Gene Expression Profiling of the Cellular Transcriptional Network Regulated by Alpha/Beta Interferon and Its Partial Attenuation by the Hepatitis C Virus Nonstructural 5A Protein

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Alpha/beta interferons (IFN-α/β) induce potent antiviral and antiproliferative responses and are used to treat a wide range of human diseases, including chronic hepatitis C virus (HCV) infection. However, for reasons that remain poorly understood, many HCV isolates are resistant to IFN therapy. To better understand the nature of the cellular IFN response, we examined the effects of IFN treatment on global gene expression by using several types of human cells, including HeLa cells, liver cell lines, and primary fetal hepatocytes. In response to IFN, 50 of the approximately 4,600 genes examined were consistently induced in each of these cell types and another 60 were induced in a cell type-specific manner. A search for IFN-stimulated response elements (ISREs) in genomic DNA located upstream of IFN-stimulated genes revealed both previously identified and novel putative ISREs. To determine whether HCV can alter IFN-regulated gene expression, we performed microarray analyses on IFN-treated HeLa cells expressing the HCV nonstructural 5A (NS5A) protein and on IFN-treated Huh7 cells containing an HCV subgenomic replicon. NS5A partially blocked the IFN-mediated induction of 14 IFN-stimulated genes, an effect that may play a role in HCV resistance to IFN. This block may occur through repression of ISRE-mediated transcription, since NS5A also inhibited the IFN-mediated induction of a reporter gene driven from an ISRE-containing promoter. In contrast, the HCV replicon had very little effect on IFN-regulated gene expression. These differences highlight the importance of comparing results from multiple model systems when investigating complex phenomena such as the cellular response to IFN and viral mechanisms of IFN resistance.

Alpha/beta interferons (IFN-α/β) are expressed by many cell types in response to viral or bacterial pathogens. By binding to specific transmembrane receptors, these cytokines trigger a response that culminates in the induction of a large number of genes, many of which encode proteins with antiviral or antiproliferative properties. This increase in gene expression is controlled by multiple transcription factors and regulatory elements, including signal transducer and activators of transcription 1 and 2 (STAT1 and STAT2), which, together with IFN-regulatory factor 9 (IRF9), bind to *cis*-acting IFN-stimulated response elements (ISREs) to induce the transcription of many IFN-stimulated genes (ISGs). Other members of the IFN-regulatory-factor family, such as IRF3 and IRF7, also play a critical role in IFN signaling. These latent cytosolic factors translocate to the nucleus upon phosphorylation by unknown kinases and bind to ISRE-like elements to mediate the expression of specific ISGs. Using gene expression profiling, a number of investigators have begun to explore the complexities of the IFN response (5, 7, 8, 39, 41, 49), and although these studies have revealed an ever-increasing number of IFN-reg-

ulated genes, little is known about the tissue-specific nature of the response or the impact of viral infection on ISG expression.

Many viruses have evolved strategies to evade the antiviral effects of the IFN response (18, 22, 26). These strategies differ greatly, but it is common for a virus to encode one or more proteins that interact directly with an IFN-induced gene product or that interfere with some aspect of IFN signaling. Since the IFN response is a central component of innate immunity, a better understanding of the mechanisms used by viruses to counteract this response would be beneficial, particularly in those instances in which IFN is also the primary therapeutic option. A case in point is hepatitis C virus (HCV). HCV has infected over 170 million people worldwide (48) and is a primary risk factor for the development of liver cirrhosis and hepatocellular carcinoma (10). Although a combination of IFN-α and ribavirin is currently the optimal choice for therapy, HCV isolates are often resistant to IFN and this regimen is ineffective in many patients (2, 25).

Much of the search for the molecular basis of HCV resistance to IFN has centered on the viral nonstructural 5A (NS5A) protein. This focus began with evidence that the amino acid sequence of a region of NS5A, termed the IFN sensitivity-determining region (ISDR), correlates with therapeutic outcome (11, 12). Subsequent in vitro studies demonstrated that NS5A from an IFN-resistant isolate inhibits the IFN-induced protein kinase PKR (16), suggesting a mechanism for IFN resistance. However, the link between NS5A and resistance to

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IFN remains hazy (44). Therapeutic outcome does not always correlate with ISDR sequence (1, 36) or with inhibition of PKR activity (13, 38), and other studies suggest that IFN resistance is related to regions of NS5A outside the ISDR (34, 35) or to the ability of NS5A to interact with one or more of a variety of cellular proteins (6, 24, 33, 45, 47).

Thus, despite all these reports, questions remain about the mechanisms by which NS5A might mediate IFN resistance. In this study, we used global gene expression profiling to evaluate the cellular transcription network regulated by IFN-α/β and to determine whether NS5A has an effect on IFN-regulated gene expression. IFN-induced changes in gene expression were examined in a variety of human cell types, including cultured primary fetal hepatocytes, HeLa cells expressing wild-type or mutant forms of NS5A, and Huh7 cells expressing an HCV subgenomic replicon. These studies revealed IFN-responsive genes that were consistently induced in all cell types examined as well as cell type-specific responses. NS5A partially blocked the IFN-mediated induction of specific ISGs as well as the IFN-mediated induction of an ISRE-driven reporter gene. Through this mechanism, NS5A may partially attenuate the IFN response, which may be an important factor in HCV resistance to IFN.

MATERIALS AND METHODS

Cell lines and IFN treatment. The construction of HeLa S3 cells expressing NS5A under the control of a tetracycline-regulated promoter was described previously (17). The cell lines used were HeLa pTRE-NS5A-1B (expressing wild-type NS5A), HeLa pTRE-NS5A-1B5 (expressing a PKR-binding-deficient mutant), and HeLa pTRE-4 (containing the pTRE vector alone). NS5A expression was induced by the removal of tetracycline from the culture medium. To measure the effects of NS5A on IFN-regulated gene expression, IFN-α/β (Hayashibara Biochemical Laboratories, Inc.; 400 IU/ml) was added to cells 8 h after induction of NS5A and RNA was isolated 16 h later as previously described (19). The optimal duration of IFN treatment was determined by a time course experiment in which HeLa pTRE-4 cells were treated with IFN-α/β (400 IU/ml) or mock treated with medium alone and incubated at 37°C for 2, 4, 6, 8, or 16 h. Total RNA was isolated from cells at each time point and used for microarray analysis. For comparison, total RNA was also isolated from HeLa pTRE-4 cells that were treated for 16 h with Intron-A (recombinant IFN-α$_{2b}$) (Schering-Plough; 400 IU/ml). Huh7 cells harboring an HCV subgenomic replicon (32) were maintained in medium supplemented with G418 (0.8 mg/ml). For gene expression studies, replicon-containing cells were grown until approximately 70% confluent and then treated with IFN (400 IU/ml) for 16 h.

Fetal hepatocytes were isolated from tissue obtained from aborted second trimester fetuses (92 to 117 days of gestation) provided by the Central Laboratory for Human Embryology at the University of Washington. Excised fetal liver (2 to 4 g) was minced into small pieces in Seglen's collagenase buffer and digested with collagenase A (Roche Diagnostics; 3 mg/ml) at 37°C. The cells were then washed twice and suspended in Dulbecco's minimal Eagle's medium. Typical cell yields were 1×10^7 cells, which were 95% viable as determined by trypan blue dye exclusion. Hepatocytes were then plated on collagen-coated dishes in medium containing 5% fetal bovine serum. After a 4-h attachment period, the medium was changed to serum-free William's medium supplemented with epidermal growth factor (Becton Dickinson; 20 ng/ml), 10 mM nicotinamide, 0.2 mM ascorbic acid-2-phosphate, 20 mM HEPES, 17 mM NaHCO₃, pyruvate (550 mg/liter), 14 mM glucose, 2 mM glutamine, 10 M dexamethasone, ITS+ premix (Becton Dickinson), and antibiotics. Cultures were maintained at 37°C at 6% CO_2.

Adult hepatocytes were obtained from surgical resections (negative for hepatitis B and C virus). Tissue wedges (approximately 50 g) were trimmed and perfused with collagenase (1 mg/ml). The yield was approximately 1.7×10^7 cells, which were 85 to 90% viable as measured by trypan blue dye exclusion. The primary hepatocytes were infected (at 72 h after plating) with a virus stock obtained from the PA317 amphotropic packaging cell line stably transfected with the plasmid pLXSN16 E6/E7, which contains the E6-E7 genes of human papillomavirus 16. Briefly, cells in 60-mm-diameter plates were infected with 250

μl of virus stock in 2.5 ml of medium containing Polybrene (4 μl/ml) and epidermal growth factor (20 μg/ml) for 24 h. The resulting cell line was designated HH2.

Microarray and Northern blot analysis. Microarrays containing 4,608 human cDNAs were constructed by the University of Washington Center for Expression Arrays. The I.M.A.G.E. identification numbers and gene identities can be found at http://ra.microslu.washington.edu/. Plates 7 through 17 plus a control were used. First-strand cDNA synthesis, microarray hybridizations, and Northern blot analyses were performed as described previously (19). For each condition, duplicate slides were hybridized with the same probes, but with the fluorescent labels reversed, to control for dye-specific effects. In addition, each analysis was repeated independently for a total of four slides per condition (eight measurements per gene). Intensity values in Cy3 and Cy5 channels were extracted from each image, and the Cy3/Cy5 ratio was determined using Spot-on Image software. Data for all replicates were combined and normalized with custom software (Spot-on Unite). Briefly, Spot-on Unite normalizes the data, rejects outliers, and calculates the mean and standard deviation for each replicate measurement (19).

Data processing and analysis. Using an Avalanche II scanner (Molecular Dynamics), microarrays were scanned at two wavelengths, and the resulting images were quantified using Spot-On Image, a spot-finding program (19). Raw data and sample information were then entered into Expression Array Manager, a custom-designed gene expression database that automatically uploads data and two-color images into the Rosetta Resolver system (Rosetta Biosoftware), a gene expression data analysis package that integrates a variety of sophisticated analysis tools, including hierarchical clustering analysis. The output from this type of analysis permits the simultaneous evaluation of multiple experiments and the visualization of coordinate patterns of gene regulation. In accordance with proposed standards (4), all data described in this report, including sample information, intensity measurements, gene lists, error analysis, microarray content, and slide hybridization conditions, are available in the public domain through Expression Array Manager at http://www.expression.washington.edu/public.

Luciferase reporter assays. HeLa pTRE-4 cells were seeded at a density of 3 $\times 10^5$ cells per 35-mm-diameter tissue culture dish and transiently cotransfected with 1.0 μg of either pISRE-Luc or pCMV-Luc (Stratagene) and with 0.2 or 1.0 μg of pTRE-NS5A-1B or pTRE-β-galactosidase (as a control). Expression of the firefly luciferase gene in pISRE-Luc is controlled by a basic promoter element (TATA box) plus 5 direct repeats of an ISRE (TAGTTTCACTTTCCC). Transfections were performed using SuperFect (Qiagen) according to the manufacturer's instructions. Cells were then treated with human IFN-α/β (400 IU/ml), incubated for 24 h, and lysed. An aliquot of each lysate was mixed with Luciferase Assay Reagent (Promega), and luciferase activity was measured in a Beckman-Coulter LS6500 scintillation system in the single-photon mode. The expression of NS5A and β-galactosidase (B-Gal) proteins was confirmed by immunoblotting using antibodies specific for NS5A (ID Labs) or β-Gal (Santa Cruz Biotechnology). Experiments were performed in triplicate.

RESULTS

IFN induces conserved and cell type-specific changes in gene expression. Since an important goal of this study was to determine whether NS5A has an effect on IFN-regulated gene expression, we began our analyses by profiling the changes in gene expression that occur in response to IFN treatment. These experiments were performed using a variety of cell types, including HeLa cells, in which our NS5A-expressing cell lines were developed; Huh7 cells, which are used for the HCV replicon system; HH2 cells, an immortalized hepatocyte line; and cultured primary human fetal hepatocytes. An initial time course of IFN treatment using HeLa cells revealed that the greatest number of gene expression changes occurred 16 h after IFN treatment (data available at http://www.expression.washington.edu/public). Although some of these changes may be due to secondary effects of IFN or to factors such as differential RNA stability, this time point was judged to provide the best snapshot of IFN-mediated changes in gene expression and was used for all subsequent experiments in which we examined the ability of NS5A to alter these gene expression patterns.
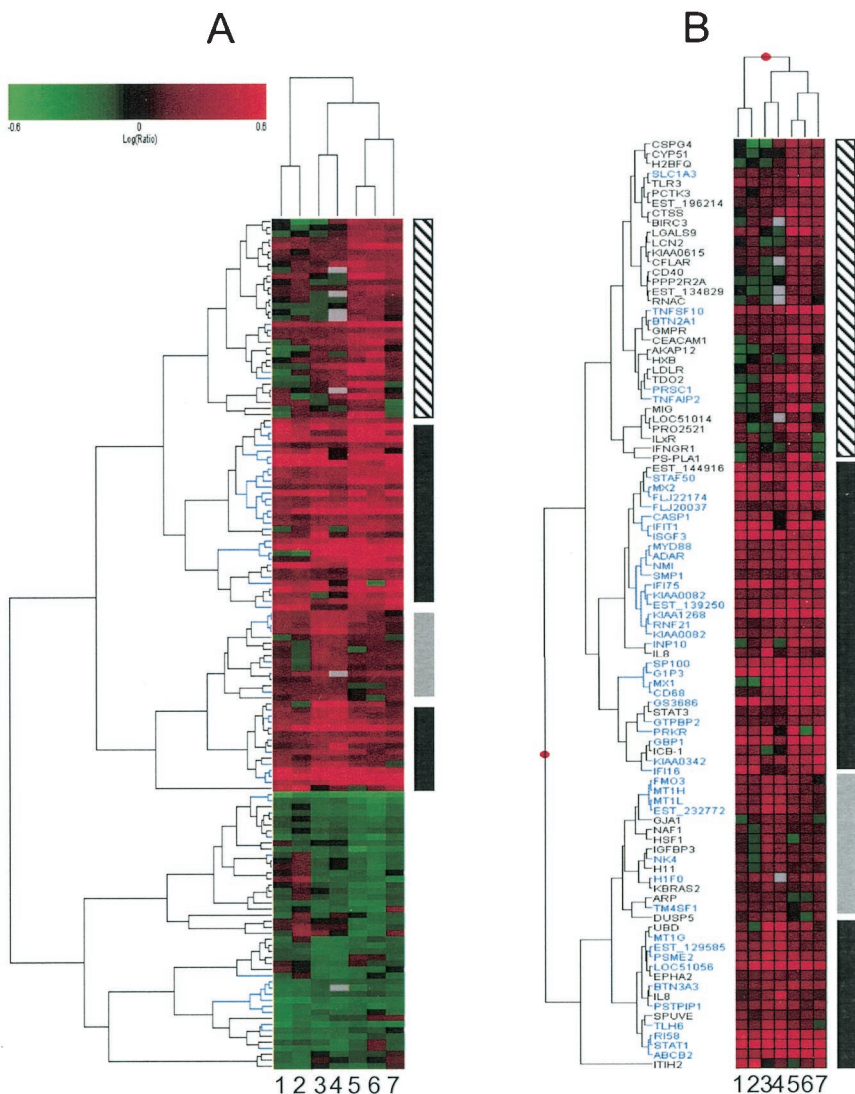
FIG. 1. IFN induces conserved and cell type-specific changes in gene expression. Lanes 1 and 2, HeLa cells; lanes 3 and 4; Huh7 cells; lanes 5 and 6, primary fetal hepatocytes; lane 7, HH2 cells. (A) Hierarchical clustering of the union set of genes that were differentially regulated in at least one cell type ($P \leq 0.005$ for each pair of duplicate experiments). The scale at top indicates the magnitude of induction or repression ($\log_{10}$). Red bars represent genes that were induced by IFN, green bars represent genes that were repressed by IFN, black bars represent genes that were not differentially expressed, and gray bars represent genes that were below the intensity threshold in that experiment. IFN-induced changes in gene expression that were conserved across all cell types are represented by the vertical black bars to the right, primary fetal hepatocyte-specific changes in gene expression are indicated by the vertical hatched bar, and Huh7 cell-specific changes are indicated by the vertical gray bar. A magnified view of genes that were down-regulated by IFN is available at http://www.expression.washington.edu/public. (B) Magnified view of ISGs detected by microarray analysis. The symbols for genes that were induced by IFN in all cell types are highlighted in blue.

Agglomerative hierarchical clustering analysis of the union set of all differentially regulated genes revealed differences in the number and identity of differentially regulated genes in each of the four cell types as well as differences in the magnitude of gene expression changes (Fig. 1; additional data available at http://www.expression.washington.edu/public). However, some changes were common to all cell types and the gene expression profiles for cells of liver origin were more similar to one another (Fig. 1A, lanes 3 to 7) than they were to those of HeLa cells, which formed their own distinct cluster (Fig. 1A, lanes 1 and 2). In general, primary human fetal hepatocytes were the most responsive to IFN treatment and HeLa cells

were the least responsive. A view of the ISGs identified by this analysis is shown in Fig. 1B, where genes that were significantly induced ($P \leq 0.005$) in all cell types examined are highlighted in blue. These genes likely represent a core response to IFN that is conserved across cell types, and subsequent bioinformatics analyses were focused on this set of genes. A complete listing of differentially regulated genes and all primary data used to generate these clusters (including severalfold change in expression and error analysis) are available at http://www.expression.washington.edu/public.

Genome analysis reveals novel ISREs. Some of the ISGs identified by our microarray analyses are known to contain

TABLE 1. Bioinformatic identification of candidate
IFN-regulatory elements[a]

Gene	Position (nt)	Strand	Locus	Sequence
ABCB2	ATG (−765)	Plus	6p21	GAAAGCGAAAGC
ABCB2	ATG (−24)	Minus		GAAATCGAAAGC
ABCB2	ATG (−18)	Minus		GAAAGCGAAATC
ADAR	ATG (−197)	Plus	1q21.1	GAAACGAAAGC
ADAR	ATG (−192)	Plus		GAAAGCGAAATT
CASP1	ATG (−438)	Minus	11q23	GAAATAGAAAC
CASP1	ATG (−42)	Minus		GAAACTGAAAGT
G1P3	ATG (−627)	Minus	1p35	GAAAAAGAAAC
G1P3	Exon 1 (−150)	Plus		GAAAATGAAACT
G1P3	Exon 1 (−109)	Plus		GAAAATGAAACT
G1P3	Exon 1 (−87)	Plus		GAAATAGAAAC
GBP1	Exon 1 (−128)	Minus	1p22	GAAACTGAAAGT
GS3686	Exon 1 (−878)	Minus	1p31	GAAATGAAAGC
IFI16	Exon 1 (−41)	Plus	1q22	GAAACGAAAGC
IFIT1	ATG (−470)	Plus	10q25	GAAACTGAAAAT
IFIT1	ATG (−208)	Minus		GAAAGTGAAACT
INP10	ATG (−287)	Plus	4q21	GAAAGTGAAACC
ISGF3	ATG (−172)	Plus	14q11	GAAAGGAAAC
KIAA1268	ATG (−79)	Plus	3q21	GAAACGAAAGC
MX1	ATG (−218)	Minus	21q22	GAAAGAGAAAC
MX1	Exon 1 (−101)	Minus		GAAACGAAAC
MX1	Exon 1 (−56)	Minus		GAAATGAAAC
MYD88	ATG (−98)	Plus	3p22	GAAAGCGAAAGC
NMI	Exon 1 (−78)	Plus	2p24	GAAAGTGAAATT
PRKR	Exon 1 (−7728)	Plus	2p21	GAAAACGAAACT
PRKR	Exon 1 (−3617)	Plus		GAAAGGAAAC
PRKR	Exon 1 (−374)	Minus		GAAAGGAAAC
PSME2	ATG (−57)	Plus	14q11	GAAAGCGAAAGC
RI58	ATG (−278)	Minus	10q23	GAAACCGAAACT
RI58	ATG (−252)	Minus		GAAATCGAAACT
RI58	ATG (−237)	Minus		GAAACTGAAACT
RNF21	Exon 1 (−107)	Minus	11p15	GAAAGTGAAATT
STAT1	Exon 1 (−492)	Minus	2q32	GAAAGCGAAACT
TNFSF10	Exon 1 (−96)	Minus	8p21	GAAATGAAAGC

[a] The consensus sequence used to identify candidate IFN-regulatory elements
was GAAA(N){1,2} GAAA(G) {0,1} C, where N equals any nucleotide and the
numbers represent the presence of 0, 1, or 2 of the preceding nucleotides.

cis-acting DNA elements that participate in IFN-regulated
transcription. However, for many of the genes we identified,
little or no information is available regarding the sequence
elements that regulate their responsiveness to IFN. As a means
both to validate our microarray results and to look for alter-
native mechanisms of regulation, we conducted a search for
ISRE-like sequences in genomic regions upstream of the 50
genes that were induced by IFN in all cell types. We reasoned
that since these genes were the most consistently induced by
IFN, they were the most likely to have recognizable IFN-
responsive elements. To account for the possibility of alterna-
tive transcription initiation sites, we examined 1,000-bp regions
upstream of both the predicted ATG start codon and the start
of the 5′ untranslated region. Because of ambiguity about what
constitutes a bona fide ISRE, we used a single consensus se-
quence that worked well in identifying known ISREs. Using
this approach, we identified all previously described ISREs
that occur upstream of known ISGs as well as new ISRE-like
sequences in alternative locations upstream of known ISRE-
containing genes such as PKR, GBP1, and MX1 (Table 1).
Previously unidentified ISRE-like sequences were also found
in the promoter regions of CASP1, GS3686, STAT1, IRF9,
KIAA1268, and others. Since these genes were induced by

IFN, it is likely that many of these sequences function as
IFN-responsive elements, although this has not been system-
atically verified. It is also possible that these sequences do not
mediate direct responsiveness to IFN but rather serve as bind-
ing sites for secondary factors, such as IRF1 (46). In all, 20 of
the 50 IFN-regulated genes examined contained ISRE-like
sequences in their promoter regions and 8 genes had more
than one element. In contrast, no consensus IFN-responsive
elements were found in genes that were down-regulated by
IFN or in random 1,000-bp fragments, suggesting that ISGs are
enriched for ISRE-like sequences. Only 10% of the more than
60 genes that were regulated by IFN in at least one cell type,
but which failed to meet statistical criteria for differential ex-
pression in all cell types, had recognizable ISRE-like se-
quences.

We also noticed that a number of ISGs were located in close
proximity to one another at several locations throughout the
genome, suggesting the possibility of a coordinate mechanism
of gene regulation based on local alterations in chromatin
structure (42). Examples of such genes include IFIT1 and RI58
on chromosome 10q23.31, BTN2A1 and BTN3A3 on chromo-
some 6p21.1, MT1G, MT1H, and MT1L on chromosome 6q13,
and STAF50/TRIM22 and RNF21/TRIM34 on chromosome
11p15. Given this observation, we devised a strategy to map
our consensus ISRE sequence to the entire noncoding region
of the human genome. This allowed us to look for additional
clusters of ISRE-like sequences as well as to visualize in detail
the positions of these sequences relative to genes in those same
regions (methods and detailed analysis are to be presented
elsewhere) (27). An example of the output obtained from this
analysis for chromosome 10q23.31 as viewed using the Univer-
sity of California Santa Cruz genome browser (http://genome
.ucsc.edu) (27) is available at http://www.expression.washing-
ton.edu/public. In addition to IFIT1 and RI58, both of which
have ISREs, there were other genes in this region that con-
tained ISRE-like sequences. For instance, IFIT4/ISG60, which
is located directly upstream of the IFIT1 gene and which is a
member of the same gene family (9), has two putative ISREs.
Indeed, an alternative form of the IFIT4 gene has been de-
scribed, which is consistent with the multiple EST species that
correspond to this region. Thus, a genome-wide scan for ISRE-
like sequences has the potential to reveal the positions of all
putative ISREs (even those located in introns), locate hotspots
of IFN-regulated genes that exhibit coordinate patterns of
regulation, and provide information regarding the potential
IFN responsiveness of genes not present on the microarray.
We caution, however, that this type of bioinformatic approach
is not meant to be a definitive analytical tool but rather is a
high-throughput method to identify potential regulatory ele-
ments.

NS5A alters IFN-regulated changes in gene expression. To
determine whether NS5A has an effect on IFN-regulated gene
expression, we performed microarray analyses on IFN-treated
HeLa cells expressing either wild-type NS5A (NS5A-1B) or a
mutant form of NS5A (NS5A-1B5) that contains multiple mu-
tations within the ISDR and that does not bind to or inhibit
PKR (15). Using hierarchical clustering analysis, we identified
14 genes that were induced more than twofold by IFN in the
absence of NS5A expression (Fig. 2A, black bars). In contrast,
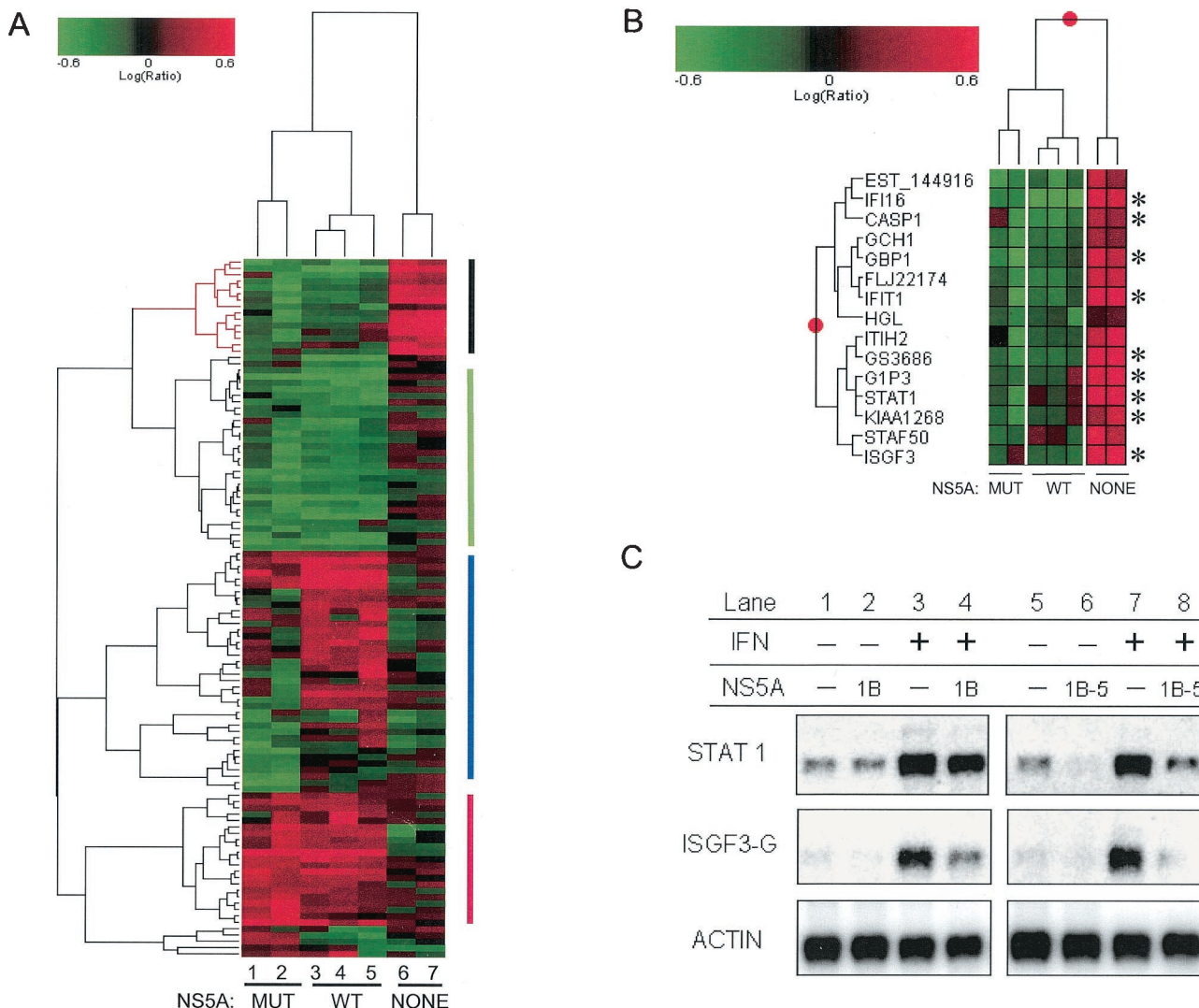when NS5A expression was induced, the IFN-mediated induc-

FIG. 2. NS5A alters IFN-regulated changes in gene expression. (A) Clustering of differentially expressed genes detected in IFN-treated cells in the presence or absence of wild-type or mutant forms of NS5A. Genes induced by IFN and down-regulated by NS5A are indicated by the vertical black bar to the right, genes down-regulated by both forms of NS5A are indicated by the vertical green bar, genes induced by both forms of NS5A are indicated by the vertical red bar, and genes that were regulated differently by NS5A-1B and NS5A-1B5 are indicated by the vertical blue bar. Genes were selected based on a ≥2-fold change and a *P* value of ≤0.005 in at least two experiments. Lanes 1 and 2, NS5A-1B5 (mutant); lanes 3 to 5, NS5A-1B (wild type); lanes 6 and 7, no NS5A. Each lane represents a unique experiment (and separate RNA isolation) using the cell line indicated. (B) Magnified view of ISGs whose expression was inhibited by NS5A. *, genes with known or putative ISREs (Table 1). Lane designations are the same as described for panel A. (C) Northern blot analysis of STAT1 and ISGF3G (IRF9) mRNAs during IFN treatment of HeLa cells expressing wild-type or mutant NS5A.

tion of all 14 of these genes was at least partially blocked (Fig. 2B). Both forms of NS5A can impose this block, although the induction of some genes, such as STAT1 and ISGF3 (IRF9), was more strongly repressed by NS5A-1B5. The results for STAT1 and ISGF3 (IRF9), both of which cooperate to bind the ISRE, were verified by Northern blot analysis (Fig. 2C). Since NS5A-1B and NS5A-1B5 had similar effects in these experiments, it is likely that regions of NS5A outside of the ISDR and PKR-binding domain are responsible for the effects of NS5A on IFN signaling.

A further analysis of the effects of NS5A on IFN-treated cells revealed that in addition to attenuating the induction of ISGs, NS5A had a broad impact on cellular gene expression.

Hierarchical clustering revealed two groups of differentially expressed genes that exhibited similar patterns of regulation in cells expressing either NS5A-1B or NS5A-1B5 (Fig. 2A, red and green bars). Conversely, other clusters contained genes that were differentially expressed in the presence of one form of NS5A but not the other (Fig. 2A, blue bar), perhaps due to differences in the PKR-binding properties of the two proteins. Gene expression profiling of NS5A-expressing cells in the absence of IFN treatment indicated that at least 200 genes were differentially regulated in response to NS5A expression (additional data are available at http://www.expression.washington.edu/public). Although a detailed analysis of these genes is beyond the scope of the work presented here, it was apparent
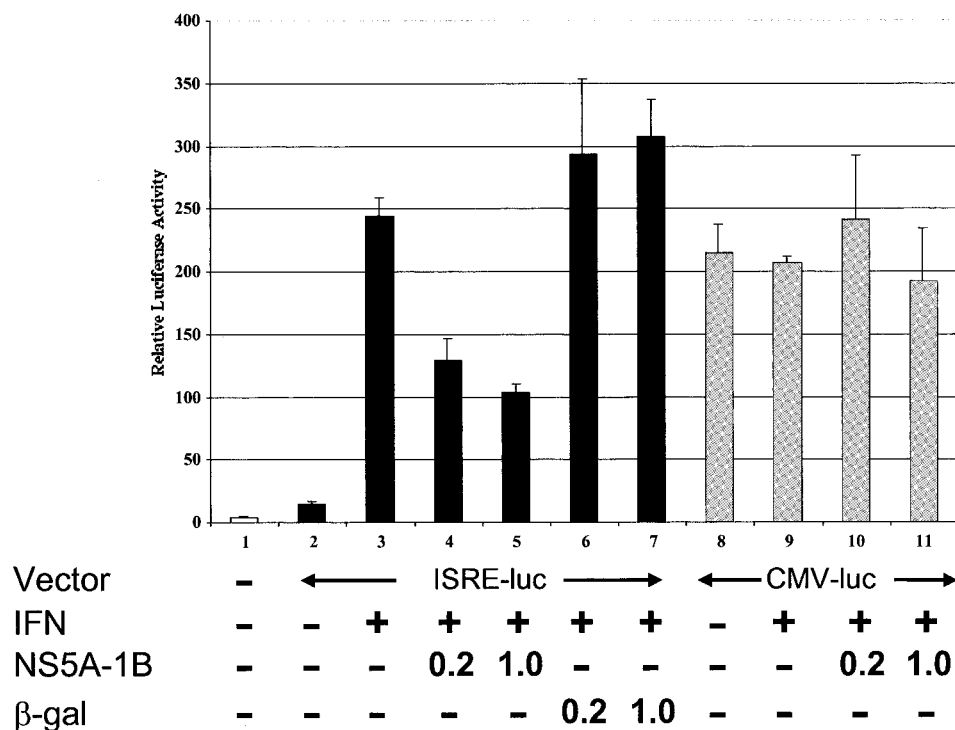
FIG. 3. NS5A inhibits ISRE-mediated gene transcription. HeLa cells were transfected with plasmid constructs as indicated. Lane 1, pTRE; lanes 2 and 3, pISRE-Luc; lane 4; pISRE-Luc + NS5A (0.2 µg of DNA); lane 5, pISRE-Luc + NS5A (1.0 µg of DNA); lane 6, pISRE-Luc + β-Gal (0.2 µg of DNA); lane 7, pISRE-Luc + β-Gal (1.0 µg of DNA); lanes 8 and 9, pCMV-Luc; lane 10, pCMV-Luc + NS5A (0.2 µg of DNA); lane 11, pCMV-Luc + NS5A (1.0 µg of DNA). Following transfection, cells were mock treated or treated with IFN for 16 h as indicated. Cell lysates were then prepared, and luciferase activity was measured. Luciferase activity is expressed as relative light units (RLU) on the y axis. All experiments were performed in triplicate. Mean values and error bars are shown.

that several signaling pathways were affected by NS5A expression. For instance, NS5A appeared to have a significant effect on calcium signaling, since the expression of multiple genes that encode for calcium-binding proteins, or which function in a calcium-dependent manner, was altered in response to NS5A expression. We also observed that NS5A expression resulted in the activation of a number of genes that cluster in a region of chromosome 19 (additional data available at http://www .expression.washington.edu/public), again suggesting the possibility of coordinate regulation related to chromatin structure.

NS5A inhibits the transcriptional activation of ISRE-containing promoters. Two of the ISGs (ISGF3 and STAT1) that were negatively affected by NS5A are transcription factors that participate in ISRE-mediated gene expression. In addition, an examination of the promoters of the 14 ISGs whose expression was attenuated by NS5A revealed that 9 of these genes contained either known or predicted ISREs in their promoter regions (Fig. 2B and Table 1). Therefore, to more directly study the effect of NS5A on ISRE-containing promoters, we used a luciferase reporter assay to examine the ability of NS5A to inhibit transcription from an ISRE-driven promoter. IFN treatment induced the transcriptional activity of an ISRE-containing reporter gene, as measured by luciferase activity, by more than 10-fold (Fig. 3, lanes 2 and 3). In contrast, in the presence of NS5A, the ability of IFN to induce ISRE-dependent transcription was partially blocked (Fig. 3, lanes 4 to 7). This block was specifically mediated by NS5A, since a control

construct encoding β-Gal failed to reduce the luciferase activity driven by the ISRE-containing promoter (Fig. 3, lanes 6 and 7). Importantly, the NS5A-mediated reduction of luciferase activity was specific to the ISRE-containing promoter, since NS5A had no effect on luciferase activity driven by a CMV promoter (Fig. 3, lanes 10 and 11). Therefore, NS5A does not appear to have a global effect on luciferase RNA translation but more likely functions to block ISRE-mediated transcription. The ability of NS5A to down-regulate ISRE-mediated transcription is consistent with our microarray results and provides independent evidence that NS5A can attenuate IFN-regulated gene expression.

An HCV subgenomic replicon has little effect on ISG expression. Given that NS5A was capable of down-regulating a subset of ISGs, we were interested in determining whether the same phenomenon occurred in Huh7 cells expressing an HCV subgenomic replicon. We began by comparing the gene expression profile of Huh7 cells expressing the replicon with that of Huh7 cells alone. Somewhat surprisingly, relatively few differences in gene expression were observed (additional data available at http://www.expression.washington.edu/public). We also noted that despite a high level of double-stranded RNA (dsRNA) production in cells expressing the replicon, there was an absence of ISG induction. The absence of ISG induction was not due to a lack of IFN responsiveness, since IFN treatment of Huh7 cells resulted in the induction of many ISGs (Fig. 1, lanes 3 and 4). However, there is a block in dsRNA
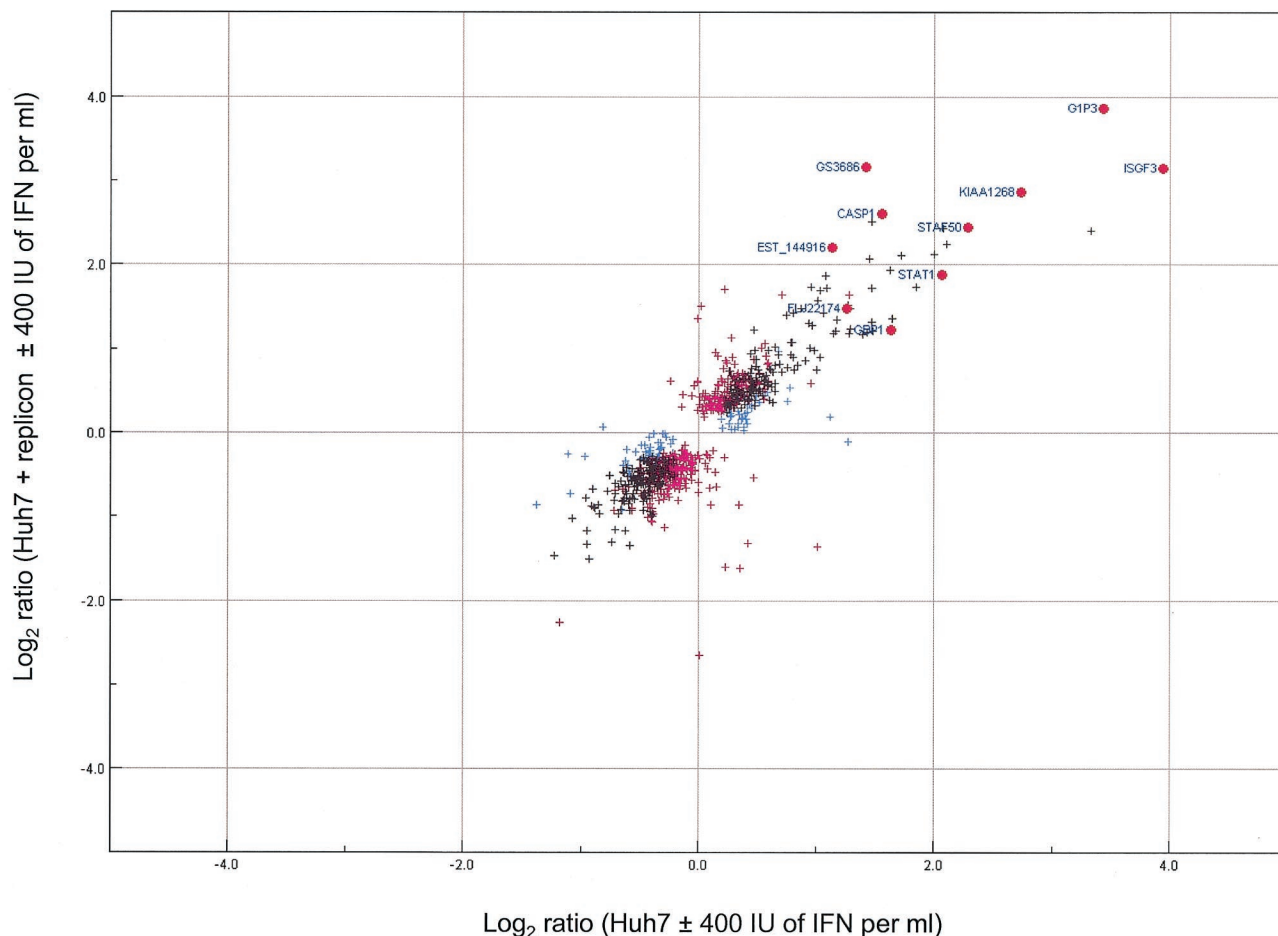
FIG. 4. IFN-regulated changes in gene expression in Huh7 cells in the presence and absence of an HCV subgenomic replicon are similar. A scatter plot of log₂ expression ratios for differentially regulated genes ($P \leq 0.01$) in Huh7 cells (x axis) and Huh7 cells harboring the replicon (y axis) in the presence or absence of IFN is shown. Blue crosses represent genes that were differentially expressed in IFN-treated Huh7 cells, magenta crosses represent genes that were differentially expressed in IFN-treated Huh7-replicon cells, and black crosses represent genes that were differentially expressed in both experiments. The ISGs that were down-regulated by NS5A in HeLa cells are highlighted by red circles. (Note that 4 of the 14 ISGs repressed by NS5A in HeLa cells were not induced by IFN in Huh7 cells).

signaling in cells expressing the replicon (37) which may inhibit the autocrine production of IFN, thereby blocking the induction of ISGs. We then examined gene expression profiles in IFN-treated cells to evaluate the effect of the replicon on the IFN-mediated induction of ISG expression (Fig. 4). For most ISGs, including the majority of those which were down-regulated by NS5A, the level of IFN-mediated induction was the same in replicon-containing cells as in cells lacking the replicon. However, the replicon did attenuate the IFN-mediated induction of ISGF3-γ and GBP1 to a level similar to that observed in NS5A-expressing cells (Fig. 4). Thus, compared to NS5A alone, the replicon had very little effect on ISG expression. The reasons for this difference are unknown but may be related to the IFN-sensitive phenotype of the replicon (14, 23) or the presence of other HCV proteins that might alter the IFN-attenuating properties of NS5A. It is also possible that the selection of replicons for replication efficiency affects nonreplicative functions of NS5A, including its effect on ISG expression.

DISCUSSION

Using global gene expression profiling, we identified a set of 50 genes that were induced by IFN in all cell types examined and another 60 genes that were induced in at least one cell type. In addition, we identified 31 genes that were repressed by IFN treatment. Different cell types differed in their response to IFN treatment, and of the cells tested, primary fetal hepatocytes exhibited the largest number of changes in gene expression in response to IFN (96 differentially regulated genes). We stress, however, that our analyses were not designed to identify all IFN-regulated genes, and despite the large number of differentially regulated genes identified in this study, these genes likely represent only a subset of the transcriptome that is responsive to IFN treatment. Analyses (using more comprehensive arrays) of other cell lines (G. K. Geiss, unpublished observations) (7, 8) or of cells overexpressing IFN-regulatory factors (21) have identified additional genes that are responsive to IFN treatment. Other variables, such as the amount or

duration of IFN treatment, also influence the number and identity of IFN-regulated genes.

Bioinformatic analysis of ISG promoter regions revealed that 26 of the 110 ISGs identified had putative ISREs and that 20 of those (Table 1) were among the 50 ISGs induced in all cell types. We not only identified known ISREs in the promoter regions of genes such as PKR, MX1, and IFIT1 but also identified novel candidate ISRE-like sequences in the promoter regions of several other genes with known roles in innate immunity, including TLR3, TNFRSF10, and CASP1. A similar analysis of 20 ISGs by Su et al. (43), using a slightly different ISRE consensus sequence that allowed a single mismatch, identified a number of ISREs not found using our search criteria. It is therefore likely that additional ISRE-containing genes remain to be identified. We again note that although this approach provides a rapid means for the identification of candidate regulatory elements, biochemical validation of the function of any specific element is required to confirm its biological activity.

A genome-wide search for common DNA elements in the promoter regions of non-ISRE-containing ISGs, and refinement of the technique to include other relevant information (such as the locations of TATA boxes relative to ISRE sites), is currently under way. ISGs that do not contain an ISRE-like sequence may include genes that contain IFN-γ-activated sites as well as genes that are not directly induced by IFN or which are induced by some other factor that is a component of a signaling pathway that overlaps with IFN signaling, such as NF-κB or TRAIL (29). In addition, we noted that some ISGs cluster with one another at various regions throughout the genome. This observation raises the possibility that alterations in chromatin structure play a role in the coordinate regulation of multiple IFN-regulated genes. This hypothesis should be testable by chromatin immunoprecipitation analysis in combination with microarrays, a procedure for studying genome-wide protein-DNA interactions (30, 40).

We used HeLa cell lines (expressing either wild-type NS5A or a mutant form of NS5A that lacks the ability to bind to or inhibit PKR) to examine the effect on ISG expression of the presence of NS5A. In the absence of NS5A, IFN treatment induced the expression of 14 ISGs by more than twofold. The expression of either form of NS5A inhibited the IFN-mediated induction of all 14 of these ISGs. This finding suggests that NS5A mediates HCV resistance to IFN by attenuating specific aspects of IFN signaling. In addition, since both forms of NS5A had similar effects on ISG expression, this property of NS5A appears to be independent of ISDR sequence or its ability to inhibit PKR. This might partly explain why ISDR sequence does not always correlate with HCV resistance to IFN. Interestingly, in a similar analysis using Huh7 cells, Girard et al. demonstrated that NS5A reduced the IFN-mediated induction of 9 of 50 ISGs (20). However, none of the ISGs that were down-regulated by NS5A in Huh7 cells were among the 14 ISGs identified in our analyses. This difference most likely reflects the cell type-specific nature of the IFN response but might also be due to other factors, including differences in the parameters used to identify and select differentially expressed genes. Still, both studies point to similar roles for NS5A in mediating IFN resistance.

NS5A also had a profound effect on overall cellular gene expression in the absence of IFN treatment. Over 200 genes were differentially regulated in at least four of nine experiments. It is likely that the large number of genes altered by NS5A expression have a profound impact on multiple cellular pathways, which might partially explain the diverse set of biological functions that have been assigned to NS5A in various systems. By combining gene expression analysis with NS5A functional studies, such as analysis of cell growth characteristics or tumorigenic potential (17), it might be possible to identify sets of genes that contribute to these phenotypes.

We also compared the expression profiles of genes induced by NS5A in HeLa cells with those of Huh7 cells expressing an HCV subgenomic replicon. The effect of the replicon on the IFN-mediated induction of ISG expression in Huh7 cells was much less pronounced than that observed for NS5A alone. However, the replicon did down-regulate two ISGs, including ISGF3-γ, a subunit of the ISRE-binding complex, and GBP1, a guanylated binding protein of unknown function. Again, the differences observed in these systems might be due to a number of factors, including the level of NS5A expression, cell-specific factors, or the presence of other HCV proteins. These factors may also contribute to the disparate results that have contributed to the controversy surrounding the role of NS5A in mediating IFN resistance (44). Moreover, the high error rate of the HCV RNA-dependent RNA polymerase introduces adaptive mutations in the replicon, which can result in distinct properties with respect to replication efficiency (3, 28, 31) and dsRNA signaling (37). Not surprisingly, some of these mutations have been mapped to NS5A. The results of this study, in conjunction with previous work, point to the need for a way of comparing results from the diverse surrogate systems used to investigate the biology of HCV. Clustering of gene expression profiles from these different systems, ideally to a standard reference sample, may eventually allow us to predict which system, if any, is most similar to a bona fide HCV infection either in tissue culture or in patients.

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