Hepatitis C Virus NS5A-Regulated Gene Expression and Signaling Revealed via Microarray and Comparative Promoter Analyses

Sophie Girard,[1]* Erik Vossman,[1]* David E. Misek,[2] Philippe Podevin,[3] Samir Hanash,[2] Christian Bréchot,[3] and Laura Beretta[1]

Most individuals exposed to hepatitis C virus (HCV) become chronically infected and are predisposed to liver disease. The mechanisms underlying viral persistence and disease progression are unknown. A role for the HCV NS5A protein in viral replication and interferon resistance has been demonstrated. To identify mechanisms affected by NS5A, we analyzed the gene expression of Huh7 cells expressing NS5A and control cells using oligonucleotide microarrays. A set of 103 genes (43 up-regulated, 60 down-regulated) whose expression was modified by at least twofold was selected. These included genes involved in cell adhesion and motility, calcium homeostasis, lipid transport and metabolism, and genes regulating immune responses. The finding of modulated expression of genes related to the TGF-β superfamily and liver fibrosis was observed. Interestingly, both the tumor necrosis factor and lymphotoxin beta receptors were down-regulated by NS5A. Similar data were obtained following expression of four NS5A mutants obtained from patients who were not responsive or were sensitive to interferon therapy. Through computational analysis, we determined that 39 of the 43 genes up-regulated by NS5A contained one or more nuclear factor κB (NF-κB) binding sites within their promoter region. Using the Gibbs sampling method, we also detected enrichment of NF-κB consensus binding sites in the upstream regions of the 43 coexpressed genes. Activation of NF-κB by NS5A was subsequently demonstrated in luciferase reporter assays. Adenovirus-mediated expression of IκBα reverted NS5A mediated up-regulation of gene expression. In conclusion, this study suggests a role of NS5A and NF-κB in HCV pathogenesis and related liver disease. *Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2004;40:708–718.)

Hepatitis C virus (HCV) is a highly prevalent pathogen associated with a high subsequent risk of progression to liver cirrhosis and hepatocellular carcinoma (HCC).[1] The HCV genome is a positive-stranded RNA of 9.6 kb that encodes for a polyprotein precursor, which is co- and posttranslationally processed to yield 10 structural and nonstructural proteins (C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B).[2]

Several studies have suggested a role of NS5A in resistance to interferon (IFN)-α treatment.[3–7] We and others reported an altered cellular response to IFN-α and a concomitant up-regulation of interleukin (IL)-8 by NS5A.[8,9] It has also been reported that NS5A participates in HCV viral replication[10–14] and is a potent transcriptional activator.[15] Therefore, identification of cellular genes modified by NS5A in hepatocytic cells may provide insight into the role of NS5A in HCV replication and pathogenesis. We used microarrays to compare the gene expression profile between control Huh7 cells and Huh7 cells expressing a NS5A mutant isolated from a patient infected with HCV-1b and resistant to IFN-α therapy. We subsequently compared the effects of four NS5A mutants iso-

Abbreviations: HCV, hepatitis C virus; NF-κB, nuclear factor κB; HCC, hepatocellular carcinoma; IFN, interferon; IL, interleukin; GFP, green fluorescence protein; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; LTBR, lymphotoxin beta receptor; IκBα, inhibitor κBα; cDNA, complementary DNA; RT-PCR, reverse-transcriptase polymerase chain reaction; PCR, polymerase chain reaction; mRNA, messenger RNA; LTBP1, latent TGF-β binding protein 1; IL-1RA, interleukin 1 receptor antagonist; TNFR, tumor necrosis factor receptor; BMP2, bone morphogenic protein 2; PLTP, phospholipid transfer protein.

From the Departments of [1]Microbiology and Immunology and [2]Pediatrics, University of Michigan, Ann Arbor, MI; and [3]INSERM U.370, Necker Institute, Paris, France.

Received January 7, 2004; accepted May 26, 2004.

This work was supported in part by National Institutes of Health grant DK59771, a grant from the University of Michigan Biomedical Research Council, and a grant from the University of Michigan Gastrointestinal Peptide Research Center (2 P30 DK34933).

Present address for P. P.: Service d'Hepatogastroenterologie, Cochin Hospital, Paris, France.

**S. G. and E. V. contributed equally to the study.*

Address reprint requests to: Laura Beretta, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N., Seattle, WA 98109. E-mail: lberetta@fhcrc.org; fax: (206) 667-2537.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.20371

lated from patients who were responsive or nonresponsive to IFN-α therapy upon transient expression in Huh7 cells.

The computational identification of transcription factor binding sites through the analysis of DNA sequence data has recently emerged as a major technology for the elucidation of transcription regulatory networks. We used computer-based approaches such as the Gibbs sampling method to determine common transcription factor motifs in the promoters of the genes up-regulated by NS5A.

Patients and Methods

Cell Culture and Transient NS5A Expression. NS5A sequences were isolated from 4 patients infected with HCV 1b, treated with standard IFN-α therapy (3 to 6 MU, 3 times per week for 6 months). Three NS5A sequences from 1 responder (R1) and 2 nonresponder (NR1 and NR2) patients to IFN-α treatment were previously described.[7] The fourth NS5A sequence, isolated from a responder (R2) patient, was amplified, sequenced, and cloned in the pCDNA3.1 vector (Invitrogen, Carlsbad, CA) using the same methodology.[7] We established Huh7 clones expressing the R1, NR1, and NR2 NS5A mutants.[7] Herein, we used the control clones T1 and T2 and two clones, F1 and F11, expressing the NS5A mutant NR1.[7] For transient NS5A expression, Huh7 cells were transfected via addition of 30 υL of lipofectamine (GIBCO BRL, Gaithersburg, MD) and 15 υg of the plasmid containing NR1, NR2, R1, and R2 NS5A sequences or the empty vector in OPTIMEM I medium (GIBCO BRL). In additional experiments, Huh7 cells were cotransfected with 4 υg of the pEGFP vector encoding the green fluorescence protein (GFP) (Clontech, La Pont de Claix, France) as a monitor for transfection efficiency. The efficiency of transfection was approximately 50%–60%.

SDS–Polyacrylamide Gel Electrophoresis and Western Blot Analysis. Total proteins (50 μg) were diluted in Laemmli sample buffer, resolved via 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dasel, Germany). The membranes were incubated with monoclonal antibodies against NS5A (Biogenesis Ltd., Poole, United Kingdom) (1:1,000 dilution) and actin (ICN, Costa Mesa, CA) (1:20,000 dilution) or with polyclonal antibodies against lymphotoxin beta receptor (LTBR) (R&D systems, Minneapolis, MN) (1:5,000 dilution) and IκBα (Santa Cruz Biotechnology, Santa Cruz, CA) (1:1,000 dilution). Immunodetection was realized using enhanced chemiluminescence reagents (Amersham, Buckinghamshire, United Kingdom).

Adenovirus Infection. F1 and F11 cells were plated at a density of 4×10^5 per well in 6-well tissue culture plates. Twenty-four hours later, the cells were infected with the adenoviruses AdIκBα and AdGFP, with a cytomegalovirus (CMV) promoter driving expression of full-length IκBα complementary DNA (cDNA) and GFP, respectively. Fluorescence microscopy confirmed that adenovirus-transferred genes were expressed in nearly 100% of cells 18 hours after infection.

Luciferase Reporter Assays. Luciferase assays were performed using an E-selectin luciferase plasmid and reagents from Promega (Madison, WI) in the single and dual format by monitoring the transfection efficiency with pRL-TK.

Preparation of Complementary RNA and Gene Chip Hybridization. Total RNA was isolated and used to generate complementary RNA probes. Preparation of complementary RNA, hybridization, and scanning of the HuGeneFL Arrays (Affymetrix, Inc., Santa Clara, CA) were performed as described previously.[8] Data analysis was performed using GeneChip 4.0 software (Affymetrix, Inc.). Genes differently modified by NS5A were compared in F1 and F11 clones with each control cell line (T1 and T2) and then selected (fold change ≥ 2, $P < .05$). For each gene, the fold change between NR1 and control clones was calculated for 2 experiments using Affymetrix software (Affymetrix, Inc.).

Reverse Transcription Followed by Polymerase Chain Reaction. Single-stranded cDNA was synthesized from total RNA using Superscript II reverse transcriptase (GIBCO-BRL) and random hexamers (GIBCO-BRL) as primers. For regular reverse-transcriptase polymerase chain reaction (RT-PCR), the reaction mixture was composed of 1 μL of cDNA template obtained from 1 μg extracted RNA; 25 pmol primers; 25 nmol each deoxyribonucleoside triphosphate; 2.5 U Taq DNA polymerase (GIBCO-BRL); and 5 μL 10$\times$ polymerase chain reaction (PCR) buffer in a final volume of 50 μL. The nucleotide sequences of primers and corresponding annealing temperatures used are as indicated (Table 1). Primers for real-time PCR were designed using the PRIMER3 program (http://www.genome.wi.mit.edu) (see Table 1). As an internal reference, actin was amplified in parallel with the genes of interest. All reactions were carried in 20 μL volumes containing 10 μL of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 1 μL (25 pmol) of each primer and 5 μL of cDNA product. Reactions were performed in the Bio-Rad iCycler iQ Optical detection system. Meltcurves were also performed to determine unique amplification products.

Table 1. Primer Sequences

Gene	Sequences	Product Size (bp)	Annealing Temperature (°C)
Actin	5′-ATCATGTTTGAGACCTTCAA-3′	644	60
	5′-TTGCGCTCAGGAGGAGCAAT-3′		
Actin	5′-TGGACTTCGAGCAAGAGATGG-3′	138	60
	5′-GGAAGGAAGGCTGGAAGAGTG-3′		
Neurotensin	5′-GCATGCTACTCCTGGCTTTC-3′	131	60
	5′-CCAGAGGGACATGTGCTT-3′		
Osteopontin	5′-CCAAGTAAGTCCAACGAAAGCC-3′	273	58
	5′-GTACTGGATGTCAGGTCTGCG-3′		
Aldore reductase	5′-AGCAGCCAGGATATGACCAC-3′	89	60
	5′-GGGTAATCCTTGTGGGAGGT-3′		
LTBP1	5′-CTTCCGCTGCCTCTGTTATC-3′	125	60
	5′-ACCCTTCCACGTTTTCACAG-3′		
GRO-α	5′-GAAAGCTTGCCTCAATCCTG-3′	107	60
	5′-CSCCSGTGSGCTTCCTCCTC-3′		
IL-1RA	5′-GCAGTCACCTAATCACTCTCC-3′	228	60
	5′-CCAAGAACAGAGCATGAGGC-3′		
LTBR	5′-CCATATGCGTCGGAGAACCAG-3′	349	59
	5′-CAGGCGGGCAGTCAGAAAGTAG-3′		
TNFR1A	5′-GACCAGGCCGTGATCTCTATGC-3′	561	60
	5′-ATGCCGGTACTGGTTCTTCCTG-3′		
Carbonic anhydrase IV	5′-AGTCCCCCATCAACATCGTCAC-3′	345	60
	5′-GCACCGCAATTTCGTCTTCAG-3′		
Trefoil factor 1	5′-GGTCGCCTTTGGAGCAGAGA-3′	205	59
	5′-CGTCGAAACAGCAGCCCTTATT-3′		
CRBP1	5′-TGTCCCGGCAGTCTCCAG-3′	380	59
	5′-CCACGCCCCTCCTTCTCA-3′		
PLTP	5′-GGCACCACCATCTCTGTCACTG-3′	344	59
	5′-AATCCCGCATGGTTCGTCAC-3′		
TGF-β1	5′-GCGCCCATCTAGGTTATTTC-3′	284	59
	5′-CCAGGCGTCAGCACCAGTAG-3′		
Collagen IV-α5	5′-TCCAGCTCTGGAAGGACCTA-3′	89	60
	5′-CCAGGATTTCCCTTCTCTCC-3′		
PLAB	5′-GACCTATGATGACTTGTTAGCC-3′	143	60
	5′-CAGGAATCGGGTGTCTCAGG-3′		
BMP2	5′-AACACTGTGCGCAGCTTCC-3′	101	60
	5′-CTCCGGGTTGTTTTCCCAC-3′		
Decorin	5′-TCAATGGACTGAACCAGATGA-3′	145	60
	5′-CCTTGAGGAATGCTGGTGAT-3′		

Quantitative Enzyme-Linked Immunosorbent Assay for TGF-β1. The amount of TGF-β1 present in the culture media of the T1, T2, F1, and F11 clones were measured with Quantikine TGF-β1 enzyme-linked immunosorbent assay (R&D Systems). The supernatants were activated with 1 N HCl for 10 minutes and neutralized with 1.2 N NaOH and 0.5 M hydroxyethylpiperazine-N-2 ethanesulfonic acid. The captured TGF-β1 proteins were quantitated at 450 nm in a spectrophotometer. Two independent experiments as well as two independent measures were performed for each sample.

Computational Promoter Analysis. Human genomic sequences were obtained from http://genome.ucsc.edu. Corresponding human messenger RNA (mRNA) sequences encoding for the N terminus of each protein were obtained from the National Center for Biotechnology Information GenBank RefSeq database (available at http://www.ncbi.nlm.nih.gov/Genbank/index.html) and subsequently aligned with the genomic sequence to identify the transcription start site. Promoter regions were then defined from this transcription start site—1 kbp upstream and 1 kbp downstream—and analyzed using MATCH, a weight matrix–based tool for searching putative transcription factor biding sites in DNA sequences.[16,17] MATCH assigns matrix and core similarity values for each transcription factor based on the position weight matrices. The cutoff values for core and matrix similarities used were 0.85. Promoter region comparison was performed by entering the defined promoter sequences into the Gibbs Motif Sampler (http://bayesweb.wadsworth.org/gibbs/gibbs.html). The Gibbs Motif Sampler is a software for locating multiple transcription factor binding sites for multiple transcription factors simultaneously in unaligned DNA sequences.[18] Motif searches were specified between 14–25 bp in length. Sequences with score values of 1.000 for core and 0.500 for matrix were selected. The internal Wilcoxon signed rank test was also used in the assessment of statistical significance of our data sets. Pictograms of the Gibbs output were generated using software available at http://genes.mit.edu/pictogram.html to show the relative frequency of each base within the returned motifs.

Results and Discussion

Gene Expression Profile of Huh7 Cells Expressing NS5A. Hepatocytic Huh7 cells expressing NS5A mutants isolated from patients infected with HCV-1b were generated as described previously.[7] We wished to compare the gene expression profile of clones F1 and F11 expressing the NS5A NR1 mutant isolated from a nonresponder to IFN-α therapy with control clones T1 and T2 expressing the empty vector using oligonucleotide arrays complementary to 6,800 genes. Expression levels of NS5A in these cells are shown in Fig. 1. Two independent experiments were performed. RNA transcript levels from both NS5A expressing cell lines F1 and F11 were compared with control cell lines T1 and T2. We selected genes that differed in their expression levels by twofold or greater in each comparison pair and in both experiments, with a P value of less than .05. The 103 genes that were identified are presented in Tables 2 and 3 for up-regulated and down-regulated genes, respectively. Changes included differential expression of genes related to cell adhesion and motility. Expression of trefoil factors 1 and 2,

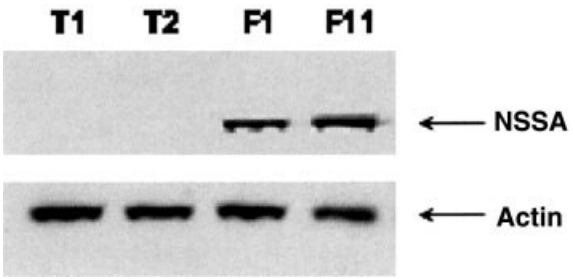

Fig. 1. NS5A expression in Huh7 cell clones. Total proteins from Huh7 clones stably expressing NS5A (F1 and F11) and from control clones (T1 and T2), were separated via SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-NS5A and anti-β-actin antibodies.

galectin 2, LI-cadherin, and hepatocyte growth factor–like protein transcripts were decreased with a concomitant up-regulation of genes of the extracellular matrix such as matrilin 3, collagen type IV, Cyr61, fibrillin-like protein, and latent TGF-β binding protein 1 (LTBP1). Several up-regulated genes were related to immune functions and inflammation, such as osteopontin, interleukin 1 receptor antagonist (IL-1RA), and the chemokines MIP-3α/CCL20, IL-8, and GRO-α. In contrast, we observed a down-regulation by NS5A of STAT6 and two members of the tumor necrosis factor receptor (TNFR) family: TNFR1A and LTBR. Finally, the regulation of a large group of genes encoding for lipid-binding proteins or enzymes involved in lipid homeostasis was observed.

Some genes reported to be differentially expressed in HCV-infected liver tissue changed their expression accordingly in our analysis. This group included the TGF-β superfamily member PLAB, HLA-DQA1, and Rho-B, which were up-regulated during acute HCV infection in chimpanzees,[19] and the CDK inhibitor Kip2, IL-8, and epidermal growth factor receptor, which were up-regulated in HCV cirrhotic liver tissue.[20]

To validate the microarray data, real-time RT-PCR analysis was undertaken. Transcript levels were determined for neurotensin, LTBP1, bone morphogenic protein 2 (BMP2), collagen IV-α5, IL-1RA, carbonic anhydrase IV, and decorin in clones T1, T2, F1, and F11 (Fig. 2). Real-time RT-PCR and microarray data were highly concordant for all of these randomly selected genes (see Fig. 2). Transcript levels were also determined using semiquantitative RT-PCR for osteopontin, aldose reductase, GRO-α, trefoil factor 1, cellular retinol binding protein 1, and phospholipid transfer protein (PLTP) in clones T1, T2, F1, and F11. In agreement with the microarray data, transcripts for osteopontin, aldose reductase, and GRO-α were increased in F1 and F11 compared with control clones, while transcripts for trefoil factor 1, cellular retinol binding protein 1, and PLTP were de-

Table 2. Genes Up-Regulated in NS5A-Expressing Huh7 Clones F1 and F11

Description	Gene Bank Accession No.	Fold Change
Hepatocarcinoma-associated		
Neurotensin	U91618	12
Epidermal growth factor receptor	X00588	2.5
Aldose reductase	X15414	2.3
Signal transduction/cell proliferation		
Adrenal-specific protein pG2	X17544	~8
Gravin	U81607	~4.5
CDK inhibitor p57/Kip2	U22398	~3.5
SH3-domain binding protein 2	AB000462	~3
Bone morphogenetic protein 2	M22489	3
Pim-2 oncogene	U77735	~2.5
Phosphodiesterase 3A, cGMP-inhibited	U36798	~2.5
Ephrin receptor EphA5	L36644	~2.5
Thromboxane A2 receptor	D38081	~2.3
Chimerin 1	**X51408**	**2.3**
Mitogen-activated protein kinase 1/extracellular signal–regulated kinase 2	Z11695	2.2
Homeobox protein Cdx1	U51095	~2.2
Guanine nucleotide binding protein	**J03260**	**~2.2**
Activin A receptor, type II	M93415	~2.2
Dual-specificity protein kinase 4	Y09305	~2.2
RhoB	M12174	2.1
TGF-Beta superfamily protein PLAB/GDF-15/MIC-1	AB000584	2
Dual-specificity phosphatase 1	**X68277**	**2**
Immune response		
Interleukin 8	**M28130**	**5**
Osteopontin	U20758	~5
GRO-α	X54489	2.2
Interleukin 1 receptor antagonist	**X53296**	**~2.2**
MIP-3α/CCL20	U64197	2
ISG20	U88964	2
TNF alpha-induced protein 2	M92357	2
Major histocompatibility complex class II (HLA-DQA1)	M34996	~2
Extracellular matrix		
Matrilin 3	AJ001047	3.5
Cysteine-rich, angiogenic inducer 61	U62015	3
Fibrillin-like protein	**U03877**	**~3**
Latent TGF-β binding protein 1	M34057	~2.5
Type IV collagen α5 chain	U04520	~2.3
Cell adhesion and motility		
α-Tubulin	X01703	4.5
Integrin-associated protein/CD47	Z25521	2.5
Vimentin	**Z19554**	**2.2**
Miscellaneous		
Reticulon 1	L10333	~3
Mg81	L08240	~3
Chloride channel 5	X81836	~2.5
Cytochrome P450, subfamily I	X02612	2.3
Glycogen phosphorylase B	**U47025**	**2.1**
Nova1	**U04840**	**2**

NOTE. mRNA from control clones T1 and T2 and from NS5A-expressing clones F1 and F11 were hybridized onto Affymetrix oligonucleotide arrays as indicated in Patients and Methods. For each gene, the fold change was calculated with Affymetrix software, and up-regulated genes were selected. The ~ indicates a fold change calculation for which the smaller value is replaced by an estimate of the minimum value for detectable transcripts. Genes up-regulated following transient expression of NSSA are shown in bold.

Table 3. Genes Down-Regulated in NS5A-Expressing Huh7 Clones F1 and F11

Description	Gene Bank Accession No.	Fold Change
Cell adhesion and motility		
Trefoil factor 2 (SP)	**X51698**	**~ −8.6**
Galectin 2	**M87860**	**~ −5.7**
Hepatocyte growth factor-like protein/MSP	**U37055**	**~ −3.3**
Trefoil factor 1 (pS2)	X52003	−2.9
Urokinase-type plasminogen activator receptor	U09937	~ −2.6
LI-cadherin	X83228	−2.5
Carcinoma-associated antigen GA733-2	**M93036**	**−2.3**
CD97	**U76764**	**~ −2.3**
Lymphocyte antigen 6 complex, locus D (E48)	**X82693**	**~ −2.2**
Signal transduction/cell cycle		
Transcription elongation factor S-II	D50495	~ −3.9
Frizzled-5	U43318	−3
Growth factor receptor-bound protein 14	L76687	−2.8
ELK3/net transcription factor	**Z36715**	**~ −2.4**
Phosphoinositide-3-kinase	Y10055	~ −2.4
Cyclin F	Z36714	~ −2.3
Mitogen-activated protein kinase kinase 3	D87116	−2.2
Calpain 1	**X04366**	**−2.2**
Decorin	M14219	−2
Immune response		
Lymphotoxin β receptor	**L04270**	**~ −7.6**
Tumor necrosis factor receptor 1A	**M58286**	**−7.3**
Complement component 4-binding protein, β	L11244	−2.6
Complement component 4-binding protein, α	M62486	−2.6
Omega Light Chain, prot 14.1	**M34515**	**~ −2.4**
STAT6	U16031	~ −2
Calcium-binding proteins		
Inositol 1,4,5-triphosphate receptor, type 3	**U01062**	**~ −2.8**
Reticulocalbin 1	**D42073**	**−2.4**
Grancalcin	M81637	−2.3
SERCA1	U96781	~ −2.2
S100 calcium binding protein P	X65614	−2
Metabolism		
Cellular retinol binding protein 2	U13831	−9
Carbonic anhydrase IV	**L10955**	**~ −6**
Glutathione S-transferase 2	X65727	−4.3
Nuclear receptor SHP	L76571	−3.1
Phospholipid transfer protein	**L26232**	**~ −2.9**
Hepatocyte nuclear factor 1β (TCF2)	**X58840**	**~ −2.5**
Fatty acid binding protein 1	M10050	−2.5
Carboxylesterase 1	L07765	−2.5
Lipoprotein-associated phospholipase A2	**U24577**	**−2.4**
Histidase	D16626	~ −2.4
Nuclear orphan liver X receptor α	U22662	~ −2.3
Glutamine transaminase K	**X82224**	**~ −2.3**
Solute carrier family 7, member 6	**D87432**	**−2.3**
Galactokinase 1	**L76927**	**~ −2.2**
Arylacetamide deacetylase	L32179	−2.2
Hydroxyacyl-CoA dehydrogenase, type II (ERAB)	U73514	−2
Apolipoprotein E receptor 2	Z75190	−2
Cellular retinol binding protein 1	**M11433**	**−2**
Miscellaneous		
Neuronal pentraxin II	U29195	−4.3
Giant larvae (*Drosophila*) homolog	**X87342**	**~ −4**
GTP-binding protein hsr1	X66436	~ −3.4
Predicted osteoblast protein (GS3786)	D87120	−2.9
H2A histone family, member A	M60752	−2.4
Carboxypeptidase N2	J05158	~ −2.3
Guanylate cyclase 2C	M73489	−2.3
Cold shock domain protein A	M24069	−2.2
Fibrinogen-like protein 1	D14446	−2.2
Hypertension-associated SA	D16350	~ −2.1
Kininogen	M11437	−2.1
LIM homeobox protein 1	U14755	~ −2
KIAA0140	D50930	−2

NOTE. mRNA from control clones T1 and T2 and from NS5A expressing clones F1 and F11 were hybridized onto Affymetrix oligonucleotide arrays as indicated in Patients and Methods. For each gene, the fold change was calculated with Affymetrix software, and down-regulated genes were selected. The ~ indicates fold change calculation for which the smaller value is replaced by an estimate of the minimum value for detectable transcripts. Genes down-regulated following transient expression of NSSA are shown in bold.

creased in NS5A-expressing clones (data not shown). Modification by NS5A of the mRNA levels for MIP-3α/CCL20, IL-8, and STAT6 was previously confirmed via PCR.[8]

Genes Dysregulated in HCC. The strongest effect of NS5A observed was a 12-fold up-regulation of neurotensin transcript. Neurotensin is transiently expressed in the fetal liver, suppressed in the adult liver, and re-expressed in certain liver cancers.[21] We identified additional genes up-regulated by NS5A that were previously reported to be overexpressed in HCC, such as epidermal growth factor receptor, aldose reductase, and osteopontin.[22–24] Decorin expression suppressed in HCC[25] was down-regulated by NS5A. Modulation of neurotensin, aldose reductase, osteopontin, and decorin by NS5A was validated via RT-PCR analysis (see Fig. 2 and data not shown).

Intrahepatic metastasis of HCC is observed frequently. Loss of cell–cell contact caused by increased cell motility is a critical step in cancer metastasis. Many genes involved in cell adhesion, motility, and cytoskeleton remodeling were regulated by NS5A. This group included vimentin, α-tubulin, scaffold protein gravin, LI-cadherin, trefoil factors 1 and 2, and RhoB. It has been reported that RhoB is involved in intrahepatic metastasis of human HCC.[26]

Finally, we observed a 2.2-fold increase of mitogen-activated protein kinase 1/extracellular signal–regulated kinase 2 mRNA expression. Mitogen-activated protein kinase/extracellular signal–regulated kinase activation may play an important role in the progression of HCC.[27]

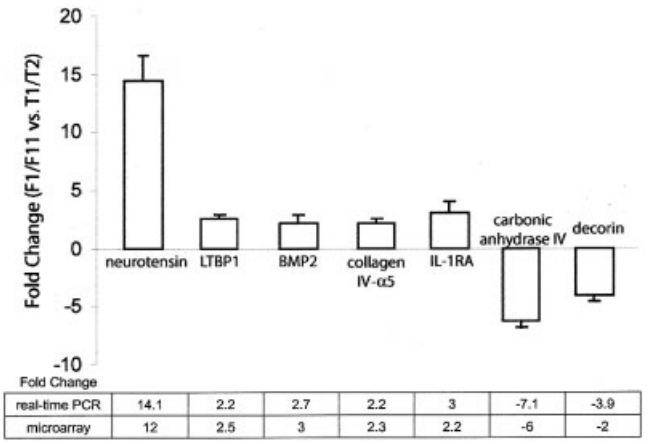

Fig. 2. Effects of NS5A on Huh7 gene expression. Total RNA from T1, T2, F1, and F11 Huh7 clones was reverse-transcribed, and real-time PCR amplification was performed for neurotensin, LTBP1, BMP2, collagen IV-α5, IL-1RA, carbonic anhydrase IV, and decorin as described in Patients and Methods. Transcript levels in F1 and F11 were normalized using actin mRNA levels and compared with T1 and T2 controls. Data are shown as mean ± SEM of the independent microarray experiments. Abbreviations: LTBP1, latent TGF-β binding protein 1; BMP2, bone morphogenic protein 2; IL-1RA, interleukin 1 receptor antagonist; PCR, polymerase chain reaction.

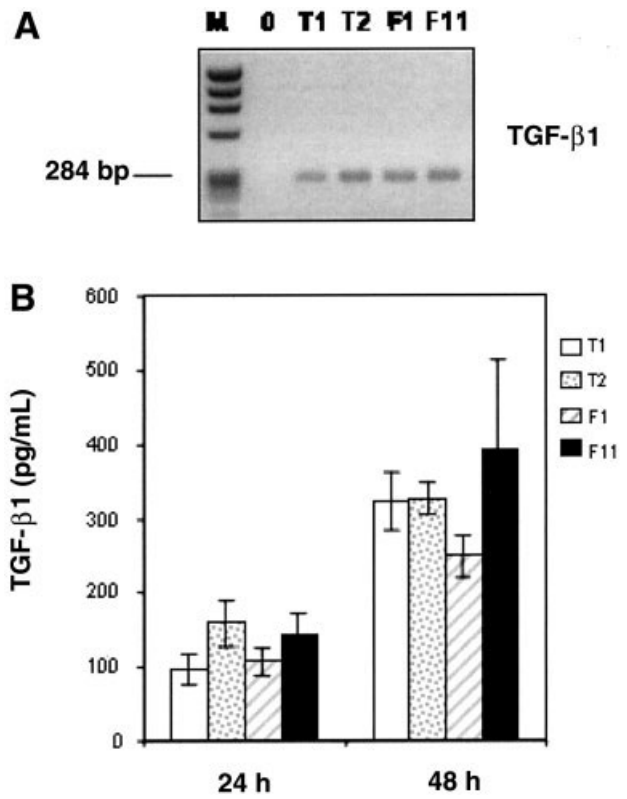

Fig. 3. Effect of NS5A on the TGF-β1 gene expression and protein secretion in Huh7 cells. (A) Total RNA from T1, T2, F1, and F11 Huh7 clones was reverse-transcribed and PCR amplification was performed for TGF-β1. (B) Secretion of TGF-β1 protein in NS5A-expressing cells as determined with enzyme-linked immunosorbent assay. T1, T2, F1, and F11 Huh7 clones were cultured for 2 days in OPTIMEM I medium (GIBCO BRL), and TGF-β1–secreted protein was measured in the supernatants every 24 hours.

TGF-β Superfamily Members and Modulators of Fibrosis. TGF-β is a potent modulator of liver fibrogenesis. In our microarray analysis, TGF-β1 mRNA levels remained unchanged between control and NS5A-expressing cells. Similar results were obtained via RT-PCR assay (Fig. 3A).

Remarkably, expression of LTBP1, a component of the latent TGF-β1 complex, was increased by 2.5-fold. LTBP1 is involved in the assembly, secretion, and targeting of TGF-β1 to sites at which it is stored and/or activated.[28] Positive staining for TGF-β and LTBP was observed in liver biopsies from HCV patients in areas with inflammation and fibrosis.[29] To determine if higher levels of LTBP1 mRNA in NS5A-expressing cells correlated with a modification of TGF-β1 protein secretion, TGF-β1 expression was determined with enzyme-linked immunosorbent assay in controls, T1, T2, and NS5A-expressing clones F1 and F11. The pattern of TGF-β1 protein expression was not significantly different in the culture supernatants between T1, T2, F1, and F11 clones, reaching 323 $\pm$ 39 (mean $\pm$ SEM) pg/mL, 327 $\pm$ 20 (mean $\pm$ SEM) pg/mL, 249 $\pm$ 28 (mean $\pm$ SEM) pg/mL, and 392 $\pm$ 121 (mean $\pm$ SEM) pg/mL in T1, T2, F1, and F11 respectively, at 48 hours (Fig. 3B).

Concomitant to the increase of LTBP1, we observed a twofold decrease of the gene coding for decorin. Decorin neutralizes TGF-β bioactivity and reduces fibrosis. In addition, transcripts for 2 members of the TGF-β superfamily, BMP2 and PLAB, were up-regulated by three- and twofold, respectively, in the NS5A-expressing clones compared with controls. PLAB is up-regulated during acute liver injury and liver regeneration.[30] The HBV pX protein enhances TGF-β, BMP2, and activin transcriptional activity, contributing to HBV-associated liver fibrosis.[31] It must be noted that the receptor of activin A, another member of the TGF-β superfamily increased in fibrotic liver, was also up-regulated by 2.2-fold in NS5A-expressing cells.

We also observed an increase in type IV collagen. High type IV collagen expression was reported in large fibrotic areas and serum type IV collagen levels correlated with fibrotic grades of HCV liver patients.[32,33] Finally, we and others reported previously that NS5A induces IL-8 expression.[8,9] Elevated IL-8 expression correlated with liver fibrosis in HCV patients.[34]

Regulation by NS5A of LTBP1, decorin, PLAB, BMP2, collagen IV-α5, and IL-8 was confirmed via RT-PCR analysis (see Fig. 2 and data not shown).[9]

Thus, the increased levels of LTBP1, PLAB, BMP2, activin A receptor, type IV collagen, and IL-8 on the one hand and the decrease of decorin on the other may contribute to hepatic fibrosis associated with HCV. In addition, NS5A may play critical roles in controlling TGF-β activity, by modulating LTBP1 and decorin gene expression.

Inflammation and Immune Response. Expression of genes implicated in inflammatory process and immune response was modulated by NS5A. TNF-related cytokines are recognized as crucial effectors of the innate and adaptive immune defenses, and targeting members of the TNF/lymphotoxin superfamily and their receptors is a strategy used by many viruses. Of great interest, therefore, was the observation that expression of two members of the TNF receptor family, TNFR1A and LTBR, were strongly down-regulated (7.6- and 7.3-fold, respectively) in the NS5A-expressing clones compared with controls. This effect was subsequently confirmed via PCR analysis (Fig. 4A). We wished to determine the protein expression levels for both genes. Although the sensitivity of Western blotting was not sufficient to detect the TNFR1A protein in Huh7 cells, the analysis of LTBR protein expression indicated that LTBR protein levels were strongly reduced in

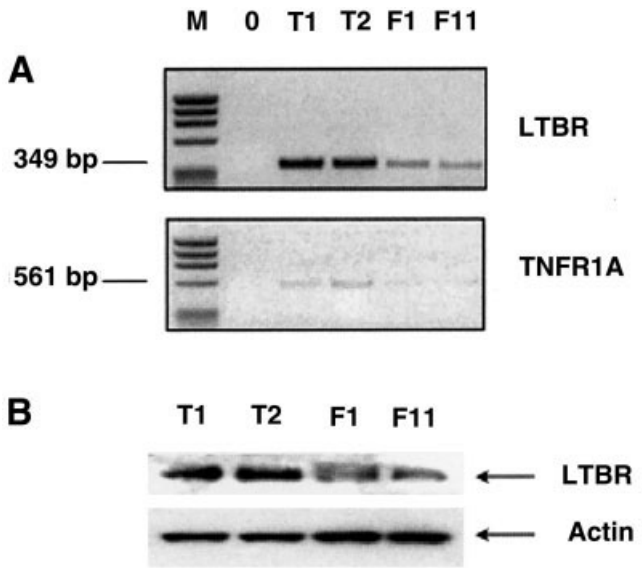

Fig. 4. Effect of NS5A on gene and protein expression of the TNFR family members LTBR and TNFR1A. (A) Total RNA from T1, T2, F1, and F11 Huh7 clones was reverse-transcribed and PCR amplification was performed for LTBR and TNFR1A. Lane 0 represents the negative control, which was the product of RT-PCR where RNA was omitted. (B) Total proteins extracted from T1, T2, F1, and F11 Huh7 clones were separated via SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-LTBR and anti-β-actin antibodies. Abbreviations: LTBR, lymphotoxin beta receptor; TNFR1A, tumor necrosis factor receptor 1A.

both NS5A-expressing clones F1 and F11 compared with control cells T1 and T2 (Fig. 4B).

Our data, which are in agreement with those of others,[35,36] support a role for NS5A as a negative regulator in TNF-α signaling cascade. Interestingly, an up-regulation of TNFR1A was observed in chimpanzee liver during acute HCV infection, correlating with viral clearance.[19] Therefore, we could speculate that the down-regulation of TNFR1A and LTBR by NS5A may confer to HCV the ability to escape immune suppression leading to persistent infection.

IL-1β is one of the most important cytokines with respect to the liver. IL-1RA counterbalances the biological activity of IL-1β by a competitive binding to IL-1 receptors. Increased levels of IL-1β and IL-1RA have been found in the sera of patients with chronic liver diseases. We observed a 2.2-fold increase in IL-1RA mRNA levels, which was confirmed via real-time RT-PCR (see Fig. 2). Other genes that are involved in inflammation and are modulated by NS5A included osteopontin, hepatocyte growth factor–like protein (which was implicated in hepatic responses to inflammatory stimuli), calpain 1, and lipoprotein-associated phospholipase A2. We also observed a down-regulation of STAT6 by NS5A. STAT6 is a mediator of IL-12 and IL-4 functions, and activation of STAT6 may regulate liver inflammation injury.[37] The NS5A–up-regulated genes also included the proinflammatory chemokines IL-8 and GRO-α.

Appropriate induction of a T helper 1 response is required for effective eradication of intracellular pathogens. IL-12, which was first described for its ability to stimulate IFN-γ production and enhance CD8 cytotoxicity, is a dominant factor in T helper 1 phenotype development. We observed an up-regulation of CD47 and a down-regulation of mitogen-activated protein kinase kinase 3, 2 regulators of IL-12 production. CD47 ligation down-regulates IL-12 production,[38] and mitogen-activated protein kinase activating kinase 3 deficiency results in a severe reduction of IL-12 production.[39] In addition, T cells from mice deficient in mitogen-activated protein kinase activating kinase 3 have a defect in IFN-γ production. It is worth noting that the potential of dendritic cells generated from HCV-infected patients to stimulate allogeneic CD4 T cells has been reported to be lower than that of dendritic cells from healthy donors, potentially because of low IL-12 expression.[40]

Complement also plays a protective role in the acute host response against several viruses. The host uses a family of proteins called *regulators of complement activation* to prevent damage to host cells by activated complement; these include the complement component 4–binding protein, an important regulator of the classical pathway of the complement system. Component 4–binding protein acts as a cofactor in degradation of complement protein C4b. Component 4–binding protein is mainly expressed in the liver and is composed of the alpha and beta subunits. Its expression increases during inflammation, infection, or tissue damage. We observed a 2.6-fold decrease of component 4–binding protein α and β in NS5A-expressing cells compared with controls.

Calcium Homeostasis and Cellular Signaling. It was reported recently that NS5A alters calcium homeostasis.[41] Expression of several members of the family of proteins known as EF-hand calcium-binding proteins such as calpain 1, grancalcin, S100P, and the endoplasmic resident protein reticulocalbin 1 were reduced in NS5A-expressing cells. Other down-regulated genes included inositol 1,4,5-triphosphate receptor type 3—an intracellular channel that mediates the release of calcium from intracellular stores—and sarco/endoplasmic reticulum calcium adenosine triphosphatase 1, the latter of which pumps calcium from the cytosol to the endoplasmic reticulum and therefore plays a major role in the control of calcium signaling.

Lipid Metabolism. Remarkably, microarray analysis revealed a dysregulation upon NS5A expression of numerous genes involved in lipid transport and metabolism. Several lines of evidence suggest that the infection cycle of HCV might involve lipoproteins and apolipoproteins. HCV associates with low-density lipoprotein, including those contain-

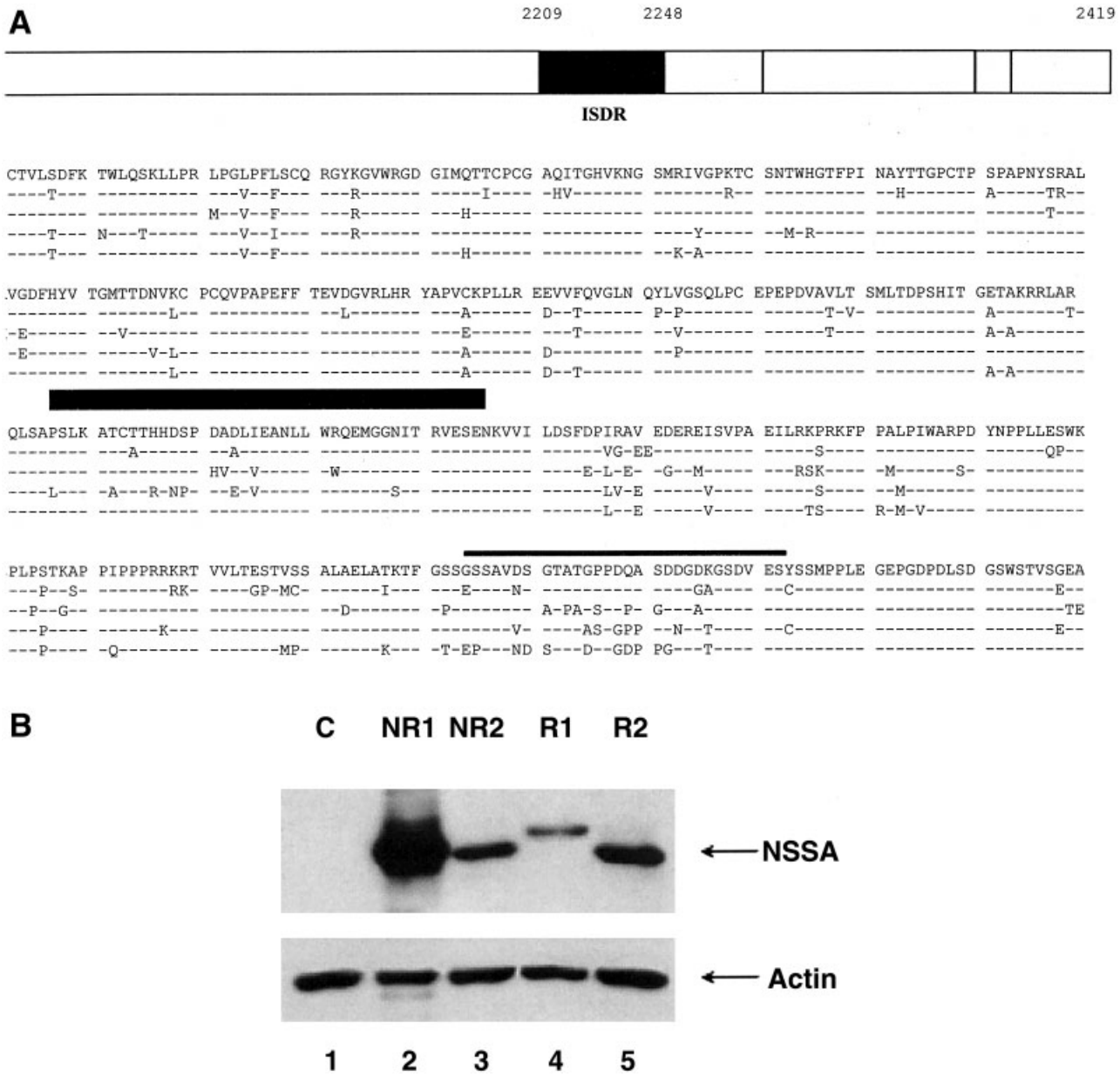

Fig. 5. NS5A expression in Huh7 cells following transient transfections. (A) Amino acid sequence alignments of NS5A sequences. Full-length NS5A sequences were cloned from 2 responders (R1 and R2) and 2 nonresponders (NR1 and NR2) to IFN-α treatment. Amino acid residues are indicated by the standard single-letter codes; dashes indicate the identical amino acid residues, with the consensus 1b sequence shown at the top. The position of the interferon-sensitivity determining region (ISDR) is depicted in black. (B) Huh7 cells were transfected with NR1, NR2, R1, and R2 NS5A-expressing plasmids or with the empty vector. After 48 hours, total proteins were separated via SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-NS5A and anti-β-actin antibodies.

ing apolipoproteins E and B. Recent studies have indicated that lipoprotein-associated HCV particles may infect cells via the low-density lipoprotein receptor.[42] Furthermore, binding of lipo-viro particles to the cell relies on apolipoproteins and their receptors, and this binding is efficiently inhibited by normal low-density lipoprotein or very low-density lipoprotein or antibodies to apolipoprotein B and apolipoprotein E.[43] Remarkably, carriage of an apolipoprotein E-ϵ4 allele may be protective against liver damage caused by HCV.[44] HCV infection is also often associated with the occurrence of liver steatosis. Interestingly, NS5A colocalizes with the HCV core protein on lipid droplets and interacts with apolipoprotein A1.[45]

Genes down-regulated by NS5A included PLTP (which mediates phospholipid transfer, high-density lipoprotein conversion, and reverse cholesterol transport), fatty acid binding protein 1, lipoprotein-associated phospholipase A2, the nuclear liver X receptor α, arylacet-

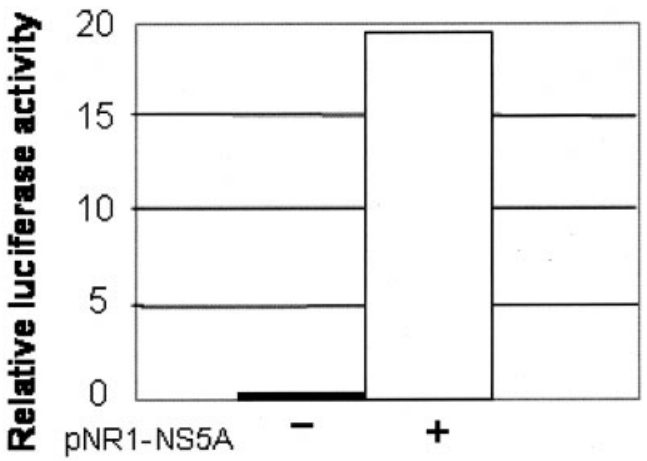

Fig. 6. Activation of NF-κB by NS5A. Huh7 cells were cotransfected with NF-κB–controlled luciferase plasmid along with NR1 NS5A expression plasmid or empty vector used as a control. Lysates from transfected cells were prepared for luciferase activity.

amide deacetylase, and apolipoprotein E receptor 2. Lipoprotein-associated phospholipase A2 is secreted by liver cells and is associated with low- and high-density lipoprotein. Liver X receptor α, PLTP, and arylacetamide deacetylase have all been associated with the production and assembly of very low-density lipoprotein. ApoE receptor 2 belongs to the low-density lipoprotein receptor family and functions in the uptake of apolipoprotein E–containing lipoproteins.

These findings support an important role for NS5A in modulating lipid homeostasis in hepatic cells that may affect the viral life cycle and/or contribute to the development of liver steatosis.

Comparison of 4 NS5A Sequences Isolated From Responders and Nonresponders to IFN-α Therapy. To discriminate between early and late events or potentially clonal effects, transient transfection assays were performed in Huh7 cells with the plasmid-expressing NR1 NS5A mutant. NS5A protein expression was determined 48 hours posttransfection via Western blot analysis (Fig. 5B, lane 2). Microarray analysis was performed for two independent experiments. Out of the 103 genes described above, 34 genes were also identified as up- or down-regulated following transient expression of NS5A ($P < .05$) as shown in bold in Tables 2 and 3. These genes included IL-8, IL-1RA, fibrillin-like protein, and vimentin, which were up-regulated, and galectin 2, trefoil factor 2, hepatocyte growth factor–like protein, inositol 1,4,5-triphosphate receptor, type 3, calpain 1, and PLTP, which were down-regulated upon NS5A expression. It is interesting to note that both TNFR1A and LTBR were also down-regulated following transient expression of NS5A. The genes identified in the clones but not verified with the transient expression system may be modulated by NS5A by less than fourfold at 48 hours and therefore are not selected because of the transfection efficiency of 50%–60%, or they may be targets whose expression is modulated by NS5A at a later time. A clonal effect resulting in the selection of these genes cannot be excluded.

We subsequently compared the effects of NS5A mutants isolated from responders and nonresponders to IFN-α therapy. In addition to the NR1 sequence, 3 full-length NS5A sequences were obtained from 1 nonresponder (NR2) and two responders (R1 and R2) (see Fig. 5A). NR1, NR2, R1, and R2 were transiently expressed in Huh7 cells (see Fig. 5B). Total mRNA was prepared from the Huh7 cells expressing transiently each of the 4 NS5A sequences or the empty vector, and transcript levels were determined using microarray analysis. Again, 2 independent experiments were performed. Out of the 34 genes

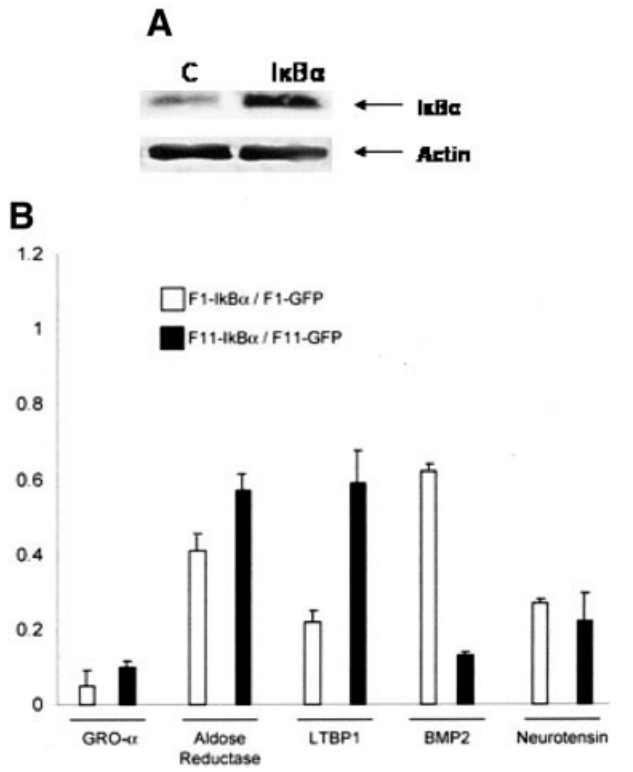

Fig. 7. Adenovirus-mediated IκBα transfer reverts NS5A-dependent gene up-regulation. F1 and F11 Huh7 clones were infected with adenovirus bearing IκBα or GFP, respectively. After 18 hours, total proteins and RNA were extracted. (A) Total proteins were separated via SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-IκBα and anti-β-actin antibodies. (B) Total RNA was reverse-transcribed and real-time PCR amplification was performed for aldose reductase, GRO-α, LTBP1, neurotensin, BMP2, and actin as described in Patients and Methods. Transcript levels in F1-IκBα and F11-IκBα were normalized using actin mRNA levels and compared with transcript levels in F1-GFP and F11-GFP, respectively. Data are shown as mean ± SEM of at least 3 independent experiments. Abbreviations: IκBα, inhibitor κBα; GFP, green fluorescence protein; LTBP1, latent TGF-β binding protein 1; BMP2, bone morphogenic protein 2.

regulated upon both stable and transient expression of NR1 NS5A, 33 were also identified as up- or down-regulated by the 3 other NS5A mutants (NR2, R1, and R2). Lipoprotein-associated phospholipase A2 transcript was found to be down-regulated specifically by NS5A NR1 and NR2 proteins. However, this latter observation was not confirmed in RT-PCR experiments (data not shown). Therefore, no difference was detected among the NS5A sequences derived from responders to IFN-α therapy and the NS5A sequences derived from nonresponders.

Computational Promoter and NF-κB Binding Site Analysis. We wished to predict putative transcription factors mediating NS5A effects on gene expression. A computational transcript mapping approach was used to locate promoter sequences from the 43 up-regulated genes (see Table 2). The promoter sequences of the 43 up-regulated genes were submitted for analysis to MATCH, a pattern matching program for searching putative transcription factor binding sites in DNA sequences.[16,17] Thirty-nine of the 43 gene promoter regions contained 1 or more binding sites for nuclear factor κB (NF-κB) (Supplementary Table 1), the large majority of them containing multiple NF-κB binding sites. The results included 6 genes known to be target genes of NF-κB: IL-8, GRO-α, vimentin, IL-1RA, MIP-3α/CCl20 and epidermal growth factor receptor (http://people.bu.edu/gilmore/nf-kb/target/).

We subsequently used the Gibbs sampling method[18] to derive overrepresented motifs in the upstream regions of these 43 coinduced genes. To determine if this set of genes may be coregulated by NF-κB, motif lengths from 14–25 bp were sampled from the 43 defined promoter regions to search for conserved alignments. Seven returned motifs fit the significant cut-off values (Wilcoxon $P < .05$), and all 43 genes were returned from the Gibbs sampler with a sequence relating to the specified consensus sequence for all 7 motifs (Supplementary Table 2). Therefore, computational analysis of the 43 coinduced genes predicts that NF-κB mediates NS5A effects on gene expression.

NF-κB Mediates NS5A Effects. To measure the transcriptional activation of NF-κB in NS5A-expressing cells, Huh-7 cells were transfected with an empty vector or NR1 NS5A expression plasmid, and induction of NF-κB was measured via cotransfection of a luciferase gene driven by the E-selectin promoter. The E-selectin promoter contains three functional NF-κB sites, yields robust induction, and is specific for NF-κB.[46] Expression of NS5A leads to activation of NF-κB as measured by the reporter readout (Fig. 6). Significant increase in NF-κB activity was also observed in NS5A-expressing clones F1 and F11 compared with control clones T1 and T2 ($P = .012$) (data not shown). This is in agreement with a recent report suggesting an activation of NF-κB by NS5A.[47] Activation of NF-κB in the liver of patients with chronic hepatitis C was also reported.[48] NF-κB is an important mediator of the inflammatory response; however, its role in liver inflammation and fibrosis remains controversial.

To confirm that NF-κB mediates NS5A-dependent gene regulation, we inhibited NF-κB activity by infecting NS5A expressing Huh7 clones F1 and F11, with adenoviruses AdIκBα or AdGFP expressing full-length IκBα cDNA and GFP, respectively. The cells were tested for IκBα expression via Western blotting (Fig. 7A). As shown in Fig. 7B, adenovirus-mediated overexpression of IκBα was effective at inhibiting the up-regulation of genes induced by NS5A for aldose reductase, GRO-α, LTBP1, neurotensin, and BMP2, all randomly selected.

In conclusion, we identified 103 genes modified by NS5A in the hepatocytic Huh-7 cells, many of which may participate in the pathogenesis of HCV infection. To this regard, the impact of NS5A protein mutations has yet to be elucidated. In addition, activation of NF-κB may play an important role in HCV pathogenesis and development of HCV-related liver diseases.

Acknowledgment: We thank Drs. D. States and R. McEachin (University of Michigan) for productive discussions; C. Logsdon (University of Michigan) for the gift of AdIκBα and AdGFP; and C. Vincenz (University of Michigan) for the gift of E-selectin luciferase. We also thank Alison Elafros, Philip Shalhoub, and Pascal Lescure for their technical support.

References

1. Alter MJ. Epidemiology of hepatitis C. HEPATOLOGY 1997;26:62S–65S.
2. Reed KE, Rice CM. Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. Curr Top Microbiol Immunol 2000;242:55–84.
3. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J Clin Invest 1995;96:224–230.
4. Gale MJJ, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, et al. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology 1997;230:217–227.
5. Paterson M, Laxton C, Thomas H, Ackrill A, Foster G. Hepatitis C virus NS5A protein inhibits interferon antiviral activity, but the effects do not correlate with clinical response. Gastroenterology 1999;117:1187–1197.
6. Pawlotsky JM, Germanidis G, Neumann AU, Pellerin M, Frainais PO, Dhumeaux D. Interferon resistance of hepatitis C virus genotype 1b: relationship to nonstructural 5A gene quasispecies mutations. J Virol 1998; 72:2795–2805.
7. Podevin P, Sabile A, Gajardo R, Delhem N, Abadie A, Lozach PY, et al. Expression of hepatitis C virus NS5A natural mutants in an hepatocytic cell line inhibits the antiviral effect of interferon in a PKR-independent manner. HEPATOLOGY 2001;33:1503–1511.
8. Girard S, Shalhoub P, Lescure P, Sabile A, Misek DE, Hanash S, et al. An altered cellular response to interferon and up-regulation of interleukin-8 induced by the hepatitis C viral protein NS5A uncovered by microarray analysis. Virology 2002;295:272–283.

9. Polyak SJ, Khabar KS, Paschal DM, Ezelle HJ, Duverlie G, Barber GN, et al. Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. J Virol 2001;75:6095–6106.
10. Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. Science 2000;290:1972–1974.
11. Krieger N, Lohmann V, Bartenschlager R. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J Virol 2001;75: 4614–4624.
12. Shirota Y, Luo H, Qin W, Kaneko S, Yamashita T, Kobayashi K, et al. Hepatitis C virus NS5A binds RNA-dependent RNA polymerase NS5B and modulates RdRP activity. J Biol Chem 2002;277:11149–11155.
13. Tu H, Gao L, Shi ST, Taylor DR, Yang T, Mircheff AK, et al. Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. Virology 1999;263:30–41.
14. Elazar M, Cheong KH, Liu P, Greenberg HB, Rice CM, Glenn JS. Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication. J Virol 2003;77:6055–6061.
15. Kato N, Lan KH, Ono-Nita SK, Shiratori Y, Omata M. Hepatitis C virus nonstructural region 5A protein is a potent transcriptional activator. J Virol 1997;71:8856–8859.
16. Kel AE, Goßling E, Reuter I, Cheremushkin E, Kel-Margoulis QV, Wingender E. MATCH^{TM}: a tool for searching transcription factor binding sites in DNA sequences. Nucleic Acids Res 2003;31:3576–3579.
17. Wingender E, Chen X, Fricke E, Geffers R, Hehl R, Liebich I, et al. The TRANSFAC system on gene expression regulation. Nucleic Acids Res 2001;29:281–283.
18. Thompson W, Rouchka EC, Lawrence CE. Gibbs Recursive Sampler: finding transcription factor binding sites. Nucleic Acids Res 2003;31:3580–3585.
19. Bigger CB, Brasky KM, Lanford RE. DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. J Virol 2001;75:7059–7066.
20. Shackel NA, McGuinness PH, Abbott CA, Gorrell MD, McCaughan GW. Insights into the pathobiology of hepatitis C virus-associated cirrhosis: analysis of intrahepatic differential gene expression. Am J Pathol 2002; 160:641–654.
21. Wood JR, Melia WM, Wood SM, Wilkinson ML, Lee YC, Portmann B, et al. Neurotensin and hepatocellular carcinoma. Lancet 1984;1:687.
22. Harada K, Shiota G, Kawasaki H. Transforming growth factor-alpha and epidermal growth factor receptor in chronic liver disease and hepatocellular carcinoma. Liver 1999;19:318–325.
23. Lee KW, Ko BC, Jiang Z, Cao D, Chung SS. Overexpression of aldose reductase in liver cancers may contribute to drug resistance. Anticancer Drugs 2001b;12:129–132.
24. Gotoh M, Sakamoto M, Kanetaka K, Chuuma M, Hirohashi S. Overexpression of osteopontin in hepatocellular carcinoma. Pathol Int 2002;52: 19–24.
25. Miyasaka Y, Enomoto N, Nagayama K, Izumi N, Marumo F, Watanabe M, et al. Analysis of differentially expressed genes in human hepatocellular carcinoma using suppression subtractive hybridization. Br J Cancer 2001; 85:228–234.
26. Genda T, Sakamoto M, Ichida T, Asakura H, Kojiro M, Narumiya S, et al. Cell motility mediated by rho and Rho-associated protein kinase plays a critical role in intrahepatic metastasis of human hepatocellular carcinoma. HEPATOLOGY 1999;30:1027–1036.
27. Ito Y, Sasaki Y, Horimoto M, Wada S, Tanaka Y, Kasahara A, et al. Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. HEPATOLOGY 1998;27: 951–958.
28. Miyazono K, Olofsson A, Colosetti P, Heldin C. A role of the latent TGF-beta 1-binding protein in the assembly and secretion of TGF-beta 1. EMBO J 1991;10:1091–1101.
29. Kinnman N, Andersson U, Hultcrantz R. In situ expression of transforming growth factor-beta1-3, latent transforming growth factor-beta binding protein and tumor necrosis factor-alpha in liver tissue from patients with chronic hepatitis C. Scand J Gastroenterol 2000;35:1294–1300.
30. Hsiao EC, Koniaris LG, Zimmers-Koniaris T, Sebald SM, Huynh TV, Lee SJ. Characterization of growth-differentiation factor 15, a transforming growth factor beta superfamily member induced following liver injury. Mol Cell Biol 2000;20:3742–3751.
31. Lee DK, Park SH, Yi Y, Choi SG, Lee C, Parks WT, et al. The hepatitis B virus encoded oncoprotein pX amplifies TGF-beta family signaling through direct interaction with Smad4: potential mechanism of hepatitis B virus-induced liver fibrosis. Genes Dev 2001;15:455–466.
32. Neubauer K, Saile B, Ramadori G. Liver fibrosis and altered matrix synthesis. Can J Gastroenterol 2001;15:187–193.
33. Yabu K, Kiyosawa K, Mori H, Matsumoto A, Yoshizawa K, Tanaka E, et al. Serum collagen type IV for the assessment of fibrosis and resistance to interferon therapy in chronic hepatitis C. Scand J Gastroenterol 1994;29: 474–479.
34. Kaplanski G, Farnarier C, Payan MJ, Bongrand P, Durand JM. Increased levels of soluble adhesion molecules in the serum of patients with hepatitis C. Correlation with cytokine concentrations and liver inflammation and fibrosis. Dig Dis Sci 1997;42:2277–2284.
35. Park KJ, Choi SH, Lee SY, Hwang SB, Lai MMC. Nonstructural 5A protein of hepatitis C virus modulates TNF-alpha-stimulated NF-kappa B activation. J Biol Chem 2002;277:13122–13128.
36. Majumder M, Ghosh AK, Steele R, Zhou XY, Phillips NJ, Ray R, et al. Hepatitis C virus NS5A protein impairs TNF-mediated hepatic apoptosis, but not by an anti-FAS antibody, in transgenic mice. Virology 2002;294: 94–105.
37. Kato A, Yoshidome H, Edwards MJ, Lentsch AB. Regulation of liver inflammatory injury by signal transducer and activator of transcription-6. Am J Pathol 2000;157:297–302.
38. Armant M, Avice MN, Hermann P, Rubio M, Kiniwa M, Delespesse G, et al. CD47 ligation selectively downregulates human interleukin 12 production. J Exp Med 1999;190:1175–1182.
39. Lu HT, Yang DD, Wysk M, Gatti E, Mellman I, Davis RJ, et al. Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. EMBO J 1999;18:1845–1857.
40. Kanto T, Hayashi N, Takehara T, Tatsumi T, Kuzushita N, Ito A, et al. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. J Immunol 1999;162: 5584–5591.
41. Gong G, Waris G, Tanveer R, Siddiqui A. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. Proc Natl Acad Sci U S A 2001;98: 9599–9604.
42. Wunschmann S, Medh JD, Klinzmann D, Schmidt WN, Stapleton JT. Characterization of hepatitis C virus (HCV) and HCV E2 interactions with CD81 and the low-density lipoprotein receptor. J Virol 2000;74: 10055–10062.
43. Andre P, Komurian-Pradel F, Deforges S, Perret M, Berland JL, Sodoyer M, et al. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. J Virol 2002;76:6919–6928
44. Wozniak MA, Itzhaki R F, Faragher EB, James MW, Ryder SD, Irving WL. Apolipoprotein E-ϵ4 protects against severe liver disease caused by hepatitis C virus. HEPATOLOGY 2002;36:456–463.
45. Shi ST, Polyak SJ, Tu H, Taylor DR, Gretch DR, Lai MM. Hepatitis C virus NS5A colocalizes with the core protein on lipid droplets and interacts with apolipoproteins. Virology 2002;292:198–210.
46. Schindler U, Baichwal VR. Three NF-kappa B binding sites in the human E-selectin gene required for maximal tumor necrosis factor alpha-induced expression. Mol Cell Biol 1994;14:5820–5831.
47. Gong G, Waris G, Tanveer R, Siddiqui A. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-KappaB. Proc Natl Acad Sci U S A 2001;98: 9599–9604.
48. Boya P, Larrea E, Sola I, Majano P-L, Jimenez C, Civeira M-P, et al. Nuclear factor-kB in the liver of patients with chronic hepatitis C: decreased RelA expression is associated with enhanced fibrosis progression. HEPATOLOGY 2001;34:1041–1048.