liver [28], HCV infection induced the up-regulation of DHCR24 expression in human hepatocytes, whereas hepatitis B virus (HBV) infection had no significant effect on DHCR24 expression [16]. The regulation of DHCR24 expression was elicited at the transcriptional level. Therefore, we cloned the 5′-flanking region of the predicted genomic promoter region of *DHCR24* (~5 kb) and characterized the promoter activity by construction of promoter reporter plasmids [27]. We transfected each HCV protein (core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B) or the full-genome HCV. The full-genome HCV induced significantly higher DHCR24 expression than other HCV viral proteins. The serial deletion mutants of the 5′-flanking region of *DHCR24* revealed that the minimum responsive element to the full-genome HCV was between −167 and −140 of the *DHCR24* gene. An electronic mobility shift assay (EMSA) identified that the specific binding factor to this element was the Sp1 transcription factor.

Transcription of *DHCR24* was induced by oxidative stress and impaired by the removal of the HCV minimum responsive element. Furthermore, the augmentation of *DHCR24* expression was impaired by treatment with a ROS scavenger, *N*-acetylcysteine. We then explored the role of the Sp1 transcription factor in the regulation of *DHCR24* expression. Phosphorylation of Sp1 at Ser101 was elevated under oxidative stress and increased by the presence of HCV. This phosphorylation of Sp1 was mediated through ataxia telangiectasia mutated (ATM) kinase [29,30]. Sustained phosphorylation of ATM and delayed de-phosphorylation of histone H2AX at Ser139 (γH2AX) were observed in HCV replicon cells [27,31], indicating that DNA repair was impaired in cells expressing or replicating HCV.

Previous studies revealed that expression of the HCV gene elevates the level of ROS via dysregulation of ER-mediated calcium homeostasis, which results in oxidative stress [32]. Also, the HCV core protein inhibits mitochondrial electron transport and increases ROS [33]. Recently, HCV infection is reported to increase ROS production through NADPH oxidase activity, especially elevated NADPH oxidase 4 (Nox4) [34]. The production of ROS can induce DHCR24 expression [27]. Thus, our results raised the possibility that DHCR24 plays a role in response to ROS generated as a consequence of HCV infection, thereby suppressing DNA repair and promoting tumorigenicity.

4. Overexpression of DHCR24 Results in Impairment of p53 Activity

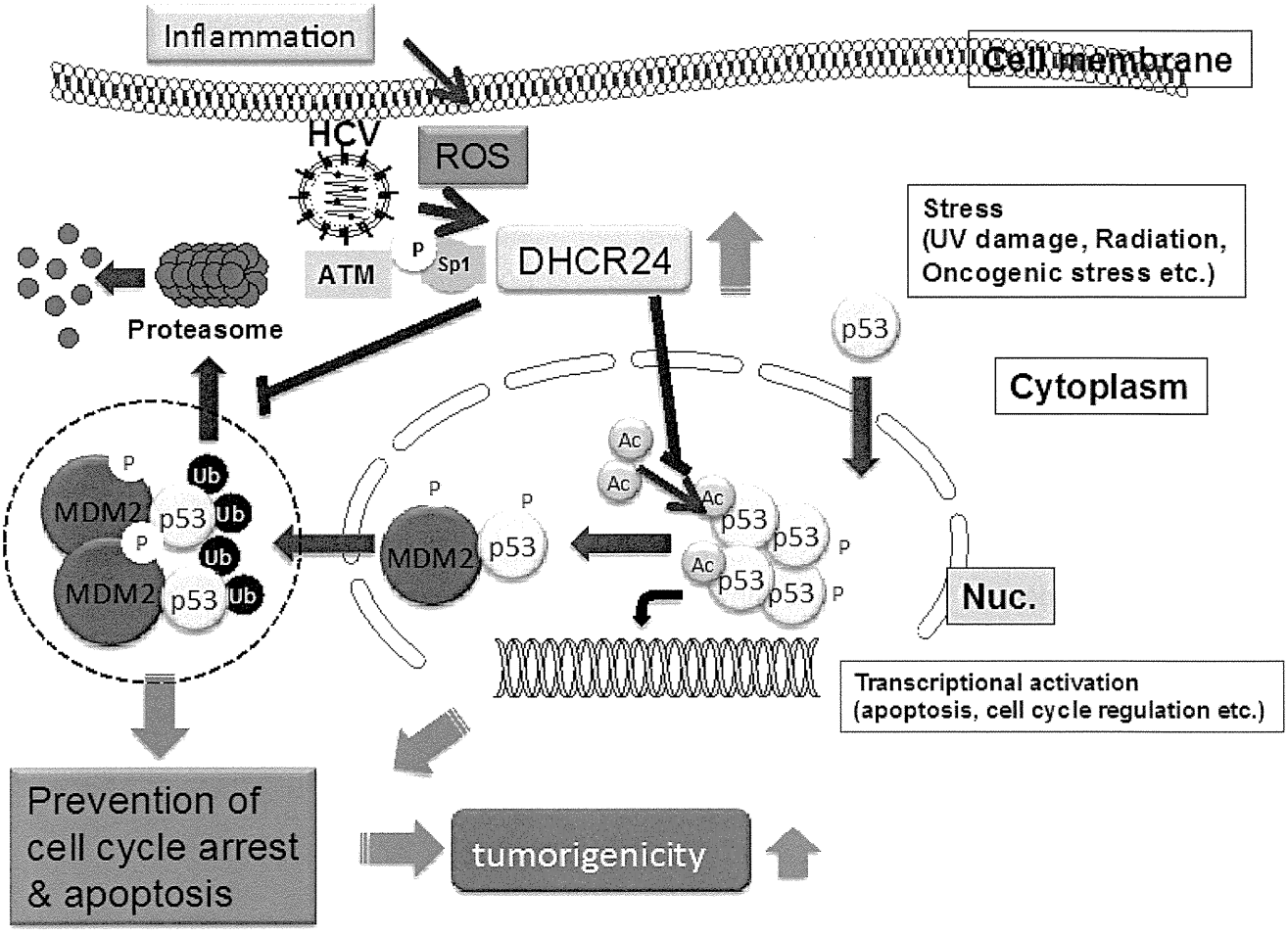
HCV gene expression or infection persistently induces over-expression of DHCR24 [16,27] in its turn induces apoptotic resistance to oxidative stress (Figure 1).

HCV gene expression elevates the levels of ROS through dysregulation of ER-mediated calcium homeostasis. This increases the level of SP1 phosphorylation by ATM kinase, and results in the transcriptional activation of the *DHCR24* gene. The augmentation of DHCR24 by HCV 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. This impairment of p53 activity may result in apoptotic resistance and increased tumorigenicity.

To further examine this mechanism, we characterized the regulatory proteins involved in the oxidative stress-induced apoptotic response and found that p53 activity was impaired in response to hydrogen peroxide, which was clarified by a $p21^{WAF1/CIP1}$ promoter reporter assay. The post-translational modification of p53 after hydrogen peroxide treatment was characterized, and we found that the acetylation of p53 at Lys^{373} and Lys^{382} was impaired by the over-expression of DHCR24. The decreased level of p53 acetylation may impair p53 sequence-specific DNA-binding activity [35] and stability [36,37].

Moreover, interaction of p53 with its specific E3 ubiquitin ligase MDM2 (also known as HDM2) in the cytoplasm was augmented. These results strongly suggest that the increased interaction between p53 and MDM2, in the cytoplasm, impaired both the nuclear translocation and the activity of p53. This interaction between p53 and MDM2 was regulated by mitogen-activated protein kinase/extracellular signal-regulated kinase kinase extracellular signal-regulated kinase (MEK-ERK)-induced phosphorylation at Ser^{166} in the MDM2 protein. Interestingly, MEK-ERK phosphorylation of MDM2 was liver specific [38].

Figure 1. Elevation of tumorigenicity in HCV infected hepatocytes through increased oxidative stress and DHCR24.



5. Conclusion

The results of our studies showed a novel HCV-induced pathway that activates DHCR24 in response to oxidative stress. Overexpression of DHCR24 by HCV contributed to the development of HCC during persistent HCV infections. Recently, we found that silencing of DHCR24 by siRNA suppresses HCV replication [39] and an inhibitor of DHCR24 (U18666A) had an anti-viral effect *in vivo*. Monoclonal antibodies to DHCR24 (2-152a) suppress HCV replication through the betaine GABA transporter-1 (BGT-1) [40]. Thus, DHCR24 is involved in HCV replication and pathogenicity. DHCR24 catalyzes the reduction of the delta-24 bond of the sterol intermediate and works further downstream of farnesyl pyrophosphate, and therefore does not influence geranylgeranylation. Our findings may indicate the

possible existence of a regulatory pathway of HCV replication by cholesterol synthesis and trafficking through DHCR24 in addition to protein geranylgeranylation. DHCR24 deficiency reduces cholesterol levels and disorganizes cholesterol-rich detergent-resistant membrane domains (DRMs) in mouse brains. Additionally, the HCV replication complex has been detected in the DRM fraction. Therefore, a deficiency in DRM, induced by silencing of DHCR24, may suppress HCV replication. In addition, BGT-1 plays a role in tonicity regulation and hyper-osmolarity [41], and recent reports show that hyperosmotic shrinkage stimulates duck hepatitis B virus replication [42]. BGT-1 is involved in sodium and chloride coupled betaine uptake and betaine levels affect lipid distribution even to such an extent that low plasma betaine levels correlate with unfavorable lipid profiles [43]. Future study will clarify the regulatory role of DHCR24 and BGT-1 in HCV replication.

In conclusion, the results of our studies suggest that HCV infected cells may become anti-apoptotic and replicate efficiently to establish chronic infection through over-expression of DHCR24. Thus, the HCV-induced oxidative stress responsive protein DHCR24 may play a critical role in the pathogenesis of HCV persistent infections.

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Impairment of interferon regulatory factor-3 activation by hepatitis C virus core protein basic amino acid region 1

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ABSTRACT

Interferon regulatory factor-3 (IRF-3), a key transcriptional factor in the type I interferon system, is frequently impaired by hepatitis C virus (HCV), in order to establish persistent infection. However, the exact mechanism by which the virus establishes persistent infection has not been fully understood yet. The present study aimed to investigate the effects of various HCV proteins on IRF-3 activation, and elucidate the underlying mechanisms. To achieve this, full-length HCV and HCV subgenomic constructs corresponding to structural and each of the nonstructural proteins were transiently transfected into HepG2 cells. IFN-β induction, plaque formation, and IRF-3 dimerization were elicited by Newcastle disease virus (NDV) infection. The expressions of IRF-3 homodimer and its monomer, Ser386-phosphorylated IRF-3, and HCV core protein were detected by immunofluorescence and western blotting. IFN-β mRNA expression was quantified by real-time PCR (RT-PCR), and IRF-3 activity was measured by the levels of IRF-3 dimerization and phosphorylation, induced by NDV infection or polyriboinosinic:polyribocytidylic acid [poly(I:C)]. Switching of the expression of the complete HCV genome as well as the core proteins, E1, E2, and NS2, suppressed IFN-β mRNA levels and IRF-3 dimerization, induced by NDV infection. Our study revealed a crucial region of the HCV core protein, basic amino acid region 1 (BR1), to inhibit IRF-3 dimerization as well as its phosphorylation induced by NDV infection and poly (I:C), thus interfering with IRF-3 activation. Therefore, our study suggests that rescue of the IRF-3 pathway impairment may be an effective treatment for HCV infection.

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1. Introduction

Hepatitis C virus (HCV), a flavivirus comprising a positive-sense, single-stranded RNA (ssRNA) of approximately 9.6 kb [1], causes persistent disease in infected individuals, possibly leading to chronic liver injury [2]. Despite the approximately 170 million individuals worldwide suffering from HCV infection that ranges from chronic hepatitis to hepatocellular carcinoma (HCC) [3,4], the exact mechanism by which the virus establishes persistent infection is not fully resolved.

The innate immune system is activated immediately upon infection as the first line of host defense against invading pathogens, with type I interferon (IFN) signaling being the crucial step in the antiviral response [5]. The IFN system is, therefore, a prime target of HCV and other viruses in order to establish persistent infections [6], wherein the disruption of the type I IFN-activation pathway forms the most efficient strategy for HCV. Studies on HCV IFN-interference mechanisms have revealed that the HCV proteins NS5A and E2 selectively inhibit the double-stranded RNA-activated protein kinase (PKR) [7,8], an IFN-inducible antiviral molecule that controls transcription and translation [6]. IFN-β, a crucial molecule in type I IFN signaling, is regulated by several cellular factors associated with the activation of interferon regulatory factor-3 (IRF-3), leading to its rapid induction following viral infection [9,10]. However, IFN-β induction is impaired in HCV-infected cells, thus resulting in the disruption of IFN downstream signaling cascade [11].

IRF-3, a key constitutively expressed transcriptional factor localized in the cytoplasm in its inactive form [9], is activated upon

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phosphorylation, whereby it translocates to the nucleus to act as a transcriptional factor for positive regulatory domain (PRD) I of the IFN-β promoter. IRF-3 activation also induces phosphorylation of Ser385 and Ser386 or the serine/threonine (Ser/Thr) cluster between amino acids (aa) 396 and 405 (located at the C-terminus of IRF-3), a step that is essential for dimerization and nuclear translocation.

A previous study by Foy et al. showed that the NS3/4A serine protease derived from a subgenomic replicon participates in the suppression of the cellular pathway that activates IRF-3 [12]. The NS3/4A protein of HCV disrupts signaling of the double-stranded RNA (dsRNA) receptors, retinoic acid-inducible gene-I (RIG-I), and Toll-like receptor 3 (TLR3) by inducing proteolysis of interferon promoter stimulator-1 (IPS-1) [13–15] and Toll/interleukin-1 receptor (TIR) domain-containing adaptor protein inducing IFN-β (TRIF) [16], as well as by suppressing the downstream activation of IFN-β [13].

In view of the above observations and the emerging data on the role of HCV in regulating the IRF-3 pathway by additional mechanisms, we aimed to investigate the effects of various HCV proteins on IRF-3 activation, and further elucidate the underlying novel mechanisms.

2. Material and methods

2.1. Transient expression of the HCV core proteins E1, E2, and NS3-4A

HepG2 cells were transfected to express E1, E2, or NS3-4A HCV core protein under the control of EF promoter (Invitrogen). The HCV core expression vectors were derived from HCR6 (genotype 1b), HCR24-12K (genotype 2a), or HCR24-12Q (genotype 2a). The E1, E2, and NS3 clones derived from HCR6 contained either the full-length cDNAs encoding the core protein or 1 of the 3 different deletions (deletion mutants), each of which lacked 1 of the 3 basic amino acid regions (BR), BR1 deletion (aa 4–14), BR2 deletion (aa 37–44), and BR3 deletion (aa 57–72) [17]. HepG2 cells were transfected with 4 μg of the core cDNA (amino acids 1–191), E1 (amino

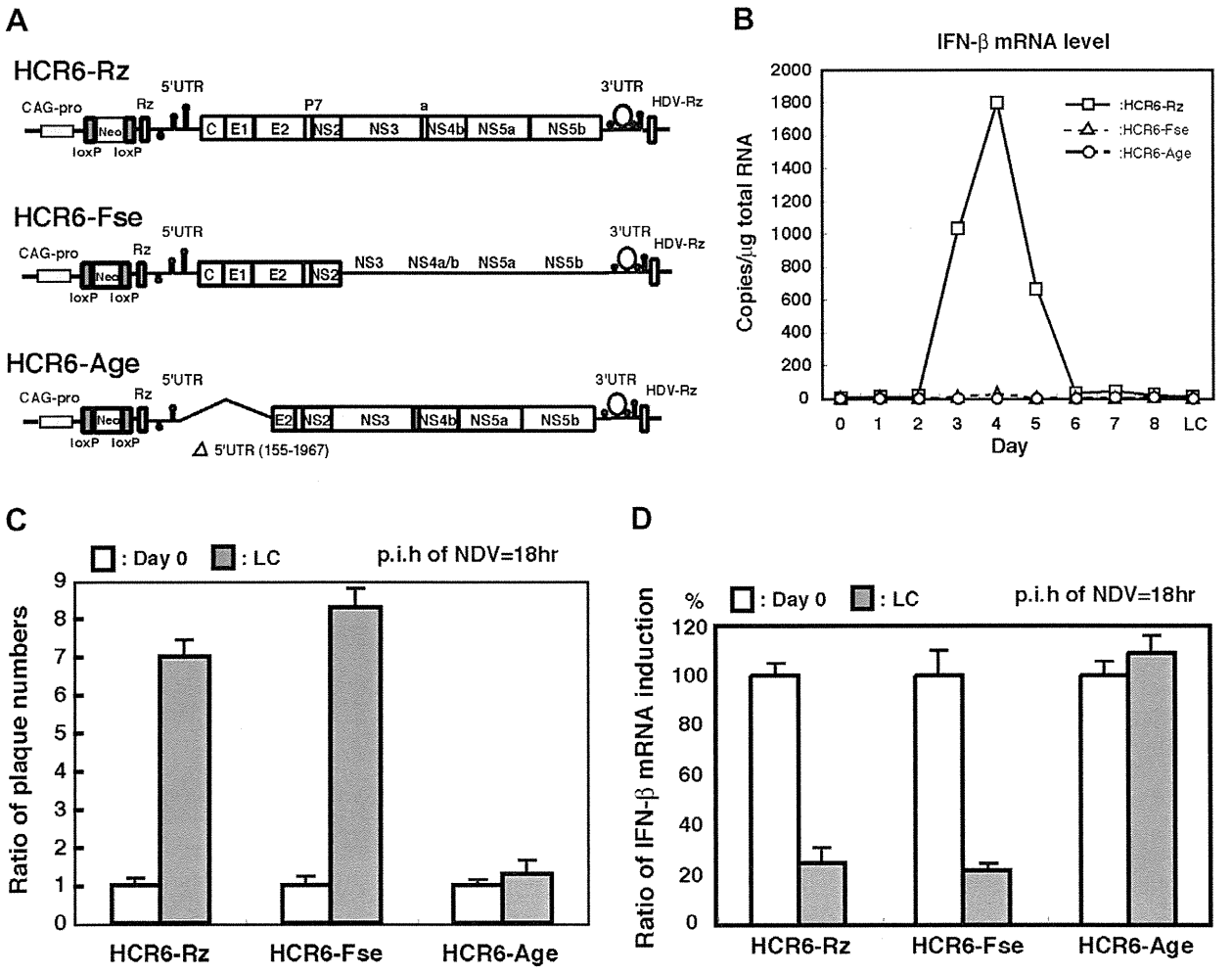

Fig. 1. (A) Structures of the conditional expression vectors for HCV RNAs and proteins. The cDNA clones that displayed highest level of homology to the consensus sequences among the 3 clones were used to construct *HCR6-Rz* (nt 1–9611). *HCR6-Fse* clone harbored a termination codon introduced at nucleotide 3606 and truncated *HCR6-Age* clone lacked nucleotides 155–1967. They were flanked with ribozyme (Rz) and hepatitis D virus ribozyme (HDV-Rz) sequences under the control of the CAG promoter in the Cre/*lox*P switching expression cassette, which consisted of the neomycin resistance gene, as a stuffer region flanked by the *lox*P sequence. (B) IFN-β mRNA levels in the cell lines *HCR6-Rz*, *HCR6-Fse*, and *HCR6-Age* before and after the expression of the HCV protein. The results are expressed as copy numbers per microgram of total RNA, as quantified by RT-PCR. (C) Efficiency of NDV plaque formation. Plaque assays were performed on Vero cells for NDV infectivity in *HCR6-Rz*, *HCR6-Fse*, or *HCR6-Age* before and after the expression of the HCV protein. The plaque numbers were counted 3 days after NDV inoculation. The ratio indicates the plaque numbers after the expression of the HCV genome divided by the plaque numbers before the expression of the HCV genome. (D) Suppression of IFN-β mRNA induction by HCV expression, 18 days after NDV inoculation. Day 0, before the expression of the HCV genome; Day 48, after the expression of the HCV genome. The results are expressed relative to the levels on Day 0 (100%) in each of the 3 HCV-expressing systems. p.i.h., post-inoculation hour.

acids 192–383), E2 (amino acids 384–809), NS3-4A (amino acids 1027–1711), or the core regions lacking the BR, in 35-mm dishes, by using Lipofectamine 2000 (Invitrogen) at 37 °C for 6 h. The medium was subsequently replaced with normal culture medium, and the cells were harvested after 48 h.

2.2. Newcastle disease virus (NDV) infection and addition of polyriboinosinic:polyribocytidylic acid [poly(I:C)]

NDV (Miyadera strain) was propagated from swabs by using the embryonated egg culture method, as described in the Supplementary methods.

Poly(I:C) (20 μg/well; GE Healthcare) was added to HepG2 cells in 35-mm dishes, 48 h after transfection with the core expression vector derived from HCR6 (genotype 1b).

2.3. Native PAGE of IRF-3 and phosphorylated IRF-3

Cells were lysed in 30 μL of lysis buffer (50 mM Tris–HCl [pH 8.0], 1% NP-40, 150 mM NaCl, 100 μg/mL leupeptin, 1 mM PMSF, 5 mM Na_3VO_4), mixed vigorously, and centrifuged at 15,000 rpm for 10 min; the supernatant was isolated. Total protein samples (10 μg) were electrophoresed on a 7.5% native PAGE gel (Bio-Rad Laboratories) and transferred onto a PVDF membrane. IRF-3 homodimer and its monomer and Ser386-phosphorylated IRF-3 were detected by western blotting using polyclonal rabbit antibodies, anti-human IRF-3 (1:1000) [18] and, anti-human Ser386-phosphorylated IRF-3 (1:10,000), respectively. Detection was achieved by enhanced chemiluminescence (ECL; Amersham, UK) according to the manufacturer's instructions. The rabbit anti-human IRF-3 and anti-human Ser386-phosphorylated IRF-3 antibodies were described previously [19].

2.4. Quantification of IFN-β gene expression

IFN-β mRNA expression was quantified by using real-time PCR (RT-PCR) as described previously [20] and Supplementary methods using the following primers and probes: sense (5′-CCATCTATGA GATGCTCCAGAA-3′), antisense (5′-TTTTCTTCCAGGACTGTCTTCA-GA-3′) and probe (5′-AGCACTGGCTGGAATGAGACTATTGTTG-3′).

3. Results

3.1. Induction of IFN and IRF-3 by HCV-Rz

To evaluate the effect of HCV gene persistent expression, cell lines expressing the HCV genomes in a Cre/*loxP* expression system [20] were established by transfecting the full-genome HCV (*HCR6-Rz*); core, E1, E2, and NS2 (*HCR6-Fse*); and E2~NS5b (*HCR6-Age*) (Fig. 1A) clones into HepG2 cells. Of the 3 HCV expression systems, only *HCR6-Rz* transiently induced endogenous IFN-β expression (Fig. 1B). No endogenous IFN-β was detected by RT-PCR on Day 0 or Day 48 (long culture; LC) [21] in any of the 3 systems (Fig. 1B).

3.2. Effect of HCV expression on NDV infection and IFN-β induction

Further, we examined whether the persistent expression of HCV genome influenced the plaque formation activity of NDV in *HCR6-Rz*-, *HCR6-Fse*-, and *HCR6-Age*-expressing cell lines. Prior to the expression of HCV proteins, the plaque numbers were similar across all the cell lines (Fig. 1C). Expression of HCV proteins for more than 48 days [21] increased plaque numbers in the *HCR6-Rz*- and *HCR6-Fse*-expressing cells from 7- to 8-fold (Fig. 1C); however, plaque numbers in the *HCR6-Age*-expressing cells remained constant. These findings thus indicate that HCV structural proteins interfered with the induction of IFN-β mRNA, after NDV infection (18 h post-infection). Therefore, we measured IFN-β mRNA levels in the 3 cell lines, *HCR6-Rz*, *HCR6-Fse*, and *HCR6-Age*, by RT-PCR before (Day 0) and after (Day 48) inoculation with NDV (Fig. 1D). The mRNA expression of IFN-β was not observed prior to NDV infection in any of the 3 cell lines (Day 0). Notably, after 18 h of NDV inoculation and prior to the expression of various HCV proteins (Day 0), the levels of IFN-β mRNA transcription were similar among *HCR6-Rz*-, *HCR6-Fse*-, and *HCR6-Age*-expressing cell lines (Fig. 1D). Following HCV protein expression, the induced IFN-β mRNA expression was reduced to 20% in both *HCR6-Rz*- and *HCR6-Fse*-expressing cell lines. However, IFN-β mRNA expression remained constant in the *HCR6-Age*-expressing cell line.

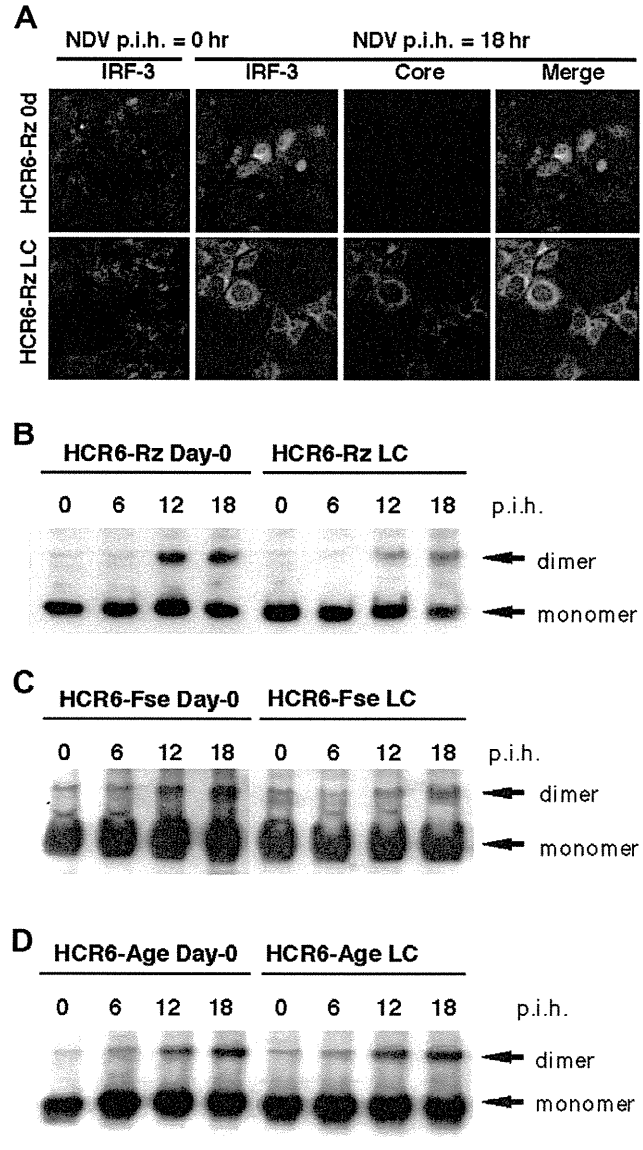

Fig. 2. (A) HCV inhibition of IRF-3 nuclear translocation. IRF-3 is a constitutively expressed transcriptional factor that localizes in the cytoplasm in a diffuse manner, when inactive. After NDV inoculation and prior to the expression of the HCV genome, IRF-3 translocated to the nucleus but was retained at a perinuclear site in the *HCR6-Rz*-expressing cells (Day 48). IRF-3 colocalized with the HCV core protein. (B–D) Suppression of IRF-3 dimerization by HCV expression. The dimeric and monomeric forms of IRF-3 were detected by western blotting on native PAGE gels. The influence of (B) *HCR6-Rz* expression, (C) *HCR6-Fse* expression, and (D) *HCR6-Age* expression on IRF-3 dimerization is shown. NDV was used to induce IRF-3 dimerization.

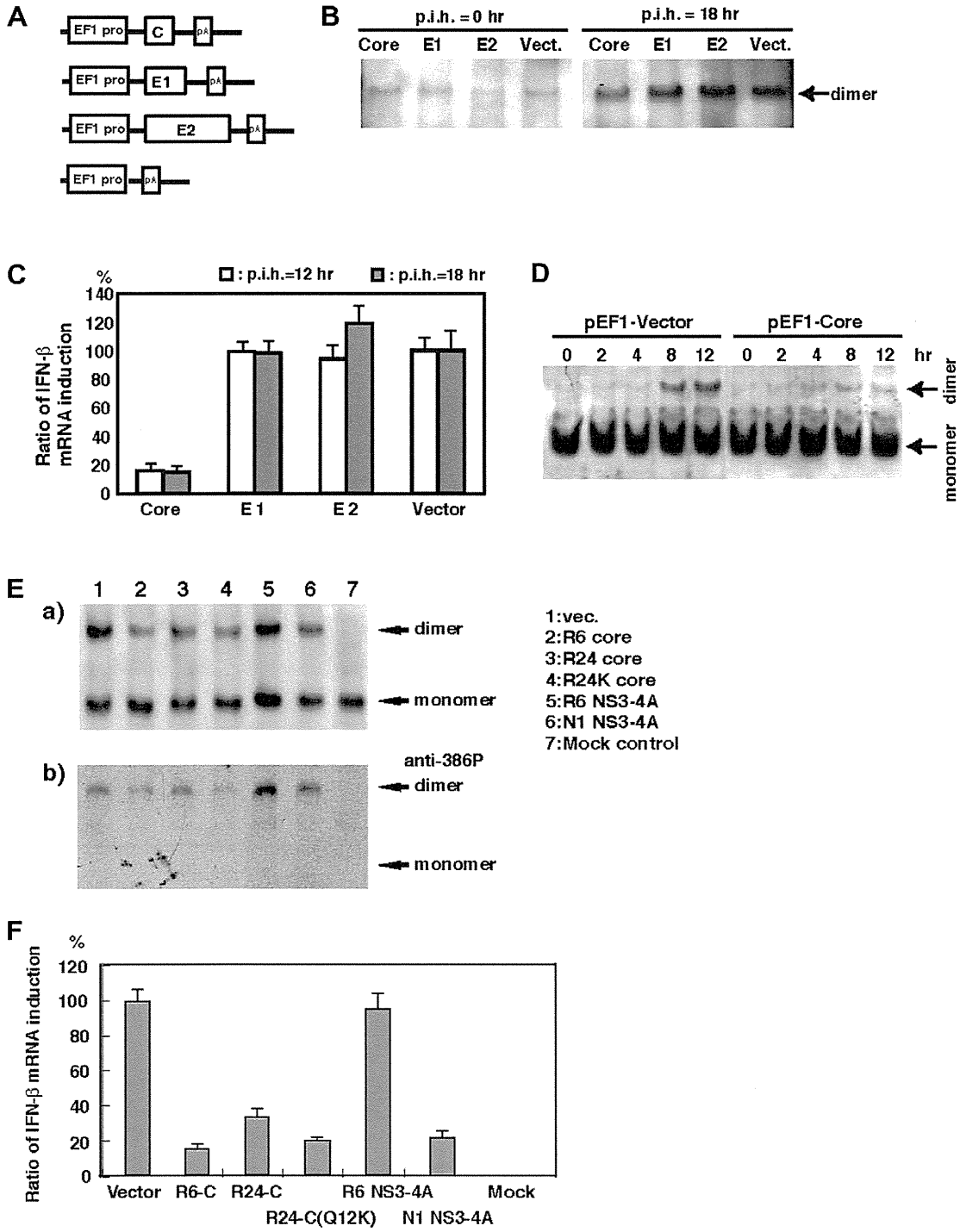

Fig. 3. (A) Structures of the HCR6 core, E1, and E2 expression vectors encoding the HCV core (aa 1–191), E1 (amino acids 192–383), and E2 (amino acids 384–809) proteins, respectively, under the control of the EF1 promoter. (B) Left panel, IRF-3 dimerization induced by NDV before transfection with the expression vectors; right panel, IRF-3 dimerization induced by NDV after transfection with the expression vectors. (C) IFN-β mRNA induction levels at 12 and 18 h after NDV inoculation into HepG2 cells transfected with the vector alone, core, E1, or E2 proteins. The results are expressed relative to the induction levels of IFN-β in HepG2 cells transfected with the vector alone (100%), for post-inoculation, each time. (D) IRF-3 dimerization on administration of 10 mg/mL poly(I:C) before the expression (Day 0), and at 2, 4, 8, and 12 h after the expression of the HCV core (pEF-Core) or vector plasmid DNA. (E) Effects of the expression of vector (lane 1), R6 core (lane 2), R24 core (lane 3), R24 core (Q12K) (lane 4), R6-NS3-4A (lane 5), N1-NS3-4A (lane 6), and mock control (lane 7) on IRF-3 dimerization (upper column a) and phosphorylation of serine residue at amino acid 386 in IRF-3 (lower column b), after infection with NDV for 18 h. F. Effects of the expressions of R6-C, R24-C, R24-C(Q12K), and R6-NS3 on IFN-β induction, 18 h after NDV inoculation. The IFN-β mRNA levels were assayed by RT-PCR. The results are expressed relative to the induction levels of IFN-β in HepG2 cells transfected with the vector alone (100%).

3.3. Effect of HCV on IRF-3 localization, nuclear translocation, and dimerization by NDV

The effect of HCV expression on cellular localization of IRF-3 was analyzed in *HCR6-Rz*-expressing cells infected with NDV before (Day 0) and after LC (Fig. 2A). Prior to NDV infection, IRF-3 was detected in the cytoplasm by immunofluorescence. Notably, after 18 h of NDV inoculation and prior to *HCR6-Rz* protein expression, when IFN-β induction and IRF-3 dimerization were maximal, a substantial amount of IRF-3 translocated to the nucleus.

However, this nuclear translocation was suppressed in the presence of HCV proteins (Fig. 2A) and resulted in the co-localization of the HCV core protein with IRF-3 at perinuclear sites (Fig. 2A, superimposed image of IRF-3 and core protein immunostaining).

To elucidate the mechanism underlying the suppression of IFN-β mRNA in *HCR6-Rz-* and *HCR6-Fse*-expressing cells, we examined the effect of HCV expression on IRF-3 dimerization after NDV infection (Fig. 2B). Interestingly, the levels of IRF-3 dimerization peaked at 12–18 h after NDV infection in the 3 cell lines lacking HCV expression (Day 0; Fig. 2). However, in the *HCR6-Rz-* and *HCR6-Fse*-expressing cell lines, IRF-3 dimerization was found to be significantly reduced, (Fig. 2B and C) when compared to that in the *HCR6-Age*-expressing cells (Fig. 2D).

3.4. Identification of the HCV genome region responsible for the inhibition of IRF-3 dimerization and IFN-β induction

To identify the HCV genome region responsible for suppression of IRF-3 dimerization, HepG2 cells were transfected to express the HCV core regions derived from HCR6, E1, or E2 (genotype 1b; Fig. 3A). Protein expression was confirmed by western blotting (data not shown). The HCV core protein suppressed IRF-3 dimerization, but E1 and E2 expressions had no effect on the dimerization (Fig. 3B). Expression of E1, E2, or the vector alone did not alter the levels of IFN-β mRNA induced by NDV infection in HepG2 cells (Fig. 3C), but significantly reduced IFN-β mRNA levels at both 12 and 18 h after infection (Fig. 3C).

3.5. Effect of HCV core protein expression on IRF-3 dimerization through TLR3

Among the synthetic dsRNAs, poly(I:C) is a potent inducer of IFN-β through TLR3. Accordingly, HepG2 cells transfected with poly(I:C) and the vector control (pEF1-vector) showed IRF-3 dimerization (Fig. 3D). In contrast, IRF-3 dimerization was suppressed in HepG2 cells expressing the HCV core protein albeit the induction of IFN-β mRNA following poly(I:C) expression (data not shown).

3.6. Effect of the HCV core protein NS3 and core proteins derived from genotype 2a on IRF-3 dimerization, compared to proteins derived from genotype 1b

Further, we investigated whether the HCV core protein NS3 and core proteins derived from other genotypes exerted the same effects on IRF-3 after 18 h of NDV infection. The core proteins derived from genotypes 1b (R6) and 2a (R24-12Q and R24-12K) suppressed IRF-3 dimerization in cells infected with NDV (Fig. 3E(a)). In contrast, IRF-3 dimerization remained unaltered in the presence of R6 clone NS3 protein, but was suppressed by the N clone NS3 protein. Thus, NS3-4A protein of R6 clone suppressed IRF-3 dimerization to a relatively lesser extent compared to that of the N1 strain. Similar results were obtained for the phosphorylation at Ser386 in IRF-3 (Fig. 3E(b)). The IFN-β mRNA transcription was quantified in HepG2 cells by RT-PCR after transfection with these expression vectors (Fig. 3F).

3.7. Identification of the HCV core region responsible for suppressing IRF-3 dimerization

We sought to identify the region of the HCV core protein responsible for suppressing IRF-3 dimerization. Expression vectors encoding the entire HCV core or the core region lacking 1 of the 3 basic amino acid regions (BR) that influenced nuclear translocation [17] were transfected into HepG2 cells, and the effects on IRF-3 dimerization were examined (Fig. 4A). Protein

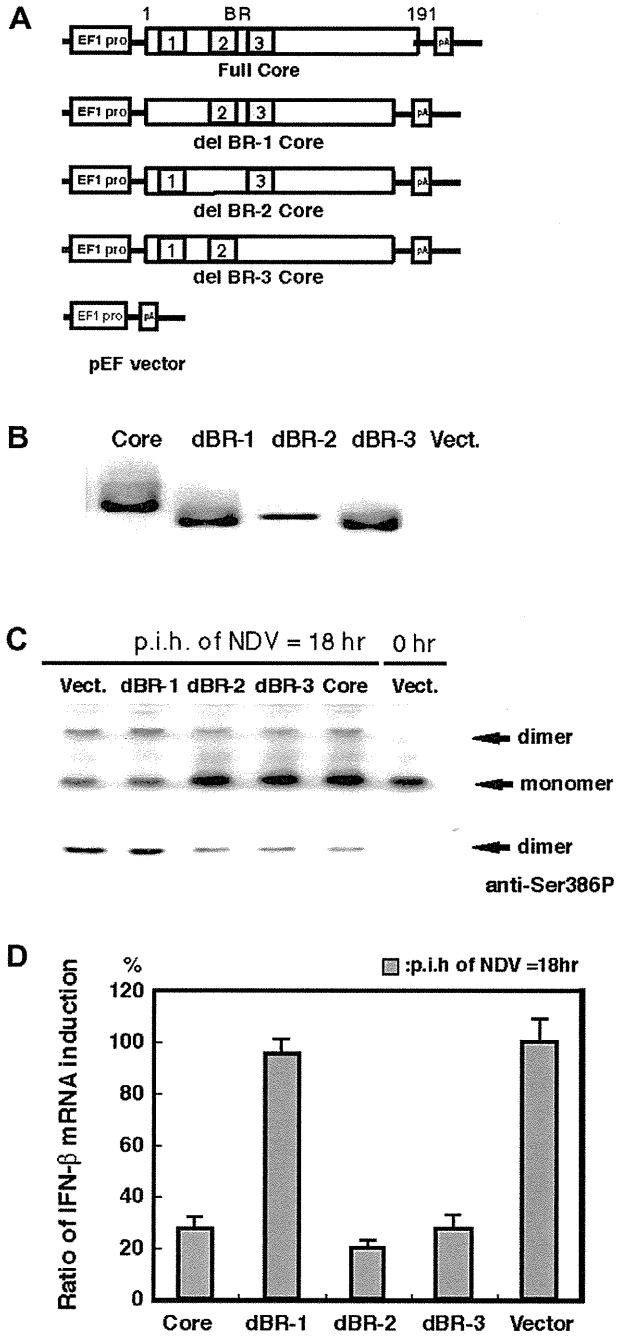

Fig. 4. (A) Structures of the HCR6 core, E1, and E2 expression vectors carrying the complete core, BR1 deletion (aa 4–14), BR2 deletion (aa 37–44), and BR3 deletion (aa 57–72). (B) Western blotting to confirmed the expression of the mutated core proteins. (C) Effects of the expression of various mutated core proteins on IRF-3 dimerization and IRF-3 phosphorylation at Ser386, 18 h after NDV inoculation. (D) Effects of the expression of each type of core protein region on IFN-β mRNA synthesis, 18 h after NDV inoculation. The results are expressed relative to the induction levels of IFN-β in HepG2 cells transfected with the vector alone (100%). IFN-β mRNA levels were assayed by RT-PCR.

expression of the core and the deletion mutants (BR1, BR2, and BR3) was confirmed by western blotting (Fig. 4B). IRF-3 dimerization, phosphorylation at Ser386 of IRF-3, and induction of IFN-β mRNA were suppressed in HepG2 cells expressing the entire core, a deletion of BR2, or a deletion of BR3 (Fig. 4C), but not in cells expressing the BR1-deleted HCV core regions (Fig. 4C and D).

4. Discussion

The present study indicates that the HCV core protein inhibits IRF-3 dimerization, IRF-3 phosphorylation at Ser386, and IFN-β induction. In addition, our study showed that the effect of the core protein derived from genotype 1b was similar to that of the core protein derived from genotype 2a, indicating that the inhibitory effect of the core protein might be effective in several genotypes of HCV. These findings are corroborated by a previous study by Foy et al. [12] who showed that HCV NS3/4 disrupts virus-associated-kinase-mediated IRF-3 activation, which further results in the suppression of IRF-3 phosphorylation, nuclear translocation, and IRF-3-dependent ISRE/PRDI activation. These findings indicate that attenuation of the IFN system was achieved through NS3/4A proteins via the interference of IRF-3 activation, thus strengthening our results, which show the potential of HCV core protein to interfere with IRF-3 activation in promoting persistent infection.

Furthermore, the present study showed that the N-terminal region of the core protein and BR-1 domain in particular are responsible for inactivating IRF-3. The N-terminal region (amino acids 1–59) of the HCV core protein has been identified as the binding region for a DEAD box protein (DDX3) [22]. Human DDX3, a putative RNA helicase, is a member of the highly conserved DEAD box subclass that includes the expression of murine PL10, *Xenopus* An3, and yeast Ded 1 proteins. Recently, expression of DDX3 was found to enhance IFN-β promoter induction by TBK1/IKKε, whereas silencing of DDX3 inhibited IFN-β promoter and virus- or dsRNA-induced IRF-3 activation [23]. It was shown that Vaccinia virus K7 protein also binds to DDX3 and inhibits pattern recognition receptor-induced IFN-β induction by preventing TBK1/IKKε-mediated IFN-β induction via impaired TBK1/IKKε-induced activation of IRF-3 [23]. A previous study by Oshiumi et al. showed that DDX3 C-terminal region (amino acids 622–662) directly binds to the IFN-beta promoter stimulator-1 (IPS-1) CARD-like domain [24] as well as the N-terminal HCV core protein [36]. The present study demonstrated that the expression of the core protein decreased the levels of DDX3 expression (data not shown). This is in agreement with the result of a previous study, which showed that DDX3 is downregulated in HCV-associated hepatocellular carcinoma (HCC) and silencing of DDX3 accelerates cell growth [25]. Collectively, these findings suggest that DDX3 may be the target of the core protein for inhibiting IRF-3 activation.

In conclusion, our study revealed a crucial region of the HCV core protein, basic amino acid region 1, to interfere with IRF-3 activation and thereby inhibit the IFN signaling cascades. Therefore, the inhibitory effects that result in the IRF-3 pathway impairment could be rescued by deleting the basic region 1 of core protein, thus suggesting that it might be an effective treatment for HCV infection. Future studies involving DDX3 modification by the HCV core protein may be interesting to explore the cell growth-dysregulation mechanisms.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.079.

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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

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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

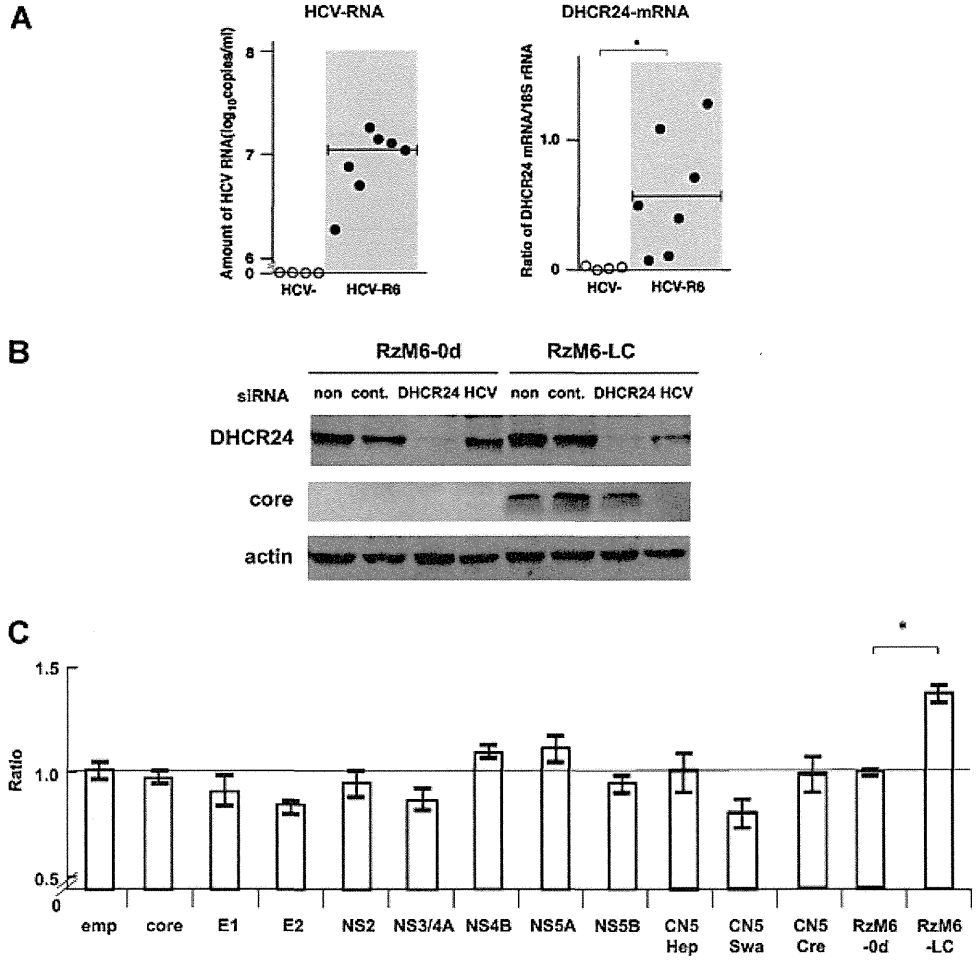

Fig. 1. *DHCR24* expression is induced in the presence of the complete HCV genome. **A**: The amount of HCV RNA in chimeric mice infected with HCV-R6 (genotype 1b) was quantified by qPCR (left panel). The amount of DHCR24 mRNA was measured, and the ratio to the amount of 18S rRNA was calculated in the 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

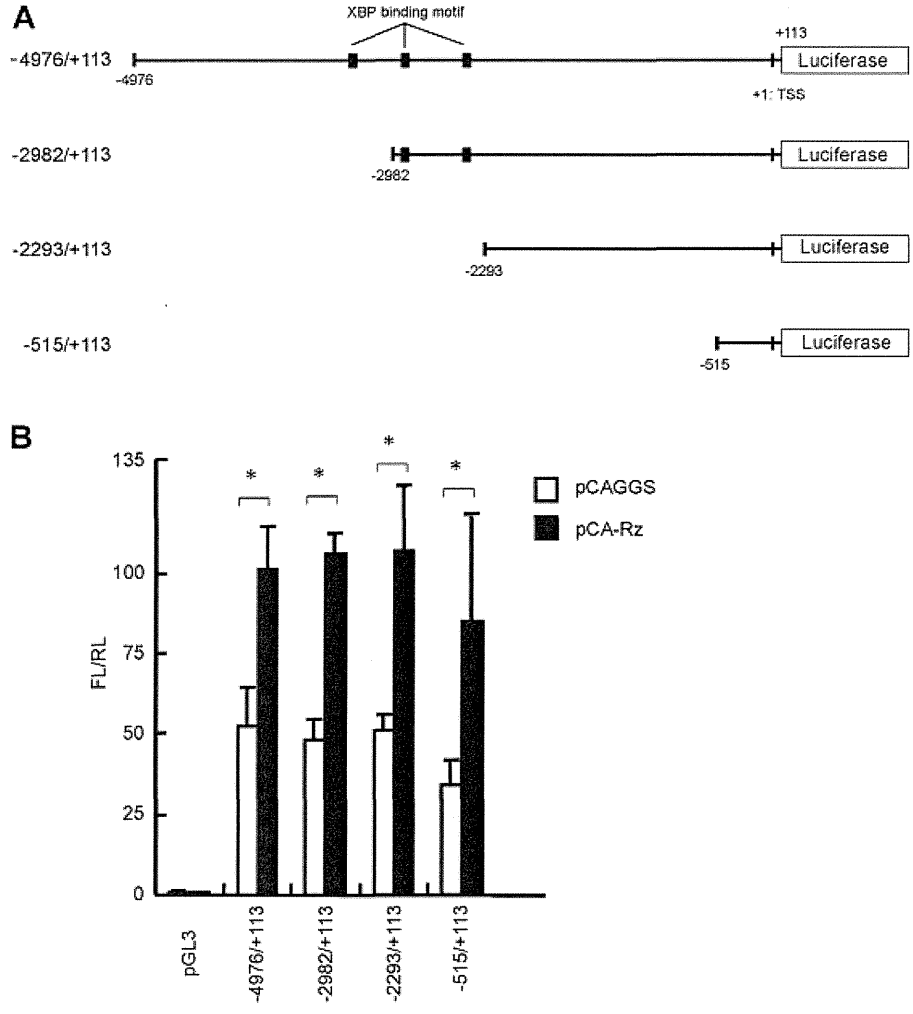

Fig. 2. *DHCR24* promoter activity is augmented by the expression of HCV. **A:** The 5′-flanking region of *DHCR24* was cloned from HepG2 and inserted upstream of the firefly luciferase gene in pGL3 (−4976/+113). A deletion series of the 5′-flanking regions was also constructed (−2982/+113, −2293/+113, 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.

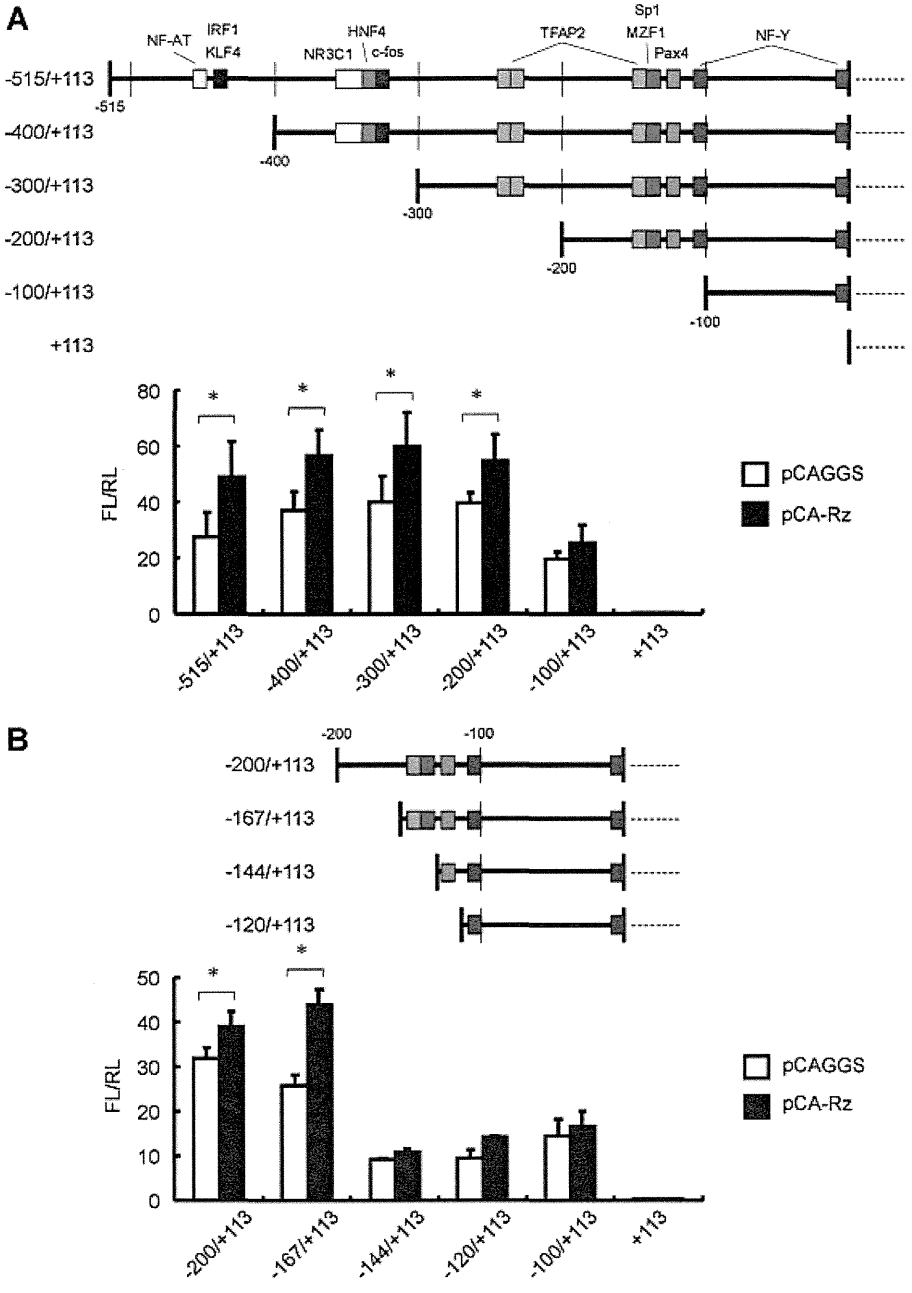

Fig. 3. Responsiveness of the *DHCR24* promoter to the expression of HCV. **A**: A *DHCR24* promoter series with sequential 100-bp deletions was constructed and analyzed as described in the legend to Fig. 2B ($^*p < 0.05$). **B**: An additional deletion series (−167/+113, −144/+113, and −120/+113) was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

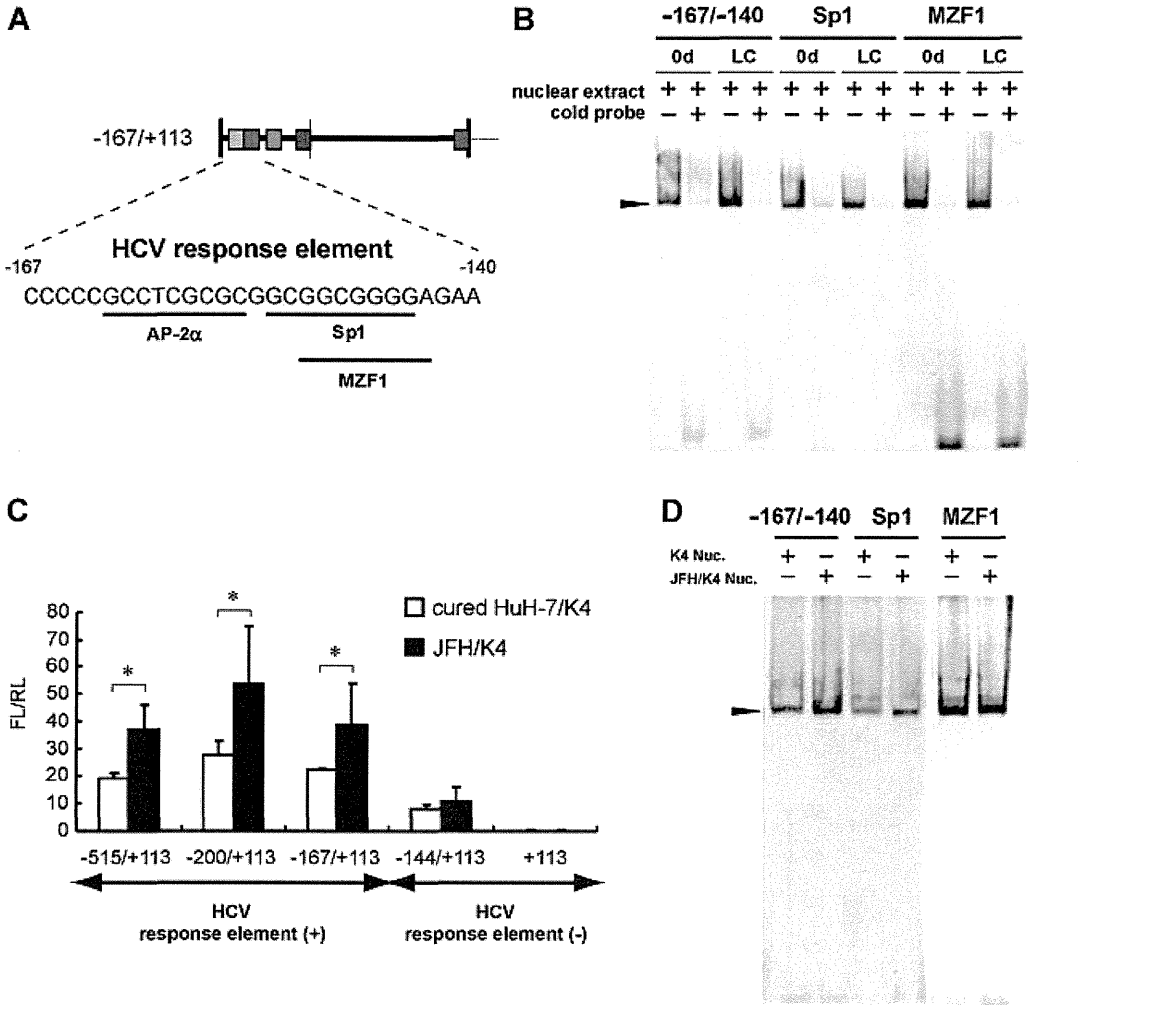

Fig. 4. The HCV response element mediates the overexpression of *DHCR24* induced by HCV. **A**: The HCV response element (−167/−140) in the 5′-flanking region of *DHCR24* includes sequences with similarity to the consensus-binding motifs for AP-2α, Sp1, and MZF-1. **B**: Nuclear extracts were prepared 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