NF-κB elements contribute to *junB* inducibility by lipopolysaccharide in the murine macrophage cell line RAW264.7

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Abstract Macrophages respond to bacterial lipopolysaccharide (LPS) by activating latent *cis*-acting factors that initiate transcription of immediate early genes. One such immediate early gene, *junB*, is induced by LPS in macrophages within 30 min. To identify elements that mediate the induction of *junB* by LPS, upstream and downstream sequences flanking the *junB* gene were examined by transient expression in the RAW264.7 murine macrophage cell line using a luciferase reporter gene vector containing the *junB* minimal promoter. A >10-fold enhancement was associated with a 222 bp region downstream of the *junB* promoter in response to LPS. Transient reporter assays demonstrated that multiple nuclear factor (NF) κB sites are required for inducibility of *junB* by LPS in RAW264.7 cells. Electrophoretic mobility shift assays confirmed binding of LPS-induced nuclear proteins included p50/p65 heterodimers at these NF-κB sites. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Lipopolysaccharide; *junB*; Macrophage; Nuclear factor κB

1. Introduction

junB is a member of the bZIP family of transcription factors, all of which encode proteins with a leucine zipper motif that mediates dimerizations to constitute active transcription factors [1]. Dimerization of *junB* with other *jun* or *fos* family members yields the formation of an active AP-1 complex [1]. Subsequent binding of AP-1 to DNA recognition sequences results in a cascade-like transcriptional activation of genes involved in cellular proliferation, differentiation, and activation. Stimulation of macrophages with bacterial lipopolysaccharide (LPS) results in an increase in *junB* mRNA transcripts and upregulation of AP-1 binding activity [2].

As many genes critical to macrophage activation contain AP-1 binding sites within their promoters [3–5], we sought to gain further insight as to how LPS-induced *junB* gene expression is controlled using the murine macrophage cell line, RAW264.7. A transient luciferase expression assay incorporating the *junB* promoter was used to screen both upstream and downstream regions of the *junB* gene. Herein we report that LPS-induced transcriptional activation of the *junB* gene in RAW264.7 macrophages is controlled by multiple nuclear factor (NF) κB binding sites within a 222 bp region downstream of the *junB* promoter. Furthermore, the cumulative effect of these NF-κB sites is due to binding of p50/p65 heterodimers.

2. Materials and methods

2.1. Cell culture and electroporation

The RAW264.7 murine macrophage cell line, obtained from the ATCC (Rockville, MD, USA), was maintained in RPMI 1640/2 mM L-glutamine/10% fetal calf serum/50 μM β-mercaptoethanol (R10). All plasmids used in electroporation experiments were purified in cesium chloride–ethidium bromide gradients. Cells were electroporated with a Cell-Porator (Life Technologies, Grand Island, NY, USA) at 200 V and 1600 μF on low resistance setting, as recently described [6]. Eighteen hours post-transfection, cells were stimulated with or without LPS (1 μg/ml; Sigma, St. Louis, MO, USA) or murine interleukin-6 (IL-6, 200 U/ml) [7] for 4 h and then harvested for luciferase and β-galactosidase assays. Luciferase expression was corrected for the expression of β-galactosidase (pADβ) as an internal control. Inducibility was calculated as the corrected activity of stimulated cells relative to that of untreated cells.

2.2. Stimulation of cells for Northern blot analysis and Western blot analysis

RAW264.7 cells were stimulated with or without LPS (1 μg/ml) or murine IL-6 (200 U/ml) as indicated and harvested by removal of all medium and subsequent lysis in the appropriate buffers. Total cellular RNA was isolated using the Qiagen RNeasy kit (Qiagen, Chatsworth, CA, USA) and Northern blot analysis performed as previously described [8]. For Western blot analysis of whole cell lysates, cells were lysed in RIPA lysis buffer (150 μM NaCl, 1% NP-40, 0.5% deoxycholate acid, 0.1% SDS, 50 μM Tris pH 8.0, and 0.5 μM AEBSF). Samples were analyzed by 8% SDS–PAGE followed by electrophoretic transfer to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). The membranes were blocked with 1% ovalbumin (Sigma) in 150 mM NaCl, 10 mM Tris, pH 8.0 with 0.05% Tween 20, incubated with a mouse monoclonal antibody directed against *junB* (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h, followed by incubation with a peroxidase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL, USA). Membranes were developed using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA).

2.3. Construction of junB plasmids

The IκB-α dominant-negative plasmid (a generous gift from Dr. Keith Brown, Laboratory of Immunoregulation, NIAID, NIH, Bethesda, MD, USA) has been described previously [9]. The plasmids used for reporter assays were constructed as previously described [7]. Briefly, an *Eco*RI site at −6500 and a *Sma*I site at +240 were used to construct pGL2/6.5 by partial *Sma*I digestion of an 8 kb *Eco*RI fragment of a clone obtained by screening a genomic BALB/c mouse library with the *junB* cDNA. The resulting 6.7 kb fragment was li-

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gated to pGL2Basic (Promega, Madison, WI, USA). A *Pst*I site at −196 was used to construct pGL2JP by ligation of a 436 bp *Pst*I–*Sma*I fragment containing the *junB* promoter (−196 to +240) to pGL2Basic. Ligation into a 2.1 kb *Eco*RI fragment containing DNA immediately downstream of the *junB* coding region in the reverse orientation into the polylinker upstream of the promoter in pGL2JP resulted in pGL2JP/2DR. Religation of pGL2JP/2DR digested with *Sma*I produced pGL2JP/1.5–2.2DR. A 1.3 kb *Bam*HI–*Eco*RI fragment was ligated to pGL2JP, resulting in pGL2JP/1.5–2.8DR. Production of pGL2JP/222DR was by ligation of a downstream 222 bp *Nla*III–*Sma*I fragment (within the 2.1 kb *Eco*RI fragment) in the reverse orientation into the polylinker upstream of the *junB* promoter in pGL2JP. The 222 bp fragment was also cloned downstream of the luciferase gene (pGL2JP/222SF). Mutations of enhancer sites within the insert of pGL2JP/222SF were constructed by mutual priming with oligonucleotides containing the desired mutations, followed by amplification by polymerase chain reaction as previously described [7].

2.4. Preparation of nuclear extracts

Cells were stimulated with or without 1 μg/ml LPS for 15 min and nuclear extracts were prepared as described previously [10]. A small aliquot was removed to determine protein concentration using Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA). Samples were frozen on dry ice, and stored at −80°C until use.

2.5. Electrophoretic mobility shift assays/supershifts

Nuclear extracts were incubated on ice for 1 h with or without the addition of normal rabbit serum (Ig control), rabbit anti-human p50 polyclonal serum, rabbit anti-human p65 polyclonal serum (a generous gift from Dr. Nancy Rice, NCI Frederick Cancer Research and Development Center, NIH, Frederick, MD, USA), or a combination of anti-p50 and anti-p65. Samples were then incubated with ^{32}P-labeled oligonucleotide probe consisting of a double stranded sequence for each of the four NF-κB sites within the downstream 222 bp region of the promoter of the *junB* gene in binding buffer (final concentration of 10 mM Tris pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 5 mM EDTA, 4% (v/v) glycerol and 0.08 mg/ml sonicated salmon sperm DNA). The sequences for these probes are as follows: NF-κB1: 5′-CGTCAGCGGGGAAAGCCCCCGGCGCACT-3′; NF-κB2: 5′-GGGCAGATTCCGGGAATCCCCTCCCCCC-3′; NF-κB3: 5′-GCCTGCTCGGGAAAATCCCGGCCCCCTT-3′; NF-κB4: 5′-CCCGGGCCTGGGGCTTTCCGCGCCAGAA-3′.

An NF-κB consensus oligonucleotide (Promega) was used as a positive control in these experiments. Samples were applied to a 6% nondenaturing PAGE gel (Novex, San Diego, CA, USA) to separate free probe from probe bound to protein.

3. Results and discussion

To document the kinetics of *junB* expression at both the transcriptional and translational levels, RAW264.7 cells were stimulated with or without 1 μg/ml LPS for different periods of time. As shown in Fig. 1A, *junB* mRNA was induced in RAW264.7 cells and peaked at approximately 1 h post-stimulation, while *junB* protein production was maximally elevated at 3–4 h (Fig. 1B). Based upon the observed kinetic pattern, a 4 h stimulation time point was chosen for subsequent reporter assays. In contrast, IL-6 (200 U/ml), another known stimulus for *junB* gene expression in macrophages [11] and plasmacytomas [12], induced both *junB* gene expression (Fig. 1C) and protein production (Fig. 1D) to a lesser extent than that induced by LPS.

To determine the regions of the *junB* promoter that were necessary for LPS-induced *junB* gene expression, RAW264.7 cells were transiently transfected with either the *junB* promoter (JP) alone, or the JP plus either a 6.5 kb upstream region, or a 2 kb downstream region. Eighteen hours post-transfection, cells were treated with or without LPS and harvested 4 h later for reporter assays. As shown in Fig. 2A, transfection with the 6.5 kb upstream region had only a modest effect on LPS inducibility of *junB* (2.4-fold increase). In contrast, transfection with the 2 kb downstream region of the *junB* promoter resulted in a 5.4-fold induction of *junB* expression compared to JP itself.

In order to locate the region responsible for LPS-induced *junB* gene expression, different fragments of the 2 kb downstream region were analyzed. As shown in Fig. 2B, the 1.5–2.8 region and the 1.5–2.2 region showed 24.0-fold and 23.3-fold inductions, respectively, suggesting that activity in this region was located within the 1500–2200 downstream fragment. The pGL2JP/222DR construct showed a 19.1-fold increase in LPS-induced *junB* promoter activity. No elevation in LPS-inducible *junB* promoter activity was observed using the pGL2JP/1547–2047DR construct, suggesting that the 1547–2047 bp downstream region does not additively contribute to activation of the promoter by LPS treatment.

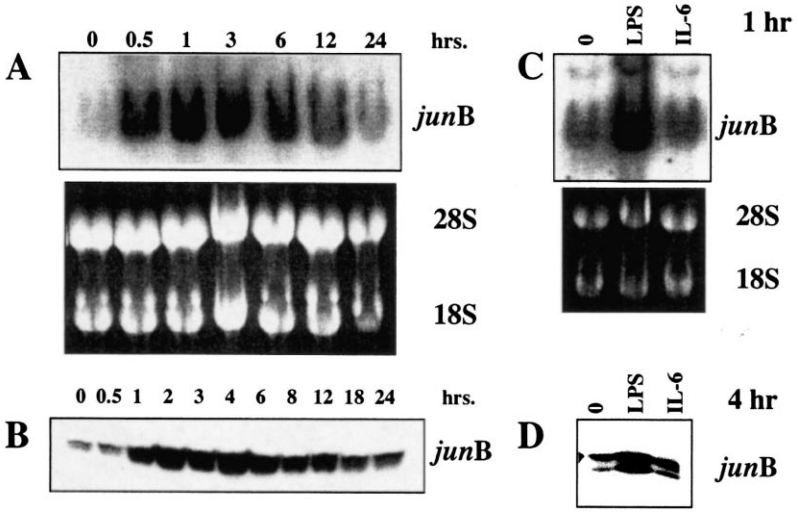

Fig. 1. *junB* induction in the murine macrophage cell line, RAW264.7. A: Northern blot analysis of the kinetics of *junB* gene expression in LPS (1 μg/ml)-stimulated RAW264.7 macrophages. B: Western blot analysis of *junB* protein production in LPS (1 μg/ml)-stimulated RAW264.7 macrophages. Northern blot analysis (C) and Western blot analysis (D) of *junB* expression in LPS (1 μg/ml)- or IL-6-treated (200 U/ml) RAW264.7 macrophages.

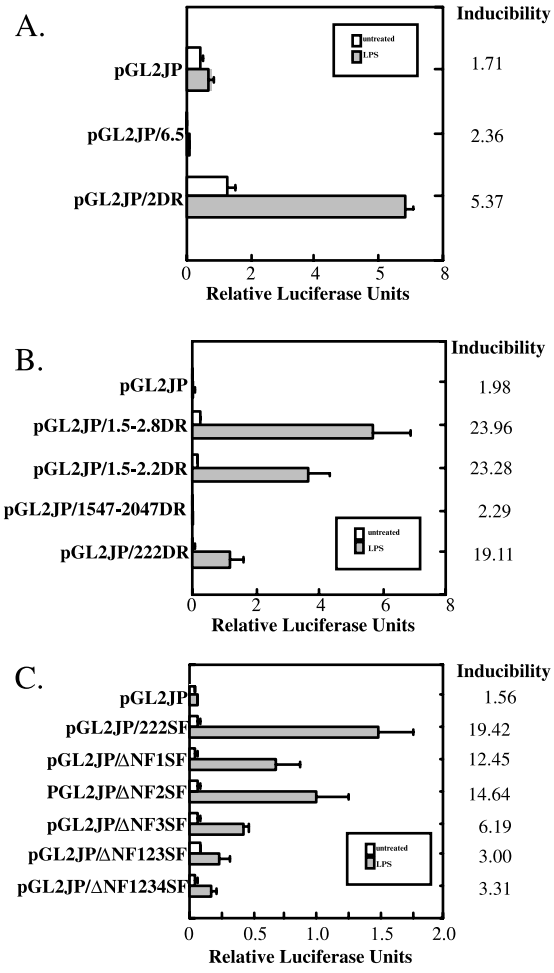

Fig. 2. LPS inducibility of the *junB* gene. RAW264.7 cells were transfected by electroporation with 1 pmol of a luciferase reporter gene vector containing the *junB* promoter alone (pGL2JP) or different flanking regions as described below. After electroporation, cells were rested overnight and two aliquots were treated with or without LPS (1 μg/ml). Luciferase expression was corrected for the expression of pADβ as an internal control. Inducibility was calculated as the corrected activity of stimulated cells relative to that of untreated cells. The results represent three independent experiments, each using separate plasmid preparations. A: LPS inducibility of the *junB* gene within a 2 kb downstream flanking sequence of *junB*. Cells were transfected with 1 pmol of a luciferase reporter gene vector containing the *junB* promoter alone (pGL2JP), the 6.5 kb upstream flanking region, or the 2 kb downstream flanking region. B: LPS inducibility of the *junB* gene within a 222 bp downstream flanking sequence of *junB*. Cells were transfected with 1 pmol of a luciferase reporter gene vector containing the *junB* promoter alone (pGL2JP), or different segments of the 2 kb downstream flanking region: pGL2JP/1.5–2.8, pGL2JP/1.5–2.2, pGL2JP/1547–2047 pGL2JP/222 (1018–2249). C: Effect of site-directed mutations of transcription factor sites contained within the 222 bp downstream flanking sequence of *junB* on LPS inducibility. Mutations of the different transcription factor binding sites within the pGL2JP/222 plasmid were created as previously described.

We previously reported that IL-6-inducible *junB* expression in murine plasmacytoma cells was due to the same 222 bp region downstream of the *junB* promoter [7]. Therefore, the 222 bp region of *junB* appears to serve as a regulatory region for multiple responses, whose specificity relates to both the stimulus and the transcription factors involved. The pGL2JP/222 region contains several different transcriptional regulation elements. In order to determine which transcription factors might be activating *junB* in response to LPS, constructs with mutated transcriptional binding sites were made and compared in their ability to activate the *junB* promoter. As shown in Fig. 2C, single mutations in either the first or third NF-κB binding sites substantially decreased *junB* promoter activity in response to LPS (12.5-fold and 6.2-fold induction by LPS vs. 19.4). A mutation in the second NF-κB site slightly reduced *junB* promoter activity (14.6-fold induction by LPS vs. 19.4). When either the first three NF-κB sites or all four sites were simultaneously mutated, LPS-induced *junB* promoter activity was reduced to almost baseline levels. Mutations in any other sites did not substantially alter LPS-induced *junB* promoter activity (data not shown). These results suggest that multiple NF-κB sites within the *junB* promoter contribute to LPS induction of *junB* gene expression in RAW264.7 cells.

To confirm that these NF-κB sites could bind NF-κB proteins, nuclear extracts were prepared from LPS-stimulated RAW264.7 cells and binding determined by electrophoretic mobility shift assay using ^{32}P-labeled oligonucleotides specific for each of the four putative NF-κB sites (Fig. 3). Oligonucleotides directed against any of the four NF-κB sites bind to proteins from LPS-activated RAW264.7 macrophages. Extracts were also incubated with polyclonal antibodies specific for the p50 and p65 subunits of NF-κB to determine which NF-κB subunits bound to the *junB* promoter. As demonstrated, these proteins are composed of p50/p65 heterodimers. While all four sites in the 222 bp region bind to p50/p65 heterodimers in LPS-activated RAW264.7 cells, the first and third NF-κB elements appear essential for the majority of LPS-mediated activation of *junB* transcription.

To determine whether NF-κB activity contributes to LPS-induced activation of *junB* transcription, RAW264.7 cells were co-transfected with the pGL2JP/222 plasmid or the pGL2JP/222 plasmid with all four mutated NF-κB sites (pGL2JP/ΔNF1234SF) on conjunction with an IκB-α construct containing point mutations in serine residues 32 and 36. These mutations prevent IκB-α from undergoing signal-induced phosphorylation and degradation, and thus the subsequent activation of NF-κB [9]. Eighteen hours post-transfection, RAW264.7 cells were stimulated with or without LPS or IL-6 and harvested 4 h later for reported assays. As demonstrated in Fig. 4, inhibition of NF-κB activation via a dominant-negative IκB-α construct substantially inhibited (approximately 60%) LPS-induced *junB* transcription in cells co-transfected with the pGL2JP/222 construct containing the four intact NF-κB sites. In contrast, cells co-transfected with pGL2JP/222 containing the four mutated NF-κB sites were not activated by LPS.

Bacterial LPS contributes to the pathology of sepsis by its ability to stimulate the functional activation and synthesis of inflammatory mediators by cells of the monocytic lineage. One of the genes induced by LPS is *junB*. JunB, itself a component of the AP-1 transcription factor complex, regulates the expression of multiple effector genes important for cell proliferation, differentiation, and survival. Our data demonstrate that LPS induces *junB* transcription via the binding of NF-κB p50/p65 heterodimers to multiple NF-κB consensus sequences within a downstream 222 bp region of the *junB* promoter. In quiescent cells, NF-κB is retained in the cytoplasm in its inactive form by the inhibitor protein, IκB-α, which strongly associates with p50/p65 heterodimers. Upon stimula-

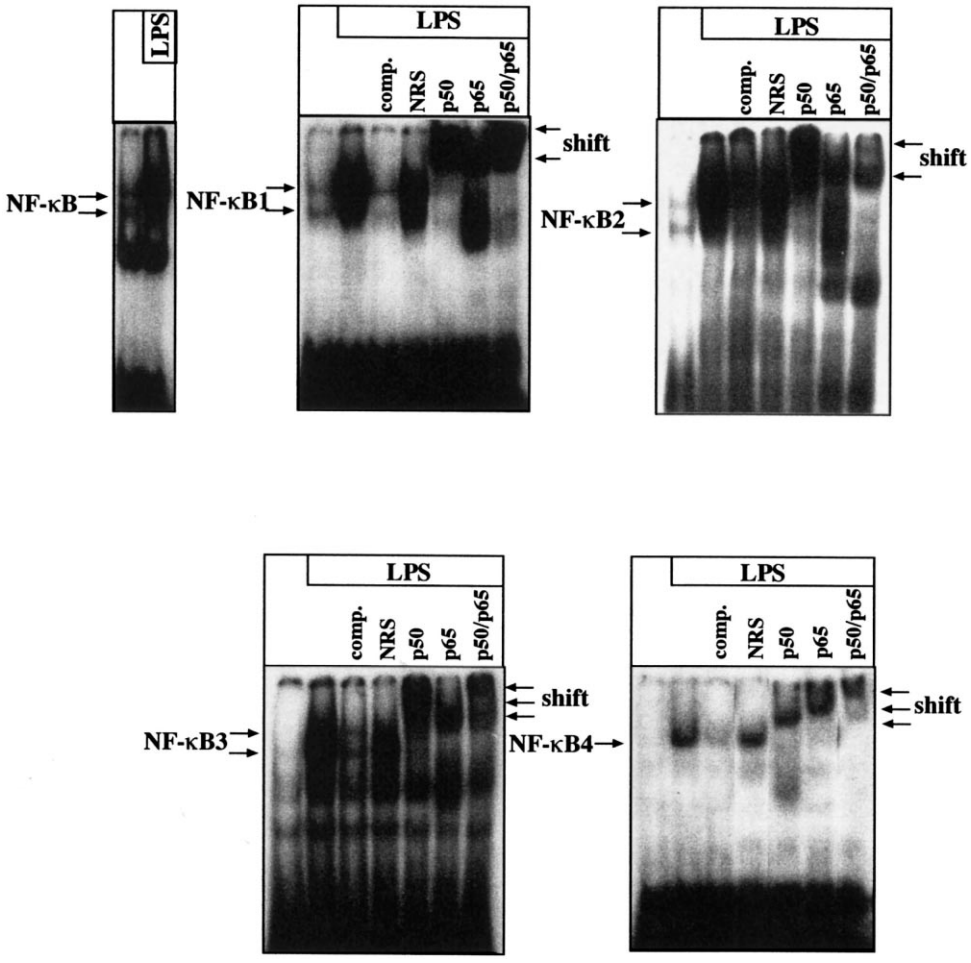

Fig. 3. Binding of NF-κB p50 and p65 subunits to NF-κB sites within the 222 bp region of the *junB* promoter. Nuclear extracts were prepared from RAW264.7 cells that were stimulated with or without LPS (1 μg/ml) for 15 min using oligonucleotide probes for each of the four NF-κB sites within the 222 bp region. Binding assays were performed with normal rabbit serum, anti-p50 antibody, anti-p65 antibody, or anti-p50+anti-p65 antibodies. The results shown are representative of four independent experiments.

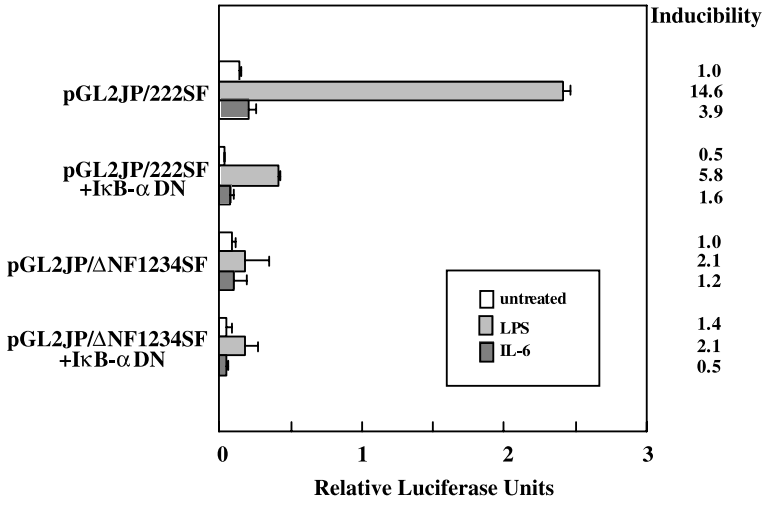

Fig. 4. A dominant-negative IκB-α inhibits LPS inducibility of the *junB* gene. RAW264.7 cells were transfected by electroporation with or without 1 pmol of the IκB-α dominant-negative vector and 1 pmol of a luciferase reporter gene vector containing the pGL2JP/222SF plasmid or the plasmid with all four mutated NF-κB sites (pGL2JP/ΔNF1234SF). After electroporation, cells were rested overnight and two aliquots were treated with or without LPS (1 μg/ml) or murine IL-6 (200 U/ml). Luciferase expression was corrected for the expression of pADβ as an internal control. Inducibility was calculated as the corrected activity of stimulated cells relative to that of untreated cells. The results are representative of three independent experiments, each using separate plasmid preparations.

tion by various pathogens or stress, IκB-α is phosphorylated and rapidly degraded, allowing NF-κB translocation to the nucleus, and initiating the transcription of target genes, such as *junB*. By use of the dominant-negative IκB-α mutant, we were able to demonstrate that LPS activates *junB* transcription in RAW264.7 cells via the NF-κB pathway. The κB DNA consensus sequence for binding of p50/p65 heterodimers (GGG ACT TTCC) [13] is recognized by both p50 homodimers and p50/p65 heterodimers and, at high concentrations, by p65 homodimers. The p50 component of this heterodimer preferentially binds to the 5′ region of the recognition sequence GGGRN, whereas the p65 component binds to the 3′ region of the recognition sequence TTCC [14,15]. Of the four different NF-κB sites within the *junB* promoter, both the NF-κB1 and the NF-κB3 sites exhibit sequence homology for a high affinity binding site for p50/p65 heterodimer complexes, consistent with our results from gel supershift assays. In contrast, the NF-κB2 and NF-κB4 sites lack the TTCC sequence for optimal p65 interaction, which could explain the limited contribution of these two sites to *junB* gene transcription. Both the NF-κB1 and NF-κB3 sites appear essential for strong activation of the *junB* gene following LPS activation, suggesting a cumulative functional interaction between the protein complexes bound to these sites. Promoter regions of other proinflammatory genes have similar multiple transcription factor binding sites that together allow for maximal transcriptional activation of these genes [16,17]. It is plausible that the multiplicity of binding sites in *junB* and other genes may help to form a multimeric complex whose tertiary and/or quaternary structure potentiates transcriptional activation.

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