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A Complex Element Regulates IFN-γ-Stimulated Monocyte Chemoattractant Protein-1 Gene Transcription

Anthony J. Valente, Jing-feng Xie, Margaret A. Abramova, Ulrich O. Wenzel, Hanna E. Abboud, and Dana T. Graves

Monocyte chemoattractant protein-1 (MCP-1) is induced in chronic osseous inflammation, and is temporally and spatially correlated with monocyte recruitment. We investigated the mechanism of MCP-1 regulation in a human osteoblastic cell line in response to IFN-γ, a potent mediator of the immune inflammatory response. Nuclear run-on and stability studies demonstrated that IFN-γ-stimulated MCP-1 transcription did not enhance mRNA stabilization. Using MCP-1 promoter/reporter gene constructs, we determined that IFN-γ-enhanced MCP-1 transcription is regulated by a 29-bp element located at −227 relative to the ATG start codon. This element contains a 13-bp CT-rich sequence (GCTTCCCTTTCCT) adjacent to a IFN-γ activation site (GAS). Since deletion of the CT sequence enhanced both the magnitude and duration of IFN-γ-stimulated, GAS-mediated transcription, we have termed it the IFN response-inhibitory sequence (IRIS). The combined IRIS/GAS sequence is highly conserved in mouse, rat, and bovine MCP-1 genes. In gel-shift assays, nuclear extracts from IFN-γ-stimulated osteoblastic cells formed two specific inducible bands with labeled IRIS/GAS DNA. Both bands were supershifted by anti-STAT1 Abs, but not by Abs to STAT2, p48(ISGF-3γ), IFN-regulatory factor-1, or IFN-regulatory factor-2. Formation of one of the bands required the presence of the IRIS motif. IRIS/GAS DNA also formed a number of specific complexes with constitutively expressed factors, none of which were affected by the above Abs. These studies establish a mechanism for IFN-γ-stimulated MCP-1 expression and identify a complex element that regulates MCP-1 gene transcription. The Journal of Immunology, 1998, 161: 3719–3728.
AP-1-like binding element has been reported to mediate fluid shear stress-induced regulation of MCP-1 expression in vascular endothelial cells (18, 19).

IFN-γ, a T and NK cell-derived cytokine, also appears to be important in bone metabolism. Although IFN-γ is thought to inhibit osteoclast formation (20), treatment of osteopetrotic patients with IFN-γ has been shown to be efficacious in treatment of the disease (21). IFN-γ also has the capacity to affect the tissue recruitment of mononuclear cells through the induction of chemokine genes. The induction of the C-C chemokine MCP-1 in response to IFN-γ stimulation has been described in a number of cell types, and typically IFN-γ rapidly and potently induces MCP-1 mRNA accumulation and MCP-1 protein production (22–25).

The secretion of IFN-γ and other lymphokines from activated T lymphocytes accumulating at sites of chronic osseous inflammation and in bone tumors may have a significant effect on the pathogenesis of these diseases. IFN-γ may act directly on local tissue to stimulate the production of MCP-1 and other proinflammatory mediators, and activate the monocyte-derived macrophages that are subsequently recruited. To investigate the mechanisms responsible for IFN-γ-regulated MCP-1 gene expression in bone cells, experiments were conducted in a human osteoblastic cell line. Our results indicate that IFN-γ rapidly increases MCP-1 mRNA and protein expression in these cells through a transcriptional mechanism, with little or no effect on MCP-1 mRNA stability. Furthermore, our functional studies suggest that transcription is regulated by a 29-bp complex element in the MCP-1 promoter located 227 bp from the ATG initiation codon, and does not require the response elements previously identified in the distal promoter.

Materials and Methods

Materials

DMEM and newborn bovine serum (NBS) were obtained from Life Technologies (Gaithersburg, MD). Abs to STAT1aβ, STAT2, p48/ISGF-3γ, IRF-1, and IRF-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Human αIFN-γ was purchased from R&D Systems (Minneapolis, MN). Oligonucleotides were synthesized by Center for Advanced DNA Technologies, University of Texas Health Science Center at San Antonio.

RNA isolation and Northern blot analysis of MG-63 osteoblastic cells

MG-63 human osteoblastic cells were purchased from American Type Culture Collection (Manassas, VA) (CRL 1427) and grown in DMEM supplemented with 10% NBS. To determine the effect of IFN-γ on MCP-1 mRNA stability, cells were incubated with the transcriptional inhibitor 5,6-dichloro-1β-ribofuranosylbenzimidazole (DRB) (20 μg/ml; Sigma, St. Louis, MO), IFN-γ (100 U/ml), or IFN-γ (100 U/ml) plus DRB (20 μg/ml) for 0, 0.5, 1, 2, 4, and 6 h. MCP-1 mRNA levels were examined by Northern blot analysis following a modification of a previously described protocol (26). A full-length human MCP-1 cDNA probe was generously provided by E. Appella (Laboratory of Cell Biology, National Cancer Institute, Bethesda, MD). Autoradiographs were examined by laser-scanning densitometry, and each band was normalized to the same blots hybridized with a CDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was generously provided by Dr. Judith A. Foster, Boston University (Boston, MA). The resulting data were expressed as the OD multiplied by the distance (OD × mm).

Isolation of nuclei and transcriptional analysis

The transcription rate of MCP-1 was determined by nuclear run-on analysis. Nuclei were isolated from the MG-63 human osteoblastic cell line incubated with IFN-γ (100 U/ml) for 6 h. Cells were washed twice with ice-cold Puck’s saline, lysed by incubation with cold lysis buffer (10 mM Tris-HCl, pH 7.6, 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40), and centrifuged at 500 × g for 10 min at 4°C. Isolated nuclei were stored at −80°C until used. The transcription assay was performed essentially as reported (27). pBluescript (Promega, Madison, WI), the vector for MCP-1 cDNA, was used as a negative control, and a plasmid containing GAPDH served as the positive control. Equal amounts of radiolabeled transcripts (2–4 × 10⁶ cpm/ml) were hybridized to nylon membranes containing 10 μg of denatured plasmid DNA per slot. Hybridization was conducted at 65°C for 72 h. The membranes were exposed to x-ray film at −80°C for 3 to 7 days. Each experiment was performed at least twice with similar results.

Sandwich ELISA for MCP-1

mAb hybridoma (E11) was donated by Dr. Edward Leonard (National Institutes of Health). Polyclonal rabbit anti-MCP-1 has been described previously (28). ELISAs were performed exactly as described (29) using a horseradish peroxidase color development kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The MCP-1 ELISAs were sensitive to ≤30 pg/ml.

MCP-1/luciferase plasmids

Heterologous promoter/luciferase constructs were prepared in the pG2L2–Basic luciferase vector (Promega). To facilitate subcloning, the Xhol/HindIII fragment from the multiple cloning site of an insert-negative (blue) colony of the T-cloning vector pCRII (Invitrogen, Carlsbad, CA) was inserted into the Xhol/HindIII site of pG2L. This transferred to pG2L the pair of nonpalindromic BstXI sites that flank the cloning site of pCRII. To prepare the −2910/+21, −248/+21, −227/+21, −214/+21, and −198/+21 MCP-1/luc plasmids (all numbers refer to nucleotide positions relative to the translation initiation codon) and are based on the nucleotide sequence for the human MCP-1 gene, GenBank Accession D26087, the MCP-1 DNA was first synthesized by the PCR. Forward oligonucleotide primers used were 5′-GAG CAT TGA CCC TTC TTT TTG-3′ for −2910/+21; 5′-AGT GTC TCG TCC TGA CCC-3′ for −248/+21; 5′-GCT TCC CTT TAC TTC CTG G-3′ for −227/+21; 5′-ACT TCC TGG AAA TTC A-3′ for −214/+21; and 5′-CAG GAT GCT GCA TTT G-3′ for −198/+21. The oligonucleotide 5′-AAG GGC GGC AGA GAC TTG TAT CAT-3′, which corresponds to the reverse and complemented first 21 nucleotides of the MCP-1 coding sequence, was used as the common reverse primer in all reactions. Bacteriophage DNA from a clone containing the human MCP-1 gene promoter region was used as a template. Products from PCR were cloned directly into pCRII (Invitrogen). Plasmid DNA from positive clones was digested with BstXI and the inserts were gel purified and cloned into the corresponding nonpalindromic BstXI sites of the modified pG2L vector. Correct nucleotide sequence and orientation with respect to the luciferase gene were confirmed by DNA sequencing using Sequenase (United States Biochemical, Cleveland, OH). Since the −2910/+21 MCP-1 DNA contains an internal BstXI site, the insert was excised from pCRII with BamHI and NotI, blunt ended with DNA polymerase I, ligated with BstXI hemiphosphorylated linkers (Invitrogen), and cloned into the modified pG2L vector, as above. Orientation and identification were confirmed by partial DNA sequencing. For transfection studies, plasmid DNA was purified using a commercial anion-exchange protocol (Qiagen, Chatsworth, CA).

Cell transfection and luciferase assay

MG-63 human osteoblastic cells (6 × 10⁵ cells/well) were plated in 12-well plates and incubated overnight. The medium was replaced with 1.5 ml of fresh complete medium, and transfection was conducted 4 to 6 h later. For transfection, 5 μg of MCP-1/luciferase plasmids was mixed with 0.5 μg of pCMV-RL, an internal control renilla luciferase expression vector (Promega), and 25 μg of pUC18 as carrier DNA, in a final volume of 0.5 ml of 0.25 M CaCl₂. This was added dropwise to 500 μl of 2× HeBS (0.275 M NaCl, 8.5 mM KCl, 1.5 mM Na₂HPO₄, 400 mM glucose, and 0.166 M HEPES, pH 6.9) with constant stirring, and the mixture was incubated at room temperature for 30 min. A total of 150 μl of transfection mix was added dropwise to the cultured cells incubated overnight at 37°C. The medium was removed, and 2 ml of fresh medium was added, and incubation continued for 48 h. To determine the effect of IFN-γ stimulation, the cells were rinsed once with DMEM and duplicate wells were incubated with human αIFN-γ (1000 U/ml) in either 1 ml of DMEM/1% NBS or 1 ml of the medium without IFN-γ (unstimulated control). Following incubation for the indicated times, the cells were rinsed once in PBS, then scraped into 1 ml of 1× passive lysis buffer supplied with the Dual-Luciferase Reporter Assay System (Promega). The extracts were vortexed for 10 s, incubated at room temperature for 10 min, then centrifuged at 12,000 × g for 5 min. Twenty microliters of the supernatant were assayed for both firefly and renilla luciferase activity using the dual-reporter assay system indicated above and a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Firefly luciferase activity was normalized to the renilla luciferase activity, and the fold stimulation was calculated as the ratio of the IFN-γ–stimulated to unstimulated values obtained at each time point.
Nuclear extracts and electrophoretic mobility shift assay (EMSA)

MG-63 cells were plated in 100-mm dishes and grown in complete medium until just confluent. The cells were rinsed with DMEM and then incubated with 6 ml DMEM 1% NBS, with or without 1000 U/ml IFN-γ. At the indicated times, the cells were rinsed twice with ice-cold PBS, and nuclear extracts were prepared as described by Schreiber et al. (30), except that in addition to 1 mM PMSF and 1 mM DTT, all cold extraction buffers contained 1 mM sodium orthovanadate to inhibit endogenous tyrosine phosphatase activity. Protein concentrations were determined by the Bradford technique, and the extracts were stored at −70°C. Complementary oligonucleotides corresponding to the MCP-1 IRIS/GAS (5′-GCT TCC CT TCC TAC TCT CTA C-3′), truncated IRIS/GAS sequence IRIS/GAS (5′-GCT TCC CT TCC TAC TCT C-3′), the MCP-1 IRIS (5′-GCT TCC CT TCC TAC TCT C-3′), and the MCP-1 GAS core sequence (5′-ACT TCC TGG AAA T-3′) were annealed and end labeled with [γ-32P]ATP and T4 polynucleotide kinase. For the assays, 4 μg of the nuclear extract was incubated for 20 min at ambient temperature with 7.5 to 10 × 10^5 cpm of the labeled DNA probe in 20 μl of binding buffer containing 10 mM Tris-Cl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 1 μg/μl BSA, and 2 μg poly(dI-dC) (Pharmacia Bio- tech, Piscataway, NJ). Samples were separated on 10% polyacrylamide gels at 200 V in 1× buffer (25 mM Tris, 190 mM glycine, and 1 mM EDTA, pH 8.3). Competition assays were conducted in the same manner, except the above reaction mixture was preincubated with competitor DNA for 15 min at 0°C before addition of the labeled probe. For supershift assays, the binding reaction was conducted as above, except that up to 2 μl Ab was added to the reaction mixture following the 20-min incubation, and the incubation continued for an additional 45 min at room temperature before separation on PAGE.

Results

MCP-1 expression in IFN-γ-stimulated osteoblastic cells is regulated at the transcriptional level

Our initial studies indicated that treatment of normal and MG-63 human osteoblastic cells with IFN-γ resulted in a rapid increase in MCP-1 mRNA levels. Since IFN-γ-induced stimulation of chemokine mRNA levels has been shown in some cell types to be regulated at the posttranscriptional level (31), mRNA stability studies were conducted in MG-63 cells. Cells were incubated with either the transcriptional inhibitor DRB, IFN-γ, or IFN-γ plus DRB. Total cell RNA was isolated, and Northern blot analysis was performed. As shown in Figure 1A, although the basal levels of MCP-1 mRNA are high in MG-63 cells, IFN-γ stimulation resulted in a marked increase in DRB-untreated cells compared with the level observed at the zero time point. The increase was observed within 1 h and increased further with longer times of incubation. In DRB-treated cells, on the other hand, IFN-γ stimulation did not increase MCP-1 mRNA levels. Comparison of mRNA levels in DRB-treated cells with or without IFN-γ showed that IFN-γ did not enhance MCP-1 mRNA stability. Under both conditions, the MCP-1 mRNA t1/2 was approximately 3 to 4 h (Fig. 1B).

Nuclear run-on assays were performed to examine the effect of IFN-γ on MCP-1 transcription. Nuclei were isolated from MG-63 cells that had been incubated with or without IFN-γ for 6 h. The nuclei were incubated with [α-32P]UTP, and RNA was isolated and allowed to hybridize with plasmid DNA containing either MCP-1 cDNA or positive or negative control cDNA. As shown in Figure 2, the resulting autoradiogram indicated that IFN-γ increased transcription of mRNA. Results of hybridization with GAPDH remained the same with or without IFN-γ treatment, demonstrating equal loading of samples. These results clearly show that IFN-γ modulates MCP-1 mRNA levels in human osteoblastic cells by increasing the rate of transcription rather than stabilization of preexisting message.

The increase in MCP-1 transcription in response to IFN-γ was also accompanied by an increase in MCP-1 protein secretion (Table I). Although the MG-63 cells secreted a high level of MCP-1 under basal conditions, consistent with the observed high basal level of MCP-1 mRNA expression, addition of IFN-γ increased MCP-1 production in a dose-dependent manner, with an increase

FIGURE 1. The effect of IFN-γ on MCP-1 mRNA. A, MG-63 cells were incubated with IFN-γ (100 U/ml), DRB (20 μg/ml), or IFN-γ + DRB for 0.5, 1, 2, 4, and 6 h, as described in Materials and Methods. Total cellular RNA was isolated, and Northern blot analysis was performed. Autoradiogram from hybridization with MCP-1 cDNA probe. Under the experimental conditions used, MCP-1 mRNA levels did not change in unstimulated cells over a 6-h period (data not shown); therefore, only total RNA from unstimulated cells at zero time point was analyzed. B, The hybridization signals from the DRB and IFN-γ + DRB treatments obtained from A were quantified by using densitometric scanning and normalized to the same blots hybridized with a cDNA probe for GAPDH. The t1/2 for each condition was calculated by linear regression from semilog plots of the percentage of mRNA remaining vs time.

FIGURE 2. Nuclear run-on analysis of MCP-1 transcription. MG-63 cells were incubated with or without (100 U/ml) IFN-γ for 6 h. Nuclei from control and IFN-γ-stimulated cells were isolated, and 32P-labeled nuclear run-on products were hybridized to denatured plasmid DNAs slot blotted on nylon membrane. Bands represent hybridization with MCP-1 (row 1), GAPDH (row 2), and pBluescript vector without insert (row 3). The result is representative of two separate experiments.
similar in magnitude to that which we have observed previously with IL-1 and TNF-α stimulation in these cells (32). Thus, treatment with IFN-γ increases both the transcription and translation of the MCP-1 gene in MG-63 cells.

Identification of functional response elements in IFN-γ-mediated transcription

The rapid transcriptional response to IFN-γ has been shown in several genes to be mediated through a transcriptional element termed the GAS. The consensus sequence (TTCCNNNA) has been defined for the GAS (33–35), and inspection of the proximal promoter region of MCP-1 identified a potential GAS element at position −214 relative to the ATG start codon (Fig. 3A). The core sequence of this element (TTCTGGAA) resembles closely the symmetrical dyad sequence defined as the binding site for the STAT family of transcription factors (with the exception of STAT2). Adjacent and 5′ to this site in the MCP-1 promoter is a CT-rich element (GCTTCCTCTTACC) that shows some homology to the consensus sequence for the IFN-stimulated response element (ISRE) (36) (Fig. 3B), which has been shown to mediate transcriptional responses to both IFN-γ and IFN-α/β.

Since mRNA stability and nuclear run-on experiments clearly demonstrated that IFN-γ rapidly induced MCP-1 through a transcriptional mechanism, transfection studies were conducted with a deletion series of MCP-1 promoter/luciferase plasmids to identify and characterize the IFN-γ-responsive elements. As shown in Figure 4, IFN-γ stimulation of MG-63 cells transfected with construct “a” (−2910/+21), the largest construct used in these studies, resulted in only a 1.7-fold increase in luciferase activity compared with unstimulated controls. Construct “a” was prepared to include the xB and other regulatory elements located in the distal enhancer region that recently have been shown to regulate MCP-1 transcription in response to IL-1β and TNF-α (9, 11). IFN-γ stimulation of cells transfected with construct “b” (−248/+21) and “c” (−227/+21), however, in which the functional xB site and the majority of the 5′ flanking sequence were eliminated, resulted in an approximately threefold increase in luciferase activity. This indicated that the distal enhancer elements in the MCP-1 promoter do not appear to be obligatory for the mediation of the IFN-γ response in MG-63 cells, and that the positive response element resided within the first 227 bp of the gene flanking the ATG start codon. Construct “c” (−227/+21) included the promoter sequence up to the start of the CT element. Since this element shows some homology to the consensus sequence for the ISRE (Fig. 3C) and is adjacent to the potential GAS element, it was possible that either or both elements may be required for mediating the IFN-γ response. Therefore, to investigate the role of the GAS element alone in mediating the response to IFN-γ, we prepared construct “d” (−214/+21), which terminated at the 5′ end of the GAS element and eliminated the CT element. Unexpectedly, this construct produced the greatest increase in the reporter gene activity observed in these experiments (≥10-fold). Thus, the CT element appears to inhibit the response to IFN-γ mediated by the GAS element. When the GAS element was deleted (construct “e,” −198/+21), responsiveness to IFN-γ was lost completely. Therefore, these experiments indicate that in the MG-63 osteoblastic cell line, the rapid IFN-γ-induced increase in transcription of MCP-1 appears to be mediated entirely through the GAS element located at position −214 to −198, whereas the adjacent CT element appears to negatively regulate this GAS-mediated response. We have termed this CT element the IFN-γ-responsive-inhibitory sequence (IRIS), which describes its observed functional activity.

The above results were substantiated by the time-course experiments shown in Figure 5. MG-63 cells transiently transfected with construct −214/+21 (IRIS+GAS) showed a substantial increase in luciferase activity following 2 h of stimulation with IFN-γ, and this increased further between 4 and 8 h. Cells transfected with the construct −227/+21 (IRIS+GAS), on the other hand, showed only a moderate increase at 2 h and did not increase further beyond this time point. At the 8-h time point, the response was approximately 25% of that observed for construct −214/+21. Thus, the IRIS appears to inhibit both the magnitude and duration of the IFN-γ-stimulated transcriptional response.

Table I. IFN-γ stimulates release of MCP-1 from human osteoblastic cells

<table>
<thead>
<tr>
<th>IFN-γ added (U/ml)</th>
<th>MCP-1 Secreted (ng/ml) (SEM), n = 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>141.0 (26.9)</td>
</tr>
<tr>
<td>1</td>
<td>185.7 (30.2)</td>
</tr>
<tr>
<td>10</td>
<td>203.4 (6.4)</td>
</tr>
<tr>
<td>100</td>
<td>348.3 (10.2)</td>
</tr>
<tr>
<td>1000</td>
<td>341.5 (39.2)</td>
</tr>
</tbody>
</table>

* Induction of MCP-1 secretion by IFN-γ was carried out in the MG-63 cell line. Cells were incubated with or without 100 U/ml IFN-γ for 24 h under serum-free conditions, and the supernatants were collected and then assayed for MCP-1 by ELISA. Each value represents the mean ± SEM (n = 6).
Evolutionary conservation of the IRIS/GAS element

Nucleotide alignments were conducted using the LALIGN program to identify potential homologous IRIS/GAS elements in the promoter regions of other chemokine genes. As shown in Figure 3B, the overall IRIS/GAS element is well conserved with respect to both sequence (92% bovine vs human; 79% murine and rat vs human) and location in the MCP-1 promoters of other species (9, 10).

<table>
<thead>
<tr>
<th>MCP-1 Promoter Construct</th>
<th>Stimulation with IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fold increase (SEM) n=3</td>
</tr>
<tr>
<td>a) -2910</td>
<td>1.7 (0.4)</td>
</tr>
<tr>
<td>b) -246</td>
<td>3.2 (0.8)</td>
</tr>
<tr>
<td>c) -227</td>
<td>3.3 (0.4)</td>
</tr>
<tr>
<td>d) -214</td>
<td>10.9 (1.8)</td>
</tr>
<tr>
<td>e) -198</td>
<td>1.1 (0.1)</td>
</tr>
<tr>
<td>f) pGL2</td>
<td>0.8 (0.3)</td>
</tr>
</tbody>
</table>

FIGURE 4. Identification of the IFN-γ-responsive region in the human MCP-1 promoter. A schematic diagram indicating the location of the deletion constructs in the MCP-1 gene 5’ flanking region and the resulting responsiveness to IFN-γ in transfected MG-63 cells. Cells were transfected with the MCP-1/luciferase constructs and the CMV-renilla plasmid (transfection efficiency control), as described in Materials and Methods, incubated for 3 days, then stimulated either with 1000 U/ml human rIFN-γ or with medium (unstimulated control) for 4 h. Luciferase activity was determined and normalized to renilla activity. No luciferase activity measured exceeded 10% of the full capacity of the assay reagent. Results are expressed as fold stimulation of luciferase activity and represent the mean of three independent experiments conducted in duplicate ± SEM.

FIGURE 5. Time course of IFN-γ stimulation of MCP-1 promoter/luciferase activity in transfected MG-63 cells. Duplicate cultures of MG-63 cells were transfected with the indicated constructs, as described, and stimulated with 1000 U/ml IFN-γ or with medium (unstimulated control) for 2, 4, and 8 h. Luciferase activity was then determined and normalized to renilla activity, as before. The results are then expressed as fold stimulation of luciferase activity and represent the mean of three independent experiments conducted in duplicate ± SEM.

FIGURE 6. Constitutively expressed and inducible factors binding the MCP-1 IRIS/GAS and GAS. MG-63 cells were either untreated or treated with 1000 U/ml IFN-γ for 30 min before extraction of nuclear proteins. A, EMSA was conducted with 5 μg nuclear extract and 32P-labeled MCP-1 IRIS/GAS, or B, GAS, or C, IRIS/GAS and GAS DNA probes, as described in Materials and Methods. The complexes formed between the constitutively expressed factors and labeled IRIS/GAS (C) and the IFN-γ-induced complexes formed with IRIS/GAS and GAS (A and B) are indicated.
IFN-γ induces binding of nuclear factors to the MCP-1 IRIS/GAS sequences

Since the above studies demonstrated that deletion of the IRIS moiety from the IRIS/GAS element significantly altered transcripational activity of MCP-1, EMSA studies were conducted to identify potential nuclear factors affected by this deletion (Fig. 6). Two oligonucleotide probes were constructed: one contained the IRIS/GAS sequence and the other contained the core GAS element alone (Fig. 3C). EMSA was conducted with nuclear proteins extracted from MG-63 cells treated with or without IFN-γ (1000 U/ml) for 30 min. Several DNA-protein complexes were formed between the IRIS/GAS probe and the nuclear extract of unstimulated MG-63 cells (Fig. 6A, bands C). Stimulation with IFN-γ resulted in the induction of two major new bands (Fig. 6A, bands A and B), in addition to the bands that were observed under basal conditions. However, using an oligonucleotide probe corresponding to the core GAS element alone, only a single band was observed corresponding to the faster migrating of the two major inducible bands that were observed with the IRIS/GAS DNA probe (Fig. 6, B and C). No DNA-protein complexes were formed between the labeled GAS probe and nuclear extract from unstimulated MG-63 cells (Fig. 6C). Thus, the IRIS moiety flanking the GAS element appears to be necessary for binding both the constitutive factors and the inducible factor with lower mobility (band B).

To determine the specificity of interaction between DNA-binding proteins and the oligonucleotide probes, competition studies were conducted (Fig. 7). The interaction between nuclear extracts of IFN-γ-stimulated cells and the labeled IRIS/GAS probe was competed with increasing concentrations of unlabeled IRIS/GAS and GAS DNA. As shown in Figure 7, the unlabeled IRIS/GAS probe competed for the two inducible factors (A and B) and six (C1-C6) of the DNA-protein complexes observed in unstimulated cells (complex C1 is obscured by band A in Fig. 7, but is clearly seen in Fig. 8). This competitive inhibition was dose dependent. The unlabeled GAS probe competed for the inducible factors, inhibiting the formation of both fast and slow migrating DNA-protein complexes (bands A and B, respectively). The unlabeled GAS probe did not compete with any of the DNA-binding factors that were constitutively expressed. Competition of the slower migrating inducible complex by the unlabeled GAS probe was surprising, since a corresponding band was not observed when the labeled GAS probe was incubated with nuclear extracts from IFN-γ-stimulated cells.

To determine whether the IRIS moiety alone is sufficient for the binding of the constitutive factors, EMSA assay was conducted using a labeled DNA probe corresponding to the sequence of the IRIS shown in Figure 3C, and nuclear extracts from unstimulated MG-63 cells. No DNA-protein complexes were observed in these experiments (data not shown), suggesting that all or part of the GAS element is also required. This was further investigated in EMSA assays using labeled IRIS/GAS DNA as the probe and competing with unlabeled DNA corresponding to IRIS/GAS, IRIS/ΔGAS, and IRIS sequences (Fig. 3C). As shown in Figure 8, deletion of the 3’ domain of the GAS element from the IRIS/GAS probe (IRIS/ΔGAS) was sufficient to reduce its ability to inhibit formation of the DNA-protein complexes. Furthermore, deletion of the entire GAS element to result in a probe consisting of the
IRIS alone resulted in a complete loss of competitive activity. Thus, these data clearly indicate that the binding of both the IRIS and GAS moieties of the IRIS/GAS element are required for the binding of the constitutively expressed factors.

A number of factors have been identified that modulate the transcription of IFN-responsive genes (33–35, 41–45). To characterize the binding proteins forming the DNA-protein complexes observed on EMSA, supershift assays were conducted with monospecific Abs to STAT1α/β, STAT2, p48(ISGF-3γ), IRF-1, and IRF-2 (Fig. 9). Ab to STAT1α/β appeared to cause a supershift of both IFN-γ-induced bands that formed with the labeled IRIS/GAS probe. In contrast, Abs to STAT2, p48(ISGF-3γ), IRF-1, and IRF-2 had no effect on any of the DNA-protein bands formed with the unstimulated IFN-γ-stimulated MG-63 nuclear extracts. Supershift EMSA was also conducted to identify the proteins in the DNA-protein complexes formed between IFN-γ-stimulated MG-63 nuclear extract and the GAS probe. As expected, the single band was supershifted by the STAT1α/β Ab, but not by any of the other Abs used (data not shown).

These data clearly suggest that the induction of MCP-1 transcription in response to IFN-γ treatment is mediated by the binding of activated STAT1 factors to the MCP-1 GAS element. However, in the EMSA studies described above, the nuclear extracts were prepared from MG-63 cells that had been stimulated with IFN-γ for 30 min, and the functional studies with the MCP-1 (−214/+21) construct (Fig. 5) clearly indicated that the reporter gene activity was actively being produced at later time points. Since a number of the transcription factors regulating IFN responsiveness are induced with time (44–46), it is possible that additional factors may have been induced during this period and positively regulated the enhanced transcription through the GAS element. Therefore, EMSA was conducted using the

![FIGURE 9. Supershift EMSA to identify specific DNA-binding proteins in IFN-γ-stimulated MG-63 nuclear extracts. Nuclear extracts were prepared from MG-63 cells stimulated with 1000 U/ml IFN-γ for 30 min. Labeled IRIS/GAS DNA probe was incubated with 5 μg of the nuclear extract, as described in Materials and Methods, and then incubated with 2 μl of the indicated Ab for 45 min before separation by PAGE.](http://www.jimmunol.org/)

![FIGURE 10. Influence of incubation time on constitutive and IFN-γ-induced IRIS/GAS-binding proteins in MG-63 cells. Nuclear extracts were prepared from MG-63 cells either untreated or treated with 1000 U/ml IFN-γ for 0.5, 2, 4, and 8 h. EMSA was conducted with 5 μg of nuclear extract and 32P-labeled MCP-1 IRIS/GAS DNA probe, as described in Materials and Methods.](http://www.jimmunol.org/)

**Discussion**

In this study, we show that IFN-γ induces MCP-1 gene expression in osteoblastic cells through a mechanism of enhanced transcription. This is supported by nuclear run-on experiments and increased reporter gene activity in transiently transfected MG-63 cells. The rapid induction of MCP-1 transcription by IFN-γ is mediated by a cis-regulatory element located between −214 and −198 of the 5′ flanking sequence. This contains a consensus GAS sequence that has been shown to confer rapid IFN-γ responsiveness in a number of genes. Deletion of this site from the MCP-1 promoter completely eliminated IFN-γ-stimulated activity, indicating that GAS is the principal element mediating the IFN-γ-induced MCP-1 gene transcription in osteoblastic cells.

A regulatory role for nucleotides immediately 5′ to the GAS was also identified. The greatest induction of transcriptional activity was observed with the deletion construct (−214/+21), which terminated at the 5′ end of the GAS element. Addition of the 13 5′ flanking nucleotides of the promoter (the IRIS moiety) resulted in a major reduction, but not elimination, in both the magnitude and duration of the IFN-γ-stimulated MCP-1 transcriptional response. Thus, the increased transcription observed in the osteoblastic cells
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Negative regulatory sequences that modulate responsiveness to the IFNs have been reported previously in the murine IFN-α11 gene and the 9/27 gene (47, 48). In both cases, however, the sequences appear to modulate an ISRE-mediated rather than a GAS-mediated IFN response, and are probably unrelated to the negative regulatory sequence that we have described in this work. Negative regulation may also be mediated by the transcriptional factor IRF-2, which acts as a repressor of the IFN-γ response and competes with the positive transcription factor IRF-1 for binding to the ISRE (43–45). However, our supershift EMSA data indicated that neither of these factors appears to bind the IRIS/GAS DNA. This is despite the fact that both IRF-1 and IRF-2 can be identified in the nuclear extract of MG-63 cells (data not shown). The lack of binding of IRF-2 suggests that the IRIS moiety of the MCP-1 IFN response element and the ISRE described in other IFN-responsive genes, although showing some degree of sequence homology, are structurally and functionally distinct. The IRIS may represent a novel regulatory sequence in a complex response element that provides a negative regulatory pathway for IFN-γ-induced gene transcription.

The complex IRIS/GAS element in the human MCP-1 promoter is highly conserved in both sequence and location in the murine, rat, and bovine MCP-1 genes. The sequence homology in the IRIS/GAS between bovine and human/mouse/rat and human is 92 and 79%, respectively. The conservation of this sequence supports our observations that it performs an important physiologic role in the regulation of MCP-1 transcription. Using a nucleotide alignment program, we were unable to identify this same combination of IRIS/GAS sequences in the known promoter sequences of IL-8, MCP-3, MIP-1β, or RANTES. This observation reinforces the concept that among the chemokine genes, MCP-1 may be induced by IFN-γ through a unique regulatory pathway. This is particularly interesting, since the functional evidence to date seems to indicate that chemokine genes may be regulated by different mechanisms in response to IFN-γ. IL-8, for instance, appears to be negatively regulated by IFN-γ in a number of cell types (49, 50), but in those cases in which it has been reported to stimulate IL-8 expression, this has been shown to result from either mRNA stabilization or increased transcription mediated through a synergistic induction of AP-1 and nuclear factor-κB (31, 51). IP-10, another member of the C-X-C subfamily of chemokines, is positively regulated by IFN-γ. The increase in IP-10 expression has been shown to be mediated through a consensus ISRE sequence located approximately 235 bases upstream of the transcription start site (52). In addition, there is evidence that for full response to IFN-γ, one or more of the κB binding sites located downstream of the ISRE may also be required (52). IFN-γ-induced transcription of the chemokine gene mig, on the other hand, appears to be mediated by a STAT1-containing factor binding a complex and imperfect palindromic sequence that bears partial homology to the GAS sequence (53, 54). Our studies reported in this work indicate that in osteoblastic cells, the positive induction of MCP-1 by IFN-γ appears to require only the discrete GAS element located between −214 and −198 of the proximal promoter, since the deletion of this element resulted in a complete loss of responsiveness to the cytokine. The positive response through this element, however, is partially repressed by the sequence adjacent to it.

Nuclear extracts from IFN-γ-treated cells formed two specific inducible DNA-protein complexes with the IRIS/GAS DNA probe. Their formation was inhibited by competition with both unlabeled IRIS/GAS and GAS DNA, indicating that both bands were dependent on the binding of nuclear factors to the GAS sequence. Supershift assay indicated that both bands contained the STAT1 protein, but not STAT2, p48(IGSF-3-γ), IRF-1, or IRF-2. However, when the EMSA was conducted using labeled MCP-1 GAS as the probe, only the faster migrating of the inducible bands (band A) was observed. Taken together, these data indicate that the rapid IFN-γ induction of MCP-1 transcription is mediated by STAT1 binding to a well-defined GAS element, as has been shown in IFN-γ induction of other genes. However, the formation of an additional slower inducible band on EMSA also suggests that a second larger complex may be formed between the IRIS/GAS element and DNA-binding factors. This must include the activated STAT1 factor, since the band was supershifted with specific anti-STAT1(α/β) Ab. It may also include other factors that bind to both STAT1 factors and the adjacent IRIS moiety.

This pattern of DNA-protein complex formation observed with the MCP-1 IRIS/GAS is analogous to the multiple DNA-protein complexes formed between the IFN-γ-responsive region of the gene for a high affinity receptor for IgG (FcγRI) and nuclear extracts of IFN-γ-treated monocytic cells (55, 56). There, like the MCP-1 IRIS/GAS, the formation of two DNA-protein complexes on EMSA was absolutely dependent on the presence of an intact GAS element located in the 3′ domain of the DNA probe, whereas the formation of a second slower migrating complex required in addition the presence of the 5′ domain. However, the influence of these 5′ sequences on function is opposite. The 5′ domain of the FcγRI response element was shown to enhance GAS-mediated transcriptional enhancement in response to IFN-γ (56), whereas as we have shown in this work, the presence of the 5′ IRIS moiety in the MCP-1 IRIS/GAS results in inhibition of induced transcription.

Nuclear extracts from unstimulated osteoblastic cells formed several specific DNA-protein complexes with the IRIS/GAS DNA. These could be competitively inhibited with unlabeled IRIS/GAS, but not by GAS or IRIS alone. In addition, they were not observed on EMSA with labeled GAS or IRIS probes. Thus, their formation required the presence of both IRIS and GAS sequences. We did not identify these constitutively expressed factors, but Abs to STAT1, STAT2, p48(IGSF-3-γ), IRF-1, and IRF-2 all failed to supershift or inhibit their complex formation on EMSA, making it unlikely they are immunologically related to these known factors. Since transcriptional studies indicated that the IRIS moiety of the IRIS/GAS element inhibits IFN-γ-stimulated MCP-1 expression, it is possible that one or more of these DNA-binding proteins is responsible for inhibiting the GAS-mediated transcription through constitutive occupation of the IRIS/GAS binding site. This could represent a mechanism for limiting MCP-1 expression under basal conditions or in response to inflammatory stimuli.

Physiologically, the diverse regulatory pathways displayed by the chemokine genes in response to IFN-γ may reflect their particular roles in IFN-γ-mediated immune events. Temporal patterns of chemokine expression have been noted in a number of pathophysiologic states of immune etiology, including tissue rejection and wound healing (57, 58). Differential regulation of chemokine genes by IFN-γ, which results in the ordered trafficking and activation of immune cells, may be an important component of these immune processes. An understanding of the molecular mechanisms that mediate IFN-γ responsiveness will help clarify the role of the chemokines in immune and inflammatory events.

Note added in proof. During submission of this paper, Zhou et al. (59) showed IFN-γ-stimulated MCP-1 transcription in astrocytoma cells through the same GAS promoter element described here.
References


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