The NF-κB Repressing Factor Is Involved in Basal Repression and Interleukin (IL)-1-induced Activation of IL-8 Transcription by Binding to a Conserved NF-κB-flanking Sequence Element*

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Interleukin (IL)-8, a prototypic chemokine, is rapidly induced by the pro-inflammatory cytokine IL-1 but is barely detectable in noninduced cells. Although there is clear evidence that the transcription factor NF-κB plays a central role in inducible IL-8 transcription, very little is known about the cis-elements and trans-acting factors involved in silencing of the IL-8 promoter. By sequence comparison with the interferon-β promoter, we found a negative regulatory element (NRE) in the IL-8 promoter overlapping partially with the NF-κB response element. Here we show that an NF-κB-repressing factor (NRF) binds to the IL-8 promoter NF-κB-NRE. Reduction of cellular NRF by expressing NRF antisense RNA results in spontaneous IL-8 gene expression. In contrast, IL-1-induced IL-8 secretion is strongly impaired by expressing NRF antisense RNA. Mutation of the NRE site results in loss of NRF binding and increased basal IL-8 transcription. On the other hand IL-1-induced IL-8 transcription is decreased by mutating the NRE. These data provide evidence for a dual role of the NRF in IL-8 transcription. Although in the absence of stimulation it is involved in transcriptional silencing, in IL-1-induced cells it is required for full induction of the IL-8 promoter.

A hallmark of inflammation is the invasion of activated leukocytes into the injured tissue. This step is critically dependent on the rapid expression of chemokines, cytokines whose main function is to attract and activate leukocytes at sites of infection or damage (1).

IL1-8 was the first identified member of the still growing chemokine family and represents the prototype human chemokine (2). IL-8 synthesis, low or undetectable in normal noninflamed tissue, can be induced in vitro as well as in a wide variety of cells in vivo by pro-inflammatory cytokines such as IL-1 or tumor necrosis factor (3, 4) or as a direct consequence of contact with pathogens like bacteria (5, 6), viruses (7, 8), and cell-stressing agents (9–12). The regulation of IL-8 by IL-1 is of particular pathophysiological importance, because blockade of IL-1 receptors by application of the IL-1 receptor antagonist consistently reduces tissue neutrophilia in a variety of disease models presumably by preventing IL-1-induced synthesis of IL-8 and related chemokines (13).

As chemokine gene expression is tightly regulated, understanding the underlying molecular basis of this control is likely to yield novel insight into the pathology of inflammation and may result, ultimately, in development of novel anti-inflammatory drugs.

In that respect the dimeric transcription factor NF-κB has emerged as a key molecule in the transcriptional regulation of genes relevant to inflammation (14), including chemokines such as IL-8 (15). The activity of NF-κB is controlled at multiple levels. First, extracellular signals activate the recently identified inhibitor of NF-κB kinase complex that induces phosphorylation-dependent proteolytic degradation of IκB proteins and allows translocation of NF-κB from the cytosol to the nucleus (16). Second, transcriptional activity of NF-κB is potentiated by inducible phosphorylation (17–23), cooperation with AP-1 (24), by binding to the CREB-binding protein/p300 coactivator (25) and integration of NF-κB into multiprotein transcriptional complexes such as the IFN-β enhanceosome (26, 27).

On the other hand, proteins have been described that repress or inhibit NF-κB activity. Recombination signal sequence-bind- ing protein Jκ (RBP-Jκ) constitutively binds to NF-κB sites in the IL-6 and NF-κB 2 (p100/p52) promoters and represses basal transcription (28–30). Upon induction RBP is displaced by NF-κB, relieving transcriptional repression. Nuclear hormone receptors physically interact with NF-κB in the absence of DNA (31) and thereby interfere with inducible IL-6 (32) or IL-8 transcription (33). The zinc finger protein A20 represses tumor necrosis factor-induced NF-κB-dependent IL-6 and granulocyte macrophage-colony stimulating factor (GMCSF) gene expression by a cytosolic pathway (34).

Previous studies have shown that a sequence spanning the forming growth factor-β-activated kinase 1; TAB1, TAK1-binding protein 1; wt, wild type; aa, amino acids; ERK, extracellular signal-regulated kinase.

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‡The abbreviations used are: IL, interleukin; AP-1, activating protein-1; C/EBP, CAAT enhancer-binding protein; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase, HA, hemagglutinin; JNK, c-Jun-N-terminal kinase; IFN-β, interferon-β; IP, immunoprecipitation; MAPK, mitogen-activated protein kinase; MKK, MAP kinase kinase; MEKK1, mitogen-activated protein kinase/ERK kinase kinase 1; NF-κB, nuclear factor-κB; NRF, NF-κB-repressing factor; NRE, negative regulatory element; Oct-1, octamer-binding protein-1; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; RLU, relative light units; TAK1, transforming growth factor-β-activated kinase 1; TAB1, TAK1-binding protein 1; wt, wild type; aa, amino acids; ERK, extracellular signal-regulated kinase.
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nucleotides –1 to –133 within the 5’-flanking region of the IL-8 gene is essential and sufficient for transcriptional induction of the gene by most stimuli including IL-1 (15). This region contains a single NF-κB element that is required for activation in all cell types studied, as well as an AP-1 and a C/EBP site (15, 35). In the absence of immune stimulation, this promoter fragment is transcriptionally silent (15, 36). This suggests that it may be under negative transcriptional control.

In a search for potential repressory mechanisms of IL-8 transcription, we found a sequence within the IL-8 promoter that showed high homology with a regulatory element of the IFN-β promoter. This 16-base pair sequence spans the decanucleotide binding site for NF-κB which overlaps with a previously characterized negative regulatory cis-element (NRE). The NRE is required for position-independent silencing of the IFN-β promoter (37). A protein called NF-κB-repressing factor (NRF) was cloned recently which specifically binds to this site (38). NRF represses basal IFN-β transcription but, although permanently bound to the promoter, is not required for virus-induced IFN-β gene expression (38).

Here we report a second gene, interleukin-8, whose basal expression is controlled by NRF. Furthermore, we provide evidence that by binding to the NRE of the IL-8 promoter, NRF plays an additional role and acts as a coactivator of IL-1-induced IL-8 gene expression.

EXPERIMENTAL PROCEDURES

Cells and Materials—HeLa cells stably transfected with the plasmid pHU1D15-1 expressing the tet transactivator protein were obtained from H. Bujard and used throughout this study (39). Cell lines were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum. E64 (trans-epoxysuccinyl-l-leucylamido(4-guanidinobutane), pepstatin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and all other chemicals were from Sigma; [γ-32P]ATP was purchased from Hartmann Analytics. Rabbit antiserum SAK9 to the C terminus of JNK2 (40) was a kind gift of J. Saklatvala. Antibodies directed against NRF were raised in rabbits immunized against five different peptides based on the antigenic index of the NRF protein sequence (aa 25–45, 175–191, 256–272, 272–288, and 364–382). Antibodies 12CA5 directed against hemagglutinin (HA) and 9E10 against c-MYC epitopes were from Roche Molecular Biochemicals. Antibodies against NF-κB p65 (sc-572) were from Santa Cruz Biotechnology. Horseradish peroxidase-coupled secondary antibodies against mouse and rabbit IgG were from Sigma. Protein A-, G-, and GSH-Sepharose were from Amersham Pharmacia Biotech. Human recombinant IL-1-α was produced as described (40).

Plasmids—The expression plasmid for GST-JUN (amino acids 1–135) was a kind gift of J. R. Woodgett. GST fusion proteins were expressed and purified from Escherichia coli by standard methods. Plasmid pMCL-AMK2K, constituting actively constitutive MKK1 (N3/S218E/S222D), was a kind gift of N. G. Ahn. Plasmids pFCHMEKK1 encoding amino acids 360–672 of MEKK1 and pSV-galactosidase were from Stratagene and Promega, respectively. pCD-SRaHATAK1 and pEPTAB1 were from E. Nishida and J. Niyomta-Tsuj1, respectively. Plasmids pCSMT-MKK7 (Ser-271, Thr-275, and Ser-277 mutated to Glu), pVHA-MKKK (Ser-207 and Thr-211 mutated to Glu), pVHA-JNK2, and the IL-8 promoter-driven luciferase reporter plasmid pHUC13–3–IL-8p (nucleotides 1348–1527 of the IL-8 gene) have been described (41). Expression plasmids p65 NF-κB, pNRFPV16, pBDVP16, pNRF, and pFFNRF have been described (38). PC5AMT-NRF encodes for N-terminally MYC-tagged NRF. The tet-off expression plasmids pTBC-NRF and pTBC-NRFas contained the full-length NRF coding region or nucleotides 1–300 of the coding region in antisense orientation, respectively. Site-directed mutagenesis of NRF and NF-κB sites was performed using the oligonucleotides described in Fig. 1. The AP-1 mutant of the IL-8 promoter plasmid has been described elsewhere (41). All mutations described above were introduced using the Quick Change™ site-directed mutagenesis kit (Stratagene). Primer sequences used for polymerase chain reaction are available upon request. Sequences were confirmed by automated DNA sequencing on an ABI 310 sequencer (Applied Biosystems).

Transfections and Preparation of Cell Extracts—Transient transfections were performed by the calcium phosphate method exactly as described (41). For determination of reporter gene activity, cells were lysed in ice-cold potassium phosphate buffer (100 mM, pH 7.8), containing 0.2% Triton X-100, 1 μg/ml pepstatin, 2.5 μg/ml leupeptin, and 1 mM PMSF. After 15 min on ice, lysates were cleared by centrifugation at 10,000 × g for 5 min at 4 °C, supernatants were taken as cytosolic extracts. Pellets were resuspended in buffer B (20 mM HEPS, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.3 mM Na3VO4, 20 μM β-glycerophosphate and freshly added 2.5 μg/ml leupeptin, 10 μM E64, 300 μM PMSF, 1.0 μg/ml pepstatin, 5 mM DTT, 400 mM okadaic acid). The pellet was resuspended in buffer A + 0.1% Nonidet P-40 and left on ice for 10 min and then vortexed. After centrifugation at 10,000 × g for 5 min at 4 °C, supernatants were taken as cytosolic extracts. Protein concentration of cell extracts was determined by the method of Bradford and samples stored at –80 °C.

Immunoprecipitation and JNK Assay—Cell extract protein (100–250 μg) from cells transfected with indicated plasmids was diluted in 500 μl of buffer C (50 mM Tris, pH 7.4, 10 mM NaCl, 50 mM MgCl2, 0.5 mM EDTA, 20 μM β-glycerophosphate, 2.5 μg/ml leupeptin, 10 μM E64, 300 μM PMSF, 1.0 μg/ml pepstatin, 5 mM DTT, 400 mM okadaic acid). After 1 h on ice nuclear extracts were vortexed and cleared at 10,000 × g for 5 min at 4 °C and supernatants collected. Protein concentration of cell extracts was determined by the method of Bradford and samples stored at –80 °C.

Electrophoretic Mobility Shift Assay (EMSA)—Double-stranded oligonucleotides corresponding to the IL-8 promoter sequences shown in Fig. 1 were end labeled using [γ-32P]ATP and T4 polynucleotide kinase and purified by gel filtration on S-200 spin columns (Amersham Pharmacia Biotech). Protein–DNA binding reactions were performed with 5–20 μg of nuclear extract protein, labeled oligonucleotide, 1 μg of poly(dI-dC) in 10 mM Tris, pH 7.5, 10 mM EDTA, 0.05% (w/v) dried low-fat milk, 50 mM NaCl, 10 mM DTT, and 10% glycerol in a total volume of 10 μl. After incubation at room temperature for 30 min, protein–DNA complexes were resolved on 5% PAGE and visualized by autoradiography. For NRF supershifts the five α-NRF antisera were mixed, and 1–2 μl of this mixture or of the corresponding preimmune sera were added at the beginning of the binding reaction.

Expression of mRNAs—HeLa-TA cells were transfected with 5 μg of pTBC, pTBC-NRF, or pTBCNRFas, 10 μg of high molecular weight DNA, and 0.5 μg of pSV2PAC encoding the puromycin acetyltransferase gene as described (38). Stably transfected cells were selected and maintained in 4.2 μg/ml puromycin and 500 μg/ml G418 in the presence of 2 μg/ml tetracycline. More than 100 clones were pooled and tested for IL-8 and NRF expression. Tetracycline was removed for 48 h to induce NRF sense or antisense mRNA. Cells were then stimulated with 24 ng/ml IL-1α or with Sendai virus (16 plaque-forming units/ml) or left untreated. After 18 h, the supernatants of HeLa-TA cells were tested for IL-8 expression or cells were harvested and subjected to immunoprecipitation experiments.

Northern Blot Analysis—Northern blots were performed as described (38, 42). cDNA probes against NRF, neomycin phosphotransferase, and interleukin-8 were generated by polymerase chain reaction.

Western Blots—Western blots were performed as described (41). The expression plasmid NRF protein was detected by immunoprecipitation using an equal mix of polyclonal antibodies directed against aa 256–272 and 272–288. Following immunoprecipitation, cleared extracts were analyzed by Western blot using a mix of polyclonal antibodies directed against aa 25–45, 175–191, and 364–382 of NRF. This procedure has been described in detail elsewhere (38).

Enzyme-linked Immunosorbent Assay—IL-8 protein concentrations in the cell culture medium were determined using the human IL-8 duo set kit (R & D Systems) exactly following the manufacturer’s instructions.
**RESULTS**

The NRE cis-Element Is Required for Basal Repression and IL-1-induced Activation of the IL-8 Promoter—Although the IL-8 and IFN-β promoters differ in most of their known positive regulatory elements, sequence comparison revealed one highly homologous region (Fig. 1). Therefore, we carried out experiments to examine a putative role of the NRE and NRF in IL-8 promoter regulation. Initially, we explored the role of the NRE in basal and IL-1-induced regulation of IL-8 transcription using a luciferase reporter gene under the control of the NRE-NF-κB site. Parallel experiments were carried out with wild type (wt) promoter or versions mutated in the NF-κB and NRE sites (Fig. 1). The NRE mutant IL-8 promoter contained two point mutations immediately adjacent to the NF-κB site, which were previously found to inactivate the NRE (37). Similarly, three distinct mutations within the NF-κB-binding site were introduced as reported by others (36) to create an inactive NF-κB-binding site. The wt IL-8 promoter displays a low transcriptional activity, which is somewhat lower when the NF-κB-binding site is mutated. Mutation of the NRE leads to a low but significant activation of the reporter (Fig. 2A). This is consistent with the role of the NRE in repression of basal transcription as previously observed for the IFN-β gene (37). IL-1 treatment of cells transfected with the wild type IL-8 promoter resulted in more than 80-fold induction of promoter activity. This was strongly reduced by mutating the NF-κB site. Surprisingly, mutating the NRE also resulted in a more than 3-fold reduction of IL-1-stimulated promoter activity (Fig. 2A).

To analyze further the requirement of the NRE site for inducible IL-8 transcription, we tested if any of the known signaling pathways triggered by IL-1 target the NRE. IL-1 induces NF-κB-dependent genes through the protein kinases MEKK1 (43) or TAK1 (44). Therefore, we cotransfected the IL-8 promoter reporter gene together with a constitutively active form of MEKK1 or of TAK1 together with its coactivator TAB1 (44). This results in strong induction of the IL-8 promoter (Fig. 2B). Mutation of the NRE reduced MEKK1- and TAK1-mediated IL-8 transcription by more than 70% (Fig. 2B). Since MEKK1 and TAK1 activate NF-κB as well as MAP kinase signaling cascades, we further delineated the involvement of individual MAPK pathways for NRE-dependent IL-8 transcription.

A significant activation of the IL-8 promoter, although lower than with MEKK1 and TAK1, was observed by selective activation of the ERK or p38 MAPK pathways. This was achieved by transfecting the constitutively active forms of MKK6 and MKK1, MKK6EG (41, 45, 46), and MKK1REF (47), respectively. However, this induction of the IL-8 promoter was not affected by the NRE mutation (Fig. 2B). Thus ERK and p38 MAPK pathways are unlikely to contribute to IL-1-, MEKK1-, and TAK1-mediated NRE-dependent IL-8 transcription.

These results suggest that the NRE is also required for transcriptional induction by stimuli that activate NF-κB, namely IL-1, MEKK1, and TAK1. It was thus necessary to characterize the role of protein(s) binding to the NRE in IL-8 gene expression.

Since the NRE in the IFN-β promoter represents the binding site for NRF (38), we examined if NRF binds to the IL-8 promoter. The minimal IL-8 promoter-luciferase reporter gene construct was cotransfected with an effector plasmid encoding an NRF-VP16 fusion protein. This fusion protein was shown to convert the NRF protein to a transcriptional activator (38). As shown in Fig. 2C, the IL-8 reporter was inducible by the simultaneous expression of the NRF-VP16 fusion protein demonstrating the ability of the fusion protein to bind to the IL-8 promoter. DNA binding of NRF to the IFN-β NRE requires amino acids 296–388 of its C-terminal end (38). This part of the NRF protein is also sufficient to recognize the IL-8 promoter, as the DBDNRF-VP16 fusion protein stimulated the IL-8 reporter to a similar extent as NRF-VP16. As a control, coexpression of an empty eukaryotic expression plasmid had no effect on the transcriptional activity of the IL-8 reporter.

The NRE Is Not Required for NF-κB p65 Binding and Transactivation—The IL-8 promoter NRE site overlaps with the NF-κB site (Fig. 1). NF-κB binding is crucial for inducible transcription of IL-8. We therefore examined if the impaired induction of the IL-8 promoter NRE mutant (Fig. 2, A and B) was caused by a loss of p65 NF-κB binding to the IL-8 promoter.

We investigated the formation of IL-1-inducible complexes between proteins and IL-8 promoter DNA by EMSA. As shown in Fig. 3, nuclear extracts from HeLa cells treated for 30 min with IL-1 formed two complexes (I and II), which were absent in untreated cells. The appearance of two IL-1-inducible complexes binding to the IL-8 promoter is consistent with the results of others (36, 49, 50). Both complexes contained p65 NF-κB as detected by supershifts (Fig. 3). This indicates that p65 is the major NF-κB subunit binding to the induced IL-8 promoter. This is explained by the fact that the IL-8 promoter NF-κB site lacks the first G of the NF-κB consensus sequence GGGRNNYYCC, resulting in preferential binding of p65 NF-κB over other REL proteins to this DNA element as determined by crystallographic studies (48).

In the presence of an NRE mutant oligonucleotide, both IL-1-inducible complexes I and II were formed and were supershifted with the anti-p65 antibodies, indicating that p65 NF-κB
binding to the IL-8 promoter is not affected by the NRE mutation in vitro (Fig. 3). In cells transfected with p65 NF-κB, the activity of the wild type IL-8 promoter was induced by 8-fold as compared with cells transfected with vector alone (Fig. 4). As expected the p65 NF-κB subunit was unable to induce the mutant NF-κB promoter (Fig. 4). However, the mutant NRE promoter was activated by p65 NF-κB to the same extent as the wild type promoter (Fig. 4). Taken together the results presented in Figs. 3 and 4 indicate that the NRE mutation does not affect DNA binding and transactivation of the IL-8 promoter by p65 NF-κB.

In the presence of NRF antiserum, but not preimmune serum, a slower migrating complex was detected in extracts from IL-1-stimulated and untreated cells. This complex failed to

form with the NRE mutant oligonucleotide. Furthermore, a prominent constitutive protein-DNA complex (labeled III) formed with the wild type IL-8 promoter oligonucleotide, but like the complexes with anti-NRF antibodies, it could not be detected with the NRE mutant oligonucleotide. Although we did not observe a classical supershift with the anti-NRF antibodies, these data suggest that NRF may be part of the constitutive protein-DNA complex III whose formation depends on an intact NRE (Fig. 3).

Therefore, in nuclear extracts of unstimulated as well as of IL-1-stimulated cells, NRF binding to the IL-8 promoter requires an intact NRE site, at least in vitro. These data also suggest that the reduction of IL-1-induced IL-8 promoter activity observed for the NRE mutation (Fig. 2, A and B) is not
by transfected p65 NF-κB (Fig. 4). Transfected nuclear p65 NF-κB was far less efficient in inducing the IL-8 promoter than IL-1 or the protein kinases MEKK1 and TAK1 (Fig. 2, A and B). This may be caused by a relatively low amount of transfected NF-κB in the nucleus, but more likely it indicates the requirement of additional signals for full IL-8 promoter induction. IL-1, MEKK1, and TAK1 activate other signaling pathways in addition to NF-κB. Any positive effect of NRF on p65-mediated IL-8 transcription might therefore depend on the activation of additional pathways that are required for maximal IL-8 transcription.

IL-1, MEKK1, and TAK1 activate NF-κB, ERK, JNK, and p38 MAPKs (44, 51–57). MKK6 and MKK1, specific activators of the p38 and ERK MAP kinases, respectively, activated the IL-8 promoter independently of the NRE (Fig. 2B). This fact and our previous finding that the JNK pathway provides a second signal, in addition to NF-κB, for IL-1-induced IL-8 transcription (41, 42), prompted us to test if the JNK pathway could provide the putative additional signal. A constitutively active mutant of MKK7, MKK73E, in which the regulatory amino acids Ser-271, Thr-275, and Ser-277 were replaced by glutamic acid (41, 58) was expressed together with JNK2 and p65 NF-κB. As previously reported by others (59, 60) active MKK7 and JNK2 translocate to the nucleus. We found that the mutant MKK73E was also present in the nucleus and efficiently activated JNK2 as assessed by immune complex protein kinase assay (Fig. 5A). Transfected p65 and NRF did not activate nuclear JNK (Fig. 5A).

Like p65 NF-κB, expression of MKK73E and JNK2 was sufficient to induce some IL-8 transcription (Fig. 5B, lane 3), which increases when a signal by p65 is provided (Fig. 5B, lane 4). Promoter activation by p65 NF-κB or MKK73E and JNK2, alone or in combination, was lost when either the NF-κB or the AP-1 sites were mutated. In agreement with our previous results (41), this indicates an essential role for both sites for NF-κB and JNK-dependent IL-8 transcription. Interestingly, cotransfected NRF enhanced p65 NF-κB and JNK-mediated transcription (Fig. 5B, lane 6). This positive effect of NRF on IL-8 transcription depends on an intact NRE site, because it was not observed when the NRF was mutated (Fig. 5B, lane 6). As the NF-κB and AP-1 promoter mutants failed to respond to NF-κB or JNK stimuli, the individual contribution of these sites to NRF coactivation could not be assessed. Together, these data suggest that simultaneous binding of NF-κB and AP-1 proteins to their respective promoter elements is a prerequisite for NRF to affect p65 NF-κB and JNK-mediated IL-8 transactivation.

**NRF Is Required for Constitutive Repression and for IL-1-induced Expression of the Endogenous IL-8 Gene**—Based on these results, we performed experiments to confirm that NRF also plays a role in regulation of endogenous IL-8 gene expression. HeLa cells were stably transfected with the sense or a 300-base pair antisense NRF cDNA under the control of a tetracycline-sensitive promoter (38). The induction of antisense RNA resulted in efficient reduction of nuclear NRF as determined by Western blot analysis of immunoprecipitated endogenous NRF (Fig. 6A). The expression of endogenous IL-8 and ectopically expressed sense and antisense NRF mRNAs was simultaneously monitored by Northern blot analysis. No IL-8 mRNA was detected in the presence of tetracycline. Omitting tetracycline led to a significant expression of the endogenous IL-8 mRNA in the NRF antisense mRNA expressing cells (Fig. 6B). In contrast, in cells expressing the full-length NRF sense mRNA, or in cells transfected with empty vector, no endogenous IL-8 mRNA expression was detectable upon withdrawal of tetracycline.

As expected from the IL-8 mRNA data, induction of the caused by an altered DNA binding and transactivation of the promoter by p65 NF-κB. Instead it correlates with a loss of NRF binding and possibly of additional proteins contained in the constitutive protein DNA complex II (Fig. 3). It is thus possible that in IL-1-stimulated cells NRF acts as a positive component in the induced promoter.

**Activation of the IL-8 Promoter by p65 NF-κB and JNK Is Enhanced by NRF**—Transfection of p65 NF-κB leads to an induction of the IL-8 promoter (Fig. 4). Cotransfection of NRF did not enhance transactivation of the IL-8 promoter mediated by transfected p65 NF-κB (Fig. 4). Transfected nuclear p65 NF-κB was far less efficient in inducing the IL-8 promoter than IL-1 or the protein kinases MEKK1 and TAK1 (Fig. 2, A and B). This may be caused by a relatively low amount of transfected NF-κB in the nucleus, but more likely it indicates the requirement of additional signals for full IL-8 promoter induction. IL-1, MEKK1, and TAK1 activate other signaling pathways in addition to NF-κB. Any positive effect of NRF on p65-mediated IL-8 transcription might therefore depend on the activation of additional pathways that are required for maximal IL-8 transcription.

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of NRF as a cofactor in IL-1-induced IL-8 transcription. First, a mutant NRE sequence leads to a substantially reduced promoter activity of the IL-8 and IFN-β promoters (38). This suggested a homology in regulation of both genes by NRF. Supershift experiments in EMSA and transfection assays demonstrate that NRF binds to the IL-8 promoter (Figs. 2 and 3). A mutant NRE sequence leads to increased transcription from the IL-8 promoter (Fig. 2). Lowering the cellular amount of NRF by antisense mRNA expression resulted in detectable levels of IL-8 mRNA and the protein, both of which are absent in unstimulated cells (Fig. 6). This derepression proves the in vivo participation of NRF in the constitutive repression of IL-8 and highlights its homologous function in the regulation of basal promoter activity of the IL-8 and IFN-β genes.

A competitive mechanism for repression of the IL-8 gene has been described earlier. Wu et al. (36) showed that the transcription factor Oct-1 binds to the C/EBP site in vitro. Converting the C/EBP-binding site to a C/EBP consensus site abolished Oct-1 binding in vitro and derepressed IL-8 transcription in reporter gene assays. In their model, transcription of the IL-8 promoter is induced by replacing Oct-1 with NF-κB and C/EBP as a consequence of IL-1 stimulation (36). Our study reveals that NRF represses the IL-8 promoter most likely by a non-competitive mechanism. NRF is not replaced by NF-κB after stimulation by IL-1 (Fig. 3), and as outlined below, it alters its function and is required for maximal promoter activity during stimulation. We therefore propose that NRF actively represses transcription factors that bind to the uninduced IL-8 promoter. Evidence for this mechanism was found for the IFN-β promoter (38).

Most interesting, several lines of evidence indicate that NRF is also required for a strong activation of the IL-8 gene by IL-1. First, a mutant NRE sequence leads to a substantially reduced IL-1-induced IL-8 transcription (Fig. 2). Second, binding of NRF to the NRE is abolished by a mutation in the NRE (Fig. 3).
Role of NRF in Basal and IL-1-induced IL-8 Transcription

Third, expression of antisense mRNA of NRF impairs IL-1-induced IL-8 secretion (Fig. 6).

The mechanism by which NRF contributes to IL-1-induced IL-8 promoter activation remains elusive. NF-κB is an essential transcription factor for IL-1-induced IL-8 gene expression (15). Overexpressed NRF did not enhance NF-κB-mediated IL-8 transcription (Fig. 4). This could indicate that the amount of NRF in the nucleus is saturating. Alternatively, NRF needs further signals from IL-1 induction to function as a coactivator for IL-8 transcription. It was recently found that the JNK signaling pathway provides an essential signal for IL-1-induced expression of NF-κB-dependent genes such as IL-8 and IL-6 (41, 42, 61). When p65 NF-κB was expressed together with active nuclear JNK, cotransfected NRF enhanced IL-8 transcription further (Fig. 5). These results suggest that either NRF is modified itself by a JNK-dependent mechanism or that it is interacting on the IL-8 promoter with kinase-activated transcription factor(s). In that context we found that NRF coactivation required not only an intact NRE site but also binding of NF-κB and AP-1 proteins to their cis-elements (Fig. 5). This suggests that the binding of all three transcription factors to their DNA-binding sites and their physical interaction is needed for maximal induction of the IL-8 promoter. The NRE overlaps with the NF-κB site, and indeed, it was shown previously that NRF is able to interact directly with members of the REL family (38). NF-κB is modified by protein kinases (19–21), interacts with various proteins that enhance its transcriptional activity (24, 25, 27, 49), and contacts through its C terminus components of the basal transcriptional machinery (62). It is thus possible that in IL-1-stimulated cells NRF enhances NF-κB activity in concert with AP-1 by acting on one or more of these mechanisms.

Induction of IFN-β is a specific response to viral infection. Most interesting, NRF in IFN-β regulation only acts as a constitutive repressor (38). After induction by virus, the presence of NRF is not required for strong IFN-β gene expression, although it remains bound to the IFN-β promoter (38). Virus induces IL-8 secretion to an extent similar to IL-1. Preliminary data indicate that virus-induced expression of endogenous IL-8 was not affected in cell lines expressing antisense or sense mRNA of NRF.2 It was recently shown that cytokines and virus induce IL-8 transcription through distinct promoter elements (63). Therefore, proteins that interact with NRF at the IL-8 promoter in an IL-1-dependent manner may be responsible for the response specificity of the coactivator function of NRF.

Several other transcriptional regulators have been described that are able to act as activators and repressors. The molecular basis of these dual roles is diverse (64). Our results show that NRF represents a novel type of repressor molecule that is able to switch to an activator. This mechanism depends on the type of inducer and the regulation and structure of the transcriptional complex. Our description of a previously undetected mechanism of IL-8 regulation adds to our understanding of the complexity of this chemokine expression. This type of regulation might also be relevant for other genes involved in inflammatory events. The combination of NF-κB and NRF sites is conserved in a variety of genes relevant to inflammation (37, 65). The NRE does not overlap the NF-κB site in all cases. However, it was shown previously that the inhibitory effect of NRF to various NF-κB elements is exhibited over distances up to 2.5 kilobase pairs (37). Thus, it is tempting to speculate that NRF constitutes a major transcription factor that prevents uncontrolled expression of proinflammatory proteins and contributes to their effective synthesis during disease.

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The NF-κB Repressing Factor Is Involved in Basal Repression and Interleukin (IL)-1-induced Activation of IL-8 Transcription by Binding to a Conserved NF-κB-flanking Sequence Element

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