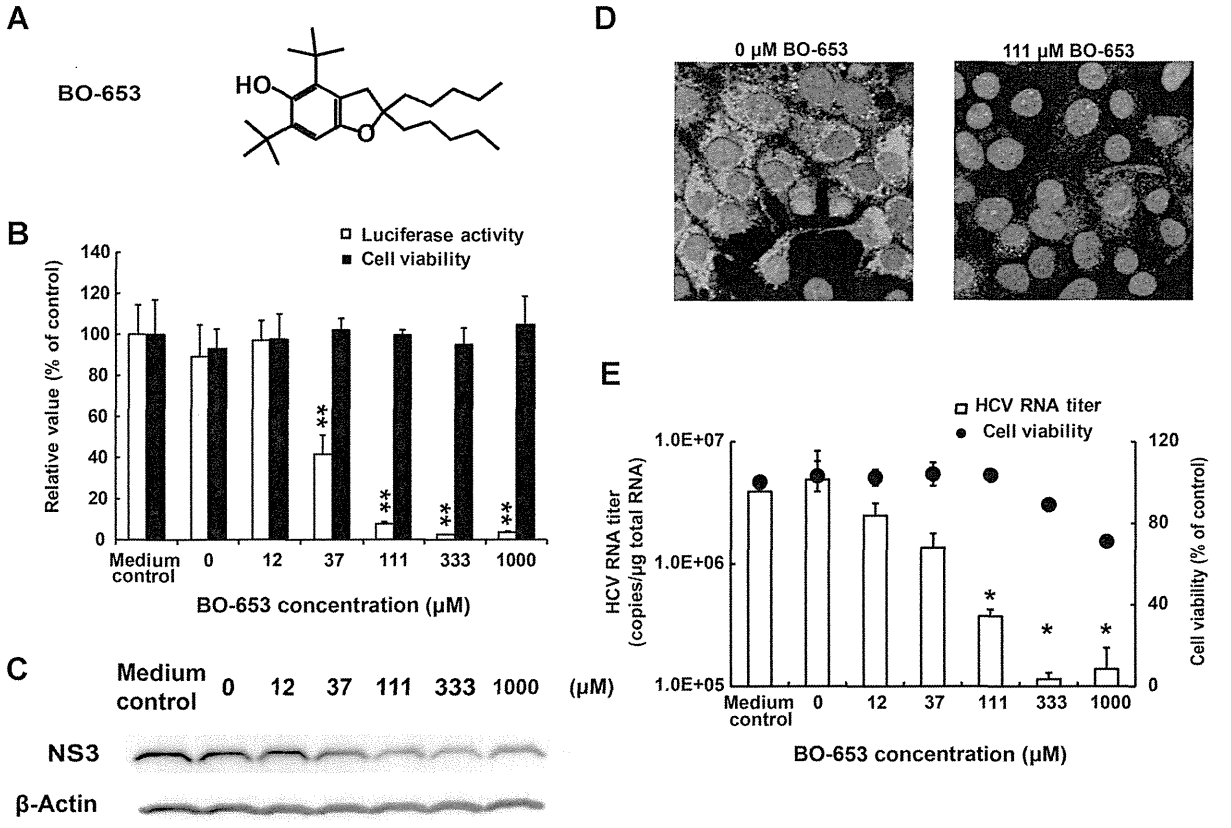

Fig. 1. In vitro anti-HCV activity of BO-653. **A**: The chemical structure of BO-653. **B**: The inhibitory effect of BO-653 on the replication of HCV subgenomic replicons in FLR3-1 cells. HCV replication (white bars) and cell viability (black bars) were determined after 72 hr of treatment; $^{**}P < 0.01$, compared with the medium control. **C**: Effect of BO-653 on the levels of HCV NS3 protein and β-actin (Western blotting). **D**: Immunofluorescent staining of HCV NS3 protein (green) in FLR3-1 cells in the absence or presence (111 μM) of BO-653; nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). **E**: Effect of BO-653 on the HCV RNA titer and cell viability of HuH-7 cells infected with HCV genotype 1a (RMT-tri) after 72 hr of treatment; $^{*}P < 0.05$, compared with the medium control. Statistical analyses were performed by using ANOVA with post-hoc Dunnett's (n = 3 replicates).

compounds had any cytotoxicity at the concentrations tested (Fig. 3B).

Anti-HCV Activity of BO-653 and PEG-IFN in Chimeric Mice Infected With HCV

As demonstrated above using an in vitro assay with FLR3-1 cells, lipophilic antioxidants, including BO-653, exhibited strong anti-HCV activity. The anti-HCV activity of BO-653 was assessed further in vivo by using the compound to treat humanized chimeric mice infected with HCV. First, to measure the pharmacokinetics of BO-653, two chimeric mice were administered orally BO-653 at 800 or 2,000 mg/kg. Twenty-four hours after administration, the mice had mean BO-653 plasma concentrations of 25.0 and 83.1 μM, respectively (Table II). Thus, the BO-653 plasma concentration at the higher dose level exceeded the IC_{50} of BO-653 (36.0 μM) demonstrated previously by the in vitro assay (Fig. 1B), suggesting that oral administration of 2,000 mg/kg BO-653 might be relevant therapeutically for chimeric mice infected with HCV. These mice were infected persistently with HCV genotype 1b by injection with the serum of a HCV-infected patient (see Materials and Methods Section). To determine the anti-HCV activity of BO-653 in early phase of treatment, BO-653 (oral) and/or PEG-IFN (subcutaneous) were then administered over a period of 14 days, according to the schedule shown in Table I, and serum and liver specimens were collected. No adverse effect of the treatment, such as loss of body weight or decreased human albumin secretion, was observed in any of the study groups (Fig. 4A and B). In the mice treated with PEG-IFN, which received a dose 20-fold higher dose than that used in the clinic, the serum HCV RNA titers fell approximately 30-fold and 50-fold in weeks 1 and 2, respectively. Treatment with BO-653 alone at 2,000 mg/kg orally once daily did not reduce the HCV RNA serum titers. However, the combination of BO-653 and PEG-IFN was effective, with the combination demonstrating 200-fold decrease in serum

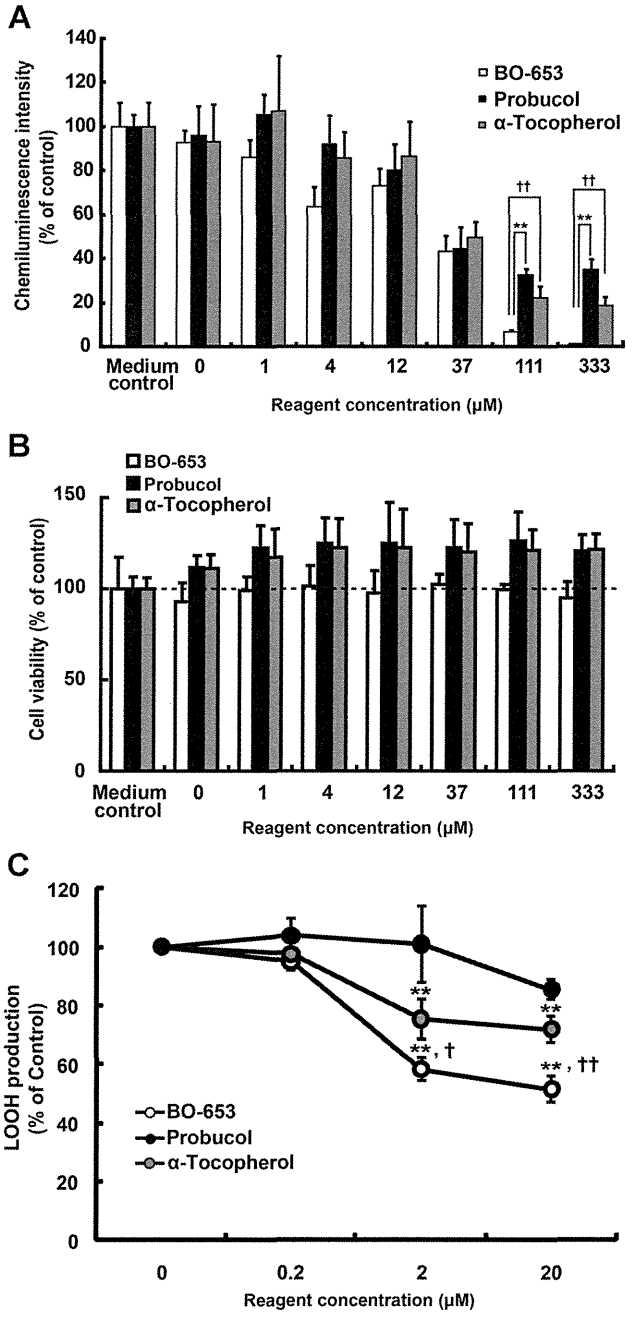

Fig. 2. Comparison of the in vitro anti-HCV activities of lipophilic antioxidants. **A**: The anti-HCV activity and (**B**) cytotoxicity of BO-653, α-tocopherol, and probucol were measured in FLR3-1 cells after 72 hr exposure to the compounds; $^{**}P < 0.01$, compared with probucol at the same concentration, $^{\dagger\dagger}P < 0.01$, compared with α-tocopherol at the same concentration. **C**: Antioxidant activity of BO-653, α-tocopherol, and probucol in the peroxidation of methyl linoleate with AMVN (FOX method); $^{**}P < 0.01$, compared with probucol at the same concentration, and $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$, compared with α-tocopherol at the same concentration. Statistical analyses were performed using ANOVA with post-hoc Tukey's.

HCV titer at 2 weeks; the effect was statistically significant compared to treatment with PEG-IFN alone (Fig. 4C). In the liver samples, a decrease in the titer of HCV RNA to 7–34% of the value in untreated mice

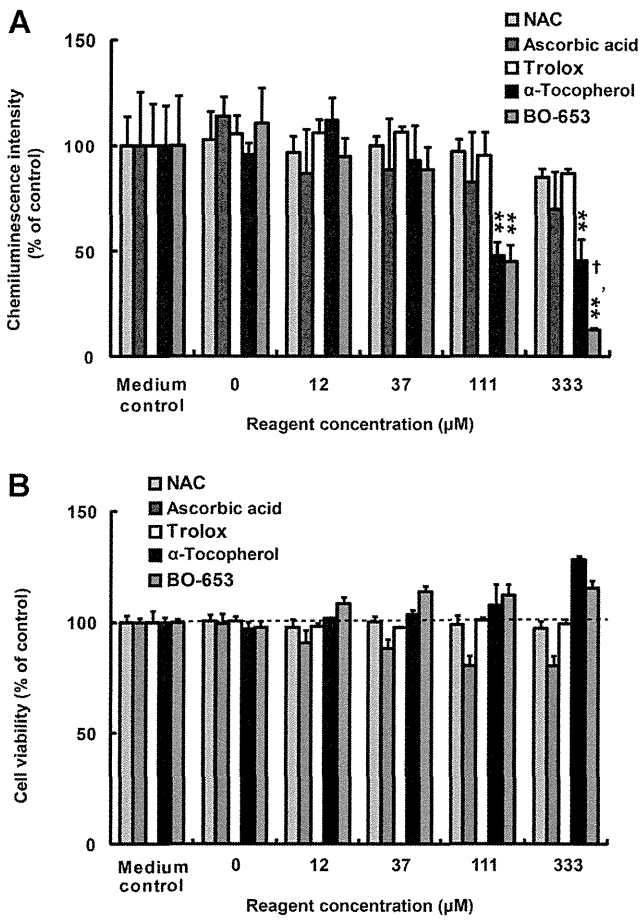

Fig. 3. Comparison of the anti-HCV activity of lipophilic and hydrophilic antioxidants. **A**: The anti-HCV activity and (**B**) cytotoxicity of BO-653 and α-tocopherol (lipophilic antioxidants) and *N*-acetyl cysteine (NAC), ascorbic acid, and trolox (hydrophilic antioxidants) in FLR3-1 cells after 72 hr of exposure to the compounds. $^{**}P < 0.01$, compared with the hydrophilic antioxidants at the same concentration, and $^{\dagger}P < 0.05$, compared with α-tocopherol at the same concentration. Statistical analyses were performed using ANOVA with post-hoc Tukey's.

was also observed in the mice given the combination therapy (Fig. 4D).

DISCUSSION

Several lines of evidence indicate that chronic HCV infection is associated with persistently elevated levels of ROS, resulting in oxidative stress and thus contributing to the development of hepatic dam-

TABLE II. Concentration of BO-653 in Plasma

Dose	Mouse ID	Concentration of BO-653 in plasma (μM)	Mean concentration (μM)
800 mg/kg	1	30.2	25.0
	2	19.8	
2,000 mg/kg	1	100.9	83.1
	2	65.2	

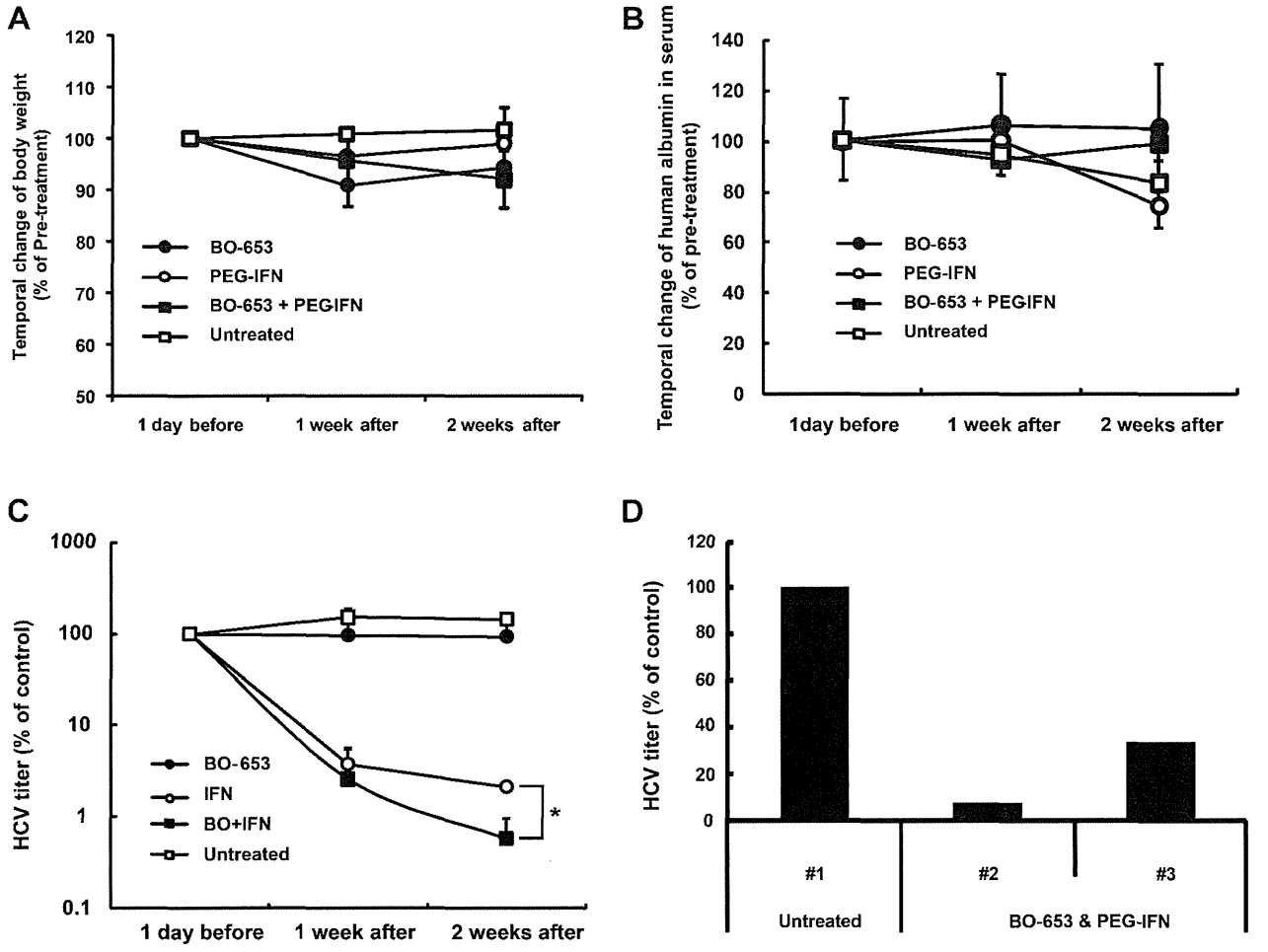

Fig. 4. Anti-HCV effect of BO-653 in chimeric mice infected with HCV. Temporal changes in the (**A**) body weight, (**B**) human albumin concentration in the serum, and (**C**) HCV RNA titer in the serum of chimeric mice infected with HCV after the indicated treatments. All groups included 3–5 mice, except for the untreated group (n = 2). The mice received once-daily oral gavage with 2,000 mg/kg BO-653 and/or twice-weekly subcutaneous injection with 30 μg/kg PEG-IFNα-2a (see Table I). The HCV RNA titers were normalized to the pre-treatment (Day-1) titer in the respective mouse. $^{*}P < 0.05$, PEG-IFN and BO-653 combination treatment (n = 5) was compared with PEG-IFN monotherapy (n = 3) of the same duration. Statistical analyses were performed using Student's *t*-test. **D**: The HCV RNA titer in the liver of chimeric mice treated with or without PEG-IFN and BO-653 combination treatment (untreated group, n = 1; combination treatment group, n = 2).

age [Shimoda et al., 1994; Bureau et al., 2001; Gong et al., 2001; Waris et al., 2005; Levent et al., 2006]. On the other hand, the influence of the intracellular redox state on HCV replication is controversial. A previous study demonstrated inhibition of HCV subgenomic replicon replication by lipid peroxidation and restoration of the replication by treatment with vitamin E [Huang et al., 2007]. A similar result was reported by Choi et al. [2004], who showed inhibition of HCV replication by exogenous hydrogen peroxide treatment. These results indicate that elevated levels of ROS, higher than those induced by natural HCV infection, can lead to inhibition of HCV replication. Yano et al. [2007] reported that several antioxidants, including vitamin E and β-carotene, enhance the replication of HCV genome-length replicons at relatively low concentrations (<10 μM). In contrast, the antioxidant pyrrolidine dithiocarbamate can suppress HCV replication via the inhibition of STAT-3 activation [Waris et al., 2005]. Notably, these studies have been performed primarily in in vitro systems, using either subgenomic replicon-containing cells, full-genome replicon-containing cells, or cells persistently infected with HCV. Therefore, clarification of the effect of antioxidants on HCV replication will require further work, including the use of in vivo models.

BO-653 (2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran), a lipophilic (hydrophobic) antioxidant, was investigated clinically for potential treatment of atherosclerosis and prevention of post-angioplasty restenosis [Cynshi et al., 1998; Meng, 2003]. Starting in November 2001, a phase II trial tested BO-653

for treatment of atherosclerosis and the prevention of post-angioplasty restenosis, but subsequent trials of the compound were suspended due to lack of efficacy. The present investigation of BO-653 as a potential anti-HCV drug reflects subsequent identification of the compound in random screening for antiviral activity.

In the present study, the anti-HCV activity of BO-653 was assessed in an in vitro system, and analyzed further in an in vivo system that used chimeric mice harboring human hepatocytes infected with HCV. In addition, the anti-HCV activity of BO-653 was compared with that of several other antioxidants, including hydrophilic and lipophilic compounds. Among these antioxidants, BO-653 had the strongest anti-HCV activity against HCV subgenomic replicons, followed by α-tocopherol. A previous study showed that the replication of a HCV replicon was increased in the presence of lower concentrations of vitamin E [Yano et al., 2007]. However, the results of the present study suggest that vitamin E exhibits anti-HCV activity when present in cell culture at concentrations exceeding 37 μM. Also, in chimeric mice infected persistently with HCV, the combination of PEG-IFN and BO-653 was more effective than PEG-IFN alone, although BO-653 alone did not have any anti-HCV activity in this mouse model. Monotherapy with DEBIO-025, a non-immunosuppressive cyclosporine A derivative and novel anti-HCV drug candidate, can decrease the viral load in patients with chronic HCV infection but not in chimeric mice infected with HCV [Inoue et al., 2007; Flisiak et al., 2009]; therefore, BO-653 monotherapy should be evaluated further in patients infected with HCV. Di Bona et al. [2006] reported impaired IFN-α signaling following oxidative stress, suggesting that oxidative stress causes resistance to the antiviral activity of IFN-α in patients infected with HCV. Taken together, these findings imply that lipophilic antioxidants such as BO-653 support the effects of PEG-IFN via antioxidant activity.

Several reports indicate that antioxidants, especially hydrophilic compounds, have antiviral activity [Docherty et al., 1999; Docherty et al., 2006; Ho et al., 2009; Geiler et al., 2010; Tian et al., 2010]. *N*-acetyl cysteine can inhibit the replication of H5N1 influenza A virus in a cell culture system [Geiler et al., 2010], and the antiviral effect of epigallocatechin gallate on enterovirus 71 may be associated with the modulation of the cellular redox state [Ho et al., 2009]. In contrast, the inhibitory effects of the lipophilic antioxidants on HCV RNA replication were stronger than those of the hydrophilic antioxidants. These lipophilic antioxidants inhibited the replication of HCV without cytotoxicity at concentrations higher than 37 μM. The role of the redox state of the lipid membrane on HCV RNA replication is of great interest, given reports of the association of RNA replication with lipid rafts [Shi et al., 2003; Sakamoto et al., 2005]. Further studies will be needed to elucidate the precise mechanism(s) underlying the suppression of HCV replication by BO-653.

In conclusion, the present study demonstrated that lipophilic antioxidants have stronger anti-HCV activity than hydrophilic antioxidants and that BO-653 has the strongest anti-HCV activity of the antioxidants tested. The combination of PEG-IFN and BO-653 was more effective than PEG-IFN alone in chimeric mice infected persistently with HCV. Further development of this compound would require additional considerations such as the route of administration and pharmacokinetics. Taken together, these findings provide insights into the influence of the intracellular redox state on the life-cycle of HCV.

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REFERENCES

Bureau C, Bernad J, Chaouche N, Orfila C, Beraud M, Gonindard C, Alric L, Vinel JP, Pipy B. 2001. Nonstructural 3 protein of hepatitis C virus triggers an oxidative burst in human monocytes via activation of NADPH oxidase. J Biol Chem 276: 23077–23083.

Choi J, Lee KJ, Zheng Y, Yamaga AK, Lai MM, Ou JH. 2004. Reactive oxygen species suppress hepatitis C virus RNA replication in human hepatoma cells. Hepatology 39:81–89.

Cynshi O, Kawabe Y, Suzuki T, Takashima Y, Kaise H, Nakamura M, Ohba Y, Kato Y, Tamura K, Hayasaka A, Higashida A, Sakaguchi H, Takeya M, Takahashi K, Inoue K, Noguchi N, Niki E, Kodama T. 1998. Antiatherogenic effects of the antioxidant BO-653 in three different animal models. Proc Natl Acad Sci USA 95:10123–10128.

Di Bona D, Cippitelli M, Fionda C, Camma C, Licata A, Santoni A, Craxi A. 2006. Oxidative stress inhibits IFN-alpha-induced antiviral gene expression by blocking the JAK-STAT pathway. J Hepatol 45:271–279.

Docherty JJ, Fu MM, Stiffler BS, Limperos RJ, Pokabla CM, DeLucia AL. 1999. Resveratrol inhibition of herpes simplex virus replication. Antiviral Res 43:145–155.

Docherty JJ, Sweet TJ, Bailey E, Faith SA, Booth T. 2006. Resveratrol inhibition of varicella-zoster virus replication in vitro. Antiviral Res 72:171–177.

Flisiak R, Feinman SV, Jablkowski M, Horban A, Kryczka W, Pawlowska M, Heathcote JE, Mazzella G, Vandelli C, Nicolas-Metral V, Grosgurin P, Liz JS, Scalfaro P, Porchet H, Crabbe R. 2009. The cyclophilin inhibitor Debio 025 combined with PEG IFNalpha2a significantly reduces viral load in treatment-naive hepatitis C patients. Hepatology 49:1460–1468.

Fontaine H, Pol S. 2011. Antiviral activity of telaprevir and boceprevir for the treatment of hepatitis C virus infection in treatment-experienced patients. Clin Res Hepatol Gastroenterol 35: S59–S63.

Geiler J, Michaelis M, Naczk P, Leutz A, Langer K, Doerr HW, Cinatl J, Jr. 2010. *N*-acetyl-L-cysteine (NAC) inhibits virus replication and expression of pro-inflammatory molecules in A549 cells infected with highly pathogenic H5N1 influenza A virus. Biochem Pharmacol 79:413–420.

Gong G, Waris G, Tanveer R, Siddiqui A. 2001. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. Proc Natl Acad Sci USA 98:9599–9604.

Ho HY, Cheng ML, Weng SF, Leu YL, Chiu DT. 2009. Antiviral effect of epigallocatechin gallate on enterovirus 71. J Agric Food Chem 57:6140–6147.

Huang H, Chen Y, Ye J. 2007. Inhibition of hepatitis C virus replication by peroxidation of arachidonate and restoration by vitamin E. Proc Natl Acad Sci USA 104:18666–18670.

Inoue K, Umehara T, Ruegg UT, Yasui F, Watanabe T, Yasuda H, Dumont JM, Scalfaro P, Yoshiba M, Kohara M. 2007. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. Hepatology 45:921–928.

Kato J, Kobune M, Nakamura T, Kuroiwa G, Takada K, Takimoto R, Sato Y, Fujikawa K, Takahashi M, Takayama T, Ikeda T, Niitsu Y. 2001. Normalization of elevated hepatic 8-hydroxy-2'-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. Cancer Res 61: 8697–8702.

Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. 2012. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. J Hepatol 56:78–84.

Lavanchy D. 2009. The global burden of hepatitis C. Liver Int 29:74–81.

Levent G, Ali A, Ahmet A, Polat EC, Aytac C, Ayse E, Ahmet S. 2006. Oxidative stress and antioxidant defense in patients with chronic hepatitis C patients before and after pegylated interferon alfa-2b plus ribavirin therapy. J Transl Med 4:25.

Meng CQ. 2003. BO-653. Chugai. Curr Opin Invest Drugs 4: 342–346.

Noguchi N, Iwaki Y, Takahashi M, Komuro E, Kato Y, Tamura K, Cynshi O, Kodama T, Niki E. 1997. 2,3-Dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran: Design and evaluation as a novel radical-scavenging antioxidant against lipid peroxidation. Arch Biochem Biophys 342:236–243.

Nourooz-Zadeh J, Tajaddini-Sarmadi J, Wolff SP. 1994. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjuction with triphenylphosphine. Anal Biochem 220:403–409.

Sakamoto H, Okamoto K, Aoki M, Kato H, Katsume A, Ohta A, Tsukuda T, Shimma N, Aoki Y, Arisawa M, Kohara M, Sudoh M. 2005. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. Nat Chem Biol 1:333–337.

Seronello S, Sheikh MY, Choi J. 2007. Redox regulation of hepatitis C in nonalcoholic and alcoholic liver. Free Radic Biol Med 43:869–882.

Shi ST, Lee KJ, Aizaki H, Hwang SB, Lai MM. 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J Virol 77:4160–4168.

Shimoda R, Nagashima M, Sakamoto M, Yamaguchi N, Hirohashi S, Yokota J, Kasai H. 1994. Increased formation of oxidative DNA damage, 8-hydroxydeoxyguanosine, in human livers with chronic hepatitis. Cancer Res 54:3171–3172.

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Muller T, Bahlo M, Stewart GJ, Booth DR, George J. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41:1100–1104.

Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, Tsukiyama-Kohara K, Kawaguchi R, Tanaka S, Kohara M. 1999. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 116:636–642.

Tamura K, Kato Y, Ishikawa A, Kato Y, Himori M, Yoshida M, Takashima Y, Suzuki T, Kawabe Y, Cynshi O, Kodama T, Niki E, Shimizu M. 2003. Design and synthesis of 4,6-di-*tert*-butyl-2,3-dihydro-5-benzofuranols as a novel series of antiatherogenic antioxidants. J Med Chem 46:3083–3093.

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41:1105–1109.

Tian Y, Jiang W, Gao N, Zhang J, Chen W, Fan D, Zhou D, An J. 2010. Inhibitory effects of glutathione on dengue virus production. Biochem Biophys Res Commun 397:420–424.

Waris G, Turkson J, Hassanein T, Siddiqui A. 2005. Hepatitis C virus (HCV) constitutively activates STAT-3 via oxidative stress: Role of STAT-3 in HCV replication. J Virol 79:1569–1580.

Yano M, Ikeda M, Abe K, Dansako H, Ohkoshi S, Aoyagi Y, Kato N. 2007. Comprehensive analysis of the effects of ordinary nutrients on hepatitis C virus RNA replication in cell culture. Antimicrob Agents Chemother 51:2016–2027.

Zhu Z, Wilson AT, Mathahs MM, Wen F, Brown KE, Luxon BA, Schmidt WN. 2008. Heme oxygenase-1 suppresses hepatitis C virus replication and increases resistance of hepatocytes to oxidant injury. Hepatology 48:1430–1439.

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Hepatitis C Virus Promotes Expression of the 3β-Hydroxysterol Δ24-Reductase Through Sp1

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Hepatitis C virus (HCV) establishes chronic infection, which often causes hepatocellular carcinoma. Overexpression of 3β-hydroxysterol Δ24-reductase (DHCR24) by HCV has been shown to impair the p53-mediated cellular response, resulting in tumorigenesis. In the present study, the molecular mechanism by which HCV promotes the expression of *DHCR24* was investigated. A significant increase in *DHCR24* mRNA transcription was observed in a cell line expressing complete HCV genome, whereas no significant difference in the expression of *DHCR24* was seen in cell lines expressing individual viral proteins. The 5′-flanking genomic region of *DHCR24* was characterized to explore the genomic region and host factor(s) involved in the transcriptional regulation of *DHCR24*. As a result, the HCV response element (−167/−140) was identified, which contains AP-2α, MZF-1, and Sp1 binding motifs. The binding affinity of the host factor to this response element was increased in nuclear extracts from cells infected with HCV and corresponded with augmented affinity of Sp1. Both mithramycin A (Sp1 inhibitor) and small interfering RNA targeting Sp1 prevented the binding of host factors to the response element. Silencing of Sp1 also downregulated the increased expression of *DHCR24*. The binding affinity of Sp1 to the response element was augmented by oxidative stress, whereas upregulation of *DHCR24* in cells expressing HCV was blocked significantly by a reactive oxygen species scavenger. Elevated phosphorylation of Sp1 in response to oxidative stress was mediated by the ATM kinase. Thus, activation of Sp1 by oxidative stress is involved in the promotion of expression of *DHCR24* by HCV. ***J. Med. Virol. 84:733–746, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: HCV; DHCR24; Sp1; oxidative stress

INTRODUCTION

Hepatitis C virus (HCV) causes chronic hepatitis and hepatocellular carcinoma [Koike, 2007]. The estimated worldwide prevalence of HCV infection is 2.2–3.0% (130–170 million people) [Lavanchy, 2009], and chronic HCV infection is a major global public health concern. The most effective current treatment for HCV infection comprises combination therapy with PEGylated interferon-α and ribavirin [Bruchfeld et al., 2001; Lu et al., 2008]. However, this therapy has limited clinical efficacy, as sustained virological responses develop in only about half of patients infected with HCV genotype 1 [Kohara et al., 1995; Nakamura et al., 2002]. Efforts to develop therapies to treat HCV are also hindered by the high level of viral variation and the capacity of HCV to cause chronic infection. Therefore, there is an urgent need to develop effective treatments against chronic HCV infection.

Additional supporting information may be found in the online version of this article.

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A cell line that expresses complete HCV genome (RzM6-LC) was established to investigate the effects of persistent expression of HCV on cell growth [Tsukiyama-Kohara et al., 2004]. A monoclonal antibody (2-152a mAb) against the RzM6-LC cell line was also developed to produce clones that recognize both cell surface and intracellular molecules. As a result, 3β-hydroxysterol Δ24-reductase (DHCR24) was identified as the target of 2-152a mAb [Nishimura et al., 2009].

DHCR24 is an oxidoreductase with a broad expression pattern and shares homology with a family of flavin-adenine dinucleotide-dependent reductases [Waterham et al., 2001]. In mammals, DHCR24 functions as an enzyme to catalyze the conversion of desmosterol to cholesterol in the post-squalene cholesterol biosynthetic pathway, and it is essential for normal tissue development and maintenance [Waterham et al., 2001; Crameri et al., 2006]. DHCR24 regulates cholesterol synthesis and promotes recruitment of domain components into detergent-resistant membrane fractions [Crameri et al., 2006]. An absence of DHCR24 leads to desmosterolosis—a rare disorder of cholesterol biosynthesis [Waterham et al., 2001]. Expression of DHCR24 is downregulated in areas of the brain affected by Alzheimer's disease [Greeve et al., 2000], suggesting that DHCR24 has alternative functions. Indeed, DHCR24 is also known as seladin-1 (the selective Alzheimer's disease indicator 1), reflecting the association between DHCR24/seladin-1 and the selective vulnerability of the neurons in the affected areas of the brain. High levels of DHCR24/seladin-1 exert protective effects, conferring resistance against oxidative stress and preventing apoptotic cell death [Greeve et al., 2000; Benvenuti et al., 2005; Di Stasi et al., 2005; Luciani et al., 2005; Lu et al., 2008]. Endogenous DHCR24/seladin-1 levels are upregulated in response to acute oxidative stress [Wu et al., 2004; Benvenuti et al., 2006; Kuehnle et al., 2008], whereas the expression declines to low levels upon chronic exposure [Benvenuti et al., 2006; Kuehnle et al., 2008]. Therefore, DHCR24/seladin-1 may be involved in integrating cellular responses to oxidative stress. DHCR24 also functions as a hydrogen peroxide scavenger [Lu et al., 2008]. Based on these findings, DHCR24 may play a crucial role in maintaining cellular physiology by regulating both cholesterol synthesis and cellular defense against oxidative stress.

HCV infection impairs apoptosis induced by oxidative stress and inhibits p53 function via overexpression of DHCR24 [Nishimura et al., 2009]. Augmented expression of DHCR24 also facilitates efficient replication of HCV [Takano et al., 2011b]. Since DHCR24 may play a significant role in viral replication and in the tumorigenicity of the hepatocellular carcinoma related to HCV, the molecular mechanism of overexpression of DHCR24 in response to HCV was examined in the present study.

MATERIALS AND METHODS

Cell Lines

The HepG2 hepatoblastoma cell line, the HepG2-derived RzM6 cell line, which is capable of conditional control of expression of HCV genome (genotype 1b) based on the Cre/*loxP* system (RzM6-0d, no switching; RzM6-LC, switching of full genome HCV induced by tamoxifen), and HepG2-derived CN5 cell line, in which all HCV proteins were expressed conditionally by cre adenovirus (CN5-Cre) [Tsukiyama-Kohara et al., 2004] were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Using a stable expression system based on lentiviral vectors, HepG2/Lenti cell lines (core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B) were established [Takano et al., 2011a]. An additional cell line—HepG2-emp—was infected with an empty lentiviral vector. Cells from the human hepatoma HuH-7 cell line were maintained in DMEM supplemented with 10% FBS and 0.4% glucose. The cell lines harboring HCV replicon, namely, R6FLR-N (genotype 1b) and FLR3-1 (genotype 1b), which are derived from HuH-7 [Takano et al., 2011b], were maintained under selective pressure with G418 (500 μg/ml for R6FLR-N and FLR3-1) in DMEM GlutaMAX (Invitrogen, Carlsbad, CA) containing 10% FBS. Cured/HuH-7 K4 cells—cured of HCV by interferon-α treatment—were maintained in DMEM GlutaMAX containing 10% FBS without G418. The JFH/K4 cell line, which shows persistent infection with the HCV JFH-1 strain, was maintained in DMEM containing 10% FBS. The human fetal hepatic cell line WRL68 was obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. The human hepatoma cell line PLC/PRF/5 was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, and cultured in Eagle's minimum essential medium supplemented with 10% FBS.

Construction of DHCR24 Promoter Reporter Plasmids

Genomic DNA was extracted from HepG2 cells, and the 5′-flanking sequence of the predicted transcription start site of *DHCR24* (~5 kb) was isolated. The genomic region was inserted upstream of the firefly luciferase gene in pGL3-Basic (Promega, Madison, WI). Deletion constructs of the *DHCR24* promoter region were constructed using restriction enzymes and PCR (sense primer for −4956, 5′-GATCCTCGAGCACTCCTGCTCACCACTGAT-3′; sense primer for −2982, 5′-GATCCTCGAGGAGGCTCACATTGTAGAAAG-3′; antisense primer, 5′-GTAGTAGATATCGAAGATAAGCGAGAGCGG-3′) and cloned into pGL3-Basic at the *Xho*I and *Nco*I sites.

Dual Luciferase Reporter Assay

HepG2 cells (1×10^4 cells/well in a 96-well plate) were transfected with each of the 3 *DHCR24* promoter reporter plasmids and their deletion constructs (0.25 μg/well) using cationic lipid (Lipofectamine LTX, Invitrogen). Samples were analyzed with the Dual-Glo Luciferase Assay System (Promega) at 48 h post-transfection, and luminescence was measured using a TriStar LB941 microplate reader (Berthold, Bad Wildbad, Germany). To account for differences in transfection efficiency, the luminescence produced by firefly luciferase (FL) was normalized to that produced by Renilla luciferase (RL), which was expressed by co-transfection with phRL-TK (Promega; 0.025 μg/well).

Electrophoresis Mobility Shift Assay

Nuclear extracts were prepared from 5×10^6 to 1×10^7 cells as described previously [Dignam et al., 1983]. Electrophoresis mobility shift assays (EMSAs) were performed by a nonradioactive method using the DIG Gel Shift Kit (Roche, Indianapolis, IN). Briefly, binding reactions were performed by mixing the following components: 1 μg of poly[d(I-C)], 0.1 μg of poly L-lysine, 40 fmol DIG-labeled double-stranded oligonucleotide probe (HCV response element −167/−140 [28-mer], 5′-CCCCCGCCTCGCGCGGCGGCGGGGAGAA-3′; Sp1 consensus sequence [22-mer], 5′-ATTCGATCGGGGCGGGGCGAGC-3′; MZF1.1-4 consensus sequence [21-mer], 5′-GATCTAAAAGTGGGGAGAAAA-3′; AP-2α consensus sequence [26-mer], 5′-GATCGAACTGACCGCCCGCGGCCCGT-3′), and 10 μg of the nuclear extract in binding buffer (10 mM Tris–HCl, pH 7.5; 50 mM NaCl; 5 mM $MgCl_2$; 0.5 mM EDTA). Where indicated, reactions were supplemented with unlabeled/competitive oligonucleotide at a 50-fold molar excess concentration before addition of the probe. Mithramycin A (MMA; Sigma, St. Louis, MO), which blocks the binding of Sp1 to target sequences, was added at different final concentrations (2.5, 5, and 10 μM) and incubated at 4°C for 1 h. For supershift assays, 1 μg of monoclonal anti-Myc Tag antibody (Upstate Biotechnology, Lake Placid, NY) was added 30 min prior to addition of the probe. Binding reactions were carried out at 25°C for 30 min and electrophoresed on 6% acrylamide–0.5× TBE gels, transferred to positively charged nylon membranes, and detected by a chemiluminescence method (Roche) and a LAS1000 scanner (Fujifilm Co., Tokyo, Japan).

Silencing of Sp1, HCV, and DHCR24 by siRNA

SP1 Validated Stealth RNAi™ siRNA (VHS40867, Invitrogen) was designed with the BLOCK-iT RNAi designer to target the human Sp1 mRNA sequence. RzM6-0d and RzM6-LC cells (1.5×10^6 cells in a 100-mm dish) were transfected with Sp1 siRNA (final concentration, 30 nM) using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM (Invitrogen) and incubated for 48 h at 37°C. The siRNAs specific for DHCR24 and HCV were designed and utilized as described previously [Nishimura et al., 2009].

Kinase Inhibitors

ATM kinase inhibitor KU55933 (Wako Pure Chemical Industry, Osaka, Japan; final concentration, 10 μM), PI3K inhibitor LY294002 (Cell Signaling Technology, Beverly, MA; 50 μM), and MEK1 inhibitor PD98059 (Cell Signaling Technology; 50 μM) were added to cell cultures, which were incubated for 8 h at 37°C.

Western Blotting

Western blotting was performed as described previously [Tsukiyama-Kohara et al., 2004] with the following primary antibodies: rabbit monoclonal anti-DHCR24/Seladin-1 (C59D8; Cell Signaling Technology); rabbit polyclonal anti-Sp1, anti-phospho-Akt (Ser473), and mouse monoclonal anti-phospho-ERK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse monoclonal anti-HCV core (clone 31-2), E1 (clone 384), E2 (clone 544), NS4A (c14II-2-1), NS5A (32-2), NS5B (14-5), rabbit polyclonal anti-NS2, NS3 (R212), and NS4B (RR10) [Tsukiyama-Kohara et al., 2004]. Phosphorylation of Sp1 was investigated by 5% SDS–PAGE and immunoblotting with a polyclonal antibody against Sp1 phosphorylated at Ser101 (Active Motif, Carlsbad, CA) or Thr453 (Abcam, Cambridge, MA). Detection of γH2AX was performed by 15% SDS–PAGE and immunoblotting with mouse monoclonal anti-phospho-histone H2AX (Ser139) (JBW301; Upstate Biotechnology). Phosphorylated ATM (Ser1981) and ATR (Ser428) were detected by specific antibodies (Cell Signaling Technology). Monoclonal anti-actin (Sigma), anti-histone H1 (Santa Cruz Biotechnology, Inc.), anti-HAUSP (Calbiochem, San Diego, CA), and anti-heat shock protein 90 (Stressgen, Victoria, BC, Canada) primary antibodies were used for normalization of Western blotting. Bound antibody was detected with a horseradish peroxidase-conjugated secondary antibody and visualization using ECL reagents (GE Healthcare, Piscataway, NJ) and an LAS1000 scanner (Fujifilm). Densitometric analysis of protein bands was performed with Image Quant software (Molecular Dynamics, Sunnyvale, CA).

Quantitative PCR and HCV Infection

Total RNA was extracted from cell lines using ISOGEN, and reverse transcription of total RNA (125 ng) was performed with SuperScript III Reverse Transcriptase and Random Primers (Invitrogen). Synthesized cDNA samples were subjected to a TaqMan gene expression assay (Applied Biosystems, Foster City, CA), and the level of expression of *DHCR24* mRNA in each sample was normalized to the level of expression of *GAPDH* mRNA and represented as a ratio of the control (Hep-emp, CN5-Hep, or RzM6-0d). Infection of the human hepatocytes from human liver-uPA/SCID chimeric mice with HCV was performed,

and HCV RNA, *DHCR24* mRNA, and 18S rRNA were measured by quantitative PCR (qPCR), as described previously [Takano et al., 2011a].

Statistical Analysis

The Student's *t*-test was used to analyze the statistical significance of the results; P values < 0.05 were considered statistically significant.

RESULTS

DHCR24 Expression Is Upregulated by the Complete HCV Genome But Not by Individual Viral Proteins

Overexpression of *DHCR24* in human hepatocytes from human liver-uPA/SCID chimeric mice has been observed after HCV infection (Fig. 1A). The overexpression of *DHCR24* in cells expressing HCV decreased to a similar extent as that observed in control cells following treatment with HCV siRNA (Fig. 1B). Since these findings suggest that overexpression of *DHCR24* is associated with the expression or infection by HCV, the identity of the viral factor involved in the augmentation of expression of *DHCR24* was examined. The level of expression of *DHCR24* mRNA was measured by quantitative RT-PCR (Fig. 1C) in HepG2-derived cell lines that stably express individual HCV proteins (core, E1, E2, NS2, NS3/4A, NS4B, NS5A, or NS5B; Supplementary Fig. 1). The level of expression of *DHCR24* mRNA was slightly higher in the cells expressing NS4B and NS5A than in control cells; however, there was no significant difference in the expression of *DHCR24* mRNA among these cell

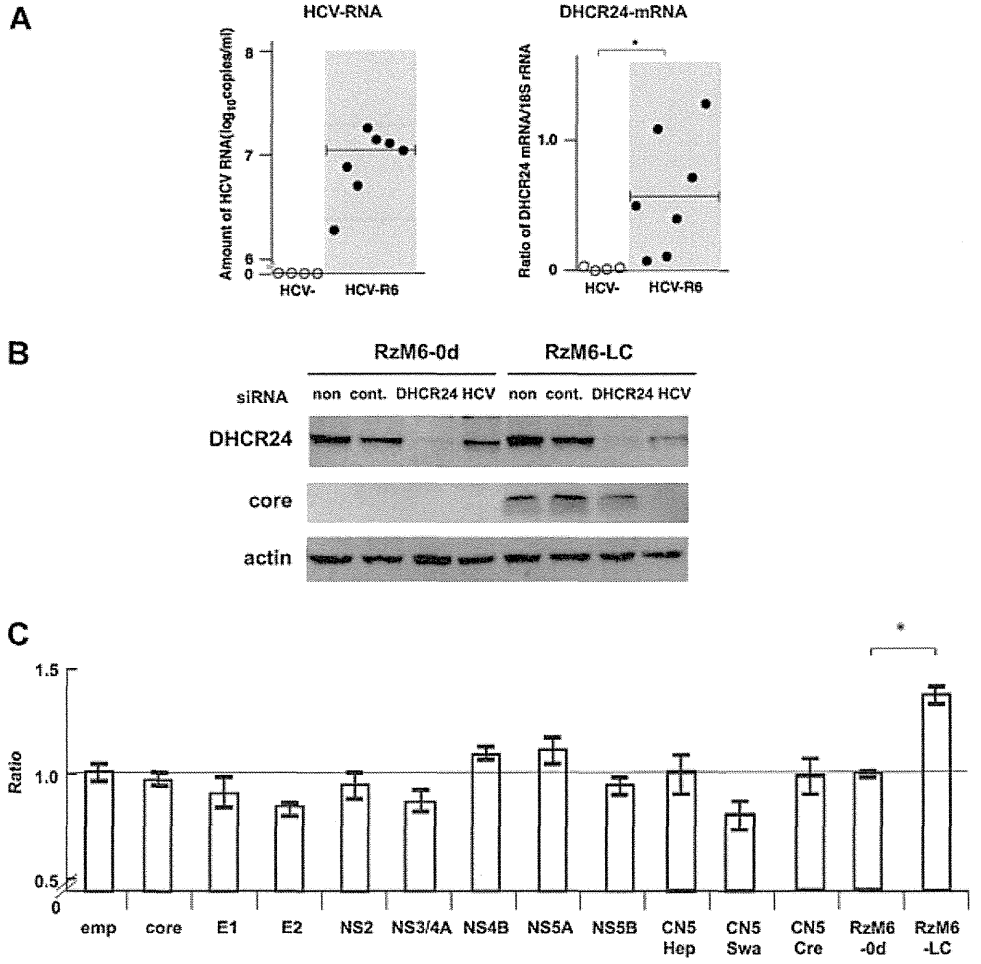

Fig. 1. *DHCR24* expression is induced in the presence of the complete HCV genome. **A**: The amount of HCV RNA in chimeric mice infected with HCV-R6 (genotype 1b) was quantified by qPCR (left panel). The amount of DHCR24 mRNA was measured, and the ratio to the amount of 18S rRNA was calculated in the tissues (right panel). **B**: Western blotting of DHCR24, HCV core, and actin protein in RzM6-0d and LC cells following treatment with the indicated siRNA. **C**: Level of *DHCR24* mRNA expression in cell lines with stable expression of individual HCV proteins, the HCV open reading frame, or the complete HCV genome. Total RNA from HepG2/Lenti cell lines (emp, core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B), CN5 cell lines (CN5-Hep, CN5-Swa, and CN5-Cre), or RzM6 cell lines (RzM6-0d and RzM6-LC) were prepared, and reverse transcription was performed. Synthesized cDNA was subjected to quantitative PCR. The level of expression of *DHCR24* mRNA for each sample was normalized to that of *GAPDH* mRNA and represented as a ratio of HepG2-emp ($^*P < 0.05$).

lines. No significant upregulation of *DHCR24* mRNA was observed in the CN5-Cre cell line, which expresses all HCV proteins and is negative for viral replication [Tsukiyama-Kohara et al., 2004]. In contrast, significant upregulation of *DHCR24* was observed in a cell line that expresses the complete HCV genome (RzM6-LC) compared with the expression in HCV-negative control cells (RzM6-0d). Thus, expression of viral proteins alone is insufficient to reproduce the augmentation of expression of *DHCR24* induced by HCV.

DHCR24 Promoter Activity Is Potentiated by the Expression of HCV

The 5′-flanking region contains a number of possible transcriptional regulatory elements, including three candidate-binding motifs for the endoplasmic reticulum (ER) stress-responsive transcription factor, XBP1. Cellular ER stress is induced in response to the expression of the HCV gene and infection by HCV [Tardif et al., 2005]. Thus, to explore host factors involved in the transcriptional regulation of *DHCR24*, the 5′-flanking genomic region (~5 kb) of *DHCR24* was isolated. Subsequently, *DHCR24* promoter reporter plasmids that contain the 5′-flanking region of *DHCR24* and the firefly luciferase gene were constructed (Fig. 2A). Relevant regions of the promoter were defined by constructing deletion mutants of the 5′-flanking regions, which were analyzed by a dual luciferase reporter assay in the presence or absence of full-length HCV genome expression—resulting from transfection with pCA-Rz [Tsukiyama-Kohara et al., 2004] or the control pCAGGS vector, respectively (Fig. 2B). Progressive shortening of the 5′-flanking regions did not

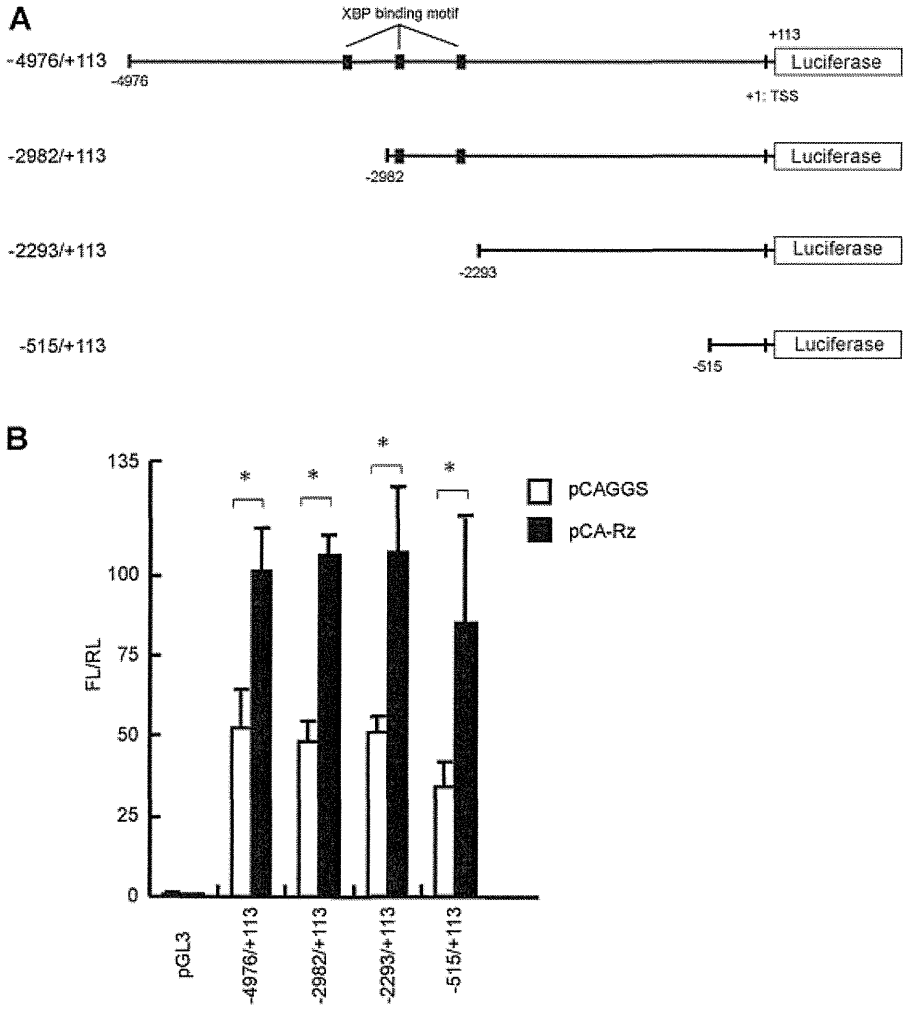

Fig. 2. *DHCR24* promoter activity is augmented by the expression of HCV. **A:** The 5′-flanking region of *DHCR24* was cloned from HepG2 and inserted upstream of the firefly luciferase gene in pGL3 (−4976/+113). A deletion series of the 5′-flanking regions was also constructed (−2982/+113, −2293/+113, and −515/+113). The black boxes indicate potential binding sites for the ER stress-responsive transcription factor, XBP-1. TSS, transcription start site (+1). **B:** HepG2 cells (1×10^4 cells/well in a 96-well plate) were co-transfected with each *DHCR24* promoter reporter plasmid (0.25 μg/well), a Renilla luciferase expression vector (phRL-TK; 0.025 μg/well), and either an expression vector containing the HCV full-length genome (pCA-Rz; 0.5 μg/well) or an empty expression vector (pCAGGS). Luciferase activity at 48 h post-transfection is shown as the ratio of firefly luciferase (FL) to Renilla luciferase (RL). Data are shown as the mean ± SD from 2 representative experiments performed in triplicate ($^*P < 0.05$).

result in significant differences in the basal promoter activity (Fig. 2B). The −515/+113 construct also produced a significant response in the presence of full-length HCV genome expression.

Additional reporter deletion mutants were constructed to define the region in the *DHCR24* promoter that is responsive to HCV expression. To this aim, potential binding motifs for transcription factors were predicted in the minimized *DHCR24* promoter sequence (nucleotides –515/+113; Fig. 3A), and a series of promoter mutants containing sequential 100-bp deletions was constructed. As shown in Fig. 3A, while the promoter activity of −515/+113, −400/+113, −300/+113, and −200/+113 constructs was increased significantly by expression of HCV ($^*P < 0.05$), the promoter activity of the −100/+113 construct was unchanged. Therefore, an HCV-responsive sequence appears to be located in the upstream region (−200 to −100 bp) from the transcriptional start site of *DHCR24*, which includes sequences with similarity to the consensus-binding motifs for AP-2α, Sp1, MZF-1, Pax-4, and NF-Y.

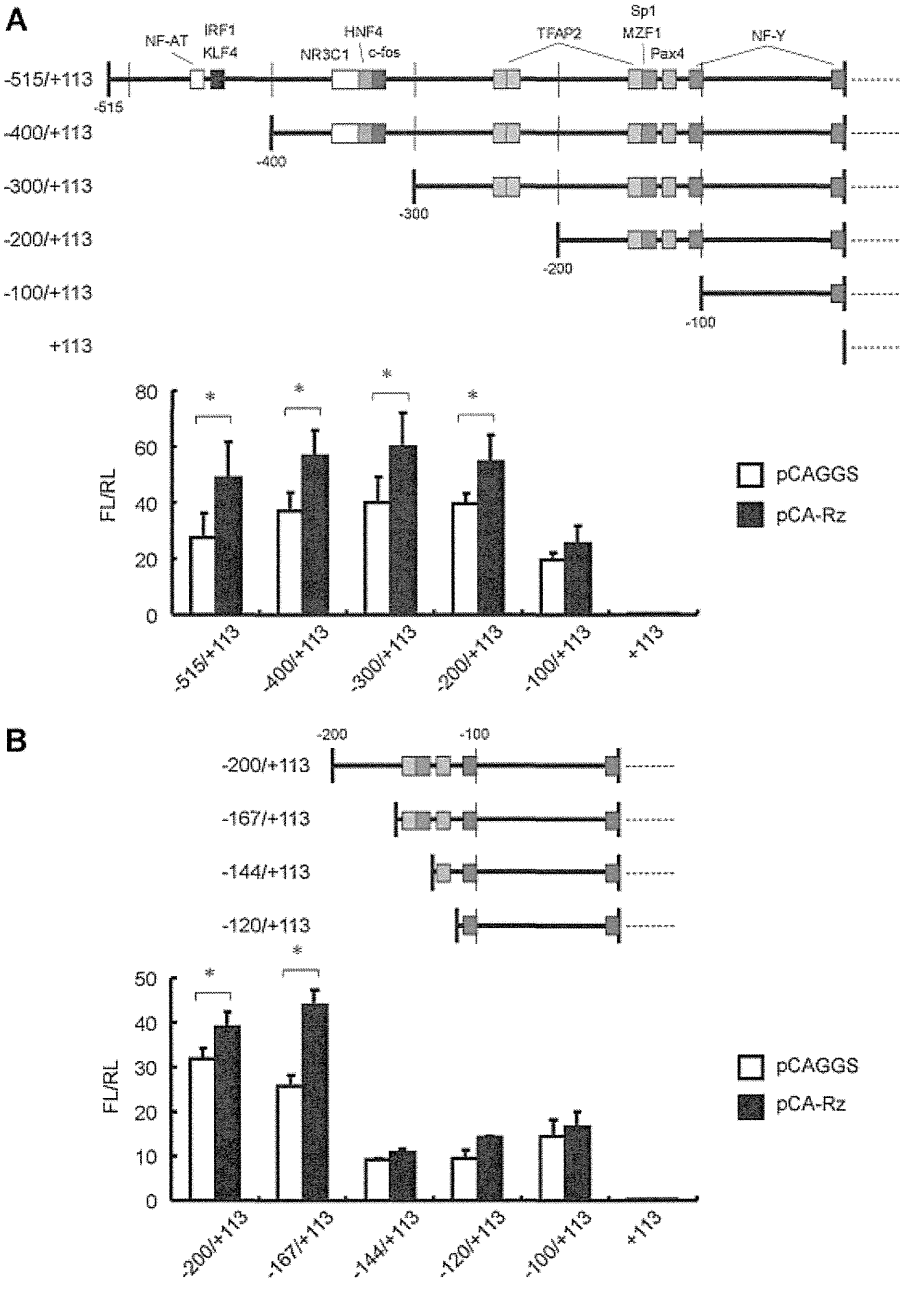

Fig. 3. Responsiveness of the *DHCR24* promoter to the expression of HCV. **A**: A *DHCR24* promoter series with sequential 100-bp deletions was constructed and analyzed as described in the legend to Fig. 2B ($^*p < 0.05$). **B**: An additional deletion series (−167/+113, −144/+113, and −120/+113) was constructed and analyzed as described in (A).

A more detailed deletion series (−167/+113, −144/+113, and −120/+113) was constructed (Fig. 3B) to determine the minimum-binding motif that responds to HCV expression. The responsiveness to the expression of HCV was lost with the removal of the proximal portion (−167 to −145), which includes candidate-binding motifs for AP-2α, Sp1, and MZF-1. Thus, the identified HCV response element in the *DHCR24* promoter represents the minimum element of DNA sequence required for the promotion of the expression of DHCR24 induced by HCV.

HCV Expression Augments the Interaction Between the HCV Response Element and the Binding Molecule(s)

Transcription of *DHCR24* is upregulated significantly in RzM6-LC cells that show persistent expression of HCV [Nishimura et al., 2009]. Therefore, the effect of expression of HCV on the interaction between the HCV response element and its related transcription factor(s) was examined. Nuclear extracts were prepared from RzM6-LC cells, and an electrophoretic mobility shift assay (EMSA) using a DIG-labeled double-stranded oligonucleotide corresponding to the response element (−167/−140, 28 bp; Fig. 4A) was performed. The interaction between the response element and the nuclear factor was increased significantly in nuclear extracts from RzM6-LC cells compared with that in RzM6-0d cells (Fig. 4B). Thus, the binding affinity or quantity of the nuclear factor may be increased by the expression of HCV. The shifted band corresponding to the Sp1 consensus sequence also increased in RzM6-LC cells compared with that in control RzM6-0d cells, whereas no difference was

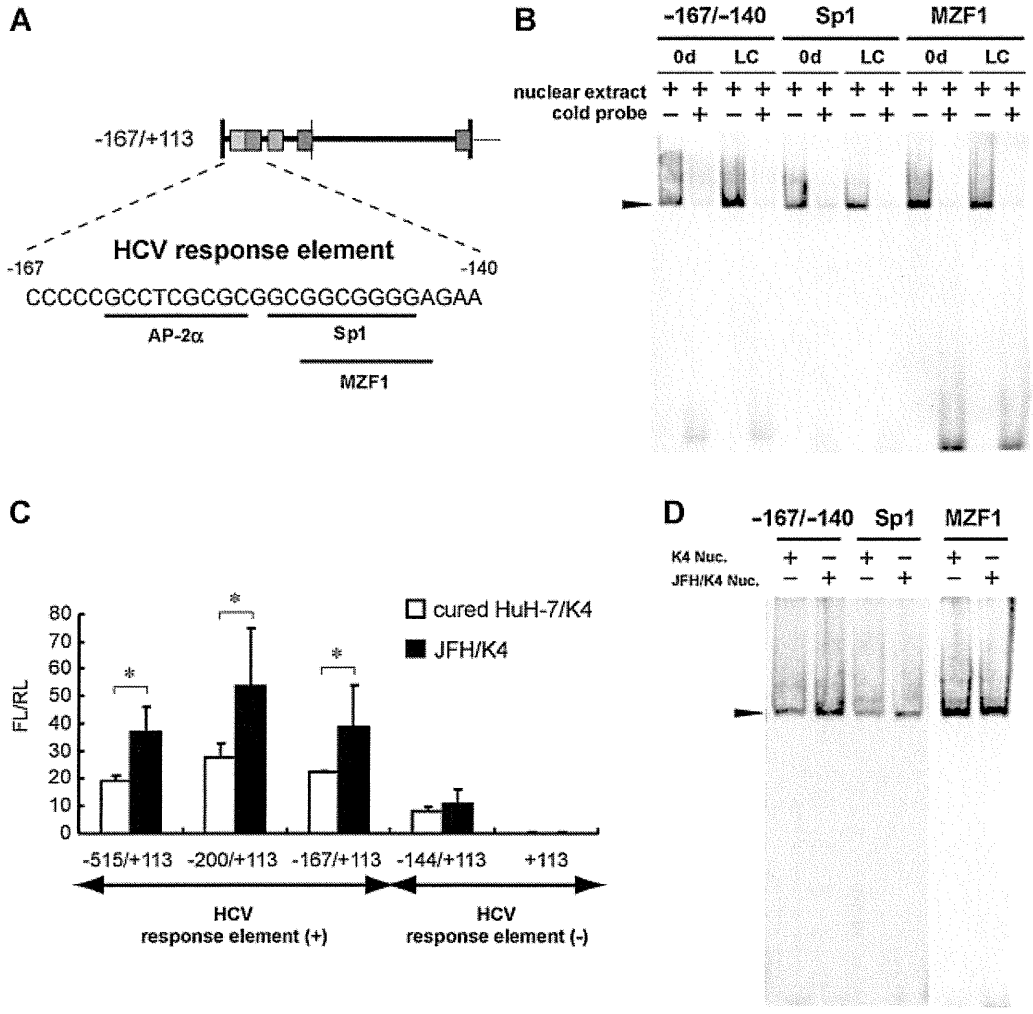

Fig. 4. The HCV response element mediates the overexpression of *DHCR24* induced by HCV. **A**: The HCV response element (−167/−140) in the 5′-flanking region of *DHCR24* includes sequences with similarity to the consensus-binding motifs for AP-2α, Sp1, and MZF-1. **B**: Nuclear extracts were prepared from RzM6-0d and RzM6-LC cells and subjected to an electrophoresis mobility shift assay (EMSA; 10 μg/sample) using the DIG-labeled HCV response element (28-bp), Sp1 (22-bp), or MZF-1 (21-bp) probes. Cold probe indicates unlabeled oligonucleotides. The arrowhead indicates the interaction between the binding factor(s) and each oligonucleotide. **C**: Cured HuH-7/K4 cells and JFH/K4 cells were co-transfected with each *DHCR24* promoter reporter plasmid (0.5 μg/well) and phRL-TK (0.05 μg/well) and analyzed as described in Fig. 2B ($^*P < 0.05$). (**D**) Nuclear extracts prepared from cured HuH-7/K4 cells or JFH/K4 cells were subjected to EMSA (25 μg/sample) using the DIG-labeled HCV response element, Sp1, or MZF-1 probes.

noted in the intensity of the shifted band for the MZF-1 sequence between the RzM6-LC and RzM6-0d cells (Fig. 4B). In contrast to Sp1, the ability of endogenous MZF-1 to bind to its target sequence (affinity and/or amount) in the RzM6-LC cells was approximately equivalent to that observed in the RzM6-0d cells. Thus, MZF-1 is not likely to be involved in the increase, mediated by HCV expression, in the shifted band corresponding to the response element.

HCV Infection Upregulates the Transcriptional Activity of the DHCR24 Promoter Through HCV Response Element

An in vitro model of HCV infection that replicates the entire HCV life cycle [Wakita et al., 2005] was used to confirm that transcription of *DHCR24* was mediated through the HCV response element. JFH/K4 cells, which show persistent infection with the HCV JFH-1 strain [Wakita et al., 2005], and control cells (cured HuH-7/K4) were transfected with the *DHCR24* promoter reporter plasmids, and promoter activity was measured. While *DHCR24* promoter reporters that included the HCV response element (−515/+113, −200/+113, and −167/+113) displayed significantly higher activity in JFH/K4 cells that in control cells, no difference was seen between the JFH/K4 cells and control cells transfected with the reporter lacking the HCV response element (−144/+113; Fig. 4C). These results suggest that the transcriptional activity of the *DHCR24* promoter was upregulated by HCV infection in a manner dependent on the response elements. Furthermore, augmentation of complex formation with the response element and the Sp1 probe was confirmed by EMSA using nuclear extracts from JFH/K4 and cured HuH-7/K4 cells (Fig. 4D).

Sp1 Binds to the HCV Response Element

The HCV response element (−167/−140) includes candidate-binding motifs for Sp1, MZF-1, and AP-2α (Fig. 4A). However, expression of AP-2 is repressed in the HepG2 cell line from which RzM6-LC cells are derived [Williams et al., 1988]. Thus, binding of AP-2α to the response element was investigated by a supershift assay using anti-Myc and nuclear extract from HepG2 cells transfected with a Myc-tagged AP-2α expression vector (Fig. 5A). The mobility of the DNA-AP-2α complex was supershifted by the addition of anti-Myc (lane 6) but not control IgG (lane 5), whereas an additional shifted band corresponding to the response element was not observed after addition of anti-Myc (lane 3). Therefore, although exogenous AP-2α protein expressed in HepG2 cells binds to the AP-2α consensus sequence, it does not bind to the HCV response element.

The ability of Sp1 to form a DNA–protein complex with the HCV response element was investigated by performing EMSAs in the presence of mithramycin A (MMA)—a GC-specific DNA-binding antibiotic that binds to the GC-box in the promoter to block binding of Sp1 or other Sp family proteins [Blume et al., 1991]. As shown in Fig. 5B, MMA (2.5, 5.0, and 10 μM) inhibited complex formation in a dose-dependent manner. In contrast, the formation of DNA–protein complexes with the MZF-1 probe was not affected by the addition of MMA, suggesting that the inhibition mediated by MMA was specific for the GC box-Sp1, and that complex formation with the response element requires the Sp1 binding site. A supershift assay using nuclear extract from HepG2 cells transfected with a Myc-tagged Sp1 expression vector and anti-Myc was also performed (Supplementary Fig. 2). The mobility of the HCV response element and the Sp1 consensus sequence was supershifted partially by addition of anti-Myc (lanes 3 and 6). The effect of silencing the expression of Sp1 with small interfering RNA (siRNA) was analyzed by EMSA using nuclear extracts from Sp1-knockdown RzM6-0d and RzM6-LC cells (Fig. 5C). DNA–protein complexes with the response element or the Sp1 probe were not observed (lanes 2, 4, 6, and 8); however, formation of DNA–MZF-1 complexes was not influenced by siRNA treatment (lanes 9–12). Immunoblotting was used to confirm efficient silencing of the Sp1 protein in cells used to generate the nuclear extracts (Fig. 5D). A significant decrease in the expression of DHCR24 was observed in the cytosolic fraction from RzM6 cells transfected with siRNA specific for Sp1 (Fig. 5D). Thus, these results indicate that Sp1, but neither AP-2α nor MZF-1, bound to the HCV response element, and that Sp1 may play an important role in the transcriptional regulation of *DHCR24*.

Transcriptional Regulation of DHCR24 Through the HCV Response Element Is Mediated by Oxidative Stress

DHCR24 functions as a mediator of the cellular response to oxidative stress [Greeve et al., 2000; Benvenuti et al., 2005; Di Stasi et al., 2005; Luciani et al., 2005; Lu et al., 2008] and is a hydrogen peroxide scavenger [Lu et al., 2008]. Expression of the *DHCR24* gene is also induced in response to oxidative stress [Wu et al., 2004; Benvenuti et al., 2006; Kuehnle et al., 2008]. Expression of the HCV gene elevates the level of reactive oxygen species (ROS) via dysregulation of ER-mediated calcium homeostasis, which results in oxidative stress [Tardif et al., 2005]. Therefore, the role of oxidative stress induced by HCV in the regulatory mechanism of the expression of *DHCR24* was examined. HepG2 cells were treated with hydrogen peroxide (H_2O_2) and transfected with reporter plasmids containing the *DHCR24* promoter deletion mutants. Measurement of promoter activity revealed a significant increase in transcription in response to oxidative stress (H_2O_2) for *DHCR24* promoters containing the HCV response element (−4976/+113, −2982/+113, −515/+113, and −167/+113) but not for the promoter lacking the response element (−144/+113; Fig. 6A). Therefore, enhanced transcription in response to oxidative stress by reporter constructs

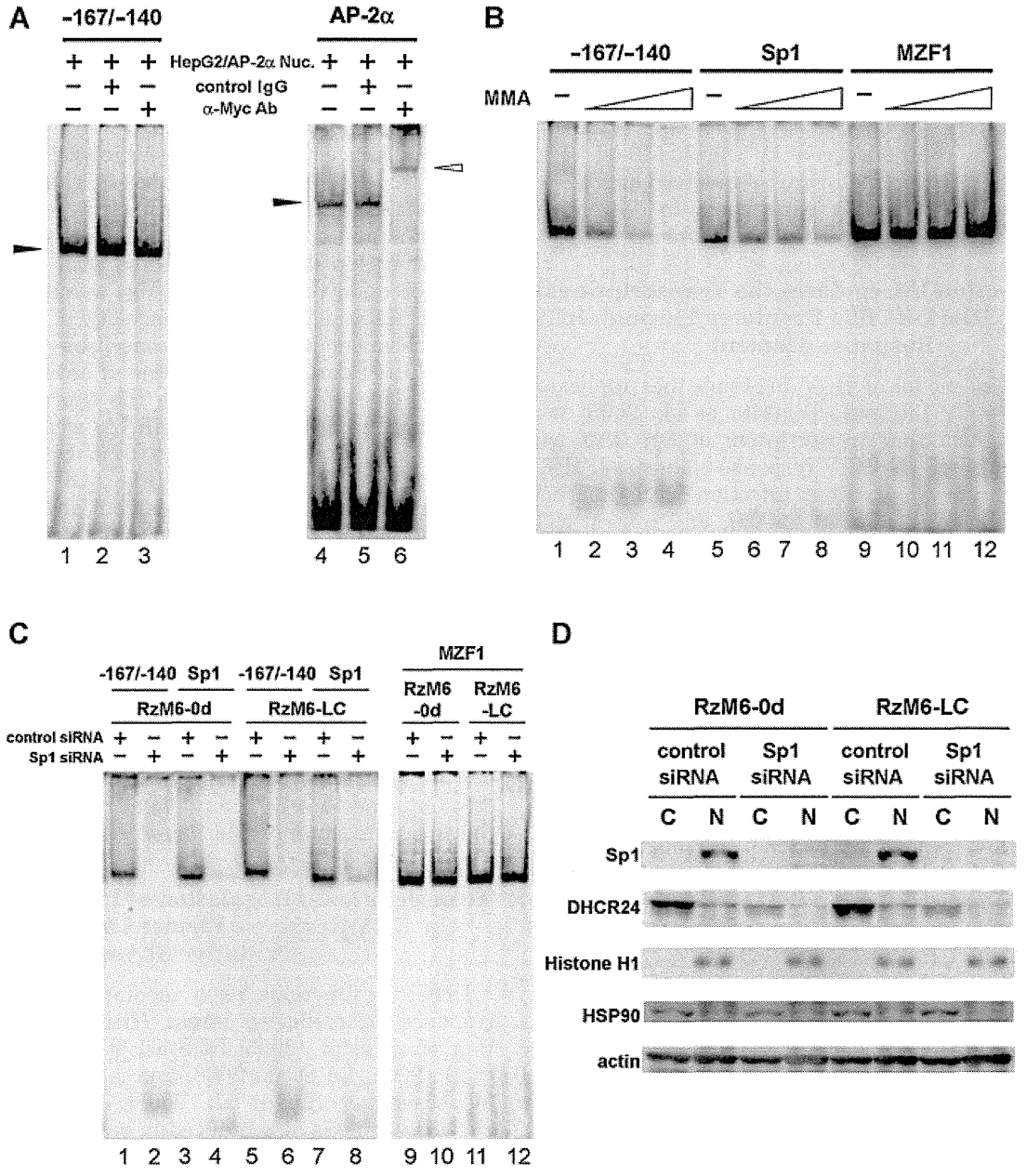

Fig. 5. Sp1 binds to the HCV response element. **A**: Nuclear extract was prepared from HepG2 cells transfected with pcDNA6-AP-2α-myc and subjected to EMSA (10 μg/sample) using DIG-labeled HCV response element or AP-2α probes (26-bp). For a supershift analysis of myc-tagged AP-2α, anti-Myc, or control IgG was added to the binding reaction. The closed arrowhead indicates the interaction between the binding factor(s) and each oligonucleotide, and an additional interaction with antibody is indicated by an open arrowhead. **B**: Nuclear extract from HepG2 cells was pre-incubated at 4°C for 1 h with different concentrations (2.5, 5, and 10 μM) of mithramycin A (MMA) and subjected to EMSA (10 μg/sample) using the DIG-labeled HCV response element, Sp1, or MZF-1 probes. **C**: Nuclear extracts were prepared from RzM6 cells transfected with Sp1 siRNA or control siRNA and subjected to EMSA (10 μg/sample) using the DIG-labeled HCV response element, Sp1, or MZF-1 probes. **D**: Expression of Sp1, DHCR24, and other proteins was detected in both the nuclear fraction (N), used for the EMSA shown in Fig. 4C, and in the cytosolic-membrane fraction (**C**).

containing the *DHCR24* promoter may be mediated through the HCV response element. The formation of complexes containing the response element or Sp1 probe was increased markedly in the nuclear extracts from the H_2O_2-treated HepG2 cells (Fig. 6B) or other hepatic cell lines (Supplementary Fig. 3), suggesting that oxidative stress enhances the binding affinity of Sp1 to the HCV response element.

Overexpression of DHCR24 in M6-LC Cells Is Blocked by an ROS Scavenger

The increase in the expression of DHCR24 induced by oxidative stress can be blocked by treatment with an ROS scavenger, *N*-acetylcysteine (NAC) [Wu et al., 2004], which is a precursor of the potent biological antioxidant glutathione. The H_2O_2-induced overexpression

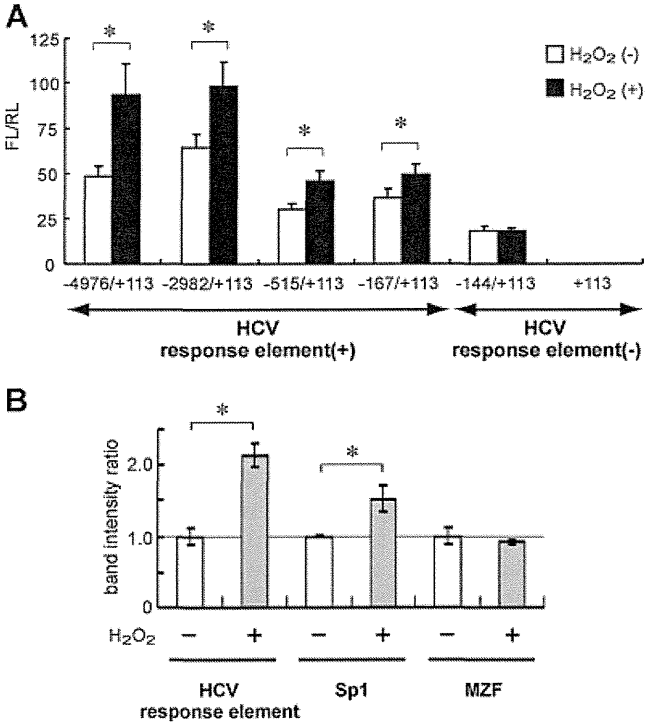

Fig. 6. Oxidative stress increases the transcription of *DHCR24* through the HCV response element and Sp1. **A**: HepG2 cells (1×10^4 cells/well in a 96-well plate) were co-transfected with individual *DHCR24* promoter reporter plasmids (0.5 μg/well) and phRL-TK (0.05 μg/well). Forty-four hours post-transfection, cells were treated with or without 1 mM H_2O_2 for 4 h and analyzed as described in Fig. 2B (*$P < 0.05$). **B**: Nuclear extracts prepared from H_2O_2-treated (1 mM, 4 h) or untreated HepG2 cells were subjected to EMSA (10 μg/sample) using the DIG-labeled HCV response element, Sp1, or MZF-1 probes. Densitometric analysis of shifted bands was performed using the Image Quant software. Data are shown as the mean + SD from triplicate quantifications of two representative experiments (*$P < 0.05$).

of DHCR24 was inhibited by pre-treatment with NAC and blocked partially by NAC treatment after the induction of oxidative stress (~50% suppression; Fig. 7A). The enhanced expression of DHCR24 in RzM6-LC cells decreased after 12 or 24 h of treatment with NAC without influencing the level of expression of HCV, suggesting that overexpression of DHCR24 in cells expressing HCV is mediated through oxidative stress.

Overexpression and Enhanced Phosphorylation of Sp1 in the Cells Expressing HCV

Sp1 is a transcription factor that is activated in response to a variety of cellular stressors, including oxidative stress [Schafer et al., 2003; Chu and Ferro, 2006; Dasari et al., 2006; Qin et al., 2009; Lin et al., 2011]. Thus, Sp1 may play an important role in linking oxidative stress and augmentation of *DHCR24* transcription in cells expressing HCV. Sp1 was overexpressed significantly in RzM6-LC cells treated with H_2O_2 compared with the control cells (Fig. 8A). Phosphorylation of Sp1 at Ser101 was also elevated under oxidative stress. Both the basal level and phosphorylation status of nuclear Sp1 were higher in the presence of HCV (RzM6-LC cells) than in the absence of HCV (RzM6-0d cells; Fig. 8B).

Phosphorylation of Sp1 at Ser101 is a target of the DNA damage signaling pathway mediated by ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) kinases [Olofsson et al., 2007; Iwahori et al., 2008]. As shown in Fig. 8C, phosphorylation of Sp1 at Ser101 was no longer detectable following pretreatment with an ATM kinase inhibitor (KU55933) before exposure to H_2O_2. In contrast, phosphorylation was not affected by other kinase inhibitors (phosphatidylinositol-3 kinase inhibitor, LY294002 or MEK1 inhibitor, PD98059). Similarly, phosphorylation of Sp1 at Thr453, which is important for transcriptional activation of Sp1 [Milanini-Mongiat et al., 2002; D'Addario et al., 2006; Hsu et al., 2006; Lin et al., 2011], was not seen in response to oxidative stress following treatment with KU55933 (Fig. 8C). The induction of expression of DHCR24 after H_2O_2 exposure was suppressed significantly by treatment with KU55933 or NAC, which corresponds with inhibition of Sp1 phosphorylation. In the presence of MMA, the phosphorylation of Sp1 was not inhibited. However, since MMA blocks the binding of Sp1 [Blume et al., 1991], the induction of expression of DHCR24 by H_2O_2 was inhibited. Impairment of DHCR24 induction by H_2O_2 was also observed after treatment with siRNAs targeting ATM (Supplementary Fig. 4).

Studies on the relationship between HCV and ATM have reported that the interaction of NS3/4A with ATM results in delayed de-phosphorylation of both phosphorylated ATM and phosphorylated histone H2AX at Ser139 (γH2AX), which acts as a substrate for ATM in response to DNA damage [Lai et al., 2008]. In the present study, delayed de-phosphorylation of γH2AX was also observed in HCV replicon cells (Supplementary Fig. 5), which corresponded with increased phosphorylation of the H2AX Ser139 residue in cells expressing HCV (Fig. 8). Similarly, phosphorylation of ATM was sustained in HCV replicon cells (Supplementary Fig. 6). Therefore, DNA repair may be impaired in cells expressing or replicating HCV, resulting in sustained DNA damage. As a result, downstream substrates such as Sp1 Ser101 and Thr453 residues or the H2AX Ser139 residue may be phosphorylated to a greater extent in cells expressing HCV compared with control cells in the basal state or cells under oxidative stress (Fig. 8A and B).

Taken together, these results indicate that the oxidative stress induced by HCV may produce quantitative as well as qualitative activation of Sp1, thereby resulting in augmentation of *DHCR24* transcription.

DISCUSSION

HCV establishes chronic infection and induces persistent overexpression of DHCR24 in human hepatocytes [Nishimura et al., 2009]. HCV also confers

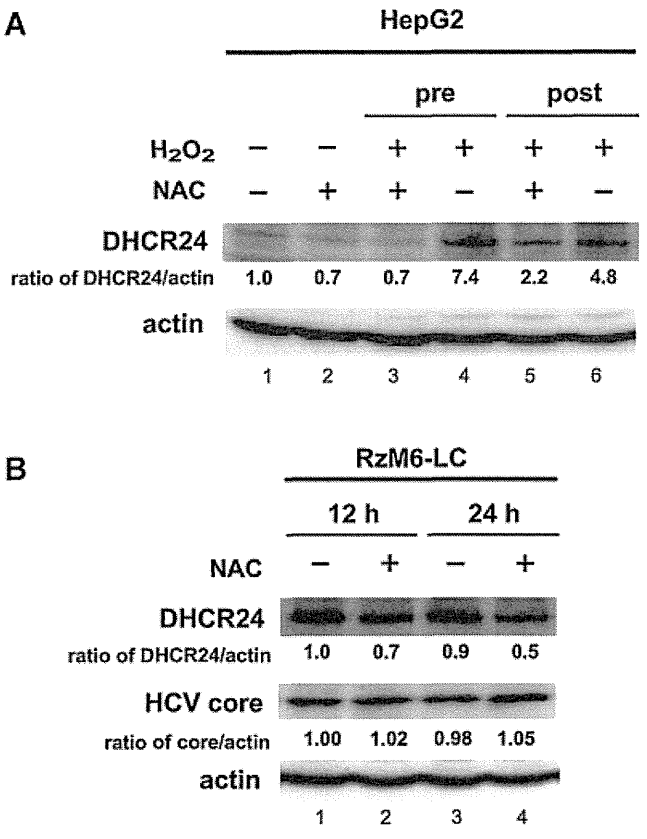

Fig. 7. Overexpression of DHCR24 in the cells expressing HCV is blocked by treatment with an oxidative stress scavenger. **A**: HepG2 cells were treated without (lanes 1, 4, and 6) or with (lanes 2 and 5) NAC (10 mM, 8 h). Cells treated with H_2O_2 (1 mM, 4 h) were also treated with 10 mM NAC for 8 h either before (pre; lanes 3 and 4) or after (post; lanes 5 and 6) H_2O_2 exposure. Whole-cell lysates (40 μg/lane) were analyzed by 10% SDS–PAGE and immunoblotting using a DHCR24/Seladin-1 mAb. Immunoblotting with an actin mAb served as the internal loading control. The ratio of DHCR24/actin was normalized to that of untreated cells (lane 1). **B**: RzM6-LC cells were treated with NAC (10 mM) for 12 h (lane 2) or 24 h (lane 4). Whole-cell lysates were analyzed as described in (A). The ratio of HCV core to actin protein was also calculated. Experiments were performed three times, and representative results are shown.

resistance to the apoptosis induced by oxidative stress and suppresses p53 activity by blocking nuclear p53 acetylation and increasing the interaction between p53 and HDM2 (p53-specific E3 ligase) in the cytoplasm, which may be mediated by inhibition of p53 degradation. Thus, the augmentation of DHCR24 by HCV reflects the tumorigenicity of hepatocytes. The present study identified the genomic region of *DHCR24* that is responsive to HCV, and showed that this response is mediated through the activation of Sp1 induced by oxidative stress. In general, expression of the HCV gene elevates the levels of ROS through dysregulation of ER-mediated calcium homeostasis [Tardif et al., 2005]. In healthy cells, ROS usually exist in equilibrium with antioxidants that scavenge ROS and prevent cellular injury. However, this critical balance may be disrupted in the cells infected with HCV, resulting in the accumulation of ROS and the development of constitutive oxidative stress.

Sp1 is a member of the Sp/KLF family of transcription factors that bind to GC elements of promoters [Black et al., 2001; Kaczynski et al., 2003; Chu and Ferro, 2005; Li and Davie, 2010]. Under a variety of endogenous and exogenous stimuli—including oxidative stress and DNA damage—activation of Sp1 may be mediated via induction of expression of Sp1 and post-translational modifications such as acetylation, sumoylation, O-linked glycosylation, and phosphorylation. Sp1 is phosphorylated by several kinases, including DNA-dependent protein kinase, casein kinase II, and cyclin A/cdk2, which exert both positive and negative effects on transcription [Jackson et al., 1990; Armstrong et al., 1997; Fojas de Borja et al., 2001; Ryu et al., 2003]. Sp1 is the only Sp/KLF family member to contain putative consensus SQ/TQ cluster domains within the transactivation domains, which suggests that Sp1 is a substrate of the PI3K-related kinases, for example, ATM, DNA-dependent protein kinase, and ATR. Indeed, Sp1 is a target of the ATM-dependent DNA damage response pathway [Iwahori et al., 2007, 2008; Olofsson et al., 2007]. ATM plays a central role in orchestrating molecular events involved in double-strand break signaling, which is mediated via the phosphorylation of a variety of substrate proteins—including p53 and BRCA1 transcription factors—involved in the DNA damage response. As a result, these phosphorylation events lead to cell cycle checkpoint activation, DNA repair, altered gene expression patterns, and/or apoptosis [Shiloh, 2006].

Given the role of Sp1 in oxidative stress [Schafer et al., 2003; Chu and Ferro, 2006; Dasari et al., 2006; Rojo et al., 2006; Qin et al., 2009; Lin et al., 2010], Sp1 may be regulated by the oxidative stress induced by HCV and the subsequent phosphorylation, which depends on ATM. However, little is known regarding the regulation of Sp1 in response to DNA damage. Although the precise role of phosphorylation of Sp1 at Ser101 in the DNA damage response is unclear, the similar kinetics of Sp1 and γH2AX phosphorylation [Olofsson et al., 2007] suggest that Sp1 is an early target of the DNA damage response pathway. Thus, Sp1 may be involved in modulating the cellular response to DNA damage to prevent cell death [Ryu et al., 2003]. Phosphorylation of Sp1 at Ser101 and histone H2AX, which occurs in parallel in response to oxidative stress, was enhanced in cells expressing HCV compared with that observed in control cells (Fig. 8A). Interestingly, augmentation of Sp1 phosphorylation in parallel with histone H2AX phosphorylation was also detected for cells expressing HCV in the basal state (Fig. 8A and B), which may be primarily due to the increase in endogenous Sp1 protein (Fig. 8A and B). In support of these results, enhanced phosphorylation of Ser101 on Sp1 occurs upon HSV-1 infection, and is mediated by ATM [Iwahori et al., 2007]. Thus, increased phosphorylation of Sp1 and γH2AX in cells expressing HCV is likely to reflect the higher activity

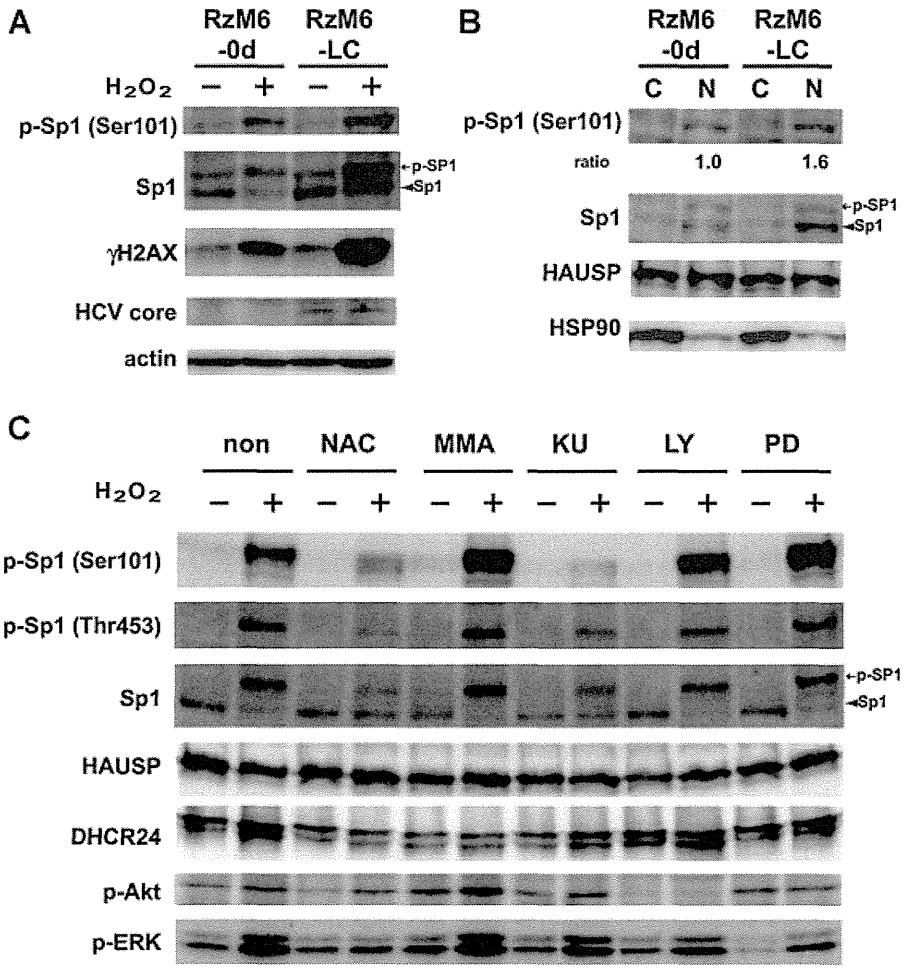

Fig. 8. Overexpression and elevated phosphorylation of Sp1 in the cells expressing HCV. **A**: RzM6-0d and RzM6-LC cells were treated with or without H_2O_2 (1 mM, 4 h). Whole-cell lysates (15 μg/lane) were analyzed by 15% SDS–PAGE and immunoblotting using phospho-H2AX (Ser139) (γH2AX) and HCV core mAbs. An actin mAb served as an internal loading control. Whole-cell lysates (25 μg/lane) were analyzed by 5% SDS–PAGE and immunoblotting using anti-Sp1 (phosphorylated Sp1 and native Sp1, as indicated) and anti-phospho-Sp1 (Ser101) was performed. **B**: RzM6-0d and RzM6-LC cells were fractionated to produce nuclear (N) and cytosolic-membrane fractions (**C**). Fractionated samples (15 μg/lane) were analyzed as described in (**A**). The ratio of phosphorylated Sp1 to Sp1 protein is indicated. Immunoblotting using anti-HAUSP served as a high-molecular-weight loading control. **C**: RzM6-0d cells were pre-treated for 8 h with NAC (10 mM), MMA (10 μM), KU55933 (KU; 10 μM), LY294002 (LY; 50 μM), or PD98059 (PD; 50 μM) and incubated for 4 h in the absence or presence of H_2O_2 (1 mM). Whole-cell lysates (40 μg/lane) were analyzed by 5% SDS–PAGE and immunoblotting using anti-phospho-Sp1 (Ser101), (Thr453), and polyclonal anti-Sp1 (white arrowhead, phosphorylated Sp1; black arrowhead, native Sp1). Detection of HAUSP was performed to confirm the quantity of loaded protein in each lane. Whole-cell lysates (25 μg/lane) were analyzed simultaneously by 10% SDS–PAGE and immunoblotting using anti-DHCR24/seladin-1 mAb, anti-phospho-Akt (Ser473), and anti-phospho-ERK antibodies.

of ATM, which may result from the accumulation and frequency of DNA damage caused by increased generation of endogenous ROS.

Oxidative stress is a common mechanism of liver injury [Loguercio and Federico, 2003] and is mediated by the direct effects of ROS on signal transduction pathways, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinases (MAPKs), which act as downstream kinases in the MAPK cascade to phosphorylate Sp1 Thr453/739 residues [Milanini-Mongiat et al., 2002; D'Addario et al., 2006; Hsu et al., 2006; Chuang et al., 2008; Lin et al., 2011]. These signal transduction pathways are also stimulated by oxidative stress in the hepatic cells expressing or replicating HCV, [Qadri et al., 2004; Burdette et al., 2010; Lin et al., 2010]. Therefore, oxidative stress in response to HCV may induce downstream signaling pathways, such as ERK1/2, JNK, and p38 MAPK as well as ATM/ATR, to activate Sp1 via post-translational modifications.

Sp1 is a host factor activated by several viral proteins, including HIV-1 Vpr, and HTLV-1 Tax [Peng et al., 2003; Amini et al., 2004; Chang et al., 2005; Zhang et al., 2009]. The HCV core and NS5A proteins also activate Sp1 [Lee et al., 2001; Xiang et al., 2010]. The HCV core upregulates the DNA-binding activity and phosphorylation of Sp1 [Lee et al., 2001], and NS5A may also exert a similar effect on Sp1 activity. However, a physical interaction between these

proteins and Sp1 has not yet been demonstrated. Both HCV core and NS5A proteins have a high potential for oxidative stress induction [García-Mediavilla et al., 2005; Dionisio et al., 2009], which may mediate activation of Sp1. On the other hand, individual viral proteins were insufficient to increase the expression of DHCR24 (Fig. 1A). Therefore, in addition to induction of oxidative stress by each viral protein, the persistence of the signaling pathways induced by oxidative stress, for example, ATM (Supplementary Fig. 6), may also be required for the Sp1-mediated increase in the expression of DHCR24.

The results of the present study revealed that knockdown of expression of Sp1 almost completely blocked the enhanced expression of DHCR24. Sp1 is expressed ubiquitously in various mammalian cells and is involved in regulating the transcriptional activity of genes implicated in many cellular processes [Black et al., 2001; Kaczynski et al., 2003; Chu and Ferro, 2005; Li and Davie, 2010]. Thus, Sp1 may represent an essential master regulator among the myriad of transcription factors involved in the direct regulation of *DHCR24* transcription.

In conclusion, HCV was shown to enhance the expression of DHCR24 via the activation of Sp1, which may shed light on the mechanism of tumorigenesis associated with HCV.

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REFERENCES

Amini S, Saunders M, Kelley K, Khalili K, Sawaya BE. 2004. Interplay between HIV-1 Vpr and Sp1 modulates p21(WAF1) gene expression in human astrocytes. J Biol Chem 279:46046–46056.

Armstrong SA, Barry DA, Leggett RW, Mueller CR. 1997. Casein kinase II-mediated phosphorylation of the C terminus of Sp1 decreases its DNA binding activity. J Biol Chem 272:13489–13495.

Benvenuti S, Luciani P, Vannelli GB, Gelmini S, Franceschi E, Serio M, Peri A. 2005. Estrogen and selective estrogen receptor modulators exert neuroprotective effects and stimulate the expression of selective Alzheimer's disease indicator-1, a recently discovered antiapoptotic gene, in human neuroblast long-term cell cultures. J Clin Endocrinol Metab 90:1775–1782.

Benvenuti S, Saccardi R, Luciani P, Urbani S, Deledda C, Cellai I, Francini F, Squecco R, Rosati F, Danza G, Gelmini S, Greeve I, Rossi M, Maggi R, Serio M, Peri A. 2006. Neuronal differentiation of human mesenchymal stem cells: Changes in the expression of the Alzheimer's disease-related gene seladin-1. Exp Cell Res 312:2592–2604.

Black AR, Black JD, Azizkhan-Clifford J. 2001. Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer. J Cell Physiol 188:143–160.

Blume SW, Snyder RC, Ray R, Thomas S, Koller CA, Miller DM. 1991. Mithramycin inhibits SP1 binding and selectively inhibits transcriptional activity of the dihydrofolate reductase gene in vitro and in vivo. J Clin Invest 88:1613–1621.

Bruchfeld A, Stahle L, Andersson J, Schvarcz R. 2001. Ribavirin treatment in dialysis patients with chronic hepatitis C virus infection—A pilot study. J Viral Hepat 8:287–292.

Burdette D, Olivarez M, Waris G. 2010. Activation of transcription factor Nrf2 by hepatitis C virus induces the cell-survival pathway. J Gen Virol 91:681–690.

Chang LK, Chung JY, Hong YR, Ichimura T, Nakao M, Liu ST. 2005. Activation of Sp1-mediated transcription by Rta of Epstein–Barr virus via an interaction with MCAF1. Nucleic Acids Res 33:6528–6539.

Chu S, Ferro TJ. 2005. Sp1: Regulation of gene expression by phosphorylation. Gene 348:1–11.

Chu S, Ferro TJ. 2006. Identification of a hydrogen peroxide-induced PP1-JNK1-Sp1 signaling pathway for gene regulation. Am J Physiol Lung Cell Mol Physiol 291:L983–992.

Chuang JY, Wang YT, Yeh SH, Liu YW, Chang WC, Hung JJ. 2008. Phosphorylation by c-Jun NH_2-terminal kinase 1 regulates the stability of transcription factor Sp1 during mitosis. Mol Biol Cell 19:1139–1151.

Crameri A, Biondi E, Kuehnle K, Lutjohann D, Thelen KM, Perga S, Dotti CG, Nitsch RM, Ledesma MD, Mohajeri MH. 2006. The role of seladin-1/DHCR24 in cholesterol biosynthesis, APP processing and Abeta generation in vivo. EMBO J 25:432–443.

D'Addario M, Arora PD, McCulloch CA. 2006. Role of p38 in stress activation of Sp1. Gene 379:51–61.

Dasari A, Bartholomew JN, Volonte D, Galbiati F. 2006. Oxidative stress induces premature senescence by stimulating caveolin-1 gene transcription through p38 mitogen-activated protein kinase/Sp1-mediated activation of two GC-rich promoter elements. Cancer Res 66:10805–10814.

Di Stasi D, Vallacchi V, Campi V, Ranzani T, Daniotti M, Chiodini E, Fiorentini S, Greeve I, Prinetti A, Rivoltini L, Pierotti MA, Rodolfo M. 2005. DHCR24 gene expression is upregulated in melanoma metastases and associated to resistance to oxidative stress-induced apoptosis. Int J Cancer 115:224–230.

Dignam JD, Lebovitz RM, Roeder RG. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489.

Dionisio N, Garcia-Mediavilla MV, Sanchez-Campos S, Majano PL, Benedicto I, Rosado JA, Salido GM, Gonzalez-Gallego J. 2009. Hepatitis C virus NS5A and core proteins induce oxidative stress-mediated calcium signalling alterations in hepatocytes. J Hepatol 50:872–882.

Fojas de Borja P, Collins NK, Du P, Azizkhan-Clifford J, Mudryj M. 2001. Cyclin A-CDK phosphorylates Sp1 and enhances Sp1-mediated transcription. EMBO J 20:5737–5747.

García-Mediavilla MV, Sánchez-Campos S, González-Pérez P, Gómez-Gonzalo M, Majano PL, Lopez-Cabrera M, Clemente G, García-Monzon C, González-Gallego J. 2005. Differential contribution of hepatitis C virus NS5A and core proteins to the induction of oxidative and nitrosative stress in human hepatocyte-derived cells. J Hepatol 43:606–613.

Greeve I, Hermans-Borgmeyer I, Brellinger C, Kasper D, Gomez-Isla T, Behl C, Levkau B, Nitsch RM. 2000. The human DIMINUTO/DWARF1 homolog seladin-1 confers resistance to Alzheimer's disease-associated neurodegeneration and oxidative stress. J Neurosci 20:7345–7352.

Hsu MC, Chang HC, Hung WC. 2006. HER-2/neu represses the metastasis suppressor RECK via ERK and Sp transcription factors to promote cell invasion. J Biol Chem 281:4718–4725.

Iwahori S, Shirata N, Kawaguchi Y, Weller SK, Sato Y, Kudoh A, Nakayama S, Isomura H, Tsurumi T. 2007. Enhanced phosphorylation of transcription factor sp1 in response to herpes simplex virus type 1 infection is dependent on the ataxia telangiectasia-mutated protein. J Virol 81:9653–9664.

Iwahori S, Yasui Y, Kudoh A, Sato Y, Nakayama S, Murata T, Isomura H, Tsurumi T. 2008. Identification of phosphorylation sites on transcription factor Sp1 in response to DNA damage and its accumulation at damaged sites. Cell Signal 20:1795–1803.

Jackson SP, MacDonald JJ, Lees-Miller S, Tjian R. 1990. GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. Cell 63:155–165.

Kaczynski J, Cook T, Urrutia R. 2003. Sp1- and Kruppel-like transcription factors. Genome Biol 4:206.

Kohara M, Tanaka T, Tsukiyama-Kohara K, Tanaka S, Mizokami M, Lau JY, Hattori N. 1995. Hepatitis C virus genotypes 1 and 2 respond to interferon-alpha with different virologic kinetics. J Infect Dis 172:934–938.

Koike K. 2007. Hepatitis C virus contributes to hepatocarcinogenesis by modulating metabolic and intracellular signaling pathways. J Gastroenterol Hepatol 22:S108–S111.

Kuehnle K, Crameri A, Kalin RE, Luciani P, Benvenuti S, Peri A, Ratti F, Rodolfo M, Kulic L, Heppner FL, Nitsch RM, Mohajeri MH. 2008. Prosurvival effect of DHCR24/Seladin-1 in acute and chronic responses to oxidative stress. Mol Cell Biol 28:539–550.

Lai CK, Jeng KS, Machida K, Cheng YS, Lai MM. 2008. Hepatitis C virus NS3/4A protein interacts with ATM, impairs DNA repair and enhances sensitivity to ionizing radiation. Virology 370: 295–309.

Lavanchy D. 2009. The global burden of hepatitis C. Liver Int 29: 74–81.

Lee S, Park U, Lee YI. 2001. Hepatitis C virus core protein transactivates insulin-like growth factor II gene transcription through acting concurrently on Egr1 and Sp1 sites. Virology 283:167–177.

Li L, Davie JR. 2010. The role of Sp1 and Sp3 in normal and cancer cell biology. Ann Anat 192:275–283.

Lin W, Tsai WL, Shao RX, Wu G, Peng LF, Barlow LL, Chung WJ, Zhang L, Zhao H, Jang JY, Chung RT. 2010. Hepatitis C virus regulates transforming growth factor beta1 production through the generation of reactive oxygen species in a nuclear factor kappaB-dependent manner. Gastroenterology 138:2509–2518.

Lin HH, Lai SC, Chau LY. 2011. Heme oxygenase-1/carbon monoxide induces vascular endothelial growth factor expression via p38 kinase-dependent activation of Sp1. J Biol Chem 286:3829–3838.

Loguercio C, Federico A. 2003. Oxidative stress in viral and alcoholic hepatitis. Free Radic Biol Med 34:1–10.

Lu X, Kambe F, Cao X, Kozaki Y, Kaji T, Ishii T, Seo H. 2008. 3Beta-hydroxysteroid-delta24 reductase is a hydrogen peroxide scavenger, protecting cells from oxidative stress-induced apoptosis. Endocrinology 149:3267–3273.

Luciani P, Gelmini S, Ferrante E, Lania A, Benvenuti S, Baglioni S, Mantovani G, Cellai I, Ammannati F, Spada A, Serio M, Peri A. 2005. Expression of the antiapoptotic gene seladin-1 and octreotide-induced apoptosis in growth hormone-secreting and nonfunctioning pituitary adenomas. J Clin Endocrinol Metab 90: 6156–6161.

Milanini-Mongiat J, Pouyssegur J, Pages G. 2002. Identification of two Sp1 phosphorylation sites for p42/p44 mitogen-activated protein kinases: Their implication in vascular endothelial growth factor gene transcription. J Biol Chem 277:20631–20639.

Nakamura H, Ogawa H, Kuroda T, Yamamoto M, Enomoto H, Kishima Y, Yoshida K, Ito H, Matsuda M, Noguchi S. 2002. Interferon treatment for patients with chronic hepatitis C infected with high viral load of genotype 2 virus. Hepatogastroenterology 49:1373–1376.

Nishimura T, Kohara M, Izumi K, Kasama Y, Hirata Y, Huang Y, Shuda M, Mukaidani C, Takano T, Tokunaga Y, Nuriya H, Satoh M, Saito M, Kai C, Tsukiyama-Kohara K. 2009. Hepatitis C virus impairs p53 via persistent overexpression of 3beta-hydroxysterol Delta24-reductase. J Biol Chem 284:36442–36452.

Olofsson BA, Kelly CM, Kim J, Hornsby SM, Azizkhan-Clifford J. 2007. Phosphorylation of Sp1 in response to DNA damage by ataxia telangiectasia-mutated kinase. Mol Cancer Res 5:1319–1330.

Peng H, He H, Hay J, Ruyechan WT. 2003. Interaction between the varicella zoster virus IE62 major transactivator and cellular transcription factor Sp1. J Biol Chem 278:38068–38075.

Qadri I, Iwahashi M, Capasso JM, Hopken MW, Flores S, Schaack J, Simon FR. 2004. Induced oxidative stress and activated expression of manganese superoxide dismutase during hepatitis C virus replication: Role of JNK, p38 MAPK and AP-1. Biochem J 378:919–928.

Qin K, Zhao L, Ash RD, McDonough WF, Zhao RY. 2009. ATM-mediated transcriptional elevation of prion in response to copper-induced oxidative stress. J Biol Chem 284:4582–4593.

Rojo AI, Salina M, Salazar M, Takahashi S, Suske G, Calvo V, de Sagarra MR, Cuadrado A. 2006. Regulation of heme oxygenase-1 gene expression through the phosphatidylinositol 3-kinase/PKC-zeta pathway and Sp1. Free Radic Biol Med 41:247–261.

Ryu H, Lee J, Zaman K, Kubilis J, Ferrante RJ, Ross BD, Neve R, Ratan RR. 2003. Sp1 and Sp3 are oxidative stress-inducible, antideath transcription factors in cortical neurons. J Neurosci 23: 3597–3606.

Schafer G, Cramer T, Suske G, Kemmner W, Wiedenmann B, Hocker M. 2003. Oxidative stress regulates vascular endothelial growth factor-A gene transcription through Sp1- and Sp3-dependent activation of two proximal GC-rich promoter elements. J Biol Chem 278:8190–8198.

Shiloh Y. 2006. The ATM-mediated DNA-damage response: Taking shape. Trends Biochem Sci 31:402–410.

Takano T, Kohara M, Kasama Y, Nishimura T, Saito M, Kai C, Tsukiyama-Kohara K. 2011a. Translocase of outer mitochondrial membrane 70 expression is induced by hepatitis C virus and is related to the apoptotic response. J Med Virol 83:801–809.

Takano T, Tsukiyama-Kohara K, Hayashi M, Hirata Y, Satoh M, Tokunaga Y, Tateno C, Hayashi Y, Hishima T, Funata N, Sudo M, Kohara M. 2011b. Augmentation of DHCR24 expression by hepatitis C virus infection facilitates viral replication in hepatocytes. J Hepatol 55:512–521.

Tardif KD, Waris G, Siddiqui A. 2005. Hepatitis C virus, ER stress, and oxidative stress. Trends Microbiol 13:159–163.

Tsukiyama-Kohara K, Tone S, Maruyama I, Inoue K, Katsume A, Nuriya H, Ohmori H, Ohkawa J, Taira K, Hoshikawa Y, Shibasaki F, Reth M, Minatogawa Y, Kohara M. 2004. Activation of the CKI-CDK-Rb-E2F pathway in full genome hepatitis C virus-expressing cells. J Biol Chem 279:14531–14541.

Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 11:791–796.

Waterham HR, Koster J, Romeijn GJ, Hennekam RC, Vreken P, Andersson HC, FitzPatrick DR, Kelley RI, Wanders RJ. 2001. Mutations in the 3beta-hydroxysterol Delta24-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. Am J Hum Genet 69:685–694.

Williams T, Admon A, Luscher B, Tjian R. 1988. Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements. Genes Dev 2:1557–1569.

Wu C, Miloslavskaya I, Demontis S, Maestro R, Galaktionov K. 2004. Regulation of cellular response to oncogenic and oxidative stress by Seladin-1. Nature 432:640–645.

Xiang Z, Qiao L, Zhou Y, Babiuk LA, Liu Q. 2010. Hepatitis C virus nonstructural protein-5A activates sterol regulatory element-binding protein-1c through transcription factor Sp1. Biochem Biophys Res Commun 402:549–553.

Zhang L, Zhi H, Liu M, Kuo YL, Giam CZ. 2009. Induction of p21(CIP1/WAF1) expression by human T-lymphotropic virus type 1 Tax requires transcriptional activation and mRNA stabilization. Retrovirology 6:35.

Self-Enhancement of Hepatitis C Virus Replication by Promotion of Specific Sphingolipid Biosynthesis

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Abstract

Lipids are key components in the viral life cycle that affect host-pathogen interactions. In this study, we investigated the effect of HCV infection on sphingolipid metabolism, especially on endogenous SM levels, and the relationship between HCV replication and endogenous SM molecular species. We demonstrated that HCV induces the expression of the genes (*SGMS1* and *2*) encoding human SM synthases 1 and 2. We observed associated increases of both total and individual sphingolipid molecular species, as assessed in human hepatocytes and in the detergent-resistant membrane (DRM) fraction in which HCV replicates. SGMS1 expression had a correlation with HCV replication. Inhibition of sphingolipid biosynthesis with a hepatotropic serine palmitoyltransferase (SPT) inhibitor, NA808, suppressed HCV-RNA production while also interfering with sphingolipid metabolism. Further, we identified the SM molecular species that comprise the DRM fraction and demonstrated that these endogenous SM species interacted with HCV nonstructural 5B polymerase to enhance viral replication. Our results reveal that HCV alters sphingolipid metabolism to promote viral replication, providing new insights into the formation of the HCV replication complex and the involvement of host lipids in the HCV life cycle.

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Introduction

Lipids have long been known to play dual roles in biological systems, functioning in structural (in biological membranes) and energy storage (in cellular lipid droplets and plasma lipoproteins) capacities. Research over the past few decades has identified additional functions of lipids related to cellular signaling, microdomain organization, and membrane traffic. There are also strong indications of the important role of lipids in various stages of host-pathogen interactions [1].

Sphingomyelin (SM) is a sphingolipid that interacts with cholesterol and glycosphingolipid during formation of the raft domain, which can be extracted for study as a detergent-resistant membrane (DRM) fraction [2]. Recently, raft domains have drawn attention as potential platforms for signal transduction and pathogen infection processes [3,4]. For instance, raft domains may serve as sites for hepatitis C virus (HCV) replication [5,6]. Additionally, *in vitro* analysis indicates that synthetic SM binds to the nonstructural 5B polymerase (RdRp) of HCV [7]. This association allows RdRp to localize to the DRM fraction (known to be the site of HCV replication) and activates RdRp, although the degree of binding and activation differs among HCV genotypes [7,8]. Indeed, suppression of SM biosynthesis with a serine palmitoyltransferase (SPT) inhibitor disrupts the association between RdRp and SM in the DRM fraction, resulting in the suppression of HCV replication [7,9].

Multiple reports have indicated that HCV modulates lipid metabolism (e.g., cholesterol and fatty acid biosynthesis) to promote viral replication [10–12]. However, the effect of HCV infection on sphingolipid metabolism, especially on endogenous SM levels, and the relationship between HCV replication and endogenous SM molecular species remain to be elucidated as there are technical challenges in measuring SM levels (for both total and individual molecular species) in hepatocytes.

To address these questions, we first utilized mass spectrometry (MS)-based techniques and analyzed uninfected and HCV-