Promoter of the Mouse Gene Encoding Calcium-independent Nitric Oxide Synthase Confers Inducibility by Interferon $\gamma$ and Bacterial Lipopolysaccharide

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Summary

Inducible nitric oxide synthase (iNOS) can be expressed by many types of mammalian cells in response to diverse signals acting synergistically, including cytokines and microbial products. We previously showed that induction of iNOS in mouse macrophages by interferon $\gamma$ (IFN-$\gamma$) and lipopolysaccharide (LPS) was at the transcriptional level. From a mouse genomic library, we now cloned a 1,749-bp fragment from the 5'-flanking region of the iNOS gene, and used S1 nuclease mapping and primer extension to identify the mRNA transcription start site within it. The mRNA initiation site is preceded by a TATA box and at least 22 oligonucleotide elements homologous to consensus sequences for the binding of transcription factors involved in the inducibility of other genes by cytokines or bacterial products. These include 10 copies of IFN-$\gamma$ response element; 3 copies of $\gamma$-activated site; 2 copies each of nuclear factor-kB, IFN-\$\alpha\$-stimulated response element, activating protein 1, and tumor necrosis factor response element; and one X box. Plasmids in which all or the downstream one half or one third of this region of iNOS were linked to a reporter gene encoding Chloramphenicol acetyltransferase were transfected into cells of the RAW264.7 macrophage-like line. All these constructs conferred inducibility of the iNOS promoter by LPS, but only the construct containing all 1,749 bp conferred synergistic inducibility by IFN-$\gamma$ plus LPS.

Nitric oxide (NO), a short-lived, radical gas, acts as an intercellular messenger in most or all mammalian organs, participating in vascular homeostasis, neurotransmission, and antimicrobial defense (1). Three genes encode NO synthases (NOS) of two biochemical types. Neuronal and endothelial NOS are constitutive but dormant until activated briefly by Ca$^{2+}$ transients that sustain the binding of calmodulin. These have been termed cNOS (1). A third NOS is expressed only after transcriptional induction (2). Once synthesized, this NOS is active for prolonged periods, perhaps because it binds calmodulin without a requirement for elevation of Ca$^{2+}$ above the levels in resting cells (3). Termed inducible NOS (iNOS), the latter form of the enzyme has been purified (4-6) and cloned (2, 7, 8) from mouse macrophages. iNOS mediates some of the cytotoxic action of activated macrophages, and when expressed in vascular smooth muscle, endothelium and/or liver, may play a key role in septic shock (9).

Three considerations have focused our interest on the iNOS promoter. First, immunologically inducible, high-output NO production is widely distributed, having been observed not only in macrophages, endothelium, vascular smooth muscle, and hepatocytes, but also in tumor cells, fibroblasts, mesangial cells, astrocytes, keratinocytes, and cardiac myocytes (1, 10-12). Second, in all these cells, maximal induction of iNOS depends on synergistic combinations of stimuli, the most effective ones varying with the cell type. For mouse macrophages, synergistic inductive combinations include IFN-\$\gamma\$, IFN-\$\alpha\$ or IFN-\$\beta\$ plus LPS, or IFN-$\gamma$ plus TNF-\$\alpha$ or -\$\beta\$ (13-16), whereas for rat and human hepatocytes, four signals are required: IFN-\$\gamma\$, TNF-\$\alpha\$, IL-1\$\beta\$, and LPS (17). Finally, iNOS is subject to immunologic suppression, for example, by TGF-\$\beta$ (18), macrophage deactivation factor (18), and IL-4 (19). We have taken steps toward analyzing the molecular mechanisms by which iNOS is induced and suppressed, by cloning the promoter of the mouse iNOS gene, and distinguishing regions within it that are responsive to LPS alone or to the synergistic contribution of IFN-$\gamma$.

Materials and Methods

Materials. Recombinant murine IFN-$\gamma$ (5 x 10$^6$ U/ml) was generously provided by Genentech (South San Francisco, CA). LPS was purchased from Sigma Chemical Co. (St. Louis, MO). Thioglycollate broth was from Difco Laboratories Inc. (Detroit,
Results

Cloning and Analysis of a 1,749-bp 5'-Flanking Region of iNOS. A 1,749-bp HindII fragment at the 5'-flanking region of iNOS was cloned from a mouse genomic library and sequenced (Fig. 1 A). Both S1 nuclease mapping and primer extension (Fig. 2) identified the same initiation site (designated +1 in Fig. 1 A) 30 bp downstream of a TATA box. The TATA box is preceded by a multitude of oligonucleotide elements homologous to consensus sequences for binding of transcription factors involved in cytokine or LPS induction of other genes, including sequences specified as IFN-γ response element (γ-IRE) (10 copies) (26, 27); γ-activated site (GAS) (3 copies) (26, 28); nuclear factor-kB (NF-kB) (2 copies) (29); IFN-α-stimulated response element (ISRE) (2 copies) (28); activator protein 1 (AP-1) (2 copies) (30); TNF response element (TNF-RE) (2 copies) (31); and X box (1 copy) (26, 32).

S1 nuclease mapping and primer extension (Fig. 2) reinforced the previous conclusion (2) that no detectable mRNA for iNOS accumulates in RAW 264.7 macrophage-like cells or primary peritoneal macrophages without induction. Treatment with IFN-γ and LPS led to transcription of iNOS from the same initiation site in both cell populations.

Promoter Activity of the Full-Length 1,749-bp Fragment and Its 5'-Truncated Constructs. The 1,749-bp 5'-flanking region of iNOS was fused to a promoterless CAT reporter gene in the vector pCAT-Basic to form the construct pSiNOS-CAT. This construct and the vector only were transfected into RAW 264.7 cells. As reflected by CAT activity, the 1,749-bp 5'-flanking region of iNOS conferred responsiveness to the combination of IFN-γ and LPS (Table 1 and Fig. 3). By itself, LPS induced promoter activity in a concentration-dependent manner. IFN-γ alone was ineffective. However, IFN-γ synergistically enhanced the response to LPS (Fig. 4 A).

Downstream portions of the 1,749-bp region were linked to the CAT gene to form the constructs p2iNOS-CAT and p3iNOS-CAT (Fig. 1 B). Both conferred inducibility by LPS in a concentration-dependent fashion. However, in contrast to pSiNOS-CAT, the constructs lacking the upstream half of the 1,749-bp fragment did not respond to the addition of IFN-γ (Fig. 4, B and C).

S1 nuclease mapping confirmed that the mRNA initiation site of the CAT transcript in the cells transfected with pSiNOS-CAT was at the same location as that for iNOS transcript itself in untransfected cells, and that no detectable CAT mRNA synthesis was initiated in transfected cells without induction by IFN-γ and LPS (data not shown).

Discussion

The 1,749-bp 5'-flanking region identified here as the promoter of the iNOS gene contains a remarkable multiplicity of consensus sequences for the binding of transcription factors involved in the induction of other genes by cytokines and bacterial products, such as IFN-γ, IFN-α, TNF, IL-2, and LPS. In addition, regions of GT- or AC-repeats considered...
inducibility by LPS, along with synergistic responsiveness to the combination of IFN-γ plus LPS. The downstream one third, as in p2iNOS-CAT, contained a promoter responsive to LPS but unresponsive to the synergizing effect of IFN-γ.

Figure 1. (A) The 5'-flanking region of iNOS. The nucleotide sequence (numbered left) of the 1,749-bp region (~1588 to +161) is shown (upper case). The sequence (lower case) following position +161 is from the cDNA of iNOS (2). Intron 1 is inserted at the parentheses (personal communication, Dr. J. Mudgett); the start codon for iNOS (atg) is in the second exon. A complementary sequence (italics) with 5'-end at +61 specifies the primer used for Fig. 2. (Bent arrow) The mRNA initiation site (+1); (boxed) TXA sequence. Elements are designated that are homologous to consensus sequences for binding of transcription factors involved in conferring responsiveness to cytokines or LPS in other genes, with the homology ratios in parentheses: (horizontal underlined arrows) γ-IRE; (starred) AP-1; (double underlined) TNF-RE; (overlined) NF-κB; (underlined) IRSE; (overlined arrow) GAS; (triple underlined) X box. (Vertical arrows) Restriction sites. (These sequence data are available from GenBank under accession number L09126). (B) CAT constructs. (Dashed line) Deleted portions.

The whole 1,749-bp region, as in p1iNOS-CAT, conferred susceptiblity to formation of Z-DNA and sometimes conferring enhancer activity (33) occupied positions ~1314 to ~1245 and ~645 to ~600.

The 26-mer indicated in Fig. 1A was used for the sequence ladders (lanes G, A, T, and C). The same 26-mer was also used in primer extension after labeling the 5'-end. Single-stranded DNA complementary to nucleotides ~300 to +61 was labeled at the same 5'-end and used in S1 nuclease mapping. (Lanes 1 and 2) S1 mapping with total RNA from RAW 264.7 cells, without (lane 1) or with (lane 2) prior treatment of the cells for 8 h with IFN-γ (50 U/ml) and LPS (1 μg/ml). (Lanes 3–6) Primer extension using RNA from RAW 264.7 cells (lanes 3 and 4) or from primary macrophages (lanes 5 and 6). The cells had been treated with (lanes 3 and 5) or without (lanes 4 and 6) IFN-γ and LPS (8 h for RAW 264.7 cells; 15 h for primary macrophages).
Table 1. Inducibility of iNOS Promoter by LPS plus IFN-γ

| Plasmid       | No. of Expts. | LPS plus IFN-γ | Fold induction |
|---------------|---------------|----------------|---------------|
| p1iNOS-CAT    | 8             | -*             | 0.25 ± 0.03t  |
|               |               | +              | 10.94 ± 2.31  |
| pCAT-Basic    | 5             | -*             | 0.19 ± 0.05   |
|               |               | +              | 0.19 ± 0.03   |

* LPS (1 μg/ml) plus IFN-γ (50 U/ml).
† CAT activity in each transfectant is expressed as percent acetylation of chloramphenicol when 4 μg cell lysate protein was assayed at 37°C for 2 h (mean ± SEM).

The critical contribution of the upstream portion for responsiveness to IFN-γ may relate to its content of four γ-IRE, all of which match the consensus sequence perfectly (8/8). By contrast, the downstream half of the 1,749-bp fragment contains six γ-IRE, but each matches the consensus sequence less well (7/8). The upstream half also contains two ISRE lacking from the downstream half; two GAS (11/15, 8/10) versus one (7/10) in the downstream half; and one site each homologous to NF-xB, AP-1, and TNF-RE. Yet the upstream half tested alone (construct p5iNOS-CAT) was devoid of promoter activity, with or without LPS plus IFN-γ (data not shown).

Figure 3. Demonstration of iNOS promoter activity. The construct p1iNOS-CAT (lanes 1 and 2) or the vector itself, pCAT-Basic (lanes 3 and 4), was transfected into RAW 264.7 cells. The cells were cultured in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of IFN-γ (50 U/ml) and LPS (1 μg/ml). Cell lysates were then assayed for CAT and the ethyl acetate extract of the reaction mixture subjected to TLC followed by autoradiography.

Figure 4. Concentration dependence of activation of iNOS promoter by LPS, and interaction with IFN-γ. CAT activity in RAW 264.7 cells transfected with (A) p1iNOS-CAT, (B) p2iNOS-CAT, or (C) p3iNOS-CAT. In each case, transfected cells were treated with the indicated concentrations of LPS with (●) or without (O) IFN-γ (50 U/ml). CAT activity was measured as described in Table 1. In each panel, all treatments were carried out with cells from the same transfected pool.

RAW 264.7 macrophage-like cells have been a useful tool for transfection with reporter constructs to demonstrate promoter activity in genes that are inducible in macrophages (34, 35). Both RAW 264.7 cells (2, 4, 13) and primary macrophages (14–16) produce iNOS when treated with combinations of cytokines and LPS. However, RAW 264.7 cells do not perfectly mirror the patterns of induction seen with primary mouse peritoneal macrophages. RAW 264.7 cells produce NO after exposure to LPS alone (13), whereas primary mouse peritoneal macrophages do so little or not at all (15). Conversely, primary mouse peritoneal macrophages respond to IFN-γ alone by producing NO (15), but early passage RAW 264.7 cells do not (our unpublished observations). Thus, a full analysis of synergistic induction and suppression of iNOS will require transfection of promoter-reporter constructs into additional cell types, including primary macrophages.

Perhaps the best understood example of transcriptional induction by IFN-γ involves a primary response gene coding for a guanylate binding protein of unknown function (28, 36, 37). The GAS in the guanylate binding protein promoter binds a 91-kD protein termed γ-activated factor (GAF). GAF has recently been cloned by virtue of its participation in the heterotetrameric transcription complex activated by IFN-γ.
After treatment of human fibroblast FS2 cells with either IFN-γ or IFN-α, GAF is phosphorylated on tyrosine and translocates to the nucleus (39). Our preliminary experiments (data not shown) suggest that tyrosine phosphorylation may also participate in the induction of iNOS. GAF or a similar protein may undergo IFN-γ-induced tyrosine phosphorylation in the cytoplasm, translocate to the nucleus, and induce the expression of a transcription factor that binds the iNOS promoter. LPS may synergize with IFN-γ by augmenting this pathway, or by activating or inducing a distinct transcription factor(s) that may cooperate with proteins that bind the iNOS promoter in response to IFN-γ.

We thank J. Mudgett (Merck Research Laboratories) for the mouse genomic library and information on intron 1; M. Tocci, A. Ding, and M. Fuortes for review of the manuscript; J. Schmidt and P. Davies for advice; Genentech, Inc. for recombinant mouse IFN-γ; and T. Troso for technical assistance during screening of the genomic library.

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Received for publication 23 February 1993.

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