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NF-κB Subunit-specific Regulation of the IκBα Promoter*

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Stimulation of endothelial cells by cytokines and bacterial lipopolysaccharide leads to activation of the transcription factor NF-κB. NF-κB in turn regulates the expression of several genes involved in the inflammatory reaction, including cell adhesion molecules, interleukins, and transcription factors. One of these induced genes encodes an inhibitor of NF-κB, ECI-6/IκBα, that contains in its 5′ regulatory region six consensus binding sites for NF-κB. We demonstrate here that these sites display striking differences in their ability *in vitro* to bind to various NF-κB subunits. *In vivo*, all six sites contribute, though to varying degrees, to transcription from the ECI-6/IκBα promoter, as demonstrated by deletion and mutation analysis. Among the NF-κB subunits tested p65, the p65/p50 heterodimer and, to a lesser extent, c-Rel, are able to activate transcription, whereas p50 or p50/RelB were inactive. Since many genes regulated by NF-κB contain only one or two DNA-binding sites for this transcription factor, the presence of six functional NF-κB-binding sites in the ECI-6/IκBα promoter represents a unique feature of this gene.

The NF-κB/rel family of transcription factors plays a pleiotropic role in the regulation of gene expression in a wide variety of cell types, including T and B cells, fibroblasts, EC,[1] keratinocytes, and cells of the monocyte/macrophage lineage (for reviews, see Refs. 1–4). Members of the family in mammals include p65 (RelA; 5–7), p50 (NF-κB-1; 8–11), RelB (I-Rel; 12, 13), c-Rel (14), and p52 (NF-κB-2, p50B; 15–18), some of which are able to form homo- or heterodimers with different binding specificity toward variants of the decameric DNA recognition site ("consensus" GGGRNNTYCC), and with different transactivating properties (1, 19). Activation of NF-κB occurs in response to widely divergent stimuli, including LPS, viruses, inflammatory cytokines, T and B cell mitogens, or physical and oxidative stress, and leads to transcription of genes encoding cytokines, cell surface receptors, and acute phase proteins, as well as transcription factors, most interestingly including those of the p50 precursor and of c-*rel* (20–22).

Regulation of NF-κB/rel activity is mediated by complex formation of the potentially active factor with an inhibitory subunit termed IκBα that sequesters the transcription factor(s) in the cytoplasm. IκBαs (MAD-3, pp40, RL/IF-1, and ECI-6) have been cloned from various species and shown to interact with the p65 subunit (23–26). *In vitro*, IκBα also blocks binding of NF-κB to DNA (27). Upon stimulation of the cell, IκBα is modified, most likely by phosphorylation, leading to dissociation from the NF-κB complex and subsequent translocation of the transcription factor to the nucleus. The signals leading to modification of IκBα presumably involve the generation of reactive oxygen intermediates (28). This system allows, without the need for prior RNA or protein synthesis, a very rapid, and usually transient response of the cell to environmental stimuli.

While the mechanisms leading to activation of NF-κB have been studied extensively, until recently very little was known about how NF-κB could be down-regulated to prestimulation levels once the responsive genes have been transcribed. We have recently cloned a gene termed *ECI-6* from cytokine-stimulated porcine aortic EC (24) encoding IκBα. Interestingly, ECI-6/IκBα mRNA is up-regulated by tumor necrosis factor α, IL-1 or LPS, the same agents that activate NF-κB. The up-regulation of IκBα is antioxidant sensitive (24, 29), suggesting the involvement of NF-κB in its regulation. Indeed, ectopic expression of p65 in several cell types leads to expression of endogenous IκBα (29–32). The mutual regulation of NF-κB and IκBα has led to the concept of a regulatory circuit, where the expression of IκBα by NF-κB leads in turn to inhibition of NF-κB (24, 29, 30, 32).

In this report, we demonstrate that this expression is mediated through the direct interaction of NF-κB with six specific binding sites in the ECI-*6/IκBα* promoter. Various *in vitro* translated members of the NF-κB family as well as NF-κB from nuclear extracts of stimulated EC bind to different degrees to these sites in EMSA. The availability of a highly efficient transfection method for EC (transferrinfection) has enabled us to evaluate the *in vivo* contribution of each site, as well as the ability of individual members of the NF-κB family to activate transcription from the *ECI-6/IκBα* promoter in this cell type.

EXPERIMENTAL PROCEDURES

DNA Sequencing—Genomic clones containing the 5′ regulatory region of *ECI-6/IκBα* were obtained by screening a porcine genomic library (Clonetech) as previously described (24). A 5.5-kb *Eco*RI fragment containing 2.1 kb upstream from the transcription start site was subcloned and sequenced using a commercial kit (United States Biochemicals Chemical Corp.).

Plasmid Constructs—Expression plasmids pCMV4TΔp65 and pC4–85 for human p65 and c-*rel*, respectively, were obtained from Dr. W. C. Greene. cDNAs for human p50 and murine *rel*B (plasmids pET3b and relB M2) were from Dr. C. Scheidereit and Dr. R. Bravo, respectively, and subcloned into a CMV expression vector. RcCMV-p65 used for *in vitro* translation was from Dr. P. A. Baeuerle. A series of deletion or substitution mutants of the ECI-6/IκBα promoter in the luciferase ex-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) Z30209.

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[1] The abbreviations used are: EC, endothelial cells; LPS, lipopolysaccharide; kb, kilobase(s); CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; BrdU, bromodesoxyuridine; IL, interleukin; PAEC, porcine endothelial cells; PAGE, polyacrylamide gel electrophoresis.

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-2114                                                        GAAT TCCTTATCAG
-2100 TGAAAATATC ATTTAAAAAT AAAGTAATAG TAGTTGTTTC CCCAGGTAAT CACTGAAAAT
-2040 CCATTGCCTG TTGACCAGAA CCAAAATGTG TATTAAAGGA AGTTCTTCAG GCAAAAATTT
-1980 AGAACTATAA CCAAAAAAAT GAGGAGTATT GGAAAGATCA TGAATGGAGG AAATAATGTT
-1920 TTAATTATCT TGATGTTTAT TAGTCTATTC AAAATGTTAT TCAAAAACAA ATATATGGAG
-1860 ATTTAAAGAA GGCTAATCCT TATGTCAGGA AAGAAGACCT CACTCATAGG CTTGTCTCTC
-1800 ATGATAACCC AAGAGATAAC ATTTTTTGAA CATTTCAACA ATGCAATTAG AGAATTATAG
-1740 GGTTTAGCCC CAGAATCAGA AAGAGGTATT CAGAAAAATC TATTTTCAAG TTACACAAGT
-1680 TTAATTGTTG GCATTTTGAA GATGCCCACT TAATGGTACT AGAGAAGTTT CAAGGCAAAC
-1620 TTACAAAAAT CTTATATGAT TATTTGATAC TGCAACAACT TGGCTAGTCT ACTCAGTCAA
-1560 CATTGTGCAT GAAAACTTAC TAAAACTGCT GATGATATTT TTTTATCCAT TTATTCTGCT
-1500 AATTTTGGTA AAAAACTATA AATTTGAGAC TTGGACAAGA TCCCAAACCT CACATTTCCT
-1440 TTGAATATCT ACCCATGGTT ACATGTAACT ACACTCCTAT GTGCTGGCCA ATGTTGTATA
-1380 ATCACAGGAT GAACTGAAGC TTTTCCTTGC TAAAGAGTTT TAGCCCTCCA AGAGTAAGTT
-1320 ATTCCAATTT ATCTCTGCAG CAACAGCAAC ATTAATTTTC ATTCAGCTCT CATAACATAG
-1260 CTTTTTAAAT ACTTGCCATT TTTGAAAAGA TCCAAGTTGC TTTATTAGGG CCTGGACCAT
-1200 TTCTAGAAGT AGATGAATGC ATTCCTTTCA TTGGCTAGGA GGTGGGGATG GGGCAGAGAG
-1140 CATACTTCTG TTTCTGCAGC TGAGACCTGG ACATGGTGAA CCTGGAGTAG CTACCCATAT
-1080 GGCATGGACA GGTCCAACTG CTGCCCCCTC CTTTGTCCCC CAAGAAGCCA GCAGGGGCAG
-1020 GATGAAGGCC ACCTTGGGCT GCCCTGAGCC TCCTGCAGTA TGCCTGGCAA CTACTTTCTT
 -960 AGCCATCTTT AAGGCCCAAT CTTGGGTAAA ATACTACTCA ACCCATTCTT TAGCCACCTT
 -900 CTCCAAATGC TTCTAGAAAG CGGCCCCCAC AAGTAGGTTC TCTGCAGCAG CACAGTGCAA
 -840 ATGGAGGAAC ACGACCTCAG TAATTATTTT GTCACTGCAA AGTATCTACA ACCTTTGCTA
 -780 TAAAAATTAA CACCTTGCTT TCCCTGAAAA ATAGCCCAGT CATATCCAGC ATTTTCCAGC
 -720 ATCCAGGGCA GAGTGCTTGC TCCTCCCCCA GTCAACAGGA CTGTTCATAC CGAGNAAATG
 -660 ATTTGAGGGT TCTCTAAGCA TTTACGCTGT TAATGCTAAA GCTTTCACGA CTTCTACCTG
 -600 AGGGGGGCTT GAGGGAGGGG GGAGGTTTAT GTCCCTGCAC TGCCAGGAGC CTGGTCTTTG
 -540 GTAGGAACGC AGAGGCAGCC GGCGACCTTC CACCCTCAGT GTGTCCTTCC CCAGGAGTTT
 -480 AGGGAAGTGA ATCCCTAGAT CCAGCCAACA TTTCCACTCC CATTTTCAAG AGATTAAAAA
 -420 AAAAAAAAAA AAAAAAAAAG GAAAGCATCG GCAGGTCAGC AAACCAGCAG TTCTCCATCC
 -360 TTGGGATCTT AGCAGCCGAC GACCCCAAAT CAAATCGATC GTGGGAAACC CCAGGGAAAA
        BS 6                                          BS 3
 -300 TAAGGTTCCA TGCAGAGGGC CAGGATTACT GACTGCAGGC TGCAGGGAAG TACCGGGGGA
                                                          BS 7
 -240 GGGGGCCGGG TCGGGAGGAC TTTCCAGCCA CTCAGCGTGC ATTAAAAAGT TCCCTGTACA
                    BS 1                                  BS 8
 -180 TGACCCCAGT GGCTCATCGC AGGGAGTTTC TCTGATGAAC CCGGGCGCGG GGTTTAGGCT
                              BS 5
 -120 TCTTTTTCCC CCAGCAGAGG ACGAGGCCAG TTCTCTTTTC TGGTCTGACT GGCTTGGAAA
  -60 TTCCCCGAGC TTGACCCCGC CCAGGAGAAA TCCCCTGCCA GCGTTTATAG GGCCGCGGCG
       BS 2                       BS 4                             +1
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FIG. 1. **Nucleotide sequence of the upstream regulatory region of *ECI-6/IκBα*.** Numbering of nucleotides is shown on the *left* and starts at the transcription start site (*+1*). The eight motifs (*BS1–8*) with homology to the NF-κB consensus sequence that were further analyzed are *underlined*, and the TATA box is indicated in *bold*.

pression vector UBT.Luc (33) was prepared by subcloning at appropriate restriction sites or by polymerase chain reaction. All constructs were confirmed by DNA sequencing.

Nuclear Run Off Transcription Assay—Nuclear run off experiments were performed as described (34). Nuclei were isolated from 3 × 10^7 porcine endothelial cells (PAEC), either unstimulated or stimulated with LPS (100 ng/ml) for 1 and 3 h. Equal numbers of counts/minute of radiolabeled RNA were hybridized to cDNA probes (ECI-6/IκBα, human β-actin, rat glyceraldehyde-3-phosphate dehydrogenase, and ECI-12, a gene from PAEC which is not regulated at the transcriptional level)[2] immobilized on nylon membranes (Hybond-N, Amersham Corp.).

In Vitro Transcription and Translation—For *in vitro* transcription, the cDNAs for p50 and c-*rel* were subcloned into pKSM13, and for p65 the plasmid RcCMV-p65 was used. Ten μg of each plasmid were linearized and transcribed using T7 or T3 RNA polymerase (Stratagene) in a

[2] Q. Cheng, F. H. Bach, and R. de Martin, unpublished results.

TABLE I
Sequences and locations of consensus NF-κB binding motifs in the ECI-6/κBα promoter

Site	Location	Sequence	Homology
			%
BS 1	–215	AGGACTTTCC	90
BS 2	–55	GGAAATTCCCC	90
BS 3	–305	GGGAAACCCC	100
BS 4	–30	GAGAAATCCCC	90
BS 5	–150	GGGAGTTTCTC	90
BS 6	–350	GGGATCTTAGC	80
BS 7	–245	GGGAAGTACC	90
BS 8	–185	AAAAGTTCCC	70
NF-κB consensus		GGGRNNYYCC [a]	

[a] R = A or G; Y = C or T. The NFκB consensus sequence is taken from Baeuerle (1991).

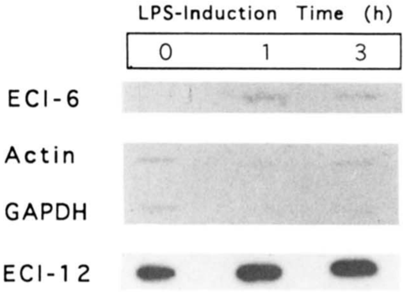

FIG. 2. **Nuclear run off analysis.** Radiolabeled RNA was prepared from nuclei isolated from porcine aortic endothelial cells (PAEC) stimulated with LPS (100 ng/ml) for 0, 1, and 3 h, and hybridized to immobilized cDNA probes for ECI-6/IκBα and, as controls, for human β-actin, rat glyceraldehyde-3-phosphate dehydrogenase, and ECI-12 (an uncharacterized gene from PAEC that is not regulated at the transcriptional level).[2]

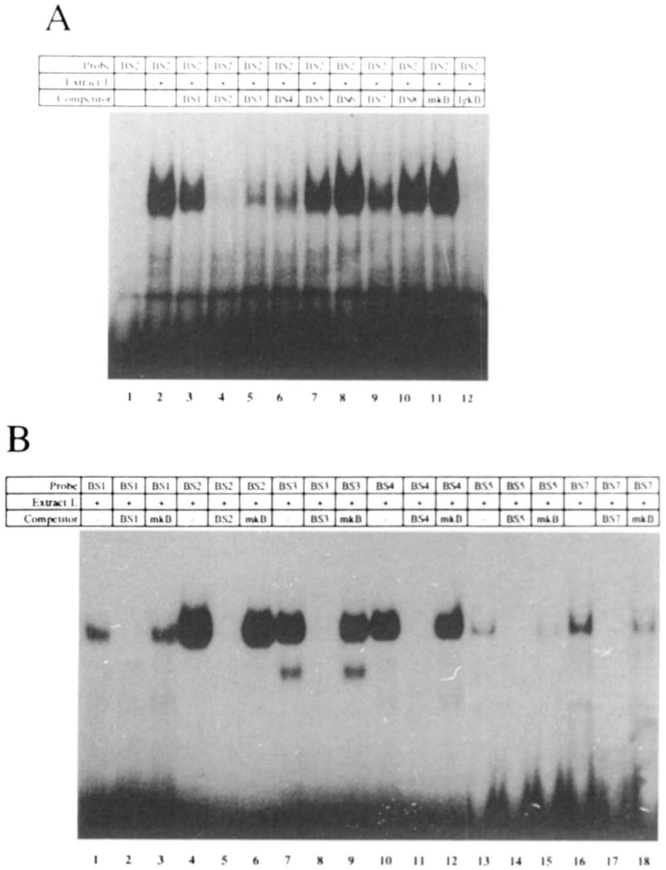

FIG. 3. **Electrophoretic mobility shift assay of NF-κB binding to the BS sequences in the ECI-6/IκBα promoter.** *A*, competition experiments: nuclear extract from porcine aortic endothelial cells (PAEC) stimulated with LPS for 2 h (*Extract L*) was probed for binding to the labeled NF-κB binding site BS2 (*lane 2*). *Lane 1*, labeled BS2 probe alone. Binding was competed with a 100-fold molar excess of unlabeled oligonucleotides representing binding sites 1– 8 as indicated in *lanes 3–10* (BS1–BS8, see Table I). Specificity of binding was demonstrated by competition with a mutated NF-κB site (*mκB*, *lane 11*) or with a NF-κB site from the immunoglobulin κ light chain enhancer (*lane 12*). *B*, binding of proteins from nuclear extract from LPS-stimulated PAEC to individually labeled oligonucleotides BS1–5 and 7 (*lanes 1, 4, 7, 10, 13,* and *16*). Competitions with the respective unlabeled probes (*lanes 2, 5, 8, 11, 14,* and *17*) and with a mutated NF-κB site mκB (*lanes 3, 6, 9, 12, 15,* and *18*) are shown.

total volume of 100 µl, including 0.25 mM m^{7}G(5′)ppp(5′)G. After DNase I treatment, 1/20 of the purified RNA was translated in 50 µl of wheat germ extract (Promega). Parallel reactions including [^{35}S]methionine were carried out to confirm the correct size of the translated products by analysis on SDS-PAGE (data not shown). For cotranslation of p50 and p65, a coupled transcription-translation system (TNT, Promega) was used.

Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear proteins from porcine aortic endothelial cells stimulated with 100 ng/ml LPS for 2 h or from non-stimulated cells were extracted as described (35). The double-stranded oligonucleotide probes were labeled by filling in the *Eco*RI overhangs with Klenow enzyme in the presence of [^{32}P]dATP, and 0.2 ng (30,000 counts/minute) used per lane in EMSA. The sequences of oligonucleotides used for EMSA were as follows (5′ to 3′, only the top strand is given): BS1, AATTCGTCGGGAGGACTTTCCAGCCAG; BS2, AATTCGGCTTGGAAATTCCCCGAGCG; BS3, AATTCGATCGTGGGAAACCCCAGGG AG; BS4, AATTCGCCCAGGAGAAATCCCCTGCCAG; BS5, AATTCATCGCAGGGAGTTTCTCTGATGG; BS6, AATTCTCCTTGGGATCTTAGCAGCCG; BS7, AATTCTGCAGGGAAGTACCGGGGG; and BS8, AATTCATTAAAAAGTTCCCTGTACG; IgκB, a NF-κB-binding site from the human immunoglobulin κ light chain enhancer (36): AATTCAGAGGGGGATTTCCCAGAGG; mκB, a mutated NF-κB site: AGCTTAGATTTTACTTTCCGAGAGGA. A 100-fold molar excess of unlabeled oligonucleotides was used for competition experiments. The resulting complexes were separated on 5% polyacrylamide gels. Polyclonal rabbit anti-p65, anti-p50, and anti-FosB antibodies were obtained from Santa Cruz Biotechnology, CA, and 1 µg added to the binding reaction 30 min before addition of the probe.

UV Cross-linking—Experiments were performed as described (37). Briefly, 15 µg of nuclear extract from EC stimulated with LPS for 2 h were incubated with 1.5 × 10^6 counts/min of a BrdU/[^{32}P]dATP-labeled (top strand) oligonucleotide representing the BS2 site. After gel electrophoresis, the protein-DNA complexes were covalently cross-linked by ultraviolet irradiation (302 nm), digested with DNase I, eluted from the gel, and analyzed by SDS-PAGE and autoradiography.

Transfections and Reporter Gene Assays—EC were isolated from pig aorta as described (38), grown in complete Dulbecco's modified Eagle's medium (2% sodium pyruvate, 1% L-glutamine (Life Technologies, Inc.), 10% heat-inactivated fetal calf serum, and antibiotics) and transfected using the transferrinfection method (39, 40). Briefly, confluent EC in 6-well plates were incubated for 2.5 h with a complex consisting of transferrin/poly(L)lysine/adenovirus containing a total amount of 3 µg of plasmid DNAs, including Rous sarcoma virus.βgal as internal control, in 1 ml of complete medium, then washed once with Dulbecco's modified Eagle's medium, and grown further for 40 h. Luciferase levels were determined and expressed as relative light units normalized for β-galactosidase expression (41).

RESULTS

Multiple NF-κB-binding Sites (BS) Are Present in the Promoter Region of ECI-6/IκBα—We have previously isolated genomic clones of *ECI-6/IκBα* and determined the transcription start site by primer extension and RNase protection (24). 2.1 kb of the upstream regulatory region are shown in Fig. 1. By searching for NF-κB-binding sites using the consensus motif GGGRNNTYCC (1), eight potential sites (designated BS1–8) were found within the first 400 base pairs upstream of the transcription start site; no NF-κB binding motifs were found further upstream. Sequences and location of these sites are given in Table I.

Induction of ECI-6/IκBα Is Regulated at the Transcriptional Level—ECI-6/IκBα specific mRNA is inducible by tumor necro-

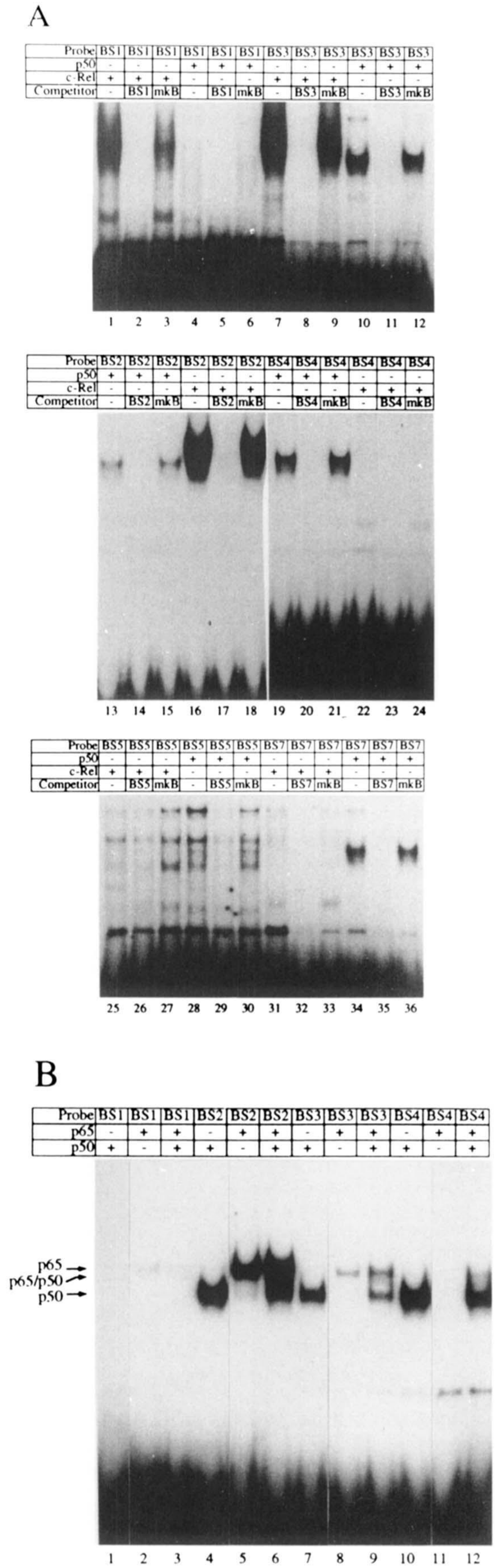

FIG. 4. **Differential binding of *in vitro* translated members of the NF-κB family to the different NF-κB-binding sites.** *A*, p50 and c-Rel were produced by *in vitro* translation in a wheat germ extract, incubated with labeled BS1–5 or 7 probes and separated on polyacrylamide gels (*lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31,* and *34*). Specificity of the binding was demonstrated by competition with the respective unlabeled probes (*lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32,* and *35*) and with a mutated NF-κB site (*lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33,* and *36*). *B*, binding of BS1 (*lanes 1–3*), BS2 (*lanes 4–6*), BS3 (*lanes 7–9*), and BS4 (*lanes 10–12*) to *in vitro* translated p50 and p65. p50 and p65 were translated either individually or cotranslated in wheat germ extract and included in the binding reaction as indicated.

TABLE II
Summary of the binding properties of BS sites to nuclear extract and individual NF-κB subunits

Binding site	Nuclear extract	p50	c-rel	p65	p65/p50[a]
1	+	−	+	(+)	(+)
2	+	(+)	+	+	(+)
3	+	+	+	+	(+)
4	+	+	−	(+)	(+)
5	+	−	−	ND	ND
6	−	−	−	ND	ND
7	+	+	−	ND	ND
8	−	−	−	ND	ND

[a] No quantification is given; ND, not determined; (+) weak binding.

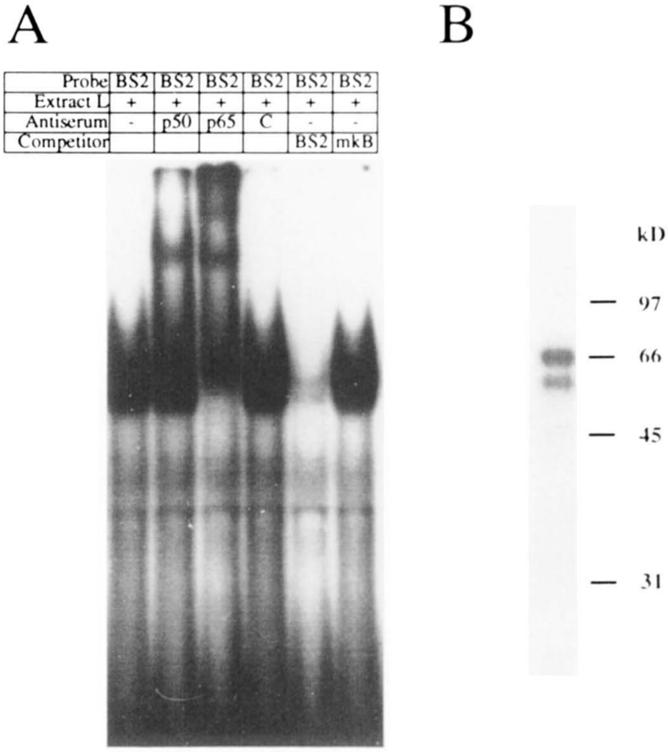

FIG. 5. **p65 and p50 are present in nuclear extracts of LPS-stimulated EC.** *A*, partial inhibition of the binding of nuclear extract to the BS2 probe by anti-p65 and anti-p50 antibodies. Before the addition of the labeled BS2 probe, the binding reaction was preincubated either with anti-p65 (*p65*), anti-p50 (*p50*), or with a control antibody (*C*). Competitors BS2 and mκB were as in Fig. 3*A*. *B*, cross-linking of proteins binding to the BrdU/^{32}P-labeled double-stranded BS2 oligonucleotide. After preparative EMSA, the gel was irradiated with UV light, protein complexes linked to the oligonucleotide recovered, digested with DNase, and analyzed by SDS-PAGE.

sis factor α, IL-1, and LPS (24). To determine whether this inducible mRNA accumulation is due to transcriptional activation, nuclear run off experiments were performed using nuclei from porcine EC treated with LPS (Fig. 2). *ECI-6/IκBα* is actively transcribed in LPS-stimulated EC, whereas no specific transcripts are detectable in the nuclei of unstimulated cells. Scanning of the nuclear run off and the Northern blot (24) at 1-h post-stimulation revealed a 8.1- and 8.5-fold increase of the newly synthesized and of the steady-state levels, respectively, indicating that ECI-6/IκBα mRNA accumulation is almost exclusively due to transcriptional regulation (data not shown).

Multiple Sites in the ECI-6/IκBα Promoter Can Bind NF-κB in Vitro—BS2 has already been established as a functional *in vitro* binding site for NF-κB by EMSA studies (24). To evaluate the other BS sites as functional elements, we have used unlabeled oligonucleotides representing each BS sequence to compete with the labeled BS2 site in EMSA. Fig. 3*A* shows that BS1, 3, 4, 5, and 7, as well as a NF-κB site from the immunoglobulin κ light chain enhancer (IgκB) are able to compete with the labeled oligonucleotide BS2 site to varying degrees. BS6, BS8, and a mutated NF-κB (mκB) site do not compete. Individual labeling of BS1, 3, 4, 5, and 7 confirmed their ability to bind NF-κB from nuclear extracts, with the respective binding capacities corresponding to the results from the competition experiments.

The BS Sequences in the ECI-6/IκBα Promoter Display Different Binding Specificity Toward Individual Members of the NF-κB Family—Different NF-κB subunits have been shown to specifically bind to certain target sequences but not others (42). Therefore, p50 and c-Rel were *in vitro* translated in wheat germ extract and probed for binding to labeled sites BS1, 2, 3, 4, 5, and 7. Fig. 4*A* shows that p50 specifically binds to BS2, 3, 4, and 7, but not to BS1 and 5 (the weak and atypical interaction with BS5 was not scored as positive). c-Rel binds preferentially to BS1, 2, and 3, with no detectable binding to BS4, 5, and 7.

Since in EC as well as in many other cell types the predominant species of NF-κB is the p50-p65 heterodimer, we have translated these two subunits either individually or cotranslated them *in vitro* in a coupled transcription-translation reaction, and assayed for their binding ability to BS1 to 4. Binding of p65 was observed predominantly to the BS2 site (Fig. 4*B*); BS1, 3, and 4 displayed weak binding, which, in the case of BS1 and 4, was visible only after longer exposure. Only low levels of the heterodimer were observed at all sites tested; this however, might rather reflect the smaller extend of heterodimer formation between p65 and p50 than the affinity of binding (see below). A compilation of the results from the EMSA studies is given in Table II.

Nuclear Extract from LPS-stimulated EC Contains p65 and p50—To address the question, which members of the NF-κB family are present in the nuclear extracts from LPS-stimulated EC, supershift, and UV-cross-linking experiments were performed. As shown in Fig. 5*A*, binding of NF-κB from nuclear extract to the BS2 site is partially inhibited both by anti-p65 and anti-p50 antibodies, but not by a control (anti-FosB) antibody, suggesting that the extract from LPS-stimulated endothelial cells contains p65 and p50. Upon UV-cross-linking (Fig. 5*B*) of bound proteins from EC nuclear extract to the BS2 site, DNase digestion, and resolution on denaturing PAGE, two bands of 50 and 65 kDa are detected, indicating the presence of p65 and p50 NF-κB subunits in LPS-stimulated EC.

Expression of an ECI-6/IκBα Promoter-Reporter Fusion Gene Is Dependent on the Presence of the p65 Subunit or c-Rel—The distinct *in vitro* binding activity of each BS to different members of the NF-κB family prompted investigation of the *in vivo* interaction between the ECI-6/IκBα promoter and various NF-κB proteins. An ECI-6/IκBα promoter-luciferase reporter fusion plasmid (p600) containing all of the BS sequences was cotransfected into EC together with CMV promoter-driven expression vectors carrying genes for either p50, p65, c-Rel, or RelB, or with a combination of p50 plus each of the other members (Fig. 6). In EC, transfection with p50 or relB did not induce the expression of the reporter gene, whereas p65 alone was able to confer a more than 300-fold increase of luciferase activity as compared with control levels. Addition of p50 resulted in a further 2-fold increase. A lower (60-fold) stimulation of luciferase activity was induced by c-Rel. Cotransfection of

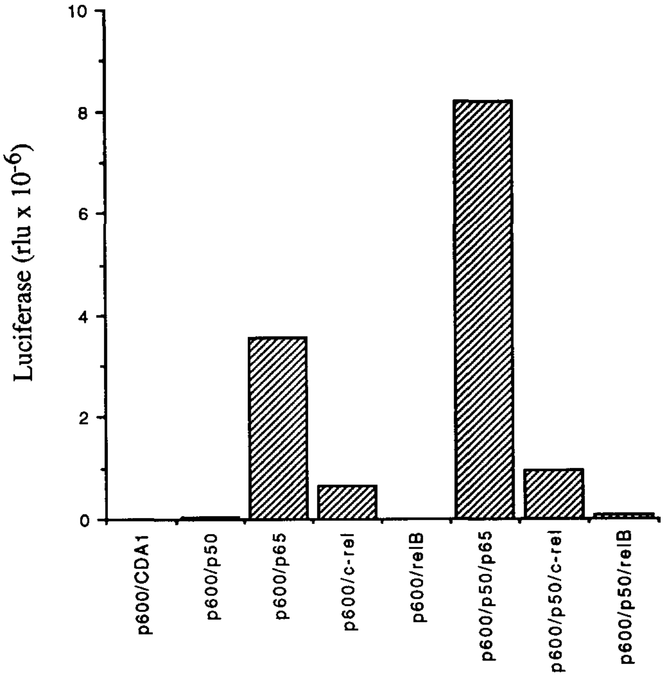

FIG. 6. **Expression of an ECI-6/IκBα promoter-reporter gene by different members of the NF-κB family.** An ECI-6/IκBα promoter-luciferase reporter gene construct, p600 (see Fig. 7) was cotransfected into EC together with expression vectors for various NF-κB subunits as indicated. All transfections included an Rous sarcoma virus.βgal expression vector as internal control. *CDA1*, control expression vector. Luciferase activity was determined 40 h later and normalized for β-galactosidase expression.

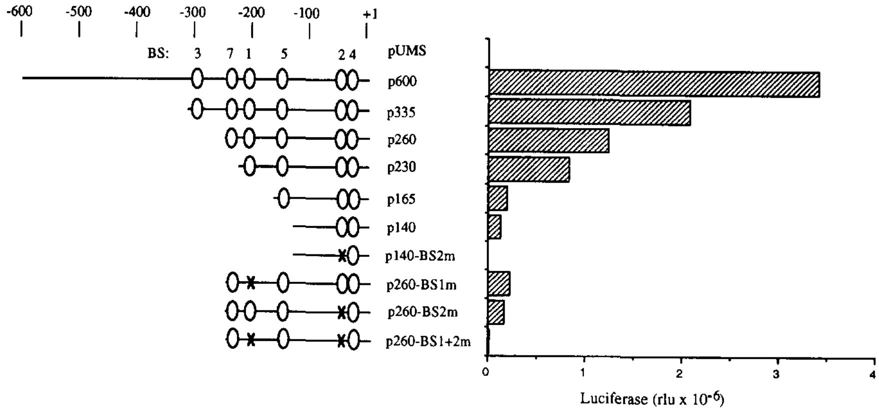

FIG. 7. **Mutation analysis of the BS sequences in the ECI-6/IκBα promoter.** Various ECI-6/IκBα promoter deletion and substitution mutants were prepared as described under "Materials and Methods" and are presented schematically on the left with the positions of the BS motifs indicated. All constructs were cotransfected together with an expression vector for p65 into EC. *BS1m* and *BS2m*, mutated BS1 and BS2 sites, respectively; *BS1+2m*: BS1/BS2 double mutation; *pUMS*, control luciferase vector. Luciferase values were analyzed as in Fig. 6.

p50 with c-Rel provided a further 1.5-fold activation above that seen with c-Rel alone.

Deletion and Mutation Analysis of Different NF-κB-binding Sites—To define the contribution of each BS to NF-κB-mediated activation, a series of deletion or substitution mutants was prepared. The resulting plasmids are presented schematically in Fig. 7. Each construct was cotransfected with an expression vector for p65 into EC. Sequential deletion of BS3, 7, and 1 resulted in a gradual loss of p65-mediated expression of the reporter gene, with less additional reduction when deleting the BS5 site. Deletion of the 300-base pair fragment upstream of the BS3 site also resulted in a 40% reduction of luciferase expression. This effect might be due to deletion of the BS6 site. BS6 by itself did not show detectable binding in EMSA but could still be functional *in vivo*. Additional mutation of BS2, leaving only BS4 intact, almost completely abolished expression. In the p260 construct, mutation of either BS1 or BS2 caused a more marked decrease in expression than deletion of BS7. Mutation of both BS1 and 2, leaving BS4, 5, and 7 intact, reduced expression to base-line levels, indicating that these two sites are of particular importance.

DISCUSSION

NF-κB is a key transcription factor involved in both transient and constitutive expression of various genes elicited by diverse external stimuli and in a variety of cell types. While constitutive expression in lymphoid tissues correlates with the presence of p50/RelB heterodimers, the p65-p50 complex is usually involved in transient gene expression (43). The activation of p65/p50-NF-κB is achieved by phosphorylation, dissociation, and subsequent degradation of a cytoplasmic inhibitor, IκBα (1, 44). Recent studies on IκBα have shown that, in various cell types, transfected NF-κB is able to subsequently induce the endogenous mRNA and protein synthesis of its own inhibitor, IκBα (29, 30, 32), and that the reappearance of IκBα protein correlates with the disappearance of nuclear NF-κB binding activity (29). These findings have led to the concept of a regulatory circuit between NF-κB and IκBα that provides a dynamic feedback mechanism to ensure the transient nature of NF-κB induction.

In support of this view, we demonstrate here that induction of the ECI-6/IκBα promoter is directly regulated by distinct members of the NF-κB family. We characterized six potential NF-κB-binding sites (BS1, 2, 3, 4, 5, and 7), which bind to varying degrees to LPS-induced NF-κB from nuclear extracts of EC and to *in vitro* translated NF-κB subunits in EMSA (Figs. 3 and 4). *In vivo*, all six BS sites contribute to different degrees to expression of the ECI-6/IκBα gene.

Cotransfection of expression vectors for p50 plus p65 induced strong transcriptional activation of the ECI-6/IκBα promoter-reporter gene (Fig. 6), consistent with the view that p50/p65 is the physiologically most relevant factor for transient transcriptional activation in most cell types. Transfection of the p65 subunit was able to evoke 40% of the expression seen with p65 plus p50. This could be due to an excess of endogenous p50 constitutively present in the cells. However, we have not detected p50-NF-κB binding activity in the nuclear extracts of non-stimulated cells by EMSA (data not shown). Alternatively, p65 could lead to expression of p50, as this has been demonstrated in Jurkat cells (20) and subsequent formation of the heterodimer. In the case of the VCAM-1 enhancer, both p65 and p65/p50 can activate transcription: low concentrations of p65 were found to act in concert with p50, whereas at high concentrations, p65 alone could stimulate transcription (45). A similar mechanism could be operative in the ECI-6/IκBα promoter.

It is worth noting that in our experiments NF-κB, either in the form of the p65-p50 heterodimer, or as the p65 homodimer, was alone sufficient to induce expression from the ECI-6/IκBα promoter. Other transcription factors, such as AP-1, HMG I(Y), or C/EBP, that have been described to act in concert with NF-κB in the regulation of other genes (46–48), seem not to be necessary. SP-1 might be an exception, since a potential binding site for this factor is located at position −44 to −39; In the HIV-1 enhancer, SP-1 has been demonstrated to interact with NF-κB to mediate inducible transactivation (49).

One or two NF-κB-binding sites are present in the 5′ regulatory regions of several genes relevant for EC activation, *e.g.* those encoding the cell adhesion molecules ELAM-1 and VCAM-1 (45, 50–52) or the cytokines IL-6 and IL-8 (53–56). Therefore, the presence of multiple NF-κB-binding sites in the ECI-6/IκBα promoter is unexpected; all six sites are functional *in vitro* and contribute, although to different degrees, to p65- and p50-p65-mediated *ECI-6/IκBα* expression in EC. Since the NF-κB/IκBα system is not restricted to EC, the presence of these multiple sites offers the possibility, that in other cell types, or in response to different stimuli, other members of the NF-κB family (including not yet discovered members) might be operative that preferentially utilize certain of the BS sites. In support of this view, we find that c-Rel, as compared with p65, displays a different preference toward certain sites *in vitro*.

Alternatively, the number of binding sites in a promoter might influence the quality of the response to a transcription factor. In a different system, transcription levels from five tandem GAL4-binding sites followed a sigmoid curve in response to increasing amounts of GAL4-VP16, showing a "threshold" effect in response to low levels of the transcription factor (57), whereas one or two sites yielded a linear response. Correspondingly, the presence of multiple κB-binding sites in the ECI-6/IκBα promoter might result in a more pronounced yes/no response of the gene depending on the concentration of NF-κB.

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