Hepatitis C Virus Core Protein Modulates the Interferon-Induced Transacting Factors of Jak/Stat Signaling Pathway But Does Not Affect the Activation of Downstream IRF-1 or 561 Gene

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INTRODUCTION

Hepatitis C virus (HCV) often causes a prolonged and persistent infection in humans. The most important feature of persistent HCV infection is the development of chronic hepatitis in half of the infected individuals, and the potential for disease progression to hepatocellular carcinoma (Saito et al., 1990; DiBisceglie et al., 1998; Hayashi et al., 1999; Jeffers, 2000). This has been attributed in part to the effect of viral gene product(s) on the expression of host cellular genes. Unfortunately, a number of important issues related to HCV persistence and disease progression are unknown at this time. Furthermore, neither a vaccine nor any other means of an effective chemotherapy is available to eradicate HCV infection. HCV genotypes 1a and 1b are predominant in patients with chronic hepatitis C in the United States (Zein et al., 1996), and interferon-α (IFNα) alone or in combination with ribavirin is the current available therapy (Martin et al., 1998). However, a significant number of cases do not respond well to therapeutic treatment, and the reason is not clear (Moradpur and Blum, 1999; Guidotti and Chisshani, 2000).

Signal transducers and activators of transcription (STATs) have drawn considerable attention due to their unique mode of action, and diversity of biological effects, which include the ability to control an immune response and an antiviral effect and to participate in cell transformation (Ihle, 1996; O’Shea, 1997). IFNα and IFNγ bind to their respective cell surface receptors and activate distinct but related signal transduction pathways, culminating in the activation of an overlapping set of interferon-sensitive genes (ISGs). Upon binding of IFNα/β to receptors, their associated protein kinases (Jak1 and Tyk2) phosphorylate Y701 of Stat1 and Y690 of Stat2. Stat1 and Stat2 then heterodimerize, translocate to the nucleus, and associate with p48 to form ISG factor 3 (ISGF3). ISGF3 activates the transcription of genes containing IFN-stimulated response elements (ISREs) within their promoters. The full activity of Stat1 also depends upon the phosphorylation of S727 via the mitogen-activated protein kinase pathway. On the other hand, IFNγ upon binding to specific receptors will similarly phosphorylate Y701 of Stat1 through their associated protein tyrosine kinases (Jak1 and Jak2). Phosphorylated Stat1 homodimerizes to form γ-activated factor (GAF), which activates the transcription of genes containing γ-activated
sequence (GAS) elements within their promoters. Phosphorylation of Stat1 on S727 is also required for the full activity of GAF (Darnell et al., 1994; Stark et al., 1998).

MHC class II expression is controlled predominantly at the level of transcription (Boss, 1997). IFNα/β and IFNγ has been observed to enhance the expression of MHC class I and MHC class II molecules (Taniguchi et al., 2001). The expression of MHC class II molecules is also uniquely regulated by IRF-1, via an indirect mechanism. The expression of CIITA, a critical transcription factor for MHC class II gene induction by IFN, is also uniquely regulated by IRF-1, via an indirect mechanism. The expression of CIITA, a critical transcription factor for MHC class II gene induction by IFN, is also uniquely regulated by IRF-1, via an indirect mechanism.

RESULTS

Effect of HCV protein on Stat1 expression in IFN-treated cells

Interferon treatment of cells causes immediate activation of the transacting factor GAF and ISGF3. Once activated they translocate from cytoplasm to nucleus, bind to the ISRE or GAS element of IFN inducible genes, and activate their transcription. Since Stat1 is a component of both the activation factors GAF and ISGF3 complex, the distribution of Stat1 was investigated by an indirect immunofluorescence using mock transfected control, or stable transfectants of HeLa cells expressing core protein alone or together with other HCV proteins in untreated and IFN-treated cells. Additionally, stable transfectants of HeLa cells expressing CD4 antigen (HeLaT4) was included in this experiment to verify the effect of an unrelated ectopic protein expression upon translocation of Stat1 following IFN treatment. Untreated cells exhibited a weak intracellular localization of the Stat1 protein (Figs. 1a, 1d, 1g, and 1j). However, a bright nuclear fluorescence of Stat1 was observed upon treatment of control HeLa or HeLa T4 cells with IFNα or IFNγ (Figs. 1b, 1c, 1k, and 1l). In contrast, a diffuse and predominantly perinuclear localization of Stat1 was observed in IFN-treated HeLa cells expressing core protein alone or together with other HCV proteins (Figs. 1e, 1f, 1h, and 1i). IFNα or IFNγ treatment of HCV protein expressing HT1080 or HepG2 cells exhibited a relatively weak nuclear translocation of Stat1 when compared to the mock transfected control (data not shown). Thus, HCV core protein is probably modulating the Jak/Stat pathway at a stage prior to GAF or ISGF3 activation.

HCV core protein expression reduces the level of IFN-induced GAF and ISGF3 transacting factors

To investigate the possible role of HCV core protein in GAF or ISGF3 activation, a gel retardation assay was performed. IFN-treated or untreated HeLa, HepG2, and HT1080 cells stably expressing core protein alone or together with other HCV proteins were used for this purpose. Mock transfected cells were separately used as control. Formation of a DNA–protein complex with the mobility and specificity of GAF was observed in both HeLa and core expressing HeLa cells following IFNγ treatment. The image of the separation profile is shown in Fig. 2A. The specificity of GAF complex was verified using an anti-Stat1 antibody which disrupted the intensity of the GAF–DNA complex. While use of an anti-Stat2 antibody
or unrelated antibody (data not shown in figure) as control did not inhibit GAF–DNA complex formation. In some cases, addition of antibody disrupts the formation of the complex rather than further changing its mobility (Sambrook and Russell, 2001). This may be due to high affinity of the antibody to Stat1, resulting in competition of the DNA binding sites for complex formation. Our observation of a decreased intensity of the GAF complex using an antibody to Stat1 is similar to those reported earlier by other investigators (Li et al., 1998, 1999). The uncharacterized band below GAF appeared in extracts of both untreated and IFN-treated cells, as observed earlier (Levy et al., 1990; Li et al., 1998). This complex was specifically inhibited by antibody to Stat1 or Stat2; however, anti-Stat2 antibody did not inhibit GAF formation. These complexes may represent inactivated heteromeric form of the Stats and awaits further characterization.

Densitometric scanning of the autoradiogram suggested that the GAF–DNA complex is ~4.8-fold higher in mock transfected control cells as compared to the core protein transfected HeLa cells (Figs. 2A and 2B). The GAF–DNA complex formation was also examined in HeLa cells stably transfected with HCV1-2962 plasmid. Mock transfected control HeLa cells exhibited a ~2.5-fold higher level of GAF–DNA complex formation than HeLa cells expressing core along with other HCV proteins (Figs. 2A and 2B). A similar finding was observed in HT1080 and HepG2 cells. The GAF–DNA complex formation was ~fourfold higher in mock transfected HT1080 control cell line than in cells transfected with

FIG. 1. Effect of HCV protein on Stat1 expression in IFN-treated HeLa cells. Mock transfectants (a, b, and c), stable transfectants expressing core protein alone (d, e, and f), or together with other HCV proteins (g, h, and i), and HeLa cells expressing unrelated CD4 protein as an additional control (j, k, and l) were either left untreated (a, d, g, and j) or treated with IFNγ 1000 U/ml (b, e, h, and k) or 500 U/ml IFNα (c, f, i, and l) for 30 min at room temperature. Cells were incubated with a monoclonal antibody to Stat1-α. A second antibody, goat anti-mouse Ig, conjugated to fluorescein isothiocyanate was added for cell staining. Treatment with IFNγ or IFNα exhibited a strong and distinct nuclear immunofluorescence in control HeLa cells. In contrast, cells expressing core protein alone or together with other HCV proteins displayed a weak Stat1 expression, predominantly localized in the perinuclear regions. Arrows indicate the distribution of Stat1 in IFN-treated control and HCV gene transfected cells.
FIG. 2. Inhibition of GAF–DNA complex formation by HCV protein(s) in IFN-γ-treated cells. Mock transfected control or experimental cells (as marked on top) were either treated with IFN-γ (500 U/ml for 90 min) or left untreated. Nuclear extracts from cells were examined for GAF–DNA complex formation by a gel retardation assay with a probe corresponding to the GAS element of the IRF-1 gene (a, c, and e). The addition of an antibody to Stat1 in the nuclear extract–DNA reaction mixture inhibited GAF–DNA complex formation. The amount of complex formation was quantitated by densitometric scanning and presented as a bar diagram (b, d, and f).
HCV core or HCV\textsubscript{1–2962} plasmid DNA (Figs. 2C and 2D).

Similarly, an increased GAF–DNA complex formation (~twofold) was observed in mock transfected HepG2 cells as compared to HepG2 cells transfected with core or HCV\textsubscript{1–2962}-expressing plasmid (Figs. 2E and 2F). Further analysis with an antibody to Stat1 verified inhibition of GAF–DNA complex formation in both HT1080 and HepG2 cells and identified Stat1 as a component of the complex.

ISGF3–DNA complex formation was also analyzed using ISRE as a probe in IFN\textsubscript{α}-treated control and core-expressing HeLa cells (Fig. 3A). The mobility and specificity of the ISGF3–DNA complex was verified using Stat-specific antibodies which competed with the protein–DNA complex formation. The ISGF3–DNA complex was ~threefold higher in mock transfected HeLa control as compared to core or HCV\textsubscript{1–2962}-transfected HeLa cells (Fig. 3B). A similar result of ~threefold higher ISGF3–DNA complex formation was observed in the control over core-transfected HT1080 cells (Figs. 3C and 3D) and in HepG2 cells (data not shown). The gel shift assays for GAF and ISGF3 DNA complex formation were reproducible from at least three different experiments. Together, our results indicated that the HCV core protein modulates both GAF and ISGF3 protein complex formation in IFN-treated cells.

**FIG. 3.** Inhibition of ISGF3–DNA complex formation by HCV protein(s) in IFN\textsubscript{α}-treated cells. Mock transfected control or experimental cells (as marked on top) were treated with IFN\textsubscript{α} (1000 U/ml for 90 min) or left untreated. Nuclear extracts from cells were examined for ISGF3–DNA complex formation by gel retardation assay with a probe corresponding to the ISRE element of the 561 gene (a and c). Addition of antibodies to Stat1 or Stat2 in the nuclear extract–DNA reaction mixture inhibited ISGF3–DNA complex formation. The amount of complex formation was quantitated by densitometric scanning and presented as fold difference of the ISGF3 DNA complex in different cell lines (b and d).
Effect of IFN on expression of the signaling proteins

Our results indicated that HCV core-protein expression lowers the level of GAF and ISGF3 in IFN-treated cells. Thus, we investigated the protein expression level of Stat1 and p48 in control and core-expressing HeLa cell lysates by Western blot analysis using specific antibodies. Results suggested an approximately threefold decrease in Stat1 protein expression in core-expressing HeLa cells than mock transfected control cells (Fig. 4A). However, the level of p48 remained similar in both the experimental and the control cells (Fig. 4C).

The phosphorylated Stat1 level was also investigated in IFNα-treated control and experimental HeLa cells by immunoprecipitation. Cell lysates were immunoprecipitated by a Stat1 monoclonal antibody and subjected to Western blot analysis using an antibody directed to the phosphotyrosine of Stat1α. Phosphorylated tyrosine moieties of Stat1α were detected by chemiluminescence (b). Expression levels of Stat1α/β and p48 were analyzed by Western blot using a monoclonal antibody to Stat1α (a, e, and h) and a polyclonal antibody to p48 (c, f, and i). An antibody to cellular actin was used as an internal control (d, g, and j). Arrows on the right indicate the respective polypeptides, and their molecular sizes correlated with the migration of standard molecular weight markers (Gibco BRL).

FIG. 4. Effect of core protein on Stat1 and p48 expression in IFNα-treated cells. Mock transfected control (left lanes) or core gene transfected (right lanes) cells were treated with 1000 U/ml IFNα for 30 min. HeLa cell lysates were immunoprecipitated with a monoclonal antibody to Stat1α. Immunoprecipitates were separated by SDS–8% PAGE and transferred onto nitrocellulose for probing with a monoclonal antibody to phosphotyrosine conjugated with horseradish peroxidase. Phosphorylated tyrosine moieties of Stat1α were detected by chemiluminescence (b). Expression levels of Stat1α/β and p48 were analyzed by Western blot using a monoclonal antibody to Stat1α (a, e, and h) and a polyclonal antibody to p48 (c, f, and i). An antibody to cellular actin was used as an internal control (d, g, and j). Arrows on the right indicate the respective polypeptides, and their molecular sizes correlated with the migration of standard molecular weight markers (Gibco BRL).

twofold following IFNα treatment in mock transfected HeLa cells than HCV core-expressing cells (Fig. 5). Similar observations were made from cells treated with IFNγ (data not shown). All of the experiments presented in Figs. 4 and 5 are representative of at least three independent experiments exhibiting similar results. These results suggested that HCV core protein modulates Stat1 expression at the transcriptional level. Thus, the core protein appears to modulate the Jak/Stat pathways by lowering the abundance of Stat1 protein.

HCV protein(s) does not inhibit induction of IFN stimulated genes

ISRE-mediated signaling was examined from the level of gene induction of two alternative IFN signaling pathways. IRF-1 mRNA is induced by IFNγ with GAS as a cis element and GAF as the transcription factors. On the other hand, 561 mRNA is induced by IFNα using ISRE as a cis element and ISGF3 as cognate transcription factor. Cellular levels of the IFNγ-inducible IRF-1 mRNA and IFNα-inducible 561 mRNA were analyzed by RNase protection assay. Activation of the IRF-1 mRNA upon IFN treatment (500 U/ml) was observed at a similar level for at least up to 12 h in both mock transfected and core or HCV protein expressing HeLa cells (Figs. 6A and 6B), and a similar result was observed using a lower dose (100 U/ml) of IFNγ (data not shown). Activation of IRF-1 mRNA was also observed following IFNγ treatment of HepG2 cells (Figs. 6C and 6D). Investigation for 561 gene induction suggested an increase in mRNA level within
6 h of IFNα treatment, which then decreased slightly between 6 and 12 h in HepG2 cells (Fig. 6E). The reason for this decline of 561 mRNA is unclear at present. Time-dependent activation of 561 was also observed in HeLa cells (data not shown). These results suggested that even a reduced level of GAF or ISGF3 complex is sufficient to activate the interferon-stimulated downstream effector genes of Jak/Stat signaling pathways, and this effect appeared to be dependent upon IFN stimulation.

**DISCUSSION**

Results from this study indicate that expression of core protein alone or together with other HCV proteins modulates the transacting factors GAF or ISGF3 of the IFN-induced Jak/Stat signaling pathway. This may be related to a decreased abundance of the Stat1 protein and/or a weak translocation of Stat1 to the cell nucleus. However, these modulatory effects do not interfere with IFN-induced downstream activation of the IRF-1 and 561 gene expression. We have used ectopic expression of HCV protein in this study as that will be more relevant to the nature of chronic HCV infection, when IFNα treatment is normally offered. Our results from expression of HCV proteins agree in part with the reduced GAF or ISGF3 formation observed following inducible expression of the entire HCV open reading frame in osteosarcoma cells (Heim *et al.*, 1999), although a difference in Stat1 expression level was observed in our study. This difference in results could be attributed to inducible vs stable expression system and the nature of the cell lines used. Our study also suggests a difference in expression level based on the cell types used. However, our results indicate that even a reduced level of GAF or ISGF3 complex in IFN-treated cells is sufficient to activate IS genes, IRF-1 and 561. Thus, we find evidence to suggest a role for the HCV core protein in the inhibition or impairment of the Stat1 expression, which can be augmented by IFN treatment for induction of the downstream effector gene of Jak/Stat pathway in HeLa, HT1080, or HepG2 cells.

IFNα regulates the function of cytokines, their receptors, and other molecules of immune importance (Goodbourn *et al.*, 2000). A large number of genes are induced by IFNα, but only a few of these gene products have been associated with an intrinsic antiviral activity. Those identified include the p68 protein kinase (PKR) and 2′-5′-oligoadenylate synthetase (2′-5′-OAS), which are both dependent upon double-stranded RNA for activity, certain MxA family proteins, and the most recently identified promyelocyte leukemia protein (Goodbourn *et al.*, 2000).

While IFN-treated cells are resistant to many different viruses, individual overexpression of these intrinsically antiviral proteins confers host resistance only against some viruses. HCV E2 and NS5A proteins have been implicated in IFN resistance through inhibition of PKR (Gale *et al.*, 1997, 1998; Taylor *et al.*, 1999). Inhibitory properties of HCV E2 glycoprotein may occur by mimicking the autophosphorylation site via a pseudosubstrate mechanism (Taylor *et al.*, 2001). PKR inhibition by E2 or NS5A protein may contribute but do not solely explain the resistance to sustained IFN treatment (Polyak *et al.*, 2000; Francois *et al.*, 2000; Gerotto *et al.*, 2000). In chronic HCV patients, the expression of 2′-5′-OAS and MxA are not significantly different at the hepatic mRNA level when compared with chronic HBV or other liver diseases of different etiology (Yu *et al.*, 2000). HCV core protein also activates 2′-5′-OAS gene in hepatocyte cell lines, which is further enhanced upon treatment with interferon (Naganuma *et al.*, 2000). Unfortunately, the lack of a suitable virus-cell culture system for study of HCV growth does not allow the study the contribution of IFN induced proteins for antiviral response at this time.

The function of Stat1 is to regulate a set of genes that collectively provide innate immunity (Ihle, 1996). The homozygous mutant cells and animals devoid of functional Stat1 protein revealed that Stat1 is indispensable for the IFN pathway, but may not be necessary for other signaling systems for normal development (Durbin *et al.*, 1996).
However, mutant animals demonstrated the essential nature of Stat1-dependent system for survival in the face of otherwise innocuous viral pathogens. This may result from the combined loss of both type I and type II IFN responses, which has been shown to impair some anti-viral responses (van den Broek et al., 1995). Stat1-dependent innate immunity, presumably produced in response to IFN, appears to be a crucial host defense mechanism combating mouse hepatitis virus (MHV) disease. A majority of humans fail to develop protection against HCV infection and Stat1 modulation by core protein may contribute to this defect.

Although Stats are translocated to the nucleus after activation, neither the mechanism underlying this translocation nor the reason for their retention in the cytoplasm prior to stimulation is well understood (O'Shea,
contain highly GC-rich sequences and consensus binding sequences for several known transcription factors, including NF-κB (Harada et al., 1994). However, our observations do not support such an alternative effect, especially when results from IFNα and IFNγ are considered, since both of these pathways are partially overlapping through Stat1. Even though our results suggest that IFN-stimulated genes of the Jak/Stat pathway are not impaired or blocked by HCV proteins, the relative weakness of IFNα therapy may arise from virus-mediated interruption of other gene products with antiviral activity or from the nature of poor antiviral activity associated with human hepatocytes.

MATERIALS AND METHODS

Cell lines, plasmid construct, and protein expression

Human cervical carcinoma (HeLa) and human hepatoma (HepG2) cells were obtained from the American Type Culture Collection (Rockville, MD). The human fibrosarcoma epithelial-like HT1080 cells were kindly provided by G. R. Stark (Cleveland Clinic Foundation, OH). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum. A full-length HCV clone (kindly provided by C. M. Rice, Washington University, St. Louis, MO) was digested with suitable restriction enzymes and the genomic region representing the structural and nonstructural proteins (amino acids 1–2962) was subcloned into a pcDNA mammalian expression vector, introducing a stop codon at the translational end. Cloning of the HCV genomic region representing the core protein (amino acids 1–191) has been described earlier (Ray et al., 1995). Subconfluent cell monolayers were transfected with a mammalian expression vector containing HCV (pcDNA/ HCV1–2962) or HCV core (pcDNA3/Core 1–191) gene using lipofectamine (Gibco BRL, MD). An empty vector DNA was transfected into cells for use as a control. Neomycin-resistant stable transfectants were pooled to avoid clonal selection and tested for integration of HCV gene and protein expression. Stable cell transfectants exhibited NSSA and/or core protein expression (depending on the plasmid transfected) separately by Western blot analysis and indirect immunofluorescence (Ray et al., 1996b) using monoclonal antibodies (mAbs) to NSSA protein (Biogenesis, Inc., MA) or core protein (C7-50, kindly provided by Jack R. Wands, Harvard Medical School, MA). Additionally, cells transfected with the pcDNA/ HCV1–2962 gene exhibited distinct immunofluorescence with monoclonal antibody to E1 or E2. Transfected cells did not exhibit unusual growth characteristics and were analyzed for the role of viral protein(s) in IFN signaling pathways.
Immunofluorescence

Stable cell transfectants were examined for subcellular localization of Stat 1 expression by a specific mAb (Santa Cruz Biotechnology, CA). Briefly, cells grown overnight on coverslips were left untreated or treated with IFNγ (500 U/ml) or IFNα (1000 U/ml) for 30 min. Cells were washed and then fixed with methanol/acetone (1:1) for 2 min. After washing, cells were incubated with the mAb at room temperature for 1 h, washed, and stained with a goat anti-mouse IgG conjugated to fluorescein isothiocyanate. A human CD4 gene stably transfected into HeLa cells was also included as an unrelated control in similar immunofluorescence study.

Immunoprecipitation and Western blot analysis

Cells were treated with 1000 U/ml IFNα or 500 U/ml of IFNγ for 90 min, washed, and lysed in 1× lysis buffer (0.5% NP-40, 50 mM Tris–HCl, pH 8.0, 10% glycerol, 0.1 mM EDTA, 300 mM NaCl, 1 mM DTT, and a cocktail of protease inhibitors) for 1 h on ice. Cell lysates were incubated with a monoclonal antibody to Stat1-α at 4°C and the immunoprecipitate was immobilized onto protein A–Sepharose CL-4B (Pharmacia, NJ). The sample was subjected to electrophoresis on a SDS–polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane and blocked with 5% low-fat milk. HRP-conjugated second antibody to mouse Ig was used to visualize the protein bands by enhanced chemiluminescence (ECL; Amersham, IL). A–Sepharose CL-4B (Pharmacia, NJ). The sample was subjected to electrophoresis on a SDS–polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane and blocked with 5% low-fat milk. HRP-conjugated second antibody to mouse Ig was used to visualize the protein bands by enhanced chemiluminescence (ECL; Amersham, IL).

Gel retardation assay

The gel retardation assay for GAF and ISGF3 was performed as previously described (Leonard and Sen, 1996). Briefly, cells were treated for 30 min with 1000 U/ml of IFNα or 500 U/ml of IFNγ and untreated cells were used as control. Cells were lysed with NP-40 in low-salt buffer (10 mM HEPES, pH 7.9; 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and a cocktail of protease inhibitors) on ice for 5 min. The nuclear pellet was collected by centrifugation and suspended in a high-salt buffer (20 mM HEPES, pH 7.9; 0.4 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 1 mM PMSF) for 30 min at 4°C. Nuclear extracts were obtained by centrifugation at 12,000 g, and aliquots were flash frozen immediately for storage at −70°C. The protein concentration of the nuclear extracts was determined using protein assay reagent (Bio-Rad, CA). The GAS element located between −129 and −106 of the IFNγ-inducible IRF1 gene, and ISRE element located between −125 and −93 of the IFNα 561 gene (Leonard and Sen, 1997), were used as probes (kindly provided by G. C. Sen, Cleveland Clinic Foundation, OH) for our studies. The annealed double-stranded probes were end labeled with [α-32P]dCTP using a DNA polymerase to fill in the ends. Gel retardation assay was initially standardized by varying the quantity of the nuclear extracts in obtaining a linear range of detection of the specific complexes. Finally, 10 μg of nuclear proteins were incubated with 32-P-labeled oligonucleotide probe in the presence of poly[dI-C] in a binding buffer (50 mM HEPES, pH 7.5; 250 mM NaCl, 2.5 mM EDTA, 1 mM MgCl₂, 2.5 mM DTT, and 1 mM PMSF, 5 mM MgCl₂, 20% glycerol, and a cocktail of protease inhibitors) for 30 min at room temperature. The specificity of the Stat1 protein in DNA–protein complex was identified by adding a murine monoclonal antibody to Stat1 (Santa Cruz) to the reaction mixture before formation of the complex. A rabbit polyclonal antibody to Stat2 (Santa Cruz), unrelated murine monoclonal antibodies, or calf serum as negative controls, were used separately for determining the inhibitory effect on DNA–protein complex formation. The samples were electrophoresed until the free radiolabeled probe was removed from the nonadenating 5% polyacrylamide separation gel and exposed to X-ray film for autoradiography. The autoradiogram was scanned densitometrically for a comparison of band intensity.

RNase protection assay

The induction of Stat1, IRF-1, and 561mRNAs were analyzed using specific probes as described earlier (Kalvakolanu et al., 1991; Leonard and Sen, 1997). A 409-nucleotide fragment of the Stat1 gene (Pharmingen, CA), a 175-nucleotide fragment (nucleotide positions 192–367) of the IRF-1 gene, or 142-nucleotide fragment (nucleotide positions 1369–1511) of the 561 gene (kindly provided by G. C. Sen) was used as a probe. A 113-nucleotide-long L32 and 128-nucleotide-long γ-actin probe was used for internal control. Briefly, total RNA was isolated from cells, with or without IFN treatment, using a RNA isolation kit (Gentra Systems, MN). An antisense RNA probe was labeled with [32P]UTP and cold NTPs using SP6 or T7 RNA polymerase. The probe was hybridized in excess to target cellular RNA (2 μg) overnight. Free probe and single-stranded RNA were digested with RNAseA and RNAseT1, followed by proteinase K treatment. The protected RNA was recovered by phenol/chloroform extraction, ethanol precipitation, and identified by resolving on a 5% polyacrylamide gel by electrophoresis, followed by autoradiography. The protected RNA band in the autoradiogram was densito-
metrical scanned to quantitate intensity after normalization against the L32 or γ-actin as an internal control.

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