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Genomic Structure and Functional Characterization of the Promoter Region of Human I κ B Kinase-related Kinase IKKi/ IKK ϵ Gene

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Abstract

The inducible I κ B kinase (IKKi/IKK ϵ) is a recently described serine-threonine kinase that activates the transcription factors NF κ B, interferon regulatory factor-3 (IRF3) and CCAAT/enhancer-binding protein (C/EBP δ). Several inflammatory agents have been shown to induce the expression of the IKKi gene in macrophages and other cell types but the mechanism is unknown. We have found that the IKKi expression was constitutive in human chondrocytes from OA cartilage and a human chondrocytic cell line C28/I2 but was up-regulated by the inflammatory cytokines TNF α or IL-1 β in an NF κ B dependent manner. To understand the constitutive and inducible expression of the IKKi gene we localized the transcription start site (TSS), cloned and sequenced a 2 kb genomic DNA fragment 5' of the TSS and characterized the putative promoter region (PPR), and identified the motifs therein that are required for basal and cytokine-induced IKKi gene promoter activity. We found that IKKi core promoter was TATA-less and by using PCR generated deletion mutants of the PPR we found that the cis-elements responsible for basal transcriptional activity were located between –51 to –100 bp upstream of the TSS while the cytokine response elements were located distally between –501 and –1000 bp upstream of the TSS. The DNA region containing the cytokine response elements had two κ B sites as the most relevant regulatory motifs. The results of site-directed mutagenesis revealed that the κ B site located between –833 and –847 bp upstream of the TSS was biologically functional and required for cytokine-induced IKKi promoter activity in human chondrocytes and HeLa cells. The silencing of the other κ B site (–816/–802) was positional, rather than sequence-specific. Over-expression of NF κ B p65 mimics the TNF α -induced activation of the IKKi promoter. In addition, the gel shift assay suggested that NF κ B p65 is responsible for activation of the IKKi promoter. These data for the first time characterize the promoter region of IKKi and provide further insights into its transcriptional regulation in human chondrocytes and other cell types.

Keywords

IKKi/IKK ϵ Promoter; cis-acting element; Transcriptional regulation; NF κ B, Chondrocyte

Abbreviations

5'-RACE, 5'-rapid amplification of cDNA ends; ActD, actinomycin D; bp, base pair(s); BRE, TFIIB responsive element; CHX, cycloheximide; DPE, downstream promoter element; EMSA, electrophoretic mobility shift assay; EMSSA, electrophoretic mobility supershift assay; I κ B, inhibitor of NF κ B; IKKi/IKK ϵ , inducible I κ B kinase; IL, interleukin; Inr, initiator; NF κ B, nuclear

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factor kappa B; nt, nucleotide(s); PPR, putative promoter region; RT-PCR, reverse transcriptase-polymerase chain reaction; SEAP, secreted form of human placental alkaline phosphatase; TBP, TATA-binding protein; TNF, tumor necrosis factor; TSS, transcription start site

1. INTRODUCTION

Transcription factor NF κ B is a strong transcriptional activator that plays an important role in the expression of genes involved in diverse cellular functions including growth, survival, immune and inflammatory responses (Karin and Ben-Neriah, 2000). In resting cells NF κ B is sequestered in the cytoplasm in association with proteins of the I κ B family of inhibitory proteins (I κ B α , I κ B β , or I κ B ϵ). Stimulation of cells by cytokines or other agonists results in phosphorylation, ubiquitination, and degradation of I κ Bs thus exposing the nuclear localizing signals (NLS) on NF κ B, leading to its nuclear translocation where it binds to and activate the promoters of NF κ B responsive genes (Ghosh et al., 1998; Karin and Ben-Neriah, 2000; Ghosh and Karin, 2002). The two I κ B kinases (IKKs) which specifically phosphorylate the critical serine residues in I κ Bs (IKK α or IKK-1 and IKK β or IKK-2) have been cloned and characterized (DiDonato et al., 1997; Zandi et al., 1997). Both IKK-1 (85-kDa) and IKK-2 (87-kDa) are activated by phosphorylation and contains an N-terminal serine/threonine kinase catalytic domain, a leucine zipper-like amphipathic helix, and a C-terminal helix-loop-helix domain (DiDonato et al., 1997; Connelly and Marcu, 1995). A third member of this group called NEMO (NF- κ B essential modulator or IKK γ) has no kinase activity but is essential for IKK phosphorylation and activation by upstream kinases (Yamaoka et al., 1998; Mercurio et al., 1999). The IKKs are ubiquitously expressed in most human tissues and serve as the converging point in the activation of NF κ B by a wide variety of agents (Zandi et al., 1997).

Recently, a new homolog of the IKK-1 and IKK-2 kinases was found to be induced in response to stimulation with LPS and designated inducible I κ B Kinase (IKKi/IKK ϵ) and was shown to activate NF κ B in macrophages (Shimada et al., 1999; Peters et al., 2000). The IKKi protein shares 31% amino acid identity with IKK-2 in the highly conserved N-terminal kinase domain but differs from IKK-2 in several important aspects. For example, it is now known that recombinant human IKKi phosphorylates I κ B α at S36 but not at S32 in vitro while IKK-2 phosphorylates both of these residues. Also, IKKi is predominantly expressed in cells and tissues of the immune system such as peripheral blood leukocytes, thymus, and spleen (Shimada et al., 1999; Peters et al., 2000), while the expression of IKK-2 is ubiquitous. Third, IKKi shows significant kinase activity when over-expressed and isolated from un-stimulated cells and was found to be associated with I-TRAF/TANK proteins within the cells (Pomerantz and Baltimore 2002; Nomura et al., 2000), while activation of IKK-2 is stimulus-dependent. Perhaps most importantly, IKKi mRNA, but not IKK-2 mRNA or protein, can be induced in multiple cell types in response to cytokines and LPS indicating that pro-inflammatory agents-mediated stimuli are required for its expression (Shimada et al., 1999; Kravchenko et al., 2003). Although initially identified as an inducible gene, more recent studies found that the expression of IKKi was constitutive in human fibroblast-like synoviocytes from RA patients (RASf), and in agreement with previous studies in which stimulation with TNF α or IL-1 β also increased the IKKi mRNA and protein expression in RASf, suggesting a role for IKKi in the chronic activation of the inflamed synovium in an arthritic joint (Aupperle et al., 2001).

Despite rapid advances in exploring the expression and biological functions of IKKi (Kishore et al., 2002; Huynh et al., 2002; Sharma et al., 2003; Kravchenko et al., 2003; Mori et al., 2004; Perry et al., 2004; Ehrhardt et al., 2004), the genomic structure of the promoter region and the identity of motifs present therein that regulate the inflammatory cytokine-induced IKKi gene expression have not been reported. In results reported here, we identified the transcription start site (TSS), characterized the DNA regions responsible for the basal transcriptional activity

and cytokine-induced expression, and demonstrated the functional and silent κ B sites on the IKKi promoter. Furthermore, we show that the NF κ B p65 protein is the transcription factor that interacts with the κ B site on the promoter region of the human IKKi gene suggesting that IKKi is the target gene of NF κ B activation. Altogether, these data extend the previous findings substantially and also provide a foundation for exploring further the biological role and regulation of IKKi in the cell type of interest.

2. MATERIALS AND METHODS

2.1. Cell culture, transfection, treatment, and reporter activity assay

Human OA cartilage samples were procured through the Cooperative Human Tissue Network/ Human Tissue Procurement Facility of the University Hospitals of Cleveland and Case Western Reserve University, with prior approval of the Institutional Review Board of University Hospitals of Cleveland. Chondrocytes were prepared by the enzymatic digestion of knee cartilage as previously described (Ahmed et al., 2004) and maintained in DMEM/F12 (Mediatech) supplemented with 10% FBS. HeLa cells were purchased from American Type Culture Collection and maintained in MEM supplemented with 10% FBS. Immortalized human chondrocyte cell line C28/I2 cells (courtesy of Dr. Mary B. Goldring, Harvard medical School) were maintained in DMEM/F12 supplemented with 10% FBS. Cells (1.5×10^5 HeLa or 2×10^5 C28/I2 cells) were cultured in humidified air with 5% CO₂ at 37 °C for 24 h in 12-well culture plates and transiently transfected with 0.5 μ g of IKKi promoter constructs or the empty vectors using the FuGENE 6 reagent (Roche). A 1/50 ratio (0.01 μ g) of phRL-TK (Promega) containing a Renilla luciferase reporter gene was co-transfected with luciferase reporter constructs as an internal control to normalize the transfection efficiency. Twenty-four hours after the transfection, cells were treated with 50 ng/ml of TNF α or 10 ng/ml of IL-1 β (Roche). Twenty-four hours after the cytokine treatment, conditioned media were collected for measurement of SEAP activity (BD Biosciences Clontech), and the cells were lysed for measurement of dual luciferase activity (Promega).

2.2. RNA isolation, reverse transcription, and TaqMan real-time PCR

Total RNA was prepared using the RNeasy Mini kit (Qiagen) and the first-strand cDNA was generated using SuperScriptII reverse transcriptase (Invitrogen). The first-strand cDNA was used to quantitate the IKKi mRNA levels in the presence or absence of cytokines and/or mRNA synthesis inhibitor (actinomycin D) and protein synthesis inhibitor (cycloheximide) by TaqMan real-time PCR system (Applied Biosystems). Briefly, the reaction mixture contained 50 ng of reverse transcribed cDNA, TaqMan universal master mix, and gene specific primers and the FAM labeled probe supplied in the Assays-on-Demand gene expression kit for human IKKi (Applied Biosystems). Amplification parameters were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C for 1 min. Expression level of house keeping gene β -actin was used as reference and fold induction was calculated by relative standard curve method.

2.3. 5'-rapid amplification of cDNA ends (5'-RACE)

The 5'-rapid amplification of cDNA ends (5'-RACE) method was used to identify the transcription start site of the human IKKi gene using GeneRacer kit according to the instructions of the manufacturer (Invitrogen). Briefly, 5 μ g of total RNA isolated from human chondrocytes using RNeasy Mini kit (Qiagen) was pretreated with calf intestinal alkaline phosphatase (CIP) and tobacco acid pyrophosphatase (TAP), followed by the first-strand cDNA synthesis with the kit supplied RT and random primers. To amplify 5' end of the IKKi cDNA, nested PCR was performed using antisense IKKi gene specific primer followed by nested antisense IKKi gene specific primer towards the 5' end of the message (Table 1). First round of PCR consisted of 5 cycles (94 °C for 30 sec, and 72 °C for 1 min), then 5 cycles (94

°C for 30 sec, and 70 °C for 1 min), followed by 25 cycles (94 °C for 30 sec, 65 °C for 30 sec, and 68 °C for 1 min). For the second round of amplification, 1 µl of reaction mixture from the first round was used as a template and the PCR was performed for 25 cycles (94 °C for 30 sec, 65 °C for 30 sec, and 68 °C for 1 min). The amplified fragment (~ 300 bp) was gel purified and cloned directly into pCR4-TOPO vector (Invitrogen) and completely sequenced.

2.4. PCR mapping of the putative IKKi promoter region

To verify the result from 5'-RACE experiment, PCR assisted mapping of the region around the putative transcription start site was performed. Total RNA isolated from human chondrocytes was first treated with DNase I (Promega) to remove the potential genomic DNA contamination, and then the first-strand cDNA was reverse transcribed using superScriptII RT and random primers (Invitrogen). Genomic DNA was isolated from human chondrocytes using DNeasy Tissue kit (Qiagen) with RNase A treatment (Invitrogen). Genomic or cDNA (100 ng) was used as templates for PCR reaction using primer pairs (F1, F2, F3, F4 and R1) flanking the proposed transcription start site (Table 1). After 35 cycles of amplification (94 °C for 30 sec, 60 °C for 30 sec, and 68 °C for 1 min), the PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining.

2.5. Promoter-reporter constructs and mutagenesis

Approximately 2 kb of the 5'-flanking DNA region was amplified by PCR using human chondrocyte genomic DNA as template and cloned into pSEAP2-Basic (Secreted alkaline phosphatase) vector (BD Biosciences Clontech). The cloned fragments were completely sequenced on both strands and sequences were analyzed for the TF binding sites using the MatInspector program v7.2 (www.genomatix.de) (Quandt et al., 1995). Deletion mutants of the cloned DNA fragment were generated by PCR using one of the following forward primers corresponding to position relative to the transcription start site: IKKi-1976-F, IKKi-1500-F, IKKi-1000-F, IKKi-500-F, IKKi-300-F, IKKi-100-F, IKKi-50-F, and reverse primer IKKi-1-R (Table 1). All the forward primers contained the recognition sequence for restriction endonuclease KpnI while the reverse primer had an EcoRI restriction site. Thirty cycles of PCR (94 °C for 30 sec, 60 °C for 30 sec, and 68 °C for 2 min) were carried out using Platinum Taq Polymerase HF (Invitrogen). The PCR products were digested sequentially and cloned into the KpnI and EcoRI sites of the pSEAP2-Basic vector. The SEAP reporter constructs containing nt -1976/+1, -1500/+1, -1000/+1, -500/+1, -300/+1, -100/+1, and -50/+1 region of the putative human IKKi promoter region were named as p1976IKKi-SEAP, p1500IKKi-SEAP, p1000IKKi-SEAP, p500IKKi-SEAP, p300IKKi-SEAP, p100IKKi-SEAP, and p50IKKi-SEAP, respectively. All constructs were verified by restriction map analysis and sequencing both strands.

The two NFκB binding sites identified by in silico analysis were mutated in the p1000IKKi-SEAP construct using mutant oligonucleotides and QuikChange II Site-Directed Mutagenesis kit (Stratagene) according to manufacturer's instruction. Briefly, the p1000IKKi-SEAP construct was subjected to PCR with 2 sets of primer pairs: Mut^{κB(-833/-847)}-F/Mut^{κB(-833/-847)}-R and Mut^{κB(-816/-802)}-F/Mut^{κB(-816/-802)}-R (Table 1) and PfuUltra HF polymerase (Stratagene), and parental DNA was removed by digestion with DpnI. Plasmid DNA was prepared as above and the presence of the mutated sites was verified by sequencing and the plasmids were named as p1000IKKi^{MutκqB(-833/-847)}-SEAP, p1000IKKi^{Mut κB(-816/-802)}-SEAP, and p1000IKKi^{Mut κB(-833/-847, -816/-802)}-SEAP.

The IKKi promoter-luciferase reporter constructs were subcloned from p1000IKKi-SEAP to pGL3-Basic with Xho I and HindIII digestion sites. Two κB sites were exchanged by nested overlapping PCR (Hughes and Andrews, 1996) using IKKi-1000L-F, IKKi-1L-R, Ex-R, Ex-F, and Ex^{Mut}-F primers (Table 1) and AccuPrime Pfx DNA Polymerase (Invitrogen). The PCR

products were digested and then ligated to XhoI and HindIII sites of pGL3-Basic vector (Promega). The resulting constructs were verified by sequencing and named as p1000-IKKi-Luc, p1000-IKKi^{Mut κB(-833/-847)}-Luc, p1000-IKKi^{Ex κB}-Luc, and p1000-IKKi^{Ex Mut κB(-833/-847)}-Luc.

2.6. Electrophoretic Mobility Shift Assay (EMSA) and Electrophoretic Mobility Supershift Assay (EMSSA)

Nuclear protein extracts of C28/I2 cells were prepared based on the method previously described (Schreiber et al., 1989). Briefly, cells were resuspended and incubated in 400 μl of cytoplasmic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF) for 15 min at 4 °C. After adding 25 μl 10% NP-40, cell lysates were vortexed and centrifuged at 12,000 x g for 30 sec. The pellets were then disrupted by adding 50 μl ice-cold nuclear buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF), rocked vigorously for 15 min at 4 °C, and centrifuged at 18,000 x g for 15 min. Supernatant was collected as nuclear protein extract for the electrophoretic mobility shift assay (EMSA) with 5' end-labeled double-stranded oligonucleotides or unlabeled oligonucleotides: Consensus^{κB}, IKK^{κB}, or IKKi^{Mut κB} (Table 1). The 20 μl binding reaction mixture contained 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 0.05% NP-40, 5 mM MgCl₂, 5 mM EDTA, 50 ng/μl poly (dI-dC), 15 μg of nuclear protein extract, and 1 pmol of biotin-labeled DNA. The reaction was incubated at RT for 30 min. The competition reactions were performed by adding 100-fold excess unlabeled double-stranded oligonucleotides. The electrophoretic mobility supershift assay (EMSSA) was performed by pre-incubating nuclear protein extract with 2 μg antibody against NFκB p65 or NFκB p50 (SantaCruz Biotechnology) for 1 h at 4 °C prior to the binding reaction. The reaction mixtures were subjected to electrophoresis on a 6% pre-casted DNA retardation gel (Invitrogen) at 100V in a 0.5X Tris-borate-EDTA buffer, transferred to a nylon membrane, and cross-linked. The biotin-labeled DNA was detected using the LightShift Chemiluminescent EMSA kit (Pierce).

3. RESULTS

3.1. The transcription start site (TSS) of the human IKKi gene

To localize the putative promoter of the human IKKi gene on the basis of its proximity to the 5' cDNA end in silico (Suzuki et al., 2001), we experimentally determined the transcription start site (TSS) by employing the 5'-RACE method and PCR mapping of the human genomic DNA region surrounding the putative TSS. 5'-RACE was performed on the cDNA synthesized from total RNA isolated from human chondrocytes and a single DNA fragment of 354 bp was produced (Fig. 1A, lane 2). No DNA fragment was amplified using the same primer combination without template DNA (Fig. 1A, lane 1, no template control). The IKKi fragment generated using the 5'-RACE method was cloned and both strands were sequenced and mapped to the NCBI reference sequence (GenBank accession number NM_014002). Our results of sequence analysis showed that the cDNA end of the IKKi was 339 bp upstream of the translation initiation codon (ATG) though the ATG is not embedded in the optimal Kozak sequence (Kozak, 1996). To further confirm these results we performed PCR amplification of the cDNA and the genomic DNA using primers flanking the putative transcription start site. As shown in Fig. 1B, DNA fragments of 204 and 217 bp, amplified by using the primer pairs F1/R1 and F2/R1, were amplified both from genomic DNA and cDNA preparations. On the other hand, DNA fragments of 237 and 693 bp, amplified by primer pairs F3/R1 and F4/R1 were only observed from genomic DNA samples but not from cDNA preparations. These results indicated that the region upstream of F3 primer was absent in IKKi mRNA, thus confirming the localization of transcription start site (TSS) identified above and its first base is referred to as nt +1 in all of the results reported herein.

3.2. IKKi core promoter activity is located between -51 and -100 bp upstream of TSS

The nucleotide sequence obtained from above experiments was used to query the finished human genome sequence using the BLAST program at the NCBI and the sequence proximal to the TSS in the genomic DNA region was identified. This sequence information was used to design PCR primers and a 1976 bp DNA fragment of the IKKi gene upstream of the TSS was amplified and cloned into pSEAP2-Basic vector and sequenced. A detailed *in silico* analysis using MatInspector v7.2 (www.genomatix.de) (Quandt et al., 1995) of the nucleotide sequence obtained (Table 2 and Fig. 2) showed that the major eukaryotic promoter elements [TATA-box (-608 to -592), CCAAT-boxes (-1732 to -1722; -1606 to -1596; -559 to -549), and GC-boxes (-1637 to -1623; -892 to -878; -887 to -873; -510 to -496)] usually located within 200 bp upstream of the TSS were located at least 500 bp upstream from the TSS indicating that the IKKi gene had a TATA-less promoter (Strachan and Read, 2004).

Sequence alignment of the promoter region of the human IKKi gene with the DNA sequence from other mammalian species using Genome browser (<http://genome.ucsc.edu>) based on hidden Markov model (HMM) and phylogenetic approaches (Siepel and Haussler, 2003) revealed the presence of evolutionarily highly conserved regions among human, chimpanzee, mouse, rat, and dog. These conserved regions corresponding to nt -51 to -69 (Fig. 3A) and nt -802 to -847 (Fig. 3B) upstream of the IKKi TSS in the human genome were later experimentally found to be essential for the basal and cytokine-induced activity of the IKKi promoter respectively (see below). Interestingly, the DNA sequence located between nt -802 to -847 upstream of the TSS in the human gene encompass two putative and highly conserved κ B sites.

The cloned DNA fragment (nt -1976 to +1) designated as p1976IKKi-SEAP was used as a template for generating progressively deleted fragments of the flanking region upstream of experimentally established TSS (Fig. 4). All the constructs were transiently transfected into HeLa cells and human chondrocytic C28/I2 cells. We used C28/I2 cells for some of these studies as they faithfully maintain the chondrocyte phenotype, are well characterized and have been used by others in a number of published studies (Goldring et al., 1994; Finger et al., 2003; Loeser et al., 1999; Sen et al., 2004; Pufe et al., 2004). Significant transcriptional activity was observed with p1976IKKi-SEAP through p100IKKi-SEAP when compared to promoter-less vector (Fig. 4). No significant activity was observed when p50IKKi-SEAP was transfected into either HeLa or C28/I2 cells. Transfection of HeLa cells with the same set of promoter-SEAP constructs displayed the similar pattern as observed in C28/I2 cells, except that the transcriptional activity of these constructs was lower in HeLa cells (Fig. 4A) as compared to C28/I2 cells (Fig. 4B) indicating possible cell type-specific regulation. Despite the relative difference of promoter strength between the two cell lines, the minimal 5'-flanking region required for constitutive expression of the human IKKi gene was found to be located approximately between -51 and -100 bp 5' upstream of the TSS.

3.3. Transcriptional regulation of IKKi mRNA expression in human chondrocytes

It was previously shown that the expression of IKKi mRNA was induced when cells were stimulated with TNF α or IL-1 β (Aupperle et al., 2001) but in this study *cis*-regulatory elements were not identified. Since the focus of our studies was the regulation of IKKi, we studied whether the human IKKi gene responds to pro-inflammatory cytokines in different cell types. In these experiments, nearly confluent human chondrocytes were stimulated with TNF α or IL-1 β and the IKKi mRNA expression was quantified by TaqMan real-time PCR. Our results showed that the endogenous IKKi expression was dramatically increased (3 folds after TNF α treatment, and 50% increment after IL-1 β treatment) over the basal level in all of the samples treated with cytokines (Fig. 5) indicating that the IKKi gene responds both to TNF α and IL-1 β in human chondrocytes as well, although the level of expression was always higher

in TNF α stimulated cells. To determine whether the cytokine-stimulated expression of IKKi gene require de novo protein synthesis, cells were pre-treated either with mRNA synthesis inhibitor ActD (1 μ g/ml) or protein synthesis inhibitor CHX (10 μ g/ml) for 30 min, and were then stimulated with TNF α or IL-1 β for 24 h. Cells stimulated with TNF α or IL-1 β in the presence of CHX showed super elevated levels of IKKi mRNA, while ActD inhibited the cytokine-induced up-regulation of IKKi mRNA expression suggesting that the regulation of IKKi mRNA induction may be at the transcription level and does not require de novo protein synthesis.

3.4. Cytokine-induced expression of IKKi mRNA is regulated by cis-regulatory elements located between -501 and -1000 bp 5' upstream of TSS

To precisely define the human IKKi DNA region 5' of the TSS that is required for the cytokine-induced activation, progressive deletion constructs of the IKKi-promoter region were generated by PCR and cloned into the SEAP reporter vector. These chimeric gene constructs were used for the transient transfection of HeLa and C28/I2 cells which were then stimulated with TNF α or IL-1 β . Results of these studies are presented in Fig. 6 and showed that the TNF α or IL-1 β inducible promoter activity was chiefly dependent on the cis-elements located in the DNA region present in the p1000IKKi-SEAP plasmid. This is supported by the observations that further deletion of the IKKi promoter DNA between -501 and -1000 bp abrogated the responsiveness of the constructs to TNF α and IL-1 β without affecting the basal promoter activity (Fig. 6).

3.5. Biologically functional κ B site exists in IKKi promoter

We analyzed the sequence of IKKi promoter region which was shown in the above experiments to be essential for TNF α and IL-1 β induced activity in human chondrocytes and HeLa cells. In silico analysis showed that there were two putative κ B binding sites present at -833/-847 and -816/-802 within this region. Using the human genome browser and the BLAT program (<http://genome.ucsc.edu/>) these sites were found to be highly conserved across the species (Fig. 3B) indicating an important role of these motifs in the regulation of the IKKi gene. To test this hypothesis experimentally, we generated three distinct mutants of the IKKi promoter having either one or both of the following double base substitutions: GA(-837, -838)TC, and/or TT(-808, -807)GG. The resulting three IKKi promoter-SEAP chimeric gene constructs were classified as p1000IKKi^{Mut κ B (-833/-847)}-SEAP, p1000IKKi^{Mut κ B (-816/-802)}-SEAP, and p1000IKKi^{Mut κ B (-833/-847, -816/-802)}-SEAP (Fig. 7A). These DNA constructs (0.5 μ g) were used to transfect human chondrocytic C28/I2 cells and HeLa cells which were then stimulated with TNF α or IL-1 β for 24 h and then the SEAP activity was measured in the culture supernatant. Our results showed that there was a ~ 3.5 fold increase in the reporter activity with TNF α and ~ 2.5 fold increase with IL-1 β in both HeLa (Fig. 7B) and C28/I2 (Fig. 7C) cells compared to the untreated samples. In cells transfected with the plasmids where the first κ B site (-833/-847) was mutated, the promoter activity was dramatically reduced and was similar to the levels present in untreated samples. In contrast, there was no significant change in the IKKi promoter driven expression of SEAP when the second κ B site (-816/-802) was mutated. There was no significant difference in the promoter activity in cultures transfected with constructs carrying the first mutated κ B site (-833/-847) or both mutated κ B sites (-833/-847 and -816/-802), suggesting that the second κ B site (-816/-802) was not active. These results clearly define the binding site for NF κ B located at position (-833/-847) as essential, and perhaps the only one, required for conferring the cytokine responsiveness of the IKKi promoter.

3.6. Positional silence of the κ B (–816/–802) site on the IKKi promoter assessed by exchange of the two κ B sites.

To distinguish whether the silent κ B site (–816/–802) was due to a “positional” or “sequence specific” effect, we generated new sets of IKKi promoter-luciferase reporter constructs in which the –833/–847 sequence (active) was exchanged with the –816/–802 sequence (silent), and in which the –816/–802 sequence (silent) was exchanged with the –833/–847 sequence (active), with or without a mutation of the wild-type –833/–847 motif. We transfected the C28/I2 cells with these new sets of constructs, treated with TNF α or IL-1 β , and compared the reporter activities to their un-exchanged counterparts. When the –833/–847 active motif replaced the –816/–802 silent motif and the –816/–802 silent motif replaced the –833/–847 active motif (Fig. 8A), the IKKi promoter activity in response to TNF α or IL-1 β was not significantly changed. This result suggests that either the –833/–847 active motif is position-independent, and/or the –816/–802 silent motif is position-dependent. However, when the two κ B sites were exchanged with each other in context with a mutation of the wild-type –833/–847 motif (Fig. 8B), the –816/–802 silent motif was no longer silent, and the IKKi promoter activity in response to TNF α or IL-1 β was significantly increased, indicating that the silencing effect of the –816/–802 κ B site was positional, rather than sequence specific.

3.7. NF κ B p65 transcription factor is involved in the activation of IKKi promoter

To verify the biological significance of NF κ B transcription factors on the activation of the IKKi gene, we co-transfected the IKKi promoter-luciferase reporter constructs, with constructs over-expressing NF κ B p65 (Panomics, Redwood City, CA), NF κ B p50 (Panomics), or dominant negative I κ B α S32A/S36A (BD Biosciences Clontech) in the absence of cytokine stimuli. As shown in Fig. 9A, our results indicated that over-expression of NF κ B p65 increased the IKKi promoter activity which was completely abolished when the –833/–847 active κ B motif was mutated, but was not abolished when the –816/–802 silent motif was mutated. NF κ B p50 had no effect on the activation of IKKi promoter in either wild-type, or κ B-mutated constructs; and dn I κ B α carrying two mutations (S32A/S36A) was unable to abolish the basal promoter activity. The function of NF κ B in mediating pro-inflammatory cytokine-induced IKKi expression was further proven by the experiment in which we co-transfected the IKKi promoter-luciferase reporter constructs containing the intact κ B sites, with constructs over-expressing the wild-type or dn I κ B α followed by the treatment of TNF α or IL-1 β . The results shown in Fig. 9B indicated that both wt and dn I κ B α completely abolished the cytokine-induced IKKi promoter activity. The wt I κ B α is a NF κ B inhibitor which prevents the NF κ B from translocating to the nucleus; the dn I κ B α is also a NF κ B inhibitor because it has two mutations (S32A, S36A) and is unable to be phosphorylated. These results implied that the cytokine-induced IKKi expression is NF κ B-dependent although the basal IKKi expression is NF κ B-independent.

3.8. NF κ B p65 transcription factor binds the –833/–847 κ B site on the human IKKi promoter.

To further investigate the role of the κ B site on the IKKi promoter in cytokine-induced IKKi promoter activation, we performed the EMSA and EMSSA assays. Biotin labeled 22-mer nucleotide containing the –833/–847 κ B site was incubated with nuclear protein extract obtained from TNF α -treated or untreated C28/I2 cells. As shown in Fig. 10A, the DNA-protein complex could only be observed in TNF α -treated cells (Lane 1), but not in un-treated cells (lanes 3 and 4). In addition, when the nuclear protein extract was incubated with the probe carrying two nucleotide substitutions on the κ B site, no shifts were observed (lanes 2 and 4). Competition assay was performed to determine the specificity of the shifted complex (Fig. 10B). The shifted complex could be competed out by using the excess oligonucleotides containing consensus κ B site (lane 2) or the wild-type IKKi κ B site (lane 3), but not by the oligonucleotide containing the mutated IKKi κ B site (lane 4). These results indicate that the

–833/–847 κ B site on the IKKi promoter is authentic, and specific. To determine which NF κ B protein binds to the –833/–847 κ B site on IKKi promoter, we also performed an EMSA assay by using antibodies directed against either NF κ B p65, or NF κ B p50 (Fig. 10C). The DNA-protein complex was migrated slowly by incubating nuclear protein extract only with anti-NF κ B p65 (lane 2), but not with anti-NF κ B p50 (lane 3). Although these experiments did not exclude the possibility that other NF κ B family members (NF κ B p52, Rel B, and c-Rel) might also be able to bind the κ B motif in the IKKi promoter, they did pinpoint that NF κ B p65, but not NF κ B p50, was the protein that binds to the κ B site on the IKKi promoter and was required for TNF α induced transactivation of the IKKi gene.

4. DISCUSSION

Studies reported here localize for the first time the correct putative transcription start site of the IKKi mRNA, and identify the promoter regions required for the basal expression and cytokine-induced up-regulation of the IKKi gene. These studies also identify and delineate the functional role of NF κ B binding sites present within the region of the IKKi promoter that is associated with the cytokine-induced up-regulation of IKKi mRNA in human chondrocytes and HeLa cells.

Eukaryotic gene transcription driven by RNA polymerase II involves core promoter, proximal promoter, and distal promoter elements of the gene (Orphanides et al., 1996; Roeder, 1996). By using MatInspector (Quandt et al., 1995) with default criteria (0.75 for core similarity and optimized for matrix similarity), we found that there were no obvious TATA-box, Inr, DPE, and BRE, nor the GC-boxes and CCAAT-boxes within 200 bp upstream of the transcription start site of the human IKKi gene (Table 2 and Fig. 2). However, the IKKi gene promoter region further upstream of the transcription start site contained typical eukaryotic promoter elements but these were located at least 500 bp upstream of the transcription start site. Based on the results of our experiments we conclude that the TATA-box located between –608 to –592 is unlikely to be active as the reporter constructs without this TATA-box (p500IKKi-SEAP, through p100IKKi-SEAP) were as efficient in driving SEAP expression as the full-length promoter containing this TATA-box (p1976IKKi-SEAP through p1000IKKi-SEAP) (Fig. 4). Also, this is in agreement with previous studies showing that the most active TATA-box is usually located very close to (mostly 25 to 30 bp upstream of) the transcription start site (Sawadogo and Sentenac, 1990). Taken together, our data indicated that the human IKKi gene promoter is a “TATA-less” promoter – a situation prevalent in many housekeeping genes (Azizkhan et al., 1993; Weis and Reinberg, 1997) and TNF-receptor family genes (Santee and Owen-Schaub, 1996) but less common in other inducible genes.

Computational analysis of the promoter region (nt –501 to –1000) shown to be required for TNF α - or IL-1 β -mediated activation of IKKi promoter activity in human chondrocytes revealed the presence of two highly conserved NF κ B binding sites (κ B^(–833/847) and κ B^(–816/802)) in this region of the human IKKi gene promoter (Fig. 7A). The first one is reverse oriented, and the second one is forward oriented but both of these sites are nearly a perfect match to consensus κ B binding sites (Faisst and Meyer, 1992). By performing site-directed mutagenesis of one or both the putative κ B binding sites in the DNA region of the IKKi gene shown to be essential for its up-regulation we could deduce that only the κ B site located distally to the TSS (–833/–847) was functional and required for the cytokine-induced IKKi promoter activity. Mutation of both of these κ B sites had no additive or subtractive effect on the level of IKKi mRNA expression compared to the first site mutagenesis, indicating that these sites behave independently, and that the second site is not required for the other one to be functional. These observations are also consistent with the notion that the transcription factors act in an orientation-independent manner (Faisst and Meyer, 1992). Thus it appears that the integrity of the distal κ B site (–833/–847) is required to respond to the TNF α - or IL-1 β -

induced activation of IKKi promoter in human chondrocytes and HeLa cells. By exchanging the two κ B sites and mutating the active one (–833/–847), we further determined that the silence of the –816/–802 κ B site on the IKKi promoter was positional, rather than sequence-specific. Whether this phenomenon is due to the fact that the two κ B sites are located in close proximity, or the binding affinity of the –833/847 site is higher than that of the –816/–802 site, has not been tested in this study. The role of the NF κ B protein in the activation of the IKKi promoter was also verified by co-transfecting the reporter vector with expression constructs for NF κ B p65, NF κ B p50, and dn I κ B α (S32A and S36A) in the absence of TNF α stimulation. Over-expression of NF κ B p65 mimics the effects of TNF α or IL-1 β induced IKKi activation, that suggests its involvement in the activation of IKKi by cytokines. We further investigated the physical interactions of cis and trans factors in response to the TNF α treatment. Our gel shift and supershift assays, as well as the competition experiment, provides the further evidence that the interaction of the NF κ B p65 protein and –833/–847 κ B site on the IKKi promoter leads to the activation of the IKKi gene. NF κ B p50 was excluded by both the co-transfection experiment and the gel supershift assay. Based on the observation that the NF κ B p65 physically interacts with the promoter region of the IKKi gene, our study suggests that IKKi is the target gene of the “canonical” NF κ B pathway. A previous study reported that the authors did not find any NF κ B binding site within the promoter region analyzed (Kravchenko et al., 2003), thus the presence of these κ B motifs in the IKKi promoter region was neither localized nor studied in detail. The reasons for this discrepancy in our results of promoter analysis and those reported previously are not clear but may be due to the different methods used for isolating the promoter region or the conclusions were based on the analysis of the DNA region shorter than the region analyzed in this study. This is likely to be the reason as the region containing the elements essential for the minimal promoter activity as well as the region containing the two κ B sites (–802 through –847) was found to be highly conserved across the species. Thus our results showing that up-regulation but not the basal level of IKKi mRNA expression is an NF κ B dependent event not only confirms the previous findings but extend these further by locating the κ B sites in the IKKi promoter region and by identifying the cytokine responsive site that is required for the up-regulation of the IKKi mRNA expression in TNF α - or IL-1 β -stimulated cells. Since over-expression of IKKi activates NF κ B, and IKKi is the target gene of NF κ B, we reasonably hypothesize that cytokine-induced IKKi expression could trigger the positive feedback amplification mechanism that could account for sustained activation of NF κ B and expression of downstream NF κ B-responsive genes. However, this was not investigated in the present study.

In summary, we have identified and characterized the promoter region and the promoter activity of the human IKKi gene in human chondrocytes and other cell types. We have experimentally located the transcription start site of the human IKKi gene and the minimal 5' flanking region required for the basal promoter activity of the gene. We have also located and mapped the promoter region of the human IKKi gene and elucidated the role of a specific κ B motif in the promoter region that is required for the pro-inflammatory agents-regulated IKKi promoter activity. Finally, we have confirmed the involvement of NF κ B p65 subunit that physically interacts with the functional κ B site on the IKKi promoter and consequently mediates the cytokine-induced up-regulation of the human IKKi gene in different cell types. The potential knowledge of the identified molecular mechanisms might ultimately result in a better understanding of the role and regulation of this kinase in an inflammatory response and in the development of new treatment options for inflammatory and degenerative joint diseases through targeting IKKi.

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Fig. 1A.

Fig. 1B.

Fig. 1. (A) 5'-rapid amplification of the cDNA end of the human IKKi gene.

Five μ g of human chondrocyte total RNA was treated with calf intestinal phosphatase (CIP) and tobacco acid pyrophosphatase (TAP) according to the GeneRacer instructions. cDNA generated with the random primer was PCR amplified with the GeneRacer 5' primer and the IKKi gene-specific primer for 35 cycles (touchdown), followed by the GeneRacer 5' nested primer and the IKKi gene-specific nested primer for another 25 cycles. Twenty μ l of PCR reaction was run on a 2% agarose gel and visualized by ethidium bromide staining. MW: 100 bp ladder (Invitrogen); Lane 1: non-template control; Lane 2: IKKi fragment; Lane 3: β -actin fragment (positive control). **(B) PCR mapping of the transcription start site of the IKKi mRNA.** Upper Panel: Schematic description illustrating the relative location of the primer pairs

used for PCR mapping of the transcription start site of the human IKKi gene. Lower Panel: PCR mapping of the IKKi DNA region upstream and downstream of the transcription start site. The primer pairs F1/R1 and F2/R1 amplified 204 and 217 bp fragments, respectively, both from human chondrocyte cDNA and genomic DNA. However, the primer pairs of F3/R1 and F4/R1 amplified 237 and 693 bp fragments, respectively, only from genomic DNA, but not from cDNA. MW: 1 Kb Plus DNA Ladder (Invitrogen); TSS, transcription start site.

-1976	GATGGG	ATGCCCACCT	TTACATCCTG	AGCACACAGT	GCCTGCCTCA	TAGCAGGTGC	TAGACACATG
-1910	<u>GTAGTTTCCT</u>	<u>TTGAATGTGC</u>	<u>TAAATATGCT</u>	<u>GGGAAAGAGA</u>	<u>TTGACGGAGA</u>	<u>GAGGAAAGTA</u>	<u>AGAGTGCTGG</u>
	kB						
-1840	TGAGGACCCA	GCAGGTTTCAT	CAGGGAAAAA	AGTGGGTAA	CATTACGGAT	TGTCTAAGTT	ATGTAGCAAA
-1770	CCTCGGTGCC	CTAAAGCAAC	AAAGGCTGAT	TTCTCACCAA	<u>AGCCAATGTC</u>	CCTTGTGGAC	CAGCAGGGGA
	CCAAT-Box						
-1700	ACTCTGCTCA	GGACAGTCAC	TCAAGGACCC	AGACTAACAG	AATCACACTG	CCAGAAAGCA	GAGTTCTGGG
-1630	<u>AGGGTCTTGC</u>	<u>ATCAGGAAAT</u>	<u>AAATGCTTTG</u>	<u>GTGGGGAAT</u>	<u>CACATGCTCC</u>	<u>CCCTATCACA</u>	<u>AAGTAACTAG</u>
	GC-Box	C/EBP	CCAAT-Box				
-1560	GATGTGCACT	TCTCCCATGT	GCCAGGCAGG	CAGAAACCTG	GGAAGACTAG	GCTGGGTGCA	GTGGCTCACG
-1490	CCTGTAAATCC	CGGCACCTTG	GGAGACTGAG	GCAGGTGGAT	CAGGAGGTG	GGAGATCTAG	ACCAACCTGC
-1420	TCAATCCACC	TCACCAACCA	CACAGTGA	CCCCATCTCT	ACTAAAAATA	CAAAAAATTA	GCCACTGGCT
-1350	CACGCTGTGTA	ATCCCGGCAC	TTTGGGAGGC	TGAGGCAGGT	GGATCAGGAG	GTTGGGAGAT	CAAGTCCAAC
-1280	CTGTTCAATC	CACCTCACCA	ACCACACAGT	GAAACCCCAT	CTCTACTAAA	AATACAAAAA	GTTAGCCGGG
-1210	CATGGTGGTG	CATGCCTGTA	GTCCAGATA	CTTGGGAGGC	TGAGGCAGAA	GAATCACTTG	AACCCAGGAG
-1140	GTGGAGGTTG	CAGTGAGCTA	ACATCATGCC	ACTGCACCTC	AGCCTGGGTG	ACAGAGTGAG	ACTCCATCTC
-1070	AAAAAAAAAA	AGCAAGCTGG	GAAGACTGGG	TGGAACATGA	GGATCTCAGG	CACTCAGGCA	CATTCTCTCT
-1000	GGTCCTGATG	TGAGAAGTTG	CAGCAACTCC	TCCAGCTGGA	GGTGTTATGG	GGAGTTCTCT	TTATCTGAGC
-930	ACTAGGCAGT	TCTCAGCTCT	GCTCCTGTTT	TAGTGTCACT	<u>GTGGGAGGGG</u>	<u>TGGGGCTGGC</u>	<u>GGAGGAAACC</u>
	GC-Box GC-Box						
-860	ACCAGGTGTG	GCCATGGGAA	<u>AGTCCCTCCA</u>	ACATTCTATA	<u>GAAACATGGA</u>	<u>ATTTCCTGGT</u>	TTTAACCATT
	kB						
-790	TCATATCCAA	CTGCCATCTC	TGAGCTGACC	AGAGCAGGAC	AGACCCCTCC	AGCCTCCTAG	GACATTGTAT
-720	GTGGGGCATC	GAGTCCTGGC	TGGGAAGTTG	GCACCTGGGT	GCCAGCCCCA	GCAGTCTCTG	AAGAGCATGG
-650	GTAAATTACT	GAAGTGTGTG	GGCCTCCATG	CCTTCAATGC	<u>CTTCTGTAA</u>	<u>AATGCTTTCC</u>	<u>AAGCTCCCAA</u>
	TATA-Box						
-580	ACAATTCCAA	ATATATCCAT	<u>GTCATCCAAT</u>	<u>CAACCCACAG</u>	<u>AGTAGCTGTT</u>	<u>ACTCTGCGGG</u>	<u>TGAGTGAGCT</u>
	CCAAT-Box						
-510	<u>GGCTGGGAGG</u>	<u>GGCTGCATGC</u>	<u>TGTGTGCTCA</u>	<u>GGTCACACTG</u>	<u>GAAGGCATCA</u>	<u>GCTGGGTCCC</u>	<u>CGCGCTTGCT</u>
	GC-Box						
-440	CTCGGACCTC	TGCAGGCCTC	CTGGGAAGGT	<u>GACTCAGATC</u>	<u>CAACTTCTAG</u>	<u>GAAGGCATCA</u>	<u>GCTGGGTCCC</u>
	AP1						
-370	CACACTTCCT	TGGGTTTCCT	GGGTGTGGGA	<u>GCATGGACTT</u>	<u>CCCAGGCAGC</u>	<u>TTTCCACTTA</u>	<u>GTAACTTGC</u>
	kB						
-300	<u>CCAACCTGCT</u>	<u>CTGTTGACTC</u>	<u>TGGGAAGAGC</u>	<u>CCTGAACTTC</u>	<u>CCTGCTCCAG</u>	<u>TGAACTGTGG</u>	<u>GAGATGCCAA</u>
	kB						
-230	AACAGCACCC	AGCTGCCCCAT	CAAGATTACC	AGAAGGGCCC	CAGTTCCTGG	CTTCTCTGTG	GCTCACACTC
-160	TGGTCTTATT	GCCTACCTTT	GGGAAGTCTG	AGCATCCTCT	TTCTCTTTTC	TGCTGAGCTG	GTGGCTGCAC
-90	CGAGTTGCCT	CTGTCTCTTT	AAGAGAGAAA	GAGAGAGCGA	GAGACGGAAA	TCAGGAAGTG	CGAGAGAGCT
	TSS +1						
-20	GAGAGCCAGG	ACTCAGTGCT	GAGCTTGGTG	TCCCACCGCC	ACAAGGAGGC	AGGGAAGAAA	CCCACTAGTC
+51	CCAGCTCCTG	GGGTGGCACA	GACATTGCAA	CTGGCCCTGC	CTGTGGGTCC	TAGGGGCCCT	TGGCTACCAG
+121	GAGGCTAAGA	ACACTGCTCA	TGAATGACAG	TGAGCCCTGA	AAGCTCTGGG	GGTGTACACC	AGTCCACAA
	Intron1						
+191	GCCTGCATCC	CCTGCAGTGG	AGATGGG-----CTC	AGCTCCTGGA	CGTGCCACAG	ACAGAAAGCA	
	Intron2						
+251	TAACATACAC	TCGCCAGGAA	GAGCCTTTGC	CTGACTCAGG	GCAGCTCAGA	GTGTGGG-----GCA	
+311	GAAGGTGACC	AGCCAGCTCA	GGGCAGGAGA	<u>TG</u>			

Fig. 2. Nucleotide sequence and schematic representation of promoter region and 5' UTR of the human IKKi gene.

The transcription start site (TSS, bent arrow) is designated +1. The base pairs downstream from the TSS of IKKi are given positive numbers. The base pairs upstream of TSS of IKKi are labeled as negative numbers. The translation start codon (ATG) is boxed. Putative core and proximal promoter components, as well as cytokine (TNF α , IL-1 β) response elements determined by MatInspector v7.2 (www.genomatix.de) program, are underlined.

Fig. 3A.

HUMAN	AGAGAGAAAGAGAGAGCGA	-51
CHIMPANZEE	AGAGAGAAAGAGAGAGCGA	-51
MOUSE	AGAGAAAGAGAGAGAGAGA	-136
RAT	AAAGAGAGAGAGCGAGAGA	-134
DOG	AGAGAGAGAAAGAACGAG-	-38

Fig. 3B.

HUMAN	ATGGGAAAGTCCCTCCAACATTCTATAGAAACATGGAATTCCT-GG	-802
CHIMPANZEE	ATGGGAAAGTCCCTCCAACATTCTATATAAACATGGAATTCCT-GG	-802
MOUSE	AGGGGAAAGTCCCAACCAACATTCTAGAGAAACATGGAATTCCTGGG	-784
RAT	AGGGGAAAGTCCCAACCAACCTTCTTGAGAAACATGGAATTCCTGGT	-804
DOG	ATGGGAAAGTCCCTCTGACATTCTCTAGAAATGTGGAGTTTCCT-GG	-673
	*****	*****

Fig. 3. Evolutionary conservation of IKKi promoter region among human, chimpanzee, mouse, rat, and dog.

Human IKKi promoter regions (A) nt -51 to -69 responsible for basal promoter activity, and (B) nt -802 to -847 containing two κB sites are compared to those of chimpanzee, mouse, rat, and dog using Genome browser (<http://genome.ucsc.edu>). Sequences are derived from chromosome 1 of human genome assembly 17 (May 2004), chromosome 1 of chimpanzee genome assembly 1 (Nov. 2003), chromosome 1 of mouse genome assembly 5 (May 2004), chromosome 13 of rat genome assembly 3 (Jun. 2003), and chromosome 38 of dog genome assembly 1 (July 2004). Two κB sites are marked by asterisks.

Fig. 4A.

Fig. 4B.

Fig. 4. IKKi core promoter activity was located between nt -51 and -100 upstream of TSS. A series of truncated IKKi promoter constructs were generated by PCR, cloned into reporter vector, and transiently transfected into (A) HeLa and (B) chondrocytic C28/I2 cells to determine the minimal requirement for basal promoter activity. SEAP activity was measured by the chemiluminescence SEAP detection method (BD Biosciences Clontech) and was represented relative to the activity obtained from a pSEAP2-Basic promoter-less vector. Results shown are Mean $\pm$ S.D. of three experiments each performed in triplicate.

Fig. 5. Quantitation of IKKi mRNA induction by cytokines in human chondrocytes.

Subconfluent human chondrocytes were pretreated with actinomycin D (1 μ g/ml), or cycloheximide (10 μ g/ml) for 30 min, followed by stimulation with TNF α (10 ng/ml) or IL-1 β (5 ng/ml) for 24 h. Total RNA isolated from human chondrocytes was reverse transcribed and subjected to TaqMan real-time PCR using a commercially available kit containing gene specific primer pairs and a 6'-FAM labeled probe for IKKi. IKKi mRNA expression was normalized to β -actin mRNA expression as a reference gene, and fold induction relative to untreated samples was plotted. Results shown are mean $\pm$ S.D. of three experiments each performed in triplicate.

Fig. 6A.

Fig. 6B.

Fig. 6. Elements controlling the cytokine inducibility of IKKi gene were located between nt -501 and -1000 upstream of TSS.

(A) HeLa or (B) C28/I2 cells were transiently transfected with sequentially deleted IKKi promoter-SEAP reporter constructs in the presence or the absence of TNF α (50 ng/ml) or IL-1 β (10 ng/ml) for 24 h. SEAP activity was measured by the chemiluminescence SEAP detection method and was represented relative to the activity obtained from a pSEAP2-Basic promoterless vector. Results shown are Mean $\pm$ S.D. of three experiments each performed in triplicate.

Fig. 7A.

Element	κB (-833/-847)	κB (-816/-802)
Consensus κB	5'-NNGGGACTTTCCNNN-3'	5'-NNGGGRATTTCCNNN-3'
IKKi κB	5'-GAGGGACTTTCCCAT-3'	5'-CATGGAATTTCTGG-3'
IKKi ^{Mut} κB	5'-GAGG <u>T</u> CCTTTCCCAT-3'	5'-CATGGAAT <u>G</u> CCTGG-3'

Fig. 7B.

Fig. 7C.

Fig. 7. Only one κ B binding site was required for cytokine-induced IKKi promoter activity. (A) Site-directed mutagenesis of the two κ B binding sites in the human IKKi promoter region. Putative κ B binding sites were identified as described under Materials and Methods in the promoter region of IKKi using the MatInspector v7.2 (www.genomatix.de) program and were mutagenized. The element positions relative to the transcription start site are in brackets. Mutated residues are underlined. **(B) HeLa and (C) Chondrocytic C28/I2 cells** were transiently transfected with the wild-type p1000IKKi-SEAP construct or mutated constructs p1000IKKi Mut κ B (-833/-847) -SEAP, p1000IKKi Mut κ B (-816/-802) -SEAP and p1000IKKi Mut κ B (-833/-847, -816/-802) -SEAP. The cells were then treated with TNF α (50 ng/ml), IL-1 β (10 ng/ml), or left untreated (UT) for 24 h. SEAP activity was measured by the chemiluminescence SEAP detection method and was represented as fold induction relative to the activity obtained from the untreated samples. Results shown are Mean $\pm$ S.D. of three experiments each performed in triplicate.

Fig. 8A.

Fig. 8B.

Fig. 8. Positional silence of κB (-816/-802) site on IKKi promoter assessed by exchange of the two κB sites.

C28/I2 cells were transiently transfected either with (A) wild-type p1000-IKKi-Luc vector, and its κB sites exchanged vector p1000-IKKi^{Ex κB} -Luc, or (B) mutated vector p1000-IKKi^{Mut κB (-833/-847)}-Luc, and its κB sites exchanged vector p1000-IKKi^{Ex Mut κB (-833/-847)}-Luc. The cells were then treated with $TNF\alpha$ (50 ng/ml), IL-1 β (10 ng/ml), or left untreated (UT) for 24 h. The luciferase activity was normalized to renilla luciferase activity using dual luciferase assay method and was represented as fold induction relative to the activity obtained from the untreated samples. Results shown are Mean $\pm$ S.D. of three experiments each performed in triplicate.

Fig. 9A.

Fig. 9B.

Fig. 9. NFκB p65 is involved in activation of IKKi promoter.

(A) C28/I2 cells were transiently transfected with the wild-type p1000IKKi-Luc construct, mutated constructs p1000IKKi Mut κB (-833/-847) -Luc, p1000IKKi Mut κB (-816/-802) -Luc, or p1000IKKi Mut κB (-833/-847, -816/-802) -Luc, together with expression vectors for NFκB p65, NFκB p50, dominant negative IκBα (S32A/S36A) or an empty vector. The luciferase activity was normalized to renilla luciferase activity using the dual luciferase assay method and was represented as fold induction relative to the activity obtained from the untreated samples. Results shown are Mean ± S.D. of three experiments each performed in triplicate. (B) C28/I2 cells were transiently transfected with the wild-type p1000IKKi-Luc construct, together with expression vectors for wild-type IκBα, dominant negative IκBα (S32A/S36A) or an empty

vector. The cells were then treated with TNF α (50 ng/ml), IL-1 β (10 ng/ml), or left untreated (UT) for 24 h. The luciferase activity was normalized to renilla luciferase activity using dual luciferase assay method and was represented as fold induction relative to the activity obtained from the untreated samples. Results shown are Mean $\pm$ S.D. of three experiments each performed in triplicate.

Figure 10. Interaction of NFκB p65 transcription factor and IKKi Promoter determined by EMSA and EMSSA.

(A) Nuclear protein extracts obtained from C28/I2 cells treated with TNFα for 30 min (lanes 1 and 2) or left untreated (lanes 3 and 4) were incubated with biotin labeled oligonucleotides containing the wild-type κB binding site (IKKi^{κB}) (lanes 1 and 3) or mutated κB binding site (IKKi^{Mut κB}) (lanes 2 and 4). (B) Nuclear protein extracts obtained from C28/I2 cells treated with TNFα for 30 min were incubated with biotin labeled oligonucleotides containing the wild-type κB binding site (IKKi^{κB}) as probe (Lane 1), or unlabeled 100-fold excess competitors: Consensus^{κB} (lane 2), IKKi^{κB} (lane 3), or IKKi^{Mut κB} (lane 4). (C) Nuclear protein extracts obtained from C28/I2 cells treated with TNFα for 30 min were pre-incubated with antibodies against NFκB p65 (lane 2), or NFκB p50 (lane 3) prior to incubation with biotin labeled IKKi^{κB}.

Table 1
Oligonucleotides Used in the Functional Analysis of the Human IKKi Gene Promoter

Name	Sequence (5' → 3') ^a	Position ^b
IKKi-GSP ^c	CCAGCAGGTCATCTGTGTGCCACAGGTA	+385/+358
IKKi-GSP-Nested ^c	GGCTGGTCACCTTCTGCCCCACACT	+324/+300
F1	CACCGCCACAAGGAGGCAG	+14/+32
F2	GAGCTTGGTGTCACCGC	+1/+19
F3	GAGAGCCAGGACTCAGTGCT	-20/-1
F4	ACACTGGAAGGCATCAGCTGG	-476/-456
R1	CCCATCTCCACTGCAGGGGA	+217/+198
IKKi-1976-F	CGGGGTACCGATGGGATGCCACCTTTACATCC	-1976/-1953
IKKi-1500-F	CGGGGTACCGTGGCTCACGCCTGTAATCCCGGC	-1500/-1477
IKKi-1000-F	CGGGGTACCGGTCTGTATGTGAGAAGTTGCAGC	-1000/-977
IKKi-500-F	CGGGGTACCGGTCTGTATGTGTGCTCAGGTC	-500/-477
IKKi-300-F	CGGGGTACCCCAACCTGCTCTGTTGACTCTGGGA	-300/-276
IKKi-100-F	CGGGGTACCGTGGCTGCACCGAGTTGCCTCTGT	-100/-77
IKKi-50-F	CGGGGTACCGAGACGGAATCAGGAAGTGCAGAGA	-50/-26
IKKi-1-R	CCGGAATTCCAGCACTGAGTCTCTGGCTCTCAGC	+1/-23
Mut ^{kB} (-833/-847)-F	GTGTGGCCATGGGAAAGGACCTCCAACATTCTATAGAAAC	-855/-816
Mut ^{kB} (-833/-847)-R	GTTTCTATAGAATGTTGGAGGTCCTTTCCCATGGCCACAC	-816/-855
Mut ^{kB} (-816/-802)-F	CAACATTCTATAGAAACATGGAAATGGCTGGTTTAAACCATTTCATATCC	-832/-783
Mut ^{kB} (-816/-802)-R	GGATATGAAATGGTTAAACCAGGCCATTCCATGTTTCTATAGAATGTTG	-783/-832
IKKi-1000L-F	CCGCTCGAGGGTCCTGTATGTGAGAAGTTGCAGC	-1000/-977
IKKi-1L-R	CCCAAGCTTCAGCACTGAGTCTCTGGCTCTCAGC	+1/-23
Ex-R	TTTCTATAGAATGTTGCCAGGAAATCCATGGGCCACACCTGGTGGTTTCC	-848/-867
Ex-F	CAACATTCTATAGAAAATGGGAAAGTCCCTCTTTTAAACCATTTCATATCCA	-801/-782
Ex ^{Mut} -F	CAACATTCTATAGAAAATGGGAAAGGACCTCTTTTAAACCATTTCATATCCA	-801/-782
Consensus ^{kB} -F	AGTTGAGGGGACTTTCCAGGC	-828/-849
Consensus ^{kB} -R	GCCTGGGAAAGTCCCTCAACT	-849/-828
IKKi ^{kB} -F	TGTTGGAGGGACTTTCCCATGG	-828/-849
IKKi ^{kB} -R	CCATGGGAAAGTCCCTCCAACA	-849/-828
Mut ^{kB} -F	TGTTGGAGGTCCTTTCCCATGG	-828/-849
Mut ^{kB} -R	CCATGGGAAAGGACCTCCAACA	-849/-828

^a Mutated nucleotides are underlined.

^b Positions are relative to IKKi transcription start site.

^c GSP is gene specific primer.

Table 2

Putative Core and Proximal Promoter Components as well as Cytokine (TNF α , IL-1 β) Response Elements in the Human IKKi Gene Promoter

Element ^a	Position ^b	Orientation	Similarity
κ B	-1913/-1899	forward	0.825
CCAAT-Box	-1732/-1722	forward	0.900
GC-Box	-1637/-1623	forward	0.881
C/EBP	-1627/-1613	reverse	0.921
CCAAT-Box	-1606/-1596	reverse	0.834
GC-Box	-892/-878	forward	0.902
GC-Box	-887/-873	forward	0.955
κ B ^c	-847/-833	reverse	0.989
κ B ^c	-816/-802	forward	0.981
TATA-Box	-608/-592	forward	0.893
CCAAT-Box	-559/-549	forward	0.957
GC-Box	-510/-496	forward	0.923
AP1	-413/-403	forward	1.000
κ B	-339/-325	forward	0.898
C/EBP	-307/-293	reverse	0.968
κ B	-281/-267	reverse	0.926
κ B	-244/-230	forward	0.896

^a Elements were identified using the MatInspector v7.2 (www.genomatix.de) program (Quandt et al, 1995).

^b Positions are relative to IKKi transcription start site.

^c Those two putative κ B sites were located in the TNF α /IL-1 β response region of IKKi promoter and tested for their functionality.