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Molecular Mechanisms of Inducible Nitric Oxide Synthase Gene Expression by IL-1β and cAMP in Rat Mesangial Cells

Wolfgang Eberhardt,* Christoph Plüß,† Richard Hummel,† and Josef Pfeilschifter2*

Expression of the inducible nitric oxide synthase (iNOS) gene in rat mesangial cells is differentially triggered by IL-1β and cAMP predominantly at the transcriptional level. The 5′-flanking region of the rat iNOS gene contains several binding sites for transcription factors potentially involved in cytokine and cAMP signaling such as nuclear factor-κB/Rel, CCAAT/enhancer-binding protein (C/EBP), and cyclic AMP-responsive element-binding protein/ATF. We tested promoter activities of serial and site-directed deletion mutants of iNOS-chloramphenicol acetyltransferase reporter genes after transient transfection and stimulation of mesangial cells. A region between bp −277 and −111 bearing a CCAAT/enhancer-binding protein-response element was found to be critical for cAMP-mediated gene induction but dispensable for IL-1β inducibility. Moreover, a minimal promoter ranging from the transcriptional start site to −111 containing a κb site is sufficient to confer IL-1β-mediated iNOS promoter activation. Consistent with these findings, an electrophoretic mobility shift assay shows the appearance of an IL-1β-inducible nuclear factor-κB p50/p65 heterodimeric complex. Using probes containing C/EBP-binding sites from the iNOS gene revealed further binding of different complexes, all of which were strongly inducible by cAMP and to a lower extent also by IL-1β. Abs against cyclic AMP-responsive element-binding protein, C/EBPβ, and C/EBPδ were able to partially supershift single complexes, suggesting the participation of these transcription factors in the regulation of iNOS gene expression by cAMP and IL-1β. Finally, we show that both cAMP and IL-1β strongly induce steady-state levels of C/EBPβ and C/EBPδ mRNA levels. These data demonstrate that IL-1β and cAMP use distinct as well as partially overlapping sets of transcriptional activators to modulate iNOS gene expression in rat mesangial cells. The Journal of Immunology, 1998, 160: 4961–4969.

Nitric oxide (NO) is a pleiotropic molecule involved in a variety of biologic processes including neurotransmission, vascular homeostasis, and effector functions of mouse macrophages. NO is synthesized in vivo from L-arginine by at least three different isoforms of nitric oxide synthase (NOS). The activities of the constitutively expressed neuronal NOS and endothelial NOS are dependent on elevated levels of Ca²⁺ and calmodulin. The inducible NOS (iNOS) is synthesized in a variety of cell types from several mammalian species and produces high concentrations of NO upon induction with inflammatory cytokines and/or bacterial LPS (1–3). Moreover, evidence has been presented that iNOS expression in rat mesangial cells is triggered by IL-1β, TNF-α, as well as cAMP (4, 5).

The diversity of the pathologic conditions in which iNOS is maximally expressed is based on the existence of different signals inducing iNOS and implies the involvement of different transcriptional activators to control its transcription. Transcriptional regulation of the mouse iNOS gene by IFN-γ, LPS, IL-1β, and TNF-α and the identification of cis-acting elements responsible for induction by these agents has been elucidated by several groups (3, 6, 7). They showed that consensus sequences for the binding of IFN regulatory factor-1 and nuclear factor-κB (NF-κB) within a 1.6-kb fragment of the mouse iNOS promoter region are required for transcriptional activation by cytokines and LPS, respectively. Cloning of the 5′-flanking region of the rat iNOS gene has opened a molecular route to evaluate the mechanism of the synergistic iNOS gene expression triggered by IL-1β and cAMP in rat mesangial cells (8). By using pyrrolidine dithiocarbamate, a specific inhibitor of NF-κB, we obtained evidence that NF-κB is a crucial transcription factor for the IL-1β-mediated iNOS gene induction, but is not involved in the cAMP-dependent pathway of iNOS transcription (9). In an attempt to identify additional transcriptional activators regulating iNOS expression, we focused on the role of cyclic AMP-responsive element-binding (CREB) protein and CCAAT/enhancer-binding protein (C/EBP) transcription factors. The cAMP response element (CRE)-binding protein CREB pre-exists in cells as an inactive protein that supports gene transcription following protein kinase A-mediated phosphorylation. CREB belongs to the immediate early transcription factors and mediates cAMP-triggered expression of a large variety of genes (10). C/EBP transcription factors comprise a family of isoforms encoded by separate genes. Expression of two of these family members, C/EBPβ and C/EBPδ, is induced in hepatic and several nonhepatic tissues by LPS and inflammatory cytokines (11). Moreover, a cAMP-dependent activation of C/EBP gene expression was reported for induction of c-fos gene transcription (12). Most interestingly, a synergistic interaction between CREB and C/EBP transcription factors has been shown to mediate a tissue-specific response to cAMP (13). In this study we demonstrate involvement of NF-κB, CREB, and C/EBP transcription factors in modulating...
iNOS gene expression in response to IL-1β and cAMP in rat mesangial cells.

Materials and Methods
Cell culture
Rat mesangial cells were cultured and cloned as described (14). Passages 9 to 15 of mesangial cells were used for the experiments. All media and supplements were purchased from Life Technologies (Basel, Switzerland).

cDNA clones and plasmid constructs
Expression clones coding for pMSV-C/EBPβ and pMSV-C/EBPβ were a kind gift from Steven L. McKnight (Tularik, San Francisco, CA). Rec-RSV-CREB was kindly provided by Richard H. Goodman (Oregon Health Sciences University, Portland, OR), and Rec-RMV-p65 was a gift from Patrick Baeuerle (Tularik).

The clone piNOS-chloramphenicol acetyltransferase (CAT), which contains 1.7 kb of the 5′ region and 133 bp of exon 1 of the iNOS gene was generated as described previously (8). piNOS-CAT-II containing 526 bp of the 5′-region and 133 bp of exon 1 of the iNOS promoter was generated by using XbaI/HinIII restriction sites. Two 5′ deletion mutants (piNOS-CAT-mut1, encompassing a promoter region from bp −277 to bp +133, and piNOS-CAT-mut2, ranging from −111 to +133) were constructed by PCR using two oligonucleotides corresponding to bp −115 to +133 of the antisense strand as primers and oligonucleotides corresponding to a promoter region from −277 to −259 (piNOS-CAT-mut1) and from −111 to −94 (piNOS-CAT-mut2) as 5′ primers. For the cloning strategy, “5′ primers” were “tailed with a HinIII restriction site at the 5′ end, and ‘3′ primers’ with an XbaI restriction site at the 5′ end. Different PCR fragments were cut simultaneously with XbaI and HinIII and inserted in corresponding orientation into an XbaI/HinIII-digested pCAT-basic vector.

The gene-specific antisense primers used for site-directed mutations were as follows: ΔNF-κB, 5′-TGGAGATCCGCTGAGAGGA AAC-3′ (wild-type: 5′-TGGAGATCCGCTGAGAGGA AAC-3′); ΔC/EBP-2, 5′-TCTCGGTGAAACCATTACGTATGCTGA-3′ (wild-type: 5′-TCTCGGTGAAACCATTACGTATGCTGA-3′).

The vector-specific primers were primer I (corresponding to the T3 promoter region of Bluescript KSvector), primer II (corresponding to the T7 region of Bluescript KS), and primer III (5′-GAACAAAAGCTGTGACCTCC TTT-3′; C/EBP-1, 5′-CTGGGGCGTGAGGGATTGGA AAG-3′; C/EBP-2, 5′-ACAGAGTGGC AGGATTAATGCA-3′).

The double-stranded oligonucleotides used for EMSA labeling with [γ-32P]ATP (3000 Ci/mmol). Reaction mixtures were incubated for 20 min on ice with 5 to 10 μg of protein in 20 μl of binding buffer containing 4% Ficoll; 20 mM HEPES, pH 7.9; 50 mM KCl; 1 mM EDTA; 1 mM DTT; 1 mM PMSF; 0.25 mg/ml BSA; 2 μg of poly(dI-dC); and 20,000 to 25,000 dpm of 32P-labeled oligonucleotide. DNA-protein complexes were separated from unbound oligonucleotide by electrophoresis through native 4% polyacrylamide gels using 0.5× Tris-borate-EDTA. Gels were fixed in 10% acetic acid/10% isopropanol for 20 min and were vacuum dried afterward. Gels were finally exposed to Bio Max film (Kodak, Rochester, NY) at −80°C for 12 to 48 h.

Competition experiments were done by coinubation of a 50- to 100-fold excess (5–10 pmol) of unlabeled double-stranded oligonucleotide in the DNA-protein binding reaction.

For supershift analysis, polyclonal Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and 2 μg of polyclonal Ab were preincubated for 30 min at room temperature before the binding reaction.

Transfection and CAT assay
Mesangial cells were transfected by a modified calcium-phosphate precipitation technique as described by Chen and Okayama (17) using the BES reagent from Sigma (St. Louis, MO). Cells that had reached approximately 70% of confluency were transfected with 15 μg of reporter plasmids and 3 μg of pcDNA-lacZ for monitoring transfection efficiency. DNA for transfection was isolated by the Qiagen-Maxi-prep system (Qiagen, Basel, Switzerland). The quality of plasmid DNA preparations used for transfections was monitored by checking for equivalent content of supercoiled DNA by agarose gel electrophoresis. Cells were treated with IL-1β (2 nM) or with dibutyryl-cyclic AMP (5 mM) 24 h after transfection and incubated for an additional 24 h before being harvested for CAT assay.

For CAT assays, the cells were washed in PBS, harvested in 0.25 M Tris, pH 7.8, pelleted by centrifugation, and lysed by three sequential freeze-thaw cycles. The protein content of cell lysates was determined by a Microprotein assay (Bio-Rad). CAT activities were determined by incubating cell extracts with 14C-chloramphenicol (Amersham, Arlington Heights, IL; sp. act. 55 mCi/mmol) and acetyl coenzyme A (Sigma) for 0.5 to 4 h at 37°C. Chloramphenicol derivatives were separated by thin layer chromatography. The activity of cotransfected pcDNA-lacZ to control transfection efficiency by the β-galactosidase assay was done following the protocol of Berger and Kimmel (18). CAT activities were expressed as the percent conversion of chloramphenicol to acetylated chloramphenicol after quantification of the radioactivity on a PhosphorImager using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Results
A promoter region of the rat iNOS gene downstream of −526 is sufficient for gene induction by cAMP and IL-1β

We recently reported molecular cloning of a 1.8-kb fragment containing the rat iNOS promoter region (8). We have demonstrated inducibility of a CAT reporter fusion gene of the 5′ regulatory region (−1713 to +133; piNOS CAT) by IL-1β and cAMP after transient transfection into 3T3 fibroblasts. Transfection of rat mesangial cells by the same transfection procedure revealed lower transfection efficiencies compared with 3T3 fibroblasts, but the CAT assays indicated a comparable iNOS-CAT promoter activation in response to IL-1β and cAMP (data not shown).
We now used an internal XbaI restriction site at −526 (Fig. 1) to create a large scale deletion mutant encompassing a promoter region from −526 to +133 (piNOS-CAT-II). This construct was tested for promoter inducibility by cAMP or IL-1. The importance of a homologous promoter region within the mouse iNOS gene for mediating the response to LPS in the RAW macrophage cell line was shown by Xie, Whisnat, and Nathan (1). Lowenstein et al. (2) demonstrated that in the mouse iNOS promoter a maximal induction as caused by the synergistic interaction of LPS and IFN-γ is dependent on two discrete regulatory regions residing between position −248 to −229 and between position −2913 to −2102 upstream from the putative TATA box. Comparison of CAT activities of corresponding CAT reporter constructs (piNOS-CAT and piNOS-CAT-II) from the rat iNOS gene revealed that piNOS-CAT-II retained a promoter activity nearly indistinguishable from that of the full-length iNOS rat promoter fragment (2.8-fold induction by cAMP in piNOS-CAT compared with a 3.5-fold induction in piNOS-CAT-II; 2.5-fold induction by IL-1β in piNOS-CAT compared with a 3.0-fold induction in piNOS-CAT-II). These results suggest that in the rat iNOS gene, promoter elements residing between −526 and the start site of transcription are functionally sufficient for the induction of iNOS gene expression by IL-1β or cAMP. Analysis of the corresponding promoter sequence revealed the presence of several putative regulatory elements for binding of transcription factors, including those that might possibly be activated by both stimuli (Fig. 1). These include three copies of C/EBP-response elements (C/EBP-RE), one of them sharing a close homology to CRE and one copy of a κB site.

Identification of promoter regions critical for trans activation

To further delineate the cis-acting elements required for promoter induction by IL-1β and cAMP, we generated two 5′ deletion mutants of iNOS-CAT-II by use of PCR (Fig. 2A). The plasmid piNOS-CAT-mut1 generated by truncation of piNOS-CAT-II upstream from bp −277 lacks an upstream C/EBP-RE (which we termed C/EBP-1). Deletion of this part did not affect IL-1β-induced CAT expression (3.0-fold induction compared with the corresponding unstimulated controls), indicating that regulatory elements crucial for promoter induction by IL-1β are located downstream from −277 (Fig. 2B).

In contrast, piNOS-CAT-mut2 conferred a 25% reduced cAMP inducibility when compared with the CAT activity from piNOS-CAT (piNOS-CAT-II, Fig. 2C). A second deletion mutant (piNOS-CAT-mut2) in which the deletion from the 5′ end of piNOS-CAT-II was extended up to bp −111 was assayed. The level of promoter activity following treatment of cells with IL-1β still remained unchanged (3.3-fold induction in piNOS-CAT-mut2 compared with 3.1-fold induction in piNOS-CAT-II, respectively, Fig. 2B). This suggests that piNOS-CAT-mut2 bears all the important regulatory elements required for iNOS promoter induction by IL-1β. In contrast, piNOS-CAT-mut2 did not confer any cAMP inducibility.

FIGURE 2. A, Schematic representation of wild-type iNOS (piNOS-CAT-II) and two deletion-mutant iNOS CAT-reporter constructs. Binding elements for NF-κB and C/EBP transcription factors that both contribute to iNOS gene induction by cytokines or cAMP are designated as boxes and ovals, respectively. Deletion analysis of iNOS promoter/enhancer activities in response to IL-1β (B) and Bt2 cAMP (C). Mesangial cells were transfected with 15 μg of the indicated CAT-reporter plasmids for 16 h and either not treated (open bars) or stimulated with 2 nM of IL-1β (solid bars in B) or 5 mM of Bt2 cAMP (solid bars in C) for an additional 24 h before being harvested for CAT assays. CAT activities were quantitated by use of a PhosphorImager. Results were calculated relative to CAT activity of wild-type (piNOS-CAT-II)-transfected cells upon stimulation with IL-1β (Bt2 cAMP), which were set at 100% relative CAT activity. Results are means ± SE of three independent experiments.
Role of κB site in IL-1β-mediated induction of iNOS

The maintenance of IL-1β inducibility in piNOS-mut2 implies that the downstream κB site at −107 to −97 is critical for IL-1β-mediated promoter activation. To determine the role of this proximal κB site, we generated a site-specific mutation by changing one nucleotide within the κB site to generate piNOS-ΔκB-κB (Table I). This base transition is sufficient to destroy binding capacity and trans activation properties of NF-κB proteins (19). The remaining transcriptional activity of the mutated promoter construct in response to IL-1β and cAMP was tested by CAT assay. As shown in Figure 3B, disruption of κB results in a total loss of IL-1β inducibility, but has almost no effects on induction by cAMP (2.7-fold induction compared with a 3.0-fold induction in piNOS-CAT-II).

Role of C/EBP-RE for iNOS promoter induction by cAMP

The difference shown between cAMP-mediated CAT activities of the piNOS-CAT-II and piNOS-CAT-mut2 constructs (Fig. 2C) implies that the κB site region shares a strong homology to a CRE (parentheses). Nucleotides that had been mutated in the corresponding piNOS CAT constructs are underlined. Numbers in parentheses indicate the number of bases that match with corresponding core sequences. H = A, T, C, K = T, G, R = A, G, Y = T, C.

To detect NF-κB binding activity in mesangial cells, we performed EMSA analysis using a 32P-labeled, double-stranded oligonucleotide encompassing the iNOS-κB site from −106 to −96 (Table II). Induced binding of NF-κB proteins to this κB element in the homologous region of the mouse iNOS gene after LPS or cytokine treatment was observed (Fig. 3).

**FIGURE 3.** A, Schematic representation of wild-type iNOS promoter (piNOS-CAT-II) and site-specific mutations for a κB site at −107 to −97 (piNOS-ΔκB-κB or a C/EBP-RE at −171 to −163 (piNOS-ΔC/EBP-2). Substitution mutations were introduced into the κB binding site or the C/EBP-2-designed binding site by changing single bases (underlined letters in Table I). B, The κB site at −106 to −96 is critical for IL-1β- but not cAMP-mediated iNOS promoter activation. Glomerular mesangial cells were transfected with 15 μg of the indicated reporter plasmids for 16 h. Thereafter, cells were treated with control medium (open bars) or medium containing 5 mM of Bt, cAMP (gray bars) or medium containing 2 mM of IL-1β (black bars). Results were calculated relative to CAT activity of piNOS-CAT-II-transfected but unstimulated cells, to which a value of onefold induction was assigned. Results are means ± SE of three independent experiments.

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### Table I. NF-κB and C/EBP consensus sequences and their corresponding sequences within the rat iNOS promoter

<table>
<thead>
<tr>
<th>Site</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK-κB consensus sequence</td>
<td>5′-GGGRHTYCCC-3′</td>
</tr>
<tr>
<td>NK-κB in rat iNOS WT promoter (11/11)</td>
<td>−106 5′-GGGACTCTCCC-3′ −96</td>
</tr>
<tr>
<td>Mutated NF-κB in piNOS-ΔNF-κB</td>
<td>−106 5′-GGGACTCTCCC-3′ −96</td>
</tr>
<tr>
<td>C/EBP consensus sequence</td>
<td>5′-TNNNGYAAK-3′</td>
</tr>
<tr>
<td>(CRE consensus sequence (7/8))</td>
<td>(5′-TGAGCTCA-3′)</td>
</tr>
<tr>
<td>C/EBP-2 in rat iNOS promoter (9/9)</td>
<td>−171 5′-TGAGCTAAAT-3′ −163</td>
</tr>
<tr>
<td>Mutated C/EBP-2 in piNOSCAT-mut2</td>
<td>−171 5′-CCAGTGGGT-3′ −163</td>
</tr>
</tbody>
</table>

* The C/EBP site at −171 to −163 shares a strong homology with a CRE (parentheses). Nucleotides that had been mutated in the corresponding piNOS CAT constructs are underlined. Numbers in parentheses indicate the number of bases that match with corresponding core sequences. H = A, T, C, K = T, G, R = A, G, Y = T, C.
treatment was demonstrated by several authors by gel shift analysis and reporter gene assays (3, 6) and by in vivo footprinting (20). To test whether cAMP or IL-1β could induce NF-κB binding activity in mesangial cells, nuclear extracts were prepared from cells treated with IL-1β, dibutyryl cAMP (Bt2 cAMP), or medium, respectively. As shown in Figure 4A, this regulatory element is bound constitutively by two different migrating complexes. Incubation with nuclear extracts from IL-1β-treated cells revealed binding to an inducible complex. The time-course experiment shows that this inducible complex is maximally detectable after 30 min of cytokine treatment, remains at these high levels between 30 min and 1 h, and is maintained at reduced levels for at least 8 h (Fig. 4A and B). By contrast, testing binding properties of extracts from cAMP-treated cells did not differ from those of untreated control cells, thus indicating that cAMP in our cell system does not influence binding activities of NF-κB (Fig. 4A).

To further characterize DNA-binding complexes, we performed supershift and competition experiments (Fig. 4B). As demonstrated by the immunoreactivity of antisera specific for NF-κB/p50 and NF-κB/p65, the IL-1β-inducible protein-DNA complex contained p50 and p65 subunits, as both anti-p50 and anti-p65 Abs impaired DNA binding. A second, lower shifting, constitutive complex was supershifted by anti-p50 but not by anti-p65 Abs, indicating that this complex consists of p50 homomeric subunits.

Table II. Oligonucleotides used for EMSA

<table>
<thead>
<tr>
<th>Site</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK-κB (iNOS) (11/11)</td>
<td>-114 5′-CCCTACTGGGGACTCTCCC-3′ -93</td>
</tr>
<tr>
<td>C/EBP-1 (iNOS) (8/9)</td>
<td>-499 5′-CTGGGGGTGGGAAATTGGG-3′ -479</td>
</tr>
<tr>
<td>C/EBP-2 (iNOS) (9/9)</td>
<td>-177 5′-ACAGAGTGTTGGAATATTGGA-3′ -157</td>
</tr>
<tr>
<td>C/EBP-3 (iNOS) (7/9)</td>
<td>-94 5′-TTGGGAACAGTGACTTTAT-3′ -76</td>
</tr>
</tbody>
</table>

*Number in parentheses indicate number of corresponding bases to the core sequence (underlined). The position corresponding with the rat iNOS promoter region is indicated by numbers.

![FIGURE 4](http://www.jimmunol.org/) DNA binding activity of NF-κB in rat mesangial cells after treatment with IL-1β or Bt2 cAMP. A, EMSA analysis of the time-course of induction of NF-κB-binding to an oligonucleotide containing the proximal κB site at -106 to -96 of the rat iNOS promoter (as shown in Table II) after treatment with cAMP or IL-1β for the indicated time periods. Positions of specific DNA-protein complexes formed are indicated. B, Supershift analysis identifying p50/p65 heterodimers of IL-1β-inducible complexes. The 32P-endabeled NF-κB oligonucleotide described in Table II was incubated with nuclear extracts (5 μg) from either unstimulated or IL-1β-stimulated mesangial cells in the presence of specific antisera raised against p50- and p65-NF-κB subunits as indicated. Furthermore, coinubation with the unlabelled NF-κB probe in a 100-fold molar excess results in a complete competition of all DNA-binding complexes (NF-κB). In contrast, incubation with an unlabeled iNOS-specific TNF-RE oligonucleotide (TNF-RE) does not affect binding of p50/p65-specific complexes, demonstrating specificity of the formed DNA-binding complexes.
The formation of all complexes was competed by a 100-fold excess of unlabeled, iNOS-specific NF-κB consensus oligonucleotide. Incubation with a 100-fold excess of unlabeled oligonucleotide encompassing a candidate TNF-RE located upstream of the κB site (−130 to −123 within the rat iNOS promoter region) did not compete for DNA binding of either low mobility complexes but prevented binding of the “constitutive complex.” This result indicates that a non-NF-κB-related DNA-binding protein that constitutively binds to an iNOS-κB site shares binding affinities to a putative iNOS-TNF-RE. In addition, in EMSAs performed with a consensus NF-κB oligonucleotide, the constitutive complex with the highest migration properties is not detectable (data not shown), thus indicating that the proteins of this complex are not related to NF-κB.

**cAMP and IL-1β promote binding of nuclear proteins to a C/EBP-RE at −171 to −163**

To test the involvement of members of the C/EBP transcription factor family in the induction pathway of iNOS gene transcription, we performed EMSA using an oligonucleotide (depicted in Table II) encompassing an iNOS-specific C/EBP-binding site at bp −171 to −163 (termed C/EBP-2). Mesangial cells cultured in control medium showed a constitutive binding activity of three complexes (complexes II, III, and constitutive complex in Fig. 5A). The intensity of the constitutive, high mobility complex (constitutive complex) displayed a certain variability when tested in different experiments (compare Fig. 5, A and B). Treatment with cAMP or IL-1β strongly increased the binding of complex II and III and caused the formation of an additional complex displaying the lowest migration properties (inducible complex I, Fig. 5A). As determined by time course experiments, binding of all complexes is maximal between 2 and 4 h of treatment with cAMP or IL-1β (Fig. 5B). Supershift analysis revealed the involvement of C/EBPβ, C/EBPδ, and CREB transcription factors (Fig. 5, B and C) as Abs to each of these transcription factors reduced the binding activity of parts of the formed complexes. In contrast, Abs against NF-κB subunits p50 and p65 did not influence the binding capacities of any complex bound to this oligonucleotide (data not shown). C/EBPβ and C/EBPδ isofoms were shown to be involved in transcriptional induction of several acute phase and cytokine genes. In contrast to C/EBPβ and C/EBPδ, C/EBPα in most cell lines shows a constitutive expression and is not inducible by cytokine treatment (11). It was therefore unexpected that anti-C/EBP, which was raised against an epitope from the C/EBPα isoform, causes a supershift identical to that obtained with anti-C/EBPβ (Fig. 5B) as could be clearly shown by overexposure of the autoradiogram (data not shown). One explanation for this behavior is that C/EBPα and C/EBPβ form a heteromeric complex that is recognized and supershifted by both Abs. Alternatively, we cannot exclude cross-reactivity between these Abs. Therefore, an induced DNA-binding capacity of C/EBPα after stimulation with IL-1β or cAMP remains uncertain. In contrast, overexposure of an autoradiograph of a second EMSA gel clearly demonstrates the different positions of the bands as caused by supershifts with C/EBPβ and C/EBPδ antisera (arrowheads in Fig. 5C). This observation excludes the possibility of cross-reactivity between these Abs. Incubation with anti-CREB Ab in the binding reaction results in a complete loss of complex I and II. Moreover, similar to anti-C/EBP sera, this Ab also reduced binding of complex III and that of the constitutive complex (Fig. 5B). This indicates that C/EBPβ, C/EBPδ, and CREB participate in transcriptional activation of the iNOS gene promoter most likely by binding to an identical promoter binding site. Binding of members of both transcription factor families to a common promoter binding site has also been described for activation of several gene promoters such as the androgen-regulated C3 gene and the phosphoenolpyruvate carboxykinase gene (13, 21, 22).

To further delineate the role of a second C/EBP binding site ranging from bp −492 to −483 within pNiNOS-CAT-II (which we
termed C/EBP-1), we performed an EMSA using an oligonucleotide with the corresponding sequence (Table II). As shown in Figure 6A, cAMP and IL-1β both equally induce DNA binding of three complexes (complexes I-III in Fig. 6A). The kinetics of induction under each stimulatory condition are slower than that found for the binding activity to C/EBP-2. Supershift experiments revealed that DNA binding of all three complexes is reduced to a comparable extent by anti-C/EBPδ and anti-p65 Abs but remained unaffected by the antisera against p50, C/EBPα, C/EBPβ, CREB, and CRP-1, a further member of the C/EBP transcription factor family (Fig. 6B). This EMSA pattern significantly differs from the one we obtained with the C/EBP-2 oligonucleotide, indicating that both C/EBP regulatory regions are occupied by nonidentical sets of transcription factors. Together with the data obtained from mutational and deletion analysis, we suggest that the C/EBP-1 RE is partially involved in the cAMP-mediated regulation of iNOS gene transcription and to a lesser extent also for promoter induction caused by IL-1β. This is somewhat of a discrepancy compared with the data obtained from EMSA, as both stimuli, cAMP and IL-1β, induced binding of nuclear proteins to C/EBP-1 equally well. We cannot give a complete explanation for this phenomenon but we refer to the fact that EMSA analysis is based on the in vitro binding capacity of an oligonucleotide and does not take into account effects of DNA topology present in the in vivo situation. Thus, DNA binding as assayed by EMSA is not necessarily a proof for a real trans activation.

Testing DNA-binding activities of an oligonucleotide that encompasses a further cognate recognition site for C/EBP, which is located in a promoter region from −94 to −76 (termed C/EBP-3), showed DNA binding neither under control nor under stimulatory conditions (data not shown). However, this putative binding site shows only a small degree of homology to the consensus C/EBP-binding site (Table II), and we assume that this sequence is not of functional importance for the regulation of the rat iNOS gene.

**Effects of IL-1β and cAMP on the mRNA levels of C/EBPβ and C/EBPδ**

To evaluate whether the cytokine- or cAMP-induced DNA-binding capacities of C/EBPβ and C/EBPδ transcription factors is accompanied by an increase in the levels of corresponding mRNA, we performed a Northern blot analysis. Total RNA was prepared from noninduced and IL-1β- or cAMP-stimulated mesangial cells, respectively. Time course induction verified that the steady-state levels of mRNA of both C/EBP isoforms are indeed affected by both stimuli, each isoform following a different induction profile. As shown in Figure 7, untreated mesangial cells constitutively express low amounts of transcripts of both isoforms. Treatment of cells with 2 nM of IL-1β caused a rapid increase of C/EBPβ mRNA, which peaked at 4 h and remained at high levels for the following 20 h of stimulation. In contrast, the level of C/EBPδ transcripts displayed a more gradual accumulation, which reached a maximum after 16 h of stimulation before it declined again between 16 and 24 h of stimulation (Fig. 7A). Bt2 cAMP also increased the mRNA levels of β and δ isoforms with a gradual increase of C/EBPβ and a more delayed rise of C/EBPδ mRNA. Both isoforms reached maximal levels between 16 and 24 h of stimulation (Fig. 7B). In summary, these data demonstrate that stimulation of mesangial cells with either IL-1β or cAMP results in a rapid increase of C/EBPβ mRNA levels followed by a delayed elevation of C/EBPδ mRNA. These differences in the induction profiles may result in a timely regulated sequence of availability of
nently NF-κB and AP-1. Increased NF-κB activity has been found to be an absolute requirement of iNOS activation elicited by proinflammatory cytokines such as IL-1β or TNF-α and LPS in different cell lines. In contrast, molecular mechanisms of cAMP-mediated iNOS gene expression are poorly defined and the identification of corresponding transcription factors shows a strong species and cell type specificity. Recent data also emphasize a role of transcription factor NF-κB in the cAMP-mediated signaling pathway of iNOS in rat hepatocytes and mouse fibroblasts (27). However, these data were derived from promoter studies using the mouse iNOS promoter. We recently have shown that despite a high extent of nucleotide sequence homology between the mouse and rat iNOS promoters, only the latter contains typical binding sites for the transcriptional activator CREB (8). In rat vascular smooth muscle cells, a promoter region extending from bp −890 to −1002 was shown to be essential for a maximal gene induction stimulated by exposure of cells to cytokines (3). This region contains consensus sequences for binding of NF-κB, IFN regulatory factor-1, and one γ-IFN-activated site. Spink et al. (3) used a cytokine mixture containing IL-1β, TNF-α, and IFN-γ for stimulation of cells but did not test the effect of each single cytokine, which of course limits the analysis of the molecular mechanisms used by different activators of iNOS gene expression. Using mesangial cells as an experimental model system offers the advantage of studying isolated signaling events triggered by each one of these stimuli, as these cells have retained their ability to express iNOS in response to a single stimulus such as IL-1β, TNF-α, IFN-γ, or cAMP.

The major finding of our analysis is that a promoter fragment that encompasses a region up to 500 bp upstream from the transcriptional start site of the gene retained a full promoter inducibility caused by treatment with IL-1β or cAMP when compared with a large promoter fragment of 1.7 kb. Therefore, we conclude that, in the rat, promoter binding sites lying upstream of this minimal promoter region (ranging from +1 to −526) have only marginal importance for the regulation of the gene by IL-1β and cAMP. However, we cannot exclude the possibility that negative regulatory elements situated in the rat iNOS promoter upstream of −526 may mask any transcriptional contribution made by the upstream κB site, or other IL-1β-responsive elements (2). A recent analysis of the human iNOS promoter has demonstrated that functional regulatory regions of cytokine induction depend strongly on cis-regulatory elements upstream of −3.8 kb (24). This is in marked contrast with the mouse and rat iNOS promoter and indicates complexity and species specificity of iNOS gene regulation. Further studies are in progress in our laboratory to determine whether, in a way similar to the human system, the rat and mouse iNOS promoters bear additional regulatory regions upstream from −1.7 kb relevant for iNOS gene regulation.

Our analysis of the functionality of single promoter binding sites within this minimal iNOS promoter region further implicated a differential set of transcription factors used by IL-1β and cAMP, respectively. EMSA analysis confirmed that in our cell system the binding of NF-κB to its cognate binding site is exclusively activated by IL-1β but does not respond to stimulation with cAMP. We further could identify enhanced binding of a p50/p65 heterodimeric complex to this site after cytokine stimulation. As p50 itself is known to possess only weak trans activating properties, a full NF-κB trans activation potency is assumed to need the participation of p65 (19). Transfection of mesangial cells with a p65 expression plasmid resulted in a strong increase of the iNOS-CAT-II reporter activity whereas p50 alone did not alter basal promoter activity significantly (data not shown). These results suggest that p65 alone is able to act as a transcriptional activator of the rat iNOS gene. The trans activation capacity of p65 is due to specific sequences in the C-terminal potion of this NF-κB subunit (19).

While we were searching for functional regions important for promoter induction by cAMP, site-directed mutagenesis of single promoter binding sites revealed a crucial CRE that shared a strong homology to a C/EBP binding site (C/EBP-2). Several findings indicate that there is a significant functional redundancy among transcription factors that bind to CRE (21). An example for this redundancy was given for the regulation of the phosphoenolpyruvate carboxykinase promoter, where C/EBPβ can stimulate transcription primarily by binding to a CRE (22). Interestingly, stimulation of mesangial cells with cAMP results in the increased
binding capacities of CREB-, C/EBPβ-, and C/EBPδ-containing complexes to this consensus sequence as tested by EMSA. As the panel of Abs tested did not cause a supershift of a single band but decreased the intensity of all DNA-bound complexes equally, we conclude that the complexes seen in the gel shifts represent different associations between CREB and C/EBP isoforms. C/EBPβ and δ are involved in the cytokine-dependent regulation of acute-phase proteins in the liver (28). Whether the increased binding activities of these transcription factors in mesangial cells are accompanied by an increase in phosphorylation has yet to be elucidated. In vivo phosphorylation of CREB most prominently by protein kinase A seems to be necessary for its transcriptional activation capability (10).

A similar pattern of complexes that can bind to the C/EBPβ-2 site of the iNOS promoter was obtained when we tested nuclear extracts from IL-1β-treated mesangial cells. The intensity of these complexes, however, did not reach the level obtained in cells treated with cAMP. In addition, supershift experiments indicate that the complexes seen in the extracts from IL-1-induced cells predominantly consist of C/EBP family members and are only marginally affected by incubation with anti-CREB Abs (data not shown), thus indicating that cAMP and IL-1β trigger differently composed complexes of transcription factors binding to a C/EBP-2CRE binding site in the iNOS promoter. All of these observations suggest considerable cooperative interactions among transcription factors induced by these two signaling pathways that may account for the synergistic transcriptional up-regulation of iNOS expression (5).

A role of C/EBP transcription factors in regulation of iNOS gene expression was further demonstrated by IL-1β- and cAMP-induced changes in steady-state levels of C/EBPβ and C/EBPδ mRNAs. Interestingly, the expression of both isoforms is dramatically up-regulated by IL-1β and cAMP, which may explain the increased pool of C/EBP proteins in stimulated cells. In this context it is worth noting the recent finding that members of the C/EBP transcription factor family contribute to the signaling of dexamethasone-mediated gene expression (29, 30). The synthetic glucocorticoid dexamethasone also has pronounced inhibitory effects on iNOS expression in rat mesangial cells (31). Whether C/EBPs are involved in dexamethasone-mediated iNOS gene repression is under investigation in our laboratory. The elucidation of the mechanisms of IL-1β and cAMP-mediated iNOS gene regulation and the crucial role of transcription factors such as NF-κB, C/EBP, and CREB provides attractive new targets for pharmacologic intervention aimed at reducing iNOS gene expression in inflammatory glomerular diseases and other pathophysiologically relevant conditions (32).

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References