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In Vivo Binding of NF κ B to the I κ B β Promoter is Insufficient
for Transcriptional Activation

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Running Title: Regulation of I κ B β gene expression by NF κ B

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SYNOPSIS

Despite certain structural and biochemical similarities, differences exist in the function of the NF κ B inhibitory proteins I κ B α and I κ B β . The functional disparity arises in part from variance at the level of gene regulation, and in particular from the substantial induction of I κ B α , but not I κ B β , gene expression post-NF κ B activation. Here we probe the differential effects of IL-1 β on induction of I κ B α and I κ B β , and more especially perform the first characterisation of the human I κ B β promoter. A consensus NF κ B-binding site, capable of binding NF κ B both *in vitro* and *in vivo* is found in the I κ B β gene 5' flanking region. However, the I κ B β promoter was not substantially activated by pro-inflammatory cytokines, such as IL-1 β and TNF α , that are known to cause strong activation of NF κ B. Furthermore, in contrast to I κ B α , NF κ B activation did not increase expression of endogenous I κ B β as assessed by analysis of mRNA and protein levels. Unlike κ B-responsive promoters, I κ B β promoter-bound p65 inefficiently recruits RNA polymerase II, which stalls at the promoter. We present evidence that this stalling is likely due to the absence of TFIIH engagement, a prerequisite for RNA polymerase II phosphorylation and transcriptional initiation. Differences in the conformation of promoter-bound NF κ B may underlie the variation in the ability to engage the basal transcriptional apparatus at the I κ B β and κ B-responsive promoters. This accounts for the differential expression of I κ B family members in response to NF κ B activation and furthers our understanding of the mechanisms involved in transcription factor activity and I κ B β gene regulation.

Key words: nuclear factor κ B (NF κ B), inhibitor of κ B (I κ B), transcription, chromatin immunoprecipitation (ChIP), RNA polymerase II, *cis*-element.

Abbreviations used: CTD, carboxy terminal domain; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RFP, red fluorescent protein; TAD, transcriptional activation domain; TNF, tumour necrosis factor; TSS: transcription start site; UTR, untranslated region.

INTRODUCTION

The inducible expression of a wide range of immune and inflammatory response genes is controlled by the action of NF κ B (nuclear factor κ B) [1]. NF κ B is a dimeric factor, comprised of members of the Rel family of proteins, some of which are transcriptionally active - p65, c-Rel, RelB, while others lack TADs (transcriptional activation domains) and are synthesised as longer inactive precursors – p105 (p50) and p100 (p52) [2]. Rel proteins share a conserved 300 amino acid N-terminal region known as the RHD (Rel homology domain) that contains regions responsible for dimerisation, nuclear localisation and site-specific DNA binding. The RHD is also responsible for mediating interactions with members of the I κ B (inhibitor of κ B) family of proteins [3]. I κ B α and I κ B β , the most extensively characterised members of this family, display numerous structural similarities. They contain a central ankyrin repeat domain responsible for association with NF κ B with 1:1 stoichiometry [4, 5]. The interaction maintains NF κ B in an inactive state in unstimulated cells. In addition, I κ B α and I κ B β contain specific N-terminal serine residues that are phosphorylated in response to NF κ B-inducing stimuli [6, 7]. This tags the I κ B protein for polyubiquitination and proteasome-mediated degradation, allowing NF κ B to enter the nucleus and bind to target promoters.

I κ B α and I κ B β respond differently to the various inducers of NF κ B. Whereas I κ B α is degraded by all stimuli known to activate NF κ B, I κ B β proteolysis only occurs in response to a subset, those causing long-term activation of the transcription factor [8]. As the I κ B α gene is positively regulated by NF κ B, its expression is rapidly induced following initial NF κ B activation [9]. The newly synthesised protein can enter the nucleus, remove NF κ B from its target promoters and return it to the cytoplasm [10]. This inhibitory feedback accounts for the transient nature of the NF κ B response produced by certain stimuli. The importance of I κ B α for regulating NF κ B was highlighted by studies on I κ B α ^{-/-} mice, which display developmental defects postnatally, dying within 8 days. The hematopoietic tissue of these animals show increased levels of nuclear NF κ B and mRNAs of some NF κ B-responsive genes. Furthermore, in I κ B α ^{-/-} deficient embryonic fibroblasts, where signal-dependent NF κ B-activation is retained due to inhibition by I κ B β , nuclear NF κ B remains elevated in response to TNF α treatment relative to wild-type cells [11]. Interestingly, knock-in mice, in which the I κ B α gene is replaced by the I κ B β gene under the control of the I κ B α promoter, do not display postnatal developmental abnormalities and show an inducible NF κ B activation similar to wild-type

animals [12]. This indicates that I κ B β can functionally compensate for I κ B α , and suggests that it is the strong NF κ B-mediated upregulation of I κ B α expression that accounts for the main difference in I κ B α and I κ B β activity. However, I κ B β is also likely to play unique roles in temporal regulation of NF κ B in some cell systems. We have previously shown that IL-1 β causes sustained activation of NF κ B in human astrocytes and that this coincides with strong induction of I κ B α but lack of resynthesis of I κ B β [13]. In the present study we further probe the mechanisms underlying the differential induction of the two I κ B forms by IL-1 β in this system.

In contrast to I κ B α , I κ B β expression in mouse is not strongly upregulated by NF κ B, although it does contain a κ B site in its promoter. Studies on the murine I κ B β promoter demonstrated that this κ B site confers modest inducibility to NF κ B activation [14]. Such findings coupled to our previous demonstration of the lack of resynthesis of I κ B β in response to IL-1 β in human astrocytes, prompted us to perform the first characterisation of the human I κ B β promoter and more especially explore its regulation by NF κ B. In the present study we report that p65 binds to the I κ B β promoter *in vivo* in an IL-1 β -dependent fashion. However, this binding fails to initiate efficient recruitment of the basal transcriptional apparatus or promoter firing. Given the critical role of I κ B proteins in regulating NF κ B, and the importance of gene regulation in dictating I κ B activity, probing the mechanisms underlying the differential expression of I κ B α and I κ B β is of clear importance. This study advances our understanding of those mechanisms, and offers an explanation for the failure of NF κ B to cause an induction of I κ B β expression.

MATERIALS AND METHODS

Materials - The human astrocytoma cell line, 1321N1, and human embryonic kidney cell line, HEK 293, were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). DMEM (Dulbecco's modified Eagle's medium), FCS (foetal calf serum), penicillin-streptomycin and trypsin were from Gibco BRL (Paisley, UK). Human IL-1 β , and TNF α were purchased from R&D Systems Europe (Oxon, UK). The double-stranded oligonucleotide containing the HIV-LTR κ B site and T4 polynucleotide kinase were supplied by Promega (Madison, WI). (γ -³²P)-ATP was purchased from Amersham International (Amersham, Buckinghamshire, UK). PCR primers and oligonucleotides containing other κ B site sequences were obtained from MWG (Ebersberg, Germany). GeneJuice transfection reagent was from Novagen (Madison, WI). Primers and probes for quantitative real-time PCR analysis of I κ B β and 18S rRNA were supplied by Applied Biosystems (CA94404, U.S.A.). Rabbit polyclonal antibodies against I κ B α (sc-203), I κ B β (sc-945), p50 (H-119), p65 (sc-372), c-Rel (sc-70X), RNA Pol II (sc-899) and p89 (sc-293) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti- β -actin antibody was from Sigma (Dublin, Ireland). Affinity-purified rabbit polyclonal antiserum against I κ B ϵ was a gift from Nancy Rice (NCI-Frederick Cancer Research and Development Center, MD, USA).

Cell Culture - The human astrocytoma cell line, 1321N1, and the human embryonic kidney cell line, HEK 293, were cultured in DMEM supplemented with 100 U/ml of penicillin, 100 μ g/ml streptomycin and 10% (v/v) of FCS. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. All cells were passaged using 1% (w/v) of trypsin in PBS. Cytokine stimulation was performed on cells in serum-containing medium at 37°C for all experiments.

5'-RACE – RNA was isolated from 1321N1 astrocytoma using Tri-Reagent (Sigma). RNA ligase-mediated rapid amplification of 5' cDNA ends (RLM-RACE) was performed using the GeneRacer kit (Invitrogen, CA 92008) according to the manufacturer's instructions. Purified RACE products were TOPO cloned into pCR2.1-TOPO (Invitrogen) and sequenced to identify the 5' end of the transcripts.

Electrophoretic mobility shift assay – Double-stranded oligonucleotides containing the I κ B β , I κ B α - κ B₁ and I κ B α - κ B₂ NF κ B binding sites were created by annealing the oligonucleotides 5'-AGTTGAGGGGAATTTCCAGGC-3', 5'-AGTTGAGGGGAAATTTCCAGGC-3' and 5'-

AGTTGAGGGGAAACCCCCAGGC-3' to their respective complementary strands (putative κ B sites are underlined). Annealing was performed by incubating 100 pmoles of each strand in 10 mM Tris-HCl, pH 7.9, containing 2 mM $MgCl_2$, 50 mM NaCl, 20 mM EDTA at 90°C for 5 min. The incubation was cooled slowly to 50°C, maintained at 50°C for 5 min and then allowed to cool to room temperature. Double-stranded oligonucleotides were labeled with (γ - ^{32}P)-ATP (10 mCi/mmol) by T4 polynucleotide kinase [15]. In EMSA analysis, nuclear extracts (10 μ g of protein, generated as previously described [16]), were incubated with 30000 dpm of probe. Incubations were performed for 30 min at room temperature in 10 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 5 mM DTT, 4% (w/v) glycerol, 4 μ g of poly (dI-dC) and 1 mg/ml nuclease-free BSA. In the supershift analysis, polyclonal antibody (1 μ g) against the NF- κ B subunits p50, p65 or c-Rel, or non-immune IgG was added to the extracts and incubated for 30 min on ice prior to incubation with labeled oligonucleotide. In competition analysis, cold oligonucleotide was added to the extracts and incubated for 20 min at room temperature in a total volume of 25 μ l before probe addition. All incubations were subjected to electrophoresis on 4% nondenaturing polyacrylamide gels, which were subsequently dried and autoradiographed.

Promoter-reporter constructs and cell transfections – The HIV-LTR κ B-, β - κ B- and α - κ B₁-luciferase constructs consisted of 5 consecutive copies of the HIV-LTR, I κ B β or I κ B α - κ B₁ κ B sites cloned into XhoI/HindIII sites in the pGL3-Basic reporter plasmid (Promega). To generate “ β -Prom”, a 1 kb fragment of the I κ B β gene 5' flanking region was amplified from 1321N1 genomic DNA by PCR using the GC-Rich PCR System (Roche, Mannheim, Germany). The primers used were 5'-CCGCTCGAGCGGGATGTGGAACAAGTCCGTCTCTCTC-3' and 5'-CCCAAGCTTGGGTAAAGTTCACTCACGTGTCCCCATC-3' (restriction sites and clamps underlined). This fragment was also cloned into XhoI/HindIII sites in the pGL3-Basic firefly luciferase plasmid (Promega). For transfections using these constructs, HEK 293 cells (2×10^5 cells/ml) were plated into 24-well plates and allowed to grow for 24 h. Cells were then transfected using GeneJuice transfection reagent with constitutively expressed renilla-luciferase reporter construct (phRL-TK) (50 ng) and with promoter-firefly luciferase construct or empty pGL3-Basic vector (390 ng). Cells were allowed to recover overnight before treatment with IL-1 β or TNF α (10 ng/ml) for 6 h. Cell extracts were generated using the Reporter Lysis Buffer (Promega) and extracts were assayed for firefly and renilla luciferase activity using the Luciferase Assay system (Promega) and coelenterazine (Sigma, 1 μ g/ml), respectively.

Quantitative real-time PCR analysis of *IκBβ* expression – 1321N1 astrocytoma (2×10^5 cells/ml; 3 ml) were plated into 6-well plates and grown for 48 h. Cells were treated with IL-1 β (10 ng/ml) for various time periods. Cells were washed with PBS, and RNA was extracted using Tri-Reagent (Sigma). After DNase I digestion, cDNA was generated from normalised RNA using Superscript II Reverse Transcriptase. Samples were assayed by quantitative real-time PCR for levels of *IκBβ* cDNA using the ABI PRISM 7900HT thermal cycler. Reactions were performed using pre-validated primers and probes (Applied Biosystems).

p65 overexpression and Western Immunoblotting – HEK 293 cells (2×10^5 cells/ml; 3 ml) were plated into 6-well plates and allowed to grow for 24 h. Cells were transfected using GeneJuice transfection reagent with a p65 expression vector (p65-RFP) or with empty RFP vector (1 μ g). After 24 h, medium was removed and cells washed with ice-cold PBS. The cells were scraped into PBS (1 ml) and pelleted by centrifugation at 20000 *g* for 5 min at 4°C. After discarding the supernatant, cells were resuspended in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% glycerol, 50 mM DTT, 0.01% (w/v) bromophenol blue; 100 μ l per sample) and incubated on ice for 10 min. Resuspended cells were then boiled for 5 min. Cell debris was pelleted by further centrifugation at 20000 *g* for 10 min. The supernatant containing the cell extract was removed to fresh eppendorf tubes. Extracts were separated by SDS-PAGE using a 12% (w/v) resolving gel and proteins were transferred electrophoretically to nitrocellulose. Immunodetection of proteins was conducted as previously described [13]. Blots were blocked in 20 mM Tris HCl buffer, pH 7.5, containing 0.15 M NaCl (TBS) and 5% (w/v) powdered milk (Marvel). Incubations with primary antibodies against p65, *IκBα*, *IκBβ*, β -actin (all 1 μ g/ml) or *IκBε* (1:2000 dilution) were for 2 h at room temperature. Horseradish-peroxidase-conjugated sheep anti-rabbit IgG and horseradish-peroxidase-conjugated goat anti-mouse IgG (Upstate) were used at concentrations of 1 μ g/ml and 0.5 μ g/ml respectively. Immunoreactive bands were detected using the Enhanced Chemiluminescence (ECL) detection system from Pierce (UK) according to the instructions of the manufacturers.

Chromatin Immunoprecipitation - 1321N1 astrocytoma cells were grown to confluency in 90 mm dishes and stimulated with or without IL-1 β (10 ng/ml) for 1 h. Chromatin immunoprecipitation assays were performed as previously described with some modifications [17]. Following stimulation, cells were cross-linked with 1% formaldehyde for 10 min at 37°C. Isolated nuclei were subjected to seven

10 s sonication pulses from a Sanyo/MES Soniprep 150 at one-third of total power. Separate aliquots from each chromatin preparation were incubated overnight at 4°C with 2 µg anti-p65/Pol II antibody, or for 24 h with 8 µg anti-p89 antibody, and with non-immune rabbit IgG antibody. An aliquot was also retained as an input sample to normalise PCR reactions and analyse shearing efficiency. Chromatin used had an average size of 750 bp. After reversion of cross-links by overnight incubation at 65°C, DNA was extracted using the Qiaquick purification kit (Qiagen) according to the manufacturer's instructions.

Standard PCR was performed using 1 µl (~ 3% of total) template DNA, 500 nM primers and 0.2 U Taq DNA polymerase (Invitrogen) per 50 µl reaction. Quantitative real-time PCR reactions were performed in duplicate with 2 µl template DNA, 50 nM primers and the SYBR Green Jumpstart Taq Readymix (Sigma) in a total volume of 20 µl, using the Mx3000P QPCR System (Stratagene). Dissociation curve analysis and gel electrophoresis of the final products confirmed that only the expected specific amplicon of correct size was generated for each target promoter. Real Time PCR data analysis was performed as previously described [18]. Results for each treatment are expressed as fold differences between DNA enrichment in the p65/Pol II/p89-ChIP sample relative to IgG-ChIP sample. The sequences of primers used are as follows: IκBα Promoter 5'-GAAAGGACCGGCAGGTTGGCAAAC-3' and 5'-GGGTCAGGCTCGGGGAATTTC-3', IκBα Gene 5'-GTAGGATCAGCCCTCATTTTGTTC-3' and 5'-CTGTTACATGTCACAGGATACCACTG-3', IκBβ Promoter 5'-GGAACGGCTAGAGAGTTGTAGTCC-3' and 5'-CTTTGCCGGGAGTTCTGGAGCTTC-3', IκBβ Gene 5'-GATTCATTGCACGATGTCCAGTGTCTTC-3' and 5'-CTACTGTATGCTAGGCCTTGTGACCAC-3', IL-8 Promoter 5'-GGAAGTGTGATGACTCAGGTTTGC-3' and 5'-GATGGTTCCTTCCGGTGGTTTCTTC-3', IL-8 Gene 5'-CCAAGGGCCAAGAGAATATCCGAAC-3' and 5'-CTTCCACATGTCCTCACAACATCAC-3', IL-2 Promoter 5'-CTTGCTCTTGTCCACCACAATATGC-3' and 5'-CAAAGACTGACTGAATGGATGTAGGTG-3', GAPDH (glyceraldehyde-3-phosphate dehydrogenase) Promoter 5'-CTACTAGCGGTTTTACGGGCG-3' and 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'.

RESULTS

Identification of transcription start site of the I κ B β gene-

In order to study regulation of I κ B β transcription in human cells, we decided to characterise its promoter. However it was initially necessary to identify the TSS (transcription start site) of the I κ B β gene. We employed RNA Ligase Mediated - 5'-RACE to identify the site. This approach identified nine independent transcription start sites upstream of the translation start codon of the I κ B β gene (Fig.1). 5'-RACE is a highly sensitive technique, and the ligation of an RNA oligonucleotide to the 5' end of decapped RNA, and subsequent use of a primer specific for this 5' oligonucleotide in conjunction with a gene specific primer, ensures the amplification of only full-length transcripts. A range of transcription start sites, or "transcription window", is not unusual in TATA-less, GC-rich promoters. However the detection of numerous sites is in contrast to the murine I κ B β promoter, where a single major transcription start site was detected in an S1 nuclease protection assay [19].

The murine I κ B β gene 5' flanking region contains an NF κ B-binding site (κ B site) 35 nucleotides upstream of the first transcription start site. Analysis of the equivalent human region and alignment with the murine promoter confirmed the presence of a consensus κ B site in the human I κ B β gene 5' flanking region (Fig. 1). Between species there is variance at a single position – a change of C \rightarrow T, from mouse to human, at the third nucleotide from the 3' end of the decameric sequence. An interspecies comparison of eleven functional κ B sites has previously shown total conservation despite a divergence in the sequence of surrounding regions, indicating the importance of the *cis*-element sequence in conferring responsiveness to the appropriate *trans*-acting factor [20]. Nevertheless, given that the human I κ B β promoter κ B site retained the consensus sequence (5'-GGGRNNYYCC-3'), and that this site had been identified as a sequence capable of interacting with p65 and c-Rel in early studies on optimal NF κ B binding sites [21], we decided to examine the functional relevance of this site and probed its ability to interact with activated NF κ B in an *in vitro* assay.

NF κ B binds to the I κ B β promoter κ B site in an IL-1 β -dependent manner in vitro-

The binding of NF κ B to the I κ B β promoter κ B site was initially characterised using an *in vitro* assay. A range of oligonucleotides containing single κ B sites were radiolabeled for use in EMSA analysis. Two of these oligonucleotides contained known, functional κ B sites from the I κ B α promoter – designated α - κ B₁ and α - κ B₂ [22]. A third included the κ B site from the I κ B β promoter – β - κ B. Since

we were especially interested in examining the differential effects of IL-1 β on I κ B α and I κ B β expression in human astrocytes, 1321N1 astrocytoma were used as an accepted model astrocytic cell system in which IL-1 β is known to cause strong and sustained activation of NF κ B. Thus, the probes were individually incubated with nuclear extracts from 1321N1 cells treated with or without IL-1 β for 30 min. All three radiolabeled probes were found to bind activated NF κ B (Fig. 2A). Given the ability of the β - κ B site to bind NF κ B *in vitro*, additional EMSA analysis was conducted to compare the NF κ B binding to this site with that to the α - κ B₁ site. In a competition EMSA, effective competition of NF κ B binding to radiolabeled β - κ B probe was achieved by pre-incubating nuclear extracts from cells stimulated with IL-1 β for 30 min with increasing concentrations of unlabeled α - κ B₁ oligonucleotide (Fig. 2B). Similarly, unlabeled β - κ B oligonucleotide competed off binding of NF κ B to a radiolabeled α - κ B₁ probe. This indicates that the same form of NF κ B can bind to the β - κ B site and the α - κ B₁ site *in vitro*. In addition, activated NF κ B appears to display similar affinity for both κ B sites, as a similar concentration of one unlabeled oligonucleotide was required to eliminate binding to the other radiolabeled probe.

To further characterise NF κ B binding to the β - κ B site, nuclear extracts from IL-1 β -treated cells were pre-incubated with antisera against the Rel proteins p50, p65 and c-Rel prior to addition of radiolabeled β - κ B probe and subsequent electrophoresis. As shown above, IL-1 β induces the activation of NF κ B, observed here as two major complexes of similar electrophoretic mobility (Fig. 2C, lane 2). These IL-1 β -induced complexes were unaffected by pre-incubation with the non-immune IgG isotype control (lane 6). Complexes I and II were supershifted by pre-incubation with anti-p65 antiserum, with complex I disappearing and complex II substantially reduced in intensity (lane 4). Pre-incubation with anti-c-Rel antiserum also caused the appearance of a faint supershifted complex, although this was not accompanied by a significant decrease in the level of any IL-1 β -induced NF κ B-DNA complex (lane 5). This indicates that p65 was contained in both of the IL-1 β -induced NF κ B complexes that bound to the β - κ B site, while a complex containing c-Rel most likely constitutes a fraction of either complex I or II. No supershift was observed upon pre-incubation with anti-p50 antibody (lane 3). The ability of both the anti-p50 and anti-c-Rel antibodies to cause a supershift of NF κ B-DNA complexes was confirmed using a radiolabeled probe containing the HIV-LTR κ B site and nuclear extracts from 1321N1 astrocytoma stimulated in the presence and absence of IL-1 β (Figures 2D and E).

Interestingly, an identical profile of supershifted complexes was observed when EMSAs were performed with radiolabeled α - κ B₁ following pre-incubation with anti-p65 or anti-c-Rel antisera (lanes

7-12). It therefore appears that despite the difference in κ B site sequence, the α - κ B₁ and β - κ B sites bind the same range of NF κ B dimers *in vitro*. The Rel subunits p65 and c-Rel harbour substantial transactivation potential, arising from the presence of C-terminal transactivation domains TADs [23, 24]. This suggests that transcriptionally active complexes of NF κ B bind similarly to the κ B site of the I κ B α and I κ B β promoters and that any differences in IL-1 β -induced expression of I κ B α and I κ B β is not due to the differential binding of NF κ B to the two promoters. However, while the EMSA procedure offers information on the ability of NF κ B to bind to oligonucleotides *in vitro*, transcription factor interaction with *cis*-elements in the intact cell depends on chromatin structure and promoter accessibility. Therefore we next investigated whether NF κ B could bind to the I κ B α and I κ B β promoters *in vivo*.

p65 binds to the I κ B β promoter in vivo-

Chromatin immunoprecipitation studies were employed to examine whether IL-1 β -mediated NF κ B activation in human astrocytes led to the association of p65 with the I κ B β promoter *in vivo*. As NF κ B is known to mediate the induction of I κ B α following activation [9], binding to the I κ B α promoter was used as a positive control for this assay. 1321N1 astrocytoma were stimulated with or without IL-1 β for 1 h prior to immunoprecipitation of nuclear lysates with anti-p65 antisera. Intriguingly, analysis of immunoprecipitated DNA by PCR indicated that p65 was present at both the I κ B α and I κ B β promoters 1 h post-IL-1 β stimulation (Fig. 3A). This was corroborated by real-time PCR analysis of immunoprecipitated DNA, where statistically significant levels of p65 binding to both promoters were recorded in response to IL-1 β (Fig. 3B). The specificity of I κ B α and I κ B β promoter enrichment was confirmed by the absence of GAPDH promoter in p65 immunoprecipitates. In addition, p65 binding was not detected at the IL-2 promoter by standard or real-time PCR of immunoprecipitated DNA. The IL-2 gene is regulated by NF κ B, but is only expressed in T-cells. Therefore, the appearance of p65 at a promoter in response to IL-1 β is not a general phenomenon at all promoters containing κ B sites, but rather is specific to a subset, including the I κ B β promoter. As IL-1 β stimulation induced the binding of NF κ B to the κ B site of the I κ B β promoter *in vitro*, and resulted in p65 engagement of the I κ B β promoter *in vivo*, we next assessed the ability of the human I κ B β κ B site to confer inducibility to IL-1 β in functional assays.

The β - κ B site confers low inducibility to IL-1 β in transient transfection assays-

Promoter-reporter constructs containing five consecutive β - κ B or α - κ B₁ sites upstream of a luciferase reporter gene (β - κ B-luciferase and α - κ B₁-luciferase) were generated as described in Materials and Methods. HEK 293 cells transfected with one of these constructs or empty pGL3-Basic vector were treated with or without IL-1 β for 6 h before assessing reporter gene activity. IL-1 β stimulation caused a 10-fold induction of α - κ B₁-luciferase expression. In contrast, only 2-fold induction of β - κ B-luciferase expression was observed upon IL-1 β treatment (Fig. 4A). Together with the EMSA analysis this data suggests that while both the β - κ B and α - κ B₁ sites bind identical forms of NF κ B, stronger transactivation is seen from α - κ B₁-bound NF κ B. The reason for the high basal level of β - κ B-luciferase activity relative to α - κ B₁-luciferase is presently unknown. However, EMSA analysis detected an unidentified complex binding to the β - κ B, but not α - κ B₁, probe, which was unaffected by IL-1 β treatment or pre-incubation with any anti-Rel subunit antisera (Fig. 2C, denoted by “*”). While sequence analysis did not reveal the presence of any known *cis*-elements within the β - κ B site that were absent from the α - κ B₁ site, it is possible that this complex represents a factor involved in stimulating basal levels of reporter gene activity.

In order to determine whether the modest IL-1 β -mediated inducibility seen with the β - κ B-luciferase construct was retained within the context of the intact promoter, we next cloned the human I κ B β 5' flanking region. The I κ B β gene had previously been assigned to chromosome band 19q13 by fluorescence in situ hybridisation [25]. A search of the NCBI database using the I κ B β cDNA sequence identified a contig (CTC-360G5) containing the I κ B β gene. A ~1 kb fragment of the 5' flanking region was amplified by PCR and cloned into the pGL3 basic vector upstream of the luciferase gene as described in Materials and Methods. HEK 293 cells transfected with the I κ B β -promoter-reporter (β -Prom) or empty pGL3-Basic vector were stimulated in the presence or absence of IL-1 β or TNF α . β -Prom displayed a high level of basal activity, approximately 20-fold over empty vector (Fig. 4B). Treatment with IL-1 β and TNF α , another pro-inflammatory cytokine known to activate NF κ B, caused a 1.3-fold and 1.5-fold induction of β -Prom activity respectively. In order to verify the responsiveness of HEK 293s to these cytokines, cells were transfected with a promoter-reporter construct containing a luciferase gene immediately downstream of five tandemly repeated κ B sites from the HIV long terminal repeat (HIV-LTR κ B-luc). IL-1 β and TNF induced a 9-fold and 13-fold induction of reporter gene activity, respectively.

Thus, transient transfections using β - κ B-luciferase and the I κ B β -promoter-reporter indicated that NF κ B activation does not lead to substantial transactivation at the I κ B β promoter. As these constructs constitute artificial extrachromosomal templates, we also examined the effect of NF κ B activity on endogenous I κ B β expression at the level of both mRNA and protein.

Activation of NF κ B does not induce endogenous I κ B β gene expression-

To examine whether I κ B β gene expression was induced by NF κ B at the transcriptional level, 1321N1 astrocytoma were treated with or without IL-1 β , a strong activator of NF κ B, for various time periods. mRNA levels were then quantitated by real-time PCR. No significant up-regulation of I κ B β mRNA was recorded following IL-1 β stimulation for 3, 8 or 24 h (data not shown). This confirms our earlier Northern Blot analysis of I κ B β transcript levels in response to activators of NF κ B [8, 13]. As a positive control, IL-8 mRNA levels were also monitored by quantitative real-time PCR, with a robust upregulation of mRNA expression observed in response to IL-1 β (data not shown). The effect of NF κ B activation on I κ B β protein levels was also assessed. p65 was overexpressed in HEK 293 cells, before monitoring levels of I κ B α , I κ B β and another I κ B family member, I κ B ϵ , by Western immunoblotting (Fig. 5). The expression of I κ B α and I κ B ϵ is known to be up-regulated by NF κ B [13], and this was reflected by increased levels of these proteins detected in cells expressing the p65-RFP fusion protein. However, levels of I κ B β were equivalent in cells transfected with and without the p65 expression vector. Together this data demonstrates that expression of endogenous I κ B β is not increased by activation of NF κ B, despite the binding of p65 to the I κ B β promoter in an IL-1 β -dependent manner.

As shown above, p65 was found to be present at the I κ B β promoter following 1 h stimulation with IL-1 β . The I κ B β mRNA 3' UTR (untranslated region) does not contain an AU-rich sequence. This indicates that the transcript is not subject to rapid turnover and, were mRNA levels to be up-regulated, this would most likely be detectable at 3 h. In addition, previous Northern blots performed by this group have demonstrated the absence of an I κ B β mRNA induction after 1 h IL-1 β treatment [13]. The question thus remained as to how binding of p65 at the I κ B β promoter failed to lead to transcriptional activation. To address this issue, we returned to chromatin immunoprecipitation analysis of the I κ B β promoter, monitoring the recruitment of a critical component of the transcriptional machinery – RNA polymerase II.

p65 binding to the I κ B β promoter results in relatively inefficient recruitment and stalling of RNA polymerase II at the promoter-

Transcriptional activators operate by binding to a gene promoter and increasing the rate of transcriptional initiation. Following site-specific DNA binding, p65 can recruit components of the basal transcription apparatus either directly or indirectly, via coactivators such as CBP/p300 [26-28]. Following initiation of transcription, RNA polymerase II moves along the gene in the elongation phase. The presence or absence of RNA polymerase II at a gene promoter, and within the gene at sites distant from the TSS, provides a picture of the transcriptional status of that gene. We chose three target genes in which to monitor RNA polymerase II activity – I κ B α , I κ B β and IL-8. p65 binds to the promoters of all three targets in an IL-1 β - dependent manner (Fig. 3 and [16]).

As for p65-ChIP analysis, 1321N1 astrocytoma cells were treated with or without IL-1 β for 1 h. Following anti-pol II immunoprecipitation of nuclear lysates, immunoprecipitated DNA was analysed for the presence of target sequences by standard and quantitative real-time PCR (Fig. 6). For each gene, amplification of two target sequences was performed. The first was located in the promoter, just upstream of the TSS. The second was at least 2000 bp downstream from the TSS. As the average size of sonicated chromatin was 750 bp, this second target was considered to be sufficiently far from the TSS to avoid false positive results, otherwise attainable by the immunoprecipitation of RNA polymerase II stalled at the upstream promoter. While absent from the IL-8 promoter in the basal state, RNA polymerase II binding occurred following IL-1 β stimulation. Furthermore, the enzyme was detected downstream in the IL-8 gene, in response to IL-1 β , demonstrating the effectiveness of pol II-ChIP as a method to study IL-1 β -induced changes in transcriptional activity at target genes. Some RNA polymerase II was present at the I κ B α promoter in the basal state, but an increase in binding was observed in response to IL-1 β , and this increase was deemed statistically significant by analysis of quantitative real-time PCR results. In addition, examination of a downstream region of the I κ B α gene showed that active transcription is in progress 1 h post-IL-1 β stimulation.

As shown earlier, IL-1 β induces binding of p65 to the I κ B β promoter *in vivo*, but this is not accompanied by an upregulation of gene expression. Pol II-ChIP showed that RNA polymerase II is found at the I κ B β promoter in unstimulated cells. IL-1 β treatment tended to cause some further recruitment of the enzyme. While quantitative real-time PCR indicated that the increase was ~2-fold, it was not found to be statistically significant at $p < 0.05$. No RNA polymerase II was detected downstream within the I κ B β gene sequence in the presence or absence of IL-1 β . This indicates that while IL-1 β induces the binding of NF κ B to the I κ B β promoter, p65 is relatively inefficient at

recruiting RNA polymerase II to the TSS, and furthermore, the recruited polymerase stalls at the promoter.

p65 binding to the $I\kappa B\beta$ promoter is not followed by TFIIH recruitment-

In the stepwise assembly model of transcriptional initiation, pre-initiation complex formation is completed by the loading of TFIIH [29]. This multi-subunit general transcription factor possesses a DNA helicase and a kinase activity, the latter of which phosphorylates the evolutionarily conserved heptapeptide repeat in the CTD (carboxy-terminal domain) of RNA polymerase II. This modification appears necessary for transcriptional initiation, possibly by inducing a conformational change that allows the polymerase to disengage from the pre-initiation complex [30]. In order to probe the mechanism underlying the failure of $I\kappa B\beta$ promoter-bound RNA polymerase II to initiate transcription, ChIP assays were performed to assess the genomic location of p89, the largest TFIIH subunit, following IL-1 β treatment of 1321N1 astrocytoma. Standard and real-time PCR of p89-immunoprecipitated DNA demonstrated that IL-1 β caused loading of p89 on the IL-8 promoter, with real-time PCR recording a 9-fold induction of binding. However, no binding was detectable at the $I\kappa B\beta$ promoter (Fig. 7A & B). This strongly suggests that the absence of $I\kappa B\beta$ promoter firing in response to NF κ B activation stems from the failure of DNA-bound p65 to efficiently recruit RNA polymerase II and to directly or indirectly engage TFIIH, with a resulting lack of RNA polymerase II CTD phosphorylation and promoter clearance. This is a highly credible mechanism underlying the non-inducibility of the $I\kappa B\beta$ gene by NF κ B, and thus IL-1 β , in human astrocytes.

DISCUSSION

The activity of transcription factors must be tightly marshalled to avoid inappropriate gene expression. I κ B proteins are charged with regulating NF κ B activity. A notable disparity in the regulation of I κ B α and I κ B β expression is the strong induction of the former, but not the latter, in response to NF κ B activation. As functional compensation by I κ B β did not occur in I κ B α ^{-/-} mice, it has been proposed that it is the upregulation of I κ B α , but not I κ B β , following activation of NF κ B that underlies the difference in activity of the two I κ Bs. In a study of the murine I κ B β promoter, Budde *et al* identified a κ B site upstream of the TSS and demonstrated that NF κ B could bind to the site and cause modest transcriptional activation [14]. The human I κ B β promoter has a κ B site in a similar position. We examined the human I κ B β κ B site at the level of NF κ B binding, and assessed the functional consequences of NF κ B activation on I κ B β promoter activity. We were especially interested in understanding the role of NF κ B in the differential induction of I κ B α and I κ B β by IL-1 β in human astrocytes, a situation that leads to sustained activation of NF κ B and likely chronic neuroinflammation.

EMSA analysis demonstrated that NF κ B, activated in response to IL-1 β , could interact with the I κ B β - κ B site in addition to two functionally competent κ B sites from the I κ B α promoter. The consistent electrophoretic mobility of the NF κ B-DNA complexes detected with different probes indicated that a similar form of NF κ B was mediating binding in each case. This likelihood was strengthened through competition EMSAs, where the NF κ B complex was found to bind to both the β - κ B and α - κ B₁ probes with equal affinity. Further support came from the immunodetection of the same Rel subunits in NF κ B complexes bound to both sites *in vitro*. p65 was found to be contained in all IL-1 β -induced NF κ B complexes, with the presence of c-Rel also apparent, though to a lesser extent. Thus, EMSA analysis showed that a form of NF κ B with substantial transactivation potential can bind to the I κ B β promoter κ B site *in vitro*. ChIP analysis was performed to investigate whether this interaction had any relevance in living cells. p65 was chosen as the target Rel subunit since it had been identified in the NF κ B complexes as the predominant subunit to interact with both the β - κ B and α - κ B₁ probes *in vitro*. Furthermore, its presence at a promoter could point towards an upregulation of transcriptional activity. Interestingly, like the I κ B α promoter, p65 was detected at the I κ B β promoter in an IL-1 β -dependent manner.

Previous studies have suggested that NF κ B activation has, at best, a modest effect on I κ B β expression. Nevertheless, given the binding of p65 to the I κ B β promoter induced by IL-1 β , we

examined the effect of NF κ B activation on I κ B β promoter activity in transient transfection assays, where it was shown to confer only a low level of inducibility. The effect of NF κ B activation on endogenous I κ B β gene expression was then assessed at the level of both mRNA and protein, where no upregulation was observed. This indicated that the binding of p65 to the I κ B β promoter was insufficient to cause transcriptional activation of the gene.

p65 contains two independent TADs in its C-terminal portion [31]. Through these TADs, p65 can interact with coactivators and components of the basal transcription apparatus [27, 28]. p65 has in fact been shown to interact directly with general transcription factors, thereby increasing the rate at which transcription is initiated at the relevant promoter [26]. ChIP studies with anti-RNA polymerase II allowed us to monitor the recruitment of the enzyme to the I κ B β , I κ B α and IL-8 promoters. In addition, by selecting a second target sequence downstream of the promoter, within which to probe for the presence of RNA polymerase II, it was possible to ascertain whether the gene was being actively transcribed after 1 h IL-1 β . IL-8 is known to be strongly induced by IL-1 β , while not being expressed in basal state. Indeed, a major recruitment of RNA polymerase II to the IL-8 promoter was recorded in response to IL-1 β . As the enzyme was also detected within the IL-8 gene sequence in an IL-1 β -dependent manner, this appeared to be followed by transcriptional activity. While RNA polymerase II was detected at the I κ B α promoter in unstimulated cells, further enzyme was recruited in response to IL-1 β and, as for IL-8, RNA polymerase II-mediated transcription resulted. RNA polymerase II was also detected at the I κ B β promoter in the basal state. While IL-1 β appeared to cause some further engagement, no RNA polymerase II was detected downstream within the I κ B β gene sequence, in the presence or absence of IL-1 β stimulation. It should be noted that the target sequences in both the I κ B α and IL-8 genes were within exons, while the I κ B β gene target was within intronic DNA. It has been suggested that RNA polymerase II accumulates at exons during transcription, making coding DNA a preferable target in pol II-ChIP [32, 33]. However, technical difficulties precluded the use of a sequence within the sufficiently large exons 3 or 5 in the I κ B β gene. The target sequence used was just 5' of exon 2. As the average chromatin size was 750 bp, it was expected that pol II – immunoprecipitated DNA would have been enriched for this sequence, were the enzyme present in exon 2 during transcription.

p65 can therefore bind to the I κ B β promoter in response to IL-1 β , though this does not result in efficient recruitment of RNA polymerase II. In addition, the modest levels of recruited polymerase appear to stall at the promoter. As promoter clearance requires the phosphorylation of serine 5 of the RNA polymerase II CTD heptapeptide sequence by the Cdk7 subunit of TFIIF [30, 34], we finally

investigated whether this general transcription factor was recruited to the I κ B β promoter post-IL-1 β stimulation. However, unlike the κ B-responsive IL-8 promoter, p65 binding to the I κ B β promoter was not followed by loading of TFIID. A mechanism may thus be proposed whereby p65 binding to the I κ B β promoter causes a slight recruitment of RNA polymerase II but fails to facilitate TFIID loading, with a resulting absence of transcriptional initiation and gene expression.

The failure of I κ B β promoter-bound p65 to interact efficiently with the basal transcription apparatus, either directly or indirectly through coactivators, may occur for various reasons. NF κ B can bind to a range of κ B site sequences and it has been proposed that the nucleotide sequence of a κ B site dictates the conformation of the bound NF κ B dimer. This allosteric role for DNA has a precedent in the binding characteristics of the nuclear receptor protein family [35]. The NF κ B conformation induced by the κ B site has functional consequences. An alteration of the site sequence can impact transcriptional activation mediated by bound NF κ B, most likely by modifying the ability of NF κ B to interact with other *trans*-acting factors or with coactivators [20, 36]. Thus, an induced conformation that does not favour recruitment of coactivators or basal transcription factors is one possible explanation for the failure of I κ B β promoter-bound p65 to activate transcription. However, an identical decameric κ B site supports transactivation in the MIP (macrophage inflammatory protein)-2 and MCP (monocyte chemoattractant protein)-1 promoters [37, 38]. Furthermore, in the case of the MCP-1 promoter, p65/p65 homodimers and p65/c-Rel heterodimers, the Rel subunits found to bind to the I κ B β promoter κ B site *in vitro*, display the ability to activate transcription through this site [37]. However, it should be noted that the sequence flanking the κ B site also influences the conformation of NF κ B-DNA complexes, as the structure of DNA is sequence-dependent [39-41]. As the flanking sequences differ between MCP-1 and I κ B β , this could lead to the same NF κ B complex binding to identical decameric κ B site sequences, but assuming different conformations due to the divergence in κ B site DNA structure imposed by surrounding bases.

The characterisation of many promoters in which NF κ B is known to play an important role in transcriptional activation has revealed the requirement for numerous other *trans*-acting factors and coactivators, combining to form a gene-specific enhanceosome [42, 43]. p65 binding to the I κ B β promoter may provide just one part of the combinatorial complex necessary for gene expression. Other signals may be provided in a stimulus- and cell-type specific manner. In addition, post-translational modifications of p65 have been shown to regulate interactions with coactivators. Such modifications

are stimulus-dependent and may even occur in a promoter-specific manner, highlighting the complexities of gene regulation at the level of the promoter [44, 45].

The failure of NF κ B activation to elicit a substantial increase in I κ B β gene expression has previously been demonstrated [8, 13]. Here the model is advanced to show that NF κ B can in fact bind to the I κ B β promoter, but that this doesn't trigger efficient general transcription factor or RNA polymerase II recruitment or promoter firing. The present findings also provide a potential mechanistic understanding of the basis for sustained activation of NF κ B in some systems. We have previously shown that IL-1 β causes sustained activation of NF κ B in human astrocytes and this is likely to play a key role in promoting chronic inflammation in the brain [46]. Such sustained activation coincides with strong induction of I κ B α , but lack of re-synthesis of I κ B β , suggesting the latter to be a key regulator of NF κ B in this system. We now define the molecular basis to the differential effects of IL-1 β on I κ B α and I κ B β expression in human astrocytes, in that IL-1 β fails to stimulate efficient recruitment or processing of the basal transcriptional machinery at the I κ B β promoter. The delineation of mechanisms that increase such efficiency may be of great value in upregulating I κ B β expression and controlling chronic activation of NF κ B in the brain.

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FIGURE LEGENDS

Figure 1: Nucleotide sequence of the I κ B β gene 5' flanking region.

A number of transcription start sites for the I κ B β gene were identified by 5'-RACE and are denoted by bold capitals. The most 5' site identified is designated +1. The translation start codon is in bold type and is underlined. A putative κ B site is located 5' of the transcription window.

Figure 2: NF κ B binds to the κ B site of the I κ B β promoter *in vitro*.

1321N1 astrocytoma were treated with or without IL-1 β (10 ng/ml) for 30 min before examining NF κ B DNA-binding activity by EMSA. (A) Nuclear Extracts (10 μ g protein) were incubated with radiolabeled oligonucleotides containing the I κ B β , I κ B α - κ B₁ and I κ B α - κ B₂ sites. (B) Nuclear extracts (10 μ g protein) were incubated with increasing amounts of cold I κ B α - κ B₁ oligonucleotide (0.07–1.75 pmoles) or with an excess of cold I κ B β - κ B oligonucleotide (1.75 pmoles) before addition of radiolabeled I κ B β - κ B probe. Also, nuclear extracts were incubated with increasing amounts of cold I κ B β - κ B oligonucleotide (0.07–1.75 pmoles) or with an excess of cold I κ B α - κ B₁ oligonucleotide (1.75 pmoles) before adding radiolabeled I κ B α - κ B₁ probe. (C) Nuclear extracts were pre-incubated with anti-p50, anti-p65, anti-c-Rel or non-immune (IgG) antibody. (D-E) Nuclear extracts were pre-incubated with non-immune (IgG) and anti-p50 (D) or anti-c-Rel (E) or antibody before addition of radiolabeled probe containing the HIV-LTR κ B site. The arrows indicate the mobility of supershifted complexes.

Figure 3: IL-1 β induces p65 binding to the I κ B β gene promoter *in vivo*.

1321N1 astrocytoma were treated with or without IL-1 β (10 ng/ml) for 1 h. Nuclear lysates were generated and immunoprecipitated with anti-p65 or control IgG antibodies. Immunoprecipitated DNA was analysed for I κ B α , I κ B β , IL-2, and GAPDH promoters by PCR and gel electrophoresis (A) and I κ B α , I κ B β and IL-2 promoters by quantitative real-time PCR (B). Data from real-time PCR is expressed as fold differences between DNA enrichment in the anti-p65 ChIP sample relative to IgG ChIP sample and is displayed as mean \pm S.E.M. of 3-5 independent experiments. * $p < 0.05$, ** $p < 0.01$ versus unstimulated control (unpaired t-test).

Figure 4: NF κ B causes strong activation of the I κ B α promoter but weak activation of the I κ B β promoter in 1321N1 cells.

(A) HEK 293 cells were co-transfected with α - κ B₁-luciferase, β - κ B-luciferase or empty pGL3 Basic vector (EV), as well as phRL-TK (constitutively expressed *Renilla* luciferase). After overnight recovery, cells were stimulated in the presence or absence of IL-1 β (10 ng/ml) for 6 h. (B) HEK 293 cells were co-transfected with β -Prom, HIV-LTR κ B-luc or empty pGL3 Basic vector (EV), and phRL-TK. After overnight recovery, cells were stimulated in the presence or absence of IL-1 β or TNF α (10 ng/ml) for 6 h. Cell lysates were assayed for firefly and *Renilla* luciferase. Data are presented relative to untreated cells transfected with empty vector. Results represent mean \pm S.E.M. of three independent experiments.

Figure 5: NF κ B causes strong induction of endogenous I κ B α but fails to up-regulate endogenous I κ B β gene expression in HEK 293 cells.

HEK 293 cells were transfected with a plasmid expressing a p65-RFP fusion protein or empty RFP vector. Cell lysates were generated after 24 h and subjected to SDS-PAGE and Western immunoblotting to measure levels of p65, I κ B α , I κ B β , I κ B ϵ and β -actin. These results are representative of three independent experiments.

Figure 6: Stimulation of 1321N1 cells with IL-1 results in relatively inefficient recruitment and stalling of RNA polymerase II at the I κ B β promoter.

1321N1 astrocytoma were stimulated in the presence or absence of IL-1 β (10 ng/ml) for 1 h. Nuclear lysates were generated and immunoprecipitated with anti-Pol II or control IgG antibodies. Immunoprecipitated DNA was analysed for IL-8, I κ B α , and I κ B β promoter and gene sequences by PCR and gel electrophoresis (A) and by quantitative real-time PCR analysis (B). Data from real-time PCR is expressed as fold differences between DNA enrichment in the anti-Pol II ChIP sample relative to IgG ChIP sample and is displayed as mean \pm S.E.M. of three independent experiments. * $p < 0.05$, ** $p < 0.01$ versus unstimulated control (unpaired t-test).

Figure 7: TFIID is not recruited to the I κ B β promoter in response to IL-1 stimulation of 1321N1 cells.

1321N1 astrocytoma were stimulated in the presence or absence of IL-1 β (10 ng/ml) for 1 h. Nuclear lysates were generated and immunoprecipitated with anti-p89 or control IgG antibodies.

Immunoprecipitated DNA was analysed for IL-8 and I κ B β promoter sequences by PCR and gel electrophoresis (A) and by quantitative real-time PCR analysis (B). Data from real-time PCR is expressed as fold differences between DNA enrichment in the anti-p89 ChIP sample relative to IgG ChIP sample and is displayed as mean \pm S.E.M. of 3-4 independent experiments. * ** $p < 0.001$ versus unstimulated control (unpaired t-test).

Figure 1

```

-150  gcccgga aaa  tgtgggaacg gctagagagt ttagtcctc ccgaatactc

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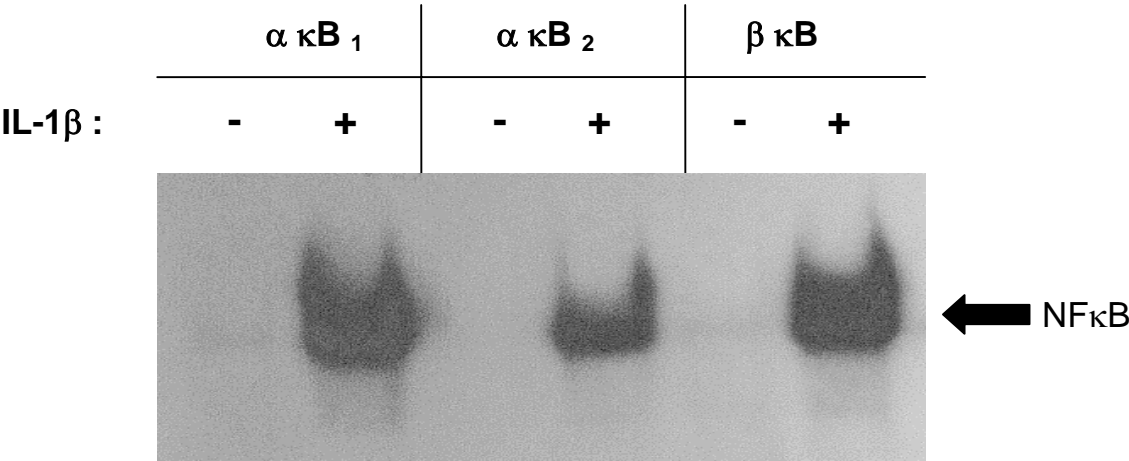
                                κB
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+51   gCcCctgagg cggcggggGc cATGgctggg gtcgcgtgct tgggaaaagc
    
```

Figure 2

(A)



(B)

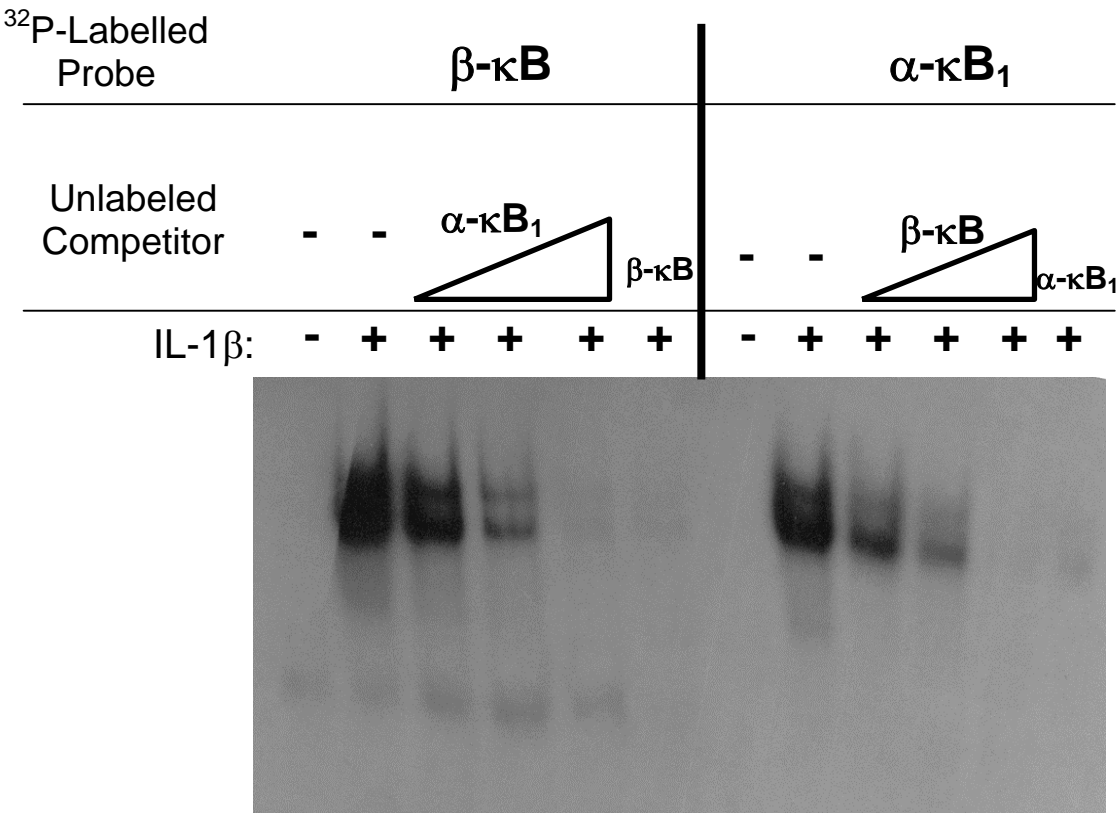


Figure 2

(C)

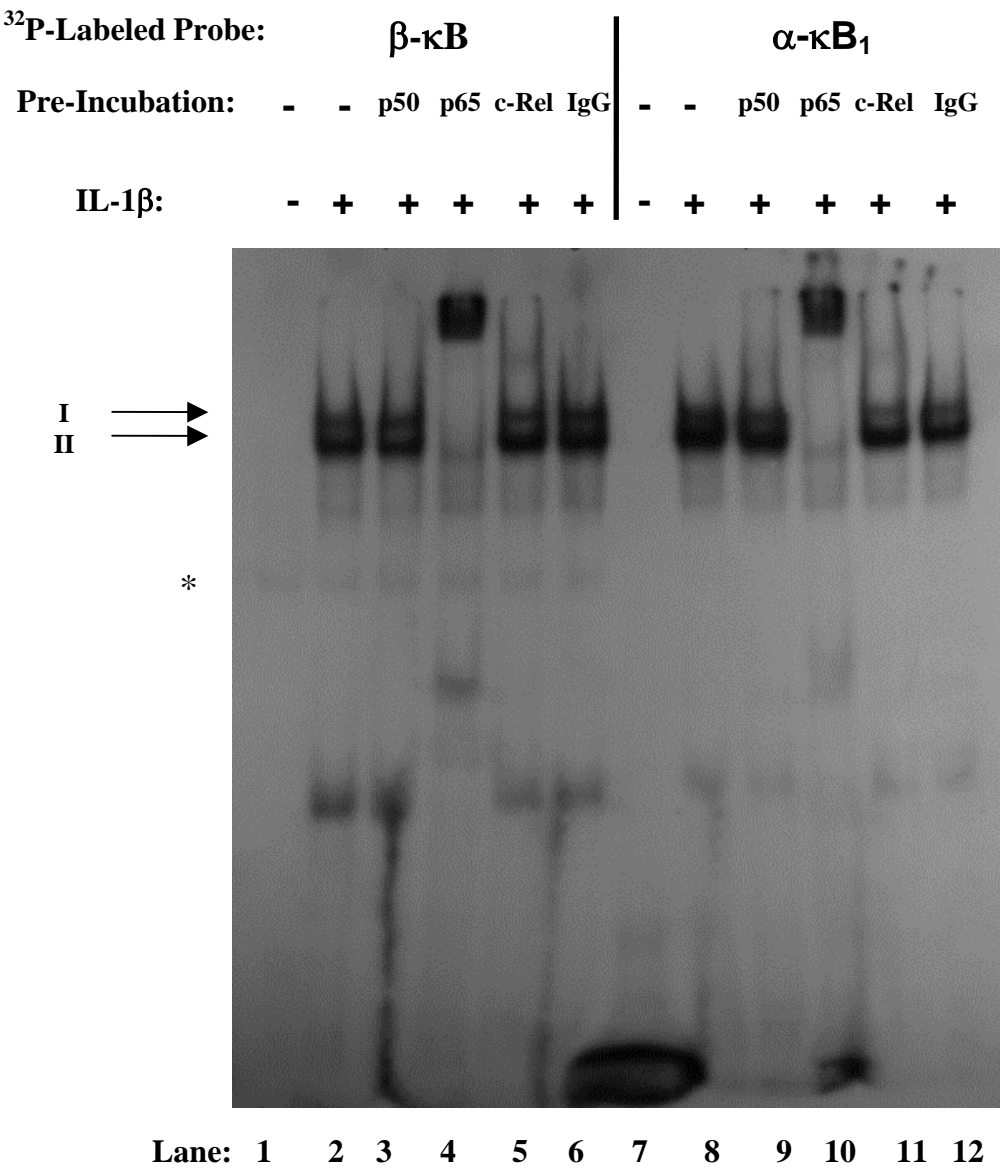
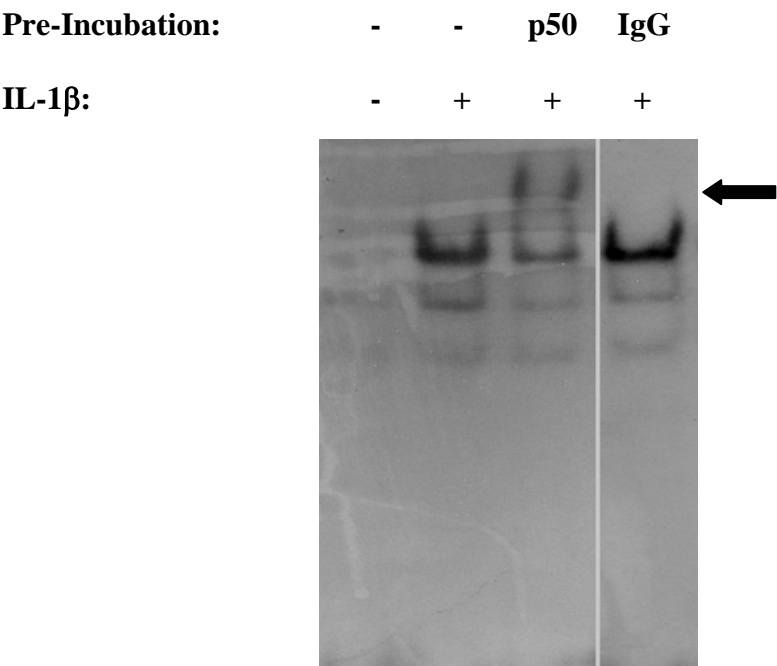


Figure 2

(D)



(E)

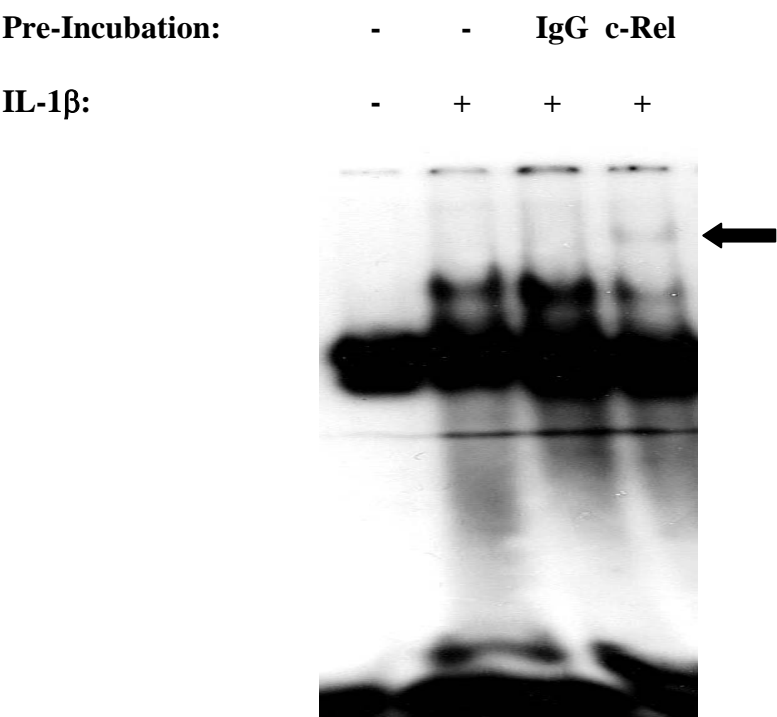


Figure 3

(A)

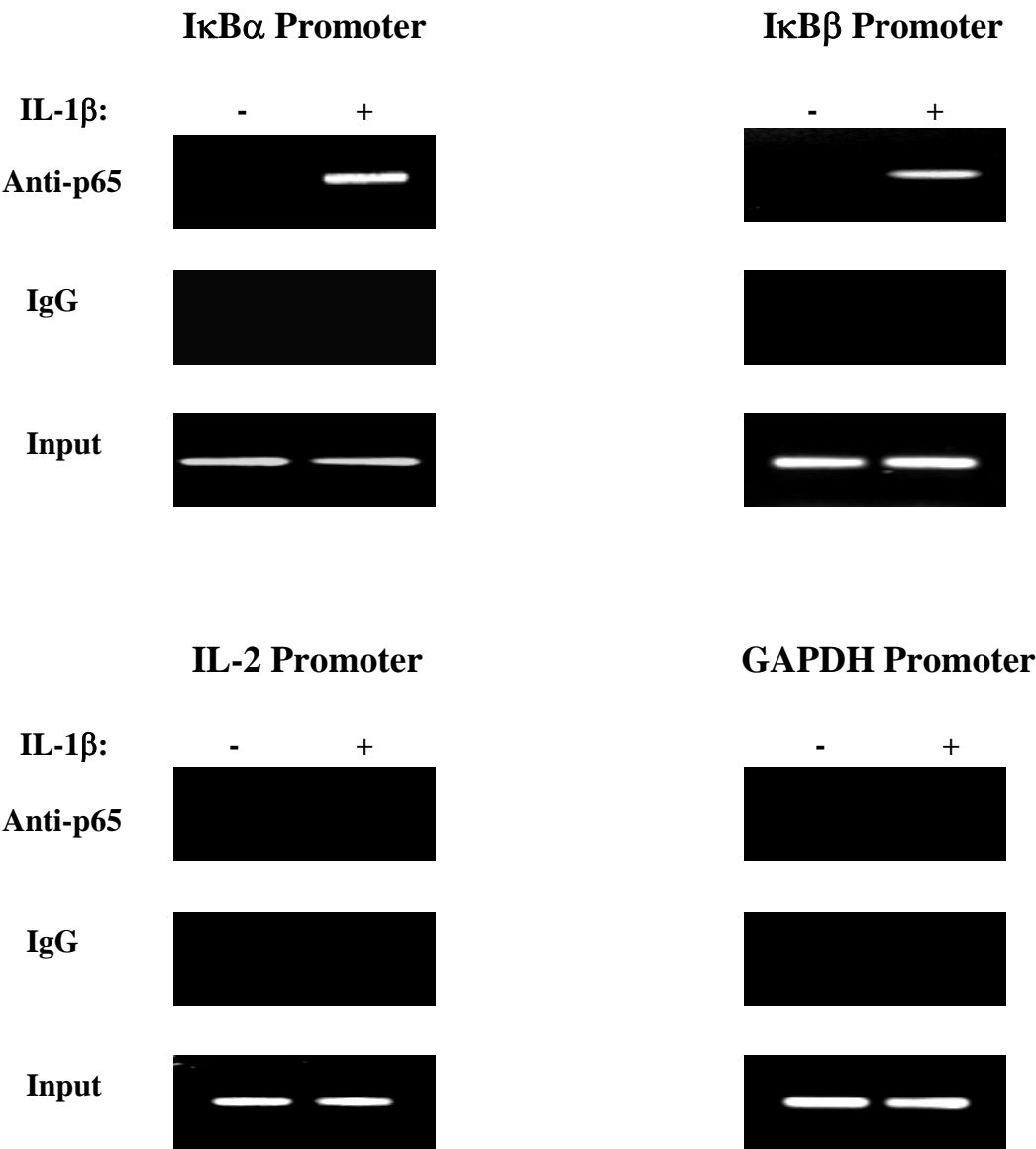


Figure 3

(B)

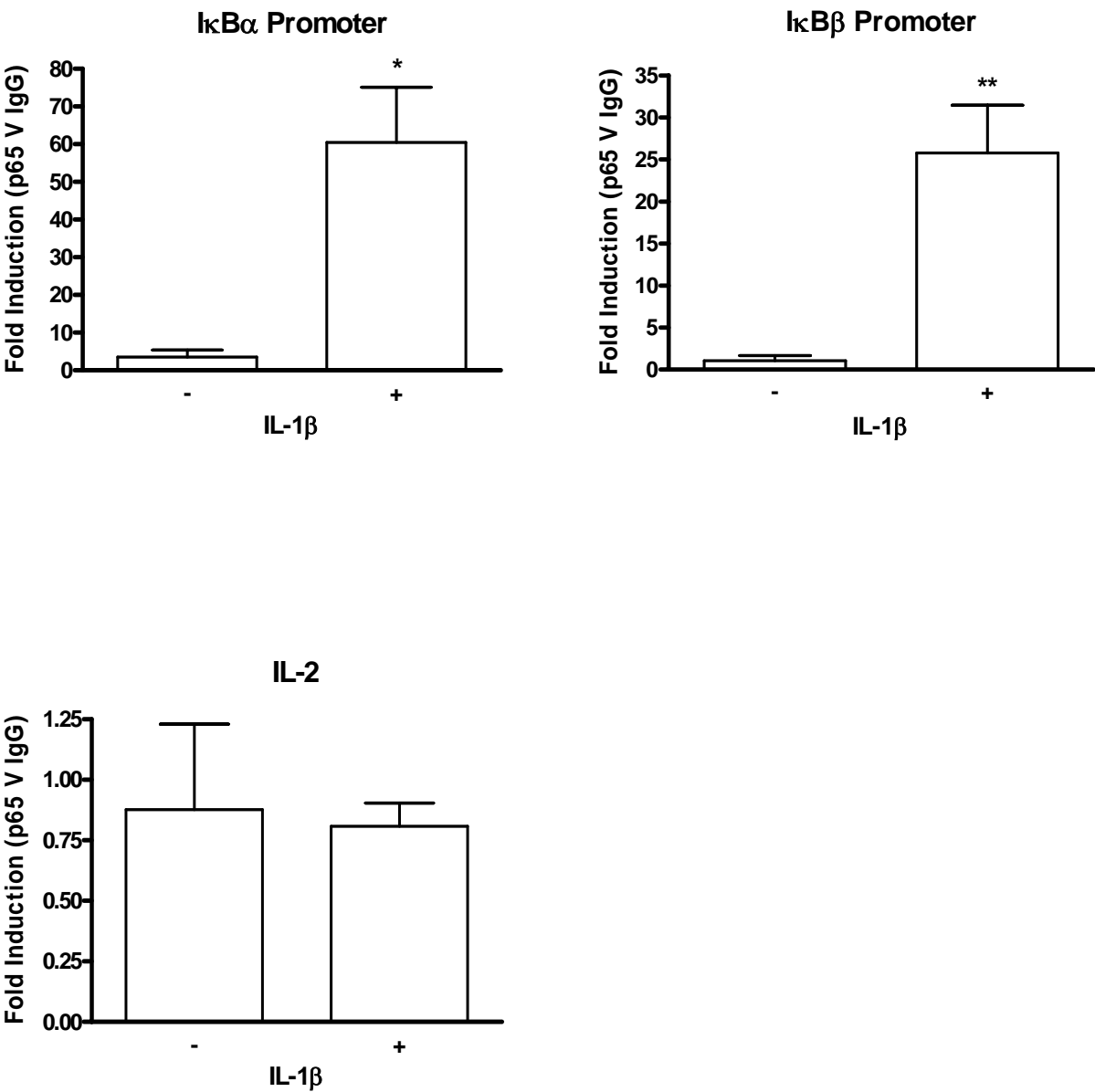
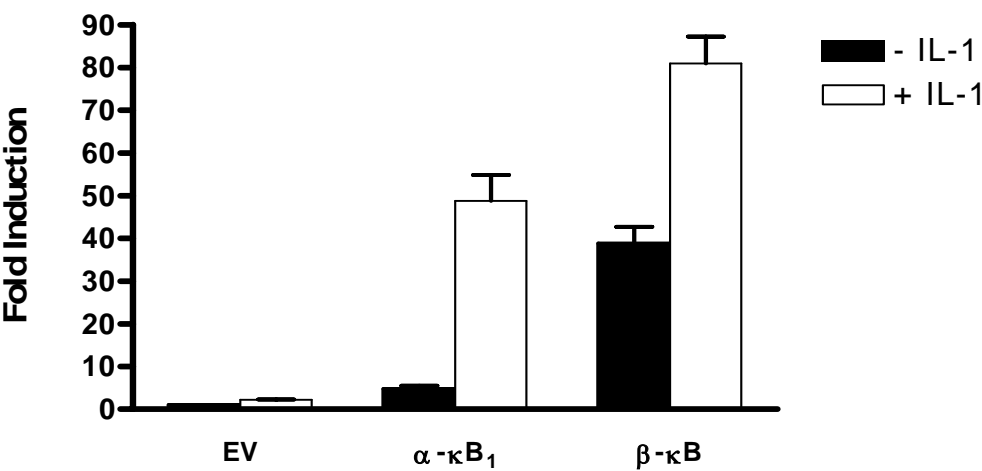


Figure 4

(A)



(B)

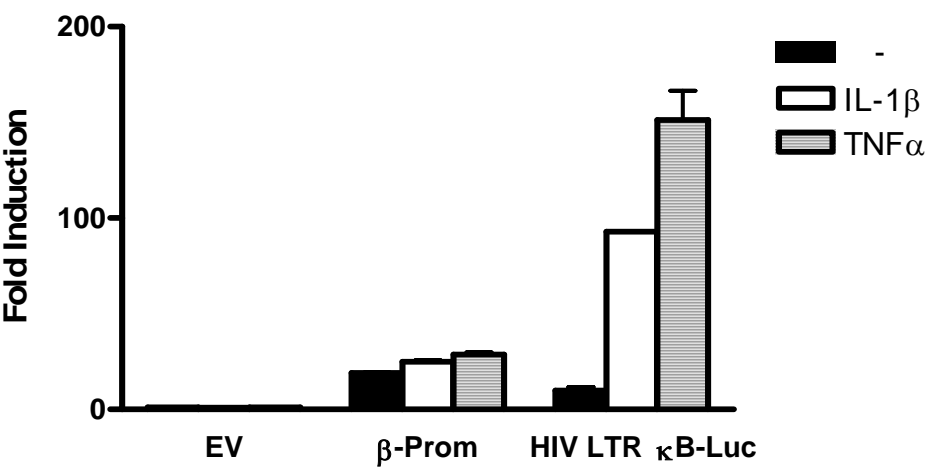


Figure 5

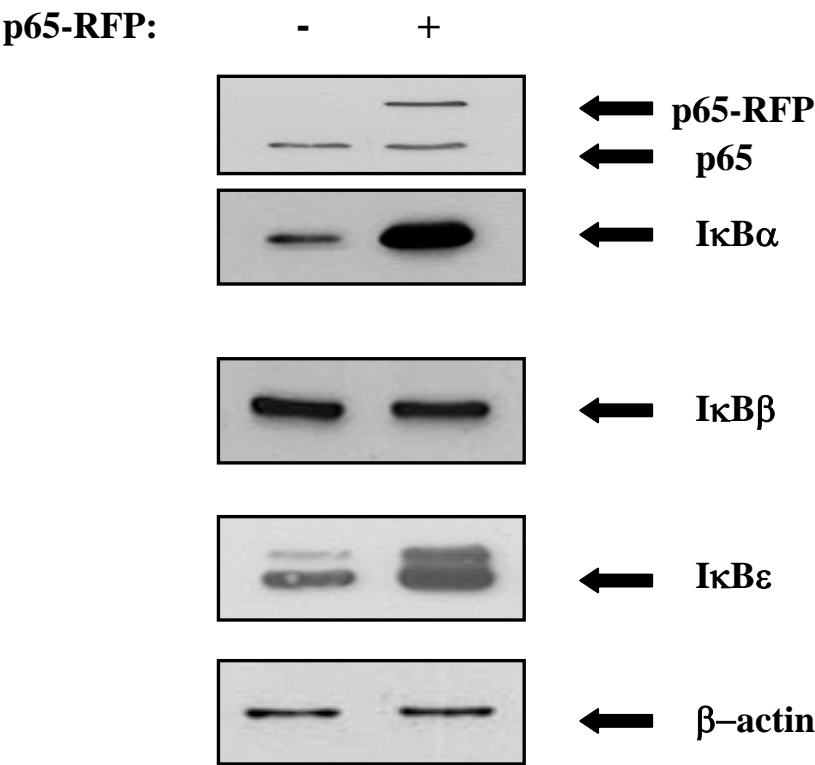


Figure 6

(A)

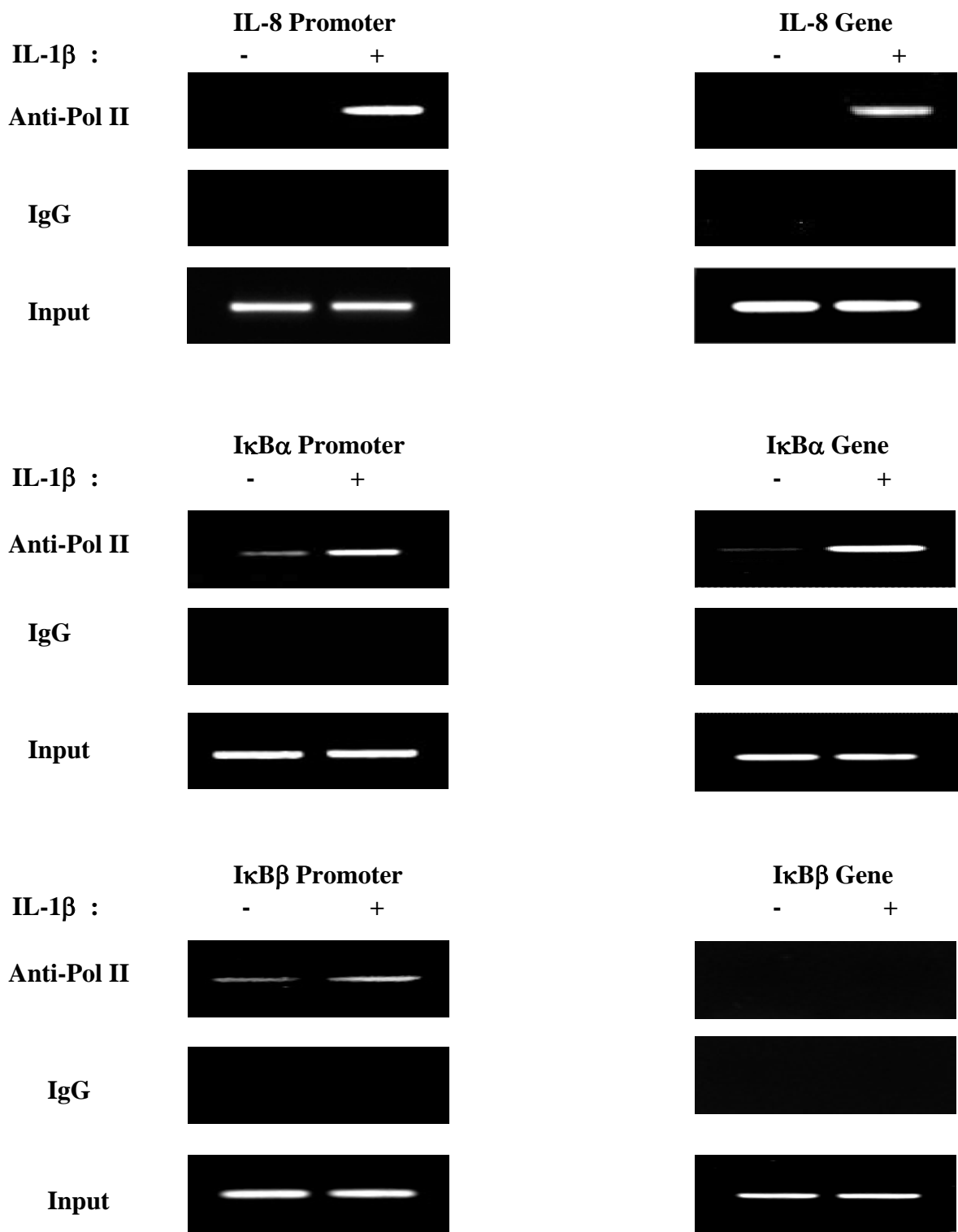


Figure 6

(B)

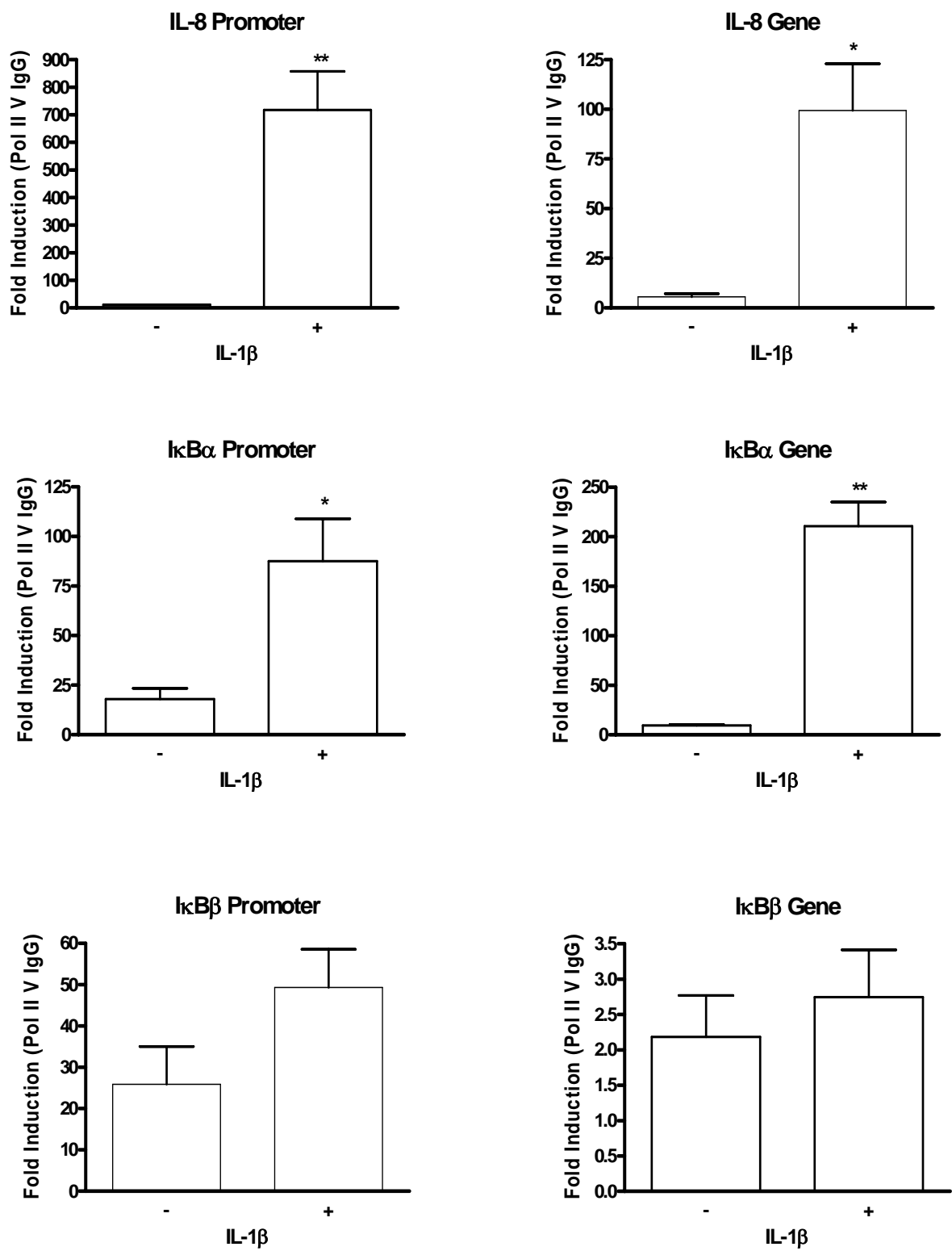
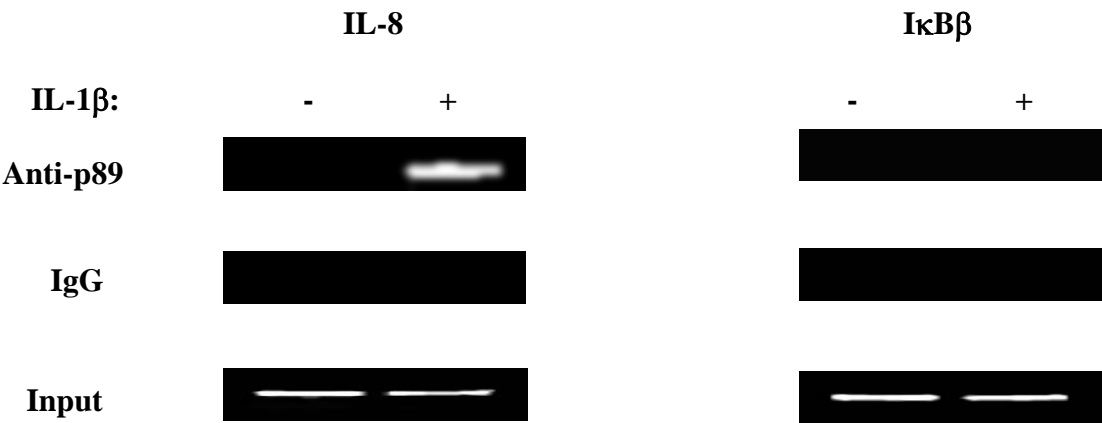


Figure 7

(A)



(B)

