TABLE 1: Synergistic effects of cytokines on Fas-induced apoptosis in *irf-1*$^{-/-}$ CN2 mice.

	None	IL-2 + IL-10	IL-2 + IL-12	IL-10 + IL-12
		WT mice		
Bcl-2 fold increase	–	–	–	–
Annexin V + percentage	****	****	****	****
Caspase-9	**	**	**	**
Caspase-3/7	***	***	***	***
		irf-1$^{-/-}$ mice		
Bcl-2 fold increase	–	–	–	–
Annexin V + percentage	***	***	***	***
Caspase-9	**	**	**	**
Caspase-3/7	**	**	**	**
		CN2-29 mice		
Bcl-2 fold increase	–	+	–	–
Annexin V + percentage	***	**	***	***
Caspase-9	**	*	*	**
Caspase-3/7	**	*	*	*
		irf-1$^{-/-}$ CN2-29 mice		
Bcl-2 fold increase	+	+++	++	++
Annexin V + percentage	**	–	*	*
Caspase-9	**	–	*	*
Caspase-3/7	*	–	*	*

Bcl-2: –, less than 2-fold increase; +, more than 2-fold increase; ++, more than 4-fold increase; +++, more than 6-fold increase (in comparison with mock treatment).
Annexin V: –, less than 20% decrease; *, up to 20% decrease; **, up to 40% decrease; ***, up to 60% decrease; ****, more than 60% decrease (in comparison with mock treatment).
Caspase-9: –, less than 10-fold decrease; *, up to 50-fold decrease; **, up to 200-fold decrease; ***, up to 400-fold decrease; ****, more than 400-fold decrease (in comparison with mock treatment).
Caspase-3/7: –, less than 200-fold decrease; *, up to 500-fold decrease; **, up to 1000-fold decrease; ***, more than 1000-fold decrease (in comparison with mock treatment).

of pro-B-lymphocyte death [17]. *Bcl-2* transgene expression increases the oncogenic potential [18] and is linked with B cell neoplasm and t(14;18) translocation [19]. We therefore examined the level of Bcl-2 protein and found that it was upregulated in the lymph nodes of *irf-1*$^{-/-}$ CN2 mice after 400 days (Figure 3(c)).

IL-10 treatment in the presence of IL-2 greatly inhibited Fas-induced apoptosis in *irf-1*$^{-/-}$ CN2 mice compared with other groups (Table 1). Furthermore, *irf-1* disruption accelerated the resistance of splenocytes to Fas-induced apoptosis in the presence of IL-2, IL-10, and/or IL-12. In particular, IL-2 plus IL-10 treatment produced the strongest upregulation of the Bcl-2 mRNA levels in splenocytes of *irf-1*$^{-/-}$ CN2 mice. This indicates that IL-2, IL-10, and/or IL-12 contribute to upregulation of *bcl-2* expression, which subsequently inhibits Fas-induced apoptosis. Caspase-9 and caspase-3/7 activities were inversely correlated with the level of *bcl-2* expression. These results indicate that aberrant cytokine expression and disruption of IFN signaling affect *bcl-2* expression synergizing with HCV proteins, which is associated with the inhibition of caspase expression.

TABLE 2: Synergistic effects of cytokines on Bcl-2 expression in WT and *irf-1*$^{-/-}$ mice in the presence of HCV core protein.

	WT mice	*irf-1*$^{-/-}$ mice
Mock	–	–
IL-2 + IL-10	+	+++
IL-2 + IL-12	+	++
IL-10 + IL-12	+	++
IL-2	–	–
IL-10	+	++
IL-12	–	–
IL-2 + Il-10 + IL-12	–	+

Bcl-2: –, less than 2-fold increase; +, more than 2-fold increase; ++, more than 4-fold increase; +++, more than 5-fold increase (in comparison with mock treatment).

HCV core protein induced IL-2 and IL-10. Envelope protein E2 induced IL-12 expression. These results indicate that the HCV core and E2 proteins are responsible for IL-2, IL-10, and IL-12 expression. Core protein expression and IL-10 stimulation most strongly induced Bcl-2 expression

(Table 2). From these results, core protein contributes significantly to the induction of Bcl-2 in the presence of cytokines.

4. Conclusion

Our results show that the conditional expression of HCV proteins induces inflammation and lymphoproliferative disorders. Furthermore, established animal models will probably provide critical information for the elucidation of the molecular mechanism(s) underlying the spontaneous development of B cell non-Hodgkin lymphoma after HCV infection. The disruption of *irf-1* enhances lymphoproliferative disorders. Therefore, IRF-1-inducible genes probably play essential roles in suppressing HCV-induced lymphoma and in eliminating HCV protein-expressing cells. The overexpression of apoptosis-related proteins (including Bcl-2) and/or aberrant cytokine production are the primary events in HCV-induced lymphoproliferation.

The *HCV* gene has the potential to induce B cell lymphomas in RzCD19Cre mice, without inducing host immune responses against *HCV* gene product. This is in agreement with the results of a previous study, which indicate that viral elimination reduces the incidence of malignant lymphoma in patients infected with HCV [20]. The incidence of B cell lymphoma in the HCV transgenic mouse strain (MxCre/CN2-29) is high, and this strongly suggests that development of B cell lymphomas occurs via expression of the *HCV* transgene.

Recent findings indicate a link between sIL-2Rα levels and hepatocellular carcinoma in Egyptian patients [21]. The level of IL-2Rα was higher in splenocytes of RzCD19Cre mice than in those of CD19Cre mice; however, the differences in the serum concentrations of sIL-2Rα between RzCD19Cre mice without B cell lymphomas and other control groups (Rz, CD19Cre, and WT) were insignificant. These results indicate that HCV increases IL-2Rα expression in B cells; proteolytic cleavage of IL-2Rα increased after B cell lymphoma development in the RzCD19Cre mice. The detailed mechanism by which HCV expression induces IL-2Rα remains unclear, but HCV core protein induces IL-10 expression in mouse splenocytes [12]. IL-10 upregulates the expression of IL-2Rα (Tac/CD25) in normal and leukemic B lymphocytes [22]. Therefore, through IL-10, the HCV core protein might induce IL-2Rα in B cells of the RzCD19Cre mouse.

Disruption of *irf-1* enables the persistent expression of HCV protein. This leads to lymphoproliferative diseases resulting from reduced apoptosis (i.e., lower levels of caspase-1, caspase-6, and caspase-7 expression). HCV CN2 transgenic (Tg+) mice are resistant to Fas-induced apoptosis because of the inhibition of cytochrome *c* release from mitochondria [11]. Mice with disruption of *irf-1* have several defects in their innate and adaptive immunities, including lineage-specific defects in thymocyte development, and the development of immature T cells into mature $CD4^+$ cells but not $CD8^+$ T cells [16, 23]. IRF-1 controls the positive and negative selection of $CD8^+$ thymocytes [24] and is required for the development of the Th1-type immune response. The absence of IRF-1 induces Th2-type immune response [16, 25]. The number of natural killer cells is dramatically reduced in *irf-1*$^{-/-}$ mice [16]. This defect may markedly increase viral protein expression and inhibit tumor surveillance mechanisms, leading to the development of non-Hodgkin lymphoma. Expression of the IL-12 p40 subunit is defective in *irf-1*$^{-/-}$ mice [16].

Hypermutation of the immunoglobulin genes in B cells induced by HCV infection is the cause of the lymphomagenesis observed in HCV infection [16, 26]. This finding may provide a more direct insight into lymphoma production, because HCV-induced hypermutation causes genetic instability and chromosomal aberrations, possibly resulting in neoplastic transformation [27]. In addition, the anti-apoptotic phenotype resulting from sustained viral protein expression may enhance the survival of lymphocytes and inhibit activation-induced cell death to turn off the activated lymphocytes. The dysregulated cytokine profiles and sustained lymphocyte survival may alter the fates of regulatory T cells and dendritic cells [28].

In summary, the mouse model of B cell lymphoma and lymphoproliferative disorder represents a powerful tool to address the molecular mechanism of lymphoma development by HCV.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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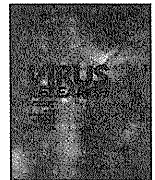

Short communication

Translocase of outer mitochondrial membrane 70 induces interferon response and is impaired by hepatitis C virus NS3

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ABSTRACT

Hepatitis C virus (HCV) elevated expression of the translocase of outer mitochondrial membrane 70 (Tom70). Interestingly, overexpression of Tom70 induces interferon (IFN) synthesis in hepatocytes, and it was impaired by HCV. Here, we addressed the mechanism of this impairment. The HCV NS3/4A protein induced Tom70 expression. The HCV NS3 protein interacted in cells, and cleaved the adapter protein mitochondrial anti-viral signaling (MAVS). Ectopic overexpression of Tom70 could not inhibit this cleavage. As a result, IRF-3 phosphorylation was impaired and IFN-β induction was suppressed. These results indicate that MAVS works upstream of Tom70 and the cleavage of MAVS by HCV NS3 protease suppresses signaling of IFN induction.

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Type I interferon (IFN) induction is the front line of host defense against viral infection. Intracellular double-stranded RNA is a viral replication intermediate and contains pathogen-associated molecular patterns (PAMPS) (Saito et al., 2008) that are recognized by pathogen-recognition receptors (PRRs) to induce IFN. One PRR family includes the Toll-like receptors (TLRs), which are predominantly expressed in the endosome (Heil et al., 2004). Another route of IFN induction takes place in the cytosol through activation of specific RNA helicases, such as retinoic acid-inducible (RIG)-I and melanoma differentiation associated gene 5 (MDA5). The ligand for RIG-I is an uncapped 5′ triphosphate RNA, which is found in viral RNAs of the *Flaviviridae* family, including hepatitis C virus (HCV), paramyxovirus, and rhabdoviruses (Kato et al., 2006). MDA5 recognizes viruses with protected 5′ RNA ends, for example, picornaviruses (Hornung et al., 2006). The adapter protein that links the RNA helicase to the downstream MAPK, NF-κB, and IRF-3 signaling pathways is referred to as the mitochondrial anti-viral signaling (MAVS) protein (Seth et al., 2005); alternative names include IPS-1, interferon-promoter stimulator 1; VISA, virus-induced signaling adaptor; and CARDIF, CARD adapter inducing IFN. HCV nonstructural protein 3 (NS3) possesses a serine protease domain at the N terminus (amino acids (aa) 1–180) and has been found to cleave adaptor proteins, MAVS at aa 508 (Meylan et al., 2005) and Toll/IL-1R domain-containing adapter inducing IFN-β-deficient (TRIF at aa 372; Ferreon et al., 2005). These cleavages provoke abrogation of the induction of the IFN pathway.

The translocase of the outer membrane (TOM) is responsible for initial recognition of mitochondrial preproteins in the cytosol (Baker et al., 2007; Neupert and Herrmann, 2007). The TOM machinery consists of 2 import receptors, Tom20 and Tom70, and, along with several other subunits, comprises the general import pore (Abe et al., 2000). Recently, Tom70 was found to interact with MAVS (Liu et al., 2010). Ectopic expression or silencing of Tom70, respectively, enhanced or impaired IRF3-mediated gene expression and IFN-β production. Sendai virus infection accelerated the

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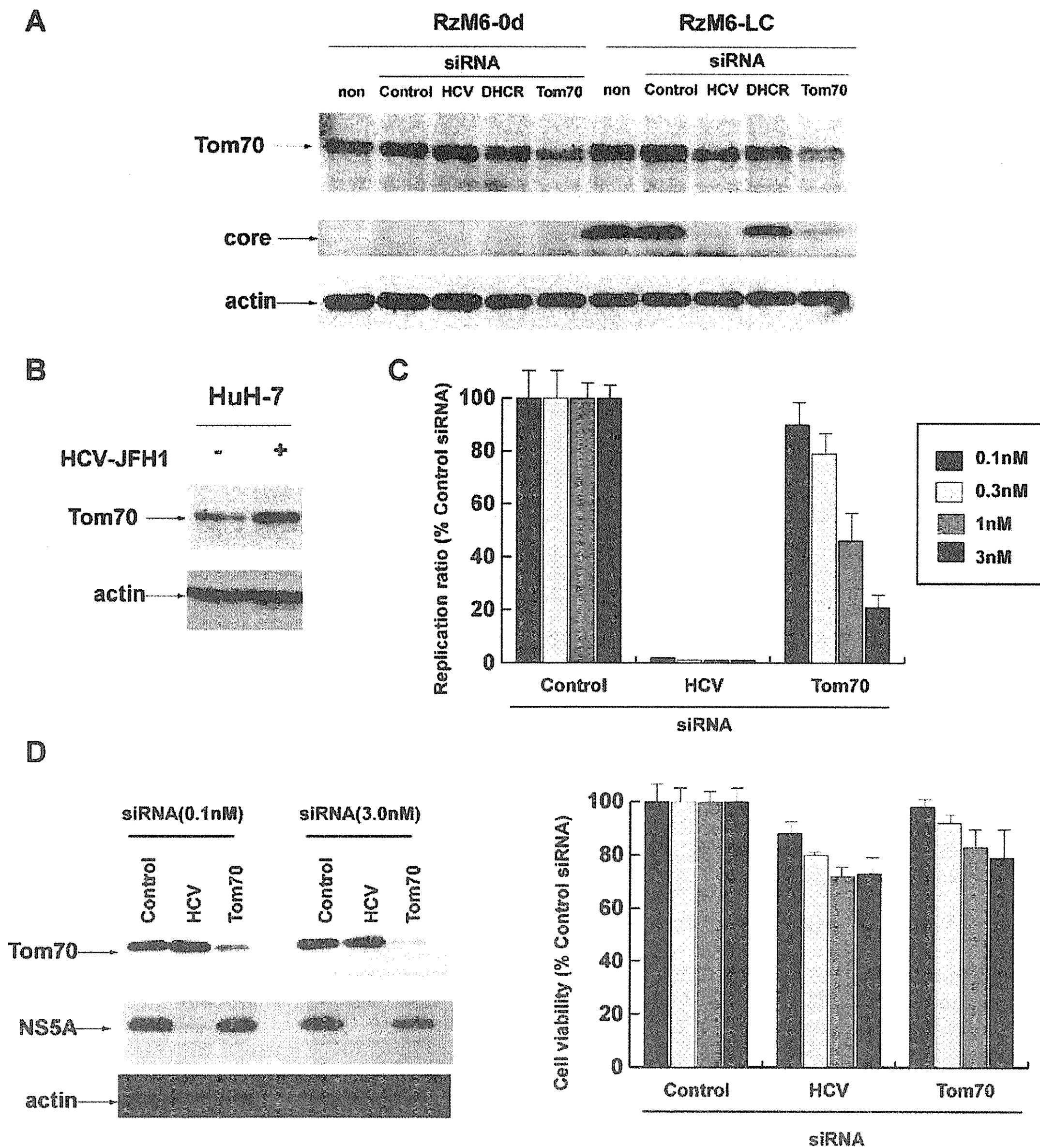

Fig. 1. HCV induces overexpression of Tom70 but impairs Tom70-induced IFN synthesis. (A) RzM6 cells (HCV-) and RzM6-LC cells (HCV+) were transfected with siRNAs of control (non-target siRNA#3: Thermo Fisher Scientific), HCV (R5: 5′-GUCUCGUAGACCGUGCAUCAuu-3′), DHCR24 (Nishimura et al., 2009), and Tom70 (Takano et al., 2011a). Control cells were mock-transfected. Tom70 protein was detected with MAb2-243a (Takano et al., 2011a) and actin protein was detected as an internal control (lower column). (B) HuH-7 cells were infected with HCV JFH-1 strain; Tom70 protein and actin protein were detected. (C) The HCV replicon cells (FLR3-1; Takano et al., 2011b) were transfected with siRNAs (control, HCV (R7: 5′-GUCUCGUAGACCGUGCACCAuu-3′), Tom70; 0.1, 0.3, 1, 3 nM) and HCV replication activity was measured with luciferase activity using the Bright-Glo luciferase assay kit (Promega). Cell viability was measured using WST-8 (Dojindo) reagent. Ratio with those of control siRNA treatment was calculated. Vertical bars were S.D. (D) HCV replicon cells (FLR3-1) were transfected with control, HCV (R7) and Tom70 siRNAs (0.1, 0.3 nM) and Tom70, NS5A and actin proteins were detected.

Tom70-mediated IFN induction and the interaction of Tom70 with MAVS. These recent findings indicated that Tom70 might be a critical mediator during IFN induction (Liu et al., 2010).

We previously observed that HCV induces Tom70 and is related to the apoptotic response (Takano et al., 2011a). However, no synergistic effect was observed for IFN induction by Tom70 and HCV. Therefore, in the present study, we have investigated the mechanism of modification of the Tom70-induced IFN synthesis pathway by HCV and clarified a finely balanced system regulated by viral protein.

The expression of Tom70 protein was examined using western blotting and modification by HCV was characterized (Fig. 1A). The level of Tom70 protein was increased in RzM6-LC cells compared with that in RzM6-0d cells (Tsukiyama-Kohara et al., 2004). The full-length HCV-RNA expression was induced by 4-hydroxytamoxifen (100 nM) and passaged for more than 44 days in RzM6-LC cells, and HCV expression was not induced in RzM6-0d cells. Silencing of HCV expression by siRNA (R5; Thermo Scientific) abolished core protein expression, and decreased the level of Tom70 protein expression in RzM6-LC cells (Fig. 1A). Silencing of Tom70 by siRNA significantly decreased the level of HCV core protein expression in RzM6-LC cells (Fig. 1A). The siRNA against 3-beta-hydroxysterol-delta24 reductase (DHCR24) slightly decreased the level of Tom70 protein. In contrast, the

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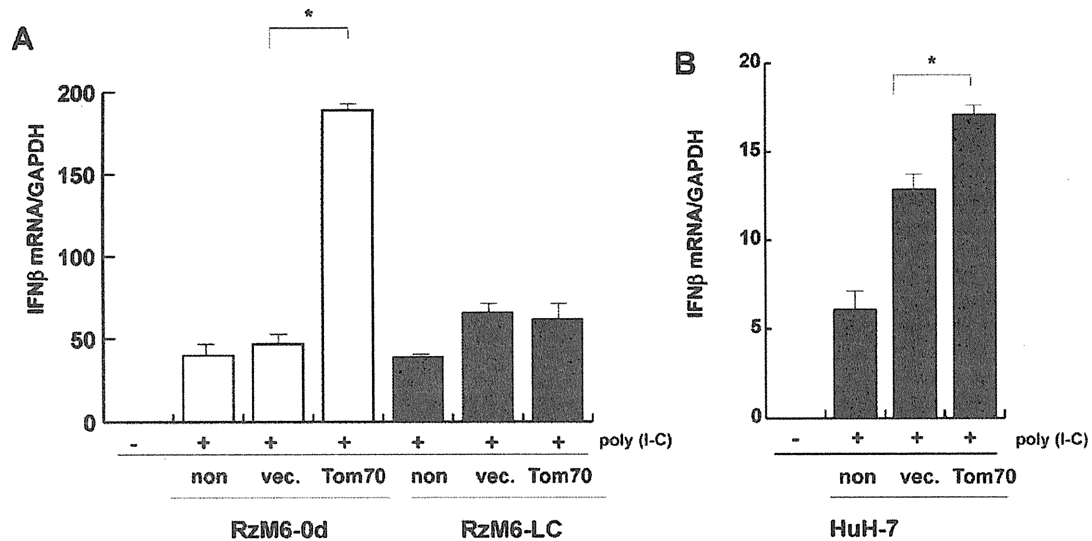

Fig. 2. Tom70-induced IFN synthesis was impaired by HCV. (A) RzM6-0d cells and LC cells were transfected with mock-vector, control pcDNA vector (vec.), or pcDNA-Tom70 expression vector, and the amount of IFN-β mRNA was measured by RTD-PCR and normalized to the amount of GAPDH mRNA using Gene expression assay kit (GE-Healthcare). Poly(I–C) (GE Healthcare) (5 μg) was transfected with RNAi Max reagent (Invitrogen) and IFN-βmRNA was measured after 6 h of poly(I–C) treatment. Vertical bars indicate S.D. *$p < 0.05$. (B) HuH-7 cells were transfected with mock-vector, control vector, or Tom70 expression vector, and the amount of IFN-β mRNA was measured by RTD-PCR and normalized to the amount of GAPDH mRNA. Vertical bars indicate S.D. *$p < 0.05$.

control siRNA did not have a significant effect on Tom70 protein expression.

We next examined the effects of HCV JFH-1 (Wakita et al., 2005) infection on Tom70 expression (Fig. 1B). Infection with HCV significantly increased the level of Tom70 protein expression. We also examine the role of Tom70 in HCV replication (Fig. 1C and D). Silencing of Tom70 by siRNA decreased the HCV replication in a dose dependent manner.

Thus, HCV induces Tom70 expression, and Tom70 is involved in viral replication.

It was recently shown that Tom70 recruits TBK1/IRF3 to mitochondria by binding to Hsp90 and inducing IFN-β synthesis (Liu et al., 2010). Therefore, we examined the effects of Tom70 overexpression on IFN synthesis and modification by HCV (Fig. 2). Level of IFN-β mRNA synthesis was quantitated by real-time detection (RTD) PCR. Overexpression of Tom70 by transfection of pcDNA6-Tom70 (Takano et al., 2011a) induced IFN-β mRNA synthesis in the absence of HCV after poly(I–C) treatment (RzM6-0d cells). However, the Tom70-mediated induction of IFN-β mRNA transcription was impaired in the presence of HCV (RzM6-LC cells) (Fig. 2A). Overexpression of Tom70 induced IFN-β mRNA synthesis in HuH-7 cells (Fig. 2B). Induction of IFN-β mRNA was lower in HuH-7 cells than HepG2 based RzM6 cells, which might be due to the defect in IFN induction system in HuH-7 cells (Preiss et al., 2008).

We have further addressed the mechanism of impairment of IFN-β mRNA transcription by HCV.

To identify the viral protein that was responsible for the induction of Tom70, we examined the Tom70 protein expression levels in HCV core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B protein-expressing cells (data not shown), and Tom70 protein expression level was highest in the NS3/4A-expressing cells than was observed in cells expressing other proteins (Fig. 3A, data not shown), indicating an effect of HCV NS3/4A protein on Tom70 expression.

The expression vector of Myc- and His-tagged Tom70 was transfected into the empty control or NS3/4A-expressing cells and immunoprecipitated with anti-Myc antibody (Suppl. Fig. 1A). Results showed that Myc-Tom70 was precipitated in both cells (right panel) and NS3 protein was specifically precipitated by anti-Myc antibody in the NS3/4A-expressing cells (left panel). NS4A protein could not be detected (data not shown).

We next stained the NS3/4A-expressing cells with anti-NS3 and -Tom70 antibodies, and observed with confocal microscopy (Suppl. Fig. 1B). The signal of NS3 protein was clearly merged with that of Tom70, strongly supporting the possibility that the NS3 protein co-localizes with the Tom70 protein.

To clarify the effect of Tom70 on NS3, we transfected NS3/4A-expressing cells with the siRNA of Tom70 (Fig. 3A). Silencing of Tom70 decreased the level of NS3 protein in cells, but did not influence the levels of the MAVS and NF-κB proteins. These results suggest the possibility that Tom70 may increase the stability of NS3 protein in cells.

Tom70 reportedly interacts with MAVS during viral infection (Liu et al., 2010). Therefore, we examined the MAVS protein in cells expressing either the control empty or NS3/4A lenti-virus vector (Fig. 3B). Cleavage of MAVS (indicated as ΔMAVS) was observed in NS3/4A protein-expressing cells, as was reported previously (Meylan et al., 2005). Overexpression of Tom70 did not have a significant effect on the MAVS expression level and did not prevent MAVS cleavage by NS3. IRF-3 phosphorylation was suppressed in NS3/4A-expressing cells and was not influenced by Tom70 overexpression. The induction of IFN-β was impaired in NS3/4A-expressing cells, even in the presence of Tom70 overexpression (Fig. 3C). These data may indicate that MAVS exists upstream of Tom70 and that cleavage of MAVS by NS3/4A impaired the downstream signaling activation of IRF-3 phosphorylation (Suppl. Fig. 2).

Mitochondria provide a substantial platform for the regulation of IFN signaling. The MAVS adapter protein is a member of the family of RIG-I like receptors (RLRs), which links the mitochondria to the mammalian antiviral defense system (Seth et al., 2005). Proteomic studies have demonstrated that MAVS interacts with Tom70 (Liu et al., 2010). This interaction was accelerated by Sendai virus infection and synergized with ectopic expression of Tom70 to significantly increase the production of IFN-β (Liu et al., 2010). The results of the present study revealed that infection with HCV induced Tom70 expression, but the presence of HCV impaired IFN

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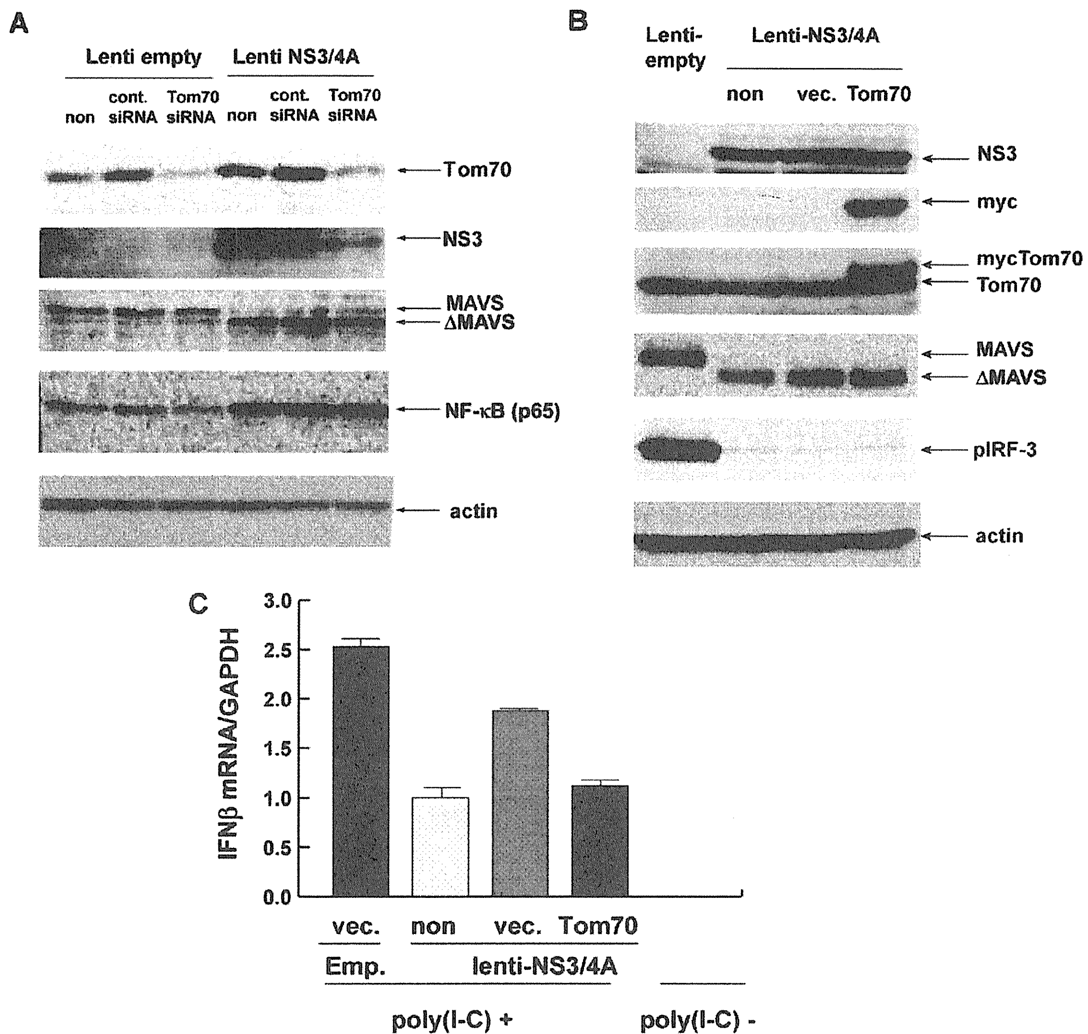

Fig. 3. Silencing of Tom70 decreased the level of NS3 and cleavage of MAVS by NS3/4A impaired IRF-3 phosphorylation even in the presence of Tom70. (A) Empty or NS3/4A-lenti virus vector expressing HepG2 cells were transfected with control siRNA and Tom70 siRNA or mock-transfected (non) as a control. MAVS, NS3, Tom70, and actin proteins were detected by western blot. (B) Empty or NS3/4A-expressing HepG2 cells were transfected with control pcDNA vector (vec.) and pcDNA6 (Invitrogen)-Tom70 or mock-transfected (non) as a control. NS3, Tom70, phosphorylated IRF-3, MAVS, and actin proteins were examined by western blot. (C) IFN-β mRNA was measured by RTD-PCR and normalized with GAPDH mRNA amount in empty or NS3/4A expressing cells with transfection of mock (non), pcDNA-vector (vec.) or pcDNA-Tom70 (Tom70). Poly(I–C) was treated, as described in the legend of Fig. 2.

induction. It has been reported that the C-terminal transmembrane domain (TM) of MAVS interacts with the N-terminal transmembrane domain of Tom70 (Liu et al., 2010). The HCV NS3 protein cleaves MAVS at residue 508 (Meylan et al., 2005), which should impair the interaction of MAVS and Tom70. This may attenuate the downstream signaling pathway (TBK-IRF3) and the induction of IFN synthesis (Suppl. Fig. 2). In our study, the level of NF-κB protein was not significantly influenced by Tom70 in the presence or absence of NS3. This may indicate that other pathways, such as TLR3 and downstream pathways, might compensate to maintain the NF-κB protein expression level in the absence of the MAVS-Tom70 signaling pathway.

Infection with HCV induced expression of Tom70, but the activation of the IFN signaling pathway was abrogated by the HCV NS3 protease. These findings indicate that recovery of the MAVS-Tom70 pathway may be a means to increase the efficacy of IFN therapy against HCV infection.

Recently, we observed that overexpression of Tom70 increased the resistance to the TNFα-induced apoptotic response (Takano et al., 2011a), indicating that Tom70 overexpression might contribute to the apoptotic resistance of HCV-infected cells and the establishment of persistent HCV infection. Thus, Tom70 might be a novel target for the regulation of HCV infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2011.10.009.

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Hepatitis C virus promotes expression of the 3β-hydroxysterol Δ24-reductase through Sp1

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Abstract

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.

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.

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). By 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, USA) 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 non-essential 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, USA). 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 by 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

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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 5×10^6–1×10^7 cells as described previously [Dignam et al., 1983]. Electrophoresis mobility shift assays (EMSAs) were performed by a non-radioactive method using the DIG Gel Shift Kit (Roche, Indianapolis, IN, USA). 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, USA), 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, USA) 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, USA; 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, USA); 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, USA) or Thr453 (Abcam, Cambridge, MA, USA). 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, USA), 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, USA) and an LAS1000 scanner (Fujifilm). Densitometric analysis of protein bands was performed with Image Quant software (Molecular Dynamics, Sunnyvale, CA, USA).

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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, USA), 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; Suppl. 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 lines. No significant

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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 3 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 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

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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.

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 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.

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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