Complex Formation of the Interferon (IFN) Consensus Sequence-binding Protein with IRF-1 Is Essential for Murine Macrophage IFN-γ-induced iNOS Gene Expression*

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This study describes the role of the interferon (IFN) consensus sequence-binding protein (ICSBP or IRF-8) in iNOS gene expression by murine macrophages. An ICSBP binding site in the iNOS promoter region (−923 to −913) was identified using an electrophoretic mobility shift assay and chromatin co-immunoprecipitation. Overexpression of ICSBP greatly enhanced IFN-γ-induced iNOS promoter activation in RAW264.7 cells, and IFN-γ-induced iNOS promoter activation was abolished in ICSBP−/− macrophages. Furthermore, transduction of retrovirus-ICSBP in ICSBP−/− macrophages rescued IFN-γ-induced iNOS gene expression. However, transduction of retrovirus-ICSBP in the absence of IFN-γ activation did not induce iNOS expression in either RAW264.7 cells or ICSBP−/− macrophages. Interestingly, ICSBP alone transduced into ICSBP−/− macrophages did not bind to IFN-stimulated response element site (−923 to −913) of the iNOS promoter region, although following activation with IFN-γ, a DNA-protein complex was formed that contains ICSBP and IRF-1. Co-transduction of ICSBP with IRF-1 clearly induces nitric oxide production. In addition, interleukin-4 inhibits IFN-γ-induced iNOS gene expression by attenuating the physical interaction of ICSBP with IRF-1. Complex formation of ICSBP with IRF-1 is essential for iNOS expression, and interleukin-4 attenuates the physical interaction of ICSBP with IRF-1 resulting in the inhibition of iNOS gene expression.

Nitric oxide (NO) has been identified as an important signaling molecule involved in regulating a wide range of biological activities in the immune, vascular, and neural system (1–9). NO and its metabolites are central to the antimicrobial and tumoricidal activity of activated macrophages and are implicated in the pathogenesis of tissue damage associated with acute and chronic inflammation (4–9). Macrophages generate NO from l-arginine through the inducible isofrom of nitric oxide synthase (iNOS), which is regulated transcriptionally. iNOS gene expression is induced by a variety of stimuli, including lipopolysaccharide (LPS), bacterial wall products, and cytokines. In particular, interferon-γ (IFN-γ) is a potent inducer of iNOS gene expression (10, 11), although the molecular mechanism for the effect is still not understood fully.

The murine iNOS promoter region contains several binding sites for transcription factors implicated in iNOS regulation, including two NF-κB binding sites. The NF-κB site situated closer to the transcriptional start site is required for iNOS gene induction by LPS, because deleting the downstream site essentially abolishes iNOS transcription (12). Deletion of the upstream NF-κB site reduces but does not abolish iNOS transcription (12–14). The upstream portion of the iNOS promoter contains an enhancer region, with several transcription factor binding sites that mediate transcriptional responses to IFN-γ. These sites include a γ-interferon-activated site element and two IFN-stimulated response elements (ISRE) (15, 16). Deletion and mutational analysis have shown the importance of the promoter sequence from −1029 to −913 in the induction of iNOS by IFN-γ (13). Site-directed mutagenesis and competition experiments have shown that the ISRE site between −923 and −913 is required for the full synergistic effects of IFN-γ and LPS in the transcription of the iNOS gene (16).

The cytokine IFN-γ plays an essential role in innate and adaptive immunity (17). IFN signaling, which involves a variety of trans- and cis-acting factors, is mediated through DNA motifs, designated ISRE and IFN-γ-activated sequence, which are found in promoters of IFN-inducible genes. The interferon consensus sequence-binding protein (ICSBP or IRF-8) is a transcription factor belonging to the IFN regulatory factor (IRF) family, which also includes the IRF-1, IRF-2, IRF-3, interferon-stimulated gene factor 3γ, and pip/IRF-4 proteins (18, 19). Unlike the other members of the IRF family, ICSBP expression is limited to activated macrophages, B cells, and T cells (20–23). Proteins of the IRF family, including ICSBP, bind to the ISRE (18). The tyrosine-phosphorylated form of ICSBP does not bind to DNA independently but requires complex formation with other members of the IRF family (IRF-1 and IRF-2) or with other transcriptional elements (24). Previous studies found that ICSBP represses transcriptional activity of ISRE promoters in various cell types. However, recent studies suggest that ICSBP can activate genes required for the development and function of macrophages (25, 26). ICSBP−/− mice were shown to be highly susceptible to infection with several...
patogens including *Listeria monocytogenes* (27, 28) and are impaired in the production by macrophages of IFN-γ-induced NO (29). However, the molecular mechanism for the regulation of NO by ICSBP is not clear.

In the present study we have analyzed iNOS gene expression in macrophages induced by IFN-γ and have identified an ICSBP binding site in the iNOS promoter region. Transduction of ICSBP with a retroviral vector in ICSBP-/− macrophages rescues IFN-γ-induced iNOS expression. Surprisingly, iNOS expression is not induced by transduction of ICSBP alone but requires co-transduction of ICSBP with IRF-1. This study provides new insights into the molecular mechanism of iNOS gene induction by IFN-γ, which indicates the importance of an ICSBP-IRF-1 complex in the regulation of iNOS gene expression.

**MATERIALS AND METHODS**

**Plasmid Constructs**—iNOS promoter fragments were inserted into the luciferase reporter vector pGL2B (Promega) as described previously (13). The IRF-1 expression plasmid was a gift from T. Taniguchi (University of Tokyo, Tokyo, Japan). All site-directed mutant plasmids were generated by two-step polymerase chain reaction using overlapping internal primers containing the mutant sites (30).

**Cell Lines and Reagents**—The RAW264.7 murine macrophage cell line (American Type Culture Collection) was maintained in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin-streptomycin. RAW267.4 cells were transiently transfected using calcium phosphate with a mixture of 2.5 µg of the retroviral expression vector pMD.OGP encoding gag-pol, and 10^9 g/ml) was added to the culture for 6 h. Then cells were washed with cold phosphate-buffered saline and lysed for 15 min on ice in 0.5 ml of lysis buffer (50 mM Tris, pH 8.0, 280 mM NaCl, 0.5% Nonidet P-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, and 1 mM dithiothreitol) containing protease inhibitors. Cell lysates were clarified by centrifugation at 4°C for 15 min at 14,000 rpm. Cell lysates were incubated with 2 µg of IRF-1 antibody (Santa Cruz Biotechnology) in the presence of 1% formaldehyde for 30 min at room temperature, and immunoprecipitated with anti-iNOS, anti-ICSBP, anti-IRF-1, or anti-IRF-2 antibodies (16000) for 1–2 h, washed with TBST, and stained with anti-rabbit or anti-mouse IgG conjugated to peroxidase (1:6000). Immunoreactivity was visualized by enhanced chemiluminescence reaction (enhanced chemiluminescence kit; Santa Cruz Biotechnology).

**RESULTS**

**Characterization of ICSBP Binding Site in the iNOS Promoter Region**—Macrophages from ICSBP-/− mice are impaired in IFN-γ-induced NO production (29), which suggests that ICSBP is involved directly in the regulation of iNOS gene expression. Because ICSBP is a transcription factor, we hypothesize that the murine iNOS promoter region may have an ICSBP binding site to which ICSBP can bind, resulting in the IFN-γ-induced activation of the iNOS promoter. The mouse iNOS promoter has an enhancer and basal promoter, within which a number of response elements have been localized. Those known to be active include NF-κB sites located both in the enhancer and basal promoter and two ISRE. The distal ISRE element (−923 to −913) is a strong activator, whereas the proximal one has no significant effect on iNOS promoter activation (16, 35). In the present study, the ISRE site between −923 and −913 was selected for the analysis, because this site is important for full induction of iNOS gene expression by IFN-γ. This site was also reported previously (16) as a binding site for IRF-1. RAW264.7 cells were activated with IFN-γ, LPS, or IFN-γ plus LPS for 4 h, and nuclear proteins were extracted for EMSA, which was performed by using probe spanning −935 to −905 of the iNOS promoter region containing −923 to −913 of the
ISRE site. A protein:oligonucleotide complex was formed that is inducible by IFN-γ and unaffected by LPS (Fig. 1A). When we used probe (−935 to −913) with mutations of ISRE site (TTCACTTTC to GCCGTAGGCA), there was no DNA-protein complex formation (data not shown). Furthermore, ICSBP antibody blocked the formation of DNA-protein complex for transfection of ICSBP. We first examined whether ICSBP can bind to this ISRE site. To confirm this result, we performed chromatin co-immunoprecipitation experiments. PCR analysis showed that ICSBP antibody precipitated the ISRE site of the iNOS promoter region (−2000 to −788) from RAW264.7 cells activated with IFN-γ for 4 h. Western blotting experiments revealed that IFN-γ-induced ICSBP protein expression in RAW264.7 cells was activated with IFN-γ, LPS, or IFN-γ/LPS for 4 h, and nuclear protein was extracted for Western blotting. 20 μg of nuclear protein was subject to 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with an antibody against ICSBP.

An ICSBP expression plasmid was co-transfected with an iNOS promoter luciferase construct or an iNOS promoter with a mutated ISRE site (−932 to −913) into RAW264.7 cells and incubated for 18 to 24 h. The transfected cells were activated with IFN-γ (10 ng/ml), LPS (5 μg/ml), or IFN-γ/LPS for 12 h. Extracts from activated cells were analyzed by luciferase assay. Results (normalized for β-galactosidase activity) are expressed as the luciferase activity (A) or percentage of wild-type iNOS promoter activity (B). Each result represents the mean of data from four to five experiments.

The above data indicate that ICSBP is important for IFN-γ-induced iNOS promoter activation.

iNOS Promoter Activity Is Impaired in ICSBP−/− Macrophages—To analyze further the importance of ICSBP in the activation of the iNOS promoter, we tested ICSBP−/− macrophages for IFN-γ-induced NO production and iNOS mRNA and protein expression. Both RAW264.7 cells and CL-2 cells were activated with IFN-γ (10 ng/ml) for 12 h (protein) or 72 h (nitrite). CL-2 cells had a reduced iNOS protein expression and nitrite accumulation induced by IFN-γ compared with RAW264.7 cells (Fig. 3, A and B). To determine iNOS promoter activation in CL-2 cells, RAW264.7 cells and CL-2 cells were transfected with an iNOS promoter luciferase reporter construct. iNOS promoter activation induced by IFN-γ was strongly impaired in CL-2 cells (Fig. 3C). However, co-transfection of ICSBP moderately rescued the iNOS promoter activation induced by IFN-γ in CL-2 cells. All these results suggest that ICSBP is important for iNOS expression induced by IFN-γ.

Transduction of ICSBP in ICSBP−/− Macrophages Rescues IFN-γ-induced iNOS Gene Expression—The data above showed that CL-2 cells were deficient in NO production induced by IFN-γ. To demonstrate the importance of ICSBP for iNOS gene expression, we transduced ICSBP into ICSBP−/− macrophages to rescue iNOS gene expression. The transduction efficiency judged by GFP fluorescence was around 70–80% by fluorescence-activated cell sorter (data not shown). Cells were activated with IFN-γ and analyzed for iNOS mRNA and protein and nitrite accumulation. After transduction of ICSBP, IFN-γ-induced iNOS expression and nitrite accumulation were rescued (Fig. 4, A–C). In addition, the synthesis of IL-12 expression, another IFN-γ-regulated gene, was rescued, as well (Fig. 4D). Unexpectedly, transduction of ICSBP alone did not rescue iNOS expression, although ICSBP protein was clearly expressed (Fig. 4, A–C). These results suggest that ICSBP alone is not sufficient for iNOS gene expression.

ICSBP-IRF-1 Complex Formation Is Required for iNOS Gene Expression—To explain the requirement for both IFN-γ and ICSBP to rescue iNOS transcription and NO production in CL-2 cells, we suggest that either another transcription factor may be needed or that IFN-γ induces post-translational modification of ICSBP. We first examined whether ICSBP can bind to the ISRE site of the iNOS promoter. After transduction with
Fig. 3. iNOS promoter activation is impaired in ICSBP−/− macrophages. A, nitrite accumulation was strongly reduced in ICSBP−/− macrophages. Both RAW264.7 cells and ICSBP−/− macrophages were activated with IFN-γ (10 ng/ml) for 48 h. Supernatants were then collected, and nitrite accumulation was determined using Griess reagent. B, iNOS protein expression was impaired in ICSBP−/− macrophages. Both RAW264.7 cells and ICSBP−/− macrophages were activated with IFN-γ for 12 h, and cellular protein was extracted for Western blotting. 20 μg of cellular protein was subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with iNOS antibody. C, IFN-γ-induced iNOS promoter activity was strongly impaired in ICSBP−/− macrophages. Both RAW264.7 cells and ICSBP−/− macrophages were co-transfected with an iNOS promoter luciferase plasmid and an ICSBP expression plasmid. The cells were incubated for 18 to 24 h and activated with IFN-γ for 12 h. Extracts from the activated cells were analyzed by luciferase activity. Results (normalized for β-galactosidase activity) were expressed as luciferase activity. Each result represents the mean of data from four to five experiments.

Fig. 4. Transduction of ICSBP into ICSBP−/− macrophages rescues IFN-γ-induced iNOS expression. CL-2 cells were transduced with retrovirus-ICSBP or retrovirus-GFP as control. A, the transduced cells were activated with IFN-γ for 48 h. Supernatants were then collected, and nitrite accumulation was determined using Griess reagent. B, the transduced cells were activated with IFN-γ for 8 h. Total cellular RNA was then extracted, and RT-PCR was performed for the detection of iNOS mRNA expression. C, the transduced cells were activated with IFN-γ for 12 h, and Western blotting was performed for the detection of iNOS protein expression. D, the transduced cells were activated with IFN-γ-LPS for 8 h, total cellular RNA was extracted, and RT-PCR was performed for the detection of IL-12 mRNA expression.

ICSBP or GFP as a control, CL-2 cells were incubated with or without IFN-γ for 4 h, nuclear protein was extracted, and EMSA was performed with a probe (~935 to ~905) containing the iNOS promoter ISRE site (~923 to ~913). Interestingly, EMSA experiments indicated that transduction with ICSBP alone did not result in DNA-protein complex formation, which was, however, seen after activation with IFN-γ (Fig. 5A). Furthermore, cycloheximide prevented the formation of this complex (Fig. 5B), suggesting that synthesis of another IFN-induced transcription factor is needed for ICSBP to bind to the ISRE site of iNOS promoter.

ICSBP is known to form complexes with IRF-1 or IRF-2. When RAW264.7 cells were activated with IFN-γ for 4 h, Western blotting results indicated that IFN-γ strongly induces IRF-1 protein expression (Fig. 6A). However, because IFN-γ has no effect on IRF-2 protein expression (Fig. 6A), IRF-1 is therefore the more likely candidate as the ICSBP partner. The ISRE site in the iNOS promoter region (~923 to ~913) was identified as an IRF-1 binding site in previous studies (16), and IRF-1−/− mice are defective in NO production. In addition, IRF-1 binds to the same ISRE site that we identified as an ICSBP site. To confirm the binding of IRF-1 to the ISRE site, we transduced ICSBP−/− macrophages with ICSBP and activated the cells with IFN-γ for 4 h. Nuclear protein was extracted, and EMSA was performed using a probe (~935 to ~905) bracketing the ISRE site (~923 to ~913). Both ICSBP and IRF-1 antibodies prevented the DNA-protein complex formation (Fig. 6B), showing the presence of both proteins in the DNA-protein complex. In addition, co-immunoprecipitation experiments demonstrated that a complex formed between ICSBP and IRF-1 in nuclear proteins from IFN-γ-activated RAW264.7 cells (Fig. 6C). When RAW264.7 cells were co-transfected with an iNOS luciferase reporter and both ICSBP and IRF-1, strong luciferase activity was observed, whereas transfection with ICSBP or IRF-1 alone only weakly induced iNOS promoter activation (Fig. 6D). Co-transduction with ICSBP and IRF-1 induced iNOS expression and nitrite accumulation, whereas transduction of either ICSBP or IRF-1 alone was not sufficient to induce iNOS expression (Fig. 6F). These re-
The complex formation of ICSBP with IRF-1 is important for iNOS expression. A, RAW264.7 cells were activated with IFN-γ for 4 h, and nuclear protein was extracted for Western blotting. 20 μg of nuclear protein was subjected to 10% SDS-PAGE, transferred to nitrocellulose, and blotted with ICSBP or GFP as a control, and the cells were activated with IFN-γ (10 ng/ml) for 4 h. The nuclear proteins were then extracted, and electrophoretic mobility shift assay was performed using a probe between −935 and −905 of the iNOS promoter. 5 μg of nuclear extracts was incubated with either 2 μg of ICSBP or 2 μg of IRF-1 antibody for 40 min before adding ³²P-labeled probe (−935 to −905). B, RAW264.7 cells were activated with IFN-γ for 4 h, and nuclear protein was extracted. 500 μg of nuclear protein was immunoprecipitated with 2 μg of IRF-1 antibody. The immunoprecipitated protein was subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with IRF-1 antibody. C, the iNOS promoter luciferase reporter was co-transfected with ICSBP and IRF-1 into RAW264.7 cells. The cells were incubated for 24 h, and the nuclear extracts were analyzed for luciferase activity. E, RAW264.7 cells were co-transduced with ICSBP and IRF-1, and the cells were incubated for 48 h. Supernatants were collected for the determination of nitrite accumulation using the Griess reagent, and cellular protein was extracted for 48 h. Total cellular RNA was then extracted, and RT-PCR was performed for the detection of iNOS mRNA expression. C, RAW264.7 cells were pretreated with IL-4 (10 ng/ml) for 1 h and activated with IFN-γ for 12 h. Total cellular protein was then extracted for Western blotting. 20 μg of protein were subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with an antibody against ICSBP. D, RAW264.7 cells were pretreated with IL-4 (10 ng/ml) and activated with IFN-γ for 4 h. The nuclear proteins were then extracted, and electrophoretic mobility shift assay was performed using a probe between −935 and −905 of the iNOS promoter. ³²P-labeled probes were incubated with 0.5 μg of poly(DI-DC) and 5 μg of nuclear extract. E, RAW264.7 cells were pretreated with IL-4 (10 ng/ml) for 1 h and activated with IFN-γ. Nuclear extracts were prepared and immunoprecipitated with 2 μg of antibody to IRF-1 and 20 μl of protein G-agarose beads. Beads were washed extensively and boiled with 1× SDS-PAGE sample buffer, subjected to 10% SDS-PAGE, and immunoblotted with an antibody against ICSBP. For controls, 20 μg of nuclear protein was subjected to 10% SDS-PAGE and immunoblotted either with antibodies against ICSBP or IRF-1.

**DISCUSSION**

This study describes the role of ICSBP in the regulation of murine macrophage iNOS gene expression. An ICSBP binding site in the iNOS promoter region (−923 to −913) was identified, and we show that the complex formed by ICSBP and IRF-1 is essential for IFN-γ-induced iNOS gene expression. IL-4, a Th2 cytokine that inhibits IFN-γ-induced iNOS gene expression, attenuates the physical interaction of ICSBP with IRF-1. Thus, complex formation of ICSBP with IRF-1 is a central element in iNOS gene expression and offers a novel example of cooperation among transcription factors resulting in the regulation of target gene expression.

The transcriptional regulation of iNOS has been studied extensively and characterized by a promoter with numerous protein-DNA and protein-protein interactions. Although many potential *cis*-regulatory elements have been identified, six enhancer elements have been shown to be important in IFN-γ and LPS induction of iNOS. In the upstream enhancer element known as region II, located between positions −1029 and −913, one IFN-γ-stimulated response element and an IFN-γ-activated sequence have been identified (15, 16, 35). For LPS transcriptional regulation of iNOS, two NF-κB sites, one located in the basal enhancer region I between positions −48 and −209 and another located in region II, are required (36). More recently, another *cis*-regulatory element, an Oct site, which binds the basal transcriptional element octomer, was found to be required for maximal iNOS transcription by LPS. NF-κB has also been shown to be required in the regulation of the human iNOS gene.
Induces transcription of the IFN-γ/ICSBP family members, including IRF-1 and ICSBP. Both IRF-1- and ICSBP-deficient mice are vulnerable to bacterial infections (16, 27, 28). These mice do not generate a Th1 immune response against intracellular pathogens, and macrophage production of IL-12 in response to LPS and IFN-γ activation is seriously compromised (39, 40). Interestingly both IRF-1- and ICSBP-/- macrophages display selective impairment of IFN-γ-induced NO production (16, 29). Kamijo et al. (16) reported that NO production induced by IFN-γ and LPS was strongly impaired in IRF-1-/- mice, and they also identified the IRF-1 binding site in the ISRE site (−923 to −913) of the iNOS promoter region. Other reports suggest that IRF-1 is required, along with NF-kB, for the synergistic effect of IFN-γ with tumor necrosis factor-α in the induction of iNOS expression (41). However, the mechanism responsible for the IRF-1 effect on iNOS gene expression is still not understood fully.

IRF-1-/- mice and ICSBP-/- mice both display clear impairment of IFN-γ-induced NO production (29), which suggests that ICSBP is involved directly in iNOS gene regulation. Because ICSBP is an IFN-γ-induced transcription factor, we hypothesized that there should be an ICSBP binding site in the iNOS promoter region, and we have now identified ICSBP binding site between −923 and −913, which is required for maximal effects of IFN-γ induction of the iNOS gene (35). EMSA and chromatin co-immunoprecipitation experiments show that ICSBP binds to this typical ISRE site. Overexpression of ICSBP greatly enhanced IFN-γ-induced iNOS promoter activation, whereas overexpression of ICSBP had no effect on the mutant iNOS promoter with a mutated ISRE site (−923 to −913) of the iNOS promoter. Furthermore, macrophages from ICSBP-/- mice are severely compromised in IFN-γ-induced iNOS promoter activation and nitric oxide production. These results suggest that ICSBP is important for iNOS gene expression and that ISRE site at −923 to −913 is a binding site for ICSBP.

As expected, when we transduce ICSBP-/- macrophages with ICSBP, IFN-γ-induced iNOS expression was rescued. But, unexpectedly, transduction with ICSBP alone did not induce iNOS gene expression, although ICSBP was expressed. EMSA experiments indicate that without IFN-γ activation, ICSBP did not bind to the ISRE site (−923 to −913) in the iNOS promoter. Furthermore, IFN-γ-induced DNA-protein complex formation is sensitive to cycloheximide, suggesting that another transcription factor is needed to form the complex. EMSA experiments demonstrated that this complex also contains IRF-1. In addition, co-immunoprecipitation experiments indicated the presence of an ICSBP-IRF-1 complex in the nucleus of IFN-γ-activated RAW264.7 cells. All these results suggest that a complex between ICSBP and IRF-1 is essential for the iNOS expression induced by IFN-γ.

Transcription of critical cytokines and other important genes is often regulated by synergistic combination of transcription factors, which form a nuclear protein complex called an “enhanceosome” (42). For example, NF-kB alone or IRF-1 alone induces transcription of the IFN-β gene at much lower levels than both transcription factors together. In the present study we found that ICSBP and IRF-1 synergize to activate the iNOS promoter. ICSBP was cloned from a cDNA expression library screened with a labeled ISRE DNA element from a major histocompatibility complex class I promoter. Specific interaction with different IFN type I-induced genes was demonstrated by Southern-Western analysis. The stimulatory effects of ICSBP on the transcriptional activity of both IRF-1 and IRF-2 suggest that different DNA-binding heterocomplexes were involved, which led to the identification of specific protein-protein complexes between these factors. In vitro translated IRF-1, IRF-2, and ICSBP were able to form strong interacting complexes that were also identified in nuclear extracts from various cell lines. In the present study, direct physical interaction of ICSBP with IRF-1 was found in nuclear extracts from RAW264.7 cells activated with IFN-γ. This suggests that the effects of ICSBP and IRF-1 on the iNOS gene expression are based on the direct physical interaction of ICSBP with IRF-1.

ICSBP was regarded initially as a transcription repressor (21). Contursi et al. (29) also reported that ICSBP represses IFN-γ-induced ISRE activity. However, it has been demonstrated recently that ICSBP can interact with PU.1, a hematopoietic cell-specific member of the Ets family (43), resulting in the activation of transcription. Accumulated evidence indicates that ICSBP and PU.1 activate transcription through Ets-IRF DNA elements and related elements in IFN-γ-stimulated macrophages, as reported for IL-1β and the respiratory oxidase gene gp91 phox (44–46). ICSBP and PU.1 may also have a role in enhancing expression of the toll-like receptor 4 and IL-18 through a similar element (47, 48). This activation seems to involve other factors, notably the co-activator/histone acetylase cAMP-response element-binding protein-binding protein/p300, with which PU.1 has been shown to interact (49). The present study demonstrated that ICSBP and IRF-1 have a synergistic effect on the activation of iNOS promoter, which agrees with the results reported for activation of the IL-12 promoter (25). These findings suggest ICSBP has a dual role in the regulation of transcription. On one hand, ICSBP represses ISRE promoter activity resulting in the inhibition of transcription, but ICSBP can also activate iNOS, IL-12, and IL-1β promoter activities. Therefore, the exact molecular mechanism for the regulation of transcription by ICSBP still needs to be elucidated. Our future study will focus on analysis of the ISRE sites in different promoters to find differences in ISRE sequence or flanking sequence and correlate these differences with the regulation of transcription by ICSBP. We will also search for new partner that ICSBP recruits following macrophage IFN-γ activation. We hope to address the mechanism responsible for the dual role of ICSBP in the regulation of transcription.

Regulation of inflammatory responses involves intercellular communication through a network of secreted cytokines. To avoid detrimental inflammatory and cytotoxic reactions of activated macrophages, the production of proinflammatory cytokines and chemokines, as well as of reactive oxygen and nitrogen intermediates, are tightly regulated (50, 51). This regulation is at least partially dependent on the balance between proinflammatory and anti-inflammatory cytokines. Although IL-4-mediated anti-inflammatory function has been found to include both transcriptional and translational events, the transcriptional regulation represents a major target (52, 53). In the present study, we found IL-4 inhibits iNOS gene expression in part by attenuating the interaction of ICSBP with IRF-1. However, we cannot exclude that other mechanisms may play a role in the inhibition of iNOS gene expression by IL-4.

Taken together, our results provide a novel insight on the molecular mechanism for the induction of iNOS gene expression by IFN-γ. ICSBP, a member of IRF family, can form a complex with IRF-1 that is essential for iNOS gene expression. IL-4, an anti-inflammatory cytokine, inhibits iNOS gene ex-
pression by disrupting the interaction of ICSBP with IRF-1. These results may provide a framework for future anti-inflammatory therapy.

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Complex Formation of the Interferon (IFN) Consensus Sequence-binding Protein with IRF-1 Is Essential for Murine Macrophage IFN-γ-induced iNOS Gene Expression

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