Multiple NF-κB Enhancer Elements Regulate Cytokine Induction of the Human Inducible Nitric Oxide Synthase Gene

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The human inducible nitric oxide synthase (iNOS) gene is overexpressed in a number of human inflammatory diseases. Previously, we observed that the human iNOS gene is transcriptionally regulated by cytokines and demonstrated that the cytokine-responsive regions are upstream of −3.8 kilobase pairs (kb). Therefore, the purpose of this study was to further localize the functional enhancer elements and to assess the role of the transcription factor NF-κB in both human liver (AKN-1) and human lung (A549) epithelial cell lines. The addition of NF-κB inhibitors significantly suppressed cytokine-stimulated iNOS mRNA expression and NO synthesis, indicating that NF-κB is involved in the induction of the human iNOS gene. Analysis of the first 4.7 kb of the 5′-flanking region demonstrated basal promoter activity and failed to show any cytokine-inducible activity. However, promoter constructs extending to −5.8 and −7.2 kb revealed 2–3-fold and 4–5-fold induction, respectively, in the presence of cytokines. DNA sequence analysis from −3.8 to −7.2 kb identified five putative NF-κB cis-regulatory transcription factor binding sites upstream of −4.7 kb. Site-directed mutagenesis of these sites revealed that the NF-κB motif at −5.8 kb is required for cytokine-induced promoter activity, while the sites at −5.2, −5.5, and −6.1 kb elicit a cooperative effect. Electromobility shift assays using a site-specific oligonucleotide and nuclear extracts from cells stimulated with cytokine-mixture, tumor necrosis factor-α or interleukin-1β, but not interferon-γ, exhibited inducible DNA binding activity for NF-κB. These data indicate that NF-κB activation is required for cytokine induction of the human iNOS gene and identifies four NF-κB enhancer elements upstream in the human iNOS promoter that confer inducibility to tumor necrosis factor-α and interleukin-1β.

The expression of the inducible nitric oxide synthase (iNOS) gene is an important part of the immune response to infection (1, 2). Overexpression of the iNOS gene is seen in many acute and chronic human diseases including septic shock, hemorrhagic shock, multiple sclerosis, rheumatoid arthritis, ulcerative colitis, and its associated cancer diathesis (2–6). Although it is constitutively expressed in some epithelial cell types (7, 8), iNOS expression in most cell types requires exposure to inflammatory stimuli such as cytokines and/or lipopolysaccharide (LPS) (9–13). We and others have shown that iNOS up-regulation in response to LPS and cytokines is transcriptionally regulated (14–16). The nitric oxide (NO) generated by iNOS from its substrate L-arginine has beneficial effects (e.g. antimicrobial, anti-atherogenic, anti-apoptotic) (8, 17–19), whereas overproduction of induced NO can have detrimental consequences (e.g. direct cellular injury, pro-inflammatory) (20, 21). Thus, elucidating the mechanisms that govern iNOS gene expression should provide insight into the molecular mechanisms of gene regulation in several pathophysiologic states and may even lead to novel therapeutic strategies to modulate iNOS expression.

Previously, we reported that transcriptional activation of the human iNOS gene required the presence of cytokine-responsive elements upstream of −3.8 kilobases (kb) in the 5′-flanking region of the human iNOS gene (16). These findings contrast markedly with the murine iNOS promoter, where two regions within 1 kb of the transcription start site have been identified as essential for the induction of iNOS in RAW 264.7 murine macrophages by LPS and IFN-γ (14, 15, 22, 23). Deletional analysis of the murine gene identified an NF-κB element at positions −85 to −76 base pairs (bp) (24) and an interferon regulatory factor-1 (IRF-1) site at positions −923 to −913 kb (25, 26) that mediate iNOS induction by LPS and IFN-γ, respectively.

The involvement of NF-κB in the induction of the murine iNOS gene is consistent with the well described role of this transcription factor in regulating inflammation-associated genes. NF-κB has been shown to be required for iNOS induction in both rodent macrophages (24, 27) and vascular smooth muscle cells (28). NF-κB has been implicated in the induction of the human iNOS gene as well, but its role has not been clearly defined (29–31). In the A549 and DLD-1 human epithelial cell lines, inhibitors of NF-κB activation minimally decreased iNOS expression (29, 30). In contrast, others have shown that the same inhibitors do not inhibit cytokine-stimulated iNOS expression in DLD-1 cells. Failure to identify a homologous functional NF-κB site in the human iNOS promoter raises the possibility that NF-κB may not be involved in the expression of the human gene by cytokines. Therefore, studies were performed to determine if NF-κB plays a role in the transcriptional activation of the human iNOS gene in human

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) AF049872.

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The abbreviations used are: iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; kb, kilobase pair(s); bp, base pair(s); PCR, polymerase chain reaction; IFN-γ, interferon-γ; IRF, interferon regulatory factor; ANOVA, analysis of variance; IL, interleukin; TNFα, tumor necrosis factor-α; CM, cytokine mixture; EMSA, electrophoretic mobility shift assay; PDTC, pyrrolidine dithiocarbamate; DDTC, diethyldithiocarbamate.

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liver (AKN-1) and lung (A549) cell lines. In this study, we not only demonstrate that NF-κB plays a crucial role in human iNOS gene regulation, we also identify NF-κB response elements in the human iNOS promoter. Unlike the murine iNOS promoter, however, the first 1.0 kb of the human iNOS gene 5′-flanking region is not sufficient for iNOS induction. Instead, inducible NF-κB elements upstream of ~4.7 kb are required for cytokine activation of the promoter. Specifically, we have identified a cytokine-responsive enhancer region from ~5.2 to ~6.1 kb in the human iNOS gene that contains four cis-acting NF-κB elements. Furthermore, gel shift assays and mutational analysis of these regulatory elements indicate that they play a functional role in the trans-activation of the human iNOS gene by NF-κB in response to cytokines.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant TNFα and IFNγ were obtained from R&D Systems, and IL-β was provided by Craig Reynolds of the National Cancer Institute. LipofectAMINE was purchased from Life Technologies, Inc. Gel shift antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other reagents were obtained from Sigma.

**Cell Culture**—The AKN-1 human liver cell line was grown in modified HCD medium supplemented with 5% bovine calf serum as described previously (16). The A549 cells (American Type Culture Collection, Rockville, MD) were cultured in F-12k medium supplemented with 10% fetal bovine serum. AKN-1 and A549 cells were plated onto 100-mm Petri dishes (Corning Co., Corning, NY) and stimulated with a cytokine mixture (CM) of TNFα (1,000 units/ml) + IL-β (100 units/ml) + IFNγ (250 units/ml) in the presence or absence of the NF-κB inhibitors pyrrolidine dithiocarbamate (PDTC, 20 or 100 μM) or dithiylthiocarbamate (DDTC, 10 μM) at the indicated time points. NO production was quantitated by measuring nitrite plus nitrate (NO₂⁻+ NO₃⁻) in the culture supernatant by an automated procedure based on the Griess assay (23).

**Northern Blot Analysis**—RNA extraction and Northern blot analysis were performed as described (12). Northern blot hybridizations were carried out using a 2.3-kb BamHI fragment of human iNOS cDNA (10).

**DNA Sequencing and Analysis of the 5′-Flanking Region of the Human iNOS Gene**—Using a series of both sense and antisense PCR primers, an EcoRI-BamHI fragment of the human iNOS promoter extending from 7.3 to 7442 kb (GenBank accession number AF049872) was sequenced using the Sanger dideoxynucleotide sequencing method (33). Sequencing was conducted by Lark Technologies, Inc. (Houston, TX) and the University of Pittsburgh DNA Sequencing Facility. Putative cis-regulatory elements were detected by comparison with the TRANSFAC data base and the MatInspector Release 2.1 data base at the University of California at Berkeley, CA. The sequences of the oligonucleotides used in the gel shift assays are outlined in Table I. Complementary primers, an HpaI fragment of the human iNOS promoter extending from 33 to 250 bp of the 5′-flanking region of the human iNOS gene that contains four cis-acting NF-κB elements. Furthermore, gel shift assays and mutational analysis of these regulatory elements indicate that they play a functional role in the trans-activation of the human iNOS gene by NF-κB in response to cytokines.

**Oligonucleotides used in electromobility shift assays**

| Oligonucleotides used in electromobility shift assays | 15149 |
|-------------------------------------------------------|------|
| NF-κB consensus                                      | 5'-AGTGAGGGCGACCTTCACCGGC-3' |
| NF-κB mutant consensus                               | 5'-AGTGAGGGCACTTCACCGGC-3' |
| NF-κB-5.8 wild-type                                  | 5'-AGAGCGTTTTCCAGAAACA-3' |
| NF-κB-5.8 mutant                                    | 5'-AGGAGCGTTCTCCAGAAACA-3' |

**RESULTS**

**Dithiocarbamates Block Cytokine Induction of Human iNOS**—To determine whether whether iNOS induction in human liver and lung cells is NF-κB dependent, A549 cells and AKN-1 cells were treated with the CM of TNFα, IL-β, and IFNγ in the presence or absence of the established dithiocarbamate NF-κB inhibitors PDTC or DDTC (36). High levels of iNOS mRNA were elicited from the CM-stimulated cells, whereas the addition of PDTC and DDTC inhibited iNOS mRNA expression in a concentration-dependent manner (Fig. 1). As expected, nitrite and nitrate release was also inhibited in a similar manner. These data demonstrate that NF-κB is necessary for cytokine activation of iNOS gene expression in the human cell line.

**EMSA Reveals Inducible NF-κB DNA Binding Activity in Cytokine-stimulated Human Liver and Lung Cells**—To document that the transcription factor NF-κB is translocating into the nucleus of A549 and AKN-1 cells in the presence of CM, gel shifts were performed on nuclear extracts of CM-stimulated A549 and A549 cells using a consensus oligonucleotide for
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NF-κB is a key transcription factor involved in the regulation of cytokine-induced expression of the iNOS gene. The NF-κB element at -115 to -106 bp is not required for cytokine induction of the human iNOS promoter. However, proximal and distal NF-κB elements at -85 bp are essential for cytokine induction of the murine iNOS promoter.

The NF-κB Element at -115 to -106 bp Is Not Required for Cytokine Induction of the Human iNOS Promoter—An NF-κB element at -85 bp upstream in the murine iNOS promoter has been shown to confer LPS responsiveness in the murine iNOS gene (24). Analysis of the 5′-flanking region of the human iNOS gene revealed a putative NF-κB element located at -115 to -106 bp that differs by only one nucleotide from the functional murine NF-κB element at -85 bp (38). Because the first 400 bp of the human and murine iNOS promoter have 66% homology (38), and because this proximal corresponding NF-κB element in the human iNOS promoter is relatively conserved, we sought to evaluate the functional role of this putative NF-κB element. Transient transfections in AKN-1 cells were performed with a wild-type iNOS 7.2-kb promoter construct (piNOS(7.2kB)Luc) and a mutated NF-κB construct (piNOS(mut7.2kB)Luc) generated by site-directed mutagenesis bearing a 2-bp mutation of the corresponding proximal NF-κB element (Fig. 3A). CM treatment of cells transfected with the wild-type construct piNOS(7.2kB)Luc resulted in a 6-fold increase in luciferase activity. When the 7.2-kb construct containing the mutated proximal NF-κB (−115 to −106 bp) (piNOS(mut7.2kB)Luc) was transfected, there was no significant decrease in either basal (data not shown) or stimulated reporter gene activity (Fig. 3B).

In addition, the inducible activity of both the 7.2-kb wild-type and mutant constructs was inhibited by PDTC (Fig. 3D) and DDTC (data not shown). Interestingly, the addition of NF-κB inhibitors did not change basal, unstimulated luciferase activity, suggesting that NF-κB does not play a dominant role in mediating basal transcription of the human iNOS gene in this cell type. Furthermore, deletion of the region from -36 to -133 bp maintained a 3-fold induction in iNOS promoter activity, which also exhibited PDTC inhibition (data not shown). Thus, either mutation or deletion of the proximal NF-κB element failed to abrogate cytokine-induced iNOS promoter activity. These results indicate that the promoter-proximal NF-κB site...
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is not required for maximal cytokine induction, suggesting that the functional NF-κB elements are further upstream in the human iNOS promoter.

Cytokine-responsive Elements Are Localized to Regions More than 4.7 kb Upstream of the iNOS Transcription Start Site—We have previously reported that the activation of the human iNOS promoter required the presence of cytokine-responsive elements upstream of −3.8 kb in the 5′-flanking region of the human iNOS gene (16). To further localize the cytokine-responsive region an additional deletion construct containing the first 4.7 kb of the 5′-flanking region was generated. Transient transfections of the ppiNOS(4.7)Luc construct into AKN-1 and A549 cells revealed no increase in promoter activity in the presence of CM. However, transfection of ppiNOS(5.8)Luc followed by CM stimulation resulted in a 2–3-fold induction in luciferase activity in both cell types (Fig. 4). Transfection of the 7.2-kb construct (piNOS(7.2)Luc) produced a 4–5-fold increase in promoter activity, consistent with our previous findings (16). To confirm the presence of these elements upstream of −4.7 kb and demonstrate position-independence of this cytokine responsive enhancer region, the segment from −2.1 to −4.7 kb was deleted to create ppiNOS(NA)Luc. Transfection studies with this construct in CM-stimulated cells revealed the same 4-fold induction of activity, thereby demonstrating position-independence of this enhancer element and confirming the presence of cytokine-responsive cis-regulatory motifs upstream of −4.7 kb in the human iNOS promoter.

DNA Sequence Analysis from −3.8 to −7.2 kb Reveals Putative Cis-regulatory NF-κB Elements, Which May Confer Cytokine Inducibility—The DNA sequence from the transcription start site to −3761 bp of the human iNOS gene has been previously published by our group and others (38, 40, 41). Because our data indicated a strong role for NF-κB in mediating TNF-α and IL-1β-stimulated activation of the human iNOS gene, and because we were not able to demonstrate a functional role for the proximal NF-κB element at −115 bp, we sequenced the 5′-flanking region from −3761 to −7242 kb looking for other specific NF-κB sites. Computer analysis revealed five potential NF-κB sites in the functionally active upstream region. Fig. 5 depicts the sequence of the human iNOS 5′-flanking region from −3.8 to −7.2 kb and localizes the five putative NF-κB sites at −5219 (−5.2 kb), −5467 (−5.5 kb), −5808 (−5.8 kb), −6080 (−6.1 kb), and −6476 (−6.5 kb), which are located upstream of −4.7 kb. Also shown are the relevant restriction enzyme sites and putative NF-κB sites between −3.8 and −4.7 kb, which are non-functional by deletional analysis.

Mutational Analysis of Upstream NF-κB Sites Reveal That Four NF-κB Sites Are Requisite for Cytokine Induction of the Human iNOS Promoter—To determine whether any of the putative NF-κB elements in the upstream region between −4.7 and −7.2 kb were functional, we used site-directed mutagenesis to generate five additional 7.2-kb constructs, each with a 2-base point mutation in the core sequence of each NF-κB element (as shown in Fig. 6). The NF-κB mutant at site −6.5 kb retained the full CM inducibility typically seen with the 7.2-kb

![Fig. 3. Mutation of an NF-κB site at −115 to −106 does not result in a loss of iNOS promoter activity. A, schematic depicting the 5′-flanking region of the human iNOS gene showing the putative NF-κB element at −115 to −106 contained within the wild-type 7.2-kb construct and mutated (−115 to −106) 7.2-kb luciferase promoter constructs used in this experiment. B, these constructs were transfected into AKN-1 cells and stimulated with CM in the presence and absence of PDTC. Basal and stimulated luciferase activity was determined 4 h after cytokine stimulation. *, p < 0.01 versus CM by ANOVA. Figure is representative of three separate experiments performed in duplicate.](image)

![Fig. 4. Deletional analysis of the upstream 5′-flanking region of the human iNOS gene. The 5′ deletion constructs utilized in the study are shown along with specific restriction sites. A549 cells and AKN-1 cells were stimulated with CM following transient transfections of the constructs. Luciferase activity is expressed as light units/μg of protein in cell lysates. Values are expressed as mean ± S.E. (n = number of experiments).](image)
FIG. 5. The 5′-flanking region of the human iNOS gene. The sequence of the 5′-flanking region of the human iNOS gene from −3761 to −7242 bp. This region contains putative sequences for the transcription factor NF-κB (underlined). Arrows depict 5′ to 3′ orientation of each element relative to the promoter sequence. Relevant restriction enzyme sites are indicated by arrowheads.
Mutation of the cis-regulatory sites at 5.2, 5.5, and 6.1 kb resulted in loss of inducible promoter activity of 60%, 45%, and 65%, respectively. Interestingly, the mutation at 5.8 kb resulted in loss of both basal and inducible promoter activity (Fig. 6). These data indicate that, within the context of the 7.2-kb construct, the site at 5.8 kb is absolutely required for iNOS promoter activity and that the sites at 5.2, 5.5, and 6.1 kb are also functionally important and regulate iNOS gene expression. Previously, we reported a 10-fold increase in cytokine-stimulated iNOS promoter activity with a full-length 16-kb iNOS promoter construct (16). To further evaluate the role of the site at 5.8 kb in the 5'-flanking region of the human iNOS gene, we created the same 2-nucleotide point mutations in the 16-kb construct. Upon transient transfection...
and CM-stimulation, we observed a 9-fold increase in promoter activity, similar to our previous findings (16). This was reduced 40% by mutating the site at −5.8 kb, providing further evidence that this site is important for cytokine-induced iNOS expression (Fig. 7).

NF-κB Proteins Bind to the iNOS −5.8 kb Promoter Element—To determine whether nuclear proteins could bind to the sequence at −5.8 kb, EMSA was performed on nuclear extracts of CM-stimulated AKN-1 and A549 cells using an oligonucleotide containing the NF-κB sequence at −5808 kb in the human iNOS gene. In A549 cells, NF-κB DNA binding activity was detectable in control cells. The addition of single cytokines (IL-1β or TNFα) and CM (IL-1β + TNFα + IFNγ) resulted in an increase in DNA binding activity, which was inhibited by PDTC (Fig. 8A). Competition assays confirmed specificity for NF-κB. Similar results were obtained with nuclear extracts from cytokine-stimulated AKN-1 liver cells, although a smaller nonspecific protein-DNA complex was also observed. Super-shift studies revealed the presence of p65 and p50 in the NF-κB complex (Fig. 8B). Antibodies against AP1 did not result in a supershift. These data show that the CM-inducible NF-κB complex at −5808 kb is composed of both p50 and p65 proteins in AKN-1 cells.

**DISCUSSION**

The purpose of this study was to identify and characterize the NF-κB elements responsible for cytokine-induced transcriptional activation of the human iNOS gene. Our data indicate that cytokine-induced iNOS expression in human liver and lung epithelial cell lines is dependent on the transcription factor NF-κB and that the active response elements are localized in the 5′-flanking region upstream of −4.7 kb. The cytokine-responsive region from −4.7 to −2.2 kb functions in a position-independent fashion, thereby exhibiting the characteristics of an enhancer element. Of the five potential NF-κB binding sites localized from −4.7 to −2.2 kb, four were shown to be functional by mutational analysis. The site at −5.8 kb is required for both basal and cytokine-induced promoter activity, whereas the sites at −5.2, −5.5, and −6.1 kb exert a cooperative effect on cytokine-stimulated iNOS expression. This work identifies a unique far upstream cytokine-responsive enhancer region from −5.2 to −6.1 kb in the 5′-flanking region of the human iNOS gene and underscores one of the major differences between the human and murine iNOS promoters.

Contained within −1.7 kb of the murine iNOS 5′-flanking region are a number of functional cis-regulatory elements including two NF-κB elements, an IRF-1 element, a γ-interferon activated sequence (GAS) site (42), and a hypoxia-responsive element (43), which is lacking within the first 7.2 kb of the human iNOS promoter. Previous work by Xie et al. (24) and Murphy et al. (23) determined that both the proximal NF-κB at −85 bp and the upstream NF-κB at −974 bp in the murine iNOS promoter are functionally important in LPS-stimulated RAW 264.7 cells. The upstream NF-κB is also responsible for the responsiveness to triple cytokine induction in murine vas-

![Fig. 7. Site-directed mutational analysis of the NF-κB at −5.8 in the 16-kb iNOS promoter construct.](image1.png)

![Fig. 8. EMSA with site-specific −5.8 oligonucleotide for NF-κB DNA binding activity.](image2.png)
NF-κB Elements Regulate Cytokine Induction of Human iNOS

NF-κB elements are present in the 5'-flanking regions of several inflammatory response genes, including cell adhesion molecules ELAM-1, VCAM-1, and ICAM-1 (45–49) and the cytokines IL-1, IL-6, and IL-8 (50–54). However, each of these promoters has only one or two proximally-located functional NF-κB binding sites. Unique from this, Cheng et al. (55) demonstrated six functional NF-κB sites located within the first 360 bp of the porcine iLb promoter. Our data localize multiple NF-κB elements in the human iNOS gene to a segment of DNA that spans ~800 base pairs and is located more than 5.2 kb upstream of the TATA box. Therefore, the presence of multiple functional NF-κB binding sites so far upstream is unique to the human iNOS promoter. Four of the five sites in the iNOS promoter are functional, although to different degrees. Because iNOS is expressed in a number of different cell types and under different conditions, we speculate that this arrangement may serve as a means for cell type specificity and tight control in iNOS regulation. For example, the number of binding sites in a promoter may influence the intensity of the response to a given transcription factor depending on the concentration of that factor in that given cell type. In this scenario, low concentrations would be expected to evoke only a minimal response; however, with increasing levels of NF-κB translocating into the nucleus, more NF-κB sites would become occupied and a greater response elicited. Another potential mechanism may be that more NF-κB binding sites over a segment of DNA serve to recruit the transcription factor to that portion of the genome and concentrate them toward the active elements. This idea is supported by our data, which show that, with the 5.8-kb promoter construct, which contains the NF-κB sites at −5.2, −5.5, and −5.8 kb, we observe a 2–3-fold increase in luciferase activity. The addition of the functional element at −6.1 kb in the 7.2-kb construct increases this activity to 4–5-fold.

It has been shown that the mechanisms involved in functional synergy between transcription factors in promoter activation involve protein-protein interactions (56, 57). Through direct physical interactions between proteins, both DNA binding affinity and complex stability are enhanced, resulting in a highly stable multi-protein complex (58, 59). In addition, it has also been shown that the arrangement in transcription factor binding site spacing, as well as intervening sequence between consensus elements, plays an important role in promoter activation (59). The NF-κB elements from −5.2 to −6.1 kb in the human iNOS gene are spaced in approximate multiples of nucleosome units (200 bp) and this spacing may contribute to the three-dimensional structure necessary for efficient iNOS transcription.

Another noteworthy finding in this study is that a combination of three cytokines (TNFα, IL-1β, and IFNγ) was required to achieve a significant increase in iNOS promoter activity in both AKN-1 and A549 cells. Although, either TNFα or IL-1β alone induced NF-κB DNA binding activity, this induction was not sufficient to activate iNOS transcription suggesting that induction or activation of additional transcription factors are required for iNOS expression. For example, members of the κB and STAT family have been shown to exhibit both functional and physical interactions with other transcription factors, including NF-IL-6, C-EBP, Jun, and Sp1 (56, 60–63). Recently, Ohmori et al. (64) demonstrated that IFNγ-activated STAT1α can cooperate with TNFα-induced NF-κB to promote transcription of a number of inflammatory response genes, including the interferon regulatory factor-1 (IRF-1), intercellular adhesion molecule-1 (ICAM-1), monokine induced by interferon-γ (MIG), and regulated on activation normal T cell expressed and secreted (RANTES) genes. Gao et al. (42) have shown that STAT1α is also involved in mediating IFNγ inducibility in the murine iNOS promoter. Whether STAT1α plays a similar role in the human iNOS promoter is unknown and is currently being investigated. Another recent study utilizing in vivo footprinting of the murine iNOS promoter suggested a functional role for Oct-1 in mediating transcription (22). We are currently performing in vivo footprinting of the human iNOS promoter to further our understanding of the complex transcription factor synergy that regulates iNOS gene expression.

Because iNOS expression has such profound physiologic effects, its regulation is strictly controlled. The expression of iNOS and subsequent production of NO serves a protective role by increasing perfusion to the viscera and sites of inflammation. However, sustained overproduction can have detrimental effects including refractory hypotension and death. Thus, the combination of cytokine-inducible transcription factors working in synergy increases the diversity and complexity of the regulation of iNOS gene expression and reduces the chance of inappropriate transcription.
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