MOLECULAR AND CELLULAR BIOLOGY, July 1997, p. 3733–3743 Vol. 17, No. 7
0270-7306/97/$04.00+0


# Recombination Signal Sequence Binding Protein Jκ Is Constitutively Bound to the NF-κB Site of the Interleukin-6 Promoter and Acts as a Negative Regulatory Factor

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Received 4 November 1996/Returned for modification 6 December 1996/Accepted 21 April 1997

**Analysis by electrophoretic mobility shift assays (EMSA) of the different proteins associated with the κB sequence of the interleukin-6 (IL-6) promoter (IL6-κB) allowed us to detect a specific complex formed with the recombination signal sequence binding protein Jκ (RBP-Jκ). Single-base exchanges within the oligonucleotide sequence defined the critical base pairs involved in the interaction between RBP-Jκ and the IL6-κB motif. Binding analysis suggests that the amount of RBP-Jκ protein present in the nucleus is severalfold higher than the total amount of inducible NF-κB complexes but that the latter bind DNA with a 10-fold-higher affinity. A reporter gene study was performed to determine the functional implication of this binding; we found that the constitutive occupancy of the IL6-κB site by the RBP-Jκ protein was responsible for the low basal levels of IL-6 promoter activity in L929sA fibrosarcoma cells and that RBP-Jκ partially blocked access of NF-κB complexes to the IL-6 promoter. We propose that such a mechanism could be involved in the constitutive repression of the IL-6 gene under normal physiological conditions.**

Although interleukin-6 (IL-6) was originally described as a pleiotropic cytokine released by activated monocytes which plays a crucial role in the immune response, it can also be induced in many other cell types, including fibroblasts (for a review, see reference 23). IL-6 stimulates differentiation of, among others, T and B cells, production of immunoglobulins (Igs), proliferation of thymocytes, differentiation of PC12 cells, and expression of acute-phase proteins in the liver. The IL-6 gene is induced in response to bacterial endotoxins, viral infection, phytohemagglutinin, or a variety of other mediators of inflammation, including cytokines such as tumor necrosis factor (TNF) and IL-1 (reviewed in reference 1).

Loss of regulation of the IL-6 gene is involved in certain pathological conditions such as rheumatoid arthritis and different types of tumors including myelomas or after viral infection by human T-cell leukemia virus type 1 or human immunodeficiency virus type 1 (for a review, see reference 3).

It has also been reported that IL-6 exerts a negative effect on the growth of some tumor types, e.g., melanoma at the early stage and breast carcinoma (32, 43).

A 1.2-kb fragment of the 5′-flanking region of the IL-6 gene contains all of the elements necessary for its induction by a variety of stimuli (42). Promoter deletion analysis revealed the presence and the functional involvement of an NF-κB element between positions −73 and −63 (31, 52, 63). A multiple response element (MRE) between −173 and −145 has also been defined (40). This region contains a consensus binding site for the transcription factor C/EBPβ/NF-IL6, which is involved in the tissue-specific transcription of the IL-6 gene in response to TNF, lipopolysaccharides, IL-1, or IL-6 (see reference 2; for a review, see reference 1). Finally, various other elements, including an AP-1 site located between −283 and −277 (56), have been proposed on the basis of sequence analysis.

In L929sA cells, electrophoretic mobility shift assaying (EMSA) with synthetic oligonucleotides revealed the constitutive nuclear localization of all of the transcription factors mentioned above, except NF-κB (data not shown). An NF-κB complex is released from its cytoplasmic form and translocated to the nucleus after induction by TNF or IL-1. While NF-κB induction is not the only event necessary to induce expression of the IL-6 gene in L929sA cells, stimulation by TNF leads to a rapid NF-κB induction followed by detectable levels of IL-6 mRNA after 2 to 3 h (39).

EMSA with an IL6-κB probe revealed constitutive binding of a nuclear protein present in all cell extracts tested (including L929sA and many other cell types of lymphoid and nonlymphoid origin). This complex, which has a mobility higher than that of the NF-κB complex, does not contain any of the Rel proteins, as shown by supershift experiments. There have been several reports showing the binding of non-Rel peptides to κB-responsive sequences. Among these factors, the high-mobility-group (HMG) family of DNA-binding proteins are involved in the cooperative activation of several inducible promoters including beta interferon (IFN-β) and E-selectin (see references 30 and 57; for a review, see reference 58). The constitutive protein, which binds to the IL6-κB oligonucleotide, turned out to be identical to the KBF2 protein that was previously identified with oligonucleotides specific for the κB sites present in the promoter of several genes including mouse class I major histocompatibility complex (MHC-CI) (reference 27 and personal communication) and $\beta_2$-microglobulin (59). KBF2 is identical to the 60-kDa recombination signal sequence binding protein Jκ (RBP-Jκ) found in the nuclear compartment of all mammalian cell lines tested so far (21). RBP-Jκ was originally identified in vitro through its binding to the immunoglobulin recombination signal sequence flanking the κ-type J segment of the immunoglobulin gene (34). However, Tun et al. (59) demonstrated that this observation was due to an artifactual binding of the protein to the synthetic oligonucleotide used in EMSA. Furthermore, they analyzed the RBP-Jκ

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sequence requirement and defined the consensus binding site as (T/C)GTGGGAA.

In *Drosophila*, Suppressor of Hairless [Su(H)], which is a protein homologous to RBP-Jκ, is required for the normal development of sensory organs (18, 51). This result was confirmed for mice, in which knockout experiments of the RBP-Jκ gene resulted in early embryonic death (38). Cloning of cDNAs from different species revealed that *Drosophila*, rats, and humans have a high degree of RBP-Jκ sequence identity (4).

Several proteins, including the *Drosophila hairless* product (10), the Epstein-Barr virus nuclear antigen 2 (EBNA-2), and the Notch protein, have recently been shown to interact in vitro and in vivo with the RBP-Jκ protein. In Epstein-Barr virus (EBV)-infected cells, EBNA-2 is responsible for viral gene expression and for cellular transformation (12). EBNA-2 regulates its own expression as well as the expression of several other genes through indirect binding to a consensus promoter sequence identical to the RBP-Jκ site. This sequence is found in a number of cellular genes, including CD21, CD23, and c-*gfr*, and in EBV and adenovirus pIX viral promoters. EBNA-2, which does not directly bind to DNA, was shown to exert its transactivating function through interaction with RBP-Jκ, which was used as a docking protein (22, 62).

The membrane-bound receptor Notch, which is found at the surfaces of vertebrate and invertebrate cells, has been implicated in cell fate decisions in the course of development. Signalling through the Notch pathway leads to gene regulation, possibly by association of the intracellular domain of Notch with RBP-Jκ and migration of this complex to the nucleus (for reviews, see references 24, 26, and 55). A truncated form of Notch (NotchΔC) is found in some human T-lymphoblastic neoplasms, and its mouse cellular homolog Int-3 is able to transform mammalian epithelial cells, suggesting that Notch may be considered as a protooncogene (17, 45).

On the other hand, a splice variant of RBP-Jκ, RBP-2N, acts as a repressor of transcription of the adenovirus pIX gene and of SP-1 or Gal4/VP16 synthetic enhancers; this effect is position independent up to a distance of 0.4 kb from the start site of transcription (16). EBNA-2 and Notch-1 can mask the negative regulatory domain of RBP-Jκ and convert this repressor into an activator, suggesting that EBNA-2 mimics the Notch cellular pathway to induce some cellular genes, which are normally suppressed by RBP-Jκ and necessary for EBV replication (25, 26).

In conclusion, data from viral, tumoral, and developmental studies all indicate that the DNA-docking protein RBP-Jκ is an important gene regulatory factor. This led us to analyze the implication of the presence of an RBP-Jκ binding site in the IL-6 promoter. Here, we show that RBP-Jκ is a constitutive silencer of the IL-6 gene in the absence of NF-κB factors and functions as a modulator of NF-κB binding and transactivation after stimulation of L929sA cells by TNF, presumably by limiting the access of NF-κB complexes to the DNA.

## MATERIALS AND METHODS

**Cell culture.** L929sA fibrosarcoma cells (60) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with antibiotics (50-μg/ml penicillin, 50-μg/ml streptomycin, 100-μg/ml neomycin), 2 mM L-glutamine, 5% newborn calf serum, and 5% fetal calf serum (all from GIBCO-BRL). Semiconfluent cultures were routinely detached with 0.25% trypsin–0.5 mM EDTA (GIBCO-BRL) and seeded at $2 \times 10^5$ cells/75-$cm^2$ flask. Murine recombinant TNF alpha (mTNF-α) or murine recombinant IL-1β (mIL-1β), both produced and purified in our laboratory, were added to culture media up to final concentrations of 500 to 2,000 IU/ml and 10 ng/ml, respectively. The biological IL-6/7TD1 growth assay was performed as originally described (61).

**Plasmid DNA and oligonucleotides.** Plasmid DNA was prepared with Qiagen (Chatsworth, Calif.) or PZ-522 (5prime-3prime, Boulder, Colo.) columns. Synthetic oligonucleotides (381A synthesizer; Applied Biosystems, Foster City, Calif.) were made in the laboratory, purified, and desalted by high-performance liquid chromatography.

The pCMV/RBP3 vector containing the cDNA specific for the human RBP-Jκ splicing variant RBP-3 was generously provided by A. Israel and has been previously described (10). The pRSV vector was constructed by replacing the *Bgl*II-*Hin*dIII cytomegalovirus (CMV) promoter fragment of pCDNA3 with the corresponding Rous sarcoma virus (RSV) fragment from pRc/RSV (both from Invitrogen). RBP-Jκ was cloned in this vector as a *Hin*dIII-*Xho*I fragment from pCMV/RBP3. Mutagenesis was performed with the Modifier kit (Clontech); the [EEF238AAA] mutation was carried out with the oligonucleotide CAT CTC GGA CTG TGG CCG CGG CTC CTT CTG ATT C introducing a unique *Not*I site.

The p1168huIL6P-luc+ reporter plasmid contains 1,168 bp of the human IL-6 (hIL-6) promoter (19), cloned as a *Hin*dIII-*Xho*I fragment at the *Hin*dIII site of the pGL3 basic reporter plasmid (Promega Biotec, Madison, Wis.). The 234huIL6P-luc+ plasmid resulted from deletion of the *Xba*I-*Nhe*I upstream promoter fragment followed by religation. The mini-IL6P-luc+ reporter plasmid was derived from this vector by removing the *Bam*HI-to-*Ssp*I promoter fragment, resulting in a 50-bp minimal IL-6 promoter coupled to luciferase. The two synthetic reporter constructs $(IL6\text{-}\kappa B)_3$-luc+ and $(T_5C\text{-}\kappa B)_3$-luc+ were obtained by replacing the *Pst*I-*Ssp*I promoter fragment by a 5′-*Pst*I-blunt-3′ synthetic double-stranded DNA, leaving the proximal 50 bp of the IL-6 promoter. $(IL6\kappa B)_3$-luc+ refers to a concatenated trimer of the wild-type sequence atgtG GGATTTTCCcatg, while $(T_5C\text{-}\kappa B)_3$-luc+ corresponds to a three-time repeat with a T-for-C substitution at position 5 (underlined) of the IL6-κB motif atgt GGGACTTTCCcatg (capital letters indicate the IL6-κB core sequence).

The vector pAB-Gal4 was used to drive expression (under the control of the RSV long terminal repeat) of fusion proteins containing the Gal4-DNA binding domain (8). pAB-p65 codes for the fusion of the Gal4-DNA binding domain with the full human RelA protein and was described previously (50). 50pAB-RBP-Jκ was constructed by cloning of the RBP3 blunted *Bam*HI fragment from the pCMV/RBP3 vector into the blunted *Xma*I site of pAB-Gal4.

The (Gal4)2 reporter element [CCG GGC GGA GGA C(A/T)G TCC TCC GGA TCC GGA GGA C(A/T)G TCC TCC GC], which contains two identical 17-mer sites, was synthesized as an *Xma*I-compatible self-complementary oligonucleotide and was cloned into the *Xma*I site of the different reporter constructs (see Fig. 8).

The pPGKβGeobpA vector constitutively expressing a [neo]r-β-galactosidase fusion protein was a kind gift of P. Soriano (54).

Restriction analysis and DNA sequencing were used to control all plasmid constructions.

**Recombinant proteins.** The recombinant glutathione *S*-transferase (GST)–RBP-Jκ fusion protein was produced in *Escherichia coli* MC1061, which had been transformed with the GST fusion protein vector pGEX-2T (Pharmacia, Uppsala, Sweden) containing the RBP3 cDNA as an insertion within the *Bam*HI site. The fusion protein was purified as described by Matthews et al. (35). Alternatively, the eukaryotic RBP3 protein was obtained by in vitro transcription of the pCMV/RBP3 vector with T7 RNA polymerase and translation in the rabbit reticulocyte system (TNT-RRL; Promega).

**EMSA.** Nuclear extracts were prepared according to a modification of the method described by Dignam et al. (15). Cells were plated in 10-cm-diameter dishes at a density of $2 \times 10^5$ to $1 \times 10^6$ cells per dish for 2 days. For induction, cells were stimulated in 10 ml of pooled, conditioned medium for the indicated times. After two washes with ice-cold phosphate-buffered saline, the cells were harvested with a rubber policeman and pelleted in 15 ml of phosphate-buffered saline by centrifugation for 5 min at $1{,}100 \times g$. Pellets were resuspended in 1 ml of hypotonic buffer no. 1 (10 mM HEPES [pH 7.5], 10 mM KCl, 1 mM $MgCl_2$, 5% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 2 mM Pefabloc [Pentafarm, Basal, Switzerland], 0.5 mM dithiothreitol [DTT], 0.15-IU/ml aprotinin, 10-μg/ml leupeptin), transferred to microtubes, incubated for 15 min on ice, and vortexed for 10 s with 0.6% Nonidet P-40. Nuclei were separated from the cytosol by centrifugation at $12{,}000 \times g$ for 60 s and were resuspended in buffer no. 2 (20 mM HEPES [pH 7.5], 1% Nonidet P-40, 10 mM KCl, 1 mM $MgCl_2$, 400 mM NaCl, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 2 mM Pefabloc, 0.5 mM DTT, 0.15-IU/ml aprotinin, 10-μg/ml leupeptin). After 10 min of vortexing at 8°C and 5 min of centrifugation at $12{,}000 \times g$, nuclear extracts were transferred to Eppendorf tubes. Protein concentrations were measured according to the method described by Bradford (9) with a commercial reagent (Bio-Rad, Richmond, Calif.).

For the binding reaction, nuclear extract (5 to 10 μg of protein) was diluted up to a total volume of 20 μl containing 20 mM HEPES (pH 7.5), 60 mM KCl, 4% Ficoll 400, 2 mM DTT, 20 μg of purified bovine serum albumin, and 2 μg of poly(dI/dC). Oligonucleotides containing different binding sites (Fig. 1) were annealed, radiolabelled with the Klenow fragment of DNA polymerase I and [α-$^{32}$P]dCTP in a fill-in reaction of the termini, and purified by reverse-phase chromatography with Elutip-D columns (Schleicher & Schuell). A 0.5-ng amount of oligonucleotide (i.e., ~30 fmol, corresponding to 50,000 to 100,000 cpm) was added to the reaction mixture followed by incubation for 30 min at room temperature. The protein-DNA complexes were separated by electrophoresis through a native 6 to 8% polyacrylamide–5% glycerol–1× Tris-borate-EDTA (TBE) gel run in 0.5× TBE buffer (1× TBE = 89 mM Tris, 89 mM boric acid,

| Substitutions | Sequence (1 5 10) | relative binding RBP-Jκ | relative binding NF-κB |
|---|---|---|---|
| **Rel oligonucleotides** | | | |
| wt Ig-κB | agctAGAG**GGGACTTTCC**GAGAGGagct. | - | ++++ |
| wt IL6-κB | agctATGT**GGGATTTTCC**CATGAGCagct | +++ | +++ |
| subst-$G_2$/A | _____A_____ | +++ | +/- |
| subst-$A_4$/C | _____C_____ | +++ | +++ |
| subst-$T_5$/A | _____A_____ | ++++ | +++ |
| subst-$T_5$/C | _____C_____ | +/- | +++ |
| subst-$T_6$/A | _____A_____ | +++ | +++ |
| subst-$T_6$/G | _____G_____ | +++ | ++ |
| subst-$T_7$/A | _____A_____ | ++ | ++ |
| subst-$T_7$/G | _____G_____ | ++ | ++ |
| subst-$T_8$/A | _____A_____ | ++ | +++ |
| subst-$T_8$/G | _____G_____ | ++ | ++ |
| subst-$C_{10}$/T | _____T_____ | +++ | ++ |
| subst-$C_{11}$/T | _____T_____ | +++ | +++ |
| **non-Rel oligonucleotides** | | | |
| HES | agctGTTACTGTGGGAAAGAAAGTCagct | +++ | - |
| AP-1 | gctcAAGTGCTGAGTCACTTTTAAAGtata | - | - |
| NF-IL6 | gtacACATTGCACAATCTTAgtca | - | - |

FIG. 1. Sequences and relative binding levels of different oligonucleotides used in EMSA. Only the positive strand of the oligonucleotides is represented. Underlining refers to unchanged base compared to the IL6-κB probe. Boldface, part of the NF-κB decameric site; lowercase, the cohesive ends of the double-stranded oligonucleotides filled in with Klenow DNA polymerase. Results shown are relative to those obtained for the same complex with the IL6-κB probe. +++, 100%; ++++, >100%; ++, >50%; +/−, <30%; −, no binding.

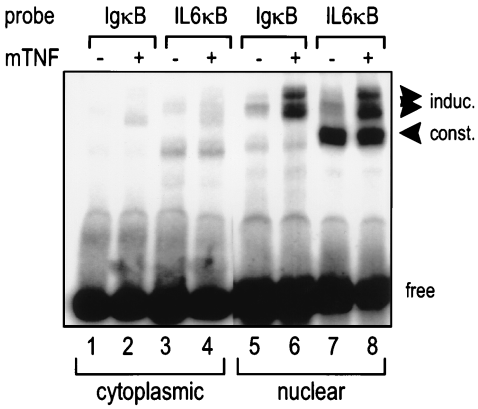



FIG. 2. NF-κB EMSA experiment with L929sA extracts. Subconfluent L929sA cells grown for 48 h were assayed for NF-κB activity with oligonucleotides specific for the classical NF-κB motif (Ig-κB [lanes 1, 2, 5, and 6]) or with the IL6-κB sequence (lanes 3, 4, 7, and 8; see Fig. 1). Equivalent cellular amounts of cytoplasmic (lanes 1 to 4) or nuclear (lanes 5 to 8) extracts from L929sA cells were analyzed on an 8% EMSA gel. When indicated, mTNF had been added to the cells at a final concentration of 1,000 IU/ml for 30 min. induc, induced; const, constitutive.

2 mM EDTA). The gels were dried, and radioactivity was revealed by phosphorimaging and computer analysis (ImageQuant software; Molecular Dynamics, Sunnyvale, Calif.).

**UV cross-linking.** Bromodeoxyuridine (BrdU)-substituted IL6-κB oligonucleotide was purchased from Eurogentech (Seraing, Belgium). The sequences of the oligonucleotides were as follows: upper, 5′-AGC TA**B** G**B**G GGA **BBB B**CC CAT GAG C-3′; lower, 5′-AGC TGC **B**CA **B**GG GAA AA**B** CCC ACA **B**-3′ (where **B** is 5-BrdU). The oligonucleotides were mixed, annealed, labelled with the Klenow fragment of DNA polymerase I, and purified as described above. A fivefold-scaled-up EMSA reaction mix was set up, incubated for 20 min at room temperature, transferred on a piece of Parafilm laid on ice, and exposed for 10 min to UV light (UV transilluminator; Bio-Rad) at a distance of 5 cm. The reaction mixture was then denatured in Laemmli buffer and fractionated in a classical sodium dodecyl sulfate (SDS)–8% polyacrylamide gel electrophoresis (PAGE) gel.

**Methylation interference assay.** The methylation interference experiment was performed essentially as described elsewhere (6, 7). Briefly, both strands of the IL6-κB oligonucleotide (Fig. 1) were separately labelled with [$\gamma$-$^{32}$P]ATP by the T4 polynucleotide kinase (GIBCO-BRL) and annealed with the unlabelled complementary strand. The resulting double-stranded DNA molecules were filled in with unlabelled deoxynucleotides by using the Klenow fragment of DNA polymerase I (Boehringer) and purified by reverse chromatography on Elutip-D columns. DNA was then precipitated and desalted. A total of $10^6$ cpm of each probe were incubated with dimethyl sulfate (Sigma Chemical Co.) for 5 min at 25°C as described in the protocol; stop solution was then added, and dimethyl sulfate was removed by two cycles of ethanol precipitation. The resulting probe was dissolved and used in a 5× scaled-up standard EMSA experiment together with 50 μg of nuclear proteins from L929sA cells. After electrophoresis in a 8% polyacrylamide gel, bound and free DNA fractions were recovered by electrotransfer on DEAE-cellulose paper (DE-81; Whatman). Methylated DNA was processed for 30 min in 1 M piperidine at 95°C (Sigma Chemical Co.), dried under vacuum, and subjected to two cycles of solubilization-lyophylization in a Speedvac (Savant). Final pellets were dissolved in 1× formamide dye and boiled for 5 min. Duplicate samples (5,000 cpm) from each fraction were loaded in a standard 15% polyacrylamide–7 M Urea–1× TBE sequencing gel. The gel was preheated for 30 min at 50 W before loading of the samples and was run at 30 W until the bromophenol blue dye reached two-thirds of the gel length. The gel was dried under vacuum and exposed for 72 h before phosphorimaging analysis.

**Cell transfection and luciferase assay.** For transient expression, murine L929sA cells were transfected with Lipofectamine according to the instructions of the manufacturer (GIBCO-BRL). Briefly, exponentially growing cells were seeded in 24-well plates at a density of $6 \times 10^4$ cells/well in 1 ml of complete medium and were grown for 24 h prior to transfection. On the day of transfection, the cells were washed twice with Optimem medium (GIBCO-BRL). A total amount of 0.5 μg of DNA was combined with 2.5 μl of Lipofectamine reagent for 30 min, which was added to the cell layer together with Optimem medium and left for 5 h at 37°C in a $CO_2$ incubator. An equal volume of DMEM containing 20% serum and antibiotics was then added to each well and left for 24 to 36 h. The cells were stimulated, washed, and lysed in 24-well plates, and lysates were transferred to 96-well plates for the luciferase and protein quantification assays.

For stable expression, L929sA cells were transfected by the calcium phosphate precipitate method (33), with 30 μg of DNA containing 1 μg of pPGKβGeobpA selection vector (encoding a [neo]$^r$-β-galactosidase fusion protein conferring resistance to G418 as well as constitutive β-galactosidase enzymatic activity), 5 μg of luciferase reporter plasmid, and either 24 μg of carrier DNA or 30 μg of expression vector. After selection for 15 days in DMEM containing 400-μg/ml G418 (GIBCO-BRL), the resistant colonies were trypsinized and pooled for use as a polyclonal population in further experiments, reducing in this way individual clonal variation and yielding an average response to the inducers.

The luciferase assay was performed as described by the manufacturer, Promega. The cells were lysed in buffer no. 3 (25 mM Tris phosphate [pH 7.8], 2 mM DTT, 2 mM cyclohexanediaminetetraacetic acid, 10% glycerol, 1% Triton X-100) for 15 min at room temperature, and cell extracts were transferred to a 96-well plate. A 20-μl volume of each extract was tested for luciferase activity by the addition of 50 μl of luciferase assay reagent (20 mM Tricine, 1.07 mM $[(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O]$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A [CoA], 470 μM D-luciferin, 530 μM ATP) and analyzed in a luminescence microplate counter (TopCount; Packard, Meriden, Conn.). Luciferase activity, which was expressed in arbitrary light units, was corrected for protein content in transient experiments or for β-galactosidase activity (Galacto-Light kit; Tropix, Bedford, Mass.) in stable transfection experiments.

## RESULTS

**The IL6-κB sequence is recognized by constitutive and inducible nuclear proteins.** The protein-DNA complexes formed with L929sA extracts were analyzed by gel shift assays (Fig. 2). Equivalent amounts of cytoplasmic and nuclear extracts from L929sA cells, either untreated or induced for 30 min with 1,000-IU/ml mTNF, were mixed with a synthetic probe identical to the NF-κB site present in the Ig κ-chain (Ig-κB) gene or with the IL6-κB probe and were separated on a native gel. A number of complexes with different mobilities were resolved, all of which were present in nuclear extracts only. Some complexes were constitutive and migrated as a broad band or a doublet, while the others were detected only in TNF-induced cells and could be resolved as doublets of variable intensities. The pattern of nuclear extracts from TNF-induced cells obtained with the classical Ig-κB probe (Fig. 2, lane 6) suggests that the upper doublet corresponded to activated NF-κB, while the lower complex, which was not detected with this reference probe, was formed by another protein.

**Identification of the IL6-κB binding proteins.** In order to identify the polypeptides involved in each of the complexes, we performed supershift analyses (Fig. 3) with antisera raised against p50 and p65 (44), RBP-Jκ (10, 27), or other members of the Rel family or other transcription factors (B-rel, c-Rel,

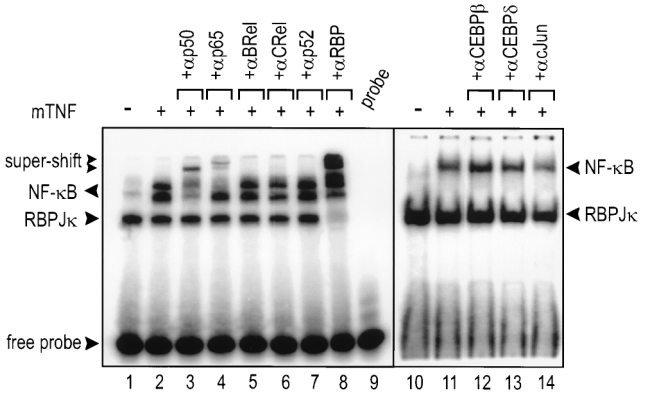



FIG. 3. Supershift EMSA experiment of the IL6-κB binding proteins. Nuclear extracts (5 μg) from mTNF-stimulated L929sA cells were preincubated for 20 min with 5 μl of specific antiserum or 2 μg of purified polyclonal antibodies and analyzed by EMSA with the IL6-κB probe. More slowly migrating complexes are visible, corresponding to antigen-antibody-oligonucleotide ternary complexes (supershift). Arrows indicate the identities of the different complexes.

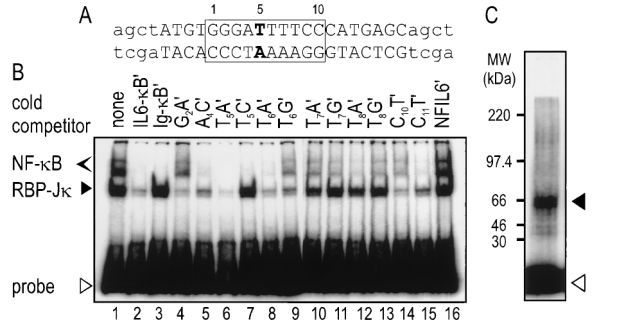



FIG. 4. Identification of the nucleotides involved in the binding of RBP-Jκ. (A) Schematic representation of the IL6-κB oligonucleotide probe (the NF-κB decamer motif is boxed); (B) 5-μg extracts of mTNF-induced L929sA preincubated for 20 min with unlabelled competitive oligonucleotides (25 ng; 50× excess), mixed for 10 min with labelled IL6-κB probe (0.5 ng), and analyzed by EMSA. Arrows indicate previously identified complexes. Legends at the top of the lanes indicate the positions of the substituted base in the unlabelled competitor. All competitor oligonucleotides contain NF-κB-like decamer motifs, except for lane 16, in which an NF-IL6-specific probe was used as control. (C) Phosphorimager analysis of a typical UV cross-linking experiment performed with a nuclear extract. Molecular mass (MW) markers included in the same gel are shown. Empty arrow, free oligonucleotide.

p52, CEBPβ/NF-IL6, CEBPδ, c-Jun [all from Santa Cruz Biotechnology]).

The results in Fig. 3 show that the two inducible NF-κB complexes (lanes 2 to 7) corresponded to the classical p50 homodimer and p50-p65 heterodimer, respectively, while the constitutive, faster-migrating complex was completely shifted by antiserum raised against the RBP-Jκ protein (lane 8). Despite many attempts and the use of different extracts, no other nuclear factors reported to associate with Rel proteins, such as NF-IL6 or c-Jun, could be demonstrated in our extracts by supershift experiments with the IL6-κB oligonucleotide, suggesting that the three identified complexes correspond to the major IL6-κB-binding compounds in L929sA cells (lanes 12 to 14).

In their study on the beta interferon (IFN-β) promoter, Thanos and Maniatis (57) have shown simultaneous binding of HMG and NF-κB proteins to the oligonucleotide, resulting in a slower-migrating NF-κB–HMG–DNA complex. We could not detect a similar difference in mobility in the case of the IL6-κB oligonucleotide compared to the Ig-κB oligonucleotide (Fig. 2), suggesting that the binding of each complex with the IL6-κB sequence was mutually exclusive. Furthermore, the supershift experiments with anti-RBP-Jκ antiserum (Fig. 3, lane 8) revealed no change in the mobility of the upper NF-κB complexes.

**The AT-rich region and especially position 5 of IL6-κB is necessary for RBP-Jκ binding.** The sequence specificities of both complexes were assessed by competition analysis with the $^{32}$P-labelled IL6-κB probe (Fig. 4A) in combination with a moderate (50×) excess of different unlabelled oligonucleotides containing one single-base exchange (see list in Fig. 1). Figure 4B shows the effects of these base substitutions and defines the critical positions for binding to the different complexes. The competition effects observed suggest that the RBP-Jκ protein contacts DNA at multiple positions, with a critical involvement of positions 5, 7, and 8.

An oligonucleotide with a substitution of T for C at position 5 (Fig. 4B, lane 7) did not compete, while the substitution of T for A (lane 6) led to an increased competitive effect. Interestingly, the T-for-C exchange at this position corresponds to the replacement of the core IL6-κB site with the classical Ig-κB site. The absence of binding of RBP-Jκ to the Ig-κB sequence (Fig. 2) is confirmed in Fig. 4B by the absence of a competitive effect with Ig-κB unlabelled oligonucleotide (lane 3). Additionally, substitution of $T_7$ and $T_8$ also led to decreased competition ability, suggesting that these bases are involved in binding of RBP-Jκ to its DNA target.

Finally, an irrelevant oligonucleotide (e.g., NF-IL6 [Fig. 4B]) did not compete for any of the complexes, confirming the specificity of the other oligonucleotides used. The results shown in Fig. 4B, which are representative of several competition experiments, were quantified by phosphorimaging analysis and are summarized in Fig. 1.

Based on cotransfection experiments, it has been proposed that RBP-Jκ bind as a monomer (11). Therefore, the observation of two complexes with slightly different mobilities (visible only in Fig. 4B because of complex intensity), both being supershifted by the anti-RBP-Jκ antiserum, may perhaps be attributed to isoforms of RBP-Jκ resulting from differential splicing or from posttranslational modification (4). In Fig. 4C, an 8% SDS-PAGE analysis of nuclear proteins from unstimulated cells, UV cross-linked to a BrdUTP-substituted IL6-κB oligonucleotide (molecular mass, ~7.5 kDa/strand) showed a band with an apparent molecular mass of ~66 kDa, which is compatible with the size of a monomer of RBP-Jκ bound to DNA. The separation of the two isoforms observed by EMSA could not be achieved by this method.

**RBP-Jκ binds to the minus strand of the IL6-κB oligonucleotide.** The RBP-Jκ consensus binding site has been shown to contain the minimal octamer motif (C/T)GTGGGAA (25, 55, 59). Interestingly, a sequence very close to this consensus is found both at the 5′ end of the IL6-κB site 5′-($_3$TGTGGGA $\underline{T}_5$)-3′ (plus strand) and at the 3′ end of this site in the minus strand 5′-($_{14}$C$\underline{A}$TGGGAA$_7$)-3′ (nonconsensus bases are underlined).

Different complementary double-stranded probes or combinations of strands from wild-type and substituted mutant $T_5$C were annealed, labelled by Klenow polymerase fill-in reactions, and used in EMSA with extracts from L929sA cells. As shown in Fig. 5A (lane 3 versus 2), the Ig-κB probe formed more-intense complexes with NF-κB factors than the IL6-κB probe but did not bind RBP-Jκ. The $T_5$-to-C-substituted IL6-κB oligonucleotide conserved full binding of NF-κB compared to that of the wild-type IL6-κB (lanes 5 versus 2) but lost almost

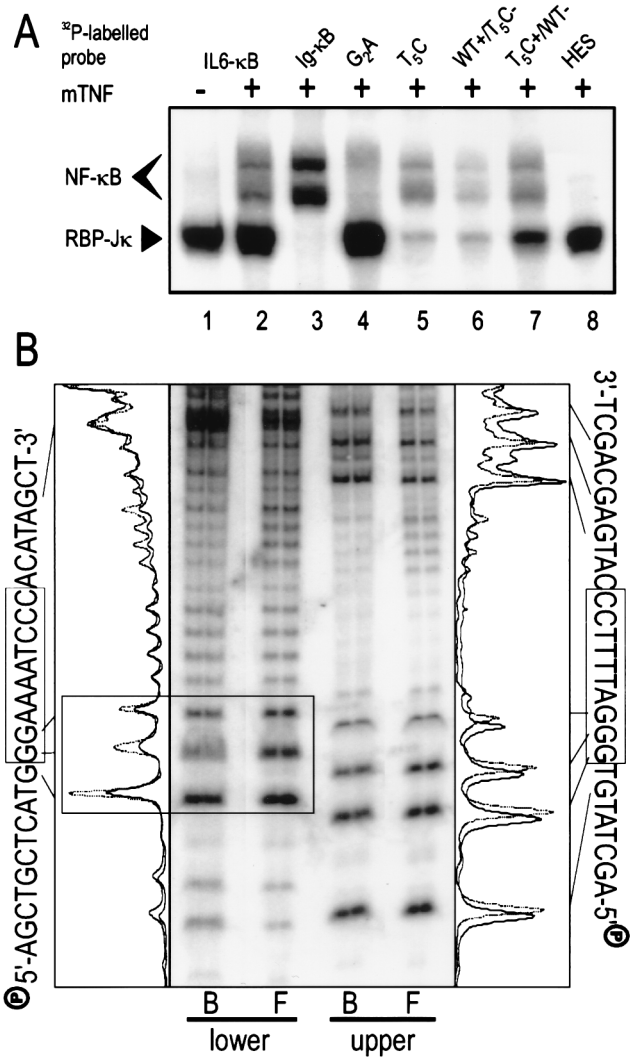



FIG. 5. EMSA analysis and methylation interference assay. (A) Different oligonucleotides or combinations of plus and minus strands from the wild-type (WT) and $T_5C$-substituted IL6-κB oligonucleotides were annealed, labelled by Klenow DNA polymerase, and mixed with nontreated (lane 1) and TNF-induced (lanes 2 to 8) L929sA nuclear extracts before EMSA analysis. (B) Nuclear proteins from noninduced L929sA cells (50 μg) were used for methylation interference analysis in combination with methylated IL6-κB probe labelled at one end by the T4 polynucleotide kinase. Bound (B) and free (F) DNAs were subjected to piperidine degradation and analyzed in duplicate by electrophoresis through a 15% polyacrylamide–7 M urea-TBE sequencing gel. Line plots corresponding to normalized bound and free profiles are shown next to the scan. Dotted lines, free probe samples; solid lines, bound fractions. The IL6-κB motif is boxed.

all binding of RBP-Jκ, as previously shown. Finally, the $G_2A$-substituted probe had largely lost the ability to bind NF-κB, while it still bound to RBP-Jκ (lane 4). Interestingly, the binding of the NF-κB complexes to the $T_5C$ mutant was not comparable to that to the Ig-κB oligonucleotide, even when the two probes shared the complete NF-κB consensus sequence. This difference probably reflects a higher amount of accessible Ig-κB probe in this experiment or a better binding of the NF-κB factor to the Ig-κB sequence due to the different flanking nucleotides. Alternatively, RBP-Jκ, while binding very weakly to the single substituted probe $T_5C$, as shown by EMSA, may still be able to compete with NF-κB factors, yielding a reduced amount of NF-κB complexes.

We then used probes resulting from annealing of oligonucleotides with a 1-base mismatch at position 5. The probes used in this experiment are comparable to the $A_6$-A central mismatched probe used by Muller et al. (37) in their crystallographic study of p50 homodimers bound to the κB motif and reported to exert little effect on protein affinity. When the T-for-C substitution occurred in the plus strand, the binding of NF-κB and of RBP-Jκ was only marginally affected (Fig. 5A, lanes 7 versus 2). On the contrary, substitution in the minus strand reduced the binding of RBP-Jκ considerably (and, to a lesser extent, that of NF-κB), leading to a similar pattern as obtained with the fully complementary ($T_5C$) oligonucleotide (lanes 6 versus 5). In summary, these results clearly indicate the major involvement of the A residue at position 5 in the minus strand of the IL6-κB sequence, which is absent in the Ig-κB site.

Furthermore, the oligonucleotide probe, which corresponds to a single RBP-Jκ site present in the *Drosophila* hairy enhancer of split (HES) promoter but which does not resemble an NF-κB motif (10), forms a complex of similar mobility as observed with the IL6-κB probe (Fig. 5A, lane 8), confirming that RBP-Jκ binds to the latter as a monomer.

To further demonstrate that the binding of RBP-Jκ occurs at the level of the minus strand, we performed a standard methylation interference experiment (Fig. 5B). As shown after phosphorimager analysis, the lower strand and, particularly, the G residues at positions −11 to −9 are involved in RBP-Jκ binding (plain line versus dotted lines in the densitometric plot in Fig. 5). By contrast, no significant difference could be observed when bound and free DNA fractions labelled at the upper strand were compared.

In summary, the results of Fig. 5 demonstrate that RBP-Jκ binds only to the minus strand of the IL6-κB sequence, with an additional requirement of $A_5$, which is not part of the reported minimal consensus site.

**Recombinant or in vitro-synthesized RBP-Jκ specifically binds to the IL6-κB site.** Different approaches were used to further confirm the identity of the constitutive protein binding to IL6-κB. Figure 6A shows that unlabelled rabbit reticulocyte lysate contains endogenous NF-κB and RBP-Jκ activities. In lane 5, the binding of rabbit RBP-Jκ was completely abolished by a 50-fold excess of unlabelled HES probe. The $T_5C$ mutation (lane 4) as well as the Ig-κB oligonucleotide (lane 2) did not compete for RBP-Jκ binding, as already shown in Fig. 4B, while the $G_2A$-substituted oligonucleotide did (lane 3).

In Fig. 6B, analysis of the binding of $^{35}$S-Met-labelled RBP3 obtained by in vitro transcription-translation (TNT-coupled rabbit reticulocyte lysate; Promega) of the human cDNA homolog of the mouse RBP-Jκ gene (pCMV-RBP3) to unlabelled oligonucleotides showed the same sequence specificity as the cellular constitutive protein, confirming the results obtained with L929sA nuclear extracts.

Furthermore, an *E. coli* recombinant GST–RBP-Jκ fusion protein also bound specifically to IL6-κB (Fig. 6C, lane 3), while GST alone or a nonrelevant fusion protein (GST-Grb2 [a kind gift of J. Vandekerckhove]) did not bind to the probe (Fig. 6C, lanes 1 and 2). The lower complex in lane 3 might be a proteolytical degradation product of full-length GST–RBP-Jκ which had retained its ability to bind to immobilized glutathione (used for purification) as well as to the IL6-κB probe used in the EMSA.

**RBP-Jκ is highly expressed but binds with an affinity lower than that of NF-κB.** Figure 7 shows a typical result obtained by analysis of the binding of the different complexes to the IL6-κB probe, in particular showing kinetic on and off rates of RBP-Jκ and NF-κB complexes, as determined by running gel shift

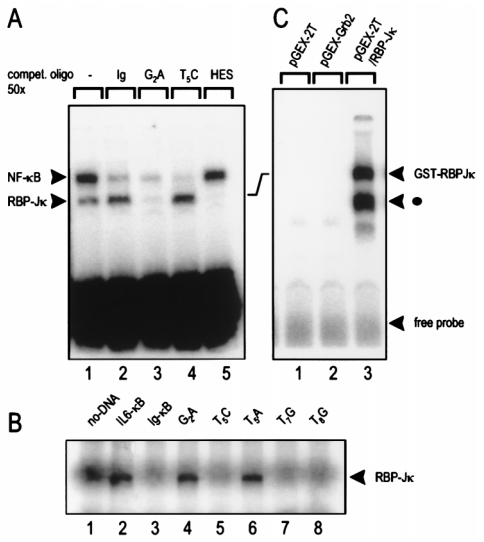



FIG. 6. Binding of rabbit or recombinant RBP-Jκ proteins to wild-type IL6-κB or substituted probes. (A) Rabbit reticulocyte extracts were used in a competition experiment with $^{32}$P-labelled IL6-κB probe and unlabelled competitor oligonucleotides (compet. oligo). A probe specific for the Su(H) RBP-Jκ homolog (HES [lane 5]) was used as a control. The NF-κB and RBP-Jκ arrows point to endogenous binding activities present in the rabbit reticulocyte extract. (B) Reverse-EMSA experiment with $^{35}$S-labelled RBP-Jκ protein produced by the in vitro transcription-translation-coupled rabbit reticulocyte system and mixed with unlabelled wild-type or mutated IL6-κB oligonucleotides. (C) EMSA with the IL6-κB probe and 2 μl of soluble extract from *E. coli* expressing recombinant GST protein, a GST–RBP-Jκ fusion protein, or an irrelevant GST-Grb2 fusion protein.

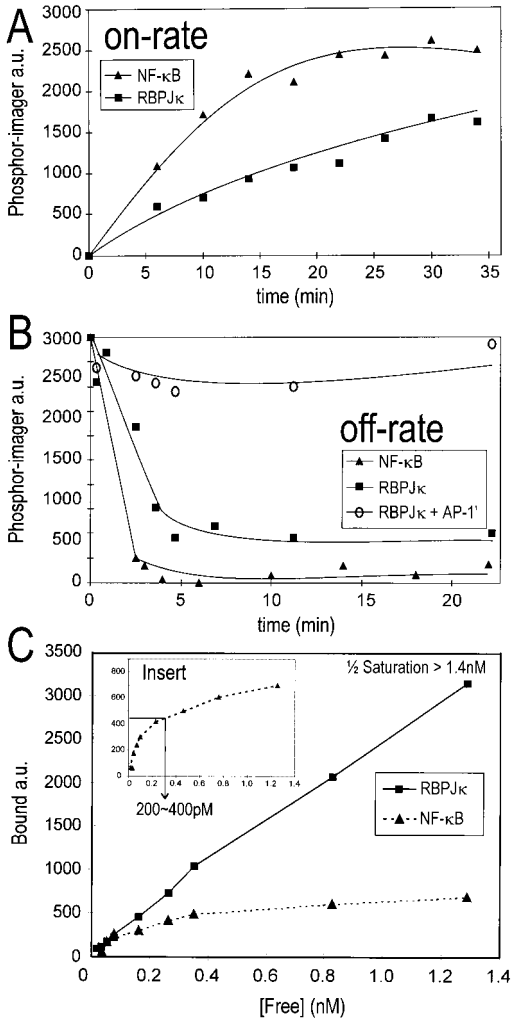



FIG. 7. Complex formation as measured by EMSA as a function of incubation time or oligonucleotide concentration. (A) On-rate analysis of RBP-Jκ (filled squares) and NF-κB (filled triangles) complexes as determined by running shift assaying. (B) Off-rate analysis of preformed complexes (20 min at room temperature), mixed with a 100× molar excess of unlabelled competitive oligonucleotide and loaded on a running gel. (C) Scatchard plot analysis. Aliquots containing 5 μg of nuclear protein extract from nonstimulated or mTNF-stimulated L929sA cells (1,000-IU/ml mTNF; 30 min) were mixed for 30 min with increasing amounts of $^{32}$P-labelled IL6-κB probe and a 200× excess of unlabelled probe specific for one species (HES for RBP-Jκ and Ig-κB for the NF-κB complexes) and analyzed by EMSA. The intensity of each complex (vertical scale) is expressed in arbitrary units (a.u.). Insert, an enlarged portion of the NF-κB plot, corresponding to the lower range of probe concentration.

analysis. For on-rate analysis, nuclear proteins from TNF-induced L929sA cells were added to the EMSA reaction mix, and aliquots were loaded on a running 8% EMSA gel at various time points. In this experiment, the two types of complexes shared a large excess of probe without competition. The results obtained show that NF-κB binding to the probe reached equilibrium after approximately 20 min, while binding of RBP-Jκ had not reached a plateau after 35 min of incubation. The off-rate study (Fig. 7B) was similar, except that the protein-DNA complexes were allowed to form for 20 min before addition of a 100× excess of unlabelled IL6-κB oligonucleotide and periodical loading on the running gel. In this experiment, NF-κB dissociation was analyzed in extracts from TNF-induced L929sA cells, while dissociation of RBP-Jκ complexes was measured in extracts from noninduced cells. Finally, incubation of RBP-Jκ with a 100× excess of unrelated oligonucleotide (AP-1 probe) did not lead to displacement, as shown by the control curve.

We finally performed a semiquantitative binding analysis of each complex by the method of Scatchard (49). RBP-Jκ and NF-κB binding were analyzed in the same extract from TNF-induced cells (Fig. 7C). In both cases, a large excess of unlabelled oligonucleotide (200×) was added to the reaction mixture. The HES oligonucleotide was used to complex RBP-Jκ in the case of NF-κB binding study, while unlabelled Ig-κB oligonucleotide could prevent binding of NF-κB proteins to the labelled probe for analysis of RBP-Jκ binding. Figure 7C shows binding of the NF-κB factor with saturation for values exceeding 1 nM free probe. By contrast, in the case of RBP-Jκ, saturation could not be obtained with a probe concentration of as high as 1.4 nM. The affinity of RBP-Jκ for its DNA site is clearly lower than that of NF-κB and could not be precisely defined due to the insufficient amount of probe used in this experiment. The highest probe concentration used here and, particularly, previously obtained results with higher concentrations of labelled probe (data not shown) suggest that the half-saturation of RBP-Jκ binding is obtained in the range of 2 to 3 nM probe, while the NF-κB affinity constant could be estimated within the range of 200 to 400 pM. On the other hand, the total amount of RBP-Jκ protein present in the nucleus is at least five- to sixfold higher than the amount of activated NF-κB, as determined by the saturation level obtained for both complexes (>3,500 versus 700 arbitrary units). The facts that the amount of RBP-Jκ is severalfold higher than that of NF-κB but that RBP-Jκ displays at least 10-fold less affinity for its target confirm that competition between the two factors can occur when low levels of NF-κB are present in the nucleus

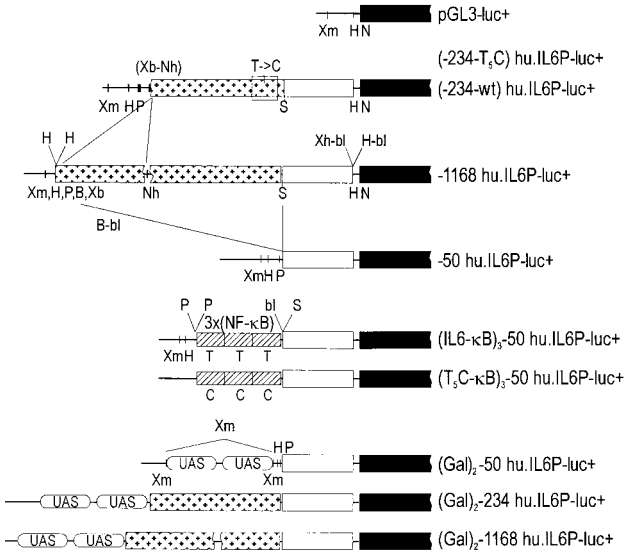


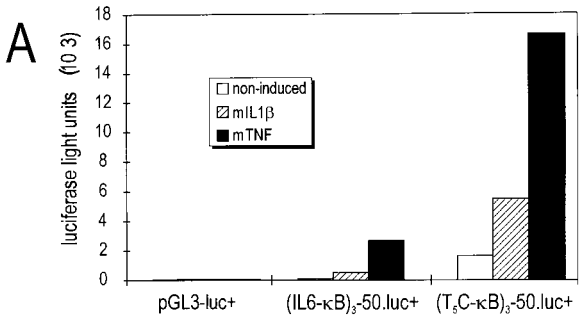

FIG. 8. Schematic maps of the different constructions used in this study. Black bars, the reporter gene coding region; dotted bars, the IL-6 promoter region between −50 and −1168 bp; white bars, the 50-bp IL-6 promoter fragment located upstream of the major start site of transcription and containing the putative TATA region; hatched bars, NF-κB sites; rounded blocks, UAS sites. Restriction enzyme sites used for construction are abbreviated as follows: B, *Bam*HI; H, *Hin*dIII; N, *Nco*I; Nh, *Nhe*I; P, *Pst*I; S, *Ssp*I; Xb, *Xba*I; Xh, *Xho*I; and Xm, *Xma*I. *bl*, blunt end generated by restriction or after fill in with Klenow DNA polymerase.

(e.g., early induction and suboptimal stimulation). Furthermore, this suggests that the full induction of NF-κB translocation leads to an influx of transactivator in the nucleus that will efficiently displace the RBP-Jκ protein from the promoter.

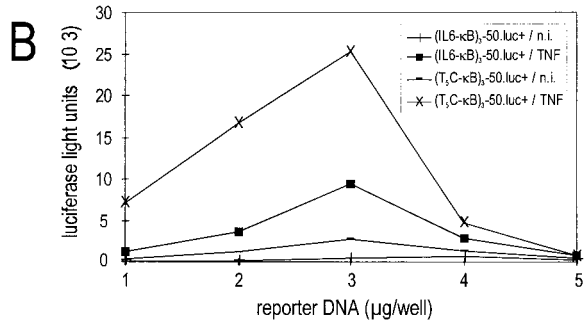
**RBP-Jκ represses transcription.** The presence of a binding site for RBP-Jκ overlapping the IL6-κB site led us to postulate a functional involvement of the RBP-Jκ protein in the transcriptional regulation of the IL-6 gene. L929sA cells were transiently or stably transfected with different luciferase reporter constructs and induced by mTNF or other stimuli for analysis in a standard luciferase assay. The various constructs are described in Materials and Methods and are schematically represented in Fig. 8.

We have already shown that the exchange of a single base at particular positions within the IL6-κB sequence is sufficient to completely abolish the recognition by the RBP-Jκ protein, while binding of NF-κB was only marginally reduced (Fig. 4B and 5A). Figure 9 shows a typical result obtained with pooled L929sA cells stably transfected by the calcium phosphate method and selected for 15 days with G418 (see Materials and Methods). As can be seen in Fig. 9A, substitution of the IL6-κB core sequence by the $T_5C$ variant was sufficient to enhance the basal level of luciferase expression by a factor of 13. Furthermore, after 6 h of induction with TNF or IL-1, the luciferase activity obtained with the $(T_5C\text{-}\kappa B)_3$-50hu.IL6P synthetic promoter was still 6- to 10-fold higher than that with the $(IL6\text{-}\kappa B)_3$-50hu.IL6P reporter construct.

These results not only confirm that RBP-Jκ competitively binds to the κB site of the IL-6 promoter, thus limiting the access of NF-κB factors translocated to the nucleus after cytokine induction, but also show that RBP-Jκ acts as a repressor of basal transcription in the absence of detectable amounts of NF-κB proteins. We conclude, therefore, that RBP-Jκ is a general silencer of the IL-6 promoter, as further documented below.




| | pGL3-luc+ | | | (IL6-kB)3-50.luc+ | | | (T5C-kB)3-50.luc+ | | |
|---|---|---|---|---|---|---|---|---|---|
| | non-ind. | mIL1 | mTNF | non-ind. | mIL1 | mTNF | non-ind. | mIL1 | mTNF |
| luciferase a.u. | 66 | 70 | 69 | 124 | 538 | 2680 | 1681 | 5463 | 16651 |
| relative units | 1 | 1 | 1 | 2 | 8 | 39 | 26 | 78 | 240 |
| (T5C vs. IL6-kB) | - | - | - | - | - | - | 13 | 10 | 6 |



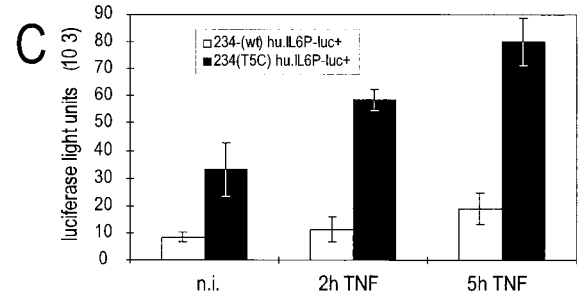




FIG. 9. Effect of RBP-Jκ binding on gene expression. (A) Pools of transfected cells were stimulated (when indicated) with 10-ng/ml IL-1 or 500-IU/ml mTNF for 6 h before extraction and luciferase assaying. The results represent more than six experiments and three different transfections. Results are normalized to the β-galactosidase activity present in the extracts (see Materials and Methods), and the relative induction factors are summarized. a.u., arbitrary units. (B) Effect of the amount of transfected plasmid on luciferase activity. L929sA cells were transiently transfected in six-well plates with Lipofectamine and increasing amounts of reporter DNA plasmid. Cells were left for 72 h before changing of the medium or stimulation with 2,000-IU/ml TNF for 6 h. Results are expressed in arbitrary light units. (C) Effect of the single-base exchange within the 234-bp promoter fragment. The $T_5C$ base substitution was introduced in the 234-bp IL-6 promoter fragment coupled to luciferase. Results are the average results and deviations for four independent assays. n.i., noninduced.

A control experiment (Fig. 9B) shows that the difference in luciferase activity observed between the two constructions $(IL6\text{-}\kappa B)_3$-50 hu.IL6P luc+ and $(T_5C\text{-}\kappa B)_3$-50 hu.IL6P luc+ was not due to a quantitative difference in the amounts of transfected reporter DNA but truly reflected the stronger promoter activity of the point-mutated sequence. The peak of activity at approximately 3 μg of DNA per six-well plate corresponds to the optimal quantity of reporter gene activity in the cells for both constructions; the further decrease in activity is probably due to toxicity at high DNA concentrations or to squelching effects.

The observations made with the synthetic reporter constructions could be reproduced with a 234-bp fragment of the IL-6 promoter bearing the single-base exchange of T for C in the κB site (Fig. 9C). The more important level of luciferase activity obtained with the mutant (~4-fold-higher) promoter probably reflects both higher basal transcription in the absense of the repressor protein and earlier induction of transcription after TNF treatment of the cells, leading to a higher accumulation of mRNA and luciferase enzyme (Fig. 9C).

To confirm the ability of RBP-Jκ to repress transcription of the IL-6 promoter, cotransfection experiments with different reporter constructs under the control of two Gal4 upstream activated sequences (UAS) in combination with eukaryotic expression vectors for Gal4-fusion proteins were performed. We cloned the two UAS sites upstream of the minimal or full-length IL-6 promoters (Fig. 8) and used Gal4 fusion vectors for expression of RBP-Jκ or for the p65 NF-κB subunit. In agreement with previously reported data on the repressor activity of RBP-Jκ (25), the results shown in Fig. 10A confirm that RBP-Jκ is a powerful repressor of basal transcription even when it is positioned more than 1 kb upstream of the start site of transcription (73 and 55% repression for the 50- and 1,168-bp constructs, respectively). On the other hand, the p65 transactivation potential became very weak at the longer distance (17- and 1.8-fold induction, respectively). These results indicate that in addition to its competitor effects with the activated NF-κB complexes for access to the IL6-κB sequence in the IL-6 gene promoter, the RBP-Jκ protein may also function on its own to repress or downmodulate IL-6 promoter activity in general.

RBP-Jκ was already shown by others to repress transcription promoted by the VP16 acidic transactivator (16). Figure 10B shows that the Gal4 N-terminal 147-amino-acid domain is responsible for a two- to threefold transactivation compared to mock-transfected cells. Others have already made this observation (25). By contrast, transient transfection of as little as 50 ng of Gal4–RBP-Jκ fusion vector led to complete repression of the promoter activity, confirming the result shown by Dou et al. with a similar construction (16). Our result confirms the high inhibitory potential of RBP-Jκ on basal transcription. Furthermore, we show that this inhibition is strong enough to repress the 234-bp IL-6 promoter after TNF induction and in the presence of upstream regulatory sequences including NF-IL6, MRE, and NF-κB binding sites, strongly suggesting that RBP-Jκ is indeed able to silence the IL-6 promoter as long as it can bind to the DNA and that this inhibition occurs even in the presence of nuclear NF-κB factor bound to a downstream sequence.

**Overexpression of an inactive RBP-Jκ positively modulates endogenous IL-6 secretion.** We described above that a mutation of the IL6-κB site, which strongly reduced RBP-Jκ binding, was associated with higher promoter activity and proposed that this was due to loss of binding of the repressor protein.

To confirm this hypothesis, we stably transfected L929sA cells with constructions coding for wild-type RBP-Jκ or its dominant-negative mutant EEF-238-AAA (RBP-AAA), which was reported by others to have lost its repression activity but which still binds to the DNA (25). Analysis of the pooled population of each transfection showed (Fig. 11) that expression of wild-type RBP-Jκ did not lead to an enhanced repression of IL-6 synthesis, probably because of the already high level of endogenous protein, but may have delayed it (see 4-h point). By contrast, constitutive expression of RBP-Jκ$^{EEF-238-AAA}$ led to a higher amount of secreted IL-6 for noninduced L929sA cells as well as to an approximately doubled amount of IL-6 protein in the supernatant after 6 h of TNF induction.

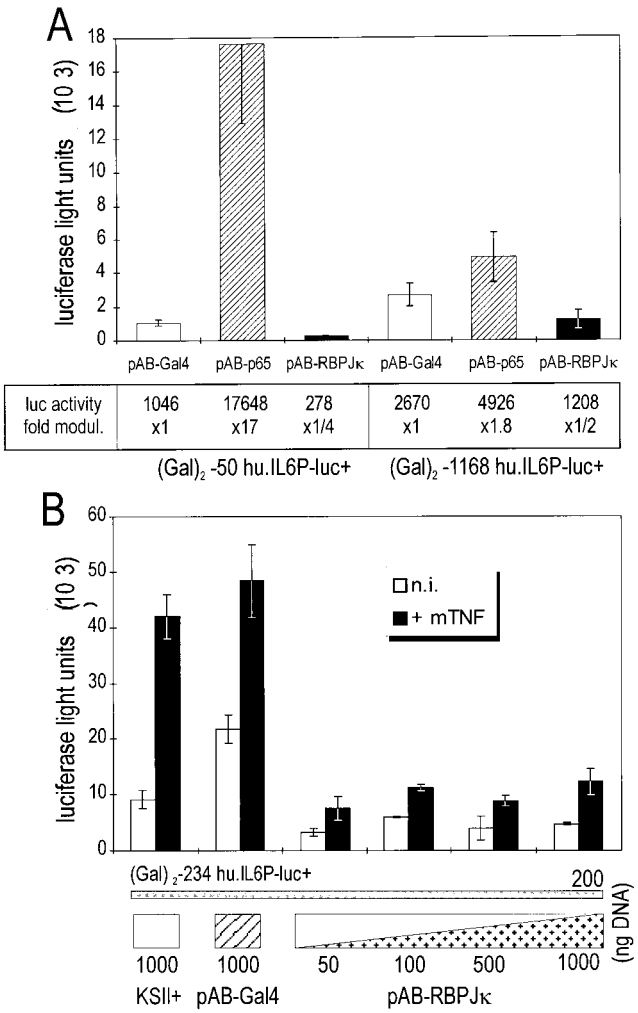



FIG. 10. One-hybrid analysis of RBP-Jκ repressor effect. (A) Transient transfection experiment showing the effect of expression of Gal4-fusion proteins on the minimal or full IL-6 promoters coupled to two Gal4 binding sites. Luciferase (luc) activity was measured 72 h after transfection and corrected for protein content. Each bar represents the average result for four independent wells. The relative modulation (modul.) (activation/repression) is indicated. (B) L929sA cells stably transfected by the calcium phosphate method with the (Gal)2-50 hu.IL6P-luc+ reporter vector. After 15 days of selection with G418, stable colonies were pooled and further transfected with DEAE-dextran with different expression plasmids (KSII+ bluescript plasmid [Stratagene] used as mock DNA). Luciferase activity was measured 48 h after transfection. Each transfection was performed four times. n.i., noninduced.

These data are complementary to the result obtained with a point mutation of the IL6-κB site (Fig. 9C). The more pronounced effect obtained by mutation of the DNA site could be due to the fact that it completely abolished binding of endogenous RBP-Jκ, while the constitutively expressed mutant protein still had to compete with the endogenous pool of wild-type RBP-Jκ.

Despite several attempts and the use of CMV and RSV promoters, overexpression of an epitope-tagged RBP-Jκ or of its inactive mutant RBP-Jκ$^{EEF-238-AAA}$ did not lead to an increased level of DNA binding activity, while the transfected protein could be shown by immunoblotting (not shown). This suggests that the overall quantity of RBP-Jκ protein in the cell might be limited by regulatory events or that the higher-level-

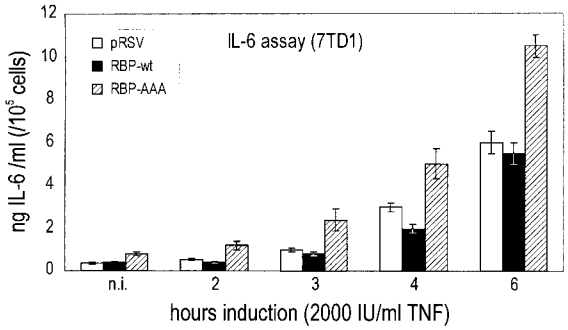



FIG. 11. Mutant RBP-Jκ competes with endogenous RBP-Jκ and releaves the silencing of the IL-6 gene. L929sA were transfected with empty vector (pRSV) or with constructs coding for wild-type (wt) RBP-Jκ or its mutant $RBP^{EEF\text{-}238\text{-}AAA}$ (RBP-AAA). After 15 days of G418 selection, the cells were transferred in 24-well plates and stimulated for the indicated time with 2,000-IU/ml TNF. Aliquots of supernatant were used in a standard IL-6 bioassay. The results are the average values and deviations for four independent assays.

expressing cells might have been counterselected following transfection.

## DISCUSSION

The complex interplay between repressors and activators defines a new level of gene expression control (see reviews in references 13 and 46). In the case of NF-κB, the major level of regulation was originally thought to be present in the cytoplasm, where the transcriptionally active form p50-p65 is trapped in an inactive complex with the inhibitory molecule IκB (for a review, see reference 5). While the NF-κB factor is a necessary and sufficient element to induce transcription of reporter genes, it is now known to synergize with other transcription factors to exert its specific gene regulatory function in vivo. NF-κB activity is directly repressed by the glucocorticoid receptor in the IL-6 (41) and IL-8 genes (36) or by the HMG protein DSP1 present at the IFN-β PRDII-NRE site (29), while it is positively regulated by the binding of other HMG proteins to the IFN-β-κB (57). Moreover, an increasing number of reports revealed the physical association of Rel proteins with other proteins, including several transcription factors (e.g., NF-IL6 and AP-1) involved in IL-6 gene expression and suggesting an in vivo function for gene regulation (20).

Additive, cooperative, and/or competitive effects of different transcription factors determine the final tissue or stage-specific expression of most, if not all, of the inducible genes. In this regard, Santhanam et al. (48) have reported the presence of a negative regulatory region between −126 and −104 (fos basal transcription element), which is responsible for the constitutive repression of IL-6 transcription. In monocytes, this region binds a repressor complex composed of the constitutive Sp1 transcription factor and retinoblastoma-like proteins (47).

Here, we have shown that a ubiquitous mammalian cell factor, RBP-Jκ, specifically recognizes and constitutively binds to the κB motif present in the IL-6 gene promoter. This binding mainly involves the minus strand of the IL6-κB site where NF-κB and RBP-Jκ factors recognize an overlapping target site and compete for access to the DNA. NF-κB has a higher affinity for the IL6-κB motif but is present in much lower abundance than the RBP-Jκ protein. RBP-Jκ functions as a negative effector in two ways. Indeed, besides its competition with NF-κB for the same binding site, thus limiting the cytokine-induced activation of the IL-6 gene promoter, our data suggest that RBP-Jκ also exerts an intrinsic repressor and silencing function in the normal physiological state.

RBP-Jκ binding to several NF-κB sequences has been described elsewhere (16, 27). However, these studies were performed at the level of protein-DNA binding in vitro in EMSA experiments and occasionally by the use of synthetic reporter constructs containing multiple RBP-Jκ sites (25). By contrast, in the context of the IL-6 promoter, we have shown that a single-base substitution within the unique NF-κB motif, while not significantly affecting the binding of NF-κB complexes, almost completely abolished the binding of the RBP-Jκ protein. This modification was accompanied by a dramatic increase in the level of basal and TNF-induced transcription. We confirmed this observation by another approach in which stable transfection of a mutant $RBP\text{-}J\kappa^{EEF\text{-}219\text{-}AAA}$ protein which had lost its repressor activity led to higher basal and TNF-induced secretion of the endogenous IL-6 in mouse L929sA cells. The concurrent results of these two experiments led us to conclude that the RBP-Jκ protein is indeed a constitutive repressor of the IL-6 promoter in vivo and that this repression is

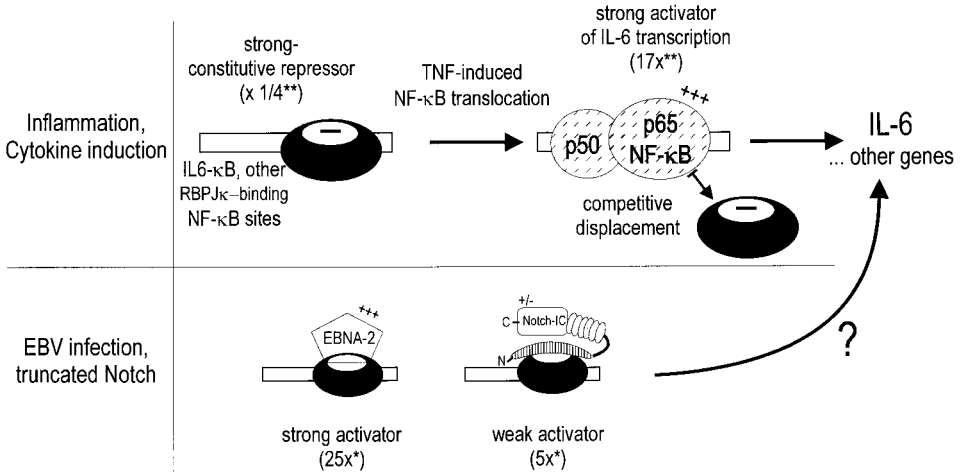



FIG. 12. Model of transcriptional regulation at the level of the IL6-κB sequence and possible relevance of known RBP-Jκ-associating factors. The competitive binding between NF-κB and RBP-Jκ may not be the only event occurring at the level of the IL-6 promoter. Interactions with EBNA-2, Notch-1, or possibly other factors could be involved in regulatory processes leading to IL-6 gene regulation as well as to the regulation of other genes. (*, reference 26; **, our results).

sufficient to maintain the IL-6 gene silent in the absence of an inflammatory stimulus.

While this paper was under revision, a similar message on the repressive function of RBP-Jκ on IL-6 gene expression was published by Kannabiran et al. (28). Whereas these authors attributed the repressive activity of RBP-Jκ to the cooperative transcriptional activation exerted by C/EBP-β and NF-κB, we clearly demonstrated by various transfection assays with recombinant, κB sequence-containing constructs, that the GATA-C/EBP site (14) is not needed for repression by RBP-Jκ and that RBP-Jκ acts as an active repressor on promoters exclusively driven by κB motifs. In contrast to the report of Kannabiran et al., in which loss of function is observed upon distancing of the RBP-Jκ binding site, we still detected repression of basal activity when the RBP-Jκ binding site was placed as far as −1168. Moreover, RBP-Jκ was able to repress the TNF-induced activity when it was present at position −234. The differences in the transfection assays and/or cell phenotypes used may account for the differences observed in repressive activity.

It is likely that the mechanism of repression studied here is not particular to the IL-6 promoter and that other NF-κB sites, which have been shown to bind RBP-Jκ, will also present the same characteristics. Hence, RBP-Jκ could be considered a transrepressor, often sharing its DNA consensus site with the NF-κB transactivator and thus preventing gene induction in the absence of NF-κB activation. Important candidates for this type of regulation include genes for which the NF-κB factor has been shown to be a main transcriptional activator and which actually bind RBP-Jκ as well, such as the genes coding for H2K (27), $\beta_2$-microglobulin (59), MHC-CI, or the IL-2 Rα (53). Indeed, uncontrolled expression of all of these genes in the absense of extracellular aggression would rapidly lead to inflammatory and immunological disorders by perturbing, for instance, the state of activation of endothelial cells or lymphoid precursors. Furthermore, demonstration of the direct effect of transactivators such as EBNA-2 or NotchΔIC through binding to the RBP-Jκ docking protein (Fig. 12) could help to explain developmental or pathological conditions leading to gene expression, independently of NF-κB activation (e.g., T-cell lymphoma, epithelial tumors, or immortalization of B cells).

## ACKNOWLEDGMENTS

We thank A. Israël for providing the pCMV-RBP3 vector, the HES oligonucleotide, and the antiserum against RBP-Jκ and for his helpful discussions; L. Schmitz for providing the Gal4 fusion vectors; J. Vandekerckhove for his gift of the pGST-Grb2-transformed bacteria; N. Rice for providing antisera against NF-κB factors; F. Molemans for DNA sequencing; and I. Van Rompaey and W. Burm for technical assistance.

Research was supported by grants from the EC-Biotech Programme (postdoctoral fellowship to S.P.), the Belgian IUAP, the FGWO, and the VIB. W.V.B. holds a grant from the Belgian IWT. G.H. is a Research Director with the FWO, Vlaanderen, Belgium.

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