness of the IP-10 gene to homodimers in the TNF α -treated cells is a consequence of the lack of a cofactor that LPS can induce.

Overexpression of IRF-3 Rescues Induction of ML I1I2 Transgene by TNF α

To identify the LPS-specific factor that allows κ B homodimers to activate the ML I1I2 transgene, we noted that LPS signals through the TLR-4 receptor (Akira, 2003; Takeda et al., 2003). TLR-4 downstream signaling is divided into two distinct pathways, one mediated by MyD88 and the other by Trif. Since IP-10 is induced by

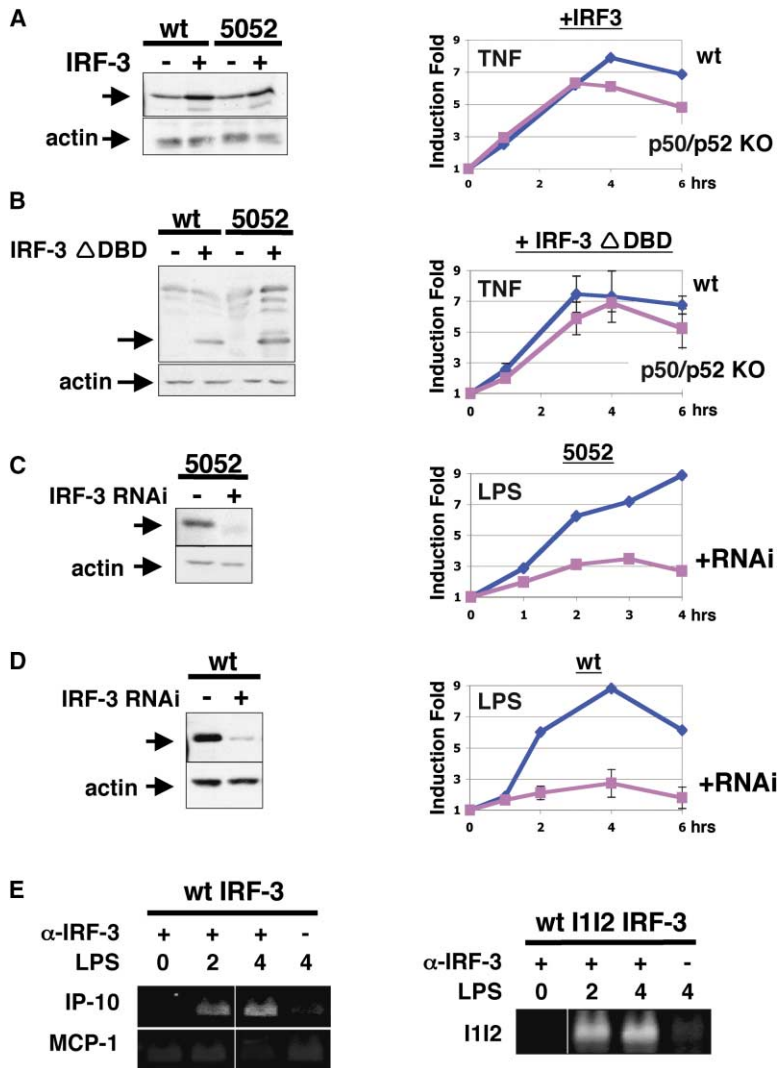


Figure 5. IRF-3 Is Recruited to the ML I112 Transgene and Required for Transgene Activation

IRF-3 and IRF-3 ΔDBD were overexpressed in wt and p50/p52 deficient cells carrying the ML I112 transgene (A and B, respectively). IRF-3 overexpressed cells were stimulated with TNF α . IRF-3 was knocked-down by RNAi in p50/p52-deficient and wt cells carrying the ML I112 transgene (C and D, respectively). IRF-3 knocked-down cells were stimulated with LPS. Chromatin immunoprecipitation assays with antibodies against IRF-3 demonstrate that IRF-3 is recruited to the IP-10 promoter but not the MCP-1 promoter in a LPS-dependent manner (E, left image). IRF-3 is recruited to the ML I112 transgene in a LPS-dependent manner (E, right image). The white line indicates where lanes irrelevant to the figure have been cropped.

LPS in MyD88-deficient fibroblasts (Yamamoto et al., 2002), we focused on signaling molecules in the Trif-dependent pathway. IRF-3 is a transcription factor induced in the Trif-dependent pathway and has been shown to play a role in IP-10 induction by LPS (Sakaguchi et al., 2003; Yamamoto et al., 2003). Furthermore, an interaction between IRF-3 and p65 has been demonstrated (Wietek et al., 2003).

A retrovirus expressing the IRF-3 cDNA was created and used to infect wt and p50/p52-deficient cells. Overexpression of IRF-3 protein was confirmed by Western analysis (Figure 5A, left image). IRF-3 overexpressing cells were then infected with the ML I112 transgene and stimulated with TNF α over a 6 hr time course. Overexpression of IRF-3 rescued the induction of the ML I112 transgene in p50/p52-deficient cells (Figure 5A, right image). This result also supports the interpretation of the ChIP data that p65 was bound to the identified functional κ B sites and not cryptic elements.

Since the MCP-1 promoter does not contain an IRF-3 binding site, it seemed likely that IRF-3 is serving as a coactivator in this situation. Previous work has shown that the c-Myc transcription factor can bind and activate a number of promoters, which contain no apparent

c-Myc binding sites (Li et al., 2003). To test this hypothesis, an expression vector containing IRF-3 with its DNA binding domain deleted was created (IRF-3ΔDBD). Expression of IRF-3 ΔDBD in wt and p50/p52-deficient cells was confirmed by Western analysis (Figure 5B, left image). The IRF-3 ΔDBD cells were then infected with the ML I112 transgene and stimulated with TNF α over a 6 hr time course. Expression of IRF-3 ΔDBD rescued the induction of the ML I112 transgene in p50/p52-deficient cells (Figure 5B, right image).

To confirm that IRF-3 is required for LPS stimulation of the ML I112 transgene in p50/p52-deficient cells, we created a retrovirus expressing an RNAi cassette against IRF-3 to silence the endogenous IRF-3 expression (Qin et al., 2003). IRF-3 protein levels were confirmed by Western analysis and were knocked down approximately 3–4-fold (Figure 5C, left image). p50/p52-deficient cells with IRF-3 knocked-down were infected with the ML I112 transgene, stimulated with LPS, and assayed for luciferase induction. The absence of IRF-3 in LPS-stimulated p50/p52-deficient cells abrogated the induction of the ML I112 transgene (Figure 5C, right image).

As mentioned earlier, endogenous IP-10 requires

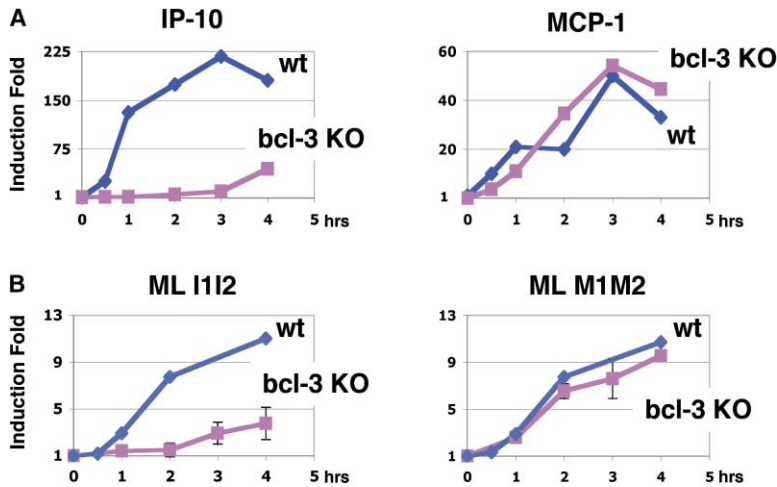


Figure 6. Induction of ML I1I2 by TNF α Requires Bcl-3

Bcl-3-deficient and wild-type (wt) cells are stimulated with TNF α or LPS over a four hour time course. Induction of endogenous IP-10 and MCP-1 RNA transcript levels are measured by quantitative PCR (A). Bcl-3 KO and wt cells carrying the ML I1I2 or ML M1M2 transgene are stimulated with TNF α over a four hour time course (B).

IRF-3 for gene activation. We have demonstrated that the κ B site sequence can alter the configuration of bound p65:p65 homodimers to implement an IRF-3 requirement for transgene activation. Does the change in κ B site sequence alter the configuration of all κ B dimers such that the ML I1I2 transgene requires IRF-3 for gene activation in wt cells, where the major species of NF- κ B is p65:p50 heterodimers? Using the same retrovirus, we knocked down IRF-3 in wt cells (Figure 5D, left image). The absence of IRF-3 in LPS-stimulated wt cells also abrogated the induction of the ML I1I2 transgene (Figure 5D, right image). To further confirm these findings, the absence of IRF-3 in TNF α -stimulated wt cells did not affect the induction of IP-10 or the ML I1I2 transgene (data not shown).

IRF-3 Is Recruited to I1I2 κ B Binding Sites

To test whether IRF-3 is directly recruited to the ML I1I2 transgene, ChIP assays were performed with an anti-IRF-3 antibody in LPS-stimulated, IRF-3 overexpressing, wt cells. We found that IRF-3 was bound to the IP-10 promoter but not the MCP-1 promoter in an LPS-dependent manner (Figure 5E, left image). Furthermore, IRF-3 was also bound to the ML I1I2 transgene (Figure 5E, right image). Therefore, IP-10's κ B site sequences are able to recruit IRF-3 to a promoter.

IP-10's requirement for IRF-3 in p50/p52-deficient cells can explain the stimulus-specific requirements for κ B family members. Under LPS stimulation, p65 homodimers appear to interact with IRF-3 to activate the ML I1I2 transgene. Under TNF α stimulation, IRF-3 is not activated and the p65 homodimers, although bound, are unable to function and the ML I1I2 transgene is not activated. For LPS-stimulated wt cells, we demonstrated that p65:p50 heterodimers appear to interact with IRF-3 to activate the ML I1I2 transgene. This result implies that the κ B site sequence can change the configuration of heterodimers, not just p65 homodimers. Finally, we show that IRF-3 is directly recruited to the ML I1I2 promoter in a stimulus-dependent manner.

IP-10 Requires Bcl-3 for Induction by TNF α

The need for IRF-3 provides an explanation for why IP-10 is induced in p50/p52-deficient cells only in response to LPS but not TNF α . Why do wt cells express IP-10 in

response to TNF α ? Might there be another coactivator that suffices in wt cells where the p65:p50 heterodimer predominates? We were drawn to the observation that Bcl-3, an I κ B-like protein that interacts preferably with p50 or p52, can serve as a coactivator (Franzoso et al., 1992; Fujita et al., 1993).

To examine the possibility that Bcl-3 is critical to IP-10 induction in wt cells, Bcl-3-deficient cells were stimulated with TNF α over a 4 hr time course. RNA transcript levels of endogenous IP-10 and MCP-1 were measured by Q-PCR. IP-10 induction was absent in Bcl-3-deficient cells, while MCP-1 induction recapitulated wt levels (Figure 6A). Thus, in TNF α -stimulated cells, IP-10 is a Bcl-3-dependent gene. As expected, when Bcl-3-deficient cells were infected with the ML I1I2 transgene and stimulated with TNF α over a 4 hr time course, minimal luciferase induction was observed, showing that the Bcl-3 dependence is a consequence of the κ B site sequence (Figure 6B, left image). In contrast, the ML M1M2 transgene was induced to similar levels in wt and Bcl-3-deficient cells (Figure 6B, right image). Finally, induction of IP-10 expression in LPS-stimulated Bcl-3-deficient cells remained intact (data not shown).

Discussion

In our previous study, no direct correlation between the κ B site sequence and κ B family member requirements for gene activation could be found (Hoffmann et al., 2003). There we compared one gene to another but when, in this study, we made interspecies comparisons of the same gene, we found a remarkable constancy of sequence, implying that the individual sequences have important characteristics. This led us to examine the role of the particular sequences found associated with particular genes. To do this we developed a lentiviral system for incorporating regulatory sequences into cellular DNA. Then, by swapping the κ B site sequences within the MCP-1 promoter to the κ B site sequences for the IP-10 gene, we found that we could impose IP-10's κ B family member requirements onto the MCP-1 promoter. Both IP-10 κ B site sequences had to be transferred to change κ B family member requirements and revealed that two κ B sites can function together as a module to regulate gene activation. This suggested that

either MCP-1 site was dominant over the two IP-10 sites. By doing chromatin immunoprecipitation experiments, we found that even though the I1 site would not work with the p65 homodimer, the IP-10 κ B sites did bind the homodimer which, in turn, even bound the coactivator CBP/p300. We then found that the IP-10 requirement for a κ B heterodimer for activation by $\text{TNF}\alpha$ is not evident after LPS stimulation. This suggested that the κ B site specificity operated by imposing on the DNA bound NF- κ B, a cofactor requirement for activation. In fact, we showed that the ML I1I2 transgene requires Bcl-3 when stimulated by $\text{TNF}\alpha$ and that IRF-3 can play this role in p50/p52-deficient cells where Bcl-3 is not able to function. Because IRF-3 is induced by LPS but not $\text{TNF}\alpha$, the role of this κ B site specificity is explained. We extended the analysis to show that the IRF-3 requirement for LPS-stimulated ML I1I2 transgene activity applies to p50:p65 heterodimers as well. Therefore, the κ B site sequence affects the configuration of both heterodimers and p65 homodimers. Finally, we showed that IRF-3 is recruited to the ML I1I2 promoter. The sequence alteration that imposes the coactivator requirement is a single nucleotide in the sixth position of the κ B site—it is quite remarkable that such a change can impose on a gene a new cofactor requirement that is fulfilled only under particular circumstances.

How Is Specificity Imposed?

There are three models for how the single nucleotide difference in the κ B site can impose such specificity. One model suggests that there is another protein bound to the DNA site that requires this particular nucleotide. It would most likely bind in conjunction with NF- κ B, just as HMG I(Y) has been shown to bind to certain κ B sites along with NF- κ B (Falvo et al., 1995). The factor would bind the distal IP-10 κ B site because that site dominantly imposes the heterodimer restriction. It seems a bit far-fetched but not impossible that a protein could bind to the κ B site and then dominantly impose a restriction on the functioning of a dimer that would be overcome by a cofactor. IRF-3 and Bcl-3 would be the responsible cofactors in LPS- and $\text{TNF}\alpha$ -stimulated cells, respectively.

Another possibility is that κ B dimers may oligomerize when bound to their respective sites. The sequence of the κ B site would determine oligomerization efficiency and precise conformation of the overall structure. The κ B tetramer would determine which cofactors would be needed for gene activation.

A likely possibility is that the particular distal IP-10 κ B site imposes a configuration on the bound heterodimers that establishes a requirement for Bcl-3. In the p50/p52-deficient cells, where Bcl-3 cannot bind the p65 homodimers, the requirement for a coactivator can be supplied by IRF-3, which can bind to p65 (Wietek et al., 2003).

Potential Model

To fully explain our data, we return to the observation that two κ B sites are needed for the MCP-1 promoter to function. The stimulus and subunit specificities have been explained by the role of one κ B site, but why then do we need two? We suspect that the two sites serve

different and nonredundant roles. This postulate leads us to a model that the two κ B sites serve different and jointly obligate functions.

In LPS-stimulated p50/p52-deficient cells infected with the ML M1M2 transgene, we know that p65 homodimers are bound to the κ B sites along with p300/CBP (data not shown) and drive luciferase expression. Our postulate that the two sites serve different functions leads us to suppose that only one κ B homodimer binds p300 and that the other binds an unknown factor X but other explanations are conceivable (Figure 7A). In LPS-stimulated p50/p52-deficient cells infected with the ML I1I2 transgene, we postulate that the I2 site continues to bind p300/CBP but the I1 κ B site sequence alters the p65 homodimer conformation such that another cofactor must take the place of X. IRF-3 can serve this function and in LPS-induced cells, it binds and cooperates to drive luciferase expression (Figure 7B). In $\text{TNF}\alpha$ -stimulated p50/p52-deficient cells with the ML M1M2 transgene, the situation is similar to that in LPS-stimulated cells (Figure 7C). However, in the $\text{TNF}\alpha$ -stimulated p50/p52-deficient cells with the ML I1I2 transgene, the change in the I1 κ B site sequence alters the conformation for p65 homodimers such that they require a cofactor of a type that is simply not present in the cells. Instead, only κ B heterodimers are able to drive transcription because of the requirement for the second activity is supplied by Bcl-3 binding to the p50 subunit (Figure 7D).

Advantages of a Lentiviral-Based Reporter System

Our lentiviral-based reporter system is a novel approach to study gene transcription. This system offers many advantages over current transcription reporter systems. (1) The promoter of the gene of interest is integrated into the cell's DNA and should therefore be subject to chromatin regulation. (2) Tandem copies of integration are avoided to allow for faithful promoter regulation. (3) Copy number of the transgene can be controlled. (4) This system can be applied to nondividing and untransfectable cells. (5) Most importantly, the system allows flexibility to conveniently modify promoter sequence by PCR. A limitation of this system is the amount of DNA sequence one can place into the vector (roughly 7 kb). In truth, it is not possible to know for sure how much regulatory DNA a particular gene requires so the optimum method for studying promoters would be to make nucleotide changes in situ in the promoter regulatory elements using "knockin" technology (Xu et al., 1996). Unfortunately, knockin or even BAC transgene experiments (Lee et al., 2003) are difficult, time-consuming, and prohibitively expensive as a method to do a systematic study, such as we have presented here. Our approach represents an effective compromise between the ideal and need for extensive experimental manipulation.

κ B Site Sequence Determines NF- κ B Family Member Requirements

In our previous study, we documented that κ B-dependent genes require specific κ B family members for functional activation (Hoffmann et al., 2003). Here, we demonstrate that the sequence of the κ B site is responsible

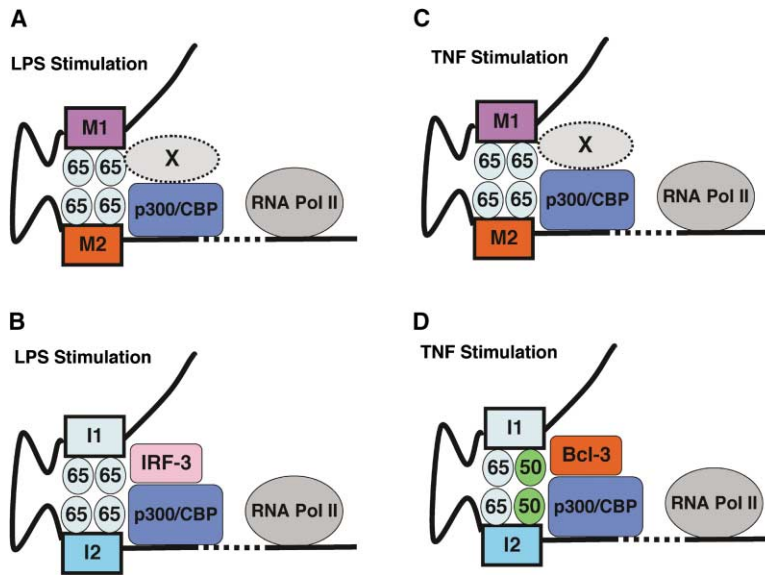


Figure 7. Two κ B Sites Function Together to Determine NF- κ B Family Member and Coactivator Specificity

A model depicting how two κ B sites could coordinate as a single module to regulate gene activation. The model postulates that the two κ B sites in a promoter/enhancer have separable functions that must be jointly provided from the module to support transcription. One is to bind p300/CBP, the other is to bind a second coactivator whose nature is dictated by the sequence of the κ B site to which that NF- κ B dimer is bound. One hypothetical coactivator is designated X, the others are known. The model is presented in the context of 4 conditions discussed in the text. In LPS-stimulated p50/p52-deficient cells, p65 homodimers bound to M1 and M2 recruit p300/CBP and X (A). In the same cells, the alteration to I1 does not allow X binding but does support IRF-3 binding (B). In TNF α -stimulated p50/p52-deficient cells infected with ML M1M2, the situation is similar to that in LPS-stimulated cells (C). However, in TNF α -stimulated p50/p52-deficient cells in-

fectured with ML I1I2, because IRF-3 is not activated, the p65 homodimers cannot bind a coactivator and gene activation does not occur (not shown). However, in wild-type cells, NF- κ B heterodimers are present and p50 is able to bind Bcl-3, providing the co-activator needed for the module to function (D).

for determining NF- κ B dimer specificity, and two κ B binding sites can function together to regulate gene activation. This regulation does not occur at the level of κ B dimer exclusion. In fact, we found inducible p65 recruitment to a functionally inactive promoter. Instead, we believe that the sequence of the κ B site contains information that is interpreted by the bound κ B dimer, changes the κ B dimer configuration, and determines which coactivators will form functional interactions with the κ B dimer.

How many functionally different κ B sites might there be? We can presently identify three different types. In our system, M1 or M2 behave identically and we postulate that they bind factor "X". A key property of this class of κ B site is that it is dominant over other sites. The second class is I1, a site that requires IRF-3 or Bcl-3 coactivators for activation when it is coupled to I2 or is used in tandem. I1 differs from M1 and M2 at only one position, nucleotide 6. Finally, the I2 site is in a category by itself. It must require a coactivator different from M1 or M2 that is not dominant over I1. It is notable that I2 has two nucleotides, at positions 5 and 8, different from both M2 and I1. There is every reason to expect that there are other κ B sites with specificities yet to be identified. From our limited evolutionary survey in Figure 1A, we see 12 different sites all of which are conserved between mouse and human. It could be that these 12 and others are all serving specific functions, going a long way to providing the breadth of diversity needed to understand how κ B sites can be found in so many different genes which are regulated in specific ways (Hoffmann et al., 2003).

κ B Site Sequence Determines Coactivator Requirements

It is impressive that changing one base pair would have such a dramatic effect on gene regulation. However,

recent structural studies support this conclusion. Comparisons of p50:p65 dimers bound to different κ B site sequences have revealed significant differences in their conformation (Chen-Park et al., 2002). These differences in conformation have been correlated to their ability to transactivate transfected reporter plasmids. Therefore, our notion that it is coactivator binding which determines specificity is supported by prior structural studies showing alternate conformations induced allosterically by different DNA binding site sequences.

In other families of transcription factors, there is also evidence that DNA can act as an allosteric regulator. It has been best studied with the glucocorticoid receptor (GR) (Lefstin and Yamamoto, 1998). The DNA binding domain of GR can bind its cognate site in a variety of conformations depending on the sequence of the site, serving to present different interfaces for the recruitment of specific cofactors and to help determine whether the bound factor will activate or repress transcription. Recent work has identified which GR surfaces are required in a gene-specific manner (Rogatsky et al., 2002, 2003). Similar findings have also been reported for the POU family of proteins (Phillips and Luisi, 2000).

The prevailing model of gene transcription is that on a given promoter multiple proteins must interact, assemble, and form an enhanceosome to activate gene transcription (Thanos and Maniatis, 1995b). If one factor is absent, the gene is not activated. Numerous examples of NF- κ B's role in this model exist, most notably on the HIV-LTR, IFN- β , and IL-2 promoters (Perkins et al., 1993, 1994; Rothenberg and Ward, 1996; Thanos and Maniatis, 1995b). Furthermore, chromatin has also been implicated as having a role in determining NF- κ B's access to DNA binding sites and may do so in a κ B dimer-specific fashion (Saccani et al., 2001, 2003). In the best-studied system, the IFN- β gene, there is only one κ B site and that is why it may be so complex. In vitro binding

and transient transfection studies demonstrated that the κ B site sequence is important, presumably to coordinate the binding of both NF- κ B and HMG (I)Y (Thanos and Maniatis, 1995a). As we imply by the models in Figure 7, where there are two κ B sites, the situation may be different because there can be two interacting complexes formed around two NF- κ B dimers.

Bcl-3 Requirement

TNF α activation of IP-10 activation requires Bcl-3, and our data implies that p50:p65 heterodimers interact with Bcl-3. Previous studies have shown that Bcl-3 preferentially interacts with p50 or p52 homodimers and not with p50:p65 heterodimers (Franzoso et al., 1992; Fujita et al., 1993). We can suggest three potential mechanisms to explain this apparent discrepancy. First, even though the major constituent of NF- κ B in wild-type fibroblasts is p50:p65 heterodimers, a significant amount of p50:p50 homodimers exist and could bind one of the two sites to interact with Bcl-3 to activate IP-10. Second, it may be possible that p50:p65 heterodimers can interact with Bcl-3 to activate IP-10 expression. Third, as mentioned earlier, p50:p65 heterodimers may oligomerize when bound to their respective sites. The p50 subunits from each heterodimer could conceivably interact and recruit Bcl-3.

Biology

MCP-1 and IP-10 are differentially regulated, presumably because they serve different functions. MCP-1 plays a significant role in innate immunity by bringing macrophages to sites of inflammation. On the other hand, IP-10 is important for lymphocytic (adaptive) immunity and regulates T cell proliferation. MCP-1 is also activated by a more diverse set of TLR agonists than IP-10. For example, TLR-2 agonists stimulate MCP-1, but not IP-10 expression (Pierer et al., 2004; Re and Strominger, 2001). Recent studies reveal that MCP-1 and IP-10 are regulated differently during TLR-4 stimulation (Sakaguchi et al., 2003; Serbina et al., 2003; Yamamoto et al., 2003). IP-10 is activated through TLR-4 via a Trif-dependent pathway. The Trif-dependent pathway activates the interferon-response pathway and is responsible for a late NF- κ B activation (Yamamoto et al., 2003). It has been shown that this pathway regulates a specific subset of TLR3/TLR4-dependent genes, and that the pathway is evolutionarily diverged from other members of the TLR family (Doyle et al., 2002). In contrast, MCP-1 is activated by both the MyD88-dependent and Trif-dependent pathways. The MyD88 pathway differs from the Trif pathway in two ways. First, it does not activate the interferon-response pathway. Second, it activates NF- κ B much earlier than the Trif pathway.

These two pathways normally work together to ready an immune response to a bacterial pathogen. A bacterial pathogen would signal the TLR-4 receptor and activate both downstream pathways. The MyD88 pathway would activate NF- κ B immediately to drive MCP-1 production and recruit macrophages to the target site. Later, the Trif pathway would activate both the NF- κ B and IRF-3 pathways to drive IP-10 production and regulate T cell proliferation. By simply changing the sequence of κ B sites in the MCP-1 promoter, we converted MCP-1's

regulation profile into IP-10's. We speculate that in a mouse this would severely disrupt the delicate balance between innate and adaptive immunity. Our results underscore the functional importance of the sequence of the κ B site and confirm why the sequence of κ B sites is strictly conserved over time. Not only does the κ B site sequence determine κ B dimer specificity, it also determines coactivator requirements.

Experimental Procedures

Cell Culture and Time Courses

Immortalized cell lines were generated and maintained as previously described (Hoffmann et al., 2003). 100% confluent cells were treated for 48 hr with 0.5% serum containing medium and then stimulated with the appropriate concentration of TNF α (4 ng/mL) or LPS (0.5 μ g/mL) over a period of 4–6 hr.

Plasmids

To generate ML M1M2, 5 kB of the MCP-1 promoter was amplified by PCR from a BAC clone. The resulting fragment was cloned into FugW (Lois et al., 2002). To generate IL, 967 bp of IP-10 promoter (–939 +28) was amplified by PCR and cloned into FugW. Variations of ML plasmid were constructed by PCR mutagenesis. IRF-3 and IRF-3 Δ DBD expression plasmids were generated by amplifying IRF-3 (amino acids 1–420) and IRF-3 Δ DBD (133–420) by PCR and cloning into the pBABE-neo vector (Morgenstern and Land, 1990). All plasmids were verified by DNA sequencing and restriction digest analysis.

Real-Time PCR

Total RNA was made from confluent and starved fibroblasts using TriReagent (Molecular Research Center, Inc.). cDNA was synthesized with Superscript II (Invitrogen) following manufacturer's guidelines and 100 ng total RNA as a starting amount. Q-PCR was performed using Sybr Green PCR Master Mix (Applied Biosystems) and a 5600 Real-Time PCR machine (Applied Biosystems). Samples were performed in triplicates, and GAPDH transcript levels were used to normalize between samples. Every experiment was performed at least twice, many three to six times with high reproducibility. Primer sequences are available upon request.

Reporter Assay

Luciferase assay was performed as described elsewhere (Pomerantz and Baltimore, 1999). Samples were normalized for protein levels by Bradford assay. Every experiment was performed at least twice, most three to five times with high reproducibility.

Chromatin Immunoprecipitation

ChIP studies were based on a combination of published protocols (Boyd and Farnham, 1999; Boyd et al., 1998; Nissen and Yamamoto, 2000; Sacconi et al., 2001). p65 and IRF-3 antibodies were purchased from Santa Cruz Biotechnologies (sc-109) and Zymed Laboratories, respectively. p300 antibody was a gift from Kevin Gardner. Sequences of promoter-specific primers and our detailed protocol are available upon request.

Lentivirus

Lentivirus was produced as described elsewhere (Lois et al., 2002).

siRNA

FG12 RNAi vector and production of siRNA was previously described elsewhere (Qin et al., 2003). The IRF-3-siRNA contains the sense targeting sequence of gacgcacagatggctgact corresponding to the 390–407 nucleotide positions of the mouse IRF-3 coding sequence.

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