5' Regulatory Region of a Novel Cytokine Gene Mediates Selective Activation by Interferon γ

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Summary
A newly described member of the platelet factor 4 family of cytokine genes, mig, is selectively induced by interferon γ (IFN-γ), and not IFN-α, in the mouse macrophage-like cell line RAW 264.7. Treatment of RAW 264.7 cells with IFN-γ activated mig gene transcription as determined by nuclear run-on assays. mig genomic clones were isolated, and constructs containing genomic fragments that included the mig promoter region and the CAT reporter gene were prepared. In RAW 264.7 cells transfected with these constructs, CAT activity was found to be selectively induced by IFN-γ. A 278-bp genomic fragment containing 235 nucleotides 5' of the transcription start site was sufficient for IFN-γ-selective induction of CAT activity. Analysis of 5' deletion mutants localized a region essential for activation by IFN-γ to within 64 nucleotides extending from −235 to −172. A genomic fragment containing this sequence was capable of conferring IFN-γ inducibility to constructs with a heterologous promoter.

IFN-γ has major effects on the state of activation and differentiation of cells participating in the immune response, particularly on cells of the monocyte/macrophage lineage (1). In addition, IFN-γ has antiviral and antiproliferative properties, some of which are shared with IFN-α and IFN-β (2). It has been recognized that many of the cellular responses to the IFNs require new RNA and protein synthesis (3) and that IFN-γ induces the expression of unique mRNAs and proteins in addition to those induced by IFN-α and IFN-β (4, 5). The study of gene expression in response to the IFNs is, therefore, of particular importance in understanding their mechanisms of action.

The investigation of transcription regulated by IFN-α, IFN-β, and IFN-γ has led to the identification of IFN-responsive cis elements (6, 7) and, more recently, to the identification of IFN-responsive DNA binding factors (8, 9). However, neither the cis elements nor the binding factors characterized thus far provide an explanation for the preferential regulation of certain genes by IFN-γ. In this report, we describe the isolation and initial characterization of a genomic sequence that confers selective transcriptional activation by IFN-γ.

Materials and Methods

Materials. Murine rIFN-γ was obtained from Amgen Biologics, Thousand Oaks, CA, and had a sp act of ≥10^7 U/mg. IFN-α was a purified natural product with a sp act of 1.4 × 10^6 IU/mg and was purchased from Lee Biomolecular, San Diego, CA. Murine TNF-α (4 × 10^7 U/mg) was obtained from Genzyme, Boston, MA. The plasmid pUMSVOCAT was the generous gift of Dr. K. Kurachi, University of Michigan, Ann Arbor. The c-fos and c-myc genomic fragments inserted into pGEM-2 (Promega-Biotec, Madison, WI) were prepared from plasmids obtained from the American Type Culture Collection, Rockville, MD, and kindly provided by M. McLane and D. Nathans, Johns Hopkins University School of Medicine. The plasmid p268CATAM-C was the generous gift of B. Christy and D. Nathans, Johns Hopkins University School of Medicine.

Cells and Cell Culture. RAW 264.7 cells were obtained from the American Type Culture Collection, and were maintained in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine.

Northern Analysis. Total RNA was isolated from RAW 264.7 cells by the guanidinium/acid phenol method and was resolved by denaturing formaldehyde agarose gel electrophoresis followed by blotting and hybridization to 32P-labeled cDNA probes prepared using random oligonucleotide primers with reagents and protocols provided by Amersham Corp., Arlington Heights, IL.

Nuclear Run-On. Nuclei were isolated from RAW 264.7 cells according to the method of Marzluff et al. (10) after a 3-h incubation in medium supplemented with IFN-γ (500 U/ml), or control medium, in the presence and absence of cycloheximide (10 μg/ml). The labeled run-on transcripts were prepared and hybridized with denatured linearized plasmid DNA (10 μg/slot) immobilized on nitrocellulose using equal amounts (cpm) of radiolabeled RNA for each filter. After hybridization at 65°C for 40 h, the filters were washed (2x SSC at 65°C for 2 h) and treated with RNase A, followed by autoradiography at −70°C with an enhancing screen.

Isolation of mig Genomic Clones. A BALB/c genomic library (Clontech, Palo Alto, CA) was screened by plaque hybridization.
with a \[^{32}P\]-labeled mig cDNA fragment using conditions for hybridization and washing as described (11).

**Plasmid Construction.** A 1.6-kb EcoRI genomic fragment containing the mig promoter region was cloned into pBluescript phagemid (Stratagene, La Jolla, CA). A fragment containing 43 nucleotides 3' of the transcription start site and 1,117 nucleotides of 5' flanking sequence was prepared by deletion using exonuclease III and the insert was ligated into the Smal site of pUMSVOCAT. Two plasmids, p(−704/+43)CAT and p(−358/+43)CAT, with major 5' deletions of the genomic fragment were made by digestion of p(−1117/+43)CAT with EcoRV or HindIII. A series of 5' deletion mutants was prepared from p(−358/+43)CAT using exonuclease III. The mig genomic fragment extending from nucleotides −235 to −148 was prepared using PCR with oligonucleotide primers extending from −235 to −218 and −165 to −148, to which were added sequences for BglII or BamHI restriction endonucleases, and the amplified fragment was inserted into the BamHI site of the reporter plasmid pBLCAT2.

**Transfection.** RAW 264.7 cells were plated at 5 × 10^6 per 100-mm tissue culture dish 2 d before transfection. The cells were transfected with \(12 \mu\)g plasmid DNA per dish using the DEAE-dextran method (12) with modification. The cells were incubated with the DNA/DEAE-dextran solution for 1 h at 37°C, followed by the addition of chloroquine (100 \(\mu\)M, final) for 1.5 h. The cells were shocked for 1 min with 10% DMSO in PBS, washed with PBS (10 ml, three times), and returned to culture medium. After \(\sim 20\) h, each dish was scraped and the cells were divided into three or four 60-mm tissue culture dishes. 2-4 h later, one dish from each transfection group received medium alone, one received IFN-\(\gamma\) (1,000 U/ml), and in some experiments, the additional dish(es) received IFN-\(\alpha\) (1,000 U/ml), 40 nM PMA, or TNF-\(\alpha\) (1,000 U/ml), as indicated. The cells were harvested 18–24 h after stimulation.

**CAT Assays.** Cells were harvested and CAT assays were performed as described by Gorman et al. (13), with the modification that butyryl coenzyme A (Pharmacia Fine Chemicals, Piscataway, NJ) was used in place of acetyl coenzyme A. In the case of cultures transfected with mig/CAT constructs, 50 \(\mu\)l of cell extract was assayed. However, due to high basal activity of the p268CATAM-C construct, only 5 \(\mu\)l of extract was tested. Activities were adjusted for differences in the protein concentration of the extracts.

**DNA Sequencing.** Sequencing of double-stranded DNA templates was performed by the dideoxy chain termination method using reagents and protocols supplied by United States Biochemical Corporation, Cleveland, OH. Both strands of the mig genomic fragment were sequenced in their entirety.

### Results and Discussion

The mig (monokine induced by IFN-\(\gamma\)) cDNA clone was obtained by differential screening of a cDNA library prepared from the mouse macrophage–like cell line RAW 264.7 treated with conditioned medium from Con A-stimulated mouse splenocytes. The mig cDNA hybridizes to a 1.6-kb mRNA selectively induced by IFN-\(\gamma\) and encodes a new member of the platelet factor 4 family of cytokines (14). The concentration dependence of mig mRNA induction by IFN-\(\gamma\) in RAW 264.7 cells is shown in Fig. 1. The expression of mig mRNA was potently induced by IFN-\(\gamma\), but not by IFN-\(\alpha\), over a wide concentration range. The absence of mig RNA accumulation in response to IFN-\(\alpha\) was not due to a failure of the RAW 264.7 cells to respond to IFN-\(\alpha\), since hybridization of the same filter, after stripping, with a cDNA probe for the IFN-responsive \(crg-2\) mRNA (11), readily demonstrated induction by IFN-\(\alpha\) (Fig. 1, bottom). The property of preferential induction by IFN-\(\gamma\) and not IFN-\(\alpha\) is consistent with previous reports that IFN-\(\gamma\) leads to the induction of a number of mRNAs and proteins not induced by IFN-\(\alpha\) or IFN-\(\beta\) (4, 5). A study of the regulatory mechanism(s) involved in the accumulation of mig mRNA should, therefore, lead to the identification of pathways selectively activated by IFN-\(\gamma\).

The effect of IFN-\(\gamma\) on the transcription of the mig gene was evaluated in nuclear run-on assays. Nuclei were isolated from RAW 264.7 cells incubated in medium alone or in

![Figure 1](image1.png)

**Figure 1.** (Top) Effect of IFN-\(\gamma\) (0.01–1,000 U/ml) and IFN-\(\alpha\) (0.1–1,000 IU/ml) on mig mRNA accumulation in RAW 264.7 cells. Shown is an autoradiograph of an RNA blot hybridization in which 20 \(\mu\)g of total RNA per lane from RAW cells treated with IFNs for 8 h were resolved by formaldehyde-agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to \[^{32}P\]-labeled mig cDNA. (Bottom) Effect of IFN-\(\gamma\) (0.01–1,000 U/ml) and IFN-\(\alpha\) (0.1–1,000 IU/ml) on \(crg-2\) mRNA accumulation in RAW 264.7 cells. After stripping of mig probe by boiling, the filter was hybridized to \[^{32}P\]-labeled \(crg-2\) cDNA.

![Figure 2](image2.png)

**Figure 2.** Nuclear run-on assay. Run-on transcripts were prepared using nuclei isolated from RAW 264.7 cells incubated in medium alone, medium containing IFN-\(\gamma\) (500 U/ml), medium containing IFN-\(\gamma\) (500 U/ml) plus cycloheximide (10 \(\mu\)g/ml), or medium containing cycloheximide (10 \(\mu\)g/ml) for 3 h. Denatured, linearized plasmid DNA (mig cDNA in pBluescript, \(c-fos\) genomic fragment in pGEM-2, \(c-myc\) genomic fragment in pGEM-2, and pBluescript without insert (BS+)) was applied to a nitrocellulose membrane and hybridized with labeled run-on transcripts as described in Materials and Methods.
medium containing IFN-γ (500 U/ml) with and without cycloheximide (10 μg/ml). Plasmids containing c-fos and c-myc genomic fragments were included as controls for gene expression. As shown in Fig. 2, transcription of the mig gene was significantly increased in cells treated with IFN-γ, and this effect was enhanced by cycloheximide. The resistance of mig transcriptional activation to protein synthesis inhibition by cycloheximide indicates that mig transcription is directly downstream of the post-receptor events that follow binding of IFN-γ. In this regard, the regulation of mig expression is similar to the regulation of "immediate early" genes whose transcription is activated in growth factor–stimulated fibroblasts (15). The activation of mig transcription by IFN-γ was first detectable 2 h after stimulation and continued for at least 9 h (data not shown). Interestingly, treatment with cycloheximide alone augmented mig transcription, although cycloheximide did not induce mig mRNA by Northern analysis (14). The observation that cycloheximide leads to mig transcription without mRNA accumulation raises the possibility that in addition to increasing mig transcription, IFN-γ may also affect the stability of mig mRNA.

Genomic clones were isolated from a BALB/c mouse genomic library by plaque hybridization using as a probe a HindIII fragment from the 5' end of the mig cDNA. Their identification as mig genomic clones was confirmed by comparing the restriction map of these clones and the restriction fragments identified by Southern analysis of restricted mouse genomic DNA hybridized with mig cDNA probes (data not shown). Southern hybridization of fragments of the genomic clones with an oligonucleotide complementary to nucleotides 45–64 of the mig mRNA identified a 1.6-kb EcoRI fragment on which was mapped the mig transcription start site (14) in appropriate relation to TATA and CAAT promoter elements (Fig. 3A and Fig. 4).

Constructs were prepared containing the mig promoter region and 5' flanking sequences inserted into the promoterless CAT reporter plasmid pUMSVOCAT. The initial mig/CAT plasmid contained an 1,160-bp mig genomic fragment extending from nucleotides -1117 to +43 relative to the transcription start site. Two additional plasmids were constructed containing the mig sequence -704 to +43 (747 bp) and -358 to +43 (401 bp), taking advantage of available restriction sites (Fig. 3A). Transfection of each of the three plasmids into RAW 264.7 cells followed by treatment with IFN-γ...
or IFN-α demonstrated that only IFN-γ was able to induce significant CAT activity (Fig. 3 B). To establish that the RAW 264.7 cells were capable of responding to IFN-α under the conditions used for transfection, cells were transfected with the plasmid p268CATAM-C, which contains the CAT gene under the control of the zif268 promoter/enhancer region. The zif268 gene is induced in quiescent fibroblasts treated with serum (16), and also in RAW 264.7 cells in response to stimulation with IFN-γ and IFN-α (J. M. Farber, B. Christy, and D. Nathans, unpublished observation). As shown in Fig. 3 B, treatment of RAW 264.7 cells transfected with the zif268/CAT construct with either IFN-γ or IFN-α induced a similar increase in the CAT activity, indicating that the lack of induction of the mig constructs by IFN-α was not due to a failure of the cells to respond to IFN-α.

To define further the sequence(s) important for the activation of mig transcription by IFN-γ, a series of 5' deletion mutants was prepared from the plasmid p(-358/+43)CAT. The results of an experiment in which RAW 264.7 cells were transfected with these constructs and tested for inducibility by IFN-γ are shown in Fig. 5. The responsiveness to IFN-γ was lost abruptly with the deletion mutant p(-172/+43)CAT.

The data indicate that the region from -235 to -172 contains one or more IFN-γ-inducible positive cis regulatory elements. As indicated in Fig. 4, within this region lies a sequence with homology to the previously reported IFN-stimulated response element (ISRE) found in the 5' regulatory regions of a number of genes whose transcription is activated by IFN-α/β and IFN-γ (6, 17). A search for related sequences in the mig promoter identified a second region with homology to the ISRE extending from -99 to -85. Neither of the homologous sequences in the mig gene match precisely the ISRE sequence found in other IFN-inducible genes (6) or the ISRE consensus sequence YAGTTTC(A/T)YTTTYCC (6), raising the possibility that the mig sequences may represent related elements that are specifically regulated by IFN-γ.

To determine whether the cis element(s) located between nucleotides -235 and -172 could function in the context of a heterologous promoter, the mig genomic fragment extending from nucleotides -235 to -148 was prepared by PCR and inserted in both orientations into the BamHI site of pBLCAT2, a plasmid that contains the Herpes simplex virus thymidine kinase (tk) promoter 5' of the CAT gene (18). As shown in Fig. 6, IFN-γ stimulated CAT expression in RAW 264.7 cells transfected with the constructs containing the mig genomic fragment in either orientation, but not in cells transfected with pBLCAT2 lacking insert. In addition, these constructs remained unresponsive to IFN-α (data not shown). Of note, the activities of these constructs were similar to the activity of p(-1117/+43)CAT determined in the same experiments (Fig. 6).

Other potential regulatory elements identified in the mig promoter region include a sequence with similarity to the NF-κB (reviewed in reference 19) binding site (noncoding, 5'-GGGAATTTCT3') located at position -145 to -154, and an AP-2 (20) binding site (noncoding, 5'-CCCCAGGC-3') located at position -214 to -221. The finding of an NF-κB-like site raised the possibility that the mig gene might be regulated by the tumor-promoting phorbol ester PMA or TNF-α, factors known to activate transcription through NF-κB (19). However, neither of these factors were able to stimulate mig expression as determined by Northern analysis and transfection studies with p(-1117/+43)CAT (14; and data not shown). While AP-2 binding sites are found in the 5' regulatory regions

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1. Abbreviation used in this paper: ISRE, IFN-stimulated response element.
of other IFN-responsive genes, such as the human metallothionein II A and mouse H-2K genes, evidence suggests that AP-2 is not involved in their regulation by IFN (20).

Additional studies are in progress to identify the specific regulatory elements responsible for the selective activation of mig transcription by IFN-γ.

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