Nitric oxide production in a variety of inflammatory conditions is dependent on the synthesis of the enzyme, inducible nitric-oxide synthase (iNOS). The gene for this enzyme is regulated by a number of inflammatory cytokines, including interferon-γ. Transcriptional activation of the gene is dependent on the interferon-γ-induced transcription factor, interferon regulatory factor-1 (IRF-1). Using a 99-base pair segment of the iNOS gene promoter encompassing nucleotides −979 to −881, a region essential for gene activation by cytokines, we show that with increasing concentrations of added IRF-1, a monomeric then a dimeric complex form. Molecular footprinting analysis shows that the factor binds initially to a canonical IRF-1 site as a monomer. The region of binding is then extended both in a 5′ and 3′ direction on formation of the dimeric complex, with additional contacts in the minor groove of DNA. Binding of the second molecule of IRF-1 is dependent on the presence of the initial bound protein. Sequential binding of IRF-1 to form a dimeric complex has not been described previously, and we show that formation of this dimeric complex is essential for full activation of the iNOS gene by cytokines in vascular smooth muscle cells.

Interferon regulatory factor-1 (IRF-1) is a transcription factor that was first identified as important in the virus-induced activation of the IFN-β gene (1). Molecular cloning revealed it to be a DNA-binding protein with a basic NH2-terminal domain linked to an acidic COOH-terminal region which is highly conserved between mice and humans (2). The NH2 terminus is responsible for DNA binding, whereas sequences within the COOH-terminal domain mediate transactivation (3–5). IRF-1 is generally present only at low levels within resting cells; but after treatment with either type I or type II IFN, transcription of the IRF-1 gene is increased, and levels of IRF-1 protein rise dramatically (2, 6). In addition, IRF-1 production is induced by other cytokines, such as IL-1, IL-6, and TNF-α (6), viral infection (2), and prolactin (7). IRF-1 enters the nucleus rapidly after synthesis, where it binds to a DNA motif with the sequence G(A)AAA(G/C)(T/C)-GAAA(G/C)(T/C) (8). This motif has been found in the upstream promoter elements of a number of IFN-inducible genes such as IFN-β (9), major histocompatibility complex class I (10), and 2′-5′-oligo(A) synthetase (11). A number of studies have shown the importance of IRF-1 in mediating transcriptional up-regulation of these and other IFN-induced genes (8).

IRF-1 has additional functions in cellular growth. Molecular cloning revealed that IRF-1 belongs to a gene family of related proteins, the closest of which is termed IRF-2 (4). This binds to the same sequence motif as IRF-1, but in general it acts as a repressor rather than a transcriptional activator. Further work has demonstrated that IRF-1 and -2 have opposing actions on cell growth, with IRF-1 acting as a growth inhibitor, whereas cells overexpressing IRF-2 showed a transformed growth phenotype and enhanced tumorigenicity (12). IRF-1 levels in the cells are linked to the cell cycle, with peak amounts in growth arrest which decline after serum stimulation (12). A role for IRF-1 as a tumor repressor is supported by the observation that the human IRF-1 gene is often deleted in patients with leukemia or myelodysplastic syndromes (13).

Recently, the gene for inducible nitric-oxide synthase (iNOS) has been shown to be regulated by IRF-1 (14, 15). Nitric oxide (NO) is a mediator of many diverse physiologic and pathophysiologic processes. It is a major determinant of resting vascular tone in mammals (16), it can act as a microbicidal and tumoricidal agent (17), and, inter alia also acts as a neurotransmitter at glutamate receptors in the brain (18). Three related enzymes can synthesize NO in different tissues. Two of these, the brain and endothelial forms, are generally constitutively present in the cells where they are found, but the third, iNOS, is usually only produced after transcriptional activation of its gene (19). Potent activators include proinflammatory cytokines such as IL-1, TNF-α, and IFN-γ, which synergize in the production of iNOS in a variety of cell types (20). Originally described in macrophages, iNOS is found in a number of different cells ranging from vascular smooth muscle (VSM) cells to astrocytes (20). Its function differs depending on the cell type. In macrophages, NO produced from iNOS is responsible for their microbicidal and tumoricidal properties. In VSM cells, NO production from iNOS produces smooth muscle relaxation. This is responsible for the hypotension seen in bacterial sepsis, a condition in which high circulating levels of endotoxin and proinflammatory cytokines combine to induce iNOS in VSM (21).

Cloning of the iNOS promoter in mice and rats has shown the presence of a number of potential IRF-1 binding sites (22, 23). Deletion and mutational analyses have shown the importance of sequences in the region of the promoter from −890 to −1002 in induction by IFN-γ (15, 24). Site-directed mutagenesis and competition experiments have suggested that IRF-1 binding at a site found between positions −913 and −923 is required for full synergistic effects of IFN-γ in induction of the
Interferon Regulatory Factor-1 DNA Binding

iNOS gene (14, 15). In addition, mice homozygous for a targeted disruption of the IRF-1 gene are unable to produce iNOS, again demonstrating the importance of this transcription factor in iNOS induction (14). The transcription factor NF-κB is also essential for cytokine activation of the iNOS gene (24).

The IRF gene family does not have any sequence similarity to those of other known transcription factors. NMR studies on IRF-2 suggest that the IRF family proteins contain a helix-turn-helix-like domain that mediates DNA binding (25). Unlike the majority of transcription factors, IRF-1 appears to bind as a monomer to a single IRF-1 site through its NH₂-terminal domain (8). Early studies have shown that contacts within its binding site are made to the major groove of DNA (4). In two studies of IRF-1 action in transcriptional up-regulation, IRF-1 has been shown to facilitate the binding of other transcription factors such as NF-κB (26) and the structural DNA-binding protein, high mobility group protein-(I)Y (27). Thus, IRF-1 binding to DNA and other proteins is central to the formation of transcription factor complexes, or enhanceosomes, which mediate the transcriptional regulation of a number of IFN-regulated genes.

Given the importance of IRF-1 in iNOS gene induction, we set out to determine how IRF-1 up-regulates transcription of this gene and possibly interacts with other transcription factors responsible for iNOS transcriptional control. We wished to determine the site or sites within the iNOS enhancer at which IRF-1 binds and its importance in regulating iNOS gene transcription. In this study, we show that binding of IRF-1 occurs initially to a single high affinity site within the iNOS promoter. This initial binding then allows the binding of a second molecule of IRF-1 to form a ternary DNA-protein complex. This second molecule of IRF-1 does not bind at a separate well defined canonical IRF-1 site but produces an extension of the area of DNA in contact with IRF-1 in both a 5’ and 3’ direction. Formation of this complex is essential for full cytokine activation of the gene.

EXPERIMENTAL PROCEDURES

Cell Culture—A7r5 rat VSM cells were obtained from the European Collection of Animal Cell Cultures at Porton Down, U. K. They were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 10% fetal calf serum, 100 units ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin at 37 °C in an atmosphere of humidified 5% CO₂.

Expression and Purification of Bacterially Expressed IRF-1—The protein coding sequence of murine IRF-1 was amplified using the polymerase chain reaction (PCR) on cDNA prepared from IFN-γ-treated L929 cells and appropriate primers derived from the NH₂ and COOH termini of the protein; these primers contained terminal BamHI sites. After amplification, the product was purified from an agarose gel, excised and the DNA eluted by incubation of the crushed gel slice in 400 μl of TE; 50 mM Tris (pH 8.0); 1 mM EDTA; 1 mM dithiothreitol, and 4, 2, 1, and 0M urea, respectively. The purity of the recombinant protein was checked by SDS-polyacrylamide gel electrophoresis and was greater than 95% in all cases. Aliquots of the pure protein were stored at −80 °C. Enterokinase (Boehringer Mannheim) digestion was performed with 1:40 w/w protease:IRF-1 in a buffer containing 10 mM Tris (pH 8.0), 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride for 16 h at 25 °C. After digestion, any remaining His-tag was removed by immobilized metal affinity chromatography, and the flow-through from the column was then renatured as described above. Complete removal of the His-tag was verified by SDS-polyacrylamide gel electrophoresis, which demonstrated that the product was all of a lower molecular weight.

The mutated derivatives of IRF-1 lacking amino acids at either the NH₂ or COOH terminus of the protein were constructed using PCR with primers designed to introduce a stop codon at the required termination point (for COOH-terminal deletions) in the IRF-1 coding sequence or to start the protein at an internal site (for NH₂-terminal deletions). Such mutated sequences were cloned into pET19b and expressed and purified as described above.

Interferon Regulatory Factor-1 DNA Binding

The 99-bp fragment of the iNOS promoter extending from position −979 to −881 was radiolabeled using the primers GTGCTAGGGGATTTTCCCTCCTC (forward) and GCATCACACATGGCAGTT (reverse) in a modified PCR in a volume of 100 μl containing reaction buffer (50 mM KCl, 10 mM Tris (pH 9.0), 0.1% Triton X-100, and 2.5 mM MgCl₂, a 0.1 mM concentration of each of dGTP, dCTP, dATP, and dTTP, 0.025 mM dCTP; 50 μg; and dATP, 0.025 μCi of [α-32P]dCTP (3,000 Ci mmol⁻¹), 50 pmol of each primer; and 2.5 μl of Taq DNA polymerase. Added template (0.1 μg) was a purified 99-bp fragment made in this reaction with no added radiolabel. The PCR was performed as follows: denaturation for 5 min at 95 °C, then 28 cycles of 30 s at 95 °C, 45 s at 55 °C, and 60 s at 72 °C, followed by one incubation of 10 min at 72 °C. Radiolabeled product was purified using a PCR purification kit (Qiagen) and required no further purification before use. For the experiment shown in Fig. 6, duplex oligonucleotides were radiolabeled with polynucleotide kinase.

EMSA’s were performed in 20-μl reactions containing 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 100 μg ml⁻¹ poly(dI·dC)·(dI·dC), 1 μl of DNA probe (≈100,000 cpm), and added DNA-binding protein as indicated. Immediately before use, an aliquot of recombinant IRF-1 was diluted to the required concentration in 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, and 1 mg ml⁻¹ bovine serum albumin. Incubations were for 15 min at room temperature. DNA-protein complexes were then separated on native 5% polyacrylamide gels in 0.5 × Tris borate-EDTA buffer and autoradiographed with an intensifying screen at −80 °C using Kodak X-Omat AR film.

Molecular Footprinting—For footprinting DNA-protein complexes, probe labeled on one strand of duplex DNA was prepared with the PCR described above, but using one of the primers already radiolabeled at the 5’ terminus with polynucleotide kinase, omitting the radiolabeled dCTP, and increasing the concentration of dCTP to 0.1 mM in the reaction mixture. dGTP and dTTP were added as described (28) and ethylation interference as described (29). Missing nucleoside analysis was performed as described (30) with the following modifications. Approximately 100 ng of radiolabeled probe was prepared in 70 μl of 10 mM Tris·Cl (pH 8.0), 1 mM EDTA (TE). In one single pipette tip the following reagents were drawn up, with an air bubble between each: 10 μl of 100 mM sodium ascobic, 10 μl of 0.6% H₂O₂, and 10 μl of a freshly prepared mixture of 10 mM iron(II) ammonium sulfate, 20 μM EDTA. These were added together to allow instantaneous mixing with the labeled DNA in the same tube. The reaction was allowed to proceed for 2 min at room temperature before quenching with the addition of 30 μl of 0.1 M thiourea, 10 μl of 0.2 M EDTA, 20 μl of 3 M sodium acetate (pH 5.2), and 40 μl of 10 mM Tris·Cl (pH 8.0), 10 mM EDTA. Gapped probe was precipitated with ethanol twice and resuspended in 20 μl of TE; 5 μl was used in one gel shift reaction.

In all cases gel shifts were performed as described above, except the autoradiograph was performed with the gel wet. Specific bands were excised and the DNA eluted by incubation of the crushed gel slice in 400 μl of 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS for 16 h at room temperature with gentle agitation. Ethanol was then extracted or phenol/chloroform/DNA precipitated with ethanol. Pellets were resuspended in 10 μl of formamide-loading dyes (95% formamide, 4% EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol). Radioactivity in each sample was determined using a scintillation counter and equal amounts of radioactivity loaded in each lane of a denaturing urea 10% polyacrylamide gel.

Image Analysis and Molecular Modeling—The density of bands on
autoradiograms was quantified by scanning the image into the program NIH Image, which calculates the band densities required in two dimensions. Intensities of bands in the unbound DNA fraction reflect the relative abundance of DNA fragments that failed to bind either one or two molecules of IRF-1. For DNA eluted from the monomeric IRF-1 complex, the usual practice of calculating the intensity of footprint by the ratio of band density from free and bound fractions of probe will therefore be inaccurate, tending to overestimate degree of protection in areas where IRF-1 in fact only contacts the probe in the dimer. Thus, quantitation of footprints was performed by calculating the ratio of band density in the bound DNA fraction compared with the same band in an equal loading of DNA eluted from a control, untreated sample (input). Figures of these gels were prepared using these scanned images transferred into MacDraw Pro and printed using the dye-sublimation technique.

Molecular modeling of the contacts made by IRF-1 onto the DNA double helix was performed using the program Insite II (Biopolymer module) obtained from BioSym/MSCI, San Diego, and run on an Indigo II Silicon Graphics workstation.

Site-directed Mutagenesis—All site-directed mutations were made using recombinant PCR (31) with the complete murine iNOS fragment spanning nucleotide 1577 to 1615 as template. Common to all of the reactions was the use of primers that amplify this whole segment, which are described in Ref. 24. For generation of the five insertion mutants, the internal mutagenizing primers were (TCAATATT-CAGCGTATCATGCTGGAAAATTCC) and its complement for the site C mutant and (CACTTTTCACATGGAAAATTC) and its complement for the mutants, the internal mutagenizing primers were (TCAATATT-CAGCGTATCATGCTGGAAAATTCC) and its complement for the site C mutant. The altered residues from wild type are underlined. Conditions for the PCRs were as described for the generation of labeled probes, except no radioactivity was added, and the concentration of all nucleotides was 0.1 mM. For the first round of PCR, annealing was at 50 °C for 28 cycles. The PCR products were purified by agarose gel electrophoresis, and one-quarter of each product was combined with the same amount of its appropriate pair and used as template in a further 12 rounds of PCR under the same conditions. Full-length mutant iNOS promoter was purified by agarose gel electrophoresis. For the five-insertion mutant a 262-bp BpuI1102I/NheI fragment was purified and cloned into BpuI1102I/NheI-digested vector pCAT-NOS (24), which contains the iNOS promoter up-stream of a CAT reporter gene. For the site B and site C mutants, an SphI/NheI fragment was used in an exactly similar fashion. All mutants were verified by DNA sequencing. For generation of mutant probes, linearized plasmid DNA from the mutant clones was used as template in the reactions as described above.

Transfections—The rat VSM line A7r5 was transfected exactly according to the method as described in Ref. 24. CAT activity was determined using the method described in the same paper. The concentrations of cytokines used were: 10 ng ml−1 IL-1, 200 units ml−1 IFN-γ, and 25 ng ml−1 TNF-α. All cytokines were of murine origin and were supplied by Genzyme or Serotec.

RESULTS

Dimeric Binding of IRF-1 to the iNOS Promoter—Recombinant bacterially expressed IRF-1 fused to an oligohistidine tag was tested for its ability to bind to the cytokine-responsive element of the iNOS gene using the EMSA. A 99-bp probe was used corresponding to position −979 to −881 of the murine iNOS promoter which encompasses the predicted IRF-1 binding sites (Fig. 1A). There are three potential IRF-1 binding sites within this region which are denoted A, B, and C. The number of nucleotides matching the canonical binding sequence is shown above each site, as a fraction of the total nucleotides comprising the potential binding site. This region of the promoter is essential for cytokine induction of the iNOS gene in VSM cells (24). Addition of increasing amounts of recombinant IRF-1 produced initially one major retarded band (Fig. 1B, lane b). With the addition of increasing amounts of IRF-1, a second, lower mobility band became apparent (Fig. 1B, lanes c and d). At the highest concentration of added IRF-1, the first complex formed largely disappeared, and a third and fourth band of still lower mobility were formed (Fig. 1B, lanes e and f). Essentially identical results were obtained after removal of the oligohistidine tag at the NH2 terminus of the recombinant protein using enterokinase (data not shown). All further experiments were performed with the oligohistidine tag attached. Protein binding to this probe was specific because bovine serum albumin or denatured IRF-1 showed no binding (data not shown). In addition, excess unlabeled probe abolished this binding; excess DNA of an unrelated sequence had no effect (data not shown).

The simplest explanation of the multiple bands seen in Fig. 1B that initially a single molecule of IRF-1 binds at one site,
followed by a second molecule at a separate site, and so on. It is well known that IRF-1 does not bind as a multimer to a single binding site (8). To determine the exact stoichiometry of binding (32), we repeated the EMSA with the same probe but adding a mixture of full-length recombinant IRF-1 (329 amino acids) and a COOH-terminal truncated IRF-1 mutein composed of amino acids 1–246, termed D3. Because the third and fourth most retarded species detected in Fig. 1 were only seen at the highest concentrations of added IRF-1, we decided to focus on the first two species formed with lower amounts of added IRF-1. The COOH-terminal truncation mutein of IRF-1 binds to the iNOS enhancer probe in a fashion similar to that of the full-length wild type protein (Fig. 2, lanes e–g), but with the shifted bands having a greater mobility than those produced by the wild type protein, consistent with the reduced molecular weight of the mutein. Binding with the mixture of proteins produces only one new species not seen in the binding of either molecule alone (Fig. 2, lanes h–k), which is intermediate in mobility between the slowest species produced by the wild type and truncated protein. This corresponds to a mixed dimer of the two species and demonstrates that the two main bands formed on IRF-1 binding in Fig. 1 correspond to monomer then dimer formation.

**Molecular Footprinting of IRF-1 Binding to the iNOS Promoter**—To determine the exact region of the iNOS enhancer which binds IRF-1, we performed molecular footprinting analyses on the monomeric and dimeric IRF-1–DNA complexes. Gel retardation with IRF-1 was performed with the 99-bp probe utilized in Fig. 1 labeled on either the top or bottom strand. Complexes corresponding to monomeric and dimeric bound IRF-1 were subjected to footprinting analysis using three separate footprinting techniques. First, we employed the technique of hydroxyl radical cleavage-based missing nucleoside analysis (30). In the monomeric IRF-1–DNA complex, residues from T914 to T916 on the coding strand (Fig. 3, A and C) and T912 to A916 on the noncoding strand (Fig. 3, B and C) were critical for IRF-1 interactions with DNA. As defined in Fig. 1A, these contacts lie at the 3′ border of IRF-1 binding site B and extend one nucleoside on the noncoding strand into adjacent site C. In the dimeric IRF-1–DNA complex, a more extensive region of protein DNA contact is evident. Surprisingly, this did not indicate binding to one of the other potential IRF-1 binding sites within the iNOS enhancer. Instead, there was an extension of the binding seen with the monomer in both the 3′ and 5′ direction. This extends from residue A918 to A912 on the coding strand and A920 to T911 on the noncoding strand (Fig. 3). On the noncoding strand, there is a clear bipartite distribution of binding strength, with two peaks of maximal interaction occurring at G917 (in site B) and T912 (in site C). In addition, compared with the monomer, the degree of binding in the dimeric IRF-1–DNA complex is considerably stronger.

To map further contacts made by IRF-1 in binding to this region of DNA, we carried out methylation and ethylation interference binding studies (Fig. 4). Partially methylated DNA bound to IRF-1 was cleaved at G residues methylated at the N-7 position, which interferes with major groove DNA binding, and A residues methylated at N-3, which interferes with binding in the minor groove (28). In the monomeric IRF-1–DNA complex, relatively few methylated residues affect IRF-1 binding, which are located on the noncoding strand; only partial interference was seen at A916 and G913 and complete interference at A911. In the dimer, however, there are more extensive interactions. On the coding strand methylation of A910 and A909 gave complete interference with IRF-1 binding. On the noncoding strand, methylation at G917 and A915 gave partial interference with binding, whereas modification of G919, A916, G913, and A911 gave complete interference with IRF-1 binding. These interactions are shown projected onto the DNA sequence of this region in Fig. 4D.

To determine the interaction of IRF-1 with the phosphate backbone of the DNA in this region, we performed ethylation interference analysis, as shown in Fig. 4. Note that because strand breakage can occur on either side of an ethylated phosphate residue, the cleaved products will run as doublets on a DNA sequencing gel which are only resolved for short fragments. As a result, the bands produced by cleavage of ethylated DNA trail behind the corresponding band obtained by cleavage of methylated DNA (29). In the monomeric IRF-1–DNA complex, no significant ethylation interference could be detected (Fig. 4). However, in the dimeric IRF-1-containing complex, significant interference was observed at positions A923 to T921, G917 to G916, C913, and A912 on the coding strand, and at positions G919 to G917 and G913 to A911 on the noncoding strand. These are shown projected onto the DNA sequence of this region in Fig. 4D.

No interactions were seen with DNA in the region of the potential IRF-1 site we have termed A.

**Computer Modeling of IRF-1–DNA Interactions**—Combining the data obtained from the missing nucleoside experiment and the methylation and ethylation interference analyses, we constructed a model of the DNA in the region of the iNOS promoter/enhancer which binds IRF-1 (Fig. 5). In this figure, the significant contacts determined by missing nucleoside analysis are shown in white, phosphate backbone contacts from ethylation interference are shown in yellow, and the N-3 and N-7 atom contacts to A and G residues, respectively, are shown in red. In the monomeric IRF-1–DNA complex, the majority of the interaction is in the most 3′ section of IRF site B, predominantly in the major groove at this site. The methylation interference data suggest that in addition there are some contacts made in the minor groove at A916 (partial interference) and A911 (complete interference). No backbone contacts are made (Fig. 5A).

In the dimeric IRF-1–DNA complex, the interactions are more extensive (Fig. 5B). In addition to the area showing interaction in the monomeric complex, there are contacts extending in both
and 3’ direction. The pattern of contacts suggests that the two molecules of IRF-1 are accommodated adjacent to one another. However, it is not possible to infer from the footprinting data the exact contacts made by each individual IRF-1 molecule; the observed contacts obviously reflect the sum of the interactions of the two IRF-1 molecules within the DNA-protein complex. There do, however, seem to be two main areas of interaction. The missing nucleoside experiments show that there are two peaks of DNA-protein interaction, one centered on the noncoding strand at G$^{917}$ in site B and the other centered on the noncoding strand at T$^{912}$, at the very 5’ portion of site C. These are shown with white arrows on Fig. 5. The first peak of interaction at G$^{917}$ on the noncoding strand lies between two areas of phosphate backbone interaction at G$^{919}$ to G$^{917}$ (non-coding strand) and C$^{917}$ to T$^{916}$ (coding strand). These areas face each other across the major groove at this point, suggesting that the IRF-1 protein binds predominantly in the major groove at this position. This is supported by the methylation interference data, which show interactions with the N-7 of G residues in the major groove at positions 2$^{917}$ and 2$^{919}$ (non-coding strand).

The second area of interaction centered on the noncoding strand at position T$^{912}$ lies between phosphate backbone contacts at C$^{913}$ and A$^{912}$ on the coding strand and at G$^{913}$ to A$^{911}$ on the noncoding strand. At the 5’ portion of this area, IRF-1 contact seems to be in the major groove, as evidenced by methylation of the N-7 of G residues in the major groove at positions −917 and −919 (non-coding strand).

Minor Groove Binding of IRF-1—The model in Fig. 5 suggests that contacts within site C made on binding the second molecule of IRF-1 are in the minor groove. Although interference of binding after methylation of A residues at the N-3 position suggests binding to the minor groove of DNA, this may not necessarily be the case, as modification of a residue may interfere with protein binding by preventing local deviations in DNA structure. An additional test of minor groove binding to DNA is to utilize mutant oligonucleotides which have I:C base pairs substituted for A:T base pairs (33). I:C and A:T pairings are identical as far as hydrogen bond donors and acceptors are concerned in the minor groove, but they differ markedly in the major groove (33, 34). Thus, if a protein binds in the minor groove of DNA, it should still bind to an oligonucleotide containing its binding site when the A:T residues are altered to I:C. This change in base pairing, however, would abolish binding of a protein which required specific major groove contacts. We generated 34-bp oligonucleotides spanning the promoter sequence from position −929 to −896, encompassing the two IRF-1 binding sites B and C. Three oligonucleotides were generated: a wild type sequence and two mutants in which I:C
base pairs were substituted for A:T base pairs either in IRF-1 binding site B or C. These oligonucleotides were radiolabeled and used as probes in a gel shift mobility assay with added IRF-1 (Fig. 6). The wild type probe produced a pattern of binding similar to that seen with the longer probe used in Fig. 1, with monomer (arrowhead) and dimer (arrow) bands (Fig. 6, lanes a–c). The dimer binding was consistently less with this probe compared with the longer probe used in Fig. 1B, suggesting that the additional sequence in the longer probe in some way facilitates dimeric binding, perhaps by allowing a greater degree of flexibility of the DNA to wrap around the IRF molecules. Using a mutant oligonucleotide with I:C substitutions in site B, all binding was abolished (Fig. 6, lanes d and e), demonstrating predominant major groove binding at this site, as suggested in the model shown in Fig. 5. However, when the oligonucleotide with I:C substitutions in site C was used as a probe, both monomer and dimer binding was preserved (Fig. 6, lanes f and g). The relative intensity of dimer binding to this...
mutant oligonucleotide was consistently about 40% of the wild type, as measured by densitometry.

**IRF-1 Binds Cooperatively to the iNOS Enhancer**—The data in Figs. 1B and 2 demonstrate that initially one molecule of IRF-1 binds to the iNOS enhancer, following which a second molecule can bind. Given that IRF-1 only ever binds as a monomer to a single IRF-1 site, even when contained within a 64-bp oligonucleotide (8), it follows that additional DNA sequences contained within the 99-bp enhancer sequence used as the probe in Figs. 1 and 2 direct the binding of the second IRF-1 molecule. The data in Fig. 5 show that in the dimeric IRF-1-DNA complex, two main areas of contacts are made, within site B and also the proximal portion of site C. Binding of the second IRF-1 molecule might be entirely independent of binding of the first molecule, or it may depend to some degree on prior occupancy of the first site by IRF-1. If binding of the two molecules is entirely independent, then in the absence of binding to the first site, there should still be detectable binding to the additional site. If there is a positive interaction between the two bound molecules, then in the absence of binding to the first site there will be reduced binding detected at the second site.

Given that adjacent to site B is a potential IRF-1 binding site (site C), we hypothesized that IRF-1 might be able to bind to this site even in the absence of binding to site B. To determine whether site C alone is able to bind IRF-1, we constructed mutants within sites B and C and assayed their ability to bind IRF-1. Mutants in site B (mB) in which the T residues at positions –916 and –915 were changed to G residues were devoid of any IRF-1 binding (Fig. 7, lanes m–p). Thus, in the absence of binding to site B, there was no detectable binding to site C, suggesting that occupancy at site B is essential for extension of IRF-1 binding into site C. The affinity of IRF-1 for the additional binding at site C is increased infinitely in the absence of binding to site B.

When site C was altered (mC) by changing the two A residues to G residues (which contact the IRF-1 in the minor groove, Fig. 5) to C residues, binding of both monomer and dimer was abolished until the amount of added IRF-1 was raised to 500 ng, when a limited amount of dimeric binding could be detected (Fig. 7, lanes i–l). This differs significantly from the wild type (Fig. 7, lanes a–d) and shows that binding of IRF-1 to site C is sequence-dependent. It is interesting to note that a mutation affecting site C also reduces apparent binding at site B (compare Fig. 7, lanes i–l with lanes a–d). This shows that diminution of binding affinity at site C in the dimeric IRF-1-DNA complex also reduces the affinity of binding at site B.

We also constructed a mutant in which the spacing between sites B and C was altered. This mutant, m5, contains a 5-bp insertion between the two sites. Interestingly, IRF-1 only binds as a monomer to this mutant (Fig. 7, lanes i–l), suggesting that the additional binding required for formation of the dimeric IRF-1 complex requires a close approximation of sites B and C. The intensity of the monomeric IRF-1-DNA complex is lower in the m5 construct. This shows that the affinity of IRF-1 binding as a monomer is reduced in the absence of the ability to form dimers, as also seen in the mC mutant described above.

**Effects of Mutations within IRF-1 Binding Sites on iNOS Promoter Activity**—To determine whether the observed binding of IRF-1 to both sites B and C was important in the function of the iNOS promoter, we cloned the mutants shown in Fig. 7 into a full-length copy of the iNOS promoter upstream of a CAT reporter gene and assayed promoter activity after cytokine stimulation in transiently transfected rat VSM cells. These cells were chosen because in this cell line the region containing the IRF-1 binding sites is essential for full activation of iNOS transcription after treatment with the proinflammatory cytokines IL-1, TNF-α, and IFN-γ (24). Sequence comparison of the rat and mouse promoters over the 99-bp region contained in the probe used in Fig. 1 shows only 6-bp changes, none of which is in the observed binding sites for IRF-1 and NF-κB (22, 23). The effects of the mutations on the ability of the promoter to respond to the mixture of proinflammatory cytokines are shown in Fig. 8. The mutations in site B and site C both reduce the activity of the iNOS promoter activity, to 15% and 30%, respectively, of the wild type sequence. This shows that a mutation in site C, which contacts IRF-1 in the dimeric complex, is important for activation of the promoter by cytokines. The m5 mutant, in which there is only binding of monomeric IRF-1 to DNA, was also tested for transcriptional activity. This was reduced to only 44% of the wild type construct.

**Domain of IRF-1 Required for Dimerization**—To determine
of A911 on the noncoding strand and A910 and A909 on the coding strand. Several pieces of evidence support this conclusion. Molecular footprinting techniques demonstrate the sequences present at this site. Fig. 2 implies that additional information specific to the sequence of this stretch of DNA allows the binding of a second molecule of IRF-1 to this region of the iNOS promoter in VSM, although immunoreactive IRF-2 is present constitutively in the nucleus of these cells.

**DISCUSSION**

This paper demonstrates that two molecules of IRF-1 can bind at closely adjacent areas of the iNOS enhancer. Binding of one molecule of IRF-1 is initially to a high affinity canonical IRF-1 binding site. This then allows a second molecule of IRF-1 to bind, which produces extension of DNA contacts in both 5′ and 3′ directions, with binding now in addition showing IRF-1 binding sites in the proximal portion of an adjacent potential IRF-1 site. Contacts within this area are made in the minor groove. Binding in this region is important for full cytokine activation of the iNOS gene within VSM cells.

**DNA Binding of IRF-1**—It has been demonstrated previously that IRF-1 only binds as a monomer to a single site, even when contained within a 64-bp oligonucleotide (8). In addition, in solution, IRF-1 is reported to exist only as a monomer (8). We also find that our preparations of IRF-1 only bind as a monomer to an oligonucleotide containing a single IRF-1 site (data not shown). Thus, the demonstration that IRF-1 can bind as a dimeric complex to the enhancer region of the iNOS promoter (Fig. 2) implies that additional information specific to the sequence of this stretch of DNA allows the binding of a second molecule of IRF-1. The data obtained from the three different molecular footprinting techniques demonstrate the sequences that are involved in binding of IRF-1 to this region of the iNOS enhancer (Fig. 5). Initial binding is to the 3′ segment of the consensus IRF-1 site termed site B, whereas more extensive contacts occur in the dimeric IRF-1-DNA complex, extending 5′ into site B and 3′ into the first part of IRF-1 consensus sequence site C (Fig. 5). The simplest model to account for these binding interactions is that of initial high affinity binding to site B, predominantly in the major groove. This binding then allows a second molecule of IRF-1 to bind to the complex. Some of the additional DNA contacts made after this binding are in the 5′ segment of site C and appear to be in the minor groove at this site. Several pieces of evidence support this conclusion. First, methylation interference studies show that the N-3 atom of A31 on the noncoding strand and A10 and A909 on the coding strand make close contact with bound IRF-1 (Fig. 4). The N-3 atom of A residues projects only into the minor groove of B form double-helical DNA. Second, when AT residues are replaced with IC base pairs in site C, binding of two molecules of IRF-1 to this region of DNA is still observed (Fig. 6). This mutation changes the spatial arrangement of the nucleosides in the major but not the minor groove of DNA and implies that contacts in site C in the dimeric IRF-1-DNA complex are predominantly in the minor groove. As discussed before, this conclusion is dependent on binding at site C having some sequence specificity. This binding to site C is shown to be sequence-specific because mutation of two residues within this site reduces IRF-1 binding (Fig. 7, lanes 1–4). In addition, when 5 bp of unrelated DNA sequence are inserted between the two sites B and C (m5) then dimeric binding is abolished, again arguing that binding within site C is sequence-dependent (Fig. 7, lanes m–p).

Most transcription factors bind DNA through contacts in the major groove of DNA (35). In B form DNA this groove can accommodate binding proteins more easily than the minor groove. In addition, the major groove pattern of hydrogen bond donors and acceptors is unique, whereas in the minor groove it is not possible to distinguish between AT and TA base pairs or GC or CG pairings. Thus, sequence-specific interactions that are required for transcription factor specificity are made more easily with contact to DNA in the major groove. However, a number of proteins do contact DNA in the minor groove in a sequence-specific manner. These include in prokaryotes the Escherichia coli integration host factor protein (36), and in eukaryotes the proteins lymphoid enhancer-binding factor-1 (34) and high mobility group protein-I(Y) (28). The three-dimensional structure of IRF-1 remains unknown, although NMR spectroscopic studies of the very closely related IRF-2 suggest that it contains helix-turn-helix-like domains with an overall folding topology similar to that of the DNA binding domains of heat shock transcription factors (25). The elucidation of the exact manner in which IRF-1 contacts DNA will require crystallographic data.

**Mechanisms of Dimeric IRF-1 Binding**—Cooperativity in binding is a general biological phenomenon that allows a small change in the concentration of an effector to produce a correspondingly larger change in the molecule to which the effector binds. We have termed the interaction between the bound molecules of IRF-1 on the iNOS enhancer as sequential, which is an extreme case of molecular cooperativity. Thus, in the absence of binding at site B in mutant mB, there is no detectable binding at any other site (Fig. 7, lanes m–p). This sequential binding prevents the formation of significant dimers of bound IRF-1 until a threshold level has been exceeded, increasing the specificity of IRF-1 DNA binding and thereby decreasing the overall number of proteins that can contact DNA in the minor groove in a sequence-specific manner. This binding to site C is shown to be sequence-dependent (Fig. 7, lanes m–p).

Given the relatively limited access to the stacked base pairs through the minor groove of DNA, binding at site C may also be influenced by factors that alter the conformation of DNA in this area. Thus, inhibition of IRF-1 binding may be achieved by binding of a factor that prevents widening of the DNA helix at this site.

We speculate that cooperative binding of the second molecule of IRF-1 to DNA is achieved mainly through protein-protein interaction. We were unable to dissociate the domain responsible for dimerization from that required for DNA binding (Table I). The COOH-terminal domain of IRF-1 was not required for dimerization; it is, however, necessary for transcriptional activation. It is of interest to know whether the closely related transcription factor IRF-2, a transcriptional repressor, binds in a fashion similar to the iNOS promoter (4). We have not been able to demonstrate IRF-2 binding to this area of the iNOS promoter in VSM, although immunoreactive IRF-2 is present constitutively in the nucleus of these cells.

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**Table I**

**Interferon Regulatory Factor-1 DNA Binding**

Using gel shift analysis, wild type (WT) and truncated IRF proteins containing the indicated amino acids were tested for their ability to form monomeric and dimeric complexes on the 99-bp fragment used in Fig. 1. +++, good binding; --, no binding.

| Mutation (amino acids present) | DNA binding |
|-------------------------------|-------------|
|                               | Monomer     | Dimer      |
| WT (1–329)                    | ++          | ++         |
| D3 (1–246)                    | ++          | ++         |
| D6 (1–132)                    | ++          | ++         |
| D7 (1–100)                    | ++          | ++         |
| D8 (20–329)                   | –           | –          |
The site C mutant mC shows reduction not only in dimer binding but also in formation of the monomeric IRF-1-DNA complex, despite the high affinity site B remaining intact (Fig. 7). This is also the case for the m5 mutant. We believe that this reflects an increased stability of the monomeric complex when the dimer is able to form, as predicted by the law of mass action. Increased affinity of a high affinity binding site in the presence of a lower affinity site has also been observed for the α repressor (37).

**Functional Significance of Dimeric IRF-1 Binding**—Why should IRF-1 binding in this fashion be necessary for transcriptional activation? Many transcription factors show oligomeric binding to DNA, either through noncovalent association of units as in the glucocorticoid receptor protein or through covalent concatenamerization of binding domains within one protein, such as in the class I zinc finger proteins (35). This probably maximizes protein-DNA interactions because one motif can only usually contact a few base pairs, so that additional DNA contacts are necessary to increase the stability of the protein-DNA complex. Indeed, in the mutants that reduce the ability of IRF-1 to bind at site C in the iNOS promoter (Fig. 7), there is a reduction in binding at the first site (site B), as discussed above.

As a test of the functional significance of this cooperative binding, a variety of mutant constructs were tested for their ability to direct cytokine-induced gene transcription, as assayed by reporter gene activity in VSM cells (Fig. 8). The most potent effect was seen with a 2-bp mutation in site B (mB), which reduced promoter activity to 15% of the wild type activity; this mutant does not bind detectable amounts of IRF-1 (Fig. 7, *lanes m–p*). This is in good agreement with experiments performed in mice with a targeted disruption of the IRF-1 gene, which do not induce iNOS (14). Mutant mC, which reduces dimeric IRF-1 binding, showed significant reduction in iNOS promoter activity, to 30% of wild type activity. Similarly, the m5 mutant in which dimeric binding is reduced also shows a reduction in promoter activity to 44% of the wild type (Fig. 8). This shows that the ability to form dimeric bound IRF-1 on the iNOS promoter is required for full cytokine activation of the gene.

How general is this unusual binding of two molecules of IRF-1 over two closely adjacent sites? A number of IFN-induced genes contain two or more binding sites in their promoter sequences. For example, the human 6–16 gene (38), the human IFN-β gene (9), and the human 2′-5′-oligo(A) synthetase gene (11) all contain closely adjacent IRF-1 binding sites. Therefore, the possibility exists that sequential DNA binding as described in this paper might be a more general phenomenon, although obviously further experiments would be required to establish this.

It is clear, however, that dimeric binding of IRF-1 in this fashion may not always be required for IRF-1 action. Thus, in the IFN-γ-inducible human IP-10 gene, there is only a single IRF-1 binding site (39). Enhancers show a considerable flexibility in their organization, with some requiring specific spatial relations between bound transcription factors, whereas others do not (27). The iNOS promoter only shows maximal transcriptional activity after stimulation with a combination of cytokines, which synergize one with another. This prevents significant iNOS transcription until a specific set of signals is received by the cell and limits the production of such a potentially toxic product, NO, only to those cells that are in immediate proximity to the focus of inflammation. We speculate that the dimeric binding of IRF-1 in the iNOS enhancer allows the specific assembly of an enhanceosome that contains other transcription factors, such as NF-κB, and is thus important in coordinating the effects of a number of cytokines to achieve regulated gene activation.

We have demonstrated here an unusual mechanism of IRF-1 transcription factor binding, which occurs initially in the major groove of DNA. Subsequently, a second molecule of IRF-1 can bind, which extends the contacts made by bound protein in both a 5′ and 3′ direction and contacts an adjacent IRF-1 site predominantly in the minor groove. This binding of IRF-1 is required for full functional activity of the iNOS gene, and, given the occurrence of multiple binding sites for IRF-1 in a number of other genes, might be a more general phenomenon.

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