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Transcription of the Interferon γ (IFN-γ)-inducible Chemokine Mig in IFN-γ-deficient Mice*

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***MuMig* or *Mig* (murine monokine induced by interferon γ) is a CXC chemokine whose induction is thought to be strictly dependent on interferon γ (IFN-γ). Here we have studied the expression of this chemokine gene in various organs of mice infected with vaccinia virus. We have employed animals deficient in either IFN-γ (IFN-$\gamma^{-/-}$), or receptors for IFN-α/β, IFN-γ, or both IFN-α/β and IFN-γ ($DR^{-/-}$) to dissect out the role of interferons in the induction of *Mig* during the host response to virus infection. Our data show that *Mig* mRNA and protein are expressed in organs of vaccinia virus-infected IFN-$\gamma^{-/-}$ mice, albeit at lower levels compared with infected, wild-type animals. In the $DR^{-/-}$ mice and in IFN-$\gamma^{-/-}$ mice treated with a neutralizing antibody to IFN-α/β, *Mig* mRNA transcripts were completely absent. Our data indicate that, in vaccinia virus-infected IFN-$\gamma^{-/-}$ mice, *Mig* mRNA expression is mediated through the interaction between IFN-γ responsive element 1 (γRE-1) and IFN-α/β-induced STAT-1 complex referred to as IFN-γ response factor 2 (γRF-2). Further, our findings support the view that γRF-2 is the IFN-α/β induced STAT-1 complex, IFN-α-activated factor. We have found that, in the absence of IFN-γ, IFN-α/β are able to induce Mig in response to a viral infection *in vivo*.**

Interferons (IFNs)[1] are an important group of cytokines that have biological activities, including antiviral effects, regulation of cell growth and cell differentiation, and modulation of the immune response (1, 2). IFNs mediate many of their biological effects through regulation of specific RNA and protein expression in the responding cell (2). Knowledge of the ways in which the IFNs regulate gene transcription is necessary to our understanding of the basic mechanisms of their action. Although type I (IFN-α and IFN-β) and type II (IFN-γ) IFNs bind to different cell surface receptors, they can induce the expression of both an overlapping as well as distinct sets of genes (2, 3). The study of genes activated by both type I and type II IFNs has led to the identification of two well-characterized promoter elements, namely, the IFN-stimulated response element (ISRE) (4) and the IFN-γ activation sequence (GAS) (5).

Both type I and type II IFNs signal through pathways that employ STAT-1. IFN-γ promotes the activation of early response genes through a STAT-1 homodimer, GAF (IFN-γ-activated factor), which binds to GAS elements (2, 6). IFN-α stimulation leads to the formation of a STAT-1 homodimer (AAF; IFN-α-activated factor) and heterodimer composed of STAT-1 and STAT-2. This STAT-1:STAT-2 dimer interacts with a "non-STAT" protein (for example, p48) to form an active transcription factor ISGF3 (7). The AAF and ISGF3 complexes translocate to the nucleus and bind to GAS elements and ISRE elements, respectively.

Differential screening of a cDNA library from IFN-γ-activated macrophages led to the identification of the murine CXC chemokine gene *Mig* (8). Chemokines constitute a family of small cytokines that are produced during the process of inflammatory and immune reactions. They regulate efficient and orchestrated recruitment of leukocytes to the site of inflammation (9–11). Mig is chemotactic for natural killer cells and T lymphocytes. Studies using macrophage cell lines and monocytes showed that both murine and human Mig were inducible only by recombinant IFN-γ in these cells (8, 12). A fragment from the 5′-flanking region of −235 to −167 of the *Mig* gene was able to mediate dramatic induction of a heterologous promoter by recombinant IFN-γ and this element termed γRE-1 has similarities to the IFN-γ activation sequence (GAS) (13). Furthermore, the presence of NF-κB sites in the *Mig* gene was shown to be important for gene transcription involving a synergistic interaction between IFN-γ and TNF (14).

It was shown recently that IFN-γ is necessary for induction of Mig in multiple organs of mice infected with *Toxoplasma gondii*, *Plasmodium yeolli*, or vaccinia virus (VV) (15). In the

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[1] The abbreviations used are: IFN, interferon; Mig, murine Mig; Crg-2, cytokine responsive gene-2; ISRE, IFN-stimulated response element; GAS, IFN-γ activation sequence; γRE-1, IFN-γ-responsive element; GAF, IFN-γ-activated factor; AAF, IFN-α-activated factor; ISGF3, IFN-stimulated gene factor 3; γRF-1/2, IFN-γ responsive factors 1 and 2; STAT, signal transducer and activator of transcription; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcriptase-polymerase chain reaction; NF-κB, nuclear factor κB; TNF, tumor necrosis factor; VV, vaccinia virus; , rVV, recombinant vaccinia virus; WT, wild-type; PFU, plaque-forming unit(s); SBE, STAT binding element.

present report, we demonstrate that *Mig* mRNA is induced in multiple organs, albeit at low levels, in IFN-γ$^{-/-}$ mice after infection with VV. Treatment of VV-infected IFN-γ$^{-/-}$ mice with an antibody to IFN-α/β completely abolished Mig induction in the liver, indicating that expression of the chemokine in VV-infected IFN-γ$^{-/-}$ mice is most likely mediated by IFN-α/β. This is further supported by our findings that *Mig* mRNA transcripts are absent in VV-infected mice lacking functional receptors for both type I and type II IFNs. Our data indicate that *Mig* mRNA induction in livers of VV-infected IFN-γ$^{-/-}$ mice is most probably mediated via a STAT-1 complex (γRF-2) binding to a STAT binding element (γRE-1) in the Mig promoter. The transcription factor (γRF-2) appears to be a STAT-1 homodimer complex.

EXPERIMENTAL PROCEDURES

Mice—Six- to eight-week-old specific pathogen-free C57BL/6J (B6) wild type (WT) mice and IFN-γ gene knockout (IFN-γ$^{-/-}$) mice (16) on the B6 background were obtained from the Animal Breeding Establishment, John Curtin School of Medical Research (ABE, JCSMR), Canberra, Australia. Mice lacking functional type I (α/β) receptors (IFN-α/βR$^{-/-}$), type II (γ) receptors (IFN-γR$^{-/-}$), or both type I and type II IFN receptors (DR$^{-/-}$) (17) on the 129/SvJ background also bred at the ABE, JCSMR, were used at 6–12 weeks of age. WT 129/SvJ mice were used as controls.

Virus—The virulent vaccinia virus, Western-Reserve strain (VV-WR ATCC VR-119), was propagated in CV-1 cells and used after purification on a sucrose density gradient as described elsewhere (18). Mice were injected intraperitoneal on day 0 with 10^6 plaque-forming units (PFU) of the virus.

Neutralization of IFN-α/β in Vivo—Purified rabbit polyclonal antibody to murine IFN-α/β was purchased from Lee Biomolecular Research Laboratories, Inc. (San Diego, CA). Groups of IFN-γ$^{-/-}$ mice were given (0.2 mg) 300 neutralizing units of anti-IFN-α/β on days −1, 1, and 2. One neutralizing unit of anti-IFN-α/β is defined as the reciprocal of the dilution of antibody, which completely neutralizes 10 units of the IFN activity *in vitro* (19). The mice were infected intraperitoneally with 10^6 PFU of VV-WR on day 0 and sacrificed on day 3. Mice similarly treated with 0.2 mg of rabbit IgG (Calbiochem, La Jolla, CA) were used as controls.

Isolation and Purification of mRNA—RNase-free plastic and water were used throughout the assay. Tissues (approximately 50 mg) were homogenized in 1 ml of RNAzol B (Biotecx, Friendswood, TX), and total RNA was isolated as recommended by the manufacturer. The RNA was resuspended in diethylpyrocarbonate-treated water containing 1 mM EDTA and quantitated spectrophotometrically. All samples were DNase-treated to remove genomic DNA. RNA gel electrophoresis was performed to confirm that RNA was intact and that the concentration had been determined correctly as described previously (20).

RT-PCR Detection of Cytokine and Chemokine mRNA Transcripts—A reverse transcriptase (RT)-PCR procedure was performed as described previously (21) with some modification (22) to determine relative quantities of mRNA for Mig, IFN-γ, IFN-α1, IFN-β, and hypoxanthine-guanine phosphoribosyltransferase. The primers and probes for all genes were prepared by the Biomolecular Resource Facility, JCSMR, on a DNA synthesizer (Applied Biosystems, Foster City, CA). Primer and probe sequences for Mig, IFN-γ, IFN-α1, IFN-β, and hypoxanthine-guanine phosphoribosyltransferase have been described previously (21, 23, 24). The cycle numbers used for amplification of each gene product are as follows: Mig, 24 cycles; IFN-γ, 25 cycles; IFN-α1 and IFN-β, 27 cycles; and hypoxanthine-guanine phosphoribosyltransferase, 23 cycles. After the appropriate number of PCR cycles, the amplified DNA was analyzed by gel electrophoresis, Southern blotting, and detected using the ECL detection system as recommended by the manufacturer (Amersham Pharmacia Biotech).

Nuclear and Cytosol Extracts—Liver tissues were obtained from groups of WT B6, IFN-γ$^{-/-}$, and DR$^{-/-}$ mice 3 days after infection with VV-WR. Liver tissues were also obtained from virus-infected IFN-γ$^{-/-}$ mice treated with antibody against IFN-α/β. To prepare cell suspension, the liver was teased apart and passed through a metal sieve into approximately 20 ml of ice-cold suspension buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). The cell suspension was centrifuged at 1500 rpm for 5 min at 4 °C, the supernatant was aspirated, and the resultant pellet was resuspended in ice-cold suspension buffer. Red blood cells were removed from the suspension using red blood cell lysis buffer, pH 7.3 (10 mM potassium bicarbonate, 0.15 M ammonium chloride, 0.1 mM EDTA, 5% fetal calf serum). The suspension was then spun at 1500 rpm for 5 min and washed twice with ice-cold suspension buffer. Nuclear and cytosol extracts were then prepared from the cell suspension according to the method of Dignam *et al.* (25).

Western Blot Analysis—Tris-glycine-SDS-polyacrylamide gel electrophoresis of protein samples from cytosolic extracts was performed with 12% acrylamide gels according to the method of Laemmli (26). As a positive control, concentrated supernatants from CV-1 cells (monkey kidney cell line) infected with recombinant VV (rVV) expressing Mig was analyzed in parallel with liver cytosolic extracts. Construction of rVV encoding Mig has been described in detail elsewhere (24). Immunoblotting was performed as described previously (27). Anti-Mig serum, JH48, was kindly provided by Dr. Joshua Farber, National Institutes of Health (Bethesda, MD).

Gel Electrophoretic Mobility Shift Assay (EMSA)—This was performed using double-stranded oligonucleotides radiolabeled by fill-in reaction using the Klenow fragment of DNA polymerase I as described previously (28). The double-stranded oligonucleotides used were the γRE-1 sequence (−200 to −167) and NF-κB sequence (−145 to −154) of the Mig promoter (28, 29) and the GAS sequence (−126 to −101) of the guanylate-binding protein promoter (5). The ISRE sequence (−224 to −211) of the murine *Crg-2* gene was used as a nonspecific competitor (30). Protein concentration was measured by the method of Bradford (31) using the protein dye reagent (Bio-Rad, Richmond, CA). Binding reaction mixtures (25 μl) contained nuclear extracts (5 μg of protein), 2 μg of denatured salmon sperm DNA in binding buffer (20 mM HEPES, pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 200 μg/ml bovine serum albumin) and were incubated for 15 min at 4 °C. The ^{32}P-labeled oligonucleotide (5×10^5 cpm) was then added to the reaction mixture and incubated for 20 min at room temperature. Where indicated, unlabeled double-stranded oligonucleotides containing γRE-1 element, GAS element, or ISRE element (competitors) were added to the binding reaction simultaneously during addition of radiolabeled fragment. In some experiments, antibody against STAT-1α (specific for p91; obtained from Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) was included in the reaction mixture. Following incubation, 5 μl of 0.1% bromphenol blue in binding buffer was then added and the mixture was immediately loaded on a 6% polyacrylamide gel. Electrophoresis was performed in 0.25× TBE buffer (TBE buffer is 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8) at 175 V with buffer recirculation. The gels were dried and analyzed by autoradiography.

RESULTS

Mig mRNA Is Expressed at High Levels in Organs of B6 WT Mice—Organs were harvested at days 3, 6, and 10 after infection of B6 WT mice with 10^6 PFU of VV-WR. Organs from uninfected mice were used as controls. Ovaries were pooled from groups of four mice for each time point, whereas other organs were analyzed individually. Fig. 1*A* shows that *Mig* mRNA is expressed at high levels in all organs examined during the course of infection but was not detected in organs of uninfected mice. The time course of infection indicates that *Mig* mRNA is expressed at high levels in all organs on days 3 and 6, but declines by day 10. Levels of expression were highest in the liver followed by ovaries, uterus, and spleen. Expression of IFN-γ was highest in the ovaries followed by uterus, spleen, and liver. Thus, although a strong correlation between the kinetics and expression of Mig induction and that of IFN-γ was observed in the spleen, ovaries, and uterus, this was not the case in the liver where we observed high levels of Mig and comparatively low levels of IFN-γ mRNA. It is possible that Mig in the liver is up-regulated by the elevated serum IFN-γ levels following infection with VV (data not shown). It is also possible that some other factor(s) are responsible for Mig induction in the liver.

Mig mRNA and Protein Are Expressed in Mice Lacking IFN-γ Function—To establish the role of IFN-γ in Mig induction during VV infection, we used IFN-γ$^{-/-}$ mice on the B6 background. Organs were harvested at days 3, 6, and 10 after virus infection while organs from uninfected mice were used as

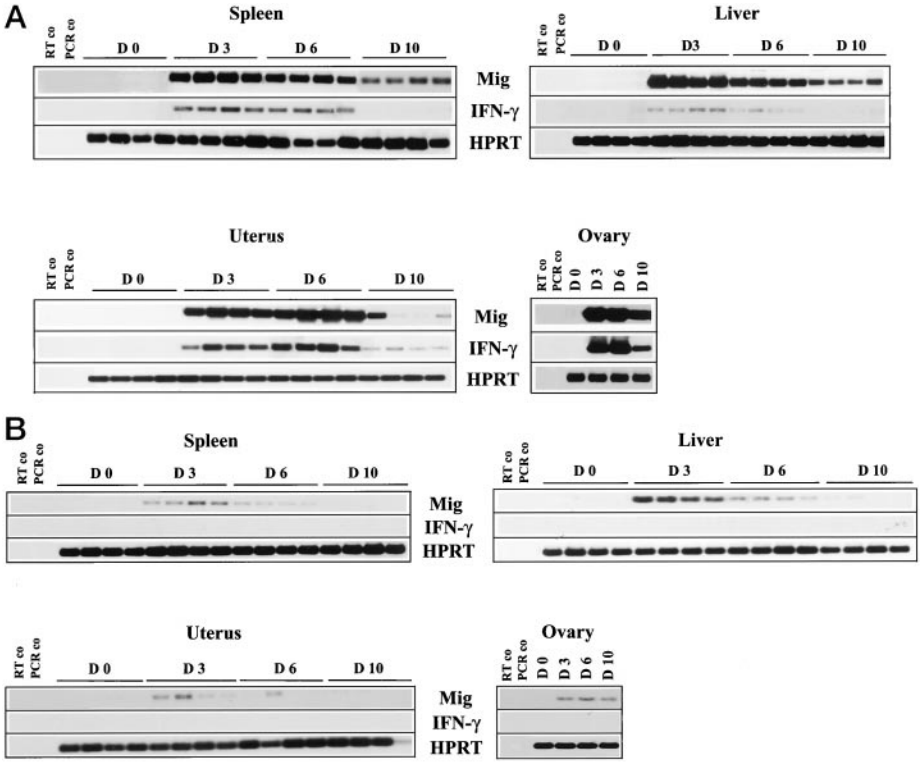

FIG. 1. **Time course expression of *Mig* and IFN-γ mRNA in (*A*) B6 WT and (*B*) IFN-γ$^{-/-}$ mice during infection with VV.** Total RNA was prepared from organs of uninfected mice (*D0*) as well as mice infected for 3 (*D3*), 6 (*D6*), and 10 (*D10*) days with VV. Specific mRNA species (Mig, IFN-γ, and hypoxanthine-guanine phosphoribosyltransferase) were amplified by RT-PCR. The amplification products were blotted onto nylon membranes and hybridized to fluorescein-labeled oligonucleotide probes specific for the PCR product. Filters were exposed for 5 min in all cases. These experiments were repeated twice with comparable results. Groups of four mice were used in these experiments. *RT co*, RT control; *PCR co*, PCR control.

controls. Fig. 1*B* shows that *Mig* mRNA was not detected in uninfected IFN-γ$^{-/-}$ mice. Unexpectedly, however, low levels of *Mig* mRNA transcripts were detected in all organs at days 3 and 6 post-infection and expression was most prominent in the liver. We next investigated the expression of Mig during the course of VV infection in IFN-γR$^{-/-}$ mice (126/SvJ background). Fig. 2*A* shows that *Mig* mRNA was expressed in the livers of VV-infected IFN-γR$^{-/-}$ mice and that the levels of expression are comparable with those in IFN-γ$^{-/-}$ mice (B6 background). This also indicates that, at least for the two strains of mice studied (B6 and 129), the genetic background of the animals did not influence Mig expression in the absence of IFN-γ function.

It was also important to determine whether the expression of *Mig* mRNA was associated with the presence of Mig protein. We performed Western blot analysis on cytosolic extracts from livers pooled from groups of four B6 WT mice and four IFN-γ$^{-/-}$ mice 3 days after infection with VV. Clearly, Mig protein was detected in cytosolic extracts of IFN-γ$^{-/-}$ mice, but the level was significantly lower compared with that of B6 WT mice (Fig. 2*B*). Culture supernatant from CV-1 cells infected with rVV expressing Mig was used as a positive control. These data show that Mig protein is expressed consonant with mRNA levels.

Mig mRNA Is Not Induced in Mice Lacking Both IFN-γ and IFNα/β Function—In the preceding section we have shown that *Mig* mRNA and protein are present in VV-infected IFN-γ$^{-/-}$ mice and that the expression of this chemokine was most prominent in the liver. Likely candidate(s) responsible for the induction of Mig in IFN-γ$^{-/-}$ mice are the type I IFNs. A time course study revealed that the expression of *Mig* mRNA also paralleled that of IFN-α1 and IFN-β in livers of VV-infected IFN-γ$^{-/-}$ mice (Fig. 3*A*). It was therefore of interest to determine whether the expression of Mig in IFN-γ$^{-/-}$ mice was mediated through the activity of IFNα/β. We used a polyclonal antibody to IFNα/β to inhibit its function *in vivo*. As expected, *Mig* mRNA expression in VV-infected IFN-γ$^{-/-}$ mice treated with an antibody to IFN-α/β was completely abolished (Fig. 3*B*). However, in VV-infected IFN-α/βR$^{-/-}$ mice, where IFN-γ function is intact but IFN-α/β function is compromised, *Mig* mRNA expression was similar to that of WT mice (data not shown). This finding is consistent with the view that IFN-γ alone is a potent inducer of *Mig* gene transcription (13), however, in the absence of IFN-γ, IFN-α/β can induce Mig expression.

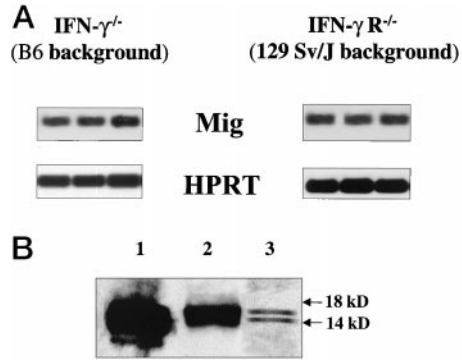

FIG. 2. ***Mig* mRNA and protein expression in mice lacking IFN-γ function.** *A*, total RNA was prepared from livers of IFN-γ$^{-/-}$ mice (*B6 background*) and IFN-γ R $^{-/-}$ (*129/SvJ*) mice 3 days after infection with VV. Specific mRNA species were amplified by RT-PCR in VV-infected IFN-γ$^{-/-}$ mice (*B6 background*) and VV-infected IFN-γ R$^{-/-}$ mice (*129/SvJ background*). *B*, cytosolic extracts were prepared from livers pooled from groups of 4 IFN-γ$^{-/-}$ mice (*lane 3*) and 4 WT B6 mice (*lane 2*) 3 days after infection. Cell culture supernatant from CV-1 cells infected with rVV expressing Mig protein was included as a positive control (*lane 1*). Samples were resolved by Tris-glycine-SDS-polyacrylamide gel electrophoresis, and Western blot analysis of Mig protein was carried out using anti-Mig serum JH48. This experiment was repeated twice with similar results.

Next, we utilized mice deficient in receptors for both IFN-α/β and IFN-γ (DR$^{-/-}$). Because the DR$^{-/-}$ mice are on a 129/SvJ background, the 129/SvJ wild-type mice were used as controls in these experiments. Livers were harvested 3 days after infection with VV and analyzed for Mig expression. Fig. 3*C* shows a complete absence of *Mig* mRNA transcripts in these mice, whereas in the control, 129/SvJ WT mice, the expression was

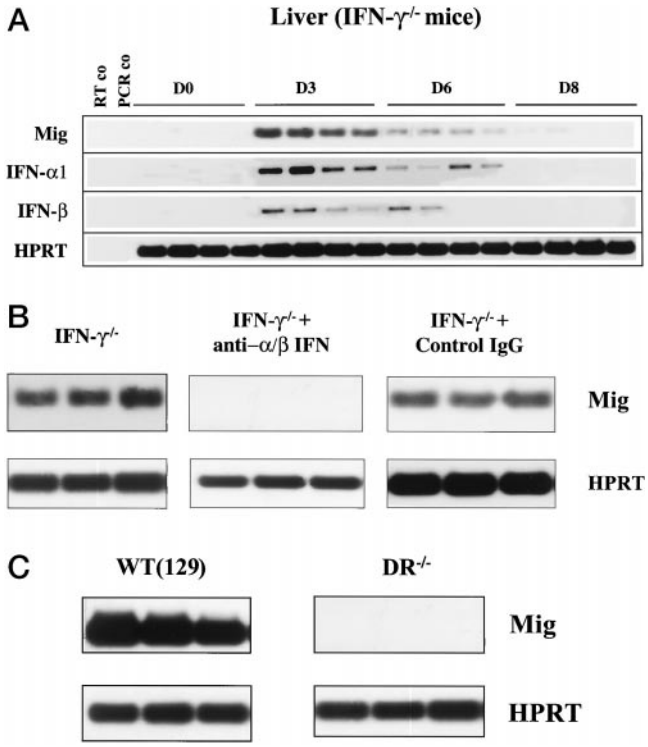

FIG. 3. *A*, time course expression of Mig, IFN-α1, and IFN-β mRNAs in livers of IFN-γ$^{-/-}$ mice 3 (*D3*), 6 (*D6*), and 8 (*D8*) days after infection with VV. Presence of specific mRNA was analyzed by RT-PCR as described in the legend to Fig. 1. Controls are as described in the legend to Fig. 1. *B*, expression of *Mig* mRNA in the livers of IFN-γ$^{-/-}$ mice and IFN-γ$^{-/-}$ mice treated with anti-IFN-α/β or control antibody (*IgG*), 3 days after virus infection. *C*, expression of *Mig* mRNA in VV-infected DR$^{-/-}$ mice. WT mice on the 129/SvJ (*129*) background were included for comparison. Total RNA was prepared from livers of mice 3 days after infection with VV. The experiment was repeated twice with comparable results.

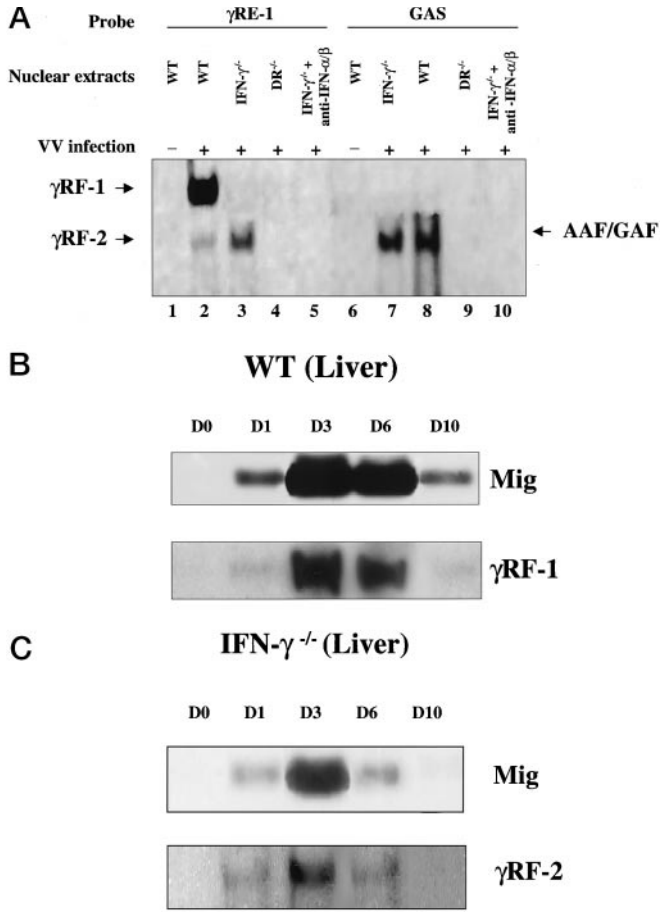

FIG. 4. *A*, DNA binding activity of γRF-1, γRF-2, and AAF in B6 WT, IFN-γ$^{-/-}$, DR$^{-/-}$ mice, and in IFN-γ$^{-/-}$ mice treated with antibody to IFN-α/β. Nuclear extracts were prepared from livers pooled from groups of three mice, either uninfected (−), or 3 days after infection (+) with VV. Gel electrophoretic mobility shift assay was performed as described under "Experimental Procedures." Kinetics of *Mig* mRNA expression and γRF-1 induction in WT mice (*B*) and IFN-γ$^{-/-}$ mice (*C*) during VV infection. Livers from three mice were pooled for each time point. *Mig* mRNA was analyzed by RT-PCR as described in Fig. 1, and γRF-1 was detected as described in *A*. Five micrograms of each nuclear extract was analyzed for the presence of these complexes by EMSA.

similar to that observed in B6 WT animals (Fig. 1*A*). These results, and those from the preceding sections, confirm that IFN-γ is important for the optimal induction of Mig expression *in vivo*. Interestingly, however, they also indicate that IFNs α/β are able to induce *Mig* mRNA, albeit at a low level, in VV-infected IFN-γ$^{-/-}$ mice.

Transcription Factor γRF-1 Is Only Detected in B6 WT Mice—Recent reports have shown that the IFN-γ-induced transcription factor, γRF-1, is involved in IFN-γ-mediated transcriptional regulation through γRE-1, a major regulatory element in the Mig promoter (13, 28). We have found that nuclear extracts from the livers of virus-infected B6 WT mice abundantly express γRF-1 (Fig. 4*A*, *lane 2*). This is consistent with the high levels of *Mig* mRNA found in this group (Fig. 1*A*). γRF-1 was not detected in nuclear extracts from livers of uninfected B6 WT mice (Fig. 4*A*, *lane 1*). Time course studies revealed that the induction of γRF-1 in the liver of virus-infected B6 WT mice paralleled the expression of *Mig* mRNA (Fig. 4*B*). Given this association of γRF-1 and Mig expression in B6 WT mice, we were interested in determining whether the expression of Mig in VV-infected IFN-γ$^{-/-}$ mice was also related to γRF-1 complex formation. However, γRF-1 was not detected in nuclear extracts of VV-infected IFN-γ$^{-/-}$ mice (Fig. 4*A*, *lane 3*), in mice lacking functional IFN receptors (DR$^{-/-}$) (Fig. 4*A*, *lane 4*), or in IFN-γ$^{-/-}$ mice treated with antibody to IFN-α/β (Fig. 4*A*, *lane 5*). This may explain, at least in part, the reduced levels or complete absence of *Mig* mRNA expression in these groups of mice.

Besides γRF-1, an additional γRE-1 binding protein was also detected by EMSA and is referred to here as γRF-2. The γRF-2 complex demonstrated higher mobility than γRF-1 and was detected in both B6 WT and IFN-γ$^{-/-}$ mice, but the levels were clearly higher in the latter (Fig. 4*A*, *lanes 2* and *3*, respectively). This complex was not detected in the DR$^{-/-}$ mice and in IFN-γ$^{-/-}$ mice treated with antibody to IFN-α/β (Fig. 4*A*, *lanes 4* and *5*). In addition, the time course studies show a similar pattern of expression of *Mig* mRNA and γRF-2 in the livers of VV-infected IFN-γ$^{-/-}$ mice (Fig. 4*C*). These findings are consistent with a role for γRF-2 in the induction of Mig expression in IFN-γ$^{-/-}$ mice infected with VV.

AAF Is Induced in IFN-γ$^{-/-}$ Mice—The preceding sections strongly suggested that IFN-α/β is responsible for the low level Mig expression in VV-infected IFN-γ$^{-/-}$ mice. It is possible that the transcription factor γRF-2, most probably induced by IFN-α/β, could have mediated this effect, because γRF-1 is not detected in these animals (Fig. 4*A*). IFN-α can directly induce some of the genes activated by IFN-γ through STAT-1 complexes such as AAF or ISGF3 (33). A comparison of the γRE-1 element, which binds to γRF-1, with the GAS element, which binds to STAT-1 complexes GAF·AAF, showed partial homology (7 of 15 base matches). It was therefore possible that AAF may also contribute to the transcriptional activation of *Mig* mRNA in the IFN-γ$^{-/-}$ mice. Indeed, we have detected AAF in

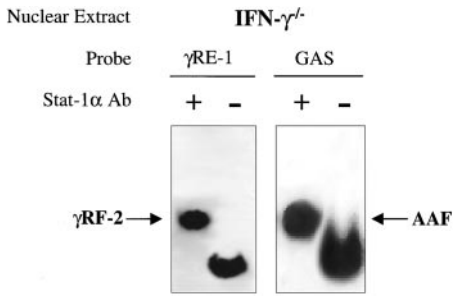

FIG. 5. **Supershift of electrophoretic mobility of γRF-2 and AAF DNA complexes by anti-STAT-1α antibody.** Nuclear extracts prepared from livers of VV-infected IFN-$\gamma^{-/-}$ mice were incubated for 2 h at 4 °C in the absence (−) or presence (+) of anti-STAT-1α antibody. These samples were then analyzed for DNA binding (to γRE-1 or GAS) activity by EMSA as described under "Experimental Procedures." The *arrows* indicate the position of the super-shifted complexes.

VV-infected B6 WT (Fig. 4*A*, *lane 8*) and IFN-$\gamma^{-/-}$ mice (Fig. 4*A*, *lane 7*) but not in the VV-infected DR$^{-/-}$ mice or IFN-$\gamma^{-/-}$ mice treated with antibody to IFN-α/β (Fig. 4*A*, *lanes 9* and *10*). In addition, STAT-1 complexes, detected by Western blot analysis, were not present in extracts obtained from VV-infected DR$^{-/-}$ mice or from VV-infected IFN-$\gamma^{-/-}$ mice treated with anti-IFN-α/β (data not shown).

γRF-2 and AAF Share a Common Component—Our results suggest that, although γRF-1 is required for optimal induction of *Mig* mRNA in B6 WT mice, γRF-2 is responsible for the low level Mig expression in VV-infected IFN-$\gamma^{-/-}$ mice. Guyer and colleagues (13) recently reported that the γRF-1 complex consists of three proteins, one of which is related antigenically to STAT-1α. It is possible that γRF-2 also contains a STAT-1 subunit, which binds to the γRE-1 element of the Mig promoter. In gel shift analysis, the addition of anti-STAT-1α antibody resulted in reduced mobility (super-shift) of γRF-2 and AAF, suggesting that these complexes are antigenically related to STAT-1α (Fig. 5). Because both AAF and γRF-2 exhibited identical mobility and are reactive to STAT-1 antibody, it is possible that γRF-2 is in fact AAF. Supporting this possibility is the recent finding of Ohmori *et al.* that the γRF-2 complex, which appears to be a STAT-1 homodimer, binds to the 3′-half-site of the γRE-1 motif (Ref. 42 and T. Hamilton, personal communication). In addition, our UV cross-linking studies on γRF-2 and AAF show two identical bands corresponding to molecular mass of 95 kDa (data not shown), consistent with the idea that γRF-2 complex may indeed be a STAT-1 homodimer. Although our data show that AAF and γRF-2 are identical (STAT-1 homodimers), we have used the term AAF in gel shift assays where the DNA binding element is labeled GAS element. On the other hand, the term γRF-2 is used in assays where the DNA binding element is γRE-1.

γRF-2 Binds γRE-1 and GAS Elements with Differential Affinity—We next examined the binding affinity of γRF-2, induced in IFN-$\gamma^{-/-}$ mice, to γRE-1 and GAS elements. A competition assay was prepared in which increasing amounts of unlabeled oligonucleotide containing one of the elements GAS (*GBP* gene), γRE-1 (*Mig* gene), or ISRE (*Crg-2* gene) were added to assays in which the complex was formed with radiolabeled oligonucleotides containing either the γRE-1 or GAS sites.

When GAS sites were used as probes, increasing amounts of the competitor (unlabeled GAS fragment) in binding reactions resulted in diminished levels of AAF-labeled GAS complex formation (Fig. 6*A*). A reduction in AAF·GAS complex formation was observed when the unlabeled γRE-1 fragment was increased by 10-fold or more molar excess. The intensity of the complex formation was quantified with a densitometer and

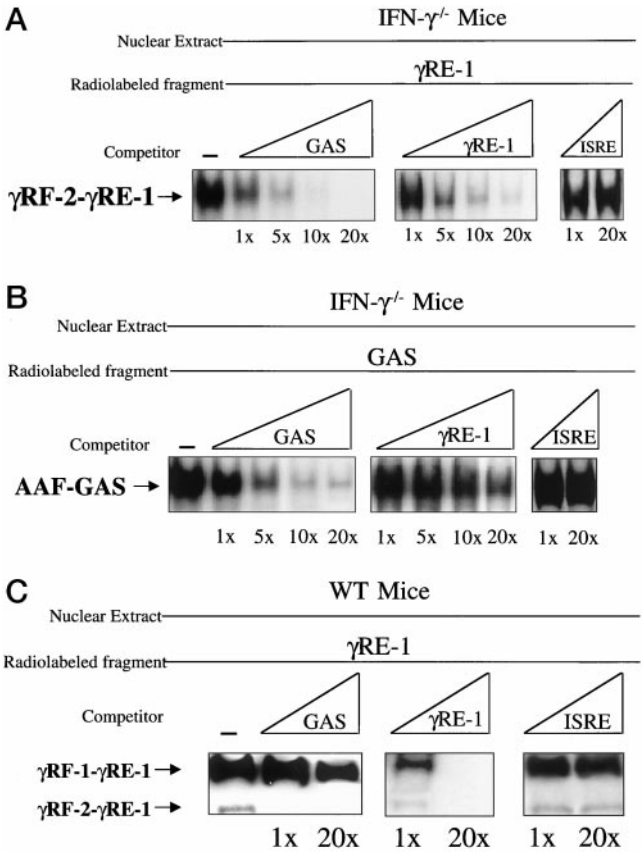

FIG. 6. **Binding affinity of γRF-1, γRF-2, and AAF to GAS and γRE-1 DNA elements.** Nuclear extracts were prepared from livers pooled from 3 IFN-$\gamma^{-/-}$ (*A* and *B*) and B6 WT (*C*) mice. All samples were taken at day 3 after infection with VV. Binding affinity to GAS (*A*) or γRE-1 (*B* and *C*) DNA elements was determined by EMSA, as described under "Experimental Procedures," using radiolabeled GAS or γRE-1 elements in a competition assay using severalfold excess of unlabeled oligonucleotides, as indicated. An ISRE element of the murine *Crg-2* gene served as a nonspecific oligonucleotide probe.

plotted against inhibitor concentration (data not shown). It was estimated that the apparent dissociation constant (the concentration of competitor DNA required to reduce AAF·GAS complex formation by 50%) for GAS was 0.4 nM and γRE-1 was 1.5 nM.

On the other hand, when the radiolabeled γRE-1 element was used as a probe and competed with a 1-fold molar excess of unlabeled GAS element, there was approximately a 50-fold reduction in γRF-2·γRE-1 complex formation as analyzed by densitometry (Fig. 6*B*). In contrast, 1-fold molar excess of unlabeled γRE-1 resulted in a 20-fold reduction in γRF-2·γRE-1 complex formation (Fig. 6*B*).

In competition experiments involving nuclear extracts from B6 WT mice probed with labeled γRE-1, a 1-fold molar excess of unlabeled GAS element resulted in loss of γRF-2·γRE-1 complex formation (Fig. 6*C*). Furthermore, a reduction in γRF-1·γRE-1 complex formation was observed when unlabeled GAS element was increased by 20-fold molar excess. When a 1-fold molar excess of unlabeled γRE-1 was used, γRF-2·γRE-1 complex formation was reduced markedly (Fig. 6C). These results demonstrate that the γRF-2 complex displays a higher binding affinity for GAS element than for γRE-1 element.

NF-κB Complex Is Induced in the IFN-$\gamma^{-/-}$ Mice—The presence of NF-κB sites in the *Mig* gene was previously shown to be important for transcription of *Mig* mRNA involving a synergistic interaction between IFN-γ and TNF (14). Because it is well documented that TNF is a potent inducer of NF-κB, it is possible that NF-κB motifs may also contribute to the low levels of

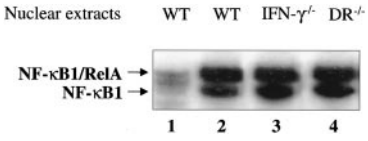

FIG. 7. **Activation of NF-κB1 and NF-κB1/RelA in livers of uninfected and virus-infected mice.** Nuclear extracts were prepared from livers pooled from groups of three mice, 3 days after infection with VV. Samples were from uninfected B6 WT mice (*lane 1*), VV-infected B6 WT (*lane 2*), IFN-$\gamma^{-/-}$ (*lane 3*), and DR$^{-/-}$ (*lane 4*) mice. Five micrograms of each nuclear extract was analyzed for the presence of these complexes by EMSA as described under "Experimental Procedures."

Mig mRNA in the IFN-$\gamma^{-/-}$ mice. However, levels of mRNA for TNF and NF-κB (data not shown) and of NF-κB protein·DNA complexes (Fig. 7) in livers of VV-infected B6 WT, IFN-$\gamma^{-/-}$, and DR$^{-/-}$ mice were similar. Treatment of VV-infected IFN-$\gamma^{-/-}$ mice with neutralizing antibodies to TNF did not result in loss of Mig expression (data not shown). These results suggest that TNF and NF-κB complexes do not play a significant role in the transcriptional activation of *Mig* mRNA in VV-infected IFN-$\gamma^{-/-}$ mice.

DISCUSSION

Cytokine-mediated intracellular signaling pathways have provided a general paradigm for the molecular mechanisms by which extracellular signals induce transcription of target genes. A number of cytokines, growth factors, and hormones trigger phosphorylation of latent cytoplasmic transcription factors termed STATs via one or more members of the Janus family of protein-tyrosine kinases (Jak) (2). Phosphorylated STATs assemble in dimeric or oligomeric form, translocate to the nucleus, and bind to specific DNA sequence motifs or SBEs (34). IFN receptors are coupled to the Jak/STAT signal transduction machinery (2, 38), which transcriptionally regulates a panel of genes that mediate the effects of IFNs, including their antiviral, antiproliferative, and immunomodulatory activities (39, 40). IFN-γ induces tyrosine phosphorylation of STAT-1α, and a homodimeric form of STAT-1α binds to GAS (33), a SBE that has been identified as a critical sequence motif involved in the transcriptional activation of many IFN-inducible genes, including the *IRF-1*, *ICAM*, and *Mig* genes (35–37). Some genes are stimulated transcriptionally by only one type of IFN, whereas others like the *guanylate-binding protein* (*GBP*) gene respond to IFN-α and IFN-γ (5, 33). The molecular pathways that allow two different ligand-receptor pairs to cause a similar biological response are of major interest in the study of intracellular signaling.

The present study was undertaken to clarify some molecular mechanism(s) involved in transcriptional regulation of *Mig* gene expression in the IFN-$\gamma^{-/-}$ mice. IFN-γ has previously been shown to be necessary for *Mig* mRNA expression in macrophages *in vitro* (8, 12). More recently, a reduction in *Mig* mRNA transcripts was demonstrated *in vivo* using a neutralizing antibody against IFN-γ in BALB/c mice infected with VV (15). In this report, we have analyzed the expression of Mig in response to VV infection in normal B6 WT mice and gene knockout mice lacking IFN function. The high levels of expression of this chemokine in spleens, ovaries, and uteri of normal, WT mice early after infection paralleled that of IFN-γ, IFN-α, and IFN-β expression (Figs. 1 and 3*A*). The high levels of Mig and IFN-γ expression in the ovaries and uterus also correlate with a previous report, which showed that VV replicates to high titers in these organs (32). These IFNs are induced early in infection and are known to play a critical role in the defense against poxvirus infections (19). Because the IFNs confer resistance to many types of viruses by activating a set of IFN-inducible genes, we speculated that Mig mediated some of the antiviral effects of IFNs. Indeed, we have recently demonstrated that Mig expressed by an rVV exhibited antiviral activity *in vivo* (24).

To define a more precise role for IFN-γ in the induction of Mig, we analyzed profiles of the chemokine in IFN-$\gamma^{-/-}$ mice after VV infection. Clearly, mRNA transcripts for Mig were detected in organs of these mice, albeit at reduced levels compared with normal B6 WT mice (Fig. 1*B*), indicating that factor(s) other than IFN-γ contributed to the expression of Mig in mice lacking the cytokine. This is the first demonstration of *Mig* mRNA expression *in vivo* in the complete absence of IFN-γ. Previous reports have shown that treatment of the macrophage cell line RAW 264.7 with cycloheximide and IFN-γ augmented Mig transcription (29), whereas treatment with cycloheximide alone does not (8). It was suggested that, in addition to increasing transcription, IFN-γ might also affect the stability of *Mig* mRNA (8, 29). Thus there was a possibility that the *Mig* mRNA expressed in the IFN-γ-deficient mice was unstable and did not translate to protein. However, our data indicate that, along with mRNA, Mig protein is also present in the liver extracts from VV-infected IFN-$\gamma^{-/-}$ mice (Fig. 2*B*).

To ascertain whether IFN-α and/or -β were responsible for induction of low levels of Mig expression in these animals, two approaches were taken. First, antibody to IFN-α/β was used to neutralize activity. In antibody-treated, VV-infected IFN-$\gamma^{-/-}$ mice, expression of this chemokine was completely abolished (Fig. 3*B*). Second, mice deficient in receptors for both IFN-α/β and IFN-γ (DR$^{-/-}$) were employed. The overall phenotype of the DR$^{-/-}$ mice is identical to that of STAT-1$^{-/-}$ mice in that they both display a global deficiency in their ability to respond to either IFN-γ or IFN-α/β (41). We confirmed the absence of STAT-1 protein complexes in the DR$^{-/-}$ mice by immunoblotting with antibody specific for STAT-1 (data not shown). We found that Mig was not induced in DR$^{-/-}$ mice following infection with VV (Fig. 3*C*). In contrast, IFN-α/β R$^{-/-}$ mice, with intact IFN-γ function, expressed Mig at similar levels to WT mice (data not shown). This was expected as IFN-γ has been shown to induce high levels of *Mig* mRNA through the induction of transcription factor γRF-1 (28). In VV-infected mice with intact IFN-α/β function, but deficient in IFN-γ receptors (IFN-γ R$^{-/-}$), liver *Mig* mRNA levels were similar to those observed in VV-infected IFN-$\gamma^{-/-}$ mice, but lower than VV-infected WT controls (Fig. 2*A*). Taken together, our data indicate that Mig can be induced *in vivo* by IFN-α/β in mice deficient for IFN-γ function, however, the levels are significantly lower.

The DNA binding factor γRF-1 is involved in IFN-γ-mediated transcriptional regulation through γRE-1, a major regulatory element in the Mig promoter (28). The γRF-1 complex is distinct from the IFN-γ-responsive transcription factor GAF but contains closely related members of the STAT protein family (13). The rapid and preferential induction of γRF-1 by IFN-γ in a variety of cells suggests that this factor is responsible for controlling a subset of IFN-γ-mediated responses, including Mig expression (13) even though, to date, the γRE-1 element has only been found in the *Mig* gene promoter. In this study, the levels and kinetic expression of γRF-1 detected in the liver is consistent with Mig expression in VV-infected B6 WT mice (Fig. 4, *A* and *B*). However, despite the absence of γRF-1 in VV-infected IFN-$\gamma^{-/-}$ mice (Fig. 4*A*), *Mig* mRNA was induced (Fig. 1*B* and Fig. 2*A*).

We have established the presence of an additional γRE-1-binding protein, which we have referred to as γRF-2 (Fig. 4*A*). This complex exhibited higher mobility than γRF-1 and was present in VV-infected B6 WT and IFN-$\gamma^{-/-}$ mice but not in VV-infected DR$^{-/-}$ or in IFN-$\gamma^{-/-}$ mice treated with antibody to IFN-α/β. Furthermore, γRF-2 induction paralleled *Mig*

mRNA expression in livers of infected IFN-$\gamma^{-/-}$ animals (Fig. 4*C*). These findings are consistent with the hypothesis that γRF-2 is responsible for *Mig* mRNA expression in the absence of IFN-γ function. Of note is the finding that the γRF-2 complex was more prominent in the IFN-$\gamma^{-/-}$ mice compared with WT mice. This may be due to the absence of γRF-1 in liver nuclear extracts of IFN-$\gamma^{-/-}$ mice, which can compete with γRF-2 for binding to γRE-1. Recently, Ohmori and colleagues (42) have reported that γRF-2 complex binds to the 3′-half-site of the γRE-1 motif and is likely to be a STAT-1 homodimer complex. In addition, our UV cross-linking studies show two bands corresponding to molecular mass of 95 kDa (data not shown) similar to AAF, suggesting that γRF-2 is also a STAT-1 homodimer.

To investigate the mechanism by which IFN-α mediates *Mig* mRNA expression in the absence of IFN-γ, candidate transcription factors ISGF3, AAF, and IRF-1 were considered. The role of ISGF3 was ruled out, because there was no cross competition between γRE-1 and ISRE of the *GBP* gene by EMSA (data not shown). Furthermore, the absence of IRF-1 binding sites in the Mig promoter excludes the involvement of this transcription factor. A comparison of the "GAS core site" consensus sequence TT(C/A)CNNNAA with γRE-1 reveals homology to both halves of the γRE-1 imperfect palindrome (28). The significance of this homology is unclear, but it may play a role in the binding to STAT-1 protein. There is a distinct possibility that the expression of Mig in VV-infected IFN-$\gamma^{-/-}$ mice is mediated through AAF, which is able to activate GAS driven genes (33). Supporting this idea is the observation that IFN-α, like IFN-γ, stimulates the appearance of a nuclear GBP-1 GAS binding activity (5, 33). This mechanism would allow IFN-α to directly induce some of the genes activated by IFN-γ. Here we have shown that AAF was activated in infected IFN-$\gamma^{-/-}$ mice but not in infected $DR^{-/-}$ mice (Fig. 4*A*). Consistent with this, low affinity binding of AAF to γRE-1 did occur between the two components as demonstrated in competition experiments using EMSA (Fig. 6*A*). We have at least four lines of evidence that suggest that γRF-2 is indeed AAF. First, γRF-2 and AAF exhibit identical mobility on EMSA (Fig. 4*A*). Second, both factors are reactive with STAT-1 antibody (Fig. 5). Third, formation of γRF-2·γRE-1 complex was abolished when competed with a 1-fold molar excess of unlabeled GAS element (which is specifically bound by AAF with high affinity) (Fig. 6*C*) and γRF-2 binds with higher affinity to GAS elements than to γRE-1 element (Fig. 6*C*). Fourth, UV cross-linking studies for both factors revealed the presence of two bands with a molecular mass of approximately 95 kDa, which correspond to that of STAT-1 (data not shown).

Other potential regulatory elements have been identified in the Mig promoter region, which includes a sequence with similarity to the NF-κB binding site located at position −145 to −154 and an AP-2 binding site at position −214 to −221 (29). The finding of an NF-κB-like site raised the possibility that the *Mig* gene might also be regulated by TNF. However, our results do not support a role for TNF with respect to transcription of Mig in VV-infected IFN-$\gamma^{-/-}$ mice. There were no differences in levels of expression of TNF mRNA, NF-κB mRNA (data not shown), or NF-κB complexes (Fig. 7) in livers of WT, IFN-$\gamma^{-/-}$, and $DR^{-/-}$ mice. These findings suggest that *Mig* mRNA expression in infected IFN-$\gamma^{-/-}$ mice is not mediated through NF-κB complexes.

Taken together, our data suggest that the STAT-1α subunit of γRF-2 binds to γRE-1 in the Mig promoter and drives the transcription of *Mig* gene in the IFN-$\gamma^{-/-}$ mice. In this regard, a recent report showed that IFN-γ treatment of fibroblasts from STAT-1-deficient mice failed to induce Mig expression, indicating a requirement for STAT-1 protein for transcription of this chemokine (14).

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