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Macrophage nitric oxide synthase gene: Two upstream regions mediate induction by interferon γ and lipopolysaccharide

(NF-κB/enhancer elements/tumor necrosis factor/transcription factors/interleukin)

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ABSTRACT **The promoter region of the mouse gene for macrophage-inducible nitric oxide synthase (mac-NOS; EC 1.14.13.39) has been characterized. A putative TATA box is 30 base pairs upstream of the transcription start site. Computer analysis reveals numerous potential binding sites for transcription factors, many of them associated with stimuli that induce mac-NOS expression. To localize functionally important portions of the regulatory region, we constructed deletion mutants of the mac-NOS 5′ flanking region and placed them upstream of a luciferase reporter gene. The macrophage cell line RAW 264.7, when transfected with a minimal promoter construct, expresses little luciferase activity when stimulated by lipopolysaccharide (LPS), interferon γ (IFN-γ), or both. Maximal expression depends on two discrete regulatory regions upstream of the putative TATA box. Region I (position −48 to −209) increases luciferase activity ≈75-fold over the minimal promoter construct. Region I contains LPS-related responsive elements, including a binding site for nuclear factor interleukin 6 (NF-IL6) and the κB binding site for NF-κB, suggesting that this region regulates LPS-induced expression of the mac-NOS gene. Region II (position −913 to −1029) alone does not increase luciferase expression, but together with region I it causes an additional 10-fold increase in expression. Together the two regions increase expression 750-fold over activity obtained from a minimal promoter construct. Region II contains motifs for binding IFN-related transcription factors and thus probably is responsible for IFN-mediated regulation of LPS-induced mac-NOS. Delineation of these two cooperative regions explains at the level of transcription how IFN-γ and LPS act in concert to induce maximally the mac-NOS gene and, furthermore, how IFN-γ augments the inflammatory response to LPS.**

Abundant evidence indicates that nitric oxide (NO) mediates the ability of macrophages to kill or inhibit the growth of tumor cells, bacteria, fungi, and parasites (1–3). The synthesis of macrophage NO synthase (mac-NOS; EC 1.14.13.39), which generates NO from arginine, is induced by stimuli such as lipopolysaccharide (LPS) or tumor necrosis factor α (TNF-α), and the level of stimulation can be augmented up to 100-fold by combining these stimuli with interferon γ (IFN-γ) (4, 5). While mac-NOS is turned on rapidly to enable the organism to respond to a wide range of pathogens, the expression of mac-NOS must be tightly controlled because NO is potentially capable of injuring host tissue indiscriminately. It is not surprising, therefore, that NO production is repressed by such agents as glucocorticoids, transforming growth factor β, interleukin (IL) 4, IL-10, and prostaglandin E_2 (6–13). Accordingly, one would expect the 5′ flanking region of the mac-NOS gene to contain multiple positive and negative regulatory elements capable of responding to numerous transcription factors.

Recently, we and others cloned the cDNA for mac-NOS (14–16). In the present study we have cloned and sequenced the promoter and adjacent regulatory region upstream of this gene.** Within that region we have identified potential response elements for regulatory transcription factors and localized them to two discrete regions involved in regulating the expression of the mouse mac-NOS gene in response to stimulation by LPS and IFN-γ.

MATERIALS AND METHODS

Cells, Culture Medium, and Reagents. The macrophage cell line RAW 264.7 (American Type Culture Collection) was cultured in spinner flasks containing 25 mM Hepes-buffered RPM1 1640 medium (GIBCO/BRL) containing 10% (vol/vol) fetal bovine serum (JRH Biosciences, Lenexa, KS), 2 mM glutamine (ICN/Flow), 100 μg of streptomycin (Sigma) per ml, and 100 units of penicillin per ml (Pfizer) for injection. The lipid A-rich fraction II of LPS phenol-extracted from *Escherichia coli* 0111:B4 was obtained from David Morrison (University of Kansas Medical Center, Kansas City) (17). IFN-γ (1.27×10^6 antiviral units/mg of protein) was obtained from Schering–Plough through the American Cancer Society (Atlanta). All reagents and media were negative for detectable endotoxin, as measured by the *Limulus* amoebocyte assay (Associates of Cape Cod) at a sensitivity level of 50 pg/ml.

Screening of Cosmid Library. All molecular biological procedures were as described (18, 19) unless otherwise noted. A genomic cosmid library derived from the spleen of a male mouse was purchased from Stratagene. It was screened by using a ^{32}P-labeled 35-base oligonucleotide (General Synthesis and Diagnostics, Toronto) complementary to the 5′ segment of the mac-NOS cDNA extending from position 33 to 67 (numbered from the transcriptional start site reported in this paper). Duplicate filters were hybridized in 6× SSPE containing 1% SDS at 60°C (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA). Filters were then washed at 60°C in 1× SSC containing 1% SDS (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7). Positive

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Abbreviations: mac-NOS, macrophage nitric oxide synthase; LPS, lipopolysaccharide; IFN-γ, interferon γ; TNF-α, tumor necrosis factor α; TNF-RE, tumor necrosis factor response element; hGH, human growth hormone; NF, nuclear factor; IL, interleukin; ISRE, IFN-stimulable response element; PIE, Pu.1/IFN-γ element.

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**The sequence reported in this paper has been deposited in the GenBank data base (accession no. L23806).

colonies were rescreened in the same fashion to obtain isolated colonies. Cosmid DNA was next isolated and subjected to Southern analysis by using the radiolabeled oligonucleotide described above. A *Hinc*II restriction fragment that reacted positively with the probe was then subcloned into pBluescript KS (Stratagene).

Sequencing and Analysis for Transcriptional Factor Motifs. The subcloned fragment of genomic DNA was sequenced on both strands by using the Sanger dideoxynucleotide protocol. The nucleotide sequence was analyzed for transcription factor motifs (20) using the transcription factor data base and the FINDPATTERNS program (copyright 1991) from the Genetics Computer Group (Madison, WI) on a Silicon Graphics supercomputer, model IRIS 4D/340VGX (20).

Mapping of the mac-NOS Transcriptional Start Site by Primer Extension. Poly(A)$^+$ mRNA was isolated (Fast Track mRNA; Invitrogen) from RAW 264.7 cells that had been stimulated for 12 hr with LPS (1 ng/ml) and IFN-γ (100 units/ml). A 25-base oligonucleotide complementary to the 5′ end of the mac-NOS cDNA (positions 88–123) radiolabeled (^{32}P) at its 5′ terminus was mixed with poly(A)$^+$ mRNA; after hybridization for 90 min at 65°C, the primer was extended with Moloney murine leukemia virus reverse transcriptase (Promega). The radiolabeled products of this reaction were separated on a 6% polyacrylamide/7 M urea sequencing gel alongside a dideoxy-sequencing ladder generated with the same oligonucleotide and autoradiographed.

Transfection and Transient Expression of Luciferase Reporter Gene Constructs. The 1.75-kilobase (kb) *Hinc*II restriction fragment upstream from the mac-NOS gene was cloned into the GeneLight luciferase vector system (Promega). Deletion constructs were created in one of two ways. One was by cloning relevant fragments into the same GeneLight vector after DNA had been subjected to digestion by specific restriction enzymes. Alternatively, the construct containing the 1.75-kb *Hinc*II fragment was digested with combinations of uniquely cleaving restriction enzymes to remove specific segments, after which the linearized plasmid was religated. All constructs were then sequenced to characterize them definitively. Plasmid DNA was purified twice by equilibrium centrifugation in CsCl/ethidium bromide gradients, once in a fixed angle rotor and once in a vertical rotor.

Constructs (2.7 pmol of each) were introduced into RAW 264.7 cells by electroporation (21) or by liposomal-mediated transfection (Lipofectin, Bethesda Research Laboratories). Because results with each approach were virtually identical, only those obtained by electroporation are reported here. To control for differences in the amounts of transfected DNA, RAW 264.7 cells were cotransfected with 0.3 pmol of pXGH5, which contains the cDNA for human growth hormone (hGH). Cells from 12 separate electroporations were pooled and distributed equally between 12 60-mm tissue culture plates, and the medium was changed 2 hr later. After 72 hr the cultures were stimulated for 8 hr with medium alone or with 100 antiviral units of IFN-γ per ml, 10 ng of LPS per ml, or both. Supernates were then radioimmunoassayed for hGH (Allegro hGH Assay System, Nichols Institute, San Juan Capistrano, CA). Concomitantly, cell monolayers were washed with phosphate-buffered saline and lysed with buffer containing 1% Triton X-100. Aliquots were assayed for luciferase activity in a luminometer (Enhanced Luciferase Assay, Monolight 2010 luminometer, Analytical Luminescence Laboratory, San Diego). Relative light units were normalized by dividing them by relevant cpm from the hGH RIA and were reported as relative luciferase activity.

RESULTS

Sequence of the 5′ Flanking Region and Mapping of the mac-NOS Transcriptional Start Site. Southern analysis of the isolated mac-NOS cosmid clone using a 35-base oligonucleotide complementary to the 5′ nontranslated region of the mac-NOS cDNA identified a 1.7-kb *Hinc*II fragment that was subcloned and sequenced (Fig. 1). A 1.2-kb intron is located between positions 170 and 171 (sequence not shown).

We identified the precise location of the transcriptional start site by primer extension analysis. The major product maps to an adenosine residue 30 base pairs downstream from a TATA box motif (Figs. 1 and 2).

Computer analysis of the sequenced region revealed numerous transcription factor motifs (Fig. 1). The locations of a select few of these thought to be potentially relevant to the regulation of this gene by LPS and IFN-γ are shown. These include motifs that may bind nuclear factors (NF) NF-κB (22) and NF-IL6 (23); sites that may bind factors induced by the IFNs, such as the IFN-stimulable response element (ISRE) and the Pu.1/IFN-γ element (PIE) (24, 25); and those that may bind factors associated with stimulation by TNF-α [TNF-responsive elements (TNF-REs); ref. 26].

Functional Analysis of Luciferase Reporter Constructs Transiently Transfected into RAW 264.7 Cells. A series of deletion constructs were made containing fragments of the mac-NOS regulatory region inserted in front of the luciferase reporter gene (Fig. 3). These were transiently transfected into macrophage RAW 264.7 cells by electroporation. When stimulated with LPS plus IFN-γ, RAW 264.7 cells that were transfected with the entire mac-NOS 5′ flanking region expressed luciferase activity. Luciferase activity increased slightly when the region from position −1589 to position −1029 (from *Hinc*II site to *Esp* I site) was deleted. When the region from −1029 to −724 was deleted (*Esp* I site to *Sma* I site), luciferase activity decreased by a factor of ≈10 in transfected macrophages stimulated with LPS and IFN-γ. Other deletion constructs made with exonuclease III (data not shown) further narrowed this region to positions −1029 to −913 (referred to as region II). As deletions were then made towards the TATA box, luciferase activity remained constant until the region from −209 to −48 was deleted (referred to as region I, from *Bst*XI to the *Pst* I site), a region adjacent to the TATA box at position −30. Deletion of region I produced a reduction in luciferase activity by a factor of ≈75. Further confirming the importance of region I, a construct with the entire 5′ flanking region except for region I (i.e., the *Bst*XI–*Pst* I deletion) showed that region II cannot act independently.

When stimulated with LPS alone, transfected cells expressed less luciferase activity than when stimulated with both LPS and IFN-γ (Fig. 3). However, the qualitative decrease when regions I and II were deleted remained the same. When stimulated with IFN-γ alone, however, transfected cells did not express more luciferase activity than did unstimulated cells. In contrast, stimulation with LPS and IFN-γ caused a significant increase in luciferase activity in all constructs tested except for the one containing the minimal promoter (*Pst* I site to *Hinc*II site).

DISCUSSION

Mac-NOS is a tightly regulated gene. We have previously demonstrated that control over mac-NOS induction in the mouse macrophage cell line RAW 264.7 is regulated at the level of transcription (5). Nascent transcripts are undetectable in nonstimulated cells, but maximal rates of transcription are reached within 1 to 2 hr after addition of LPS and IFN-γ.

The major finding of this study is that two areas upstream of the mac-NOS gene are required for maximal induction of its transcription. Deletion of either region I or II separately reduces expression of the reporter gene in stimulated cells. Region I by itself can activate expression but not to the levels obtained when both regions are present. Region II by itself

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          (-1592)GTTGACTTTGATATGCTTGAAATCCATAAGCTGTGTGTGTGT
                 ↑HincII
-1550 GCAAGTGTGCATGCGCATGTGTGCACATGAGTGTGCAGGTATATGTAGGA
-1500 GCTAGAAGACAATCTCAGCTCTTGTTTCCCAGGTTACCCAGCATCTCTCA
-1450 CCAGCCTGGAACCTGCCTAGTAGGCTAGGCTGGCTGGCCAGCAAACCCTA
                                        NF1
-1400 GGCATATTCCTGTCTTTACCTCCCCAGAACTTATTGCAAGTGTGTGTCAC
-1350 CACACCCAGCATTTTATCATTGACCTATTGACTGGTGTGTGTGTGTGTGT
-1300 GTGTGTTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT
-1250 GTGTGTAAATTCCTTATCTCACCAACCCATGCCCAGCTTTTGAACTTAGG
-1200 TCCTTGTACATGCAAGGCAAGCACTTTACCAACTGAGCCATCTCCCCAGC
-1150 CCAATTACTTGATTTGTAATTCATTTATTCAATCAACAATTTATTTGTTC
                                  AP1
-1100 TCCCAACTATTGAGGCCACACACTTTTTGGGTGACTTAGTCTGTGTACCT
Region II
-1050 CAGACAAGGGCAAAACACGAGGCTGAGCTGACTTTGGGGACCATGCGAAG
                EspI↑  TNF-RE
-1000 ATGAGTGGACCCTGGCAGGATGTGCTAGGGGGATTTTCCCTCTCTCTGTT
                EBS          NFκB
 -950 TGTTCCTTTTCCCCTAACACTGTCAATATTTCACTTTCATAATGGAAAAT
          PIE                ISRE
 -900 TCCATGCCATGTGTGAATGCTTTATTGGAAGCATTGTAAGAAATTATAAT
 -850 TTATTCGTTTTTGTTTGTTTCTCAGAACAGGGTTTTTCTGTGTAGTGTTC
 -800 CTGGCTTATCCTAGAACTTACTCTGTAGACCAGGCTAGCCCAAACTCAGG
 -750 GATCAGCCTTTCTCTGTCTCCTGAATCCCGGGATTAAAGGCTTATGCCAC
          ISRE          ↑SmaI
 -700 CACACCCAGGTAGGACATTATAATCCTATATATAAGAAGTCACCCACACA
 -650 TACAAACACACACACCACACACACACACACACACACACACACACACAC
 -600 AGAGAGAGAGAGAGAGAGAGAGGGAGAGAGAGAGAGAGAGAGAATG
 -550 CCACTGAGAAAAAAAATAAAAAGGCTTCACTCAGCACAGCCCATCCACTA
 -500 TTCTGCCCAAGCTGACTTACTACTAGTGGGGAAATGCTGGTCAGACGGCA
                                NF-IL6
 -450 TCTGTGCCCACAGCTTGCCTTCCATCCTTTCTAGAAAACCTCCTGATGAA
      NF1                  ↑XbaI
 -400 TGTGTCCTGGGCGTGTTGGAATATTGGCACCATCTAACCTCACTGAGAGA
                NF-IL6
 -350 ACAGACAGAAAGCCAGAGAGCTCCGTGCCCAGAACAAAATCCCTCAGCAG
                ↑SacI
 -300 CTGCAAGCCAGGGTATGTGGTTTAGCTAAGAAAAGCCAGCCTCCCTCCCT
Region I
 -250 AGTGAGTCCCAGTTTTGAAGTGACTACGTGCTGCCTAGGGGCCACTGCCT
                PRD1                          ↑BstXI
 -200 TGGACGGGCGACCAGGAAGAGATGGCCTTGCATGAGGATACACCACAGAG
                EBS
 -150 TGATGTAATCAAGCACACAGACTAGGAGTGTCCATCATGAATGAGCTAAC
      NF-IL6                                  TNF-RE
 -100 TTGCACACCCAACTGGGGACTCTCCCTTTGGGAACAGTTATGCAAAATAG
                NFκB    NF-IL6      Oct
  -50 CTCTGCAGAGCCTGGAGGGGTATAAATACCTGATGGCTGCTGCCAGGGTC
      ↑PstI          TATA Box
   +1 acaactttacagggagttgaagactgagactctggccccacgggacacag
  +51 tgtcactggtttgaaacttctcagccaccttggtgaagggactgagctgt
 +101 tagagacacttctgaggctcctcacgcttgggtcttgttcactccacgga
                        ▽
 +151 gtagcctagtcaactgcaagagaacggagaacg(+183)
          ↑HincII
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FIG. 1. Nucleotide sequence of the mac-NOS 5′ flanking region. The transcriptional start site is denoted as nucleotide position +1, and a portion of the transcribed sequence is indicated in small letters. The location of a 1.2-kb intron is shown between positions +170 and +171 (open triangle). A likely TATA box begins at position −30. Several potential transcription factor binding sites are underlined and labeled. The transcription factor binding sites shown either have perfect sequence identity with those previously reported or contain only a single mismatch.

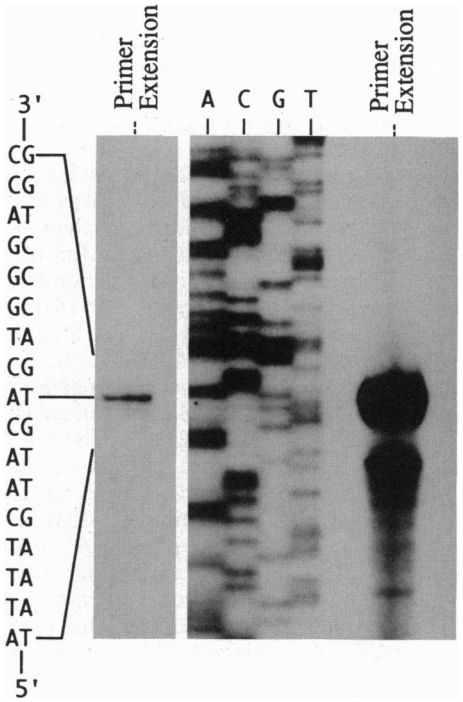
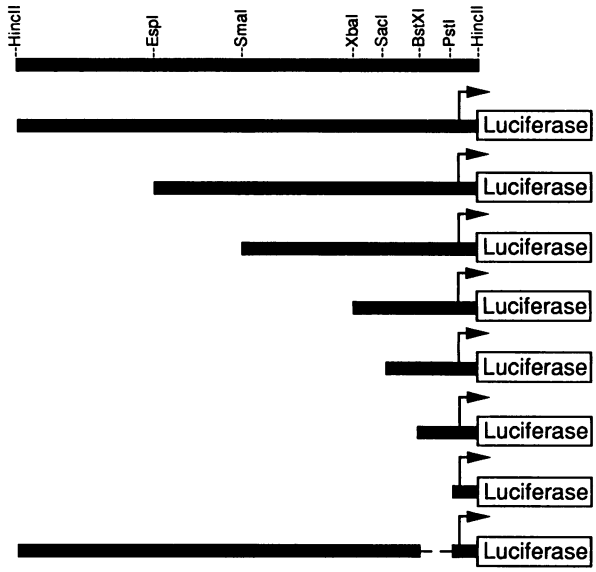


FIG. 2. Primer extension mapping of transcriptional start site. Poly(A)$^+$ RNA isolated from RAW 264.7 cells stimulated with LPS + IFN-γ was hybridized to an oligonucleotide from the 5′ end of mac-NOS cDNA and extended with Moloney murine leukemia reverse transcriptase; the products were then electrophoresed alongside a dideoxy-sequencing ladder generated with the same oligonucleotide. The primer extension lane on the left represents a shorter exposure by a factor of 2.7 than the lane on the right, which allowed precise localization of the product in comparison with the sequencing ladder.

cannot induce reporter gene expression but in conjunction with region I can augment expression. Both regions I and II are necessary for LPS-activated expression because deletion of either region decreases expression of the luciferase reporter gene regardless of whether the stimulus is LPS alone or LPS plus IFN-γ. Region II mediates IFN-γ regulation because, compared with LPS stimulation alone, IFN-γ plus LPS stimulation increases reporter expression only of constructs containing region II. Since region II alone has little independent regulatory effect on induction, it may act primarily as an enhancer. This result is consistent with our previous report that IFN-γ by itself does not induce synthesis of mac-NOS in RAW 264.7 cells (5).

LPS alone induces 60% of the reporter gene activity obtained with LPS plus IFN-γ. We have previously shown that LPS alone induces only 15% of the maximal amount of mac-NOS mRNA that is induced by costimulation with both LPS and IFN-γ (5). Possible explanations for this discrepancy between results generated from induction of mac-NOS in macrophages and induction of transfected luciferase constructs in macrophages include the following two possibilities: (*i*) DNA sequences upstream or downstream of the region tested here may negatively regulate inducibility of the mac-NOS gene in response to LPS or (*ii*) transfected construct DNA may not acquire precisely the same chromatin configuration as does the native mac-NOS gene. The reduction in luciferase activity produced by extending the regulatory construct DNA upstream of the *Esp* I site supports the former possibility.

Regions I and II contain potential binding sites for numerous transcription factors. Transcription factors constitutively expressed in macrophages include AP1, Oct-1 and Oct-2,



	Relative Luciferase Activity			
	Medium	IFN-γ	LPS	IFN-γ+LPS
Luciferase	7	6	69	117
Luciferase	6	9	92	157
Luciferase	3	2	14	12
Luciferase	3	2	17	21
Luciferase	2	3	20	13
Luciferase	3	3	15	15
Luciferase	0.1	0.1	0.2	0.2
Luciferase	0.3	0.3	1	1

FIG. 3. Luciferase activity in transfected macrophages stimulated with IFN-γ alone, LPS alone, or LPS + IFN-γ. Each regulatory region–luciferase construct was cotransfected into RAW 264.7 cells along with the hGH-expressing plasmid pXGH5. Transfected cells were stimulated with medium alone or medium with IFN-γ, LPS, or both IFN-γ and LPS. After assaying for luciferase activity and hGH, the luciferase relative light units were normalized by dividing them by cpm from the hGH RIA, and the data were reported as relative luciferase activity. Stimulation does not affect hGH production (data not shown).

Pu.1, and NF-1 (21, 27, 28). LPS induces the activity of NF-IL6 (29), Ets2 (which is related to Pu.1 by relative sequence identity) (30), NF-κB, and the AP1 (31) and ISRE binding proteins (32). IFN-γ-inducible factors include ICSBP (IFN consensus sequence binding protein; ref. 33), IRF-1/ISGF2 (interferon regulatory factor-1/interferon-stimulated gene factor 2; ref. 34), ISGF3 (interferon-stimulated gene factor 3; ref. 35), GAF (γ interferon-activated factor; ref. 36), and an NF-Y or YB1-like activity (37). In addition, TNF-α induces both (*i*) a transcription factor that is distinct from AP1 or CREBP (cAMP response element binding protein) but binds to a DNA element resembling their cognate recognition sites (26) and (*ii*) the transcription factor NF-GMa (38). Potential binding sites for many of these transcription factors are concentrated in regions I and II. Furthermore, allowing a greater number of mismatches during computer searches for transcription factor binding sites places an IFN-γ activation site [gamma activation site (GAS), which binds GAF] in precisely the same location as the PIE element in region II. Perhaps the PIE and GAS sites are variations of a consensus element that binds GAF. Whether or not these factors play a role in mac-NOS regulation is not yet known.

Region I appears to be most important for mediating LPS induction of mac-NOS, and region II is crucial for responses to IFN-γ. Region I contains more LPS-related response elements, including a particularly striking array of octamer, NF-IL6, NF-κB, and TNF-REs. It is noteworthy that the potential NF-κB and NF-IL6 sites are tandemly arranged, because these two transcription factors are known to form heterodimers (39), and their cooperative interaction is required for the induction of at least one cytokine gene, that for IL-8 (40). In contrast, IFN-responsive elements are concentrated in region II, including an ISRE and a PU-box/IFN element or PIE (25). In view of these findings, it is likely that region II is most important in mediating IFN-related responses.

Both regions I and II contain potential binding sites for NF-κB. NF-κB is a transcription factor that is induced by a number of inflammatory stimuli including LPS and IFN-γ (41). It is normally present in the cytosol bound to its inhibitor I-κB; when I-κB is phosphorylated, NF-κB is released and translocates to the nucleus, where it activates gene transcription (42). Our sequence data suggest that mac-NOS is induced by complexes of transcription factors formed in both regions I and II, each of which potentially contain NF-κB.

A striking finding is that LPS and IFN-γ responsive elements are respectively concentrated in two distinct regulatory regions. Moreover, LPS by itself stimulates mac-NOS expression, whereas IFN-γ is effective only in the presence of LPS. These findings coincide with and may explain aspects of inflammation in which multiple stimuli both limit and augment responses. In sepsis, LPS released from gram-negative bacterial cell walls circulates throughout the body. In contrast, IFN-γ is released locally to enhance inflammatory responses in discrete cell populations. Macrophages can be stimulated to a limited extent by LPS alone. IFN-γ elaborated by infiltrating lymphocytes can prime the macrophages for a maximal response to LPS. Thus, maximal production of NO is restricted to those cells needed to kill the invader, thereby minimizing damage to adjacent tissues. Systemic activation is avoided, because both LPS and IFN-γ must be present in sufficient quantity to elicit a maximal response (5). The findings reported here show at the level of transcription how such tight regulation is accomplished.

Note Added in Proof. While this work was in progress, Xie *et al.* (43) independently reported the isolation of a mac-NOS regulatory region.

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