Hepatitis C Virus Nonstructural 5A Protein Induces Interleukin-8, Leading to Partial Inhibition of the Interferon-Induced Antiviral Response

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Hepatitis C virus (HCV), a major cause of liver disease worldwide, is frequently resistant to the antiviral alpha interferon (IFN). The HCV nonstructural 5A (NS5A) protein has been implicated in HCV antiviral resistance in many studies. NS5A antagonizes the IFN antiviral response in vitro, and one mechanism is via inhibition of a key IFN-induced enzyme, the double-stranded-RNA-activated protein kinase (PKR). In the present study we determined if NS5A uses other strategies to subvert the IFN system. Expression of full-length NS5A proteins from patients who exhibited a complete response (FL-NS5A-CR) or were nonresponsive (FL-NS5A-NR) to IFN therapy in HeLa cells had no effect on IFN induction of IFN-stimulated gene factor 3 (ISGF-3). Expression of mutant NS5A proteins lacking 110 (NS5A-ΔN110), 222 (NS5A-ΔN222), and 334 amino-terminal amino acids and mutants lacking 117 and 230 carboxy-terminal amino acids also had no effect on ISGF-3 induction by IFN. Expression of FL-NS5A-CR and FL-NS5A-NR did not affect IFN-induced STAT-1 tyrosine phosphorylation or upregulation of PKR and major histocompatibility complex class I antigens. However, NS5A expression in human cells induced interleukin 8 (IL-8) mRNA and protein, and this effect correlated with inhibition of the antiviral effects of IFN in an in vitro bioassay. NS5A induced transcription of a reporter gene driven by the IL-8 promoter, and the first 133 bp of the IL-8 promoter made up the minimal domain required for NS5A transactivation. NS5A-ΔN110 and NS5A-ΔN222 stimulated the IL-8 promoter to higher levels than did the full-length NS5A protein, and this correlated with increased nuclear localization of the proteins. Additional mutagenesis of the IL-8 promoter suggested that NF-κB and AP-1 were important in NS5A-ΔN222 transactivation in the presence of tumor necrosis factor alpha and that NF-IL-6 was inhibitory to this process. This study suggests that NS5A inhibits the antiviral actions of IFN by at least two mechanisms and provides the first evidence for a biological effect of the transcriptional activity of the NS5A protein. During HCV infection, viral proteins may induce chemokines that contribute to HCV antiviral resistance and pathogenesis.

Chronic hepatitis C virus (HCV) infection is a significant clinical problem affecting an estimated 150 million individuals worldwide and 3.9 million individuals in the United States. About 85% of people infected with HCV develop chronic infection, and approximately 70% of patients develop histological evidence of chronic liver disease (41). Alpha interferon (IFN) is a Food and Drug Administration-approved treatment for chronic HCV infection. Only 8 to 12% of patients with HCV genotype 1 have a sustained clinical virological response to IFN therapy (4, 43, 61). Recently, combination therapy with interferon and the guanosine analogue ribavirin was shown to be superior to IFN monotherapy in producing sustained biochemical and virological responses (9, 45, 62). However, despite the significant improvement in rates of sustained response, as many as 60% of patients with high-titer HCV genotype 1 infection are nonresponsive to combination therapy.

When IFN binds to its receptor, two receptor-associated tyrosine kinases of the STAT/JAK family, Tyk2 and Jak1, become activated. These activated kinases phosphorylate STAT-1 and STAT-2 on a single conserved tyrosine residue (8). STAT-1 and STAT-2 form heterodimers and combine with the p48 protein to form an active transcription factor known as IFN-stimulated gene factor 3 (ISGF-3). ISGF-3 binds to a common element termed the interferon-stimulated response element (ISRE), found in the promoter regions of all IFN-stimulated genes, whereupon transcription occurs. Expression of the entire HCV polyprotein has been shown to inhibit IFN-induced STAT/JAK signaling in human U2-OS osteosarcoma cells (25). It was not reported which HCV protein was responsible for this effect.

Recent studies have led to exciting discoveries in the emerging research area of the roles of HCV proteins in antiviral resistance. Two examples are the interaction of the HCV non-
structural 5A protein (NS5A) and the second envelope (E2) glycoprotein with the IFN-induced, double-stranded-RNA-activated protein kinase (PKR). PKR is one of the major intracellular enzymes that mediate the antiviral action of IFN (32). Both the NS5A and E2 proteins inhibit PKR activity, which is postulated to allow HCV replication to continue in the presence of an IFN-induced antiviral response (19, 77). For E2, the interaction with PKR requires a 12-amino-acid domain (77), which is a highly stable element that does not mutate during antiviral therapy (1, 57). For NS5A, the interaction with PKR requires the IFN-sensitivity-determining region (ISDR) on NS5A, a region that is associated with clinical IFN resistance in Japanese and Spanish patients (6, 12, 13, 36, 67). Since accumulation of mutations in the ISDR also prevented the NS5A-PKR interaction (17), ISDR-dependent inhibition of PKR seemed to provide a molecular explanation of HCV resistance to IFN. However, subsequent studies have shown that there is no correlation between ISDR mutations and IFN response in France, Germany, Italy, and the United States (7, 11, 15, 22, 27, 56, 60, 64, 65, 72, 73, 79), which has caused considerable debate (26). In a prospective study that sequenced entire NS5A genes, we demonstrated that the response to antiviral therapy correlates with mutations in two regions in the C terminus of the NS5A protein (52), the previously described V3 region (11, 30), and a region that overlaps the proline-rich region of NS5A.

NS5A has also been intensely studied in vitro, and we (58) and others (18, 55, 71) have established in vitro bioassays showing that expression of NS5A in vitro inhibits the antiviral actions of IFN against IFN-sensitive viruses. Intriguingly, these in vitro bioassays have shown that the regions on NS5A required for inhibiting PKR activity may not be required for inhibiting antiviral actions of IFN (55, 58). Using a similar bioassay, a recent study found that expression of the entire HCV polyprotein also inhibited the antiviral actions of IFN independently of PKR (14). Cumulatively, these studies suggest that NS5A-mediated inhibition of the IFN system may involve mechanisms other than targeting of PKR.

The CXC chemokine interleukin-8 (IL-8) is a 71-amino-acid chemotactic cytokine. IL-8 is induced primarily by the cytokines IL-1 and tumor necrosis factor alpha (TNF-α) and is produced by many cells, including fibroblasts and hepatocytes. IL-8 is a proinflammatory cytokine that induces neutrophil, T-lymphocyte, and basophil chemotaxis and degranulation (49). IL-8 has been shown to be a principal mediator of the inflammatory response to many viruses and bacteria and has recently been shown to inhibit the antiviral actions of IFN-α in vitro. It was demonstrated that addition of recombinant human IL-8 could rescue the replication of encephalomyocarditis virus (EMCV) in the presence of IFN (33). In this study, we studied the interaction of NS5A with the STAT/JAK pathway and the IL-8 system as potential mechanisms for inhibition of the antiviral actions of IFN.

MATERIALS AND METHODS

Cells, cytokines, and viruses. Human HeLa Tet-Off cells were obtained from Clontech (Palo Alto, Calif.). HeLa Tet-Off cells were grown in Hela Tet-Off media: Dulbecco’s modified Eagle medium containing 10% fetal bovine serum (FBS; HyClone, Logan, Utah), 2 mM L-glutamine (Gibco-BRL, Grand Island, N.Y.), 1 × PSF (100 U of penicillin G per ml, 100 μg of streptomycin per ml, 0.25 μg of fungizone [Gibco-BRL] per ml, 200 μg of G418 (Calbiochem, San Diego, Calif.) per ml, 1 μg of tetracycline or doxycycline (Sigma, St. Louis, Mo.) per ml. In this study, two stable human HeLa cell lines expressing full-length NS5A from genotype 1b-infected IFN-nonresponsive and completely responsive patients (termed FL-NS5A-NR and FL-NS5A-CR, respectively) were used. NS5A expression in both cell lines was under the control of the tetracycline-regulated promoter (24) and was generated as described previously (58). Cells were grown in HeLa Tet-Off media supplemented with 100 μg of hygromycin B (Calbiochem) per ml. Murine L929 cells (obtained from the American Type Culture Collection, Manassas, Va.) were grown in Dulbecco’s modified Eagle medium containing 10% FBS, 2 mM L-glutamine, 1% PCF, and 25 μM aminoglycosidic amino acids (Gibco-BRL). All cells were grown in a humidified 5% CO2 atmosphere (Forma, Marietta, Ohio). Recombinant IFN (Intron A; kindly provided by Schering-Plough, Kenilworth, N.J.) or human leukocyte IFN (Sigma) was used in some experiments. The stocks had activities of 6 × 10^8 U per ml. Recombinant human IL-8 and TNF-α (R&D Systems, Minneapolis, Minn.) at stock concentrations of 100 μg/ml were also used in some experiments. For virus rescue assays, EMCV (American Type Culture Collection) was used. The virus stock had a titer of 10^9 PFU per ml.

Plasmids. A full-length NS5A construct obtained from a genotype 1b IFN-nonresponsive patient was cloned into pTRIE as described previously (58) and designated pTRIE-FL-NS5A-NR. A series of amino- and carboxy-terminal deletion mutants were generated by PCR with Phu polymerase, using pTRIE-FL-NS5A-NR as a template. The primers NS5A-1b-335 (5′-TGG CTG TGA GTG CCT CCC CCC TTC TTG-3′), NS5A-1b-671 (5′-TTC ACC ATG TCC GCC CCC CCC TTC TTG-3′), and NS5A-1b-1007 (5′-TTC ACC ATG GACC TAT CTC CTT CCG GG-3′) were used in separate PCR amplifications with NS5A-1b-1363 (5′-TGG CTA CAT TAG GAC TAT GAG CAC CAG AGC-3′) to generate NS5A proteins lacking 110, 222, and 334 amino-terminal amino acids. The primers NS5A-1b-1099 (5′-TCC CTA CAT TAG TCC GGG TCT TCG CAG GG-3′) and NS5A-1b-670 (5′-TGT CTA GAT TAC GCT GCC AGC TTA CGC TTC G-3′) were used in separate PCR amplifications with NS5A-1b-1 (5′-TGA GGA TCC ACC ATG GCC GGC TCG TGC TCT CTA-3′) to generate NS5A proteins lacking 117 and 230 carboxy-terminal amino acids. The PCR products were digested with NcoI and XbaI and cloned into the NeoI and XbaI sites of pTRIE-pUC (see below). The resulting constructs were designated pTRIE-NS5A-DN110, pTRIE-NS5A-DN222, pTRIE-NS5A-DN334, pTRIE-NS5A-ΔC117, and pTRIE-NS5A-ΔC283, respectively. A full-length NS5A construct obtained from a patient with genotype 1b who showed a complete response to IFN was cloned into pMOS Blue (Amersham-Pharmacia, Piscataway, N.J.) as described previously (11). The NS5A insert was subcloned into pTRIE as follows. First, the SpeI-SpeI multiple-cloning site from pUC21 was ligated into the XbaI site of pTRIE to generate pTRIE-pUC. NS5A was released from pMOS Blue first by SpeI digestion, which was subsequently blunted, and then by NcoI digestion. NS5A was directly ligated into pTRIE-pUC at the NeoI and EcoRV sites. This plasmid was designated pTRIE-FL-NS5A-AR. Plasmid constructs containing the full-length (542-loc) and truncated (133-loc and 98-loc) IL-8 promoters controlling the expression of the luciferase gene were generated as described previously (50). The 133-loc plasmid was also mutated at the NF-κB, AP-1, and NF-IL-6 binding sites. Adapta plasmids NS-EB-loc, NS-EB-loc, and NS-IL-6-loc, respectively, were constructed as described previously (50). All plasmids were isolated with an Endofree Mega plasmid kit (Qiagen, Santa Clarita, Calif.). pTRIE-EIL expresses the vaccinia virus EIL protein, a known inhibitor of the IFN antiviral response (5), and was cloned as described previously (58). pSVEIA expresses the adenovirus type 5 E1A protein. pcdNA/Neo/PKR expresses wild-type PKR and was cloned as described previously (46). Cells were transfected by electroporation as described previously (58).

Trans rescue assay. Cells (5 × 10^5 per well) (stable cell line or transiently transfected cells) were plated in a six-well tissue culture plate and grown in induction media in the presence or absence of 2 μg of tetracycline per ml for 24 to 48 h. Recombinant IFN (Intron A) was then added at various concentrations for 24 h to induce the IFN antiviral state. Infection with EMCV at a multiplicity of infection (MOI) of 0.01 was then performed, and supernatants were harvested at various times postinfection. Changes in infectious virus yields were measured by a standard virus plaque assay with L929 cells as described previously (58).

Western blot analysis. HeLa Tet-Off cell lines expressing FL-NS5A-NR and FL-NS5A-AR were induced and then treated with human leukocyte IFN at various time points. Protein extracts were prepared in lysis buffer I (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 400 mM NaCl, 1% [vol/vol] NP-40, 1 mM EDTA, 1 mM diithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, and 25 mM NaF). Protein concentration was determined with Coomassie Protein Assay reagent (Pierce Chemicals, Rockford, III.). Cell extracts were then separated by sodium dodecyl...
sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Proteins were detected with primary antibodies to human PKR (a gift from Ara Hovanessian, Pasteur Institute), β-tubulin (Sigma), phosphorylated STAT-1 (BioSource International, Camarillo, Calif.), and human antisemur from HCV-positive patients for the detection of NS5A (58). Secondary antibodies to mouse, rabbit, and human were conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, Pa.) and visualized with a chemiluminescence substrate (Pierce Chemicals).

Determination of IL-8 mRNA. IL-8 mRNA was detected and quantitated by reverse transcription-PCR (RT-PCR) and an RNase protection assay. For RT-PCR, cytoplasmic RNA was isolated from cells expressing or not expressing NS5A in the absence and presence of Introna A using the RNaseasy system (Qiagen). RNA samples were subsequently treated with DNase I to remove contaminating DNA. cDNA was synthesized using random hexanucleotides (pdN6) as described previously (60). To quantify the amount of IL-8 mRNA, a semi-quantitative PCR for IL-8 mRNA was performed using serial dilutions of input cDNA. The sequence of the upstream primer was 5′ ATT ACT TCC ACC ACG GCC GAT CCT CGG GAA AGG GAA ACC GAA ACT GAA GCC-3′, and that of the downstream primer was 5′ TCT ACA TCC ACC TCA AAA ACT TCT C-3′. The primers span introns on the IL-8 gene and produce a PCR fragment of 289 bp if mRNA is amplified. If genomic DNA is amplified, then a fragment of approximately 1,500 bp is generated. For RNase protection assays, the assays were performed according to protocols of the manufacturer (RiboQuant System; PharMingen). Cellular RNAs were hybridized to radiolabeled single-stranded riboprobe cocktails and digested with RNase. Protected RNAs were electrophoretically separated on denaturing urea-acrylamide gels, and autoradiography was performed.

RESULTS

Effects of NS5A expression on IFN signal transduction. Since the NS5A protein has been controversially implicated in inhibition of IFN signal transduction in both cell culture supernatants, and in intracellular levels of NS5A protein expression can be achieved by titrating the dose of tetracycline to the tetracycline-regulated promoter (24). In this system, NS5A can be selectively induced or repressed by the removal or addition of tetracycline to the tissue culture medium, and intermediate levels of NS5A protein expression can be achieved by titrating the dose of tetracycline (58). We cloned full-length NS5A proteins from two patients, one who was a complete, long-term responder (FL-NS5A-CR) and one who was a nonresponder (FL-NS5A-NR) to IFN therapy. Using FL-NS5A-NR as a template, we generated a series of amino- and carboxy-terminal deletions of the proteins, as described in Materials and Methods. Panel A depicts expression of full-length NS5A proteins from the complete responder and nonresponder patients and of mutant NS5A proteins lacking 110 (NS5A-ΔN110), 222 (NS5A-ΔN222), and 334 (NS5A-ΔN334) amino-terminal amino acids and 117 (NS5A-ΔC117) of the C-terminus. Panel B depicts expression of various NS5A proteins from the nonresponder patient, and panel C depicts expression of various NS5A proteins from the responder patient. In each panel, NS5A was transfected into HeLa Tet-Off cells, transfected with pTRE plasmids or pTRE-FL-NS5A-NR plasmids, or transfected with pTRE plasmids or pTRE-FL-NS5A-CR plasmids. Luciferase activities were determined in triplicate, and data are expressed as means ± standard deviations. Student’s t tests were used to compare differences in IL-8 levels and luciferase activities in cell cultures.

Nucleotide sequence accession numbers. The accession numbers for the sequences of FL-NS5A-NR and FL-NS5A-CR are AF034151 and AY008261, respectively.
tissue culture media. Figure 1B depicts the effects of NS5A on IFN induction of ISGF-3, the principal transcription factor induced by IFN. As can be seen, expression of FL-NS5A-NR, FL-NS5A-CR, and all amino- and carboxy-terminal NS5A mutant proteins had no effect on IFN induction of ISGF-3. For controls, we expressed the vaccinia virus E3L protein, a known inhibitor of the IFN system (5, 58), and PKR and also found no effect on ISGF-3 induction. Transfection of the parental pTRE-pUC plasmid also had no effect on IFN treatment and is indicated with arrows.

FIG. 1. Effect of NS5A expression on IFN signal transduction. (A) Tetracycline (Tc)-regulated expression of full-length and mutant NS5A proteins. FL-NR denotes a full-length NS5A construct derived from an IFN-nonresponsive patient, while FL-CR denotes a full-length NS5A construct derived from an IFN-responsive patient. ∆N-110, ∆N-222, ∆N-334, ∆C-117, and ∆C-230 represent NS5A deletion mutants lacking 110, 222, 334, amino acids from the amino terminus and 117 and 230 amino acids from the carboxy terminus, respectively. Protein positions are shown by arrows. Western blots were probed with HCV-infected patient serum as described in Materials and Methods. (B) Effect of NS5A expression on ISGF-3 induction. Plasmids were transiently transfected into HeLa Tet-Off cells, grown in the absence and presence of tetracycline to induce and repress NS5A, respectively, and treated with IFN to induce ISGF-3. Protein extracts were hybridized to a 32P-labeled oligonucleotide corresponding to the consensus ISRE. “puc” represents a control transfection with the pTRE-puc parental plasmid with no insert. “E3L,” “PKR,” and “E1A” represent expression of the E3L, PKR, and E1A proteins, respectively. The p48 monoclonal antibody used to form supershifts (denoted by “ss” and an arrow) is denoted by “p48 Mab”. “n” and “c” represent nuclear and cytoplasmic extracts, respectively. ISGF-3 is induced only with IFN treatment and is indicated with arrows.

Expression of E3L, PKR, and E1A was verified by Western blot analysis (data not shown). The specificity of the electromobility shift was verified in two ways. First, the inclusion of a monoclonal antibody to the p48 protein produced a supershift. Second, the inclusion of excess unlabelled oligonucleotide completely inhibited complex formation. Thus, these data indicate that expression of the HCV NS5A protein does not affect STAT/JAK signaling via induction of ISGF-3.

To further verify this result, we determined the effect of NS5A expression on IFN-induced changes in protein phos-
phorylation and gene expression. We performed Western blot analysis on protein extracts from cells expressing or not expressing FL-NS5A-NR or FL-NS5A-CR and treated with IFN for various times. Figure 2A depicts the results. The top panels of Fig. 2A demonstrate that expression of FL-NS5A-NR and FL-NS5A-CR proteins was repressed or induced by growing cells in the presence or absence of doxycycline (Dox) for 24 h and treated with IFN, and protein extracts were harvested at various times thereafter. Protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, respectively, followed by Western blotting with antibodies to NS5A (first panels), phosphorylated STAT-1 (second panels), PKR (third panels), and β-tubulin (fourth panels) as described in Materials and Methods. (B) Effect of NS5A expression on IFN-induced upregulation of MHC antigen. HeLa cells expressing FL-NS5A-NR (left panels) and FL-NS5A-CR (right panels) proteins for 24 h were treated with IFN, and cells were prepared for flow cytometry as described in Materials and Methods. Top panels are from cells grown in the presence of doxycycline (NS5A expression repressed), while bottom panels are from cells grown in the absence of doxycycline (NS5A expression induced). The gray histogram on the left represents a control staining with an isotype-matched control. To the right of the control histogram in each panel are the specific staining patterns. IFN-treated cells are depicted by the gray curve, while cells not treated with IFN are depicted by the black curve.
doxycycline to induce or repress NS5A, respectively, and treated with IFN, and MHC antigen was measured by flow cytometry. FL-NS5A-NR and FL-NS5A-CR proteins did not affect IFN-induced upregulation of MHC antigen expression on the surfaces of human cells. Expression of FL-NS5A-1a or NS5A-1a-DISDR also had no effect on IFN upregulation of MHC (data not shown). Collectively, the data in Fig. 1 and 2 indicate that NS5A expression does not affect the activation of the IFN system, at least at the level of IFN signal transduction through the STAT-JAK pathway involving ISGF-3 and tyrosine phosphorylation of STAT-1. NS5A does not appear to affect the expression of genes induced by IFN. These data suggested that NS5A might modulate the IFN-induced antiviral response by a postinduction mechanism. Since it had been previously demonstrated that IL-8 could inhibit the antiviral actions of IFN against certain viruses in vitro, we examined the role of IL-8 in NS5A-mediated antagonism of the IFN system.

NS5A induces IL-8 mRNA. In the absence of tetracycline, NS5A expression was detected by Western blot analysis (Fig. 3A). Following addition of tetracycline to the tissue culture medium, NS5A expression was no longer detected. The addition of IFN did not affect the level of expression of NS5A. A semiquantitative PCR for IL-8 mRNA was then performed using serial dilutions of cDNA samples. Figure 3B (top panels) indicates that NS5A expression was associated with an approximately eightfold induction of IL-8 mRNA, as detected by semiquantitative RT-PCR. IL-8 cDNA signals were not due to genomic DNA contamination, as verified by the absence of larger IL-8 PCR products and by the absence of PCR products when reverse transcriptase was omitted from the cDNA synthesis reaction mixture (Fig. 3B). The results of an RNAse protection assay (Fig. 4C) indicate that NS5A selectively induced IL-8 approximately eightfold among several chemokines. HeLa cells did not appear to express any of the other chemokines such as RANTES, IP-10, and MIP-1ß. In summary, NS5A expression was associated with increases in IL-8 mRNA expression in vitro.

NS5A, the trans rescue assay, and IL-8. Since IL-8 has been shown to inhibit the antiviral action of IFN (33) we tested the hypothesis that IL-8 is induced by NS5A in the trans rescue assay (58). The principle of the trans rescue assay is as follows. In the absence of HCV gene expression, IFN treatment of cells inhibits the replication of the IFN-sensitive virus EMCV (a positive-stranded RNA virus like HCV), measured as a reduction in viral titers. If HCV proteins inhibit the antiviral actions of IFN, higher titers of EMCV are produced in the presence of IFN. Figure 4A demonstrates titration of the amount of tetracycline, which resulted in a range of NS5A protein levels. In the absence of tetracycline, NS5A expression (determined by Western blot analysis) was repressed. (A) Tetraacycline-regulated expression of NS5A in human cells. HeLa cells expressing FL-NS5A-NR were incubated in medium with or without tetracycline for 48 h to repress or induce NS5A expression, respectively. Cells were then treated or not treated with IFN (20 U/ml) for 24 h. Cell extracts were prepared, and equal amounts of total cellular proteins were analyzed by Western blotting. The positions of the 58-kDa NS5A and positive control, NS5-(superoxide dismutase [SOD]), proteins are shown with arrows. (B) Semiquantitative detection of IL-8 mRNA by RT-PCR. cDNA was prepared from HeLa cells expressing or not expressing FL-NS5A-NR and treated or not treated with IFN. Serial dilutions of 1:2, 1:4, 1:8, 1:24, and 1:96 of cDNA (depicted above each lane with 2, 4, 8, 24, and 96, respectively) were subjected to PCR using IL-8-specific primers. Positive and negative controls (ctrl) are depicted with + and – signs, respectively. Amplification of cDNA generated without reverse transcriptase produced no PCR signal, indicating that the observed IL-8 PCR products were derived from IL-8 mRNA. Lanes M, molecular size markers. (C) Detection of IL-8 mRNA by RNAse protection assay. Equal amounts of total cellular RNA from HeLa cells expressing or not expressing FL-NS5A-NR were hybridized to a 32P-labeled riboprobe cocktail containing probes for various human chemokines, including IL-8. Hybrids resistant to RNAse digestion were separated on denaturing acrylamide gels, and autoradiography was performed. The positions of IL-8 control L32, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs are indicated with arrows. Note that the size of the protected fragment is smaller than the probe size because the probe contains vector-derived sequences that do not hybridize with target mRNA.
NS5A INDUCES IL-8 TO INHIBIT IFN

The data indicate a direct correlation between the level of EMCV replication in the presence of IFN and levels of both NS5A and IL-8 protein. We attempted to neutralize endogenous IL-8 using neutralizing anti-IL-8 monoclonal antibodies. This was not successful because prolonged incubation of significant amounts of anti-IL-8 antibodies was toxic to the cells (data not shown). Nonetheless, it was previously shown that antibodies to IL-8 reversed the IL-8-mediated inhibition of IFN antiviral action (33).

To determine if IL-8 could directly inhibit the antiviral actions of IFN in our system, EMCV replication was examined in HeLa cells pretreated with or not treated with recombinant human IL-8. Cells were then treated or not treated with IFN and then infected with EMCV. As shown in Fig. 4B, in the absence of IL-8 pretreatment, IFN inhibited EMCV replication by >3 log units. However, when cells were pretreated with IL-8, an 11.6-fold increase in EMCV yields during IFN challenge was observed. Thus, the IFN-induced antiviral activity was reduced by greater than 10-fold. This indicates that recombinant human IL-8 can partially inhibit the antiviral actions of IFN in our system.

**Transactivation of the IL-8 promoter by NS5A.** Since NS5A has been shown to be a transcriptional activator (16, 31, 75), we next determined if NS5A expression could transactivate the IL-8 promoter. For these experiments, the luciferase reporter protein under the control of the IL-8 promoter was used (50). Figure 5A indicates that NS5A expression resulted in a significant 2.5-fold increase of luciferase activity in the absence of IFN, when the reporter gene was under the control of the 546-bp IL-8 promoter. In the presence of IFN, NS5A expression was associated with a significant threelfold increase in luciferase activity. Because there are a number of response elements in the IL-8 promoter with binding sites for hepatocyte nuclear factor-1, AP-1, NF–IL-6, and NF–κB (49), we next determined the minimal promoter domains for NS5A-mediated transcriptional activation of the IL-8 gene using a set of truncated IL-8 promoters. Figure 5B indicates that NS5A transactivated both the 546- and 133-bp IL-8 promoters. NS5A transactivation resulted in higher levels of luciferase activity with the 133-bp promoter than with the 546-bp promoter. Further truncation of the IL-8 promoter to include only 98 bp abrogated the ability of NS5A to transactivate the reporter gene. These results suggest that NS5A increases IL-8 mRNA and protein expression via transcriptional activation and further suggest that the minimal IL-8 promoter required for NS5A transactivation consists of 133 bp immediately upstream of the IL-8 gene. This region of the IL-8 promoter contains binding sites for the transcription factors NF–κB, AP-1, and NF–IL-6.

**Analysis of NS5A mutants lacking the amino terminus.** The transcriptional activity reported for NS5A has been observed only when 100 or more amino acids are deleted from the amino terminus of the protein (16, 31, 68, 75). We therefore compared the effects of expressing the FL-NS5A-NR, NS5A-ΔN110, NS5A-ΔN222, and NS5A-ΔC171 proteins on transactivation of the IL-8 promoter. Figure 6A indicates that expression of NS5A-ΔN110 and NS5A-ΔN222 resulted in higher levels of luciferase activity than did expression of the full-length protein. The NS5A-ΔC171 protein did not transactivate the IL-8 promoter, suggesting a requirement for C-terminal amino acids in NS5A transactivation. We hypothesized
that these differences in transcriptional activity may be related to the subcellular localization of the full-length and truncated NS5A proteins. We therefore performed immunofluorescence experiments to localize the FL-NS5A-NR, NS5A-ΔN222, and NS5A-ΔC117 proteins in HeLa cells. Figure 6B depicts the results. Expression of FL-NS5A-NR in HeLa cells was localized to the perinuclear region (top panels), consistent with our previous report (58). Expression of NS5A-ΔN222 in HeLa cells produced a diffuse staining throughout both the cytoplasm and the nucleus (middle panels), whereas expression of the NS5A-ΔC117 protein resulted in a punctate cytoplasmic staining pattern (lower panels). Positive staining was seen only when cells were grown in the absence and not in the presence of tetracycline, indicating that the expression of each NS5A protein was tightly regulated. These results suggest that the increase in transcriptional activity by NS5A proteins lacking amino termini may be due to increased migration of the protein to the nucleus, while the lack of IL-8 transactivation by the carboxy-terminally deleted NS5A protein may be due to increased retention of the protein in the cytoplasm. Based on this result, we used NS5A-ΔN222 to determine the minimal regions of the IL-8 promoter to gain further insight into the transcription factors required for NS5A transactivation. NS5A-ΔN222 was cotransfected with wild-type and various mutant IL-8 promoters. After 48 h, cells were treated with TNF-α for 6 h and luciferase activity was measured. Similar to our previous results, the 546-luc and 133-luc constructs had increased luciferase activities when NS5A was expressed in the presence of TNF-α. Deletion of the IL-8 promoter to 98 bp inhibited reporter gene activity upon TNF-α stimulation. The expression of NS5A could not overcome the lack of a response from the 98-bp promoter, although a twofold increase in luciferase activity was noted when NS5A was expressed. Mutation of the NF-κB and AP-1 binding sites also inhibited luciferase activity in the presence of TNF-α and NS5A expression, although the effect of mutating NF-κB was more pronounced than that of mutating AP-1. In contrast, mutation of the NF-κB binding site resulted in higher levels of luciferase activity in the presence of TNF-α. In this case, NS5A expression resulted in even higher levels of luciferase activity. These data suggest that, after stimulation of HeLa cells with TNF-α or expression of NS5A, NF-κB and, to a lesser extent, AP-1 are required for transactivation of the IL-8 promoter and that the binding of NF-κB is inhibitory to this process.

**DISCUSSION**

Antiviral therapy for HCV infection is still rather primitive, with only two Food and Drug Administration-approved drugs available. While newer-generation therapies for HCV are clearly more effective than conventional IFN monotherapy, over 60% of patients with HCV genotype 1 infection are not cured of their infection. In order to improve the success of antiviral intervention in HCV, it is important to understand the molecular mechanisms of antiviral resistance. In recent years, insight into the molecular mechanisms involved in the intrinsic resistance of HCV to IFN therapy has been gained. The emerging trend in the literature is that HCV proteins, when overexpressed in vitro, disrupt normal biological processes by interaction with cellular proteins. Two examples of this is the interaction of HCV NS5A and E2 proteins with the IFN-induced enzyme PKR (19, 77). However, since the roles of mutations in the NS5A protein and in the clinical response to IFN therapy are controversial, it has been suggested that NS5A may use other mechanisms to perturb the IFN-induced antiviral response (59).

The present study provides detailed mechanistic evaluation of an HCV protein in the context of the broad IFN response pathway. The present report found no evidence for a role of NS5A in inhibiting signal transduction via the STAT/JAK pathway, at least in terms of tyrosine phosphorylation of STAT-1 and induction of ISGF-3, the principal factor induced by IFN. This finding was supported by the observations that NS5A did not affect the induction of expression of two IFN-induced genes, those for PKR and MHC antigen. It is possible that levels of serine phosphorylation on STAT proteins are affected by NS5A expression (78). Evidence was provided that NS5A can induce the expression of IL-8 mRNA and protein. IL-8 expression was associated with NS5A-induced inhibition
of the IFN-α antiviral response in vitro, and the addition of recombinant human IL-8 also partially rescued EMCV replication during IFN challenge. Using a luciferase reporter gene under the control of the IL-8 promoter, it was also shown that NS5A expression could transactivate the IL-8 promoter.

It is currently unknown if NS5A-mediated transcriptional activation of the IL-8 promoter occurs directly or indirectly. NS5A may in theory bind to the IL-8 promoter if it can enter the nucleus. Evidence discounting a direct effect of NS5A are derived from observations that full-length NS5A proteins have a cytoplasmic perinuclear distribution when expressed in human cells (29, 35, 48, 58, 76). It is possible, however, that a small amount of the NS5A protein, beyond the level of detection in these systems, may migrate to the nucleus. In this regard, it has recently been shown that processing of NS5A occurs naturally in transfected cells in vitro and that N-terminally deleted forms of the protein migrate to the nucleus (68). The C terminus of NS5A also contains a nuclear localization signal that does not by itself direct NS5A to the nucleus but is nonetheless functional in directing nuclear translocation when it is placed at the amino terminus of a reporter gene (29). In this report, we found that deletion of the amino terminus from NS5A resulted in increased transactivation of the IL-8 promoter, which was associated with increased nuclear staining of the protein. Deletion of C-terminal amino acids from NS5A created a protein that failed to transactivate the IL-8 promoter, and the protein appeared to be retained in the cytoplasm. These results suggest that the NS5A protein may be able to enter the nucleus under certain physiological instances.

Promoter mutagenesis experiments suggested that the minimal region for NS5A transactivation involved the 133 bp of the IL-8 promoter. Previous reports indicate that the transcription factors NF-κB, AP-1, and NF-IL-6 are involved in TNF-α-induced stimulation of the IL-8 promoter and that the involvement of each transcription factor can be cell line dependent (49). In this report, we found that TNF-α induction of the IL-8 promoter in HeLa cells required NF-κB and AP-1 but that NF–IL-6 appeared inhibitory to this process. NS5A expression could not overcome deletion of the NF-κB and AP-1 binding sites to activate luciferase activity. Thus, it is possible that NS5A induces IL-8 transcription by interacting with tran-

FIG. 6. Characterization of domains on NS5A and the IL-8 promoter required for transactivation. (A) Effect of NS5A mutant proteins on luciferase expression under the control of the full-length (546-luc) IL-8 promoter. Ten-microgram quantities of the 546-luc plasmid and pTRE-FL-NS5A-NR, NS5A-ΔN110, NS5A-ΔN222, or NS5A-ΔC117 were transfected into HeLa Tet-Off cells, and cells were split in triplicate and grown in media with or without tetracycline for 48 h. The amount of luciferase activity in cell lysates was determined using a luminometer. The data shown are the fold changes in luciferase activity with the gene in the induced versus the uninduced state. (B) Immunofluorescence analysis of HeLa cells expressing FL-NS5A-NR, NS5A-ΔN222, or NS5A-ΔC117. HeLa Tet-Off cells stably transfected with FL-NS5A-NR (top panels), NS5A-ΔN222 (middle panels), or NS5A-ΔC117 (lower panels) were grown in the absence or presence of tetracycline (Tc) to induce or repress NS5A expression for 48 h. Cells were fixed and stained with a monoclonal NS5A antibody and then with Cy3-linked anti-mouse secondary antibodies, as described in Materials and Methods. Images are at a ×100 amplification. (C) Effect of NS5A-ΔN222 expression on luciferase activity under the control of mutated IL-8 promoters. Ten-microgram quantities of NS5A-ΔN222 and the relevant reporter plasmid were cotransfected into HeLa Tet-Off cells; cells were split in triplicate and grown in media with or without tetracycline for 48 h. Cells were then treated with 4 ng of TNF-α for 6 h. The amount of luciferase activity in cell lysates was determined using a luminometer. 546-luc, 133-luc, and 98-luc contain 546, 133, and 98 bases of the IL-8 promoter, respectively. NF-κB–luc, AP-1–luc, and NF–IL-6–luc represent the 133-luc plasmid with point mutations in the NF-κB, AP-1, and NF–IL-6 binding sites.
scription factors. Indeed, NS5A has been shown to interact with a novel transcription factor (23). We are investigating further the domains of NS5A responsible for IL-8 transactivation and the subcellular localizations of the proteins and characterizing the transcription factors required for this process.

The results presented in this and other reports (17, 28, 63, 74) suggest that NS5A is a pleiotropic molecule, possessing multiple activities in vivo through its interaction with various cellular proteins and signaling pathways. Specifically, it appears that NS5A employs multiple mechanisms to achieve antiviral resistance, implying that inhibition of PKR by NS5A is but one way by which NS5A subverts the IFN system. This may be a plausible hypothesis since NS5A-ISDR mutations are not universally associated with IFN sensitivity (7, 11, 15, 27, 52, 56, 60, 64, 65, 72, 73, 79). More recent data suggest that a region in the C terminus of NS5A, termed the variable 3 (V3) region, may be associated with IFN resistance, at least in France and North America (11), and with a distinct region within the proline-rich domain of NS5A (52). A recent study also demonstrated that expression of the entire HCV polyprotein inhibits the activity of IFN against EMCV independently of PKR (14). Moreover, HCV replicons selected for increased replicative activity in vitro harbor mutations in NS5A, including a deletion of the entire ISDR (3). It should be emphasized that NS5A gene-encoded mechanisms for inhibition of the IFN system need not be mutually exclusive. It is possible that the different activities of NS5A are related to the location of the protein in the cell. In this scenario, cytoplasmic NS5A may inhibit PKR while nuclear NS5A may interact with transcription factors and influence gene expression.

Why NS5A would upregulate a chemokine that induces an inflammatory response is currently not known. One might speculate that NS5A-induced increases in IL-8 affects the activity of the intrahepatic immune response to HCV, through T-cell chemotaxis to the HCV-infected liver. Since T-cell infiltration is a hallmark histological finding of chronic hepatitis C, NS5A induction of IL-8 may ultimately be involved in HCV persistence and pathogenesis. Since IL-8 is elevated in alcoholic hepatitis (44), it is tempting to speculate that NS5A induction of IL-8 may exacerbate the deleterious effects of ethanol on the liver, contributing to the increase in liver disease activity and poor antiviral responses in HCV-infected patients who abuse alcohol (69). Moreover, since lymphocytes have been shown to harbor replicating HCV (37, 38, 47), NS5A induction of IL-8, followed by lymphocyte recruitment to the infected liver, may also facilitate the spread of HCV to extrahepatic reservoirs. It is also possible that NS5A-mediated induction of IL-8 may contribute to HCV antiviral resistance, through the interaction between the chemokine- and IFN-signaling pathways. Indeed, there are several reports of IFN-induced downregulation of IL-8 (2, 53, 70). Thus, NS5A may subvert the IFN signaling pathway to promote the expression of cellular genes that may counteract the IFN-induced antiviral response. These concepts will remain speculative until they can be formally addressed in a small animal model or in vitro expression and replication systems.

Currently, the mechanism of IL-8-mediated inhibition of the IFN system is not known. Khabar and colleagues (33) demonstrated that the anti-IFN effect of IL-8 was detectable as late as 20 h after the addition of IFN. This suggests that IL-8 inhibits the IFN-induced antiviral response at a posttranscriptional level and is consistent with this report showing that NS5A does not affect IFN signal transduction. Preliminary data suggest that IL-8 inhibited 2′-5′-oligoadenylate synthetase (OAS), a major pathway for IFN action against RNA viruses (34). We are currently exploring the hypothesis that NS5A induction of IL-8 perturbs the OAS and other IFN-induced effector molecule pathways. It is also possible that the anti-IFN effect of IL-8 involves the high-efficiency IL-8 receptor CXCR1. Indeed, HeLa cells which are susceptible to IL-8 proviral action express CXCR1 but not CXCR2 (K. S. A. Khabar, unpublished data; 34).

Viral modulation of chemokine expression represents the continuous battle between viral invaders and antiviral, inflammatory, and immune responses. Perhaps the best examples are the recent discoveries that human immunodeficiency virus (HIV) entry into macrophages and T lymphocytes requires interaction with distinct chemokine receptors and that soluble chemokines can inhibit the entry of HIV (20). In HIV infection, IL-8 is frequently elevated and the HIV Tat and Vpr proteins can induce IL-8 expression (54, 66). Other examples include human cytomegalovirus, which encodes a chemokine receptor that may facilitate viral replication by regulating cell cycle progression or inhibiting apoptosis (51). Recent studies have demonstrated that Sendai virus-infected cells induce the CC chemokine RANTES and that this effect is mediated by interferon regulatory factor 3 and NF-kB (21, 42). Thus, it will be important to determine the mechanisms involved in the induction of IL-8 by the HCV NS5A protein and how IL-8 inhibits the antiviral actions of IFN. If this interaction between HCV and the chemokine system can be proven to occur in vivo, then its selective disruption may increase therapeutic response rates and reduce the pathogenicity associated with chronic hepatitis C.

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