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Hepatocytes nuclear factors 1 and 4 (HNF1 & HNF4) mediates hepatic MRP2 up-regulation during hepatitis C virus gene expression

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Running title: ROS-induced HNF1, HNF4 and MRP2 activation by HCV

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Abbreviations: HCV, hepatitis C virus; NS5A, non structural protean 5A; ROS, reactive oxygen species, HNF1, hepatocytes nuclear factor 1; HNF4, hepatocytes nuclear factor 4, MRP2, multidrug resistant protein 2; tBHQ, tert 8-bromo-7-hydroxyquinoline; tH_2O_2, tert-hydrogen peroxide; NAC, N-acetylcysteine; GST, glutathione S-transferase; EMSA, Electrophoresis Mobility Shift Assays; DCFH-DA, 2', 7'-dichlorofluorescin diacetate;

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ABSTRACT

Hepatitis C virus (HCV) is known to induce hepatic oxidative stress which 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-1 (HNF-1) plays a central role in liver gene regulation during HCV gene expression and/or subgenome replication. MRP2 protein and mRNA expression was 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, thus implicating HNF1 in the induction of MRP2 by HCV. The mechanism by which HNF1-mediated activation occurs appears to be transcriptional, as the regulated expression of HNF4, that is known to control HNF1 expression, was also increased. Consistent with this, 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 NS5A and HNF1, leading to enhanced HNF1 DNA binding. In conclusion, we describe novel mechanism, by which HCV gene expression may induce adaptive responses, involving MRP2 via HNF1 activation. This in part may constitute the cellular detoxification task force during HCV infection.

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INTRODUCTION

Hepatitis C virus (HCV) infection poses a serious health problem as 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 which is co- and post-translationally cleaved to produce at least 10 polypeptides including the 3 structural components (Core, E1, and E2) and 7 nonstructural components (NS2 to NS5B) (Reed and Rice, 2000). HCV nonstructural proteins direct viral replication from a ribonucleoprotein (RNP) replication complex that is associated with the endoplasmic reticulum (ER) (Hijikata et al., 1993). Although the potential mechanism(s) of HCV induced cell injury and disease progression are unclear; emerging evidence, thus far presented, 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 (Abdalla et al., 2005; Multu-Turkoglu, 1997; Liu et al., 2003; Qadri et al., 2002; Qadri., et al., 1004).

Transcriptional regulators such as hepatocyte nuclear factors 1 and 4 (HNF1 and HNF4), Nuclear factor kappa B (NF κB), signal transducer and activator of transcription 3 (STAT 3), multidrug resistance proteins (MRPs) and glutathione (GSH) appear to be involved in this process (Geieret et al., 2003; Guo et al., 2002 Hayashi et al., 1999; Sladek & Seidal, 2001; Schrenk et al., 2001; 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, while 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 (Baily et al., 2001; Hatzis et al., 2001; Hayashi et al., 1999; Sladek & Seidal, 2001; Watt et al., 2003; Wang et al., 2001).

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Following hepatic injury and/or oxidative stress, acute-phase response genes are known to be rapidly upregulated in order to restore homeostasis and limit tissue damage (Dietrich et al., 2003). Furthermore, glucocorticoids, rifampicin, cell swelling and reactive oxidative stress (ROS) increase MPR2 expression (Geier et al., 2003; Kubitz et al., 1999; Kauffmann et al., 1998; 2002; Li and Weinman 2002; Payen et al., 2001; Reichard et al., 2003), but the molecular mechanism(s) are largely unknown. Other 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; therefore, playing a critical role in the detoxification processes (Payen et al., 2001; Reichard et al., 2003). It is widely acknowledged that oxidative stress activates multidrug resistant proteins, MDR1 (ABCC1), MRP2 (ABCC2), and MRP3 (ABCC3) involving in part nuclear factor kappa B (NF κB) and tumor necrosis factor alpha TNF-α (Geier et al., 2003). The link between MPR2 activation and oxidative stress has also been established by treating cells with sulforaphane 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-2,4dinitrophenyl glutathione, bilirubin glucuronide, and 17-beta-glucuronosyl estradiol are also substrates for the MRP2 export pump (Gerk and Vore, 2002; Kim, 2002; Schrenk et al., 2001).

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, 1997; Qadri et al., 2002; Qadri., et al., 1004; Waris et al., 2005). Since oxidative stress plays a pivotal role in the

detoxification process; the present study was designed to investigate the molecular basis of feed back control regulation of hepatocyte nuclear factors and MRP2, the major transporter involved in biliary secretion of oxidized products.

Our studies demonstrate 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-acetyl-cysteine, supporting a common link for increased MRP2 expression. Alternatively HNF1 activation may occur by direct protein-protein interaction between HCV encoded NS5A and HNF1, whereby NS5A stimulates DNA binding of HNF1 to its cognate DNA sequence.

MATERIALS AND METHODS

Cell and Culture conditions: Huh.8 cell line used in these studies contains the hepatitis C virus sub-genomic replicon expressing nonstructural component (NS); NS2, NS3, NS4A, NS4B, NS5A and NS5B (Blight et al., 2000). The coding region of HCV NS components under the control of ECMV IRES, was integrated into human hepatoma Huh-7 chromosome along with selectable G-418 resistant gene which was under the control of HCMV promoter. Huh7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose supplemented in 2mM 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 2-3 passages for the experiments in a humidified environment containing 5% CO_2.

Plasmids: NS5A expression plasmids pCDNA/NS5A (mammalian) and pGST-NS5A (bacterial) were obtained from Dr. Katze, Seattle which was originally isolated from HCV strain 1b. The cloning of human MRP2 promoter will be described in a separate paper. Briefly, a 2133 base pair (bp) human MRP2 promoter was isolated from P1 human genomic library by PCR screening. This region was cloned into pGL3 and the

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recombinant plasmid is referred to as p2.1. Series of 5' deletion were generated using exonuclease III and mungbean nuclease in order 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 are the consensus core HNF1 binding motif. Δp760-LUC contains the -760 bp of MRP2 promoter fused to luciferase gene into pGL3. Plasmid $\Delta p760^{mut}$-LUC was constructed by mutating the **GTTA** into CCCC within the HNF1 binding site sequence (5'-AAAATTAGGT**GTTA**ATCCTTGACCTTATA-3') of MPR2 promoter. Human HNF1 promoter constructs H473 and H82 contains the 473 and 82 bp region of human HNF1 alpha promoter fused to the luciferase gene and have been described (Jung and Kallak-Ublick 2003). MutH82 contains the mutation within the HNF4 binding site direct repeat unit AGTCCAAAGTTCA at position -63 to -51 which was mutated into A**TA**CCAAA**TA**TCA by site-directed mutagenesis. All promoters are cloned into pGL3 vector. pGL3-promoter vector contains the SV40 promoter and reporter luciferase (LUC) gene. Full-length human HNF4α promoter-luciferase construct (F4FLluc) containing 12,145 bp of human HNF4 were obtained from Dr. Talianidis, Crete, Greece. Mutant HNF4 promoter construct (0.65H4luc) containing the 684 bp from the ATG of human HNF4 was also obtained from Dr. Talianidis Crete, Greece.

RT-PCR Methods: Primers and probe for MRP2, HNF1 and HNF4 were designed with the assistance of the Prism 7700 sequence detection software (Primer Express, PE ABI) and will be available upon request. ABI Prism 7700 sequence detector (Perkin Elmer Corp./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 (PE ABI) in a 50 ul mix containing 8 % glycerol, 1X TaqMan buffer A (500 mM KCl, 100 mM Tris-HCl, 0.1 M EDTA, 600 nM passive reference dye ROX, pH 8.3 at room temperature), 300 uM each of dATP, dGTP, dCTP and 600uM dUTP, 5.5 mM MgCl2, 900 nM forward primer, 300 nM reverse primer, 200 nM probe , 1.25 U AmpliTaq Gold DNA Polymerase (Perkin-Elmer, Foster city CA), 12.5 U Moloney Murine leukemia virus reverse transcriptase (Life Technologies, INC. Gaithersburg, MD), 20 U RNAsin ribonuclease

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inhibitor (Promega corp. Madison, WI) and the template RNA. Thermal cycling conditions were as follows: RT was performed at 48°C for 30 min followed by activation of TaqGold at 95°C for 10 min. Subsequently 40 cycles of amplification were performed at 95°C for 15 seconds and 60°C for 1 min. After amplification, real-time data acquisition and analysis were performed. The fluorescence data were expressed as Rn or ΔRn. Normalized reporter signal (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 Ct 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 is 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 (PE ABI, P/N 4308310).

Oligonucleotides: 5'-AAAATTAGGT**GTTA**ATCCTTGACCTTATA-3' sense and 5-'TATAAGGTCAAGGAT**TAAC**ACCTAATTTT-3' antisense was synthesized encompassing to the consensus HNF-1 binding site motif (GTTA) at position 2055 of hMRP2 -2 promoter. Similarly oligonucleotides 5' TCTAGGCAAG**GTTA**ACGATTAAATGGTTG-3'sense 5'-CAACCATTTAATCGT**TAAC**CTTGCCTAGA-3' antisense was synthesized that encompass the HNF1 binding site motif (GTTA) at position 158 of hMRP2 promoter. HNF-1 mutated oligonuleotides for position 2055 were as follows. 5'-AAAATTAGGTCCCCATCCTTGACCTTATA-3' sense 5''TATAAGGTCAAGGATGGGGACCTAATTTT-3' antisense; HNF-1 mutant oligonucleotides for position 158 were as follows.
5'-TCTAGGCAAGCCCCACGATTAAATGGTTG-3' sense and 5'-CAACCATTTAATCGTGGGGCTTGCCTAGA-3' antisense.

Antibodies: Monoclonal NS-5b for NS5A was from ID Labs (London, Canada). HNF1 and HNF4 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). MRP2 antibodies were from Alexis Biochemical (M2111-6) (San Diego, CA).

Protein-Protein interactions: Cells were washed twice in cold PBS and lysed at -4^{0}C in buffer containing 150 mM NaCl, 20 mM Tris /pH 7.4, 1 mM EDTA, 0.1% SDS, 1% NP-40, 10 mM p-nitrophenolphosphate, 10 mM sodium fluoride, 40 mM glycerophosphate and 1 mM PMSF. Nuclear extracts were prepared as described (36). Both fractions were mixed and dialyzed against buffer containing 50 mM KCl, 10 mM Tris /pH 7.4, 1 mM EDTA, 1 mM DTT, 10% (vol/vol) glycerol and 1 mM PMSF. Whole cell extracts were used for immunoprecipitation and affinity chromatography. The bound fractions were resolved by 7.5 % SDS-PAGE, transferred to nitrocellulose filters (Schleicher and Schuell) and probed α–HNF1 using the ECL system (Amersham). Cell lysates were allowed to pass through GST-affinity resin (Pharmacia) which were immobilized with either 10 mg of GST or GST-NS5A protein and allowed to interact for 2 hrs at 4^{o}C, with rocking in buffer A, containing 100 mM KCl, 20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.1% NP40, 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-alpha, pMT7-rHNF4-wt pCMVHNF6-alpha and pRc/CMV/NS5A by using, T7 RNA polymerase, ^{35}S-methionine and TNT rabbit lysates (Promega). ^{35}S-labeled NS5A, HNF1, HNF4, and HNF6 proteins (5 μl of a 50 μl) were chromatographed through 20 μl microaffinity column containing immobilized (Affi-gel 10, Bio-Rad) GST and GST- fusion proteins at a 2 mg/ml protein concentration in buffer A (10 mM HEPES [pH7.5], 0.15 M KCl, 1 mM ATP, 1 mM EDTA, 10% glycerol, containing 2 mg of bovine serum albumin and 0.1% Nonidet P40. Affinity columns were washed with buffer A and bound fractions eluted in buffer A containing 0.5 M KCl and 1% SDS. GST-fusion proteins were extracted from bacterial cultures as described previously.

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Mutagenesis: HNF1 consensus DNA binding site motif 5-'GTTA-3 located at position 158 of hMRP2 promoter was mutated into 5-CCCC-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 employing oligonucleotides 5'-TCTAGGCAAGCCCCACGATTAAATGGTTG-3' sense and 5'-CAACCATTTAATCGTGGGGCTTGCCTAGA-3' as an antisense strand. Mutations were confirmed by DNA sequencing of the $\Delta p760^{mut}$-LUC.

Reactive Oxygen Species measurement: H_2O_2 production was measured within Huh-7 (control), Huh.8 (HCV replicating cells) using the well-known 2', 7'-dichlorofluorescin diaceatate (also know as 2'-7'dichlorodihydrofluorescin; DCFH-DA) (Molecular Probes) as the indicator. DCF emission is measured at 525+/-20 nm by flow cytometry. Upon cell activation NADPH oxidase catalyzes the reduction of O_2 to O_2-, which is further, reduced to H_2O_2. H_2O_2 and peroxidases are able to oxidize the trapped dye DCFH to DCF, which is highly fluorescent at 530 nm. 2 million cells were subjected for FACS analysis as described previously (Qadri et al., 2004).

Preparation of Nuclear Proteins: Nuclear proteins were prepared from liver-derived cells as described previously (7 & 8). Nuclear extracts were prepared using sucrose gradients and adjusted to 0.3% Triton X 100 (v/v). Sucrose gradients were prepared and centrifuged at 77,000-x g for 50 min at 4^0C. The nuclear pellets were resuspended in buffer and ammonium sulfate slowly added to a final concentration of 0.4M. The mixture was centrifuged at 62,000-x g for 60 minutes. The supernatant was saved and 0.33 g ammonium sulfate per ml of supernatant added. The mixture was centrifuged and protein pellets were resuspended and dialyzed aliquots were snap frozen at -70^0C.

Western Blotting: SDS-PAGE and Immunoblotting were carried out using minigels. Proteins were transferred by the procedures of Towbin and processed using ECL for detection (Amersham) with specific antibodies using 1% nonfat milk in TBS. All washes were in 0.5%Tween/TBS for 5 minutes. An analysis of autoradiograms was by Phoshoimager.

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DNA Transfections: Cultures were set up 24 hrs prior to transfections in 35 mm 6 well plates at 3 x10^5 cells/well in DMEM media with appropriate supplements. Transfections were carried out using Fugene 6 (Roche Diagonistics), 2:1 DNA/lipid ratio. 48 hrs post-transfection cells were harvested and assayed for luciferase activity using a dual luciferase reporter assay system (Promega). Luciferase activity was read in a Packard plate reader luminometer.

Electrophoresis Mobility Shift Assays (EMSA). The oligonucleotides were radio labeled with [γ^{32}P] dATP using T4 polynucleotide kinase. Binding reaction mixtures contain 20 mM HEPES (pH 7.9), 4% Ficoll type 400, 50 mM KCl, 0.5mM EDTA, 100 mg of poly (dI-dC), 50 ng of pSK+ DNA (Stratagene), 20,000 cpm of DNA probe (7, 8). Reactions were preincubated on ice for 10 min prior to the addition of ^{32}P-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 0.5X TBE (45 mM Tris borate, 1 mM EDTA) at room temperature. Reaction mixtures were incubated at 25^{o}C for 30 minutes, with DNA, nuclear extract and bacterially purified proteins. Appropriate antibodies were either purchased commercially or obtained from individual investigators. The complexes were separated by electrophoresis in 4.5%-5% native polyacrylamide gel at 120 V at 4^{o}C in 0.5 X TBE buffer. Gels were dried and autoradiographed at 70 °C for 1-2 h.

RNA Isolation and Northern Blot Hybridization. Unless otherwise indicated, Huh-7 and Huh.8 cell lines were cultured in medium containing heat inactivated FBS for 48 hr. Total RNA was isolated using SV total RNA Isolation kit (Promega, Madison WI). RNA was resolved (10 µg/lane) on a 1% agarose, 2.2 M formaldehyde gel, transferred to a nylon membrane (Hybond N+; Amersham Biosciences, Inc.), and cross-linked to the membrane with UV light. cDNA probes were radiolabeled with [^{32}P]dCTP using the RediprimeTM II labeling kit (Amersham Biosciences, Inc.). Membranes were hybridized using the QuikHyb hybridization solution (Stratagene, La Jolla, CA) according to the

manufacturer's protocol. Blots were normalized for variations of RNA loading by hybridization to a control probe, by mouse 18 S ribosomal cDNA. The RNA levels were quantitated using a Phoshoimager (ImageQuant software; Molecular Dynamics, Inc., Sunnyvale, CA).

Data Analysis: Data were expressed as Means ± SEM and analyzed statistically by using two-way ANOVA, followed by post hoc analysis with Tukey's test. Other comparisons among groups were made using the Student's t-test. A p value of <0.05 was determined to be statistically significant.

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_2O_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 to Huh-7 cells. Furthermore, addition of antioxidant N-Acetyl Cysteine (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. While very low levels of MPR2 protein expression was observed

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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 to Huh.8 cells (Fig 2B), thus 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 measured as arbitrary light unit (ALU). A 7 fold increase ($p<0.002$) in the ALU was detected in HCV replicating Huh.8 cells vs. control Huh-7 cells (Fig 2B), thus confirming increased transcriptional activity of MPR2 in Huh.8 cells. To gain further insight into the transcriptional regulation of MRP2 by HCV subgenome 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 Δp760-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 GTTA core motif of this HNF1 binding site located within the 760 bp region of MRP2 promoter. Analysis of human MRP2 promoter (p2.1), Δp760-LUC and Δp760^{mut}-LUC promoters were carried out by transient transfection into Huh-7 and Huh.8 cells (Fig 2C), and measurement of ALU. Mutant MRP2 promoter (Δp670^{mut}-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.

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Induced expression of HNF1 is coupled with increased DNA binding during HCV replication. We next determined whether HNF1 expression was increased during HCV subgenome 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 vs. 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 to control nuclear extract for Huh-7 (lanes 7-11). Cold HNF1 oligo DNA (10x) 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 super-shifted (lane 14). HNF1 antibodies had no reactivity with free probe (lane 12). Unrelated Oct-1 and STAT-5 b antibodies have no effect on the supershift (data not shown).

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; Jung et al., 2004; Eeckhoute et al., 2004). In order to assess whether HCV replication may have affected HNF4 expression, Huh.8 and Huh-7 cells were first measured by Northern blotting (Figure 4A). HNF4 expression was increased 1.7 fold in Huh.8 cells compared to control Huh-7 cells ($p<0.01$) (Fig 4A). Consistent with increased mRNA level, a ~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 super-shifted the HNF4-DNA band (lane 4), while an unrelated STAT5b antibody had no effect (lane 5). To corroborate the observation of increased HNF4 mRNA expression, HNF4-LUC construct (H4FL-LUC) was transected 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

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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 i) position -413 to -398 and ii) position -63 to -51 (Jung and Kalluk-Ublick, 2003). When a truncated 82 bp HNF-1 promoter LUC construct (H82) encompassing only one HNF4 biding 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, thus 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 following transfection of the mutant HNF1-LUC construct (Δ82) in Huh-7 and Huh.8 cells and luciferase activity measured. Mutation of the proximal HNF4 binding site in the HNF1 promoter reduced basal activity in hepatoma cells. Importantly, no activation of the mutant HNF1-LUC construct was seen in Huh.8 cells, therefore establishing a direct link of HNF4 binding site located at -63 to -51 in the activation of HNF1 gene in HCV replicating cells

HNF1 and HNF4 expression is induced by ROS. In the next series of experiments, we sought to determine the mechanism of HNF1 and HNF4 activation. We examined the possibility that increased reactive oxidative species might be responsible for signaling increase in HNF1 and HNF4 expression. Pro-oxidants tert-hydrogen peroxide (tH_2O_2) and tert-butylhydroquinone (tBHQ) were added separately to Huh-7 cells to increase oxidative stress. Huh-7 cells were then transfected with either 473 bp HNF1- LUC (H473), or the HNF4 promoter-LUC (0.65HNF4-LUC) (5A and B). A 35% increase with pro-oxidants tH2O2 ($p<0.05$) and 80% with tBHQ ($p<0.04$) in the human HNF1 promoter activity was measured in treatment conditions (Fig 5A). A 2 fold increased HNF4 promoter was seen in transfected Huh-7 cells treated with tH_2O_2 ($p<0.01$) or tBHQ

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($p<0.04$) (Fig 5B). These results are consistent with the earlier observation of induced HNF4 protein and mRNA expression in Huh.8 cells expressing HCV replicon. Figure 5C demonstrates that both treatments increased oxidative-stress as measured by the oxidative responsive ARE-LUC promoter construct. In Huh-7 cells treated with either tH_2O_2 or tBHQ, ARE-LUC activity was increased 2 ($p<0.05$) and 3 fold ($p<0.001$), respectively.

Next, we examined the ability of antioxidant NAC to repress HNF1 and HNF4 promoter activity in Huh.8 HCV cells (Fig 5D and E). HNF1-LUC (H82) and HNF4-LUC (0.065H4-LUC) were transfected into Huh.8 cells, followed by treatment with NAC (20 mM) and measurement of luciferase activity. Antioxidant NAC reduced HNF1 ($p<0.01$) and 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 (Qadri et. al., 2002; Li et al., 2002; Zhou and Yen, 1991). 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 ^{35}S-[methionine] labeled HNF1 showed direct interactions with GST-NS5A (lane 6). No interactions were seen with GST (lane 1–3). To further confirm the specificity of NS5A-HNF1 interactions, co-immnoprecipitation of Huh-7 and Huh.8 cell extracts were performed using either anti-NS5A (Fig 6B, lane 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. While, no complexes were seen in lysates without antibody (lane 1) or Huh-7 cells (Fig 6B, lanes 2 and 3), HNF1-NS5A complexes were specifically determined using either antibodies (lane 4 and 5).

In order 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 3-4 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), thus implying the specificity of this reaction. Cold competitor DNA competed with the binding (lane 6) and HNF1 antibodies specifically super-shifted the HNF1-DNA complex therefore 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; Lanford 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 employed IFN-alpha in our analysis to suppress HCV protein synthesis and RNA replication of subgenomic replicon, in order 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 75U/ml of IFNα for 96 hrs. RNA was extracted and analyzed for HNF1 and HNF4 and MRP2 expression by RT-PCR. The picogram amounts of HNF1, HNF4 and MRP2 RNA/ng rRNA is shown in Fig 7. In Huh-7 cells HNF1 (700±20), was significantly less than Huh.8 cells (1201±26) ($p<0.01$). IFN treatment reduced HNF1 in Huh.8 cells to values similar to Huh-7 cells (744±35) ($p<0.01$). A similar trend was measured for HNF4 [(406±12 vs. 396±24.56) ($p<0.03$). In addition MRP2 was increased in Huh.8 (1411+/-18) compared to Huh-7 cells (351±9.1), while IFN treatments restored 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 vs. the control Huh-7 cells. Importantly, values in Huh.8 cells were restored to control levels after IFN treatment, while no change in HNF1, HNF4, and MRP2 expression was measured in control Huh-7 cells treated with IFNα (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 multidrug resistance-associated protein (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 (Qadri et., al., 2004; Liu et al., 2003; Okuda et al., 2002). 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 glutathione (GSH) to control the oxidative imbalance (Multu-Turkoglu etal., 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 AP-1, NFκB, and STAT 3 (Qadri et al., 2004, Waris et al., 2005). Therefore the present studies were undertaken to examine whether an *in vitro* cell model of HCV replication was associated with increased expression of MRP2; and what were the molecular mechanism(s) involved in its up regulation. Using cell lines expressing the HCV replicon, our data indicate that compared to 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 studies indicate that ROS transcriptionally increased HNF1 and HNF4 expression.

Increased ROS was measured in Huh.8 compared to Huh-7 using DCF fluorescence. In addition, increased ROS in Huh.8 cells was reduced following addition of NAC, confirming previous reports that HCV is associated with elevated levels of ROS (Qadri et al., 2004). Thus, Huh.8 cells appeared 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 the adenosine triphosphate binding cassette (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 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 to 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 to 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 due to deletion of a putative silencer element (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 which we have shown is important in the basal expression of MRP2 (unpublished). Mutation of this HNF1 site resulted in significantly lower expression of MRP2 and importantly prevented the increased expression of $\Delta p760^{Mut}$ 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. Firstly, HNF1 mRNA, protein content and expression were increased in Huh.8 cells. Involvement of HNF1 during viral infection is not unprecedented, as 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). Secondly, 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 process in combination seems 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, glucuronyl transferases and glutathione transferases as well as genes involved in bile acid and lipid metabolism (Jung and Kullak-Ublick 2003; Jung et al., 2004; Shih et al., 2001). HNF1 may be regulated by development, cytokines, and other transcription factors, most importantly HNF4 (Hatzis and Taliandis 2001; Eechoune et al., 2004; Guo et al., 2004; Sladek & Seidal, 2001)

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 HNF1 α and β, HNF3 (FOXA), HNF6 (ONECUT), C/EBP, Sp1, NF1 and GATA-6 (Hayashi et al., 1999; Sladek & Seidal, 2001; Watt et al., 2003). Hormones also may

either positively regulate HNF4 such as glucocorticoids and glucagons (Bailly et al., 2001, Hatzis and Talianidis 2001) or repress its expression including insulin and lipopolysacharide (through IL-1) expression (Wang et al., 2001). Redox state, fatty Acyl-CoA derivates and protein kinase A mediated phosphorylation may modulate HNF4 transcriptional activity both positively and negatively (reviewed in Sladek & Seidal, 2001). Since 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 tH_2O_2 and tBHQ both increased transcriptional expression of HNF1 and HNF4 in Huh-7 cells, while NAC significantly decreased their expression in Huh.8 cells. Taken 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 co-ordinate regulation of γ-glutamylcysteine synthetase and MRP1 (ABCC1) and MRP2 genes (Kauffmann et al., 1998; Kauffmann et al., 2002; Payen et al., 2001; Kuo et al., 1998). 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 is 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 (Figure 8). Increased ROS may increase HNF4α, possibly involving ROS responsive transcription factors, NF-1 and/or Sp-1 interacting with putative binding sites on the human HNF4 promoter (Hartiz and Talianidis 2001; Sladek & Seidal, 2001; Watt et al., 2003). Increased HNF4α in turn increases expression of HNF1α leading to MRP2 induction, thus permitting elimination of potentially toxic intracellular compounds. Increased HNF1 and HNF4 expression during HCV replication may also be involved in other aspects of detoxification including regulation of glutathione production and glucuronic acid conjugation. However, control of conjugated transport during HCV infection is a complex process which depends upon the onset of inflammation, development of fibrosis and regulation of phase 1 and 2 by drugs, hormones and cytokines. In summary, these studies demonstrate that increased

production of ROS either associated with HCV replication or *in vitro* addition of oxidizing agents increases MRP2 expression due to expression of two key hepatic transcription factors, HNF1 and HNF4. These observations suggest that intracellular factors associated with HCV replication; in particular the non structural proteins may transcriptionally increase HNF4 and HNF1 and MRP2 as part of the expected major compensatory defense changes associated with the adaptive response to cell injury.

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FIGURE LEGENDS:

FIG 1: (A) DCF fluorescence of HCV sub-genomic replicon expressing Huh.8 cells vs. the control Huh-7 cells. Huh.8 cells show 30% increase in DCF fluorescence. Addition of 10 mM antioxidant NAC reduced the oxidative stress in Huh.8 cells, while 10 mM proxidant tH2O2 increased the ROS in Huh-7 cells

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) and cDNA probe, respectively. **(B)** Human MRP2 promoter is activated in HCV replicating cells in a 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/renilla is presented (n=5). Empty bar, Huh-7 cells and shaded bar, Huh.8 cells **(C)** Analysis of hMRP2 deletion mutant-LUC (Δp760-LUC) and HNF1 binding site mutant ($\Delta p760^{mut}$-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**.**

FIG 3: Induced HNF1 expression is coupled with stimulated DNA binding during HCV subgenome replication. (A) Northern blot analysis of HNF1 mRNA expression in Huh-7 and Huh.8 cells. Data is presented % control over 18S. Average of 4 experiments and a representative blot is shown **(B)** EMSA of Huh.8 and Huh-7, nuclear extract for binding to HNF1 consensus DNA**.** Lane 1, free probe. Lanes 2-6 and 7-11 contains 10, 20, 25, 50, 100 ug of nuclear extracts (NE) from Huh.8 and Huh-7 cells respectively. Lane12, 10 x cold HNF1 probe added Lane 13, HNF-1 antibodies added with DNA probe without NE, to show the non-binding of antibodies to DNA. Antibodies to HNF-1 specifically super shifted HNF1-DNA complex from Huh.8 and Huh-7 NE (lanes 14 and 15).

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FIG 4: Induced HNF4 expression is coupled with increased DNA binding during HCV subgenome replication. (A) Northern blot analysis of HNF4 mRNA expression in Huh-7 and Huh.8 cells. Data is presented % 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 super shifted the HNF4-DNA complex (lane 4). **(C)** HNF4-LUC expression in Huh-7 and Huh.8 cells. The ratio of luciferase/renilla 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 (D82) (n=4). Data is presented as mean SD +/-. Plasmid DNAs were transfected into Huh-7 and Huh.8 cells by Fugene 6 method. Empty bar, Huh-7 cells and shaded bar, Huh.8 cells. The ratio of luciferase/renilla is presented.

FIG 5: ROS induces the human HNF1 and HNF4 promoter in Huh.8 cells. (A & B) Pro-oxidants tH_2O_2 (tert-hydrogen peroxide) and tBHQ (tert-butylhydroquinone) induces the HNF1 and HNF4 promoter activity in control Huh-7 cells. **(C)** A known antioxidant response element ARE-LUC construct is activated in response to tH_2O_2 (tert-hydrogen peroxide) and tBHQ and tBHQ (tert-butylhydroquinone). **(D & E)** Antioxidant N-acetyl cysteine (NAC) reduced the HNF1 and HNF4 promoter activation in Huh.8 cells. Cells were transfected with either HNF1-LUC (H473), or HNF4-LUC (0.65HNF4) followed by treatment with 150 μM pro-oxidant tH_2O_2 and 20 mM anti-oxidant, NAC for 48 hrs.

Fig 6: HCV NS5A associates with HNF1 and stimulates the DNA binding of HNF1. (A) Glutathione S-transferase (GST)-pull down assay. ^{35}S[methionine]-labeled HNF6, HNF4 and HNF1 proteins immobilized on GST (lanes 1-3) or GST-NS5A (lanes 4-6). $1/10^{th}$ of input is shown**. (B)** *In vivo* coimmunoprecipiation of HNF1–NS5A complexes. First, IP was performed with anti-NS5A (lanes 2 and 4) or anti-HNF1 (lanes 3 and 5) that was followed by Immunoblotting with anti-HNF1. **(C)** Exogenous NS5A stimulates the

DNA binding of HNF1 to its cognate DNA sequence**.** Lane I, free probe. In lanes 2-5, 0.1 μg of bacterially purified GST, GST-NS5A (HCV encoded non-structural 5A protein), GST-TBP (TATA Box binding protein) and GST-STAT5 (signal transducer and activator of transcription-5) were added respectively along with 20 μg of Huh-7 Nuclear extracts. Lane 7, 100x cold competitor added. Lanes 8 and 9, Huh.8 nuclear extracts alone and with HNF1 antibodies respectively to show the super shift

Fig 7: Real time-PCR analysis of untreated Huh-7 and Huh.8 cells and Huh-7, Huh.8 cells treated with interferon alpha (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 is then used to calculate the relative amounts of MRP2, HNF1 and HNF4 in test samples. Values are shown as pg RNA/ng of rRNA for HNF1, HNF4 and MRP2**.** Quantities in test samples were normalized to the corresponding 18s rRNA (PEABI, P/N 4308310).

FIG 8: A schematic model depicting HCV induced adaptive responses involving reactive oxidative stress (ROS) and liver-enriched transcription factors (HNF1 and HNF4) and detoxifying gene (MPR2; ABCC2). 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 & Talianidis, 2001; Sladek & Seidal, 2001; Guo et al., 2002).

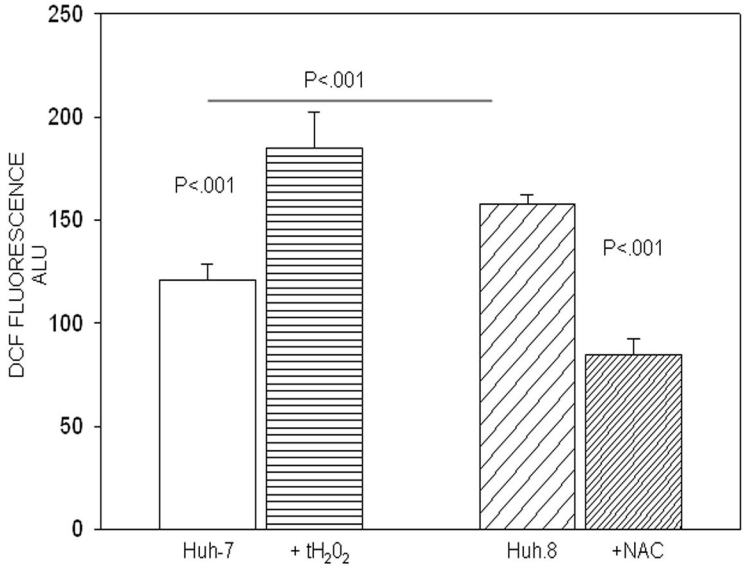
250
200
150
100
50
0
DCF FLUORESCENCE
ALU
P<.001
P<.001
P<.001
Huh-7
+ tH202
Huh.8
+NAC

Figure 1

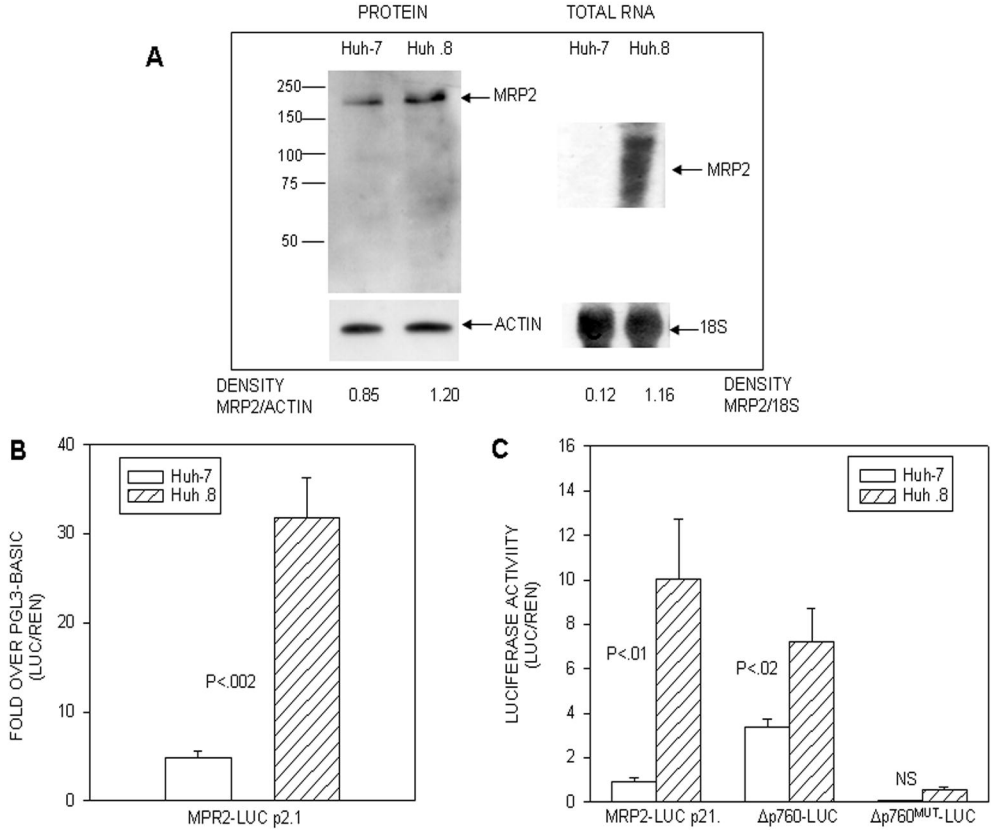
A
PROTEIN
TOTAL RNA
Huh-7
Huh .8
Huh-7
Huh.8
250
150
100
75
50
MRP2
MRP2
ACTIN
18S
DENSITY MRP2/ACTIN
0.85
1.20
0.12
1.16
DENSITY MRP2/18S
B
FOLD OVER PGL3-BASIC (LUC/REN)
Huh-7
Huh .8
P<.002
MPR2-LUC p2.1
C
LUCIFERASE ACTIVITY (LUC/REN)
Huh-7
Huh .8
P<.01
P<.02
NS
MRP2-LUC p21.
Δp760-LUC
Δp760MUT-LUC

Figure 2

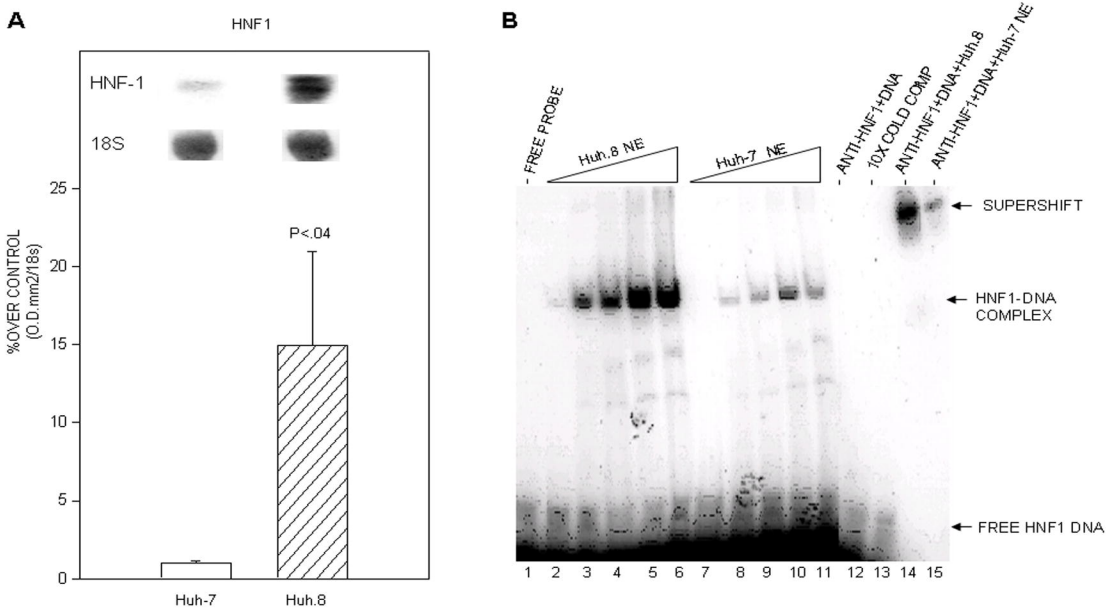
A
HNF1
HNF-1
18S
%OVER CONTROL
(O.D.mm2/18s)
25
20
15
10
5
0
P<.04
Huh-7
Huh.8
B
- FREE PROBE
Huh.8 NE
Huh-7 NE
- ANTI-HNF1+DNA
10X COLD COMP
ANTI-HNF1+DNA+Huh.8
ANTI-HNF1+DNA+Huh-7 NE
SUPERSHIFT
HNF1-DNA
COMPLEX
FREE HNF1 DNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 3

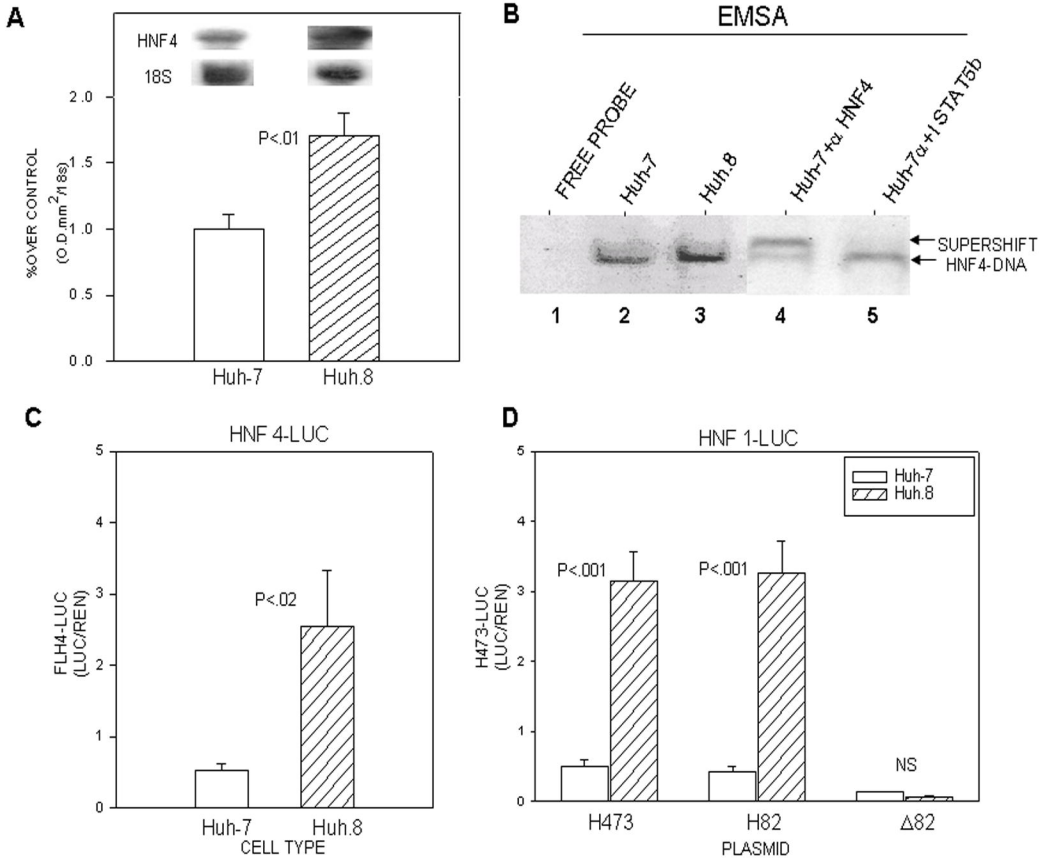
A
HNF4
18S
P<.01
%OVER CONTROL
(O.D.mm2/18s)
2.0
1.5
1.0
0.5
0.0
Huh-7
Huh.8
B
EMSA
FREE PROBE
Huh-7
Huh.8
Huh-7+α HNF4
Huh-7+α+STAT5b
SUPERSHIFT
HNF4-DNA
1
2
3
4
5
C
HNF 4-LUC
FLH4-LUC
(LUC/REN)
P<.02
5
4
3
2
1
0
Huh-7
Huh.8
CELL TYPE
D
HNF 1-LUC
Huh-7
Huh.8
H473-LUC
(LUC/REN)
P<.001
P<.001
NS
H473
H82
Δ82
PLASMID

Figure 4

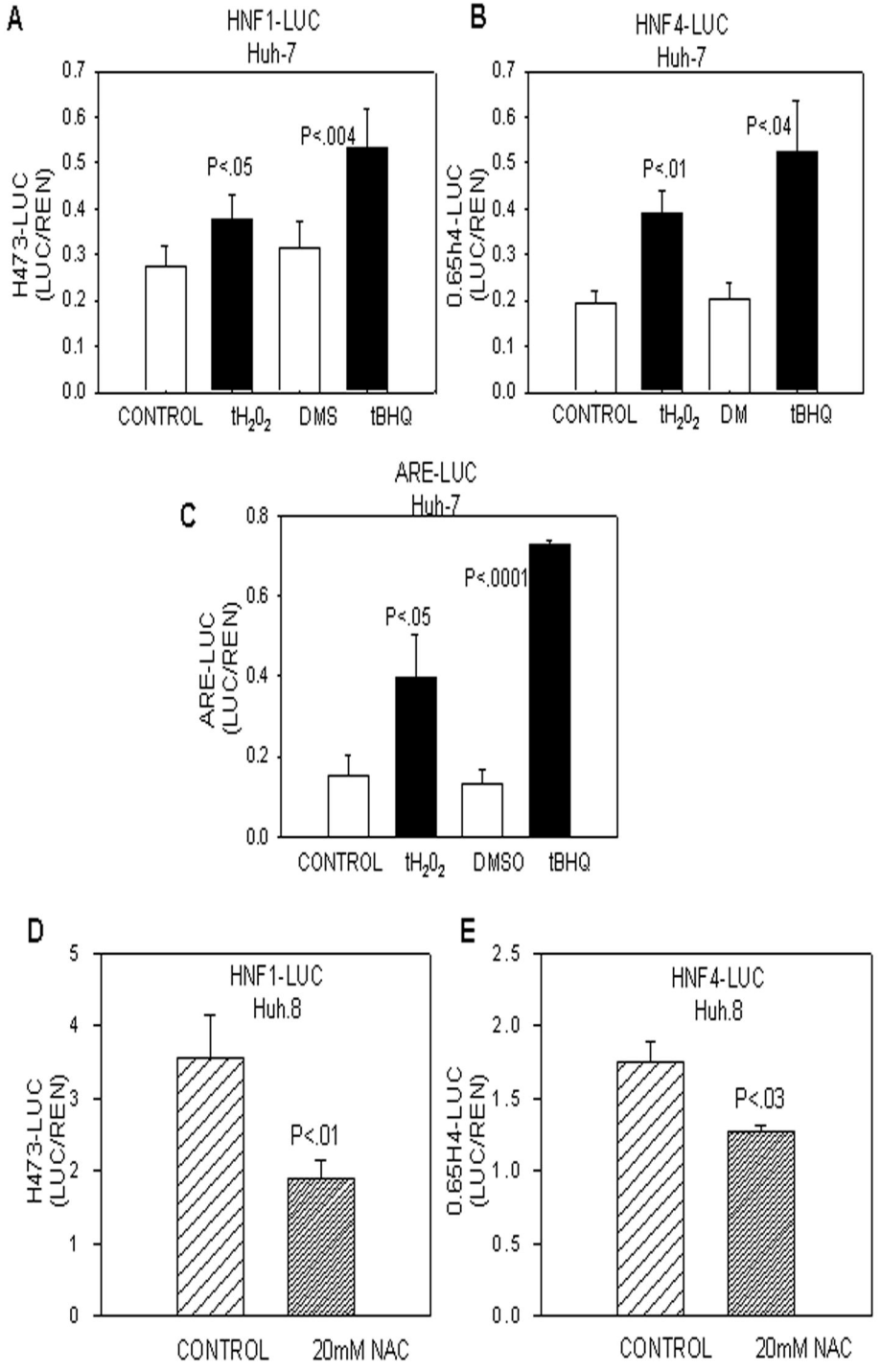
A
HNF1-LUC
Huh-7
H473-LUC (LUC/REN)
P<.05
P<.004
CONTROL
tH2O2
DMS
tBHQ
B
HNF4-LUC
Huh-7
0.65h4-LUC (LUC/REN)
P<.01
P<.04
CONTROL
tH2O2
DM
tBHQ
C
ARE-LUC
Huh-7
ARE-LUC (LUC/REN)
P<.05
P<.0001
CONTROL
tH2O2
DMSO
tBHQ
D
HNF1-LUC
Huh.8
H473-LUC (LUC/REN)
P<.01
CONTROL
20mM NAC
E
HNF4-LUC
Huh.8
0.65H4-LUC (LUC/REN)
P<.03
CONTROL
20mM NAC

Figure 5

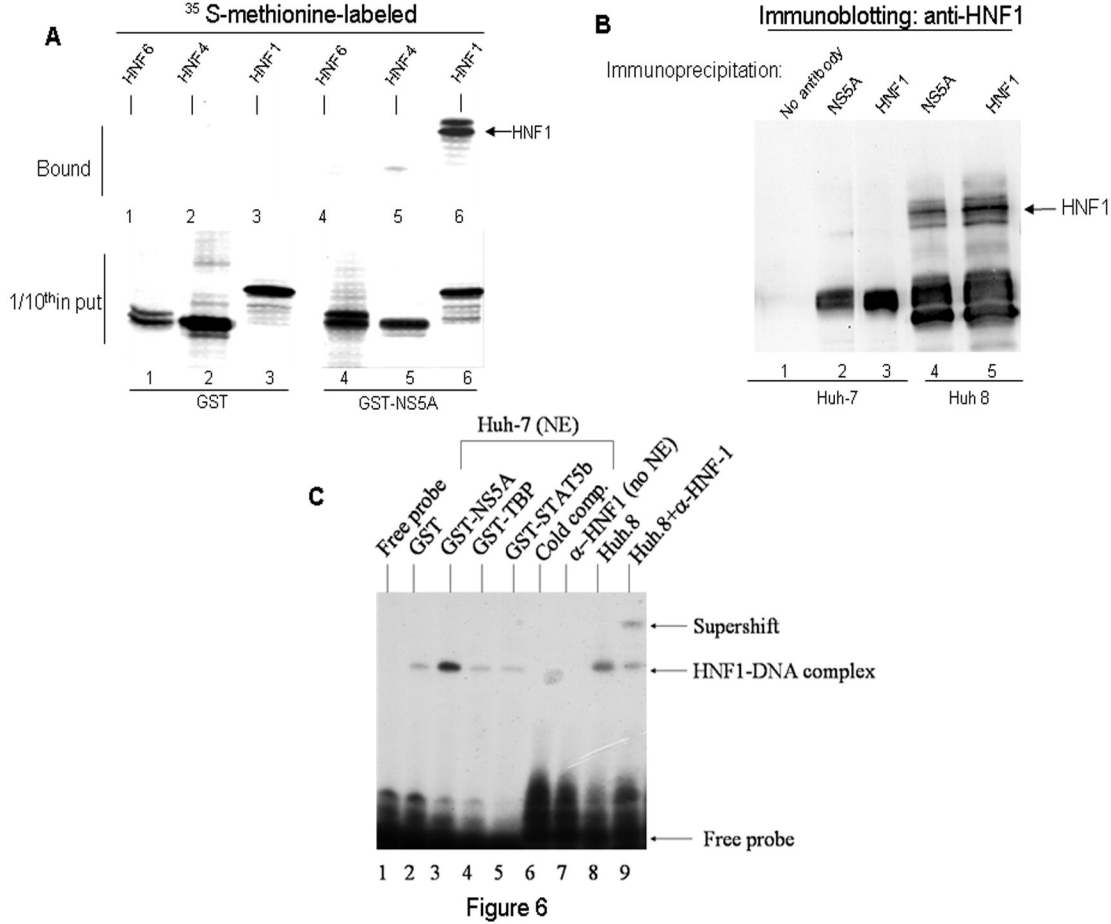
A
35 S-methionine-labeled
HNF6
HNF4
HNF1
HNF6
HNF4
HNF1
HNF1
Bound
1 2 3 4 5 6
1/10thin put
1 2 3 4 5 6
GST
GST-NS5A
B
Immunoblotting: anti-HNF1
Immunoprecipitation:
No antibody
NS5A
HNF1
NS5A
HNF1
HNF1
1 2 3 4 5
Huh-7
Huh 8
C
Huh-7 (NE)
Free probe
GST
GST-NS5A
GST-TBP
GST-STAT5b
Cold comp.
α-HNF1 (no NE)
Huh.8
Huh.8+α-HNF-1
Supershift
HNF1-DNA complex
Free probe
1 2 3 4 5 6 7 8 9

Figure 6

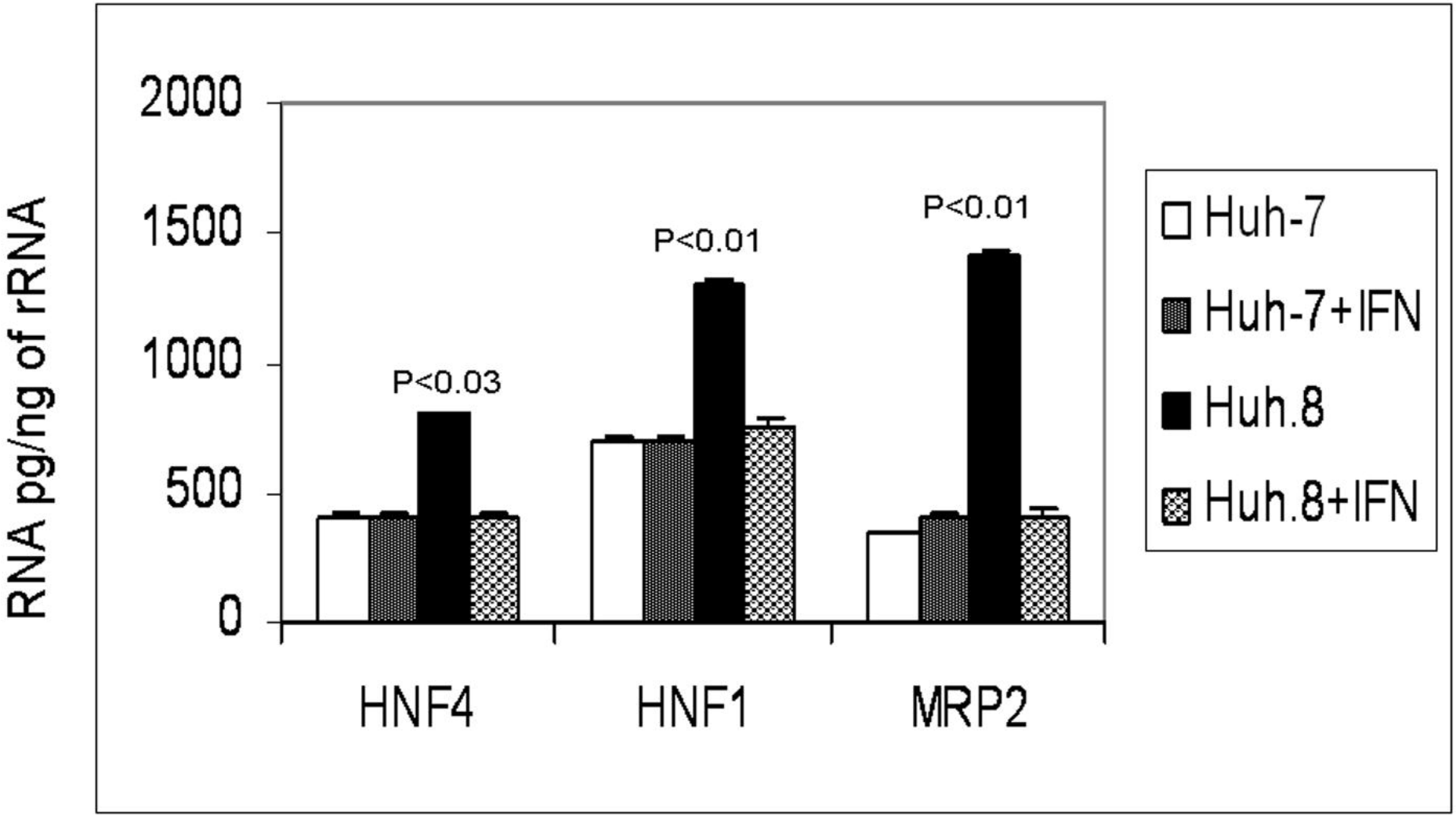
RNA pg/ng of rRNA
2000
1500
1000
500
0
P<0.03
P<0.01
P<0.01
HNF4
HNF1
MRP2
Huh-7
Huh-7+IFN
Huh.8
Huh.8+IFN

Figure 7

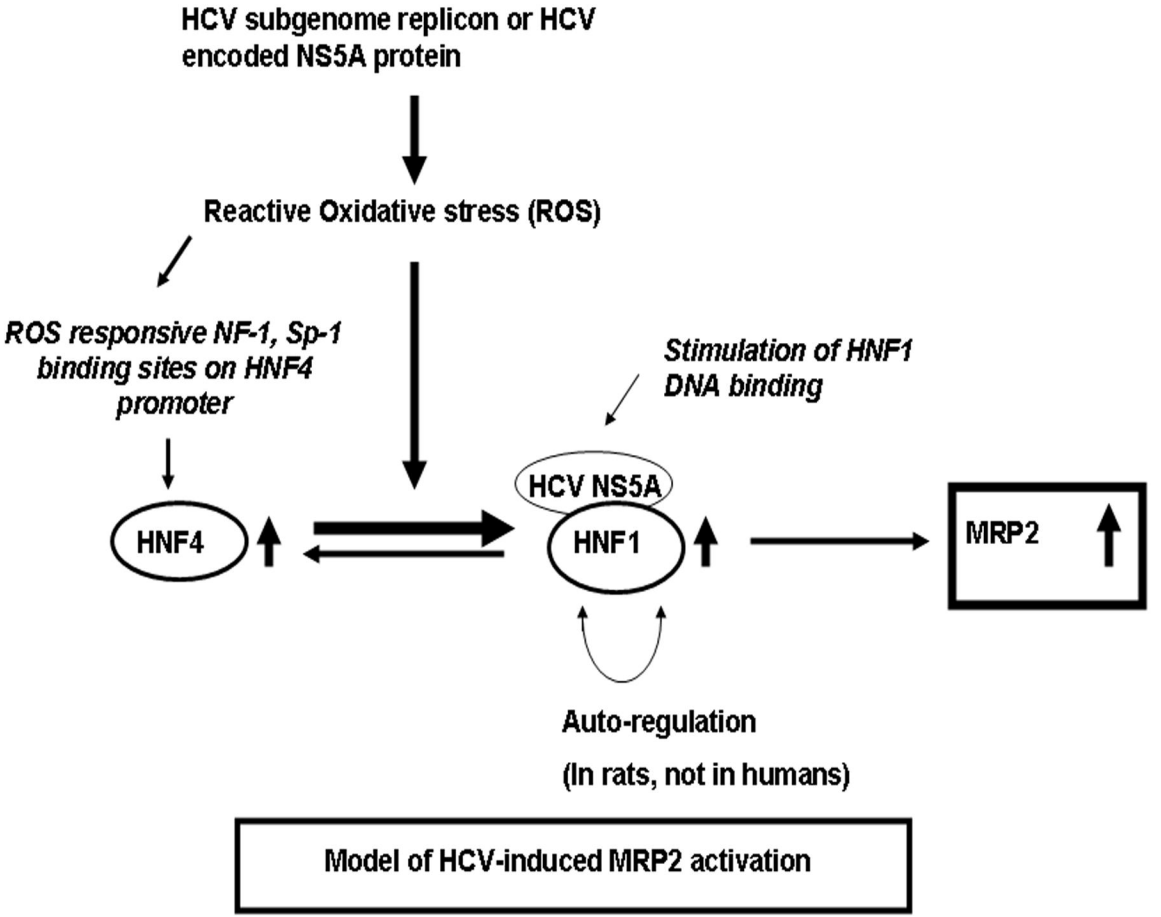
HCV subgenome replicon or HCV encoded NS5A protein
Reactive Oxidative stress (ROS)
ROS responsive NF-1, Sp-1 binding sites on HNF4 promoter
Stimulation of HNF1 DNA binding
HCV NS5A
HNF4
HNF1
MRP2
Auto-regulation
(In rats, not in humans)
Model of HCV-induced MRP2 activation

Figure 8