Proteome Analysis of Liver Cells Expressing a Full-Length Hepatitis C Virus (HCV) Replicon and Biopsy Specimens of Posttransplantation Liver from HCV-Infected Patients†

Jon M. Jacobs,^{1,‡} Deborah L. Diamond,^{2,‡} Eric Y. Chan,² Marina A. Gritsenko,¹ Weijun Qian,¹
Miroslava Stastna,¹ Tracey Baas,² David G. Camp II,¹ Robert L. Carithers, Jr.,³
Richard D. Smith,¹ and Michael G. Katze^{2,4*}

Biological Sciences Division, Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, Washington,¹ and Department of Microbiology,² Hepatology Section, Department of Medicine,³ and Washington National Primate Research Center,⁴ University of Washington, Seattle, Washington

Received 6 October 2004/Accepted 13 February 2005

The development of a reproducible model system for the study of hepatitis C virus (HCV) infection has the potential to significantly enhance the study of virus-host interactions and provide future direction for modeling the pathogenesis of HCV. While there are studies describing global gene expression changes associated with HCV infection, changes in the proteome have not been characterized. We report the first large-scale proteome analysis of the highly permissive Huh-7.5 cell line containing a full-length HCV replicon. We detected >4,200 proteins in this cell line, including HCV replicon proteins, using multidimensional liquid chromatographic (LC) separations coupled to mass spectrometry. Consistent with the literature, a comparison of HCV replicon-positive and -negative Huh-7.5 cells identified expression changes of proteins involved in lipid metabolism. We extended these analyses to liver biopsy material from HCV-infected patients where a total of >1,500 proteins were detected from only 2 µg of liver biopsy protein digest using the Huh-7.5 protein database and the accurate mass and time tag strategy. These findings demonstrate the utility of multidimensional proteome analysis of the HCV replicon model system for assisting in the determination of proteins/pathways affected by HCV infection. Our ability to extend these analyses to the highly complex proteome of small liver biopsies with limiting protein yields offers the unique opportunity to begin evaluating the clinical significance of protein expression changes associated with HCV infection.

Hepatitis C virus (HCV) is the most common blood-borne infection in the United States, infecting approximately 2% of the population. Approximately 85% of cases progress to chronic infection, which often results in liver disease, including variable degrees of hepatic inflammation and fibrosis, cirrhosis, and hepatocellular carcinoma. Since the discovery and sequencing of the virus genome in 1989 (7), studies have been performed to elucidate the host/virus interactions pertinent to persistent infection of the liver. To date, global characterization of the host cellular response to infection has centered on the use of expression microarray profiling to identify potential gene markers of HCV-associated liver disease (11, 44, 45, 48). In contrast, only limited studies describing the proteomic analysis of human liver proteins have been reported (21, 55, 56). Proteomic studies of HCV infection have been particularly limited for several reasons, including the lack of a good cell culture model and the need for large amounts of protein for conventional proteomic analysis.

Recent advances in both proteomic methodologies (43, 44, 49, 50) and cell culture models of HCV infection (5, 26) now

make it possible to perform global characterization of the host cell protein response within the context of the complete set of HCV genes in vitro. Refined multidimensional liquid chromatographic (LC) separations coupled with mass spectrometry (MS) for proteome analysis has allowed global experiments to be performed utilizing less protein and obtaining more sensitivity, throughput, and dynamic range than with previous proteomic techniques (42, 46, 47, 53, 54). Concurrently, the establishment of a highly permissive Huh-7 subline (Huh-7.5) which supports high-level replication of full-length HCV genomes allows for the analysis of host cell response in the presence of persistent viral replication and expression of all viral proteins such that the interactive potential of HCV proteins can be investigated (5). This contrasts with subgenomic replicons where sequences encoding the viral structural proteins are missing, thus precluding the ability to monitor the effect of these viral proteins on replication and pathogenesis.

We describe the first global proteomic analysis of the highly permissive Huh-7.5 cell line in the presence and absence of replication of the full-length HCV genome. Powerful multidimensional separation techniques coupled with tandem MS (MS/MS) analysis identified >4,200 cellular proteins, along with 7 HCV proteins with high confidence after the application of conservative search criteria, coupled with LC elution time information. A semiquantitative comparison of total peptide identifications was subsequently used as a first-pass means to detect changes in protein abundance associated with HCV RNA replication. Preliminary comparative analysis of protein

* Corresponding author. Mailing address: Department of Microbiology, University of Washington, Box 358070, Seattle, WA 98195-8070. Phone: (206) 732-6135. Fax: (206) 732-6056. E-mail: honey@u.washington.edu.

† Supplemental material for this article may be found at <http://jvi.asm.org/>.

‡ These authors contributed equally to this work.

abundance in the presence and absence of the full-length replicon revealed potential up-regulated proteins involved in lipid biosynthesis and down-regulated proteins involved in fatty acid oxidation. The significance of these observations is discussed within the context of the recently suggested relationship between HCV infection and lipid metabolism (2, 22–24, 28, 30, 34, 38, 43). Many of these proteins were detected in human liver biopsy tissue, now making it possible to investigate protein abundance changes involved in HCV-associated liver disease *in vivo*. These findings demonstrate the potential of proteomic analysis for assisting in the determination of proteins/pathways affected by HCV infection and provide a foundation for studies currently under way to further investigate protein abundance changes in both the full-length replicon system and serial liver biopsy specimens from patients who have undergone liver transplantation for cirrhosis secondary to chronic HCV infection.

MATERIALS AND METHODS

Cell culture and tissue samples. Huh-7.5 cells and Huh-7.5 cell populations containing a full-length HCV genotype 1b Con1 replicon (GenBank accession no. AJ238799) harboring the nonstructural protein 5A (NS5A) adaptive change S2204I [FL-Neo (S2204I)] were kindly provided by Charles Rice, Laboratory of Virology and Infectious Disease, Center for the Study of Hepatitis C, The Rockefeller University, New York, N.Y. The details of this experimental system have been previously described (5). Briefly, Huh-7.5 cells are a subline of the Huh-7 human hepatoma cell line that was established by “curing” a cell clone containing an HCV subgenomic replicon with wild-type NS5A by prolonged alpha interferon (IFN- α) treatment. In contrast to the parental Huh-7 cells, Huh-7.5 cells are highly permissive for the replication of subgenomic and full-length HCV RNAs (5). Huh-7.5 cells were maintained in Dulbecco’s modified Eagle medium supplemented with nonessential amino acids and 10% fetal bovine serum. Cells containing the FL-Neo (S2204I) replicon were maintained in the same medium plus 0.75 mg/ml G418 to select for HCV replication-competent cells.

Human liver tissue was obtained from core needle biopsy specimens from HCV-infected patients after liver transplantation at the University of Washington. All transplant recipients gave written informed consent according to protocols approved by the Human Subjects Review Committee of the University of Washington, and all samples were reviewed by both the University of Washington institutional review board and the Pacific Northwest National Laboratory (PNNL) institutional review board for human subject research in accordance with federal regulations.

Sample preparation. Preparation of Huh-7.5 cells was identical for HCV replicon-positive and -negative samples and included both a global preparation and a subcellular fractionation for increased coverage of microsomal and nuclear proteins. Global lysates were prepared by washing cells twice with ice-cold phosphate-buffered saline and then lysing the cells in buffer (10 mM Na₂HPO₄, pH 7.0; 0.5% sodium dodecyl sulfate [SDS]) using sonication for 10 min in ice-cold water. Cell debris was pelleted, and the clarified supernatant was denatured and reduced using 8 M urea and either 5 mM tributylphosphine (TBP; Sigma-Aldrich, St. Louis, MO) or 5 mM Tris(2-carboxyethyl)phosphine (TCEP; Pierce Biotechnology, Inc., Rockford, IL) at 37°C for 60 min, after which the sample was alkylated by incubation with 15 mM iodoacetamide for 1.5 h at room temperature. The alkylation reaction mixture was diluted eightfold with 50 mM ammonium bicarbonate, pH 7.8, and 1 mM CaCl₂.

For subcellular fractionation, the replicon-positive and -negative Huh-7.5 cells were first washed with ice-cold phosphate-buffered saline and incubated in hypotonic buffer (100 mM NH₄HCO₃, pH 8.4; 5 mM MgCl₂) for 30 min on ice. After incubation, cells were Dounce homogenized and centrifuged at 1,000 $\times$ g for 20 min (4°C) to pellet the nuclear fraction. The supernatant was further separated into microsomal and cytosolic fractions by ultracentrifugation at 100,000 $\times$ g for 3 h at 4°C. The resulting supernatant (cytosolic fraction) was removed, denatured, reduced, and alkylated as described above. The microsomal pellet was resuspended in ice-cold 50 mM ammonium bicarbonate, pH 7.8, by sonication and then pelleted again by ultracentrifugation at 356,000 $\times$ g for 30 min at 4°C. The protein pellet was resuspended and denatured in solubilization solution, consisting of 8 M urea, 1% CHAPS {3-[(3-cholamidopropyl)-dimeth-

ylammonio]-1-propanesulfonate} in 50 mM NH₄HCO₃, pH 7.8, by being vortexed and sonicated on ice. The denatured sample was reduced with either 5 mM TBP or 5 mM TCEP at a final concentration of 5 mM and incubated for 1 h at 37°C, followed by alkylation with 15 mM iodoacetamide during 1.5-h incubation at room temperature. The alkylation reaction mixture was diluted eightfold with 50 mM NH₄HCO₃, pH 7.8, and 1 mM CaCl₂.

The pellet containing nuclear proteins was resuspended in low-salt buffer (20 mM Tris, pH 7.5, 5 mM MgCl₂, 20 mM KCl, 1 mM dithiothreitol, 1 mM EDTA) with an equal amount of high-salt buffer (20 mM Tris, pH 7.5, 5 mM MgCl₂, 1.2 M KCl, 1 mM dithiothreitol, 1 mM EDTA) gradually added to the sample, followed by incubation at 4°C for 2 h with continuous agitation (Termomixer R; Eppendorf, Westbury, NY). The sample was centrifuged at 25,000 $\times$ g for 15 min, and the recovered supernatant was dialyzed against cold hypotonic buffer (100 mM NH₄HCO₃, pH 8.4, and 5 mM MgCl₂) for at least 8 h at 4°C prior to denaturation, reduction, and alkylation as described above.

Frozen human liver biopsy tissue embedded in optimum cutting temperature freezing medium was cut on dry ice and then thawed on ice to separate the tissue from the remaining optimum cutting temperature medium. The samples were then disrupted in 0.5 to 1.0 ml M-PER (Pierce Biotechnology, Inc., Rockford, IL) with a Polytron homogenizer (PowerGene 700; Fisher Scientific, Pittsburgh PA). The lysate was centrifuged at 15,800 $\times$ g for 15 min at 4°C, and total protein concentration of the clarified supernatant was determined by the BCA assay from Pierce Biotechnology, Inc. (Rockford, IL). The samples were denatured and reduced with 8 M urea and 5 mM TCEP at 37°C for 60 min, after which the samples were either diluted sevenfold with 50 mM ammonium bicarbonate, pH 7.8, 1 mM CaCl₂ or alkylated with 15 mM iodoacetamide during a 1.5-h incubation at room temperature. The alkylation reaction mixture was diluted eightfold with 50 mM NH₄HCO₃, pH 7.8, 1 mM CaCl₂.

Immunoblot analysis of HCV replicon-negative and -positive Huh-7.5 cells. Cell monolayers in 35-mm-diameter wells were lysed and 40 μ g of protein lysate was resolved by 4 to 20% SDS-polyacrylamide gel electrophoresis (PAGE) gel and immunoblotted as previously described (50). Briefly, blots were probed for expression of HCV proteins with primary antibodies recognizing either NS5A (ID Labs Biotechnology, London, Ontario, Canada), NS5B (ID Labs Biotechnology), Core (Research Diagnostics, Flanders, N.J.), or glycoprotein E1 (National Heart, Lung, and Blood Institute Program for Genomic Applications, University of Texas Southwestern Medical Center) followed by anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Expression of host proteins was monitored using primary antibodies to actin, catalase, and thioredoxin (all from Abcam, Cambridge, MA) and hepatoma-derived growth factor (R&D Systems, Minneapolis, MN), followed by the appropriate secondary IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc.). In all cases, the reactive proteins were then visualized by enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NY).

Trypsin digestion. Sequencing-grade modified porcine trypsin was prepared as instructed by the manufacturer (Promega, Madison, WI) and added to all protein samples at a 1:50 (wt/wt) trypsin-to-protein ratio for 5 h at 37°C. Rapid freezing of the sample in liquid nitrogen interrupted the digestion, and the sample was stored at –80°C until it was time for analysis.

Peptide separation. Huh-7.5 peptide samples were subjected to strong cation exchange chromatography (SCX) with a Polysulfoethyl A 200- by 4.6-mm column (PolyLC, Columbia, MD) preceded by a 10- μ by 4.6-mm guard column at a flow rate of 1 ml/min. The separations were performed with a Shimadzu LC-10A system utilizing a Unicam 4225 (Thermo Electron, Waltham, MA) UV/visible light detector with mobile phases as follows: solvent A consisted of 10 mM ammonium formate and 25% acetonitrile (ACN), pH 3.0, and solvent B consisted of 500 mM ammonium formate and 25% ACN, pH 6.8. Once the sample was injected, the run was isocratic for 10 min at 100% solvent A, followed by an initial gradient from 100% solvent A to 50% solvent B for 50 min. A steeper gradient to 100% solvent B lasting 10 min was then performed and then held isocratically at 100% solvent B for 15 min. A total of 70 1-ml fractions were collected with a Shimadzu FRC-10A fraction collector. Each fraction was lyophilized to dryness and stored at –80°C until time for analysis.

Reversed-phase LC separation and MS/MS analysis. This method has been previously reported (42). Briefly, the reversed-phase capillary liquid chromatography system was made in house with a 150- μ m inner diameter, 360- μ m outer diameter, and 65-cm-diameter capillary column (Polymicro Technologies, Inc., Phoenix, AZ) fitted with 2- μ m retaining mesh and packed with a 5- μ m Jupiter C₁₈ stationary-phase column (Phenomenex, Torrance, CA). Mobile phase C consisted of 0.05% trifluoroacetic acid and 0.2% acetic acid in water, and mobile phase D consisted of 0.1% trifluoroacetic acid and 90% ACN in water. The exponential gradient mixing of mobile phase C with mobile phase D (1.8 μ l/min

FIG. 1. Schematic representation of sample preparation, separation and analysis of the Huh-7.5 replicon model system. Global lysates or subcellular fractions (cytosolic, microsomal, and nuclear) were prepared from Huh-7.5 cells (negative and positive for the HCV full-length replicon) and digested with trypsin. The resulting peptides were initially separated by SCX, followed by reversed-phase capillary-to-liquid chromatography (RP-LC) coupled with mass spectrometry. MS/MS spectra were collected and analyzed by SEQUEST with stringent filtering criteria to provide confident protein identifications.

flow rate) began while constant pressure (5,000 lb/in²) was maintained for 20 min, following a 10- μ l injection of the sample (1.0 μ g/ μ l).

The capillary column was interfaced with a Finnigan LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) with an electrospray ionization source manufactured in house. The initial MS scan utilized an m/z range of 400 to 2,000 after which three of the most abundant ions were selected for MS/MS analysis using a collisional energy setting of 45%. Dynamic exclusion was used to prevent repeated analysis of the same high abundant ion. The temperature of the heated capillary and the electrospray voltage were 200°C and 2.2 kV, respectively.

LC-MS/MS data analysis. SEQUEST analysis software (9) was used to match the MS/MS fragmentation spectra with peptides from the IPI Human Database (52,830 entries) and also included the HCV strain 1b protein sequences (10 entries). The criteria selected for filtering followed methods based upon a human reverse-database false-positive model which has been shown to give ~95% confidence for the entire protein data set (37). Briefly, protein identifications were retained if their identified peptide met the following criteria: (i) SEQUEST DelCN value of ≥ 0.10 and (ii) SEQUEST correlation score (Xcorr) of ≥ 1.5 for charge state 1+ and full tryptic peptides and Xcorr of ≥ 3.1 for partial tryptic peptides; Xcorr of ≥ 1.9 for charge state 2+ and full tryptic peptides and Xcorr of ≥ 3.8 for partial tryptic peptides; and Xcorr of ≥ 2.9 for charge state 3+ and full tryptic peptides and Xcorr of ≥ 4.5 for partial tryptic peptides. In addition, an elution time constraint was placed upon peptide identification, based upon the predicted versus observed elution time in the LC separation prior to MS/MS analysis (35). Such a technique has been previously shown to increase the overall confidence of the data set (17). Proteins identified by peptides that did not elute within $\pm 10\%$ of their predicted elution time were removed as possible false identifications. A potential mass and time (PMT) tag database containing the calculated mass and normalized elution time (NET) for each identified peptide was generated to assist with subsequent high-sensitivity, high-throughput analysis of human liver biopsy samples using the accurate mass and time (AMT) tag approach.

In an attempt to remove redundant protein identifications in the reported results, the program ProteinProphet was used as a reduction tool (29). All peptides that passed our criteria were given the identical score of 1, and entered into ProteinProphet only for redundancy analysis. This condensed the number of proteins detected significantly; after analysis, a combined total of 4,214 proteins were reported as identified, with 98% of those being identified by a full tryptic peptide. Proteins were categorized based upon the Gene Ontology (GO) identifications for both the location in the cell (cellular component) and the biological process described for each protein.

Proteins (see Table 3) had to pass a minimum criterion to be used in a quantitative nature which is based upon the analysis performed in Qian et al. (36). Briefly, a protein needed to have a minimum of five peptide identifications in one of the samples to eliminate the inclusion of proteins which are not detected with enough frequency to warrant quantitation, as well as have at least

a 3.5-fold increase/decrease in the relative abundance measurements between the two samples.

Identification of human liver biopsy proteins using the AMT tag approach.

Liver biopsy peptide samples (prepared in both the presence and absence of alkylation) were analyzed by LC-Fourier transform ion cyclotron resonance MS (LC-FTICR-MS) using the same high-resolution, reversed-phase capillary LC described in the previous section, coupled to an electrospray ionization interface with a Fourier transform ion cyclotron resonance mass spectrometer (46). We used an 11.5-T FTICR instrument, designed and constructed in our laboratory at PNNL (13), to analyze 2 μ g of total protein (in both the presence and absence of alkylation) from 0-month (taken at the time of transplantation) and 6-month (posttransplantation) biopsy samples obtained from the same HCV-infected patient.

The acquired FTICR spectra (10^5 resolution) were processed and deconvoluted with ICR-2LS (software written in house at PNNL) to obtain peak lists containing the monoisotopic mass, observed charge, and intensity of the major ions in each spectrum. The masses were calibrated using the masses of internal calibrant peaks infused at the beginning and end of each LC-FTICR analysis. The peak lists for each analysis were then matched against the PMT database created by the combined previous LC-MS/MS analyses of the Huh-7.5 cell samples with VIPER (software written in house at Pacific Northwest National Laboratory). Matching involved finding the groups of ions in the data, computing a median monoisotopic mass for each group, and then comparing the mass and elution time of the group with the mass and NET of each peptide in the Huh-7.5 PMT database (match tolerance of ± 5 ppm and ± 0.05 NET), resulting in the generation of an AMT tag. Because a mass tag database for the whole genome does not exist to date, the LC-FTICR analysis could identify only AMT tags that corresponded to previously identified mass tags from the LC-MS/MS runs described above. For the purpose of deriving a final list of proteins identified by MS, we included only proteins that had been detected by at least two tags in any single experimental data set.

RESULTS

The flow of Huh-7.5 HCV replicon transfected and non-transfected samples during proteomic characterization is outlined in Fig. 1. Initial sample preparation was performed by two methods (both a global analysis and a protein-level subcellular fractionation) to essentially duplicate the analysis, as well as optimize the possibility to detect and localize the viral proteins in the HCV-positive replicon sample. Analysis of both the cells was performed by previously described multidimensional separation techniques (53), including an initial separa-

TABLE 1. Huh-7.5 peptides and proteins identified

Peptide or protein	Total HCV−	Total HCV+	Overlap, HCV+, HCV−	Total no. of unique peptides or proteins
Total peptides ^a	16,853	15,511	7,694	24,670
Virus peptides ^b	0	43	0	43
Virus proteins ^c	0	7	0	7
Total proteins ^d	3,406	3,160	2,352	4,214

^a The total number of peptides detected in Huh-7.5 cells negative or positive for the HCV replicon. A total of 7,694 peptides were detected in both samples, thus resulting in a total of 24,670 unique peptides detected in the entire study.

^{b,c} A total of 43 viral peptides, corresponding to seven HCV proteins, were detected only in Huh-7.5 cells transfected with the HCV replicon.

^d The total number of proteins identified from the peptides detected in Huh-7.5 cells negative or positive for the HCV replicon. A total of 2,352 proteins were detected in both samples, thus resulting in a total of 4,214 unique proteins detected in the entire study.

tion of tryptic peptides via SCX, to improve proteome coverage. Each SCX fraction was further separated and analyzed by reversed-phase capillary LC-MS/MS. A combined total of 370 fractions were analyzed by LC-MS/MS, 185 each for the positive and negative HCV replicon, and approximately 1,000,000 MS/MS spectra were collected and analyzed for the entire study. The number of high-confidence peptide/protein identifications observed following SEQUEST analysis and the application of stringent search criteria (see Materials and Methods) is summarized in Table 1. HCV protein sequences were combined into the IPI Human Database to search for both viral proteins and host proteins concurrently in the samples. A total of 24,670 peptides corresponding to 4,214 proteins, including 7 HCV proteins, were identified for the entire study. The ~70% overlap between the proteins of the two samples mostly reflects the identification of many less-abundant proteins detected in only one of the samples but also includes proteins whose abundances are affected by the presence of the HCV replicon as well.

Consistent with Western blot analyses demonstrating specific expression of HCV proteins only in Huh-7.5 cells transfected with the HCV replicon (Fig. 2), LC-MS/MS identifications of viral proteins were also specific to the HCV-positive sample (Table 1). The HCV proteins specifically detected in this study are summarized in Table 2. The viral proteins were detected only after subcellular fractionation and were localized to the isolated microsomal fraction, consistent with the idea that the HCV proteins remain tightly associated with membranes of the endoplasmic reticulum (4). Upon closer review of those HCV proteins not detected, viral proteins p7 and NS3/4A proteinase cofactor are relatively small in size (63 and 54 amino acids, respectively) and have in silico tryptic digestion patterns generating peptides that would be challenging to detect by the current methodology (in the case of these two proteins, in silico digestion produces either large very hydrophobic peptides or small hydrophilic peptides, 2 to 7 amino acids in length). Glycoprotein E1 (192 amino acids) was also not observed. Since this protein is known to be highly glycosylated, along with glycoprotein E2 (363 amino acids) and since no specific deglycosylation protocol or search criteria were used in targeting those types of posttranslationally modified peptides, glycosylation would reduce the possibility of identifying those modified peptides (and their corresponding pro-

FIG. 2. Western blot analysis of Huh-7.5 cells negative and positive for the HCV replicon. Protein lysates from Huh-7.5 cells negative and positive for the HCV replicon were resolved by 14% SDS-PAGE gels. Subsequent Western blot analysis using antibodies to the following HCV proteins: NS5A (predicted molecular weight [MW], 56,000 to 58,000) (A), NS5B (predicted MW, 68,000) (B), NS5B followed by core protein (predicted MW, 21,000) (C) revealed the specific detection of these proteins only in cells containing the HCV replicon. We were not able to detect glycoprotein E1 using a commercially available antibody generated against a glutathione *S*-transferase fusion protein containing the first 77 amino acids of HCV-1b E1 (data not shown). This may reflect an inability of the antibody to recognize with appreciable affinity the native (full-length, glycosylated) untagged protein. Although we cannot rule out the possibility that E1 is not expressed, this seems unlikely given that both of its flanking partners in the polyprotein precursor (core and E2) are detected.

teins) by LC-MS/MS analysis. Our ability to detect peptides corresponding to glycoprotein E2 most likely results from its higher molecular weight and hence the greater number of tryptic peptides potentially available for MS detection. These findings demonstrate the utility of LC-MS/MS for detection of both host and viral proteins and provide a foundation for the establishment of an AMT tag database essential for future proteomic studies characterizing onset and progression of HCV in infected liver biopsy tissue.

The distribution of detected proteins in HCV replicon-positive Huh-7.5 cells by either cellular location or cellular process

TABLE 2. HCV viral proteins detected in the replicon model

No. of viral proteins detected ^a	No. of peptides
HCV core protein.....	2
HCV glycoprotein E1.....	0
HCV glycoprotein E2.....	3
HCV p7 protein.....	0
HCV NS2 proteinase.....	5
HCV NS3 proteinase/helicase.....	10
HCV NS3/NS4A proteinase cofactor.....	0
HCV NS4B protein.....	3
HCV NS5A phosphoprotein.....	6
HCV NS5B RNA-dependent RNA polymerase.....	14

^a A total of 7 out of 10 described HCV proteins were identified by a minimum of two peptides, and all detections were localized to the microsomal fraction of the Huh-7.5 HCV replicon-positive sample. The recently characterized HCV F protein originates from a frameshift sequence and was not included in the database for the initial search.

FIG. 3. Graphic representation of the distribution of detected proteins from the global Huh-7.5 HCV replicon-positive sample by various cellular classifications. (A) Categorization was based upon GO identification numbers corresponding to cellular component, with approximately 56% of the proteins categorized. (B) Categorization was based upon GO identification numbers corresponding to cellular process, with approximately 55% of the proteins categorized. Values in parenthesis represent the percentage of all IPI Human Database GO identifications for comparison. Results were similar for the Huh-7.5 HCV replicon-negative cell samples.

is shown in Fig. 3, with categorization based upon GO identifications downloaded from EBI at www.ebi.ac.uk/GOA/index.html. The proteins detected in the global protein lysate show good representation across most subcellular compartments, although no specific characteristic was targeted during separation (Fig. 3A). Figure 3A also shows for comparison the percentage values of all IPI human entries assigned a GO identification. Proteins categorized as unknown correspond to only those proteins specified as such by GO annotation. Most categories do not show a significant percentage of difference, but

some categories appear to be either over- or underrepresented in this survey. For example, identification of extracellular proteins appears underrepresented here. Considering that the cells were washed extensively prior to lysis, fractionation, and analysis, it would be expected that little extracellular protein is retained. By contrast, ribosomal and mitochondrion proteins are overrepresented. The extremely high coverage for these proteins is expected, given their overall high abundance in the cell. Categorization by cellular process revealed similar findings (Fig. 3B) and comparison of positive- and negative-HCV

replicon samples showed only minor changes in the percent distribution of the cellular classifications (not shown). These findings suggest that the HCV replicon model is well suited to comparative studies aimed at providing quantitative, global protein profiles associated with HCV RNA replication and viral protein expression.

Of potential interest are those proteins that appear as either up- or down-regulated when comparing the Huh-7.5 positive- and negative-HCV replicon samples. Previous experiments with ion-trap LCQ technology demonstrated a "semiquantitative" relationship between the total number of peptide identifications and the relative abundance of the corresponding protein in a sample A versus sample B scenario (10, 25). This approach has also been verified by our laboratory with human plasma samples (36). When comparing the Huh-7.5 positive- and negative-HCV replicon sample results, total peptide identifications for a protein were used to distinguish proteins that were specifically found by numerous identifications in one analysis (sample A) but were either absent or identified with an extremely low number of peptide hits in a subsequent analysis (sample B) or vice versa. In this study, multiple protein candidates were discovered for both up- and down-regulation in Huh-7.5 cells transfected with the HCV replicon (see Table S1 in the supplemental material). Table 3 shows a selected group of these proteins and their respective total peptide identifications, and Fig. 4 provides examples of Western blot analyses confirming changes observed by the peptide hits approach. We detected abundance changes in many proteins involved in lipid metabolism, most notably an apparent down-regulation of enzymes involved in mitochondrial and peroxisomal β -oxidation of fatty acids (e.g., carnitine *O*-palmitoyltransferase II, acyl-coenzyme A [CoA] dehydrogenase, and peroxisomal long-chain acyl-CoA thioesterase) (Table 3). Interestingly, we also observed a downward trend in abundance of several cofactors of peroxisome proliferator-activated receptor α (PPAR α) (e.g., peroxisome proliferator-activated receptor-binding protein, and retinoic acid receptors RXR α and RXR β) (see Table S1 in the supplemental material), a transcriptional activator of fatty acid oxidation genes (3, 27, 58). The impact of HCV on host cell transcription is conflicting. While one study reports that the HCV core protein can enhance the transcriptional activation of RXR α /PPAR α heterodimers (51), microarray analyses of Huh-7 cells expressing the full-length genotype 1b HCV-N polyprotein have demonstrated only subtle changes in the host cell transcriptome (40). By contrast, a down-regulation of PPAR α has been reported in the early stage of HCV infection of two chimpanzees with either sustained or transient clearance of viremia (48). The exact mechanism responsible for the down-regulation of enzymes in the β -oxidation pathway observed here remains to be determined, and we cannot rule out the possibility that impaired expression and interaction of several cofactors of PPAR α transcription complex alters PPAR α -controlled gene expression. Alternatively, these changes in protein abundance may reflect virus-induced perturbations in host cell protein synthesis or stability. Future studies aimed at delineating between these possibilities might include microarray analysis of host gene expression, analysis of PPAR α -dependent promoter activity, and pulse-chase studies of protein turnover.

The impaired mitochondrial function reported here is con-

TABLE 3. Comparison of total peptide hits from select proteins in Huh-7.5 HCV replicon-positive and negative model system analyses

Name	No. of peptide hits	
	+ HCV	- HCV
Selected possible up-regulated proteins + HCV replicon		
Telomerase-binding protein p23	29	1
Fatty acid-binding protein, liver ^a	76	22
Fatty acid-binding protein, epidermal ^a	29	3
Thioredoxin ^a	177	8
Superoxide Dismutase 1, soluble ^a	68	8
Glutaredoxin (thioltransferase) ^a	6	0
Stathmin ^a	45	6
Platelet-activating factor acetylhydrolase IB, gamma subunit	39	3
Platelet-activating factor acetylhydrolase IB, beta subunit	29	3
Similar to hepatoma-derived growth factor, related protein 2	19	2
Hepatoma-derived growth factor ^a	103	17
Selected possible down-regulated proteins + HCV replicon		
Hypothetical protein FLJ33834 ^a	0	24
Carnitine <i>O</i> -palmitoyltransferase II ^a	0	12
Splice isoform 1 of P43155 carnitine <i>O</i> -acetyltransferase	0	5
Acyl-CoA dehydrogenase, short-branched chain specific ^a	5	19
Acyl-CoA dehydrogenase, medium-chain specific ^a	1	7
Acyl-CoA dehydrogenase family member 9	1	8
Acetyl-coenzyme A acyltransferase 2 ^a	8	47
Acetyl-CoA acyltransferase ^a	5	30
Peroxisomal long-chain acyl-coA thioesterase ^a	1	12
Catalase ^a	7	41
Selected unchanged proteins		
S100 calcium-binding protein A10	17	25
Keratin, type I cytoskeletal 18 ^a	136	171
ATP synthase beta chain ^a	183	202
ATP synthase alpha chain ^a	119	102
Actin, cytoplasmic 2 ^a	431	405
40S ribosomal protein S18 ^a	32	24
60S ribosomal protein L19 ^a	30	34

^a These proteins were also detected in ultrasensitive proteomic analyses of human liver biopsy samples by AMT tag technology and are currently being investigated in quantitative proteomic studies of serial liver biopsies acquired from HCV-infected patients after liver transplantation.

sistent with a previous study describing a reduction in mitochondrial metabolic processes as a result of HCV-associated oxidative stress (30). Moreover, similar perturbations in the mitochondrial proteome have been reported in a well-characterized rodent model of chronic ethanol feeding that induces oxidative stress and hepatic mitochondrial dysfunction (52). These alterations in mitochondrial physiology and accompanying cellular oxidative stress are expected to result in a concomitant increase in antioxidant proteins aimed at protecting the host cell against oxidative injury. Not surprisingly, we detected an up-regulation of several antioxidant proteins, including thioredoxin (Table 3; Fig. 4), which was previously shown to be elevated in the sera of HCV-infected patients (49). This contrasts with the observed down-regulation of catalase (Table 3; Fig. 4), a peroxisomal antioxidant protein. Although this pro-

FIG. 4. Confirmation of protein abundance changes by western blot analysis. Protein lysates from Huh-7.5 cells negative and positive for the HCV replicon were resolved by 4 to 20% SDS-PAGE gel. Western blot analysis was then performed using antibodies to the following proteins: actin (predicted MW, 41,800) (A), HDGF (predicted MW, 26,800) or thioredoxin (predicted MW, 11,700) (B), and catalase (predicted MW, 59,600) (C). These data indicate that the patterns observed by Western blot analysis are consistent with the data obtained by the peptide hit approach. Both methods detected no change in the expression of actin while catalase was down-regulated and both HDGF and thioredoxin were up-regulated in the presence of the HCV replicon. It is noted that while the predicted MW of HDGF was 26,800, our findings were consistent with the results of previous Western analyses identifying an ~40-kDa band, presumably associated with cotranslational/posttranslational modification (31).

tein does not appear to respond to PPAR α (58), the apparent decline is consistent with the impaired peroxisomal function described above.

Additional up-regulated proteins included stathmin, platelet-activating factor acetylhydrolase (PAF-AH) and hepatoma-derived growth factor (HDGF) (Table 3; Fig. 4). Stathmin is a general regulating protein integrating diverse intracellular signaling pathways; more recently, it has been shown to control microtubule dynamics by preventing assembly and promoting disassembly (8). Given that HCV replication induces microtubule aggregates essential for HCV replication (6), up-regulation of stathmin may reflect a protective host cell defense mechanism aimed at inhibiting HCV RNA synthesis. The up-regulation of PAF-AH may also reflect a protective effect against HCV. Consistent with this idea, PAF-AH activity is increased in a variety of diseases including HIV infection, where protection against the toxic effects of platelet-activating factor (a mediator of inflammation) and other biologically active oxidized phospholipids is achieved by PAF-AH-catalyzed hydrolysis (19). Finally, studies with mice that develop spontaneous fatty livers revealed that HDGF, a protein highly expressed in tumor cells, is induced prior to tumor development following the onset of steatohepatitis (57). The up-regulation of HDGF observed here may reflect a similar induction associated with HCV-induced derangements in lipid metabolism.

The findings reported above are consistent with previous studies demonstrating HCV-associated alterations in lipid metabolism (2, 23, 24, 28, 30, 34, 43, 48) and demonstrate the potential of proteomics for assisting in the determination of proteins/pathways affected by HCV infection. Our ultimate goal is to advance a systems-level understanding of virus-host interactions during the progression of liver disease by analyzing serial liver biopsies acquired from HCV-infected patients

after liver transplantation. However, this remains a significant challenge. The small size of the biopsies and resulting low protein yields (often <50 μ g of total protein from the amount of starting material available) necessitate the use of ultrasensitive proteomic analyses using nanogram to picogram amounts of protein rather than the >50 μ g of protein typically required for conventional proteomic studies. We utilized FTICR-MS 11.5-T instrumentation coupled with the AMT tag approach and an initial mass tag database prepared from the Huh-7.5/Huh-7.5 HCV replicon model system (>82,000 tryptic mass tags with an Xcorr of ≥ 2.0) to characterize two serial liver biopsies from the same patient (a baseline biopsy obtained at the time of transplantation and a biopsy obtained 6 months posttransplantation). Figure 5 shows a single-peptide example of how identification is made using accurate mass and time measurements. Briefly, Fig. 5A and 5B show the identification of a peptide by using MS/MS fragmentation spectra for the creation of a mass tag database which incorporates both mass and elution time information for a given peptide. Figure 5C and 5D show the FTICR-MS analysis of a liver biopsy sample and the identification of an accurate mass and elution time which matches with that of the previously identified peptide found in the mass tag database. Conservative identifications were made using a minimum of two or more AMT tags per open reading frame with <5 ppm mass accuracy and $\pm 5\%$ correlation with elution time constraints, resulting in a high-confidence open reading frame data set.

Using the approach described above, we identified >1,500 proteins from a total of four FTICR-MS analyses, each using only 1 μ g of liver biopsy protein lysate (Tables 3 to 5; see also Table S1 in the supplemental material). This is in stark contrast to previous studies of the liver proteome employing less-sensitive proteomic methods where milligram amounts of protein lysate are typically utilized to identify a few hundred

FIG. 5. Example of a liver biopsy peptide identified using the AMT tag approach. (A) Representative MS survey scan taken during reversed phase LC-MS/MS of Huh-7.5 samples previously fractionated by strong cation exchange. The peak shown by an asterisk was selected for further identification via collision-induced dissociation (MS/MS). (B) MS/MS scan identifying the peptide SVTELNGDIITNTMTLGDIVFKR with its accompanying theoretical mass and elution time information for placement into a mass tag database. This was one of seven peptides detected that identified protein P07148, fatty acid-binding protein in the Huh-7.5 sample. Shown are the total ion count (C) and specific LC-FTICR-MS scan (no. 1136) (D) of the liver biopsy sample detecting an accurate mass isotopic distribution and elution time (shown by the asterisk), which matches with the peptide mass tag found in the database, identifying this peptide in the biopsy sample. Such a technique removes the need for MS/MS analysis of every sample, increasing throughput and allowing protein identification of small samples.

proteins (Table 4) (20, 32, 33, 41). Among the proteins detected in human liver biopsies are many candidates of particular interest, based on previous genomic or proteomic studies indicating a potential role in HCV infection and liver disease. These include many proteins involved in pathways of lipid metabolism that were differentially expressed in the replicon model system as described above (e.g., carnitine *O*-palmitoyl-transferase II, acyl-CoA dehydrogenase short-branched and medium-chain-specific forms, and fatty acid-binding protein) (Table 3 and Fig. 5). Additional examples are summarized in

TABLE 4. Comparison of liver proteome coverage using various proteomic approaches

Technique ^a	Sample source (starting amt of protein) ^b	No. of proteins identified.	Source or reference(s)
High-resolution LC-FTICR-MS	Human liver biopsies (2 µg)	>1,500	Present study
High-resolution LC-MS/MS	Huh-7.5 cells ± HCV replicon (~5 mg)	>4,400	
ICAT + LC-MS/MS	Human liver carcinoma Huh-7 cells, primary human fetal hepatocytes, and human HH4 hepatocytes (1–2.5 mg)	~1,100–1,300 from each cell type; 2,159 unique total	55, 56
2D gel + MALDI-TOF-MS	Normal and tumor tissue of human hepatocellular carcinoma (up to 1 mg)	127 (~1,130 spots)	20
2D gel + MALDI-TOF-MS	Normal and tumor tissue of human hepatocellular carcinoma (1 mg)	>250 (>1,000 spots)	33
2D gel + MALDI-TOF-MS or LC-MS/MS	Cell culture model of hepatocellular carcinoma (0.4 mg)	301 (~2,000 spots)	32, 41

^a Protein samples were analyzed by a variety of methods including two-dimensional polyacrylamide electrophoresis (2D gel) for protein separation, followed by either matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) or reversed-phase liquid chromatography MS/MS (LC-MS/MS) for protein identification; isotope-coded affinity tag (ICAT) method for cysteine peptide enrichment, followed by LC-MS/MS for identification; or high-resolution LC-MS/MS and high-resolution LC-Fourier transform ion cyclotron mass spectrometry (LC-FTICR-MS).

^b The type of samples analyzed by each method and the amount of starting material is reported for comparison. Conventional 2D gel separations and subsequent MALDI-TOF-MS or LC-MS/MS analyses utilized milligram amounts of protein. The combination of high-resolution LC separations and ultra-high-sensitivity FTICR-MS permitted analysis of low-microgram quantities of starting material, a key requirement for analysis of small liver biopsy samples.

TABLE 5. Proteins detected in human liver biopsy tissue that are of particular interest for further investigation of HCV infection and liver disease in vivo

Protein or gene
IFN-regulated proteins ^a
Adenosylhomocysteinase
β-2-Microglobulin precursor
Electron transfer flavoprotein alpha-subunit, mitochondrial precursor
Hypothetical UPF0120 protein DKFZp564C186
Microsomal triglyceride transfer protein, large subunit precursor
Peptidylprolyl isomerase A
Fatty acid synthase
Glucosidase II alpha subunit
Ig lambda chain C regions
Symplekin
Tubulin alpha-6 chain
Proteins interacting with the HCV IRES and/or HCV NS5A protein ^b
Lupus La protein
Nucleolin
Polypyrimidine tract-binding protein 1 (splice isoform 2 of P28599)
Marker genes of HCV-associated hepatocellular carcinoma ^c
Aldehyde dehydrogenase 1
Catalase
Copper transport protein ATOX1
High-density lipoprotein-binding protein (Vigilin)
Ribosomal protein L30
Marker proteins of HCV-associated hepatocellular carcinoma ^d
Acyl-CoA dehydrogenase
Glutamate dehydrogenase 1
Marker proteins of both HBV- and HCV-associated hepatocellular carcinoma ^e
Aldehyde dehydrogenase
Annexin V
Catalase
Enoyl-CoA hydratase
Heat Shock 27-kDa protein
Tubulin β-chain
Ubiquinol-cytochrome C reductase complex core protein 1
Marker genes of HCV-associated cirrhosis ^f
DNA topoisomerase 1
Golgi autoantigen, golgin subfamily A member 5 (splice isoform 2 of Q8TBA6)
Peptidylprolylisomerase B
Protein kinase C substrate; 80 kDa
Ras-related protein Rab-18
Vimentin

^a Proteins differentially expressed during IFN treatment of Huh-7 cells and thus implicated to play a role in host antiviral defense.

^b Proteins reported to interact with the HCV internal ribosomal entry site (IRES) and/or HCV nonstructural protein 5A (NS5A), thus implying potential roles in viral protein translation, virus replication, or IFN resistance.

^{c,f} Potential markers of HCV-associated hepatocellular carcinoma or cirrhosis identified by differentially expressed mRNA in liver biopsy tissue.

^{d,e} Potential markers of HCV-associated or hepatitis B virus/HCV-associated hepatocellular carcinoma identified by differentially expressed protein in surgical liver material.

Table 5. These include IFN-regulated proteins (56), proteins implicated in internal ribosome entry site-mediated translation and/or interaction with NS5A (15, 16), and several marker genes/proteins of HCV-associated pathological processes, in-

cluding cirrhosis and hepatocellular carcinoma (20, 44, 45). The liver biopsy samples analyzed here were not fibrotic (stage 0) as determined using the Batts-Ludwig system, suggesting that comparison with the Huh-7.5 protein database was not skewed as a result of fibrotic alteration of protein levels. We do, however, anticipate that such an effect could occur; thus, efforts are now under way to supplement the Huh-7.5 AMT database with proteins detected from recently acquired normal and cirrhotic human liver tissue. We expect that this will enhance proteome coverage when utilizing the highly sensitive AMT tag strategy for identification and quantification of up- and down-regulated proteins during fibrosis progression in the liver transplant model. In the meantime, we note that preliminary quantitative studies using the Huh-7.5 AMT database together with stable isotope labeling methods ($H_2^{16}O/H_2^{18}O$) have already revealed examples of down-regulated proteins involved in lipid metabolism and oxidative stress (e.g., acetyl-CoA acetyltransferase and superoxide dismutase) when comparing biopsy samples from a patient who progressed to fibrosis (stage 4) versus one who did not (stage 0) (data not shown). Analysis of additional biopsy samples from a larger patient cohort will be necessary in order to determine whether the observed changes in relative protein abundance translate into meaningful protein profiling patterns associated with HCV infection and fibrosis progression. While such a detailed proteomic characterization of the liver transplant model is beyond the scope of this study, we believe that the findings reported here clearly demonstrate the ability of the AMT tag strategy to address the previously unmet need for ultrasensitive protein characterization of small amounts of liver biopsy tissue.

DISCUSSION

In recent genomic analyses of liver biopsies from acutely infected chimpanzees and an in vitro subgenomic replicon model, the accumulation of free fatty acids associated with transcriptional changes in host genes involved in lipid metabolism was reported to have a positive effect on the HCV replicon and may have a similar effect on HCV replication (48). Consistent with these observations, we demonstrated that transfection of Huh-7.5 cells with a full-length HCV replicon induces several protein abundance changes indicative of disturbances in lipid metabolism. The apparent down-regulation of enzymes involved in peroxisomal and mitochondrial β-oxidation would clearly contribute to the accumulation of free fatty acids proposed to benefit the virus. By contrast, an overabundance of lipid has adverse effects on the host cell. The accumulation of triglycerides promotes development of hepatic steatosis (fatty liver disease) and oxidative stress, frequent findings in patients with chronic hepatitis C infection. Studies aimed at further investigating the pathogenesis of HCV revealed that the HCV core protein and NS5A alter lipid metabolism in cell culture, and expression of the HCV core protein induces oxidative stress and steatosis (2, 23, 24, 28, 30, 34, 43, 48). A model of HCV-induced steatosis resulting from inhibition of microsomal triglyceride transfer protein and defective assembly and secretion of very low density lipoprotein has been described in studies of transgenic mice overexpressing HCV core protein alone (34). In this study, defects in mitochondrial β-oxidation did not appear to play a major role

in the development of liver steatosis. By contrast, a separate study of transgenic mice expressing all the HCV nonstructural proteins credited increased levels of reactive oxygen species and oxidative stress to a direct effect of the HCV core protein on mitochondria (30). Further impairment of mitochondrial function associated with chronic oxidative stress was proposed to contribute to the development of steatosis by inhibiting β -oxidation. Similar findings have been reported in a recent proteomic study of *S*-adenosylmethionine knockout mice that exhibit impaired mitochondrial function and spontaneously develop oxidative stress, nonalcoholic steatohepatitis, and hepatocellular carcinoma (39). The variability between studies is expected to reflect, at least in part, the net effect of viral proteins on the host cell. The down-regulation of proteins involved in β -oxidation observed here is in good agreement with the latter studies. Since the model system utilized here expresses the complete range of viral proteins, it is predicted to more closely resemble a transgenic mouse model expressing the full complement of HCV nonstructural proteins rather than just the HCV core protein alone. However, whether these alterations in lipid metabolism are a direct effect of viral RNA replication and protein expression or a secondary effect associated with mitochondrial injury remains to be determined.

Of potential interest is the elucidation of common posttranslational modifications (phosphorylation, glycosylation, etc.) involving these known proteins to determine the role of these events in HCV infection. The current study did not attempt to identify or characterize any of these types of modified peptides, but it would be of obvious interest to pursue this in future studies as little is known about how HCV infection involves not only protein/transcriptome abundance but signaling, transport, and regulatory pathways as well.

As alluded to above, the use of a full-length HCV replicon offers the advantage of allowing investigation of the potential influence of both structural and nonstructural proteins on the host cell, thus providing a more comprehensive view of the interactions among multiple pathways responding to viral infection. Our findings provide additional evidence supporting a generally emerging theme whereby disruptions in lipid metabolism induce a state of oxidative stress that contributes to the pathogenic effects of HCV. The results reported here clearly demonstrate the potential for proteomic studies of the HCV replicon system to assist in the determination of proteins/pathways affected by HCV infection. However, this model is not without limitations. The inability of the system to support production of infectious viral particles precludes analysis of the complete viral life cycle in vitro. Moreover, the highly permissive nature of the Huh-7.5 subline suggests marked differences in the host cell environment relative to the parental Huh-7 cells. A defect in cellular antiviral response of cured Huh-7 cells associated with the down-regulation of IFN regulatory factor 1 has recently been described and was suggested to result from adaptation of the host cells to increased permissiveness for the replicon during continuous selection with G418 (18). While we cannot exclude the possibility that defects in the antiviral response of Huh-7.5 cells and/or G418 selection influenced the observed changes in the host cell proteome, we emphasize that the findings reported here are consistent with previous studies describing alterations in lipid metabolism during HCV infection. Additional studies comparing changes in

protein abundance in Huh-7.5 versus parental Huh-7 cells during G418 selection of replicon-containing cells or using transient assays allowing assessment early after transfection without the need for G418 selection could be performed to confirm the results reported here. However, since our ultimate goal is to study human liver biology and disease we have focused on extending our analyses to serial liver biopsy specimens obtained from patients with recurrent HCV after liver transplantation. Serial liver biopsies represent a true system of HCV infection and liver disease. Moreover, the liver transplant model offers the unique opportunity to prospectively examine early virus-host interactions and to determine which of these factors show critical associations with severe liver injury and early progression to cirrhosis. Preliminary studies using the AMT tag approach for high-throughput, high-sensitivity Fourier transform ion cyclotron resonance mass spectrometry analysis identified >1,500 proteins from only a few micrograms of protein lysate prepared from liver biopsy tissue (Tables 3 to 5; see also Table S1 in the supplemental material). Previous studies of the liver proteome involving one-dimensional or two-dimensional polyacrylamide electrophoresis for protein separation coupled with either matrix-assisted laser desorption ionization-time of flight MS (MALDI-TOF-MS) or microcapillary high-performance liquid chromatography followed by electrospray tandem mass spectrometry (LC-MS/MS) for protein identification typically utilized milligram amounts of protein lysate to identify a few hundred proteins (Table 4) (20, 32, 33, 41). Methods that further reduced sample complexity improved the number of protein identifications. Thus, employment of either the isotope-coded affinity tag (ICAT) method for cysteine peptide enrichment (55, 56) or high-resolution microcapillary chromatographic separations (in the present study) facilitated the identification of thousands (rather than hundreds) of proteins from similar amounts of starting material (Table 4). The combination of high-quality liquid-phase separations and rare, high-resolution FTICR instrumentation provides even greater sensitivity and dynamic range, now allowing for identification of thousands of proteins from microgram rather than milligram amounts of starting material. This robust and ultrasensitive "nanoproteomic" technology was critical to our success in analyzing the highly complex proteome of small liver biopsies. The low protein yields (often <50 μ g total protein) associated with these small liver biopsy specimens precluded previous attempts to detect a broad abundance range of proteins using more conventional methods (e.g., ICAT plus LC-MS/MS). These studies lay the foundation for current efforts aimed at employing the AMT tag strategy together with stable isotope-labeling methods for quantitation of differential protein abundance during the clinical course of HCV infection. The data generated from our in vitro (HCV replicon) and in vivo (liver transplant model) proteomic studies will support on-going efforts to develop and visualize computational models that integrate mRNA and protein abundance data with the host-virus network scaffold to generate predictive models of host-virus network states. This information is expected to facilitate the identification of biomarkers for HCV infection and disease progression, as well as novel targets for future antiviral treatment.

Conclusions. Presented here is the first large-scale proteomic analysis of a full-length HCV replicon model system for

in vitro analysis of HCV replication. The utilization of refined, high-resolution multidimensional chromatographic separations that reduce sample complexity and the range of relative protein abundances enabled the identification of >4,200 proteins in the Huh-7.5 cell line. To our knowledge, this represents the most comprehensive protein database yet reported for a human cell line, providing a baseline for characterization of HCV-induced perturbations in the cellular environment, identification of potential targets for future antiviral treatment, and future comparative studies involving antiviral drug screening and evaluation for therapeutic intervention. Moreover, the proteins identified represent a large robust cellular database for application of the AMT tag approach incorporating high-throughput, high-sensitivity FTICR-MS analysis of liver biopsy specimens. The high resolution, large dynamic range, and unmatched sensitivity of this platform address the previously unmet need for robust and ultrasensitive proteomic methods where available tissue is severely limited. The power of FTICR-MS represents a significant advancement in our clinical proteomics efforts and offers the unique opportunity to begin investigating the clinical significance of protein abundance changes associated with HCV infection. We are particularly interested in further characterizing the mechanisms of HCV-induced alterations in lipid metabolism that contribute to the development of oxidative stress and steatosis. The apparent link with progression of liver injury and fibrosis (1, 12, 14) makes these cellular metabolic pathways attractive targets for discovering new antifibrotic drugs.

ACKNOWLEDGMENTS

We acknowledge Ron Moore and David Anderson for their assistance in the analyses of the Huh-7.5 cell samples.

We thank the NIH National Center for Research Resources (grant RR018522 to R.D.S.), the National Institute on Drug Abuse (grant 1P30DA01562501 to M.G.K.), and NIH National Institute of Diabetes and Digestive and Kidney Diseases (grant R01DK056388 to R.L.C.) for the support of this research.

We thank the Environmental Molecular Sciences Laboratory at Pacific Northwest National Laboratory (PNNL) for use of the instrumentation applied in this research. The Environmental Molecular Sciences Laboratory is a national scientific user facility sponsored by the Department of Energy's Office of Biological and Environmental Research and located at Pacific Northwest National Laboratory. Pacific Northwest National Laboratory is operated by Battelle Memorial Institute for the U.S. Department of Energy under contract no. DE-AC06-76RLO 1830.

REFERENCES

- Adinolfi, L. E., M. Gambardella, A. Andreana, M. F. Tripodi, R. Utili, and G. Ruggiero. 2001. Steatosis accelerates the progression of liver damage of chronic hepatitis C patients and correlates with specific HCV genotype and visceral obesity. *Hepatology* **33**:1358–1364.
- Barba, G., F. Harper, T. Harada, M. Kohara, S. Goulinet, Y. Matsuura, G. Eder, Z. Schaff, M. J. Chapman, T. Miyamura, and C. Brchet. 1997. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc. Natl. Acad. Sci. USA* **94**:1200–1205.
- Barrero, M. J., N. Camarero, P. F. Marrero, and D. Haro. 2003. Control of human carnitine palmitoyltransferase II gene transcription by peroxisome proliferator-activated receptor through a partially conserved peroxisome proliferator-responsive element. *Biochem. J.* **369**:721–729.
- Bartenschlager, R., and V. Lohmann. 2000. Replication of hepatitis C virus. *J. Gen. Virol.* **81**:1631–1648.
- Blight, K. J., J. A. McKeating, and C. M. Rice. 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J. Virol.* **76**:13001–13014.
- Bost, A. G., D. Venable, L. Liu, and B. A. Heinz. 2003. Cytoskeletal requirements for hepatitis C virus (HCV) RNA synthesis in the HCV replicon cell culture system. *J. Virol.* **77**:4401–4408.
- Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**:359–362.
- Curmi, P. A., O. Gavet, E. Charbaut, S. Ozon, S. Lachkar-Colmerauer, V. Manceau, S. Siavoshian, A. Maucuer, and A. Sobel. 1999. Stathmin and its phosphoprotein family: general properties, biochemical and functional interaction with tubulin. *Cell Struct. Funct.* **24**:345–357.
- Eng, J. K., A. L. McCormack, and J. R. Yates. 1994. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* **5**:976–989.
- Gao, J., G. J. Opiteck, M. S. Friedrichs, A. R. Dongre, and S. A. Hefta. 2003. Changes in the protein expression of yeast as a function of carbon source. *J. Proteome Res.* **6**:643–649.
- Geiss, G. K., V. S. Carter, Y. He, B. K. Kwieciszewski, T. Holzman, M. J. Korth, C. A. Lazaro, N. Fausto, R. E. Bumgarner, and M. G. Katze. 2003. 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. *J. Virol.* **77**:6367–6375.
- Gochee, P. A., J. R. Jonsson, A. D. Clouston, N. Pandeya, D. M. Purdie, and E. E. Powell. 2003. Steatosis in chronic hepatitis C: association with increased messenger RNA expression of collagen I, tumor necrosis factor- and cytochrome P450 2E1. *J. Gastroenterol. Hepatol.* **18**:386–392.
- Harkewicz, R., M. E. Belov, D. A. Anderson, L. Pasa-Tolic, C. D. Masselon, D. C. Prior, H. R. Udseth, and R. D. Smith. 2002. ESI-FTICR mass spectrometry employing data-dependent external ion selection and accumulation. *J. Am. Soc. Mass Spectrom.* **13**:144–154.
- Hourigan, L. F., G. A. Macdonald, D. Purdie, V. H. Whitehall, C. Shorthouse, A. Clouston, and E. E. Powell. 1999. Fibrosis in chronic hepatitis C correlates significantly with body mass index and steatosis. *Hepatology* **29**:1215–1219.
- Houshmad, H., and A. Bergqvist. 2003. Interaction of hepatitis C virus NS5A with La protein revealed by T7 phage display. *Biochem. Biophys. Res. Commun.* **309**:695–701.
- Izumi, R. E., S. Das, B. Barat, S. Raychaudhuri, and A. Dasgupta. 2004. A peptide from autoantigen La blocks poliovirus and hepatitis C virus cap-independent translation and reveals a single tyrosine critical for La RNA binding and translation stimulation. *J. Virol.* **78**:3763–3766.
- Jacobs, J. M., H. M. Mottaz, L.-R. Yu, D. J. Anderson, R. J. Moore, W. N. Chen, K. J. Auberry, E. Strittmatter, M. E. Monroe, B. Thrall, D. G. Camp II, and R. D. Smith. 2004. Multidimensional proteome analysis of human mammary epithelial cells. *J. Proteome Res.* **3**:68–75.
- Kanazawa, N., M. Kurosaki, N. Sakamoto, N. Enomoto, Y. Itsui, T. Yamashiro, Y. Tanabe, S. Maekawa, M. Nakagawa, C. H. Chen, S. Kakinuma, S. Oshima, T. Nakamura, T. Kato, T. Wakita, and M. Watanabe. 2004. Regulation of hepatitis C virus replication by interferon regulatory factor 1. *J. Virol.* **78**:9713–9720.
- Khovidhunkit, W., R. A. Memon, J. K. Shigenaga, M. Pang, M. Schambelan, K. Mulligan, K. R. Feingold, and C. Grunfeld. 1999. Plasma platelet-activating factor acetylhydrolase activity in human immunodeficiency virus infection and the acquired immunodeficiency syndrome. *Metabolism* **48**:1524–1531.
- Kim, J., S. H. Kim, S. U. Lee, G. H. Ha, D. G. Kang, N. Y. Ha, J. S. Ahn, H. Y. Cho, S. J. Kang, Y. J. Lee, S. C. Hong, W. S. Ha, J. M. Bae, C. W. Lee, and J. W. Kim. 2002. Proteome analysis of human liver tissue by two-dimensional gel electrophoresis and matrix assisted laser desorption/ionization-mass spectrometry for identification of disease-related proteins. *Electrophoresis* **23**:4142–4156.
- Kim, W., S. O. Lim, J. S. Kim, Y. H. Ryu, J. Y. Byeon, H. J. Kim, J. I. Kim, J. S. Heo, Y. M. Park, and G. Jung. 2003. Comparison of proteome between hepatitis B virus- and hepatitis C virus-associated hepatocellular carcinoma. *Clin. Cancer Res.* **9**:5493–5500.
- Kumar, D., G. C. Farrell, C. Fung, and J. George. 2002. Hepatitis C virus genotype 3 is cytopathic to hepatocytes: reversal of hepatic steatosis after sustained therapeutic response. *Hepatology* **36**:1266–1272.
- Lai, M. M. C. 2002. Hepatitis C virus proteins: direct link to hepatic oxidative stress, steatosis, carcinogenesis and more. *Gastroenterology* **122**:568–571.
- Lerat, H., M. Honda, M. R. Beard, K. Loesch, J. Sun, Y. Yang, M. Okuda, R. Gosert, S. Y. Xiao, S. A. Weinman, and S. M. Lemon. 2002. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* **122**:352–365.
- Liu, H., R. G. Sadygov, and J. R. Yates III. 2004. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal. Chem.* **76**:4193–4201.
- Lohmann, V., J. O. Koerner, U. Herian, L. Theilmann, and R. Bartenschlager. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**:110–113.
- Maehara, K., T. Hida, Y. Abe, A. Koga, K. Ota, and E. Kutoh. 2003. Functional interference between estrogen-related receptor alpha and peroxisome proliferator-activated receptor alpha/9-cis-retinoic acid receptor alpha heterodimer complex in the nuclear receptor response element-1 of the medium-chain acyl coenzyme A dehydrogenase gene. *J. Mol. Endocrinol.* **31**:47–60.

28. Negro, F. 2002. Hepatitis C virus and liver steatosis: is it the virus? Yes it is, but not always. *Hepatology* **36**:1050–1052.
29. Nesvizhskii, A. I., A. Keller, E. Kolker, and R. Aebersold. 2003. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **75**:4646–4658.
30. Okuda, M., K. Li, M. R. Beard, L. A. Showalter, F. Scholle, S. M. Lemon, and S. A. Weinman. 2002. Mitochondrial injury, oxidative stress, and anti-oxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* **122**:366–375.
31. Oliver, J. A., and Q. Al-Awqati. 1998. An endothelial growth factor involved in rat renal development. *J. Clin. Investig.* **102**:1208–1219.
32. Ou, K., T. K. Seow, R. C. Liang, S. E. Ong, and M. C. Chung. 2001. Proteome analysis of a human hepatocellular carcinoma cell line, HCC-M: an update. *Electrophoresis* **22**:2804–2811.
33. Park, K. S., H. Kim, N. G. Kim, S. Y. Cho, K. H. Choi, J. K. Seong, and Y. K. Paik. 2002. Proteomic analysis and molecular characterization of tissue ferritin light chain in hepatocellular carcinoma. *Hepatology* **35**:1459–1466.
34. Perlemuter, G., A. Sabile, P. Letteron, G. Vona, A. Topilco, Y. Chrétien, K. Koike, D. Pessayre, J. Chapman, G. Barba, and C. Bréchet. 2002. Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. *FASEB J.* **16**:185–194.
35. Petritis, K., L. J. Kangas, P. L. Ferguson, G. A. Anderson, L. Pasa-Tolic, M. S. Lipton, K. J. Auberry, E. Strittmatter, Y. Shen, R. Zhao, and R. D. Smith. 2003. Use of artificial neural networks for the prediction of peptide liquid chromatography elution times in proteome analyses. *Anal. Chem.* **75**:1039–1048.
36. Qian, W. J., J. M. Jacobs, D. G. Camp II, M. E. Monroe, R. J. Moore, M. A. Gritsenko, S. E. Calvano, S. F. Lowry, W. Xiao, L. L. Moldawer, R. W. Davis, R. G. Tompkins, and R. D. Smith. 2005. Comparative proteome analyses of human plasma following in vivo lipopolysaccharide administration using multidimensional separations coupled with mass spectrometry. *Proteomics* **5**:572–584.
37. Qian, W. J., T. Liu, M. E. Monroe, E. F. Strittmatter, J. M. Jacobs, L. J. Kangas, K. Petritis, D. G. Camp II, and R. D. Smith. 2005. Probability-based evaluation of peptide and protein identifications from tandem mass spectrometry and SEQUEST analysis: the human proteome. *J. Proteome Res.* **4**:53–62.
38. Ramalho, F. 2003. Hepatitis C virus infection and liver steatosis. *Antiviral Res.* **60**:125–127.
39. Santamaria, E., M. A. Avila, M. U. Latasa, A. Rubio, A. Martin-Duce, S. C. Lu, J. M. Mato, and F. J. Corrales. 2003. Functional proteomics of non-alcoholic steatohepatitis: mitochondrial proteins as targets of S-adenosylmethionine. *Proc. Natl. Acad. Sci. USA* **100**:3065–3070.
40. Scholle, F., K. Li, F. Bodola, M. Ikeda, B. A. Luxon, and S. M. Lemon. 2004. Virus-host cell interactions during hepatitis C virus RNA replication: impact of polyprotein expression on the host cell transcriptome and cell cycle association with viral RNA synthesis. *J. Virol.* **78**:1513–1524.
41. Seow, T. K., S. E. Ong, R. C. Liang, E. C. Ren, L. Chan, K. Ou, and M. C. Chung. 2000. Two-dimensional electrophoresis map of the human hepatocellular carcinoma cell line, HCC-M, and identification of the separated proteins by mass spectrometry. *Electrophoresis* **21**:1787–1813.
42. Shen, Y., R. Zhao, M. E. Belov, T. P. Conrads, G. A. Anderson, K. Tang, L. Pasa-Tolic, T. D. Veenstra, M. S. Lipton, and R. D. Smith. 2001. Packed capillary reversed-phase liquid chromatography with high-performance electrospray ionization fourier transform ion cyclotron resonance mass spectrometry for proteomics. *Anal. Chem.* **73**:1766–1775.
43. Shi, S. T., S. J. Polyak, H. Tu, D. R. Taylor, D. R. Gretch, and M. M. C. Lai. 2002. Hepatitis C virus NS5A colocalizes with the core protein on lipid droplets and interacts with apolipoproteins. *Virology* **292**:198–210.
44. Smith, M. W., N. Y. Zhaoxia, G. K. Geiss, N. Y. Sadovnikova, V. S. Carter, L. Boix, C. A. Lazaro, G. B. Rosenberg, R. E. Bumgarner, N. Fausto, J. Bruix, and M. G. Katze. 2003. Identification of novel tumor markers in hepatitis C virus-associated hepatocellular carcinoma. *Cancer Res.* **63**:859–864.
45. Smith, M. W., N. Y. Zhaoxia, M. J. Korth, H. A. Do, L. Boix, N. Fausto, J. Bruix, J. Carithers, R. L., and M. G. Katze. 2003. Hepatitis C virus and liver disease: global transcriptional profiling and identification of potential markers. *Hepatology* **38**:1458–1467.
46. Smith, R. D., G. A. Anderson, M. S. Lipton, L. Pasa-Tolic, Y. Shen, T. P. Conrads, T. D. Veenstra, and H. R. Udseth. 2002. An accurate mass tag strategy for quantitative and high-throughput proteome measurements. *Proteomics* **2**:513–523.
47. Smith, R. D., L. Pasa-Tolic, M. S. Lipton, P. K. Jensen, G. A. Anderson, Y. Shen, T. P. Conrads, H. R. Udseth, R. Harkewicz, M. E. Belov, C. Masselon, and T. D. Veenstra. 2001. Rapid quantitative measurements of proteomes. *Electrophoresis* **22**:1652–1668.
48. Su, A. I., J. P. Pezacki, L. Wodicka, A. D. Brideau, L. Supekova, R. Thimme, S. Wieland, J. Bukh, R. H. Purcell, P. G. Schultz, and F. V. Chisari. 2002. Genomic analysis of the host response to hepatitis C virus infection. *Proc. Natl. Acad. Sci. USA* **99**:15669–15674.
49. Sumida, Y., T. Nakashima, T. Yoh, Y. Nakajima, H. Ishikawa, H. Mitsuyoshi, Y. Sakamoto, T. Okanoue, K. Kashima, H. Nakamura, and J. Yodoi. 2000. Serum thioredoxin levels as an indicator of oxidative stress in patients with hepatitis C virus infection. *J. Hepatol.* **33**:616–622.
50. Tan, S. L., H. Nakao, Y. He, S. Vijaysri, P. Neddermann, B. L. Jacobs, B. J. Mayer, and M. G. Katze. 1999. NS5A, a nonstructural protein of hepatitis C virus, binds growth factor receptor-bound protein 2 adaptor protein in a Src homology 3 domain/ligand-dependent manner and perturbs mitogen signaling. *Proc. Natl. Acad. Sci. USA* **96**:5533–5538.
51. Tsutsumi, T., T. Suzuki, T. Shimoike, R. Suzuki, K. Moriya, Y. Shintani, H. Fujie, Y. Matsura, K. Koike, and T. Miyamura. 2002. Interaction of hepatitis C virus core protein with retinoid X receptor α modulates its transcriptional activity. *Hepatology* **35**:937–946.
52. Venkatraman, A., A. Landar, A. J. Davis, L. Chamlee, T. Sanderson, H. Kim, G. Page, M. Pompilius, S. Ballinger, V. Darley-Usmar, and S. M. Bailly. 2004. Modification of the mitochondrial proteome in response to the stress of ethanol-dependent hepatotoxicity. *J. Biol. Chem.* **279**:22092–22101.
53. Washburn, M. P., D. Wolters, and J. R. Yates. 2001. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* **19**:242–247.
54. Wolters, D. A., M. P. Washburn, and J. R. Yates. 2001. An automated multidimensional protein identification technology for shotgun proteomics. *Anal. Chem.* **73**:5683–5690.
55. Yan, W., H. Lee, E. W. Deutsch, C. A. Lazaro, W. Tang, E. Chen, N. Fausto, M. G. Katze, and R. Aebersold. 2004. A dataset of human liver proteins identified by protein profiling via isotope-coded affinity tag (ICAT) and tandem mass spectrometry. *Mol. Cell. Proteomics* **3**:1039–1041.
56. Yan, W., H. Lee, E. C. Yi, D. Reiss, P. Shannon, B. K. Kwieciszewski, C. Coito, X. J. Li, A. Keller, J. Eng, T. Galitski, D. R. Goodlett, R. Aebersold, and M. G. Katze. 2004. System-based proteomic analysis of the interferon response in human liver cells. *Genome Biol.* **5**:R54.
57. Yoshida, K., H. Nakamura, Y. Okuda, H. Enomoto, Y. Kishima, H. Uyama, H. Ito, T. Hirasawa, S. Inagaki, and I. Kawase. 2003. Expression of hepatoma-derived growth factor in hepatocarcinogenesis. *J. Gastroenterol. Hepatol.* **18**:1293–1301.
58. Zhou, Y. C., H. W. Davey, M. J. McLachlan, T. Xie, and D. J. Waxman. 2002. Elevated basal expression of liver peroxisomal α -oxidation enzymes and CYP4A microsomal fatty acid-hydroxylase in STAT5b $^{-/-}$ mice: cross-talk in vivo between peroxisome proliferator-activated receptor and signal transducer and activator of transcription signaling pathways. *Toxicol. Appl. Pharmacol.* **182**:1–10.