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Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: Initial analysis of the human NOS2 promoter

(nitric oxide/tumor necrosis factor α/interleukin 1β/interferon γ/gene regulation)

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ABSTRACT **The expression of inducible nitric oxide synthase (NOS2) is complex and is regulated in part by gene transcription. In this investigation we studied the regulation of NOS2 in a human liver epithelial cell line (AKN-1) which expresses high levels of NOS2 mRNA and protein in response to tumor necrosis factor α, interleukin 1β, and interferon γ (cytokine mix, CM). Nuclear run-on analysis revealed that CM transcriptionally activated the human NOS2 gene. To delineate the cytokine-responsive regions of the human NOS2 promoter, we stimulated AKN-1 cells with CM following transfection of NOS2 luciferase constructs. Analysis of the first 3.8 kb upstream of the NOS2 gene demonstrated basal promoter activity but failed to show any cytokine-inducible activity. However, 3- to 5-fold inductions of luciferase activity were seen in constructs extending up to −5.8 and −7.0 kb, and a 10-fold increase was seen upon transfection of a −16 kb construct. Further analysis of various NOS2 luciferase constructs ligated upstream of the thymidine kinase promoter identified three regions containing cytokine-responsive elements in the human NOS2 gene: −3.8 to −5.8, −5.8 to −7.0, and −7.0 to −16 kb. These results are in marked contrast with the murine macrophage NOS2 promoter in which only 1 kb of the proximal 5′ flanking region is necessary to confer inducibility to lipopolysaccharide and interferon γ. These data demonstrate that the human NOS2 gene is transcriptionally regulated by cytokines and identify multiple cytokine-responsive regions in the 5′ flanking region of the human NOS2 gene.**

Nitric oxide (NO) is a potent effector molecule which is involved in a multitude of physiological activities ranging from the regulation of vascular tone and neurotransmission to the killing of microbes or tumor cells (1). NO is synthesized by the enzyme nitric oxide synthase (NOS) from an L-arginine substrate. Three isoforms of NOS enzymes have been characterized, two or which are constitutively expressed and a third which is inducible (2). The constitutive enzymes, neuronal NOS (NOS1) and endothelial NOS (NOS3), are calcium-calmodulin dependent and intermittently produce small amounts of NO that mediate processes such as vasorelaxation and neurotransmission. The inducible NOS (iNOS, NOS2) protein has enzymatic activity that is not dependent on elevations in calcium above basal intracellular levels. NOS2 is typically not present in unstimulated cells but is expressed in response to cytokines, lipopolysaccharide (LPS), and a host of other agents (3). In contrast to the two constitutive enzymes, NOS2 produces large, sustained amounts of NO that display both cytotoxic and cytoprotective effects.

Recent reports, however, have challenged this paradigm of constitutive and inducible NOS expression. Studies have shown that the two cNOS isoforms are inducible in certain settings (4, 5). Conversely, it appears that NOS2 may also be constitutively expressed in some tissues and cell types, such as the large airways of humans and certain human mononuclear cell lines (6, 7). These observations demonstrate the complexity of gene expression of the different NOS enzymes and lend credence that NOS expression, whether constitutive or inducible, is not just limited to features of the gene for a specific isoform but also to the cell type and stimulus involved in NOS expression.

Our own studies have indicated that the regulation of cytokine-induced NO synthesis in hepatocytes is under complex control. We previously showed that tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), interferon γ (IFN-γ), and LPS synergistically induce NOS2 gene expression *in vitro* in both rat and human hepatocytes (8–10). Among the cytokines, IL-1β is the most effective single stimulus to induce NOS2 expression in primary rat or human hepatocyte cultures (11). These studies led us to clone the cDNA (12) and gene (13) for human hepatocyte NOS2, providing conclusive evidence that NOS2 can be expressed in human cells. *In vivo*, we have shown that rat hepatocytes produce NO during endotoxemia and chronic liver inflammation (14, 15). In rodent studies using a nonselective NOS inhibitor, we have found that NO exerts a protective effect in the liver during endotoxemia by preventing the formation of microthrombi and by neutralizing radical oxygen intermediates (16, 17). We have also determined that NOS2 expression in rat hepatocytes is differentially regulated from the acute phase response (14) and is inhibited by steroids (10) as well as the heat shock response (M.E.d.V., T.R.B., and D.A.G., unpublished data).

The cytokine and endotoxin induction of rodent NOS2 involves the transcriptional activation of the NOS2 gene (11, 18–21). Attempts to elucidate the molecular mechanisms underlying this induction have led to the characterization of the 5′ regulatory region of the murine macrophage NOS2 gene (22, 23). Two functional regulatory elements of the murine NOS2 promoter have recently been found to reside within 1 kb of the transcription start site (24, 25). There are, however, major differences in sequence and potential cis-acting elements between the human and rodent NOS2 5′ flanking

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Abbreviations: TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; IFN-γ, interferon γ; CM, cytokine mixture (TNF-α/IL-1β/IFN-γ); NO, nitric oxide; NOS2 or iNOS, inducible nitric oxide synthase; NO_2^- + NO_3^-, nitrite + nitrate; u, units; LPS, lipopolysaccharide.

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regions (13). It is, therefore, likely that human NOS2 utilizes a different mode of gene regulation, even though the same cytokines regulate transcription in both rat and human hepatocytes. With the increasing evidence that NOS2 is involved in numerous disease processes in humans, understanding the regulation of this gene is paramount. In this study, we demonstrate transcriptional regulation of NOS2 in a human liver epithelial cell line (AKN-1) and describe an initial functional analysis of the human NOS2 promoter. Our results reveal significant differences between human and murine NOS2 promoter-regulatory regions and provide insight into the molecular mechanisms underlying transcriptional induction of NOS2 by cytokines.

MATERIALS AND METHODS

Reagents. Human recombinant TNF-α and human recombinant IFN-γ were obtained from R & D Systems; human recombinant IL-1β was a kind gift from Craig Reynolds of the National Cancer Institute. Lipofectamine was obtained from GIBCO/BRL.

Cell Culture. AKN-1 cells were maintained in HCD medium prepared as described (26) except gentamicin (10 μg/ml; GIBCO/BRL) was used in place of penicillin and streptomycin, and insulin/transferrin/selenium was obtained as a ready mixture (Collaborative Biomedical Products, Bedford, MA). To induce NOS2 expression, TNF-α [1000 units (u)/ml] + IL-1β (100 u/ml) + IFN-γ (250 u/ml) (cytokine mixture, CM) or different combinations of these cytokines were added to culture medium for 2–24 hr.

Nitrite + Nitrate (NO_2^- + NO_3^-) Measurements. To measure NO production in the culture medium, supernatants were assayed for NO_2^- + NO_3^- levels using an automated procedure based on the Griess assay (27).

Northern Blot and Nuclear Run-on Analyses. RNA extraction, Northern blot hybridization, and autoradiography were performed as described (9). Relative mRNA levels were quantitated on a PhorphorImager scanner (Molecular Dynamics) using IMAGEQUANT software. For nuclear run-ons, nuclei were isolated and *in vitro* transcription was performed as described elsewhere (11). Sense and antisense cRNA probes were generated for the full-length NOS2 cDNA (12), a 1.0-kb *Eco*RI fragment of rat liver glutaminase (28), and a 1.3-kb *Pst* I fragment of rat argininosuccinate synthetase (29) and immobilized on a GeneScreen membrane using a Schleicher & Schuell "Minifold II" slot blotter. Equal amounts of labeled RNA were hybridized to the membrane and washed. After PhosphorImager analysis, relative transcription rates of NOS2 were determined by subtracting the sense from the antisense cRNA signal and normalizing to transcription of glutaminase, a constitutively expressed gene in these cells. These experiments were performed three times, and each study yielded similar results.

Plasmids. To construct the human NOS2 reporter gene constructs, a 598-bp *Xba* I to *Hinc*II fragment from the 5′ end of human hepatocyte NOS2 cDNA (11) was used to screen a human genomic cosmid library. A cosmid clone extending >30 kb upstream of the NOS2 transcriptional start site (+1) was isolated. Fragments of the human NOS2 5′ flanking region starting at bp +33 and extending 0.63, 1.3, 3.8, 5.8, 7.0, and 16 kb upstream were prepared using specific restriction sites and subcloned into the promoterless luciferase reporter gene vector pXP_1 or pXP_2 (30) (see Fig. 3). The regions between −3.8 to −5.8, −5.8 to −7.0, and −7.0 to −16 kb were inserted into pTK-Luc (30), a plasmid carrying a portion of the herpes thymidine kinase promoter (−109 to +52) ligated upstream of luciferase, to create pNOS2(3.8–5.8)TK-Luc, pNOS2(5.8–7.0)TK-Luc, and pNOS2(7.0–16)TK-Luc (see Fig. 5). Restriction enzyme analyses were performed to confirm the orientation and validity of all constructs. pRSV-Luc (31) and pIEP-lacz (kindly provided by H. Tahara, University of Pittsburgh) were used as controls in the transfections.

Transient Transfections and Activity Assays. DNA transfections of AKN-1 cells were carried out in six-well plates (Corning) using Lipofectamine. Cells were exposed to serum-free medium containing 1 μg of DNA and 20 μg of liposomes for 4 hr, washed, and replenished with medium supplemented with 5% calf serum. Preliminary transfection experiments showed optimal transfection efficiency and low toxicity with a DNA:liposome ratio of 1:20. Transfections were carried out using equimolar amounts of DNA based upon the size of the entire construct. To keep the total amount of DNA constant in each transfection, carrier DNA (plasmids pXP_1 or pXP_2) was cotransfected with the smaller constructs. In some experiments, to control for transfection efficiency between groups, 0.5 μg of a plasmid containing a cytomegalovirus promoter-driven β-galactosidase gene (pIEP-lacz) was added to each well. As a positive control, cells were transfected with pRSV-Luc while transfection of the promoterless plasmid pXP_1 or pXP_2 served as negative control for each experiment. To ensure that luciferase activities reflected transcriptional effects of cytokines rather than effects on transfection efficiency, transfected cells were allowed to recover overnight before exposure to cytokines for 4 hr. Preliminary studies had shown that maximal inducible luciferase activity could be detected after this 4-hr exposure. Cells were lysed with Reporter lysis buffer (Promega) or buffer containing 1% Triton X-100, 5 mM dithiotreitol, 50% glycerol, 10 mM EDTA, and 125 mM Tris-phosphate (pH 7.8). Luciferase activity was assayed with 20 μl of lysate in a Berthold (Nashua, NH) AutoLumat LB 953 luminometer using a commercially available kit (Promega). β-Galactosidase activity was determined with 30 μl of lysate and 50 μl of β-galactosidase substrate (Promega) by measuring absorbance at 405 nm in a 96-well multiplate reader (Molecular Devices) using SOFTMAX software (Molecular Devices). Protein concentration was assayed with 10 μl of lysate using bicinchonic acid protein assay reagent (Pierce) by measuring absorbance at 550 nm. Luciferase activity was normalized either to the amount of protein or to β-galactosidase activity.

Statistical Analysis. Values for NO_2^- + NO_3^- are reported as mean ± SEM. The significance of differences was determined by ANOVA using the STATVIEW statistics program (Abacus Concepts, Calabasas, CA). Statistical significance was established at $P < 0.05$.

RESULTS

Cytokines Transcriptionally Activate NOS2 in AKN-1 Cells. The AKN-1 cell line was originally isolated from cultures of liver cells obtained from sections of histologically normal human liver through serial dilutional cloning. Karyotype analysis showed abnormal changes in chromosomes 2 and 8, and injections of these cells into nude mice showed tumor growth within 2 weeks, revealing that these cells had been transformed and thus immortalized. Immunohistochemical staining was positive for CK 19 (bile duct keratin), AE1 (low molecular weight cytokeratin), and α1-antitrypsin, indicating that AKN-1 cells display both biliary epithelial and hepatocyte characteristics. Full characterization of these cells will be described elsewhere. To induce NOS2 expression, AKN-1 cells were stimulated with TNF-α/IL-1β/IFN-γ (CM). Peak NOS2 mRNA levels (≈15-fold induction) occurred 4–6 hr after CM stimulation (Fig. 1*A*). NO_2^- + NO_3^- accumulation in the culture medium lagged behind NOS2 mRNA expression but then increased up to 24 hr, the last time point measured. To determine which cytokines would most effectively stimulate NOS2 expression, AKN-1 cells were exposed to different combinations of the same concentrations of TNF-α, IL-1β, or IFN-γ for 6 hr. The combination of all three cytokines (CM) induced the highest levels of NOS2 mRNA, with evidence for

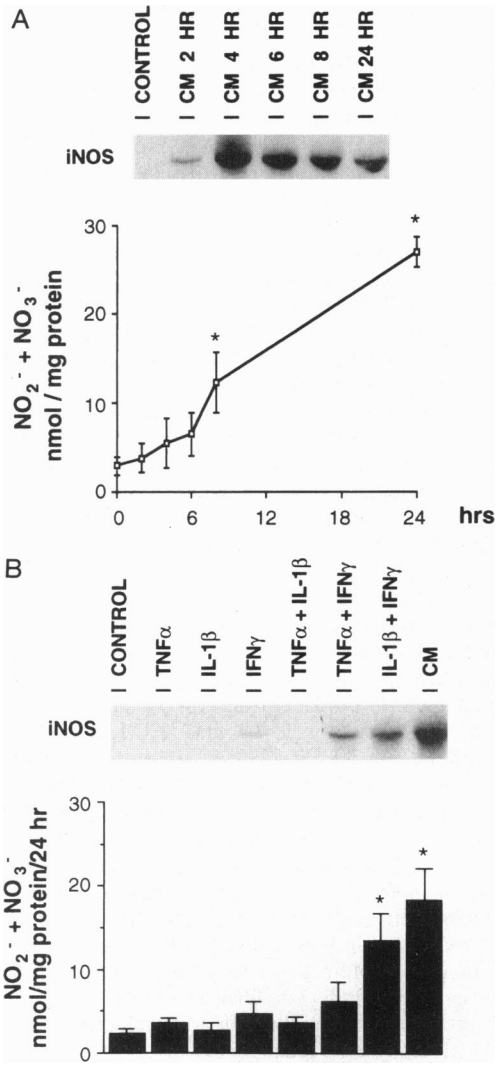


FIG. 1. Cytokine-induced NOS2 expression in AKN-1 cells. (*A*) Time course of NOS2 mRNA induction (Northern blot, *Upper*) and NO_2^- + NO_3^- production in culture supernatants (*Lower*) following stimulation with TNF-α (1000 u/ml) + IL-1β (100 u/ml) + IFN-γ (250 u/ml) (cytokine mix, CM). (*B*) Pattern of 6-hr NOS2 mRNA induction (Northern blot, *Upper*) and 24-hr NO_2^- + NO_3^- release (*Lower*) in response to various cytokines and cytokine combinations. Each Northern blot is representative of three separate experiments. Subsequent 18S rRNA probing showed equal loading in all lanes (not shown). Values for NO_2^- + NO_3^- production are expressed as mean ± SEM for three independent experiments performed in duplicate. *, $P < 0.05$ vs. control.

synergism between the three cytokines (Fig. 1*B*). IFN-γ was the most effective single agent to stimulate NOS2 expression, achieving ≈33% of CM levels while IL-1β or TNF-α alone had little effect. IL-1β/IFN-γ was the most effective double cytokine combination, achieving ≈66% of CM levels, followed by TNF-α/IFN-γ (20%). TNF-α/IL-1β failed to induce appreciable NOS2 expression and unstimulated cells did not have any detectable NOS2 mRNA. NO production measured as 24-hr NO_2^- + NO_3^- accumulation correlated with NOS2 mRNA expression, with IFN-γ and IL-1β/IFN-γ inducing NO_2^- + NO_3^- release into the medium at 4.8 and 13.4 nmol/mg of protein per 24 hr, respectively, while CM achieved levels of 18.3 nmol/mg of protein per 24 hr.

To determine whether the CM-induced increase in NOS2 mRNA levels involved transcriptional activation of the gene, nuclear run-on assays were performed. CM increased NOS2 gene transcription by ≈5-fold at the 2-hr and 4-hr time points

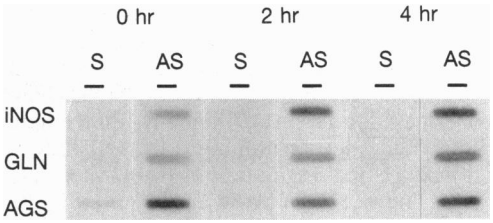
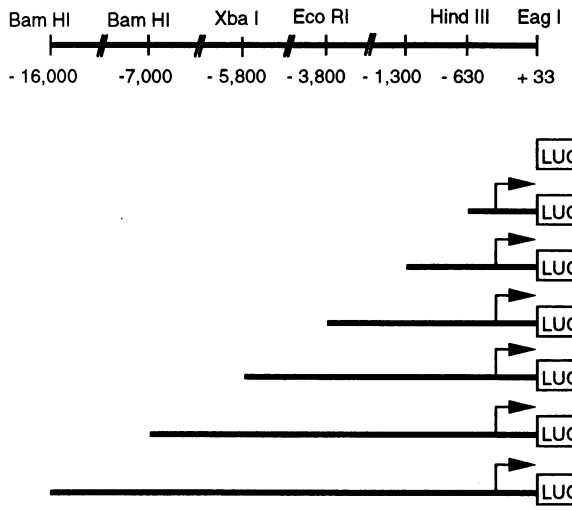


FIG. 2. Nuclear run-on analysis of cytokine-induced NOS2 in AKN-1 cells. Cells were not stimulated (control, 0 hr) or stimulated with TNF-α (1000 u/ml) + IL-1β (100 u/ml) + IFN-γ (250 u/ml) (CM) for 2 or 4 hr. Nuclei were isolated and incubated with [α-^{32}P]CTP. ^{32}P-labeled nuclear RNA was hybridized to a GeneScreen membrane containing 5 μg of immobilized sense (S) and antisense (AS) cRNA probes of iNOS, glutaminase (GLN), and argininosuccinate synthetase (AGS).

(Fig. 2). Unstimulated AKN-1 cells exhibited basal NOS2 transcription, a finding which we have previously noted in cultured rat hepatocytes (11). Consistent with the absence of NOS2 mRNA in unstimulated AKN-1 cells (Fig. 1), Western blot analysis of unstimulated AKN-1 cells showed no expression of NOS2 protein (data not shown).

Cytokine-Responsive Elements Are Localized to Regions More Than 3.8 kb Upstream of the NOS2 Transcription Start Site. To localize the transcriptional regulatory regions of the NOS2 gene, deletions of the 5′ flanking region of the human NOS2 gene were fused upstream of the luciferase reporter gene (Fig. 3). Transient transfections of the NOS2-luciferase constructs into AKN-1 cells and stimulation with CM revealed no significant induction of luciferase activity in constructs containing only the first 3.8 kb of the 5′ flanking region (Fig. 3). However, transfection of pNOS2(5.8)Luc and pNOS2(7.0)Luc followed by CM stimulation resulted in 3-fold and 5-fold induction in luciferase activity, respectively. This 5-fold induction in promoter activity is similar in magnitude to the endogenous transcriptional activation of NOS2 seen in the nuclear run-on experiments (Fig. 2). A 10-fold increase in luciferase expression was obtained when pNOS2(16)Luc was transfected into AKN-1 cells compared to unstimulated cells. These results indicate the presence of cytokine-responsive cis-regulatory elements upstream of −3.8 kb. Identification of cytokine-responsive regions was confirmed in independent experiments in which NOS2 constructs were cotransfected with the internal reference plasmid pIEP-lacz to eliminate the possibility that any cytokine-dependent increase in luciferase activity merely reflected differences in transfection efficiency rather than increases in transcription rate (not shown). We consistently noted low levels of luciferase expression in unstimulated AKN-1 cells transfected with the various NOS2 constructs (Fig. 3, compare with pXP_1), indicating basal promoter activity in the absence of cytokine stimulation. For comparison, transfection of pRSV-Luc resulted in luciferase activity averaging 15,000 u/μg of protein in unstimulated AKN-1 cells (not shown).

In order to further localize cytokine-responsive regions, AKN-1 cells were transfected with pNOS2(5.8)Luc, pNOS2(7.0)Luc, and pNOS2(16)Luc and stimulated with various combinations of cytokines at the standard CM concentrations. TNF-α, IL-1β, or IFN-γ alone did not elicit any significant changes in luciferase activity (Fig. 4). TNF-α/IL-1β or TNF-α/IFN-γ induced a 2- to 3-fold increase in luciferase activity in all three constructs. IL-1β/IFN-γ elicited a modest 2-fold increase in luciferase expression with pNOS2(5.8)Luc. However, a greater increase in luciferase activity (7-fold and 9-fold) was noted with pNOS2(7.0)Luc and pNOS2(16)Luc, suggesting the presence of IL-1β- and IFN-γ-responsive elements between −5.8 and −7.0 kb. Luciferase activity did not differ significantly between CM and the IL-1β/IFN-γ mixture with pNOS2(5.8)Luc and pNOS2(7.0)Luc. However, CM elicited a 13-fold increase in luciferase activity with



	Luciferase Activity Control	Luciferase Activity CM	Fold Induction (CM / Control)	N
pXP1 or pXP2	2.1 ± 0.8	2.5 ± 0.9	1.2	18
pNOS2(0.63)Luc	121 ± 26	119 ± 33	1	8
pNOS2 (1.3) Luc	75 ± 53	53 ± 17	0.7	12
pNOS2 (3.8) Luc	32 ± 7	31 ± 7	1	14
pNOS2 (5.8) Luc	199 ± 76	584 ± 203	2.9	6
pNOS2 (7.0) Luc	131 ± 19	631 ± 88 *	4.8	14
pNOS2 (16) Luc	319 ± 69	3,245 ± 732 *	10.2	18

FIG. 3. Deletional analysis of response of the human NOS2 5′ flanking region to cytokines. The 5′ deletional constructs utilized in the study are shown along with specific restriction sites. AKN-1 cells were stimulated with TNF-α + IL-1β + IFN-γ (CM) following transient transfections of the constructs. Luciferase activity is expressed as light u/μg of protein in cell lysates. Values are expressed as mean ± SEM (N = number of experiments). *, $P < 0.05$ vs. control.

pNOS2(16)Luc, suggesting the presence of additional cytokine-responsive elements (e.g., TNF-responsive elements) further upstream of −7.0 kb.

Regions Upstream of 3.8 kb Confer Cytokine Responsiveness to a Heterologous Promoter. To confirm that regions upstream of −3.8 kb contain cytokine-inducible elements whose function is independent of elements other than the NOS2 basal promoter, we tested the capacity of the segments between −3.8 to −5.8, −5.8 to −7.0, and −7.0 to −16 kb to confer cytokine responsiveness to the heterologous thymidine kinase promoter (Fig. 5). Reporter gene assays showed a 2-fold induction of luciferase activity with pNOS2(3.8–5.8)TK-Luc. Analysis of pNOS2(5.8–7.0)TK-Luc and pNOS2(7.0–16)TK-Luc indicated a 2-fold and 6-fold increase in luciferase expression, respectively. These results confirm the presence of functional, cytokine-responsive regulatory elements upstream of −3.8 kb.

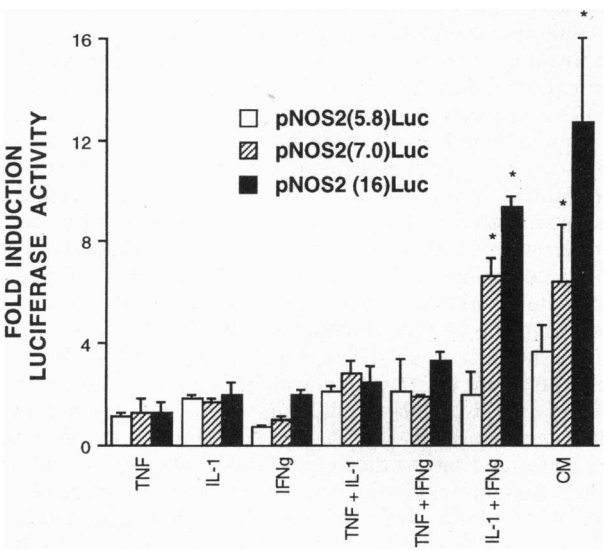
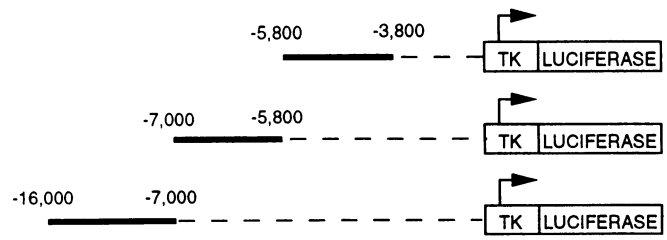


FIG. 4. Deletional analysis of response of the human NOS2 5′ flanking region to individual cytokines and cytokine combinations. AKN-1 cells were transfected with pNOS2(5.8)Luc, pNOS2(7.0)Luc, and pNOS2(16)Luc and exposed to various combinations of TNF-α (1000 u/ml), IL-1β (100 u/ml), and IFN-γ (250 u/ml). Values are expressed as mean ± SEM of the fold induction of luciferase activity (n = at least 4 per group). *, $P < 0.05$ vs. control.

DISCUSSION

In the present investigation, we describe our initial attempt to characterize the human NOS2 promoter in a cytokine-responsive human liver epithelial cell line (AKN-1). Cytokine (TNF-α/IL-1β/IFN-γ) stimulation of AKN-1 cells induced NOS2 expression in a time-dependent manner (Fig. 1*A*) similar to the time course of NOS2 induction that we previously reported in primary human hepatocytes (9). Furthermore, the use of a combination of cytokines showed additive or synergistic effects for induced NO synthesis in the AKN-1 cell line (Fig. 1*B*), a finding also seen in primary human hepatocytes (10). These results show that cytokine-induced NOS2 expression in AKN-1 cells is similar to NOS2 induction in primary human hepatocyte cultures and indicate that the AKN-1 cell line is likely to be a useful reproducible model in which to study the molecular regulation of the human NOS2 gene. It is important to point out that some qualitative differences between AKN-1 cells and primary human hepatocytes were observed. IFN-γ was the most effective single cytokine to stimulate NOS2 expression in the AKN-1 cells (Fig. 1*B*), whereas IL-1β was the most effective cytokine in the primary hepatocytes (11). Further studies are required to determine whether the differences in NOS2 induction are due to variable expression of cytokine receptors or cell-specific differences in NOS2 gene transcription factors.

Nuclear run-on assays with CM-stimulated AKN-1 cells showed an ≈5-fold increase in NOS2 transcription rates compared to unstimulated cells (Fig. 2). These data indicate that the human NOS2 gene is regulated in part at the level of transcription. Parallel reports have shown that the regulation of the rodent NOS2 gene also involves transcriptional activation (11, 18–21). Studies elucidating the molecular mechanisms involved in the transcriptional regulation of NOS2 have been reported only for murine macrophages. Two regions in the murine macrophage NOS2 promoter have been shown to be essential for conferring inducibility of mac-NOS2 to LPS and IFN-γ (22, 23). An NF-κB element at positions −76 to −85 was found to bind members of the NF-κB/Rel family of proteins in response to LPS (24). Further upstream (positions −913 to −923), an IRF-E/ISRE site was shown to bind IRF-1



	Luciferase Activity		Fold Induction
	Control	CM	(CM / Control)
pNOS2(3.8-5.8)Luc	621 ± 198	1,228 ± 355	2
pNOS2(5.8-7.0)Luc	374 ± 161	765 ± 319	2
pNOS2(7.0-16)Luc	697 ± 152	4796 ± 1026*	7

FIG. 5. Cytokine induction of luciferase activity in AKN-1 cells. Following transfection of pNOS2(3.8–5.8)TK-Luc, pNOS2(5.8–7.0)TK-Luc, and pNOS2(7.0–16)TK-Luc, AKN-1 cells were stimulated with TNF-α/IL-1β/IFN-γ (CM). Luciferase activity was measured as described in the legend to Fig. 3. Values are expressed as mean ± SEM (n = 6 per group). *, $P < 0.05$ vs. control.

upon stimulation of RAW 264.7 cells with IFN-γ (25). The latter element appears to function as an enhancer and independently is not able to confer inducibility to either IFN-γ or LPS (22). These findings have provided a reasonable molecular basis for the synergistic induction of NOS2 in murine macrophages.

Recently, we cloned the human NOS2 gene and the proximal 400 bp of the 5′ flanking region and reported a 66% sequence homology to its murine counterpart (13). However, this homology decreases to only 47% when the first 1.5 kb of both promoters are compared, suggesting that differences in gene regulation may prove to exist (data not shown). Computer analysis reveals the presence of >30 putative elements in this 1.5-kb region, including several copies of NF-κB, IFN-γ-responsive elements (IRF-1, ISRE, GAS), and TNF-responsive elements. Yet, AKN-1 cells transfected with a series of NOS2-luciferase gene constructs exhibited no significant activity in the first 3.8 kb of the 5′ flanking region upon stimulation with CM (Fig. 3), demonstrating a marked contrast with the murine NOS2 gene in macrophages, wherein only 1 kb of the proximal 5′ flanking region is required for LPS and IFN-γ induction. Cytokine-inducible activity was initially noted with pNOS2(5.8)Luc, indicating the presence of cytokine-responsive regulatory elements between −3.8 and −5.8 kb. Further induction of luciferase activity was noted in constructs extending to −7.0 and −16 kb. In addition, our transfection studies indicate that IL-1β and IFN-γ-responsive elements exist in the region between −5.8 and −7.0 kb (Fig. 4). To further confirm the presence of cytokine-responsive motifs upstream of −3.8 kb, we assessed the ability of these regions to confer cytokine responsiveness to the thymidine kinase promoter. Transfection assays demonstrated the presence of cytokine-responsive regulatory elements in the regions between −3.8 and −16 kb (Fig. 5). Thus, the cytokine-induced increase in human NOS2 transcription rates in AKN-1 cells requires elements upstream of −3.8 kb to sustain high levels of promoter activity. As yet unknown from these initial studies are the specific elements required for transcriptional activation and what regions, if any, in the proximal promoter are also required.

The promoter inducibility of human NOS2 that we obtained in this study was less than that reported for the murine NOS2 promoter in RAW 264.7 cells treated with LPS/IFN-γ (22, 23). We have carried out transfection studies with the 1.5 kb murine promoter-luciferase construct (22) in LPS/IFN-γ-treated RAW 264.7 cells and obtained 6- to 7-fold increases in luciferase expression (M.E.d.V., T.R.B., and D.A.G., unpublished data). It is unclear why we found lower levels of inducibility in the murine system, although it is most likely due to different transfection techniques, the use of different reporter genes, and different culture and stimulation conditions. Nevertheless, this level of induction of murine NOS2 promoter activity that we obtain is comparable to our 5-fold induction in promoter activity using pNOS2(7.0)Luc in AKN-1 cells. Furthermore, our transfection results are very consistent with the endogenous 5-fold increase in NOS2 transcripts that we see in our nuclear run-on assays.

We consistently measured low levels of promoter activity even in the absence of cytokine stimulation. This could simply reflect the fact that transfected NOS2 constructs do not have the chromatin structure of the endogenous NOS2 gene and also are present in much higher copy number than the endogenous NOS2 gene. However, a second possibility is that the human NOS2 gene may actually be transcriptionally activated to a small degree in resting cells. Nuclear run-on assays support this notion in that NOS2 transcripts are present in unstimulated cells, indicating a low-level, "constitutive" expression of NOS2 (Fig. 2). We have noted this phenomenon also in primary rat hepatocytes (11), and this has been demonstrated in murine peritoneal macrophages as well (32). The absence of mature NOS2 mRNA in unstimulated AKN-1 cells suggests that these transcripts are highly unstable in the absence of cytokines. Others have shown the presence of NOS2 mRNA in the human DLD-1 adenocarcinoma cell line in the absence of stimulation (33), and we have also observed a basal level of NOS2 promoter activity in transient transfection studies of DLD-1 cells (not shown). These findings are consistent with reports demonstrating NOS2 expression in the absence of cytokines *in vivo* in epithelial cells and alveolar macrophages lining the large airways of humans by immunohistochemistry (6). Whether this basal expression reflects the chronic exposure of these cells to inflammatory stimuli is not known. Furthermore, human Epstein–Barr virus-infected lymphocytes and Burkitt lymphoma cell lines also express NOS2 in the absence of cytokine stimulation (7). NO appears to play a role in inhibiting lymphocyte apoptosis and in preventing viral reactivation in these cells. Whether the regulation of NOS2 expression in these settings is different from cytokine-induced NOS2 expression is unknown and warrants separate analysis.

These data indicate that the human NOS2 gene is regulated by a complex promoter-regulatory region which, in contrast to the murine macrophage NOS2 gene, requires cytokine-responsive elements upstream of −3.8 kb to increase its transcriptional activity. It is important to note that no one has examined regions of the murine NOS2 gene that are further upstream of ≈1.5 kb to determine whether additional regions are increasing NOS2 promoter activity. Based on our observations here, it would be reasonable to speculate that such elements could exist in the murine promoter. Although other genes in the liver such as albumin and α-fetoprotein contain enhancers thousands of base pairs proximal to the gene (34, 35), these and most other genes also contain inducible promoter elements within the first 1 kb of the transcriptional start site. We are not aware of any other human gene with a promoter similar to that of NOS2. Based on the importance of NO not only in the liver but in other tissues as well, it is not surprising that complex mechanisms have evolved to regulate human NOS2 gene expression.

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