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 Biologicals, 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 ^{32}P-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 (2× 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

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Volume 173 February 1991 417–422

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 SmaI 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 μg 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 μM, 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 ∼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-γ (1,000 U/ml), and in some experiments, the additional dish(es) received IFN-α (1,000 U/ml), 40 nM PMA, or TNF-α (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 μl of cell extract was assayed. However, due to high basal activity of the p268CATAM-C construct, only 5 μl 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-γ) 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-γ and encodes a new member of the platelet factor 4 family of cytokines (14). The concentration dependence of *mig* mRNA induction by IFN-γ in RAW 264.7 cells is shown in Fig. 1. The expression of *mig* mRNA was potently induced by IFN-γ, but not by IFN-α, over a wide concentration range. The absence of *mig* RNA accumulation in response to IFN-α was not due to a failure of the RAW 264.7 cells to respond to IFN-α, 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-α (Fig. 1, *bottom*). The property of preferential induction by IFN-γ and not IFN-α is consistent with previous reports that IFN-γ leads to the induction of a number of mRNAs and proteins not induced by IFN-α or IFN-β (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-γ.

The effect of IFN-γ 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

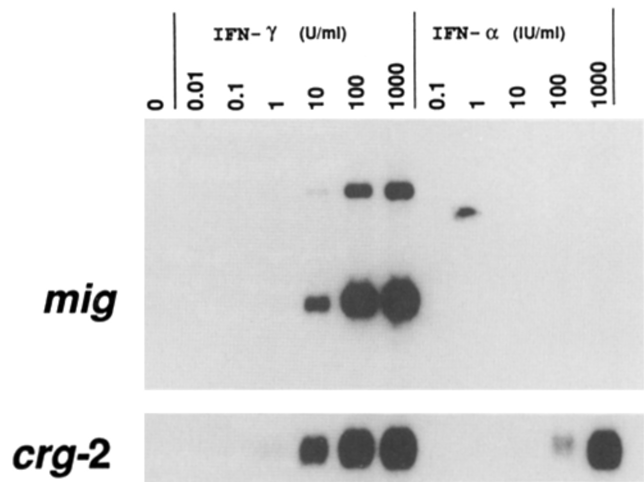


Figure 1. (*Top*) Effect of IFN-γ (0.01–1,000 U/ml) and IFN-α (0.1–1,000 IU/ml) on *mig* mRNA accumulation in RAW 264.7 cells. Shown is an autoradiograph of an RNA blot hybridization in which 20 μg of total RNA per lane from RAW cells treated with IFNs for 8 h were resolved by formaldehyde-agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to ^{32}P-labeled *mig* cDNA. (*Bottom*) Effect of IFN-γ (0.01–1,000 U/ml) and IFN-α (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.

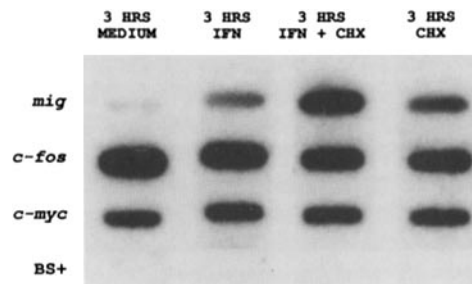
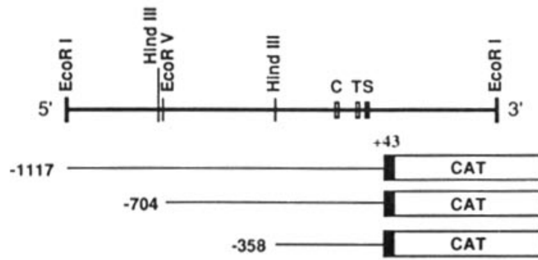


Figure 2. Nuclear run-on assay. Run-on transcripts were prepared using nuclei isolated from RAW 264.7 cells incubated in medium alone, medium containing IFN-γ (500 U/ml), medium containing IFN-γ (500 U/ml) plus cycloheximide (10 μg/ml), or medium containing cycloheximide (10 μg/ml) for 3 h. Denatured, 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.

A



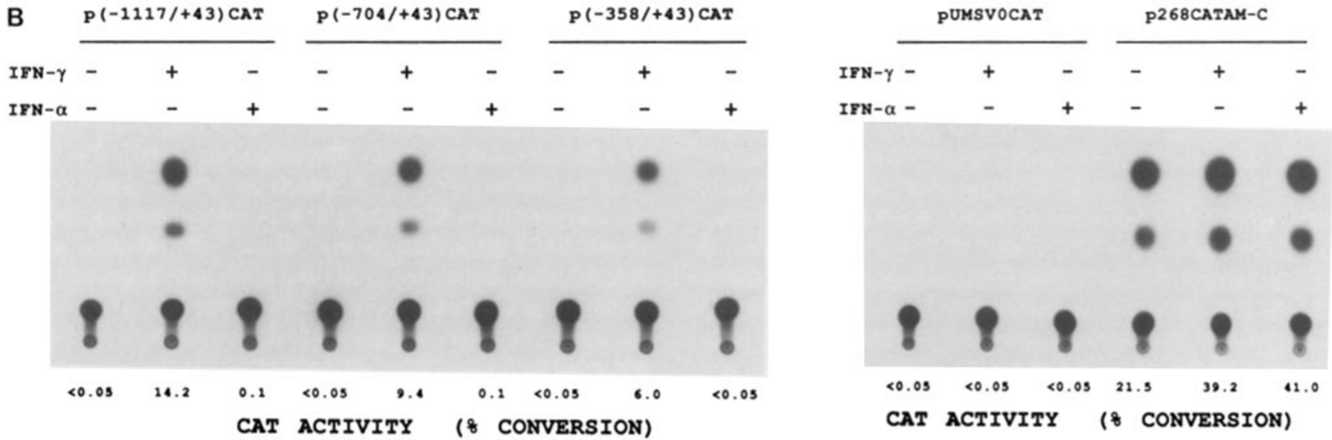


Figure 3. (*A*) Partial restriction map of the 1.6-kb EcoRI genomic fragment from the 5′ flanking region of the *mig* gene and (*below*) diagram of reporter gene plasmids containing *mig* genomic inserts ligated into pUMSVOCAT. *C*, *T*, and *S* refer to the positions of the CAAT box, TATA box, and transcription start site, respectively. The numbers indicate the number of nucleotides 5′ (−) and 3′ (+) to the transcription start site (+1). (*B*) CAT activity of cell extracts prepared from RAW 264.7 cells transfected with the indicated plasmids. Cells were treated with IFN-γ (1,000 U/ml), IFN-α (1,000 IU/ml), or medium alone for 22 h before harvest and determination of CAT activity. The percent conversion of chloramphenicol is shown below the corresponding lane of the autoradiogram. The results shown are from a single experiment and are representative of $n \geq 3$.

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 HinfI 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. 3 *A* 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. 3 *A*). Transfection of each of the three plasmids into RAW 264.7 cells followed by treatment with IFN-γ

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                                                                                -358
                                                                            5'- AGCTTTGA
-350       -340       -330       -320       -310       -300       -290
CTTGTGAGGA AAGGCAGTTT GAGTCGCCAT ATAGTGTCAT GTCCCACGTG GGAAGTACAT CTCCAACCTT
-280       -270       -260       -250       -240       -230       -220
CATTGTACAG CATTAATATA GTCATTGTAT AATCTATTCC ACATCCAGGT AGCAACTTTG CCTGGGGGTG
-210       -200       -190       -180       -170       -160       -150
GTTTCACATC CCTTACTATA AACTCCCCGT TTATGTGAAA TGGAAGTAGA ACATGCAGAA ATTCCCTGGG
-140       -130       -120       -110       -100       -90        -80
ATCTGAGAGT AGGGTTTTCC CCAGGACGAT CAATTTGTGG TTAGTTTAGT TCTTCTAGGT CAGCTGAGGA
-70        -60        -50        -40        -30        -20        -10
GACCAGCCAA TCAGAGACGG GAAGGAAAAG GGATTTCCTA AATAAATATG ATCCCCAAGA ACATGCTCTC
+1         +10        +20        +30        +40
TAAAGACATT CTCGGACTTC ACTCCAACAC AGTGACTCAA TAG -3'
transcr. start
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Figure 4. Nucleotide sequence of the 401-bp IFN-γ-responsive *mig* genomic fragment. The numbering of residues is relative to the distance from the transcription start site (indicated by the arrow). The TATA and CAAT homologies are underlined and highlighted. The boxed nucleotides indicate the two sequences with homology to the IFN-stimulated response element (ISRE).

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 *zif*268 promoter/enhancer region. The *zif*268 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 *zif*268/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.

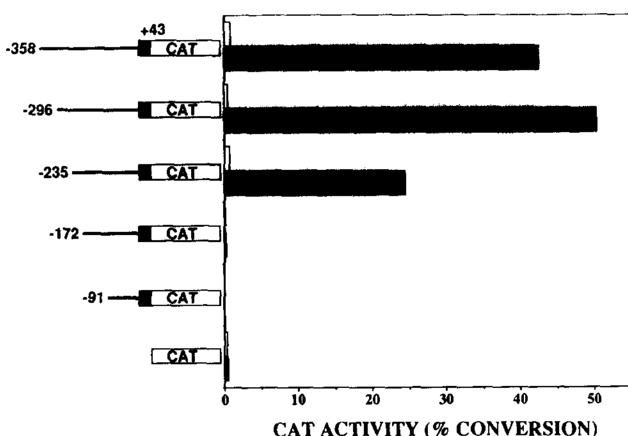

Figure 5. CAT activity of cell extracts prepared from RAW 264.7 cells transfected with *mig* promoter/CAT deletion mutants. Cells were treated with 1,000 U/ml IFN-γ (*solid bars*) or medium alone (*open bars*) for 20 h before harvest and determination of CAT activity. The results shown are from a single experiment and similar results were obtained in two additional experiments.

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)[1] 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'-GGGAATTTCT-3') 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

[1] *Abbreviation used in this paper:* ISRE, IFN-stimulated response element.

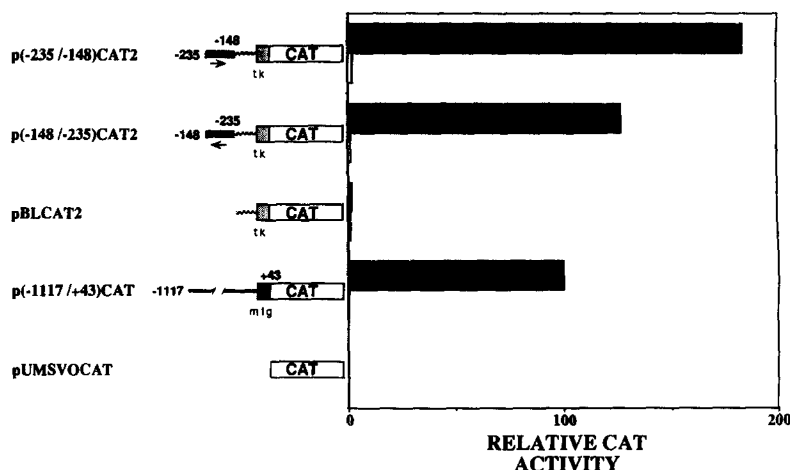

Figure 6. CAT activity of RAW 264.7 cells transfected with plasmids containing the *mig* genomic sequence −235 to −148 inserted in both orientations into pBLCAT2, pBLCAT2 without insert, p(−1117/+43)CAT, or pUMSVOCAT. Cells were treated with 1,000 U/ml IFN-γ (*solid bars*) or medium alone (*open bars*) for 20 h before harvest and determination of CAT activity. The data are expressed as "relative activity", where the activity in mock transfected cultures (< 0.05% conversion) equals 0 relative activity, and the activity of cultures transfected with p(−1117/+43) and stimulated with IFN-γ (29.8–37.0% conversion) equals a value of 100. The data are the averages from two experiments performed in duplicate.

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.

We thank S. Willenberger for his excellent technical assistance, and B. Christy, Y. Nakabeppu, and D. Nathans for their thoughtful discussions and advice.

This work was supported by grants AM-01298 and CA-48059 from the National Institutes of Health, a Biomedical Support Grant from the Johns Hopkins University, and by a grant from the Maryland Chapter of the Arthritis Foundation.

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Received for publication 30 April 1990 and in revised form 24 August 1990.

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