Hepatocyte Nuclear Factor (HNF) 1 and HNF4 Mediate Hepatic Multidrug Resistance Protein 2 Up-Regulation during Hepatitis C Virus Gene Expression

Ishtiaq Qadri, Mieko Iwahashi, Gerd A. Kullak-Ublick, and Francis R. Simon

Department of Pediatrics and Graduate Program in Toxicology (I.Q.) and Division of Gastroenterology/Hepatology (M.I., F.R.S.), University of Colorado Health Sciences Center, Aurora, Colorado; and Laboratory of Molecular Gastroenterology and Hepatology, University Hospital, Zurich, Switzerland (G.A.K.-U.)

Received February 13, 2006; accepted April 28, 2006

ABSTRACT

Hepatitis C virus (HCV) is known to induce hepatic oxidative stress that is implicated in the up-regulation of multidrug resistance proteins (MRPs). The relationship between increased prooxidant production, MRPs, and HCV has not been investigated. Here, we report that a homeodomain-containing transcription factor, hepatocyte nuclear factor (HNF) 1, plays a central role in liver gene regulation during HCV gene expression and/or subgenome replication. MRP2 protein and mRNA expression were increased and MRP2 promoter activity was increased 7-fold. Mutations within the putative HNF1 binding site of the human MRP2 promoter abrogated HCV-induced activation, implicating HNF1 in the induction of MRP2 by HCV. The mechanism by which HNF1-mediated activation occurs seems to be transcriptional, because the regulated expression of HNF4, which is known to control HNF1 expression, was also increased. Consistent with this finding, HNF1 mRNA was increased 10-fold. A promoter-luciferase construct of the human HNF1 gene was activated in an HNF4-dependent manner, and a mutant construct lacking the HNF4 binding site was not activated in HCV-positive cells. Consistent with this hypothesis, HNF4 protein and mRNA levels as well as HNF4 promoter activity and DNA binding activity were increased. The expression of HNF1 seems to play a critical role in the induction of hepatic MRP2 secondary to HCV subgenomic replication. The ability of HCV to induce HNF1 and HNF4 is attributed to 1) increased oxidative stress and 2) direct protein-protein interactions between HCV nonstructural component (NS) 5A and HNF1, leading to enhanced HNF1 DNA binding. In conclusion, we describe a novel mechanism by which HCV gene expression may induce adaptive responses involving MRP2 via HNF1 activation. This may constitute, in part, the cellular detoxification task force during HCV infection.

Hepatitis C virus (HCV) infection poses a serious health problem, because the virus is the causative agent of chronic hepatitis worldwide. HCV infection typically produces a prolonged, insidious course that may progress to liver fibrosis, cirrhosis, insulin resistance, and eventually hepatocellular carcinoma (World Health Organization, 1999). HCV is a positive-strand RNA virus that is co- and post-translationally cleaved to produce at least 10 polypeptides, including three structural components (Core, E1, and E2) and seven non-structural components (NS2 to NS5B) (Reed and Rice, 2000). HCV nonstructural proteins direct viral replication from a ribonucleoprotein replication complex that is associated with the endoplasmic reticulum (Hijikata et al., 1993). Although the potential mechanism(s) of HCV-induced cell injury and disease progression is unclear, emerging evidence includes the relationship between liver damage and production of oxygen-derived free radicals, glutathione (GSH) depletion, tumor suppressor p53 protein inactivation, and sustained release of inflammatory cytokines (Multu-Turkoglu et al., 1997; Qadri et al., 2002, 2004; Liu et al., 2003; Abdalla et al., 2005).

Transcriptional regulators such as hepatocyte nuclear factor (HNF) 1 and HNF4, nuclear factor-κB (NF-κB), signal
transducer and activator of transcription (STAT) 3, multidrug resistance proteins (MRPs), and GSH seem to be involved in this process (Hayashi et al., 1999; Schrenk et al., 2001; Sladek and Seidal, 2001; Guo et al., 2002; Geier et al., 2003; Watt et al., 2003). Both HNF1 and HNF4 are essential transcriptional factors for normal liver development. HNF1, a hepatocyte-enriched factor, is a member of a novel dimerizing homeodomain protein family with members such as Hox1.6, Paired, Pit-1, and Oct2, whereas HNF4, a member of the steroid /thyroid nuclear receptor superfamily, is expressed in kidney, intestine, and especially the liver, where it is a central regulator of liver metabolism (Hayashi et al., 1999; Bailly et al., 2001; Hatzis et al., 2001; Sladek and Seidal, 2001; Wang et al., 2001; Watt et al., 2003).

After hepatic injury and/or oxidative stress, acute phase response genes are known to be rapidly up-regulated to restore homeostasis and limit tissue damage (Dietrich et al., 2003). Furthermore, glucocorticoids, rifampicin, cell swelling, and reactive oxidative stress (ROS) increase MPR2 expression (Kauffmann et al., 1998, 2002; Kubitz et al., 1999; Payen et al., 2001; Li and Weinman, 2002; Geier et al., 2003; Reichard et al., 2003), but the molecular mechanism(s) is largely unknown. Other ATP-binding cassette (ABC) liver proteins such as MRP1(ABCC1) and MRP3(ABCC3) also demonstrate high expression in severe liver disease (Ros et al., 2003).

Multidrug resistance is generally accepted as an important cause of treatment failure for patients with neoplastic or infectious diseases. Multidrug resistance-associated genes are induced during induced oxidative stress, possibly to transport glutathione S-conjugates and glutathione disulfide into the extracellular space, thereby playing a critical role in detoxification processes (Payen et al., 2001; Reichard et al., 2003). It is widely acknowledged that oxidative stress activates MDR1 (ABCC1), MRP2 (ABCC2), and MRP3 (ABCC3), involving, in part, NF-xB and tumor necrosis factor-α (Geier et al., 2003). The link between MPR2 activation and oxidative stress has also been established by treating cells with sulfophane and t-butylhydroquinone, which are known to regulate drug metabolism through ROS formation (Payen et al., 2001). Many lipophilic compounds conjugated with glutathione, glucuronate, or sulfates and substrates of the glutathione S-conjugate leukotriene C4, S-2,4-dinitrophenyl glutathione, bilirubin glucuronide, and 17-β-glucuronosyl estriadiol are also substrates for the MRP2 export pump (Schrenk et al., 2001; Gerk and Vore, 2002; Kim, 2002).

Despite the probable importance of MRP2 in the detoxification process associated with oxidative stress, the molecular mechanisms that regulate gene expression during HCV replication have not been investigated. We and others have previously shown that HCV is associated with reactive oxidative stress (Multu-Turkoglu et al., 1997; Qadri et al., 2004; Waris et al., 2005). Because oxidative stress plays a pivotal role in the detoxification process, the present study was designed to investigate the molecular basis of feedback control regulation of hepatocyte nuclear factors and MRP2, the major transporter involved in biliary secretion of oxidized products.

Our study demonstrates that MRP2 activation is dependent on induced HNF1 and HNF4 expression. HNF1 and HNF4 expression were both increased by oxidative stress induced by hydrogen peroxide or decreased by N-acetylcycteine (NAC), supporting a common link for increased MRP2 expression. Alternatively, HNF1 activation may occur by direct protein-protein interaction between HCV-encoded NSSA and HNF1, whereby NSSA stimulates DNA binding of HNF1 to its cognate DNA sequence.

Materials and Methods

Cell and Culture Conditions. The Huh.8 cell line used in this study contains the hepatitis C virus subgenomic replicon expressing nonstructural components NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Bligh et al., 2000). The coding region of HCV NS components, under the control of encephalomyocarditis virus internal ribosomal entry site, was integrated into human hepatoma Huh-7 chromosome along with selectable G418 resistant gene, which was under the control of human cytomegalovirus promoter. Huh-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing high glucose supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, and 10% fetal bovine serum. Huh.8 cells were maintained in complete DMEM supplemented 10% heat-inactivated fetal bovine serum, nonessential amino acids, and 1 mg/ml G418. All cells were maintained at 37°C for two to three passages for the experiments in a humidified environment containing 5% CO2.

Plasmids. NSSA expression plasmids pCDNA/NSSA (mammalian) and pGST-NSSA (bacterial) were obtained from Dr. M. Katze (University of Washington, Seattle, WA), which was originally isolated from HCV strain 1b. The cloning of human MRP2 promoter will be described in a separate article. In brief, a 2133-base pair (bp) human MRP2 promoter was isolated from P1 human genomic library by PCR screening. This region was cloned into pGEM3, and the recombinant plasmid is referred to as p2.1. A series of 5’ deletions were generated using exonuclease III and mungbean nuclease to determine the important liver-specific factors. A construct containing 760 bp of MRP2 was designated as Δ760-LUC and was selected for mutagenesis. Nucleotides GTTA at position of 158 of human MRP2 gene and have been described previously (Jung and Kallak-Ublick, 2003). Mutant HNF4 promoter construct (0.65H4luc) containing 12,145 bp of human HNF4 was obtained from Dr. I. Talianidis (Institute of Molecular Biology and Biotechnology, Crete, Greece). Mutant HNF4 promoter constructs H473 and H82 contain the 473- and 82-bp region of human HNF1α promoter fused to the luciferase gene and have been described previously (Jung and Kallak-Ublick, 2003). MutHB2 contains the mutation within the HNF4 binding site direct repeat unit AGTGCAAGTTCA at position 63–51, which was mutated into ATACCAATATCATA by site-directed mutagenesis. All promoters are cloned into pGL3 vector. pGL3-promoter vector contains the simian virus 40 promoter and reporter LUC gene. Full-length human HNF4α promoter-luciferase construct (P4FLuc) containing 12,145 bp of human HNF4 was obtained from Dr. I. Talianidis (Institute of Molecular Biology and Biotechnology, Crete, Greece). Mutant HNF4 promoter construct (0.65H4luc) containing the 684 bp from the ATG of human HNF4 was also obtained from Dr. I. Talianidis.

Reverse Transcription-Polymerase Chain Reaction Methods. Primers and probe for MRP2, HNF1, and HNF4 were designed with the assistance of the Prism 7700 sequence detection software (Primer Express; Applied Biosystems, Foster City, CA) and are available upon request. ABI Prism 7700 sequence detector (Applied Biosystems) was used to measure the fluorescent spectra of all 96 wells of a thermal cycler during PCR amplification. The reactions were monitored in real time. Amplification reactions were performed in MicroAmp optical tubes (Applied Biosystems) in a 50-μl mix containing 5% glycerol; 1× TaqMan buffer A (500 mM KCl, 100 mM Tris-HCl, 0.1 M EDTA, and 600 mM passive reference dye ROX, pH 8.3, at room temperature); 300 μM each of dATP, dGTP, and dCTP; and 600 μM dUTP, 5.5 mM MgCl2, 900 nM forward primer, 300 nM reverse primer.
primer, 200 nM probe, 1.25 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems), 12.5 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA), 20 U of RNAsin ribonuclease inhibitor (Promega, Madison, WI), and the template RNA. Thermal cycling conditions were as follows: Reverse transcription was performed at 48°C for 30 min followed by activation of TaqGold at 95°C for 10 min. Thereafter, 40 cycles of amplification were performed at 95°C for 15 s and at 60°C for 1 min. After amplification, real-time data acquisition and analysis were performed. The fluorescence data were expressed as normalized reporter signal (Rn) or ΔRn. Rn is calculated by dividing the amount of reporter signal by the amount of passive reference signal. ΔRn represents the amount of normalized reporter signal minus the amount of reporter signal before PCR. The detection threshold was set above the mean baseline fluorescence determined from the first 15 cycles. Amplification reactions in which the fluorescence intensity increased above the threshold were defined as a positive reaction. Threshold cycle represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected. A standard curve was generated using the fluorescent data from the 10-fold serial dilutions of HeLa RNA. This curve was then used to calculate the relative amounts of MRP2, HNF1, and HNF4 in test samples. Quantities in test samples were normalized to the corresponding 18S rRNA (Applied Biosystems).

Oligonucleotides. 5'-AAAAATAGGTGTTAATCCCTGACCTCTATA-3' sense and 5'-TATAAGGTCAGGATTACACCTAATTTT-3' antisense were synthesized and encompass the consensus HNF1 binding site motif (GTTA) at position 2055 of hMRP2 promoter. Likewise, oligonucleotides 5'-TCTAGGCAAGGTTAACGAT- TAAATGGTTC-3' sense and 5'-CAACCATTATATCGGATACCTTG- GCCTAGA-3' antisense were synthesized and encompass the HNF1 binding motif (GTTA) at position 158 of HMRP2 promoter. HNF1 mutant oligonucleotides for position 2055 were as follows: 5'-AAAAATAGGTCCCACTCTGACCTTATA-3' sense and 5'-TATAAGGTCAGGATTACACCTAATTTT-3' antisense. HNF1 mutant oligonucleotides for position 158 were as follows: 5'-TCTAGGCAAGGTTAACGAT- TAAATGGTTC-3' sense and 5'-CAACCATTATATCGGATACCTTG- GCCTAGA-3' antisense.

Antibodies. Monoclonal NS5b for NS5A was from ID Labs (London, ON, Canada). HNF1 and HNF4 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). MRP2 antibodies were from Alexis Biochemicals (San Diego, CA).

Protein-Protein Interactions. Cells were washed twice in ice-cold phosphate-buffered saline and lysed at −4°C in buffer containing 150 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, 0.1% SDS, 1% Nonidet P-40, 10 mM p-nitrosophenol phosphate, 10 mM sodium fluoride, 40 mM glycophosphate, and 1 mM phenylmethylsulfonyl fluoride. Nuclear extracts were prepared as described previously (Qadri et al., 2002, 2004). Both fractions were mixed and dialyzed against buffer containing 50 mM KCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, and 1 mM phenylmethylsulfonyl fluoride. Whole-cell extracts were used for immunoblotting analysis using the ECL System (GE Healthcare, Little Chalfont, UK). Cell lysates were allowed to pass through GST-affinity resin (Pfizer, Inc., New York, NY), which were immobilized with either 10 mg of GST or GST-NS5A protein and allowed to interact for 2 h at 4°C, with rocking in buffer A, containing 100 mM KCl, 20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 10% glycerol, and protease inhibitors. GST affinity beads were extensively washed with buffer A containing 450 mM KCl.

In Vitro Protein Synthesis and Affinity Chromatography. HNF1, HNF4, HNF6, and NS5A were translated in vitro from plasmids and pRHNF1-α, pMT7-rHNF4-wt pCMVHNF6-α, and pRc/CMV/NS5A by using T7 RNA polymerase, [35S]methionine, and TNT rabbit lysates (Promega). [35S]-Labeled NS5A, HNF1, HNF4, and HNF6 proteins (5 µl of a 50-µl volume) were chromatographed through 20-µl microaffinity column containing immobilized (Affi-gel 10; Bio-Rad, Hercules, CA) GST and GST-fusion proteins at a 2 mg/ml protein concentration in buffer A (10 mM HEPES, pH 7.5, 0.15 M KCl, 1 mM ATP, 1 mM EDTA, and 10% glycerol), containing 2 mg of bovine serum albumin and 0.1% Nonidet P-40. Affinity columns were washed with buffer A, and bound fractions were eluted in buffer A containing 0.5 M KCl and 1% SDS. GST-fusion proteins were harvested from bacterial cultures as described previously (Qadri et al., 2002, 2004).

Mutagenesis. HNF1 consensus DNA binding site motif 5'-GTTA-3' located at position 158 of hMRP2 promoter was mutated into 5'-CCCCC-3' by site-directed mutagenesis using polymerase chain reaction. Mutations were confirmed by DNA sequencing analysis of the plasmid DNA. Plasmid Δ760-LUC DNA was used in the mutagenesis reaction by using oligonucleotides 5'-TCTAGGCAAGGTTAACGAT- TAAATGGTTC-3' sense and 5'-CAACCATTATATCGGATACCTTG- GCCTAGA-3' antisense. Strands were confirmed by DNA sequencing of the Δ760mut-LUC.

Reactive Oxygen Species Measurement. H2O2 production was measured within Huh-7 (control) and Huh.8 (HCV-replicating cells) using 2',7'-dichlorodihydrofluorescein diacetate (also known as 2',7'-dichlorodihydrofluorescein) (Invitrogen) as the indicator. DCF emission was measured at 525 ± 20 nm by flow cytometry. Upon cell activation, NADPH oxidase catalyzes the reduction of O2 to O2−, which is further reduced to H2O2. H2O2 and peroxides are able to oxidize the trapped dye 2',7'-dichlorodihydrofluorescein to DCF, which is highly fluorescent at 530 nm. Two million cells were subjected for fluorescence-activated cell sorting analysis as described previously (Qadri et al., 2004).

Preparation of Nuclear Proteins. Nuclear proteins were prepared from liver-derived cells as described previously (Qadri et al., 2002, 2004). Nuclear extracts were prepared using sucrose gradients and adjusted to 0.3% Triton X-100 (v/v). Sucrose gradients were prepared and centrifuged at 77,000g for 50 min at 4°C. The nuclear pellets were resuspended in buffer and ammonium sulfate slowly added to a final concentration of 0.4 M. The mixture was centrifuged at 62,000g for 60 min. The supernatant was saved and 0.33 g of ammonium sulfate per milliliter of supernatant added. The mixture was centrifuged, protein pellets were resuspended, and dialyzed aliquots were snap-frozen at −70°C.

Western Blotting. SDS-PAGE and immunoblotting were carried out using minigels. Proteins were transferred according to the procedures of Towbin et al. (1979) and processed using enhanced chemiluminescence for detection (GE Healthcare) with specific antibodies using 1% nonfat milk in Tris-buffered saline. All washes were in 0.15% Tween/Tris-buffered saline for 5 min. An analysis of autoradiograms was by PhosphorImager (GE Healthcare).

DNA Transfections. Cultures were set up 24 h before transfections in 35-mm six-well plates at 3 × 105 cells/well in DMEM media with appropriate supplements. Transfections were carried out using FuGENE 6 (Roche Diagnostics, Indianapolis, IN), with a DNA/lipid ratio of 2:1. Forty-eight hours after transfection, cells were harvested and assayed for luciferase activity using a dual luciferase reporter assay system (Promega). Luciferase activity was read in a Lumiscan luminometer (PerkinElmer Life and Analytical Sciences).

Electrophoresis Mobility Shift Assays. The oligonucleotides were radiolabeled with [γ-32P]dATP using T4 polynucleotide kinase. Binding reaction mixtures contain 20 mM HEPES, pH 7.9, 4% Ficoll type 400, 50 mM KCl, 0.5 mM EDTA, 100 mg of poly(dI-dC), 50 ng of pSK+ DNA (Stratagene), and 20,000 cpm of DNA probe (Qadri et al., 2002, 2004). Reactions were preincubated on ice for 10 min before the addition of 32P-labeled double-stranded oligonucleotide probe (0.2 pmol). Competitor oligonucleotides were added to the preincubation at 100-fold molar excess. Samples were held on ice for a further 20 min, and the protein-DNA complexes were resolved on a pre-electrophoresed 5% polyacrylamide gel in 0.5× Tris borate-EDTA (45 mM
Results

Measurement of Reactive Oxygen Species in Cell Lines. Cell lines expressing HCV have proven to be useful models for examining the intracellular changes associated with HCV replication. In Huh-7 cells, addition of the oxidizing agent, H$_2$O$_2$, significantly increased DCF fluorescence 30% (p < 0.001) above control values. Figure 1 also demonstrates that Huh.8 cells, expressing HCV NS proteins, have 30% (p < 0.001) increased ROS compared with Huh-7 cells. Furthermore, addition of antioxidant NAC to Huh.8 cells significantly reduced ROS to below normal values (p < 0.01), supporting the role of increased ROS with HCV replication. These results are consistent with the hypothesis that Huh.8 cells are a reasonable model of HCV-induced oxidative stress.

MRP2 Is Up-Regulated in HCV-Replicating Huh.8 Cells. MRP2 is a 190-kDa organic anion efflux pump protein that is located at the apical side of normal hepatocytes and contributes to biliary secretion and transport of conjugated oxidative metabolites. Therefore, we examined MRP2 protein and RNA expression by Western and Northern blotting, respectively (Fig. 2A). Total membrane fractions of Huh-7 and Huh.8 cells were prepared by sodium bicarbonate method and subjected to immunoblotting using MRP2 monoclonal antibodies. Although very low levels of MPR2 protein expression were observed in Huh-7 membrane fractions, a substantial increase of 1.5-fold in MRP2 expression was seen in Huh.8 cells (Fig. 2A). Northern blotting analysis of the total RNA correlates with protein expression of MRP2 in Huh-7 compared with Huh.8 cells (Fig. 2B), suggesting transcriptional regulation of MRP2 during HCV replication. The expression of HCV subgenomic (-) stranded RNA replicon in Huh.8 cells was confirmed by Northern blotting using the NS5A-specific DNA probe labeled with $^{32}$P-ATP (data not shown).

To fully assess the HCV-induced activation of MRP2, the construct p2.1 was transfected into Huh-7 and Huh.8 cells, and firefly luciferase activity was measured as arbitrary light units (ALU). A 7-fold increase (p < 0.002) in the ALU was detected in HCV-replicating Huh.8 cells versus control Huh-7 cells (Fig. 2B), confirming increased transcriptional activity of MPR2 in Huh.8 cells. To gain further insight into the transcriptional regulation of MRP2 by HCV subgenomic replication and/or gene expression, several deletion mutants of MRP2 promoter were generated and assayed by transient transfection into Huh-7 and Huh.8 cells. A minimal deletion mutant $\Delta$p760-LUC containing upstream ~760-bp sequence of MPR2 promoter was found to be responsive in Huh.8 cells (p < 0.02) (Fig. 2C). Within this 760-bp region, a key HNF1 binding site was located by DNA sequence homology. The putative role of HNF1 in activation of MRP2 was examined by mutation of the GGTTA core motif of this HNF1 binding site located within the 760-bp region of MPR2 promoter. Analysis of human MRP2 promoter (p2.1) and $\Delta$p760-LUC and $\Delta$p760mut-LUC promoters were carried out by transient transfection into Huh-7 and Huh.8 cells (Fig. 2C), and measurement of ALU. Mutant MRP2 promoter ($\Delta$p670mut-LUC) lacking HNF1 binding site did not respond to activation in Huh-7 and Huh.8 cells, suggesting that activation process requires an intact HNF1 DNA binding site within the short MRP2 promoter. These results support the conclusion that induced HNF1 expression leads to increased HNF1 binding at position 158 bp of MRP2 promoter. As a control, a known HNF1-responsive CYP2E1 promoter was also transfected into Huh.8 cells, and as expected ~10-fold increased expression of CYP2E1 promoter was observed in Huh.8 cells (p < 0.01) (data not shown). Collectively, these results strongly supported a central role of HNF1 in MRP2 increased expression.

Induced Expression of HNF1 Is Coupled with Increased DNA Binding during HCV Replication. We next determined whether HNF1 expression was increased during HCV subgenomic replication and/or gene expression. mRNA analysis was performed in Huh.8 cells by Northern blotting analysis. Figure 3A revealed 10-fold increased expression of HNF1 mRNA in HCV subgenomic replicon-expressing Huh.8 cells versus control Huh-7 (p < 0.04) (Fig. 3A). Next, we assessed the DNA binding of HNF1 by EMSA, using the HNF1 core motif oligonucleotides and nuclear extracts from
Huh.8 (HCV subgenomic replicon) and Huh-7 (control) cells (Fig. 3B). Increased DNA binding of HNF1 was observed in HCV cells (lanes 2–6), in comparison with control nuclear extract for Huh-7 (lanes 7–11). Unlabeled HNF1 oligonucleotide DNA (10×) competed with the binding, suggesting the specificity of HNF1-DNA complex (lane 13). To further show the specificity of HNF1-DNA complex, HNF1 antibodies were added, and the band was supershifted (lane 14). HNF1 antibodies had no reactivity with free probe (lane 12). Unrelated Oct-1 and STAT5b antibodies have no effect on the supershift (data not shown).

Fig. 2. Activated MRP2 expression during HCV replication. A, Western and Northern blotting of Huh-7 and Huh.8 membrane fractions and total RNA using MRP2-specific antibodies (Santa Cruz Biotechnology, Inc.) and cDNA probe, respectively. B, human MRP2 promoter is activated in HCV-replicating cells in an HNF1-dependent manner. Luciferase activity of 2133-bp human MRP2 promoter-reporter-LUC (MRP2-LUC; p2.1) constructs were transfected into Huh-7 and Huh.8 cells by FuGENE 6 method. The ratio of luciferase/β-galactosidase is presented (n = 5). White column, Huh-7 cells and hatched column, Huh.8 cells. C, analysis of hMRP2 deletion mutant-LUC (p760-LUC) and HNF1 binding site mutant (Δp760mut-LUC) constructs in Huh-7 and Huh.8 cells by transient transfection. GTTA core motif of HNF1 binding site was mutated within the 670-bp region of MRP2 promoter (n = 4). Note that hMRP2 promoter without HNF1 binding site failed to respond to activation in HCV replicating cells, suggesting the importance of HNF1 in activation process.

HNF1 Promoter Is Activated in an HNF4-Dependent Manner. The human and rat HNF1 promoters are activated by HNF4 as well as other factors (Jung and Kullak-Ublick, 2003; Eeckhoute et al., 2004; Jung et al., 2004). To assess whether HCV replication may have affected HNF4 expression, Huh.8 and Huh-7 cells were first measured by Northern blotting (Fig. 4A). HNF4 expression was increased 1.7-fold in Huh.8 cells compared with control Huh-7 cells (p < 0.01) (Fig. 4A). Consistent with increased mRNA level, an ~3-fold increased DNA binding of HNF4 to its consensus binding site was observed in HCV subgenome replicon (Fig. 4B, lane 3)
versus the control Huh-7 (lane 2). Antibodies to HNF4 supershifted the HNF4-DNA band (lane 4), whereas an unrelated STAT5b antibody had no effect (lane 5). To corroborate the observation of increased HNF4 mRNA expression, HNF4-LUC construct (H4FL-LUC) was transfected into Huh-7 and Huh.8 cells. Luciferase expression was increased ~3-fold in Huh.8 cells versus control Huh-7 cells (p < 0.02) (Fig. 4C). These results are consistent with recently published studies that have identified the direct involvement of HNF4 in regulation of human HNF1 (Jung and Kullak-Ublick, 2003; Eeckhoute et al., 2004).

Next, we examined HNF1 activation, using a 473-bp human HNF1 promoter LUC construct, H473 (Fig. 4D). H473 was transfected into Huh-7 and Huh.8 cells, and a 3-fold increased luciferase activity (ALU) in Huh.8 cells was observed (p < 0.01). To further evaluate the importance of increased HNF4 in induction of HNF1, we examined HNF1 mutant promoters. H473 contains two HNF4 binding site motifs located at 1) position −413 to −398 and 2) position −63 to −51 (Jung and Kullak-Ublick, 2003). When a truncated 82-bp HNF1 promoter LUC construct (H82) encompassing only one HNF4 binding site at −63 to −51 was transfected into Huh-7 and Huh.8 cells, this construct also responded to activation (p < 0.01) in Huh.8 cells, implying the importance of the HNF4 site at position −63 to −51 in the minimal promoter (Fig. 4D). The importance of HNF4 binding site at position −63 to −51 in the HNF1 promoter was assessed further after transfection of the mutant HNF1-LUC construct (Δ82) in Huh-7 and Huh.8 cells, and luciferase activity was measured. Mutation of the proximal HNF4 binding site in the HNF1 promoter reduced basal activity in hepatoma cells. It is noteworthy that no activation of the mutant HNF1-LUC construct was seen in Huh.8 cells, establishing a direct link of HNF4 binding site located at −63 to −51 in the activation of HNF1 gene in HCV-replicating cells.

**Fig. 4.** Induced HNF4 expression is coupled with increased DNA binding during HCV subgenomic replication. A, Northern blot analysis of HNF4 mRNA expression in Huh-7 and Huh.8 cells. Data are presented as percentage of control over 18s (n = 4). B, EMSA of Huh.8 and Huh-7, nuclear extracts for binding to HNF4 consensus DNA. Lane 1, free probe. Lanes 2 and 3, 50 μg of Huh.7 and Huh.8 nuclear extracts added, respectively. Lanes 4 and 5, antibodies to HNF4 and STAT5B (an unrelated protein) added in Huh-7 nuclear extracts, respectively. HNF4 antibodies specifically supershifted the HNF4-DNA complex (lane 4). C, HNF4-LUC expression in Huh-7 and Huh.8 cells. The ratio of luciferase/R. reniformis is presented (n = 5). D, HNF1 activation is HNF4-dependent. Luciferase activity of 473-bp human HNF1 promoter-reporter-LUC construct (H473), truncated 82-bp LUC construct (H82) and HNF4 mutant binding site construct (Δ82) (n = 4). Data are presented as mean ± S.D. Plasmid DNAs were transfected into Huh-7 and Huh.8 cells by FuGENE 6 method. White column, Huh-7 cells and hatched column, Huh.8 cells. The ratio of luciferase/R. reniformis is presented.
HNF4 (p < 0.03) promoter activation in Huh.8 cells, respectively. Other stress-inducible factors and/or cellular processes responsible for HNF4 activation remain to be investigated, but these results support the notion that reactive oxidative stress is one of the main components that contributes to induced activation of HNF1 and HNF4 expression.

HCV-NS5A Protein Directly Associates with HNF1.

Previous reports have indicated that viral proteins may interact with transcription factors leading to modulated binding to target DNA sites (Zhou and Yen, 1991; Li et al., 2002; Qadri et al., 2002). Therefore, we sought to determine whether HNF1 or HNF4 are direct targets for HCV NS5A protein interactions. We provide evidence that NS5A directly and selectively associated with HNF1. HNF1, HNF4, and HNF6 proteins were translated in vitro and allowed to interact with glutathione affinity beads immobilized with either GST (Fig. 6A, lanes 1–3) or GST-NS5A (lanes 4–6). Only [35S]methionine-labeled HNF1 showed direct interactions with GST-NS5A (lane 6). No interactions were seen with GST (lanes 1–3). To further confirm the specificity of NS5A-HNF1 interactions, coimmunoprecipitation of Huh-7 and Huh.8 cell extracts was performed using either anti-NS5A (Fig. 6B, lanes 2 and 4) or anti-HNF1 (Fig. 6B, lanes 3 and 5). The fractions were separated on SDS-PAGE, followed by immunoblotting with anti-HNF1. Although no complexes were seen in lysates without antibody (lane 1) or in Huh-7 cells (Fig. 6B, lanes 2 and 3), HNF1-NS5A complexes were specifically determined using either antibodies (lanes 4 and 5).

To assess the functional relevance of HCV NS5A association with HNF1, we examined the ability of NS5A to influence the DNA binding kinetics of HNF1. When bacterially purified GST-NS5A was added in the gel shift assay using Huh-7 nuclear extracts and HNF1 DNA probe, NS5A stimulated at least 3- to 4-fold the DNA binding of HNF1 to its cognate DNA (Fig. 6C, lane 3). No stimulation of DNA binding was seen with the addition of GST alone (lane 2) or with GST-TBP (lane 4) and GST-STAT5b (lane 5), implying the specificity of this reaction. Unlabeled competitor DNA competed with the binding (lane 6) and HNF1 antibodies specifically supershifted the HNF1-DNA complex, further establishing the authenticity of HNF1-DNA complex.

Interferon-Treated Huh.8 Cells Show Reduced HNF1, HNF4, and MRP2 Expression. Recently, several studies have reported that interferon (IFN-α and -γ) specifically inhibited protein synthesis and RNA replication of subgenomic and genomic HCV replicon (Frese et al., 2002; Landford et al., 2003). These studies further show that the inhibitory action of IFN does not rely on the production of nitric oxide or depletion of tryptophan. We have used IFN-α in our analysis to suppress HCV protein synthesis and RNA replication of subgenomic replicon to separate the effects of HCV replication from possible cell selection artifacts. To rule out the clonal effects of HCV subgenome replicon, Huh.8 cells were treated with 75 U/ml IFN-α for 96 h. RNA was extracted and analyzed for HNF1 and HNF4 and MRP2 expression by reverse transcription-PCR. The picogram amounts of HNF1, HNF4, and MRP2 RNA/ng rRNA are shown in Fig. 7. In Huh-7 cells, HNF1 (700 ± 26) was significantly less than in Huh.8 cells (1201 ± 26) (p < 0.01). IFN treatment reduced HNF1 in Huh.8 cells to values similar to that of Huh-7 cells (744 ± 35) (p < 0.01). A similar trend was measured for HNF4 (406 ± 12 versus 396 ± 24.56) (p < 0.03). In addition, MRP2 was increased in Huh.8 (1411 ± 20) compared with Huh-7 cells (351 ± 9.1), whereas IFN treatments restored MRP2 to normal values (405 ± 38.51) (p < 0.01). Collectively, in all cases the amount of RNA was significantly higher in HCV-replicating Huh.8 versus the control Huh-7 cells. It is noteworthy that values in Huh.8 cells were restored to control levels after IFN treatment, whereas no change in HNF1, HNF4, and MRP2 expression was measured in control Huh-7 cells treated with IFN-α (Fig. 7). These results clearly support the hypothesis that HCV subgenome replication directly contributed to increased expression and that HCV-induced ROS proteins play a pivotal role in the activation of HNF4 that in turn activates HNF1, which leads to downstream effects on MRP2 expression.
Discussion

Many viruses, including HCV, have been shown to induce oxidative stress during replication and/or protein expression, and chronic HCV infection in human liver as well as different experimental models is associated with excess oxidative stress (Okuda et al., 2002; Liu et al., 2003; Qadri et al., 2004). Adaptive responses to this increased oxidative burden induced by HCV includes activation of key antioxidant pathways, including enzymes such as manganese superoxide dismutase (Qadri et al., 2004), catalase, and GSH peroxidase and heme oxygenase-1; and intracellular antioxidants compounds thioredoxin and GSH to control the oxidative imbalance (Multu-Turkoglu et al., 1997; Okuda et al., 2002). Increased lipid-oxidative products, which have been implicated in tissue injury, are eliminated in large part by excretory pathways used for detoxified drugs (Borst et al., 1999; Kim, 2002). Our previous studies demonstrated a major pathway for excretion of detoxified reactive lipid products involved MRP2 (Reichard et al., 2003).

HCV replication has been shown in association with ROS production to increase several hepatic transcription factors, including activator protein-1, NF-kB, and STAT3 (Qadri et al., 2004, Waris et al., 2005). Therefore, the present study was undertaken to examine whether an in vitro cell model of HCV replication was associated with increased expression of MRP2 and to determine the molecular mechanism(s) involved in its up-regulation. Using cell lines expressing the HCV replicon, our data indicate that compared with the parental human hepatoma cell line, HCV replication in Huh.8 cells transcriptionally increased MRP2 expression primarily through increased interaction of HNF1 with the MRP2 promoter. Furthermore, our data indicate that ROS transcriptionally increased HNF1 and HNF4 expression.

Increased ROS was measured in Huh.8 compared with Huh-7 using DCF fluorescence. In addition, increased ROS in Huh.8 cells was reduced after addition of NAC, confirming previous reports that HCV is associated with elevated levels of ROS (Qadri et al., 2004). Thus, Huh.8 cells seemed to be reasonable in vitro model permitting study of the adaptive response to HCV replication.

Biliary elimination of both endogenous compounds as well as exogenous toxic substances is a major physiological defense process for hepatic function. One of the major transport system involved in this process is ABC transporter superfamily (Kim, 2002). In addition to excreting chemotherapeutic drugs, these ATP-dependent export proteins are responsible for transport of lipids, hormones, conjugated drugs, and toxins (Borst et al., 1999). MRP2, a member of this family, is involved in excretion of conjugated bilirubin, hormones, and drugs, particularly those conjugated with glucuronides and glutathione (Gerk and Vore, 2002). Similar to other ABC transporters, MRP2 is transcriptionally regulated by drugs, hormones, enzyme inducers, and oxidative products (Schrenk et al., 2001). Although previous studies have identified DNA binding sites for hormone transcription factors, and xenosensors, the molecular mechanisms are still poorly understood. In this study, we hypothesized that an efficient transport mechanism may be required to overcome the oxidative burden during HCV replication, and that the molecular process involved increased expression of specific hepatic transcription factors.

MRP2 is located principally at the liver apical surface membrane. Supporting our hypothesis that increased ROS might be associated with induction of MRP2, we demonstrated both increased protein and mRNA content (1.5- and 9-fold, respectively) in Huh.8 compared with control Huh-7.
cells. Transfection of MRP2-LUC (p2.1) and Δp760-LUC into Huh.8 cells expressing HCV demonstrated ~10- and ~3-fold increased expression compared with Huh-7 cells, indicating that increased MRP2 was regulated at the level of transcription. Δp760-LUC deletion was more active than the MRP2-LUC (p2.1), presumably because of deletion of a putative silencer element (I. Qadri and F. R. Simon, unpublished data). These studies suggested that the reactive site for increased MRP2 transcription was located in the proximal deletion fragment. This region of the MRP2 promoter contains an HNF1 site that we have shown is important in the basal expression of MRP2 (I. Qadri and F. R. Simon, unpublished data). Mutation of this HNF1 site resulted in significantly lower expression of MRP2 and importantly prevented the increased expression of Δp760mut in Huh.8 cells, indicating an important role for HNF1 in the up-regulation of MRP2 in Huh.8 cells.

Several lines of evidence in this report support the conclusion that increased HNF1 is primarily responsible for the increased MRP2 expression. First, HNF1 mRNA, protein content, and expression were increased in Huh.8 cells. Involvement of HNF1 during viral infection is not unprecedented, because HNF1 is also known to activate transcription of hepatitis B virus genes in a hepatocyte-specific manner (Zhou and Yen, 1991; Li et al., 2002). Second, in addition to increased transcription of HNF1, this study demonstrates for the first time that HNF1 DNA binding was stimulated by direct physical interaction of HCV-encoded NS5A with the homeodomain transcription factor HNF1. These two processes in combination seem to increase MRP2 transcription in Huh.8 cells. HNF1 is a constitutively expressed liver-enriched transcription factor that is necessary for the basal expression of a number of liver proteins, including fibrinogen, albumin, α1-antitrypsin, glucuronol transferases, and glutathione transferases as well as genes involved in bile acid and lipid metabolism (Shih et al., 2001; Jung and Kullak-Ublick, 2003; Jung et al., 2004). HNF1 may be regulated by development, cytokines, and other transcription factors, most importantly HNF4 (Hatzis and Talianidis, 2001; Sladek and Seidal, 2001; Guo et al., 2002; Eeckoune et al., 2004).

The present studies also found increased levels of HNF4 mRNA, protein content, and transcriptional activity present in Huh.8 cells. In addition, transfection of the Δ82 mutated HNF1 promoter activity was markedly reduced in Huh.8 cells showing the importance of increased HNF4 in up-regulation of HNF1. HNF4 regulation involves a series of complex cellular, extracellular, and hormonal events, including transcriptional factors HNF1α and -β, HNF3 (FOXA), HNF6 (ONECUT), C/EBP, Sp-1, NF-1, and GATA-6 (Hayashi et al., 1999; Sladek and Seidal, 2001; Watt et al., 2003). Hormones also may either positively regulate HNF4, such as glucocorticoids and glucagon (Bailly et al., 2001, Hatzis and Talianidis, 2001), or repress its expression, such as insulin and lipopolysaccharide (through interleukin-1) expression (Wang et al., 2001). Redox state, fatty acyl-CoA derivatives and protein kinase A-mediated phosphorylation may modulate HNF4 transcriptional activity both positively and negatively (for review, see Sladek and Seidal, 2001). Because HCV replication is associated with increased ROS, we examined the possibility that oxidative injury leads to increased expression of HNF4 and/or HNF1. Addition of H2O2 and tBHQ both increased transcriptional expression of HNF1 and HNF4 in Huh-7 cells, whereas NAC significantly decreased their expression in Huh.8 cells. Together, these observations indicated that ROS was associated with increased expression of hepatic transcription factors involved in the up-regulation of MRP2. Other studies have shown that increased ROS is associated with coordinate regulation of γ-glutamylcysteine synthetase and MRP1 (ABCC1) and MRP2 genes (Kuo et al., 1996; Kauffmann et al., 1998, 2002; Payen et al., 2001). Thus, adaptation to reactive oxygen intermediates in hepatocytes may involve coordinate increases in glutathione synthesis and conjugation of potentially toxic lipids permitting efficient biliary excretion.

Overall, our data are consistent with the hypothesis that HCV-induced ROS during viral infection leads to increased detoxification of reactive products through a process involving transcription of MRP2 (Fig. 8). Increased ROS may increase HNF4α, possibly involving ROS-responsive transcription factors, NF-1 and/or Sp-1 interacting with putative

![Fig. 7](image-url) Real-time PCR analysis of untreated Huh-7 and Huh.8 cells and Huh-7, Huh.8 cells treated with interferon-α (75 U/ml for 3 days). A standard curve was generated using the fluorescent data from the 10-fold serial dilutions of HeLa cell RNA. This curve is then used to calculate the relative amounts of MRP2, HNF1, and HNF4 in test samples. Values are shown as picograms of RNA/ng rRNA for HNF1, HNF4, and MRP2. Quantities in test samples were normalized to the corresponding 18s rRNA (PEABI, P/N 4308310).

![Fig. 8](image-url) Schematic model depicting HCV-induced adaptive responses involving ROS and liver-enriched transcription factors (HNF1 and HNF4) and detoxifying gene (MRP2, ABC2). We suggest that HCV-induced ROS (Qadri et al., 2004) may also increase HNF4α, possibly involving ROS-responsive transcription factors NF-1 and/or Sp-1 on the human HNF4 promoter (Hatzis and Talianidis, 2001; Sladek and Seidal, 2001; Guo et al., 2002).
Hatzis P and Talianidis I (2001) Regulatory mechanisms controlling human hepatic expression of the transcription factors HNF4 and HNF1 and MRP2 as part of the expected major response to injury. We thank Dr. Mizanoor Rahman for critical reading of the manuscript and statistical evaluation of the data.

Acknowledgments

We thank Dr. C. M. Rice (Rockefeller University, New York, NY) for providing the Huh.8 cell line. We thank Dr. Talianidis for the full-length HNF4 promoter-LUC (HF4F1LUC) and its deletion mutant (0.684H4-LUC) constructs. We thank Dr. Mianoor Rahman for critical reading of the manuscript and statistical evaluation of the data.

References


