25. Puchhammer-Stockl, E., W. Mor, M. Kundi, F. X. Heinz, H. Hofmann, and C. Kunz. 1994. Prevalence of hepatitis-C virus RNA in serum and throat washings of children with chronic hepatitis. J. Med. Virol. 43:143–147.
26. Qian, C., J. Camps, M. D. Maluenda, M. P. Civeira, and J. Prieto. 1992. Replication of hepatitis C virus in peripheral blood mononuclear cells. Effect of alpha-interferon therapy. J. Hepatol. 16:380–383.
27. Rey, D., S. Fritsch, C. Schmitt, P. Meyer, J. M. Lang, and F. Stoll-Keller. 2001. Quantitation of hepatitis C virus RNA in saliva and serum of patients coinfected with HCV and human immunodeficiency virus. J. Med. Virol. 63:117–119.
28. Roque Afonso, A. M., J. Jiang, F. Penin, C. Tareau, D. Samuel, M. A. Petit, H. Bismuth, E. Dussaix, and C. Feray. 1999. Nonrandom distribution of hepatitis C virus quasispecies in plasma and peripheral blood mononuclear cell subsets. J. Virol. 73:9213–9221.
29. Roy, K. M., J. Bagg, G. L. Bird, E. Spence, E. A. Follett, P. R. Mills, and J. Y. Lau. 1995. Serological and salivary markers compared with biochemical markers for monitoring interferon treatment for hepatitis C virus infection. J. Med. Virol. 47:429–434.
30. Roy, K. M., J. Bagg, B. McCarron, T. Good, S. Cameron, and A. Pithie. 1998. Predominance of HCV type 2a in saliva from intravenous drug users. J. Med. Virol. 54:271–275.
31. Sugimura, H., H. Yamamoto, H. Watabiki, B. Ogawa, H. Harada, I. Saitoh, T. Miyamura, M. Inoue, K. Tajima, and I. Kino. 1995. Correlation of detectability of hepatitis C virus genome in saliva of elderly Japanese symptomatic HCV carriers with their hepatic function. Infection 23:258–262.
32. Takamatsu, K., I. Okayasu, Y. Koyanagi, and N. Yamamoto. 1992. Hepatitis C virus propagates in salivary glands. J. Infect. Dis. 165:973–974.
33. Taliani, G., D. Celestino, M. C. Badolato, A. Pennica, A. Bozza, G. Poliandri, V. Riccieri, G. Benfari, A. Sebastiani, C. De Bac, G. Quaranta, and A. Aceti. 1997. Hepatitis C virus infection of salivary gland epithelial cells. Lack of evidence. J. Hepatol. 26:1200–1206.
34. Vincent, A. 2000. Mixed cryoglobulinemia and other extrahepatic manifestations of hepatitis C virus infection, p. 295–313. *In* T. J. Liang and J. H. Hoofnagle (ed.), Hepatitis C. Biomedical research reports. Academic Press, San Diego, Calif.
35. Wang, J. T., J. C. Sheu, J. T. Lin, T. H. Wang, and D. S. Chen. 1992. Detection of replicative form of hepatitis C virus RNA in peripheral blood mononuclear cells. J. Infect. Dis. 166:1167–1169.
36. Wang, J. T., T. H. Wang, J. T. Lin, J. C. Sheu, S. M. Lin, and D. S. Chen. 1991. Hepatitis C virus RNA in saliva of patients with post-transfusion hepatitis C infection. Lancet 337:48.
37. Wang, J. T., T. H. Wang, J. C. Sheu, J. T. Lin, and D. S. Chen. 1992. Hepatitis C virus RNA in saliva of patients with posttransfusion hepatitis and low efficiency of transmission among spouses. J. Med. Virol. 36:28–31.
38. Young, K. C., T. T. Chang, T. C. Liou, and H. L. Wu. 1993. Detection of hepatitis C virus RNA in peripheral blood mononuclear cells and in saliva. J. Med. Virol. 41:55–60.
39. Zoulim, F., M. Chevallier, M. Maynard, and C. Trepo. 2003. Clinical consequences of hepatitis C virus infection. Rev. Med. Virol. 13:57–68.

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Hepatitis C virus core protein exerts an inhibitory effect on suppressor of cytokine signaling *(SOCS)-1* gene expression

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Background/Aims: Suppressor of cytokine signaling (SOCS)-1, a negative feedback regulator of cytokine signaling pathway, also has a tumor suppressor activity, the silencing of its gene by hypermethylation is suggested to contribute to hepatocarcinogenesis. We studied the effect of the core protein of hepatitis C virus (HCV) on the expression of *SOCS-1* gene.

Methods: HCV core gene transgenic mice, which develop hepatocellular carcinoma late in life, HepG2 cells expressing the core protein, and human liver tissues were analyzed.

Results: The expression of *SOCS-1* gene was significantly suppressed in the liver of core gene transgenic mice and HepG2 cells expressing the core protein, while that of *SOCS-3* gene was conserved. *SOCS-1* expression levels also decreased in HCV-positive human liver tissues. The core protein differentially down-regulated the expression of signal transducer and activator of transcription (STAT) target genes, but rather enhanced STAT1 and STAT3 activation after interleukin-6 stimulation in mouse liver tissues and cells.

Conclusions: HCV core protein down-regulates the expression of *SOCS-1* gene. This is a mechanism leading to *SOCS-1* silencing, an alternative to the hypermethylation of the gene; this effect of the core protein may modulate the intracellular signaling pathway, contributing to the pathogenesis in HCV infection including hepatocarcinogenesis.

Keywords: Tumor suppressor gene; Hepatocellular carcinoma; Transgenic mouse; STAT3

1. Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis. A substantial proportion of patients with chronic hepatitis C eventually develop hepatocellular carcinoma (HCC), which is one of the leading causes of death worldwide [1,2]. Despite the absence of appropriate in vitro replication systems or practical infectious animal model systems, the mechanism underlying hepatocarcinogenesis in human HCV infection is gradually clarified. Both the direct and indirect effects of HCV on HCC development are demonstrated [3–6]. The accumulation of gene aberrations, such as the inactivation of tumor suppressor genes or the activation of oncogenes, which are induced through the inflammation-mediated continuous death of hepatocytes followed by regeneration, may be one of the mechanisms underlying hepatocarcinogenesis [3]. On the other hand, the viral gene products are suggested to contribute to the development of HCC by their direct effects on hepatocytes [4]. Such direct effects have been demonstrated by the use of model systems including mice

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[7–9]. HCV-infected hepatocytes produce viral structural and nonstructural proteins. Some of these confer certain phenotypes to hepatocytes and may be associated with the pathogenesis of HCV infection including the development of HCC. Among such viral proteins, the core protein of HCV has a variety of biological activities, including oncogenic activity, which substantially affects host cellular functions [7–11].

Suppressor of cytokine signaling (SOCS)-1, also called signal transducer and activator of transcription (STAT)-induced STAT inhibitor-1 or Jak binding protein-1, is a negative feedback regulator of cytokine signaling through the Jak/STAT pathway. SOCS-1 contains the SH2 domain and directly interacts with the kinase domain of Jak to suppress Jak activity. *SOCS-1* gene expression is augmented by various cytokines, such as interferon (IFN)-γ, interleukin (IL)-6 or leukemia inhibitory factor (LIF), resulting in the suppression of the signal transduction downstream pathways of these cytokines [12–14]. Moreover, SOCS-1 has been recently shown to exhibit a tumor suppressor activity. SOCS-1 suppresses the expression of several oncogenes or growth-related genes acting as a negative regulator of cell proliferation: the loss of function of SOCS-1 facilitates tumor progression [15–17]. As a mechanism underlying the loss of function of SOCS-1, a recent study has revealed a frequent silencing of the *SOCS-1* gene by CpG methylation in HCC tissues [18–20]. Alternatively, however, it may be possible that HCV infection, particularly, the proteins that the HCV genome encodes per se, may render the *SOCS-1* gene unable to exhibit its function by gene silencing.

We examined such a possibility using a mouse model for HCV infection that is destined to develop HCC [7,9], as well as cultured cells expressing the HCV core protein [21]. The core protein markedly suppressed the expression of the *SOCS-1* gene in both liver tissues and cultured cells. This silencing of the *SOCS-1* gene may be one of explanations for the pathogenicity of HCV in humans.

2. Materials and methods

2.1. Transgenic mouse and cell lines

HCV core gene transgenic mice have been described previously [7]. These mice develop HCC late in life [7,9]. The mice were cared for according to the institutional guidelines and maintained in a specific pathogen-free state. All the animals received humane care and the study protocol complied with the institution's guidelines for the care and use of experimental animals. HepG2 cell lines expressing the HCV core protein under the control of CAG promoter (Hep39J, Hep396 and Hep397) or a control HepG2 line (Hepswx) carrying the empty vector were described previously [21,22].

2.2. IL-6 Stimulation

For the in vivo experiments, 0.05–0.5 μg/g BW murine IL-6 (Diaclone, Besançon, France) was administered into 8 w.o. male mice i.p., and liver tissues were obtained 60 min later. Cultured cells were treated with human IL-6 (Diaclone) at 10–100 ng/ml or IFN-α at 1.0–10.0 ng/ml and then were harvested 60 min later.

2.3. Reverse transcription (RT)-PCR analysis

Total RNA was extracted from liver tissues or cultured cells before and after the treatment with IL-6 using TRIzol (Invitrogen). RNA was reverse-transcribed using oligo(dT) primers and Superscript II (Invitrogen). Equal amounts of cDNA were then subjected to PCR. The primer pairs used were:

5′-CACTCACTTCCGCACCTTCC-3′ (forward) and
5′-TCCAGCAGCTCGAAAAGGCA-3′ (reverse) for murine *SOCS-1*,
5′-CACGCACTTCCGCACATTCC-3′ (forward) and
5′-TCCAGCAGCTCGAAGAGGCA-3′ (reverse) for human *SOCS-1*,
5′-TCACCCACAGCAAGTTTCCCGC-3′ (forward) and
5′-GTTGACAGTCTTCCGACAAAGATGC-3′ (reverse) for murine *SOCS-3*,
5′-CACGCACTTCCGCACATTCC-3′ (forward), and
5′-GTTGACGGTCTTCCGACAGAGATGC-3′ (reverse) for human *SOCS-3*.

For the RT-PCR analysis, the quantity of cDNA template and the number of amplification cycles were optimized to ensure that the reaction was terminated during the linear phase of product amplification, so that the semiquantitative comparison of mRNA abundance between different samples was possible [23]. The intensities of the bands were determined using a densitometer. RT-PCR was also done using GAPDH primers to adjust the amounts of RNA in each experiment.

2.4. Human liver tissue samples and real-time PCR

Nine patients with HCC who had underlying chronic hepatitis C were studied for *SOCS-1* expression in noncancerous tissues. Additional nine patients, who were found to be negative for both HBsAg and anti-HCV at the time of operation, were also studied. The latter patients underwent liver resection for metastatic liver tumors from colon cancer. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethics Review Committee for Human Experimentation. Informed consent was obtained from each patient. The noncancerous liver tissues obtained from these patients were immediately frozen and stored at −80 °C until further use.

Taqman real-time RT-PCR was performed as described previously [24], using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Primers and the TaqMan probe for *SOCS-1* were as follows:

Forward primer:5′-CTGGCCCCGGAGCAT-3′
Reverse primer:5′-GTTGTGTGCTACCATCCTACAGA-3′
Probe:5′-FAM-CCGGACGCTATGGCCCA-MGB-3′

Primers and probes for *SOCS-3*, β-*actin*, interferon regulatory factor (*IRF*)-1, c-*myc* and *bcl*-X_L genes were purchased from ABI by Assays-on-Demand system.

2.5. Methylation status

The methylation status of the *SOCS-1* gene was analyzed by methylation-specific PCR as described previously [20].

2.6. Western blotting and immunoprecipitation

Nuclear and cytoplasmic fractions were prepared from HepG2 cells, and Western blotting was performed as described previously [25]. Anti-STAT1 and anti-STAT3 polyclonal antibodies (Cell Signaling Technology, Inc., Beverly, MA), anti-phosphorylated STAT3 (Tyr705) polyclonal antibody (Cell Signaling Technology), anti-phosphorylated STAT3 monoclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY), and anti-protein inhibitor of activated *STAT (PIAS)1*, *anti-PIAS3* and *anti-SOCS-1* antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were

used. Immunoprecipitation was done as described previously using antibodies followed by protein A-Sepharose [26].

2.7. Immunocytofluorescence

HepG2 cell lines with or without the core gene were grown overnight on chamber slides and treated with 10 ng/ml human IL-6 for 60 min. Cells were fixed with 4% paraformaldehyde plus methanol, and reacted with the anti-STAT3 antibody followed by incubation with a FITC-labeled secondary antibody.

2.8. Statistical analysis

The results are expressed as means±SD. The significance of the difference in means was determined by Mann–Whitney's *U*-test. $P<0.05$ was considered significant.

3. Results

3.1. HCV core protein suppresses SOCS-1 gene expression

To examine the impact of the core protein on *SOCS-1* gene expression, we analyzed mRNA expression levels by semi-quantitative RT-PCR in liver tissues from the HCV core gene transgenic and nontransgenic control mice. *SOCS-1* mRNA expression levels in mouse liver tissues of nontransgenic mice were increased in a dose-dependent manner of IL-6, but were only marginal in the liver tissues from the core gene transgenic mice even in those treated with the maximal dose of IL-6 (0.5 μg/g BW, $n=5$ each) (Fig. 1(A) and (C)). In contrast, the expression levels of *SOCS-3* mRNA in the core gene transgenic mice were comparable to or rather higher than those in nontransgenic mice, before and after stimulation with IL-6 (Fig. 1(A) and (E)) [27,28].

We then examined whether or not this observation in mice is reproducible in HepG2 cell lines that constitutively express the core protein. *SOCS-1* gene expression was suppressed in the core-expressing HepG2 cell lines Hep396, Hep397 and Hep39J, even after stimulation with IL-6, while control bulk HepG2 cells or a control Hepswx cell line expressed *SOCS-1* mRNA at high levels (Fig. 1(B) and (D)). In contrast, the levels of *SOCS-3* gene expression were similar among the core-expressing HepG2 cell lines and control HepG2 cells, and were augmented by stimulation with IL-6 (Fig. 1(B) and (F)). These observations indicate that the core protein selectively suppresses *SOCS-1* gene expression before the translational level. The SOCS-1 protein was not detectable by Western blotting either in the mouse liver or HepG2 cells using currently available anti-SOCS-1 antibodies.

These results, obtained in HepG2 cell lines constitutively expressing the core protein, were then evaluated using a transient expression system. In this system, HepG2 cells were infected with baculovirus, expressing the core protein as described previously [29], and *SOCS-1* expression was determined by semiquantitative RT-PCR. The introduction

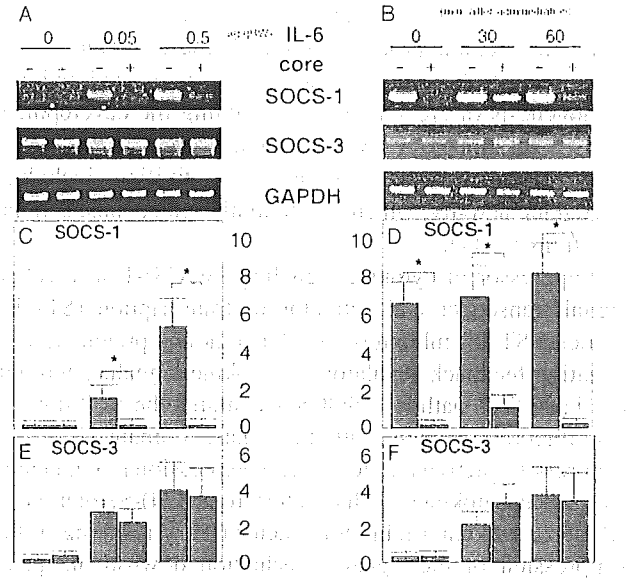

Fig. 1. Suppression of *SOCS-1* gene expression by hepatitis C virus core protein. (A) and (B) RNA from mouse liver tissues (A) or HepG2 cells (B) with or without the core protein was subjected to RT-PCR for the determination of *SOCS-1* and *SOCS-3* gene expression. Liver tissues were taken from mice one hour after inoculation of 0, 0.05 or 0.5 μg/g BW of IL-6. HepG2 cells (Hep396 and Hepswx) were treated with 10 ng/ml of IL-6 for 0, 30 or 60 min before RNA extraction. Bottom panels in (A) and (B) show the expression level of the housekeeping gene GAPDH as an internal control. (C) and (D) Represent means± SD. of five independent experiments on *SOCS-1* gene expression corresponding to the lanes in (A) and (B), respectively. *, $P<0.05$. (E) and (F) Represent means±SD. of five independent experiments on *SOCS-3* gene expression corresponding to the lanes in (A) and (B), respectively.

of the core protein selectively suppressed the expression level of *SOCS-1* mRNA even after stimulation with IL-6 (data not shown).

Modulation of expression by the core protein of STAT-target genes other than *SOCS*s was then examined by determining the mRNA levels in mouse liver tissues. Expression of *IRF-1* gene was suppressed in the presence of the core protein under the stimulation with IL-6, while that of c-*myc* was not affected (Fig. 2(A) and (B)). The expression of *bcl*-X_L gene was rather augmented by the core protein although the difference was not statistically significant (Fig. 2(C)).

The methylation status of *SOCS-1* gene was then explored in liver tissues from the core gene transgenic mice by a method described previously [20], to determine whether or not the *SOCS-1* gene expression may be suppressed by hypermethylation. No hypermethylation was observed in the *SOCS-1* gene of the core gene transgenic mice either at the 5′-noncoding region or the CpG island in the coding region (Fig. 3).

In the analysis of *SOCS-1* expression in noncancerous liver tissues from patients with HCV infection, the *SOCS-1* mRNA expression levels were 0.494±0.352 in HCV-positive patients ($n=9$) and 0.862±0.465 in the control subjects without HCV infection ($n=9$) (in arbitrary units,

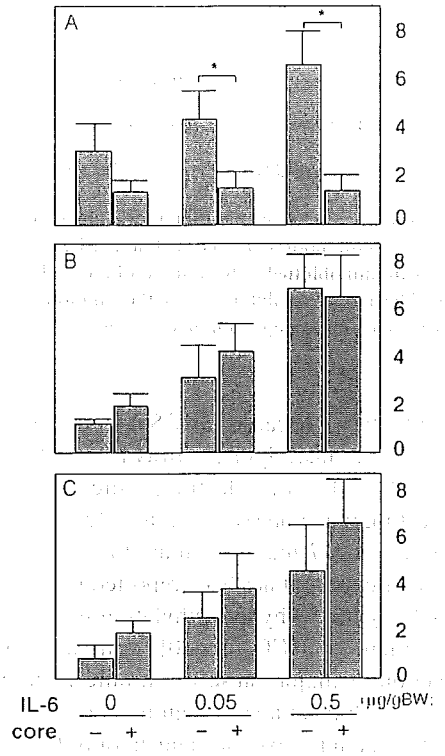

Fig. 2. Effect of hepatitis C virus core protein on the expression of STAT-target genes. RNA from mouse liver tissues with or without the core protein was subjected to RT-PCR for the determination of *IRF1* (A), c-*myc* (B) and *bcl*-X_L (C) gene expressions. Liver tissues were taken from mice one hour after inoculation of 0, 0.05 or 0.5 μg/g BW of IL-6. *, $P<0.05$.

$P=0.0345$). Thus, the SOCS-1 levels in the liver tissues of chronic hepatitis C patients were significantly lower than those of subjects without HCV infection.

3.2. The core protein did not suppress phosphorylation of STAT3 or STAT1

The activation of STAT3 enhances *SOCS-1* expression, thereby forming a negative feedback loop to the STAT3 status [17]. To determine whether or not STAT3 activation is involved in the *SOCS-1* gene suppression in this system, the tyrosine phosphorylation of STAT3 in the mouse liver was examined by Western blotting using an anti-phospho-STAT3 (tyrosine (Tyr)^{705}) antibody. At baseline, Tyr^{705} phosphorylation of STAT3 was low in both the core gene transgenic and nontransgenic mice. However, in response to stimulation with IL-6, the levels of Tyr^{705} phosphorylation of STAT3 was higher in the liver tissues from the core gene transgenic mice than that from nontransgenic mice. A representative result is shown in Fig. 4(A). Similarly, the levels of Tyr^{705} phosphorylation of STAT3 were higher in HepG2 cell lines expressing the core protein than those in control cells (Fig. 4(B)). These results observed in HepG2 cells constitutively expressing the core protein was also evaluated in a transient expression system using a recombinant baculovirus, as described above. The Tyr^{705} phosphorylation of STAT3 was enhanced in HepG2 cells infected with baculovirus expressing the core protein compared with mock-infected HepG2 cells (data not shown). The activation of STAT1 was also analyzed using HepG2 cell lines. As shown in Fig. 4(C), the levels of STAT1 phosphorylation was higher in HepG2 cells expressing the core protein than in control cells similar to the result on STAT3.

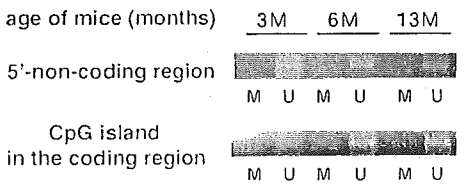

Fig. 3. Methylation status of *SOCS-1* gene in liver from hepatitis C virus core gene transgenic mice. DNA from the liver tissues of core gene transgenic mice at the age of 3 months (3M), 6 months (6M) or 13 months (13M) was subjected to methylation-specific PCR. Only PCR with unmethylation-specific primers yielded bands indicating that the *SOCS-1* gene was unmethylated in the liver tissues of core gene transgenic mice. M, methylation-specific primers; U, unmethylation-specific primers.

3.3. Subcellular localizations of STAT3 and STAT1

STAT activation by tyrosine phosphorylation results in the migration of STAT from the cytoplasm to the nucleus to bind to genomic DNA, modulating of cellular gene expression. We thereby evaluated the subcellular localization of STAT3 and STAT1 by preparing cytoplasmic and nuclear fractions from HepG2 cells followed by Western blotting. The amounts of STAT3 in the nuclei of core-expressing HepG2 cells were similar to or slightly larger

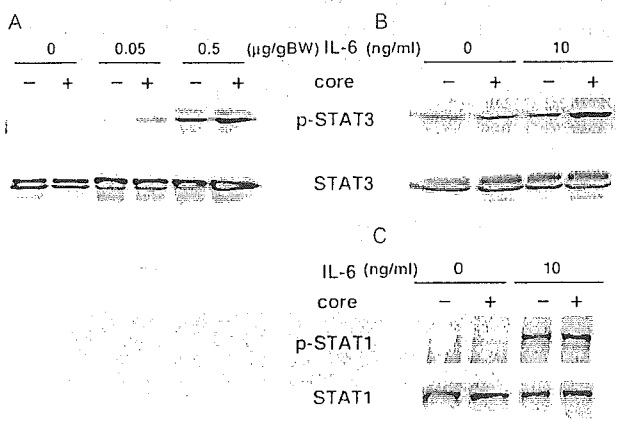

Fig. 4. Increase in the level of tyrosine phosphorylation of STAT3 and STAT1 by hepatitis C virus core protein. Whole cell lysates from mouse liver tissues (A) and HepG2 cells (B) and (C) were subjected to SDS-PAGE followed by Western blotting with anti-STAT3 and anti-*P*-STAT3 (A) and (B) or with anti-STAT1 and anti-*P*-STAT1 (C). Liver tissues were obtained from the mice treated as described in the Fig. 1 legends. HepG2 cells were treated with 10 ng/ml IL-6 or vehicle for 1 h. *P*-STAT3, phosphorylated STAT3; STAT3, total STAT3; p-STAT1, phosphorylated STAT1; STAT1, total STAT1. The experiments were repeated three times.

than those in control HepG2 cells in the presence or absence of IL-6 (Fig. 5(A)). This result was confirmed by an immunofluorescence study (Fig. 5(B)). A similar result was obtained in the analysis of STAT1 subcellular localization (Fig. 5(C)). These observations indicate that the HCV core protein does not inhibit the translocation of STAT3 or STAT1 to the nucleus, and the feedback mechanism is not the cause of *SOCS-1* gene suppression.

Because PIAS3 blocks the nuclear translocation of STAT3 or binding of STAT3 to genomic DNA [30], the expression of PIAS3 was examined by Western blotting. However, there was no significant difference in the levels of PIAS3 between core-expressing HepG2 cells and control HepG2 cells (data not shown). Co-immunoprecipitation analysis was also performed using HepG2 cell lines to know whether or not the core protein affects the association of PIAS1 with STAT1 or PIAS3 with STAT3. However, neither co-immunoprecipitation of STAT1 with anti-PIAS1 antibody nor that of STAT3 with anti-PIAS3 antibody was affected by the presence of the core protein (Fig. 6). We also examined the possibility of the interaction of the core protein with STAT3, which blocks the binding of STAT3 to the promoter of *SOCS-1* gene. For this purpose, a co-immunoprecipitation technique was utilized with whole-cell extracts of core-expressing HepG2 cells. However, no association was observed between these two proteins.

4. Discussion

In the current study, we demonstrated that the core protein of HCV suppresses the expression of *SOCS-1* mRNA in the liver tissues of mice that develop HCC late in their life [4,7]. This observation was reproduced in cultured cells that expressed the core protein. This phenomenon may contribute to the modification of the IFN signaling systems

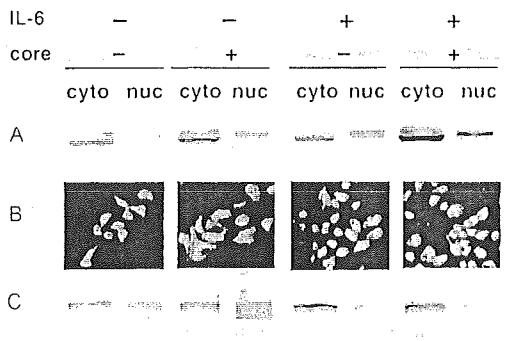

Fig. 5. Hepatitis C virus core protein did not affect subcellular localization of STAT3 or STAT1. Cytoplasmic and nuclear fractions from HepG2 cells with or without the core protein were subjected to Western blotting with the anti-STAT3 antibody (A) or anti-STAT1 antibody (C). HepG2 cells were fixed and an immunocytofluorescence study was performed using the anti-STAT3 antibody (B). Cells were processed before or 60 min after the treatment with 10 ng/ml of IL-6. cyto, cytoplasmic fraction; nuc, nuclear fraction [This figure appears in colour on the web].

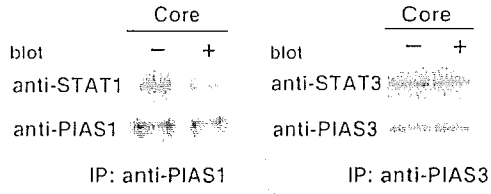

Fig. 6. Effect of the core protein on the interaction of STATs and PIASs. Cell lysates were immunoprecipitated with anti-PIAS1 or anti-PIAS3 antibody, and immunoblotted with anti-STAT1 or STAT3 antibody, respectively. There was no difference in the amounts of STAT1 or STAT3 that were co-immunoprecipitated with anti-PIAS antibodies.

in HCV infection, because SOCS-1 and SOCS-3 play central roles in the Jak/STAT pathway as negative feedback regulators [12–14]. In addition, since SOCS-1 also possesses a tumor suppressor activity [15–17], the down-regulation of *SOCS-1* may contribute to hepatocarcinogenesis in HCV infection. It has been reported that the silencing of the *SOCS-1* gene by hypermethylation is associated with the development of HCC [18–20]. Among patients with HCV infection, a major cause of chronic hepatitis worldwide, HCC develops at a very high incidence [1,2]. Hence, there may be an alternative mechanism of *SOCS-1* silencing to gene methylation in HCV infection. Our current data suggest a possibility of such a mechanism in that HCV per se acts as a negative regulator of SOCS-1, a tumor suppressor. The expression levels of *SOCS-1* mRNA in noncancerous liver tissues in chronic hepatitis C patients were also significantly lower than those in HCV-negative subjects, although the 'shut-off' of the *SOCS-1* gene observed in the experimental systems was not the case. This may be due to the presence of other factors influencing *SOCS-1* gene expression in vivo, including inflammation.

In the exploration of the mechanism underlying the down-regulation of *SOCS-1* expression, we first examined the methylation status of the *SOCS-1* gene in liver tissues from core gene transgenic mice by methylation-specific PCR. Neither the 5′-non-coding region nor the CpG island in the coding region of the *SOCS-1* gene [18–20] was hypermethylated, refuting methylation as a mechanism of SOCS-1 suppression.

We next determined whether or not STAT3, a transcription factor for the *SOCS-1* gene, is involved in the suppression of *SOCS-1* by the core protein: a decreased level or a disturbed phosphorylation of STAT3 may account for the suppression of *SOCS-1*. It was found, however, that STAT3 was rather activated by the core protein, consistent with a previous report [28]. The effect on STAT3 activation by the core protein is yet controversial [31]. Similarly, the activation and nuclear translocation of STAT1 was not disturbed by the presence of the core protein. The core protein differentially affected the expression of STAT-target genes such as *IRF1*, c-*myc* or *bcl*-X_L. The core protein suppressed *IRF1* expression in the mouse liver but did not those of c-*myc* and *bcl*-X_L genes. Regulation of *IRF1*

expression is STAT1-dependent in general, although STAT3 is also involved when stimulated by IL-6 [32]. c-*myc* and *bcl*-X_L inductions by IL-6 are chiefly mediated by STAT3 [33]. Thus, the modulation of expression by the core protein may occur in some other STAT-target genes, suggesting somewhere in Jak/STAT signaling pathway including STAT1 activation is impaired by the core protein. However, no defect was identified in the activation and nuclear translocation of STAT1 and STAT3 in the current study. Thus, although we could not define the precise role of the core protein in *SOCS-1* gene suppression, the direct effect of the core protein on the transcription of the gene is the most likely.

In summary, we found that the HCV core protein selectively suppresses *SOCS-1* gene expression in the liver tissues of animals and cultured cells. These findings may provide a basis for an alternative mechanism of the switch-off of SOCS-1 in the pathogenesis of HCV infection by modulating a tumor suppressor activity or responses to IFNs.

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References

[1] Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, et al. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci USA 1990;87:6547–6549.

[2] Simonetti RG, Camma C, Fiorello F, Cottone M, Rapicetta M, Marino L, et al. Hepatitis C virus infection as a risk factor for hepatocellular carcinoma in patients with cirrhosis. Ann Intern Med 1992;116:97–102.

[3] Umeda T, Hino O. Molecular aspects of human hepatocarcinogenesis mediated by inflammation: from hypercarcinogenic state to normo- or hypocarcinogenic state. Oncology 2002;62:38–42.

[4] Koike K, Tsutsumi T, Fujie H, Shintani Y, Moriya K. Role of hepatitis viruses in hepatocarcinogenesis. Oncology 2002;62:29–37.

[5] Block TM, Mehta AS, Fimmel CJ, Jordan R. Molecular viral oncology of hepatocellular carcinoma. Oncogene 2003;22: 5093–5107.

[6] Colombo M. Hepatitis C virus and hepatocellular carcinoma. Semin Liver Dis 1999;19:263–269.

[7] Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4: 1065–1067.

[8] Lerat H, Honda M, Beard MR, Loesch K, Sun J, Yang Y, et al. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology 2002;122:352–365.

[9] Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, et al. Oxidative stress in the absence of inflammation in the liver of a mouse model for hepatitis C virus-associated hepatocellular carcinoma. Cancer Res 2001;61:4365–4370.

[10] Tsuchihara K, Hijikata M, Fukuda K, Kuroki T, Yamamoto N, Shimotohno K. Hepatitis C virus core protein regulates cell growth and signal transduction pathway transmitting growth stimuli. Virology 1999;258:100–107.

[11] Ray RB, Meyer K, Ray R. Hepatitis C virus core protein promotes immortalization of primary human hepatocytes. Virology 2000;271: 197–204.

[12] Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, et al. A family of cytokine-inducible inhibitors of signaling. Nature 1997;387:917–921.

[13] Endo TA, Masuhara M, Yokochi M, Suzuki R, Sakamoto H, Mitsui K, et al. A new protein containing an SH2 domain that inhibits JAK kinases. Nature 1997;387:921–924.

[14] Naka T, Narazaki M, Hirate M, Matsumoto T, Minamoto S, Aono A, et al. Structure and function of a new STAT-induced STAT inhibitor. Nature 1997;387:924–929.

[15] Rottapel R, Ilangumaran S, Neale C, Rose JL, Ho JMY, Nguyen MHH, et al. The tumor suppressor activity of SOCS-1. Oncogene 2002;21:4351–4362.

[16] Ilangumaran S, Rottapel R. Regulation of cytokine receptor signaling by SOCS1. Immunol Rev 2003;192:196–211.

[17] Kishimoto T, Kikutani H. Knocking the SOCS off a tumor suppressor. Nat Genet 2001;28:4–5.

[18] Yoshikawa H, Matsubara K, Qian GS, Jackson P, Groopman JD, Manning JE, et al. SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. Nat Genet 2001; 28:29–35.

[19] Okochi O, Hibi K, Sakai M, Inoue S, Takeda S, Kaneko T, et al. Methylation-mediated silencing of SOCS-1 gene in hepatocellular carcinoma derived from cirrhosis. Clin Cancer Res 2003;9: 5295–5298.

[20] Miyoshi H, Fujie H, Moriya K, Shintani Y, Tsutsumi T, Makuuchi M, et al. Methylation status of suppressor of cytokine signaling-1 gene in hepatocellular carcinoma. J Gastroenterol 2004;39:563–569.

[21] Ruggieri A, Harada T, Matsuura Y, Miyamura T. Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. Virology 1997; 229:68–76.

[22] Aizaki H, Harada T, Otsuka M, Seki N, Matsuda M, Li YW, Kawakami H, et al. Expression profiling of liver cell lines expressing entire or parts of hepatitis C virus open reading frame. Hepatology 2002;36:1431–1438.

[23] Tsutsumi T, Suzuki T, Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, et al. Alteration of intrahepatic cytokine expression and AP-1 activation in transgenic mice expressing hepatitis C virus core protein. Virology 2002;304:415–424.

[24] Tsutsumi T, Suzuki T, Moriya K, Shintani Y, Fujie H, Miyoshi H, et al. Hepatitis C virus core protein activates ERK and p38 MAPK in cooperation with ethanol in transgenic mice. Hepatology 2003;38: 820–828.

[25] Moriya K, Fujie H, Yotsuyanagi H, Shintani Y, Tsutsumi T, Matsuura Y, et al. Subcellular localization of hepatitis C virus structural proteins expressed in transgenic liver. Jpn J Med Sci Biol 1997;50:169–177.

[26] Tsutsumi T, Suzuki T, Shimoike T, Suzuki R, Moriya K, Shintani Y, et al. Interaction of hepatitis C virus core protein with retinoid X receptor-α modulates its transcriptional activity. Hepatology 2002;35: 937–946.

[27] Bode JG, Ludwig S, Ehrhardt C, Erhardt A, Albrecht U, Schaper F, et al. IFN-α antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J 2003;17:488–490.
[28] Yoshida T, Hanada T, Tokuhisa T, Kosai K, Sata M, Kohara M, et al. Activation of STAT3 by the hepatitis c virus core protein leads to cellular transformation. J Exp Med 2002;196:641–653.
[29] Shoji I, Aizaki H, Tani H, Ishii K, Chiba T, Saito I, et al. Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. J Gen Virol 1997;78:2657–2664.
[30] Chung CD, Liao J, Liu B, Rao X, Jay P, Berta P, et al. Specific inhibition of Stat3 signal transduction by PIAS3. Science 1997;278:1803–1805.
[31] Hosui A, Ohkawa K, Ishida H, Sato A, Nakanishi F, Ueda K, et al. Hepatitis C virus core protein differently regulates the JAK-STAT signaling pathway under interleukin-6 and interferon-γ stimuli. J Biol Chem 2003;278:28562–28571.
[32] Kojima H, Nakajima K, Hirano T. IL-6-inducible complexes on an IL-6 response element of the junB promoter contain Stat3 and 36 kDa CRE-like site binding protein(s). Oncogene 1996;12:547–555.
[33] Kiuchi N, Nakajima K, Ichiba M, Fukada T, Narimatsu M, Mizuno K, et al. STAT3 is required for the gp130-mediated full activation of the c-myc gene. J Exp Med 1999;189:63–73.

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Characterization of HCV-like particles produced in a human hepatoma cell line by a recombinant baculovirus

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Abstract

Although processing of the hepatitis C virus (HCV) polyprotein and characterization of each of its viral proteins have been described in detail, analysis of the structure and assembly of HCV particles has been hampered by the lack of a robust cell culture system to support efficient replication of HCV. In this study, we generated HCV-like particles (HCV-LP) using a recombinant baculovirus encoding structural and a part of non-structural proteins in a human hepatoma cell line. The HCV-LP exhibited a buoyant density of 1.17 g/ml in CsCl equilibrium gradient and particles of 40 to 50 nm in diameter. Binding of the HCV-LP to human hepatoma cells was partially inhibited by the treatment with anti-hCD81 antibody, in contrast to the hCD81-independent binding of HCV-LP produced in insect cells. These results indicate that HCV-LP generated in different types of cells exhibit different cellular tropism for binding to target cells.

Keywords: Hepatitis C virus; Baculovirus; Virus-like particle; Human hepatoma cell line; hCD81

Hepatitis C virus (HCV) is the most important causative agent of post-transfusion and sporadic non-A non-B hepatitis, infecting at least 3% of the world population [1]. HCV infection becomes chronic in most cases and may eventually lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2]. Current treatments for chronic HCV infection are still limited, and there is no vaccine available to prevent HCV infection [3]. Although processing of the HCV polyprotein and characterization of each of its viral proteins have been described in detail [2], the infection mechanisms of HCV are still unclear due to the lack of a robust and reliable cell culture system and small animal models for HCV replication. To circumvent this problem, soluble truncated E2 protein [4,5], HCV-like particles (HCV-LP) produced by recombinants of baculovirus [6–11] and vesicular stomatitis virus (VSV) [12], an RNA replicon system of Semliki forest virus (SFV) [13], an in vitro assembly system [14], and pseudotype viruses based on VSV [15,16] and retroviruses [17,18] have been developed and used to examine the binding and entry receptors for HCV. It has been shown that the expression of the major structural proteins by various viral vectors leads to the formation of virus-like particles (VLP) [6–10,12]. Production of VLP has mostly succeeded in insect cells using a recombinant baculovirus expression system [6–10,19,20]. VLP are demonstrated to be useful not only for subunit vaccine [19] but also for study of virus–cell interactions [20]. Several groups have succeeded in producing HCV-LP in insect cells

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using recombinant baculoviruses [6,9,10], and the resulting particles have been suggested to have properties similar to those of authentic HCV particles based on morphologic, biophysical, and antigenic analyses [7,10,11], in addition to dose-dependent and saturable binding to human lymphoma and hepatoma cell lines [8]. Although HCV-LP exhibit several properties similar to those of the recombinant E2 protein and pseudotype viruses, including involvement of cell surface glycosaminoglycans and neutralization by specific antibodies for binding to target cells, the binding of HCV-LP did not correlate with the expression of hCD81, which is a candidate receptor for HCV [8,11,21]. Therefore, the interaction of putative HCV receptors with HCV-LP is still controversial.

Recombinant baculoviruses were shown to be able to deliver foreign genes not only into insect cells, but also into mammalian cells without extensive cytopathic effects [22–24], and expression of the HCV genome in mammalian cells has been reported by use of the recombinant baculovirus system [25,26]. Recently, we developed modified recombinant baculoviruses possessing the VSV envelope G protein (VSVG) on the virion surface, and enhanced gene transfer efficacy has been demonstrated in a variety of cell lines [27,28]. In this study, we produced HCV-LP in a human hepatoma cell line, FLC4, by using the recombinant baculovirus system and characterized the properties of the HCV-LP.

Materials and methods

Plasmids. The HCV cDNA (genotype 1b) used in this study was originally isolated from a blood sample of an HCV carrier, which was infectious for both humans and chimpanzees (NIHJ1 clone) [29]. The puromycin resistance gene was excised from pPUR (BD Biosciences Clontech Laboratories, Mountain View, CA) and cloned into pCAGGS [30] under the SV40 early promoter. To create multiple cloning sites, a DNA fragment was synthesized by two oligonucleotides, 5′-AATTAATT AAGTTTAAACGGCGCGCCGATATCTTAAGATCTTCG-3′ and 5′-AATTCGAAGATCTTAAGATATCGGCGCGCCGTTTAAACTTAA TT-3′; the fragment contains 8 restriction sites, *Pac*I, *Pme*I, *Asc*I, *Eco*RV, *Afl*II, *Bgl*II, *Bst*BI, and *Eco*RI, inserted into the *Eco*RI site of pCAGGS, and the plasmid was designated pCAGPM. The PCR fragment of each region indicated in Fig. 1 was amplified from pBRT703′X containing a whole HCV cDNA into pBR322 (Promega, Madison, WI) under the T7 promoter and cloned into the *Eco*RV and *Bgl*II sites of pCAGPM. The whole HCV genome was excised from pBRT703′X and cloned into the *Eco*RI and *Pme*I sites of pCAGPM.

Cells. HEK293T, HeLa, Huh7, HepG2, BRL3A, BHK, NMuLi, CHO, and RK-13 cell lines were obtained from the ATCC (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS). FLC4 cell line was maintained as described above. The *Spodoptera frugiperda* (Sf9) cell line was purchased from the ATCC (Rockville, MD) and maintained in Sf900II-SFM (Invitrogen Life Technology, Carlsbad, CA).

Construction of recombinant baculoviruses. Viral constructs were prepared with the pFastBac1 plasmid and the Bac-To-Bac baculovirus expression system (Invitrogen Life Technology) as described previously [31]. A cDNA encoding the region from the HCV core to NS2 was cloned into pFB-VSVG/CAG [31] under the CAG promoter, and a recombinant baculovirus AcVSVG-CACN2 was generated following a protocol provided by the manufacturer. AcVSVG-CAGFP [28] was used as a negative control.

Reagents and antibodies. GST fusion and purification of a large extracellular loop of hCD81 (hCD81LEL) were performed as described previously [4]. Mouse monoclonal antibody against hCD81 was purchased from BD Biosciences Pharmingen (San Diego, CA). The anti-core (c-11-7, c-11-10, and c-11-14) monoclonal antibodies have been described previously [32]. Anti-E1 (299 and 384) and anti-E2 (187) monoclonal antibodies were kindly provided by Dr. M. Kohara. The anti-E2 (AP33) monoclonal antibody has been described previously [10]. Anti-E1 (0726cb1) monoclonal antibody was prepared by immunization with cell lysates of CHO/chiJ1 [15]. Control mouse IgG1 and IgG2a antibodies, anti-E1 (JMAb80) and anti-E2 (JMAb70) human monoclonal antibodies were provided by JT laboratory (Osaka, Japan). Endoglycosidase H (Endo H) and peptide-*N*-glycosidase F (PNGase F) were purchased from Roche-Diagnostics (Mannheim, Germany).

Expression of HCV proteins in mammalian cells. Culture supernatants of cells transfected with the expression plasmids were harvested at 48 h post-transfection, and cells were lysed with TNE lysis buffer (50 mM Tris, 50 mM NaCl, 0.1% NP-40, and 0.5 mM EDTA, pH 7.5) containing protease inhibitor (Complete EDTA-free; Roche-Diagnostics). Expression of HCV proteins in supernatants and cell lysates was analyzed by immunoblotting, quantitative HCV core ELISA (Ortho-Clinical Diagnostics, Tokyo, Japan), and ELISA for E2 protein as described previously [33]. FLC4 cells [34] infected with AcVSVG-CACN2 or AcVSVG-CAGFP at an moi of 250 were harvested at 12 to 96 h post-infection, and expression of HCV proteins in supernatants and cell lysates was analyzed as described above. Furthermore, FLC4 cells infected with the recombinant baculovirus were immunoprecipitated with anti-E1 (JMAb80), anti-E2 (JMAb70), or control human monoclonal antibody, and HCV structural proteins were detected by immunoblotting using specific monoclonal antibodies.

HCV-LP. Purification of HCV-LP from FLC4 cells infected with the recombinant baculovirus was carried out as described previously [6]. Briefly, FLC4 cells infected with AcVSVG-CACN2 were homogenized and subjected to low-speed centrifugation at 15,000*g* for 30 min at 4 °C, and the supernatant was spun down through 30% (wt/vol) sucrose in a phosphate-buffered saline (PBS) cushion by centrifugation at 200,000*g* for 1.5 h at 4 °C. The pellets were resuspended in PBS containing the protease inhibitor, homogenized, and mixed with 33% (wt/wt) cesium chloride and centrifuged at 300,000*g* for 48 h at 4 °C. After centrifugation, 10 fractions (0.5 ml each) were collected from the top and pelleted through a 30% (wt/vol) sucrose cushion by centrifugation at 200,000*g* for 1 h at 4 °C. The pellets were resuspended in PBS containing the protease inhibitor and analyzed by immunoblotting, ELISA, PCR, and electron microscopy. To examine the incorporation of viral RNA into HCV-LP, each of 10 fractions was treated with 50 mU/μl DNase and 0.6 μg/μl RNase for 30 min at 37 °C to remove free RNA and DNA. Total RNA was isolated from each sample by using TRIzol Reagent (Invitrogen Life Technology) and subjected to reverse transcription by using a First-Strand cDNA Synthesis kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's protocol. Synthesized cDNA was used as a template for HCV-specific PCR.

Electron microscopy. Five microliters of purified HCV-LP was absorbed onto carbon-coated copper 200 mesh grids (NisshinEM, Tokyo, Japan) for 5 min, washed with water, and negatively stained with 2% (wt/vol) uranyl acetate. For immunogold labeling, HCV-LP absorbed on the grids were incubated with HCV-specific mouse monoclonal antibodies, washed with PBS, incubated with a droplet of 10-nm gold particles conjugated to goat anti-mouse IgG antibody (British Biocell International, South Glamorgan, UK), washed quickly with water, and negatively stained. The grids were examined with a Hitachi electron microscope (Hitachi, Tokyo, Japan) at 75 kV.

Binding assay. Binding of HCV-LP to the target cells was determined by the amount of core proteins detected by quantitative core ELISA. HepG2 or Huh7 cells were incubated with various amounts of HCV-LP for 2 h at 4 °C, harvested after extensive washing with DMEM containing 2% FBS, and examined by core ELISA. To determine the effects of chemical modification of cells on the binding of HCV-LP, Huh7 cells were pre-incubated with various concentrations of phospholipase C, Pronase, or sodium periodate as described previously [27]. Binding of HCV-LP to

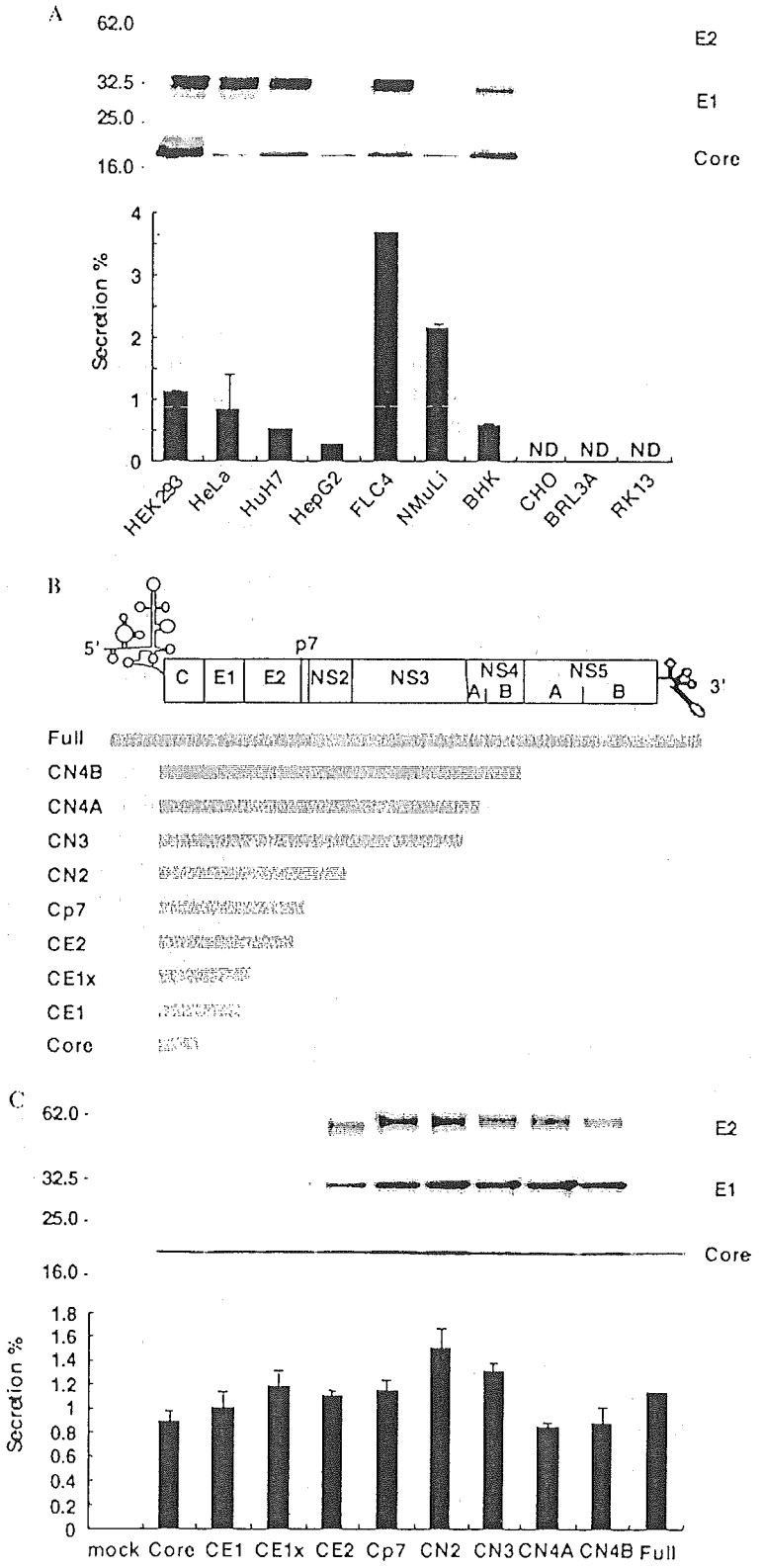

Fig. 1. (A) Expression of HCV structural proteins in various cell lines transfected with the expression plasmid, pCAG-Cp7. (top) Immunoblot analysis of structural proteins. (bottom) Secretion % of core proteins from each cell line. (B) Various cDNA clones used for transient expression in FLC4 cells. (C) Expression of HCV structural proteins in FLC4 cells transfected with the expression plasmids encoding various regions of HCV. (top) Immunoblot analysis of structural proteins. (bottom) Secretion % of core proteins in FLC4 cells transfected with each construct.

Huh7 cells was examined by the amount of core proteins in the cell lysates after 2 h incubation. Infection of AcVSVG-CAGFP was examined by the expression of GFP after 24 h incubation. Cell viabilities were determined by trypan blue staining. To determine the inhibitory effects of binding of HCV-LP to the target cells by soluble proteins or antibodies, HCV-LP were pre-incubated with soluble hCD81LEL (50 μg/ml) or anti-E2

antibody (AP33, 50 μg/ml) for 16 h at 4 °C, and the mixture was incubated with HepG2 or Huh7 cells for 2 h at 4 °C. To assess the effect of anti-hCD81 antibody on the binding of HCV-LP, HepG2 or Huh7 cells were incubated with anti-hCD81 antibody (20 μg/ml) for 1 h at 26 °C prior to the addition of HCV-LP. The binding of HCV-LP to HepG2 or Huh7 cells was examined by core ELISA as described above.

Results and discussion

Determination of a cell line and cDNA construct for the production of HCV-LP

Although previous studies have produced HCV-LP in mammalian cells using various expression systems, including a recombinant VSV [12] and an SFV RNA replicon [13] system, the levels of production of HCV-LP in mammalian cells were lower than those in insect cells [6–8,10,11]. In this study, we tried to produce HCV-LP in human hepatoma cells using a novel baculovirus vector for an efficient transduction of foreign genes into mammalian cells [22–24]. To determine a cell line suitable for production of HCV-LP, various mammalian cell lines were transfected with an expression plasmid, pCAG-Cp7, encoding HCV structural proteins (Figs. 1A and B). Among the cell lines examined, the human hepatoma cell line FLC4 was selected due to its high level expression of HCV structural proteins and its high level secretion of core proteins into culture supernatants (Fig. 1A). FLC4 cells have previously been shown to efficiently translate HCV RNA [34] and to produce infectious HCV particles in a three-dimensional radial-flow-bioreactor system [35]. We then compared expression plasmids encoding various regions of HCV proteins to determine a cDNA construct for HCV-LP production (Fig. 1B). Among the clones examined, an expression plasmid encoding the region from the core to NS2 (CN2) under the CAG promoter exhibited the highest expression of structural proteins and secretion of core proteins in FLC4 cells (Fig. 1C). Therefore, we selected the CN2 construct encoding the region from the core to NS2 for construction of a recombinant baculovirus.

Expression of HCV structural proteins in FLC4 cells infected with a recombinant baculovirus

Recombinant baculovirus possessing VSVG protein on the virion surface exhibited higher efficiency of gene transfer into a variety of cell lines, including FLC4 cells [27,28]. To achieve an efficient gene transduction of the CN2 construct into FLC4 cells, we constructed a recombinant baculovirus AcVSVG-CACN2 encoding a cDNA encoding the region from the core to NS2 under the control of the CAG promoter (Fig. 2A). HCV structural proteins properly processed as reported previously in both mammalian and insect cells [6,15,36] were detected in cells by immunoblotting analysis using specific monoclonal antibodies (Fig. 2B). To examine the kinetics of HCV-LP production, the level of secretion of core proteins in culture supernatants and the expression of core proteins in FLC4 cells infected with AcVSVG-CACN2 were determined (Fig. 2C). The expression of core proteins in cells reached the highest level (approximately 500 pg/μg of total protein) at 24 h post-infection and then decreased, whereas the level of core proteins secreted into the culture supernatants gradually increased up to 96 h post-infection (approximately 115 pg/ml) and then decreased. Similar results were obtained in the expression of envelope proteins by immunoblotting analyses (data not shown). No significant cytopathic effect was observed in the infected cells (data not shown). Co-immunoprecipitation of not only E1 and E2 proteins but also core proteins was observed by anti-E1 and -E2 monoclonal antibodies (Fig. 2D). No precipitation was detected in cells infected with AcVSVG-CAGFP or in the precipitates with a control antibody. Immunofluorescence analyses of the infected cells revealed co-localization of structural proteins in the cytoplasm, primarily in the ER (data not shown). These results indicate that properly processed HCV structural proteins were generated in FLC4 cells by infection with AcVSVG-CACN2.

Characteristics of HCV-LP generated in FLC4 cells

Although a small amount of HCV-LP was secreted into the culture supernatant of FLC4 cells, most of them were detected in intracellular organelles, primarily the ER, as previously demonstrated for HCV-LP produced in insect cells and by an SFV replicon system [6,13]. According to the purification procedure of HCV-LP in insect cells, lysates of FLC4 cells infected with AcVSVG-CACN2 were subjected to CsCl equilibrium gradient centrifugation, and each fraction was analyzed by ELISA and immunoblotting (Fig. 3A). ELISA revealed two peaks of core and E2 proteins in fractions 4 and 7/8, corresponding to buoyant densities of 1.17 and 1.29–1.40 g/ml, respectively (Fig. 3A, bottom). Immunoblotting analyses revealed core proteins clearly and small amounts of E1 and E2 proteins in these fractions (Fig. 3A, top). The buoyant density of HCV-LP in fraction 4 is consistent with that of the high-density HCV particles in patient sera [37,38], HCV-LP produced in insect cells [6,8,9], and infectious HCV particles of the JFH-1 strain recently reported [39,40]. HCV-LP produced in insect cells have been shown to incorporate HCV RNA [6]. To examine the incorporation of viral RNA in each fraction, total RNA was extracted, reverse transcribed, and amplified by PCR using four sets of primers specific for the core, E1, E2 and NS2 proteins. Viral RNA was detectable only in fraction 4, in which HCV proteins were detected by immunoblotting (Fig. 3A, middle), suggesting that HCV-LP produced in FLC4 cells contain HCV genomes, as do HCV-LP produced in insect cells [6]. HCV RNA has also been detected in the lower-density particles (1.07 g/ml) in the supernatants of FLC4 cells cultured in a three-dimensional radial-flow-bioreactor system [35] and in purified particles from the sera of hepatitis C patients [37,41]. Although we do not know the biological meaning of the presence of these particles exhibiting different densities, it may be that the higher-density fractions (over

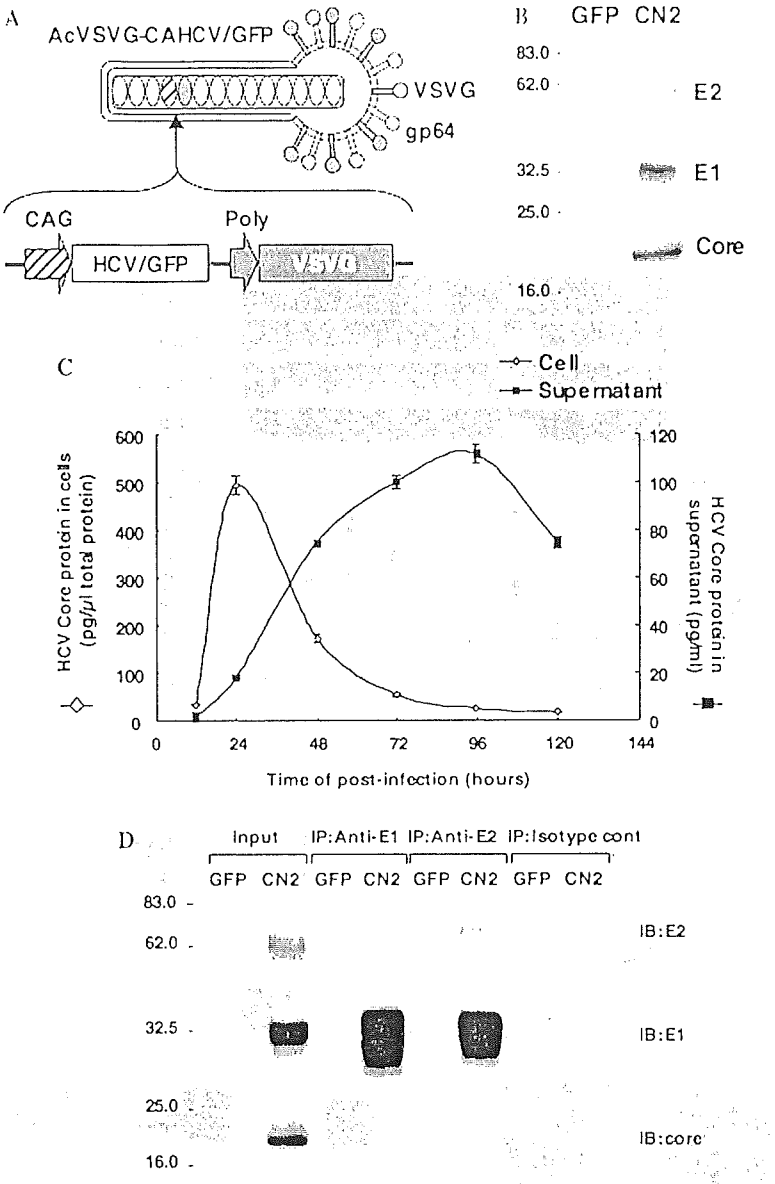

Fig. 2. (A) Schematic representation of recombinant baculoviruses used in this study. HCV or GFP and VSVG genes were inserted under the CAG and the polyhedrin (Poly) promoter, respectively. (B) Expression of HCV proteins in FLC4 cells by infection with a recombinant baculovirus. FLC4 cells were infected with AcVSVG-CAGFP (GFP) or AcVSVG-CACN2 (CN2) at an moi of 250. Expression of HCV proteins was determined by immunoblot analysis at 24 h post-infection. (C) Expression of core proteins in FLC4 cells (open diamond) and secretion into culture supernatants (closed square) upon infection with AcVSVG-CACN2. (D) Co-immunoprecipitation of HCV structural proteins in FLC4 cells. FLC4 cells were infected with AcVSVG-CAGFP (GFP) or AcVSVG-CACN2 (CN2) at an moi of 250 and cell lysates were immunoprecipitated with anti-E1 (JMAb70) or anti-E2 (JMAb80) human monoclonal antibody, or isotype control antibody (JMAb23). The immunoprecipitates were detected by immunoblotting with anti-core (c-11-7, 10, 14), anti-E1 (0726cb1), or anti-E2 (187) mouse monoclonal antibodies.

1.24 g/ml) represent empty particles or those with broken envelope proteins [37].

To examine the morphology of HCV-LP, the gradient fractions were examined by electron microscopy. HCV-LP were detected in fraction 4 by negative staining and immunogold labeling. The majority of the particles had a diameter of 40 to 50 nm (Fig. 3B). The specificities were also confirmed by immunogold affinity labeling with anti-E1 (299 and 0726cb1; Figs. 3C and D) and anti-E2 (97 and 187; Figs. 3E and F) monoclonal antibodies under the electron microscopic analyses. The morphology of HCV-LP produced in the FLC4 cells was similar to that produced in insect cells [6,8,10] and authentic HCV particles [41], suggesting that HCV-LP retain immunoreactive envelope proteins on the particles. To determine the glycosylation of HCV envelope proteins, cell lysates of FLC4 cells infected with the recombinant baculovirus and purified HCV-LP were treated with Endo H or PNGase F. E1 and E2 proteins in both cells and HCV-LP were completely digested by both of the enzymes, suggesting that E1 and E2 proteins have high-mannose-type carbohydrates (Fig. 3G). E1 protein digested with PNGase F was hardly detected by anti-E1 antibodies, probably due to reduction of affinity to the anti-E1 antibody.

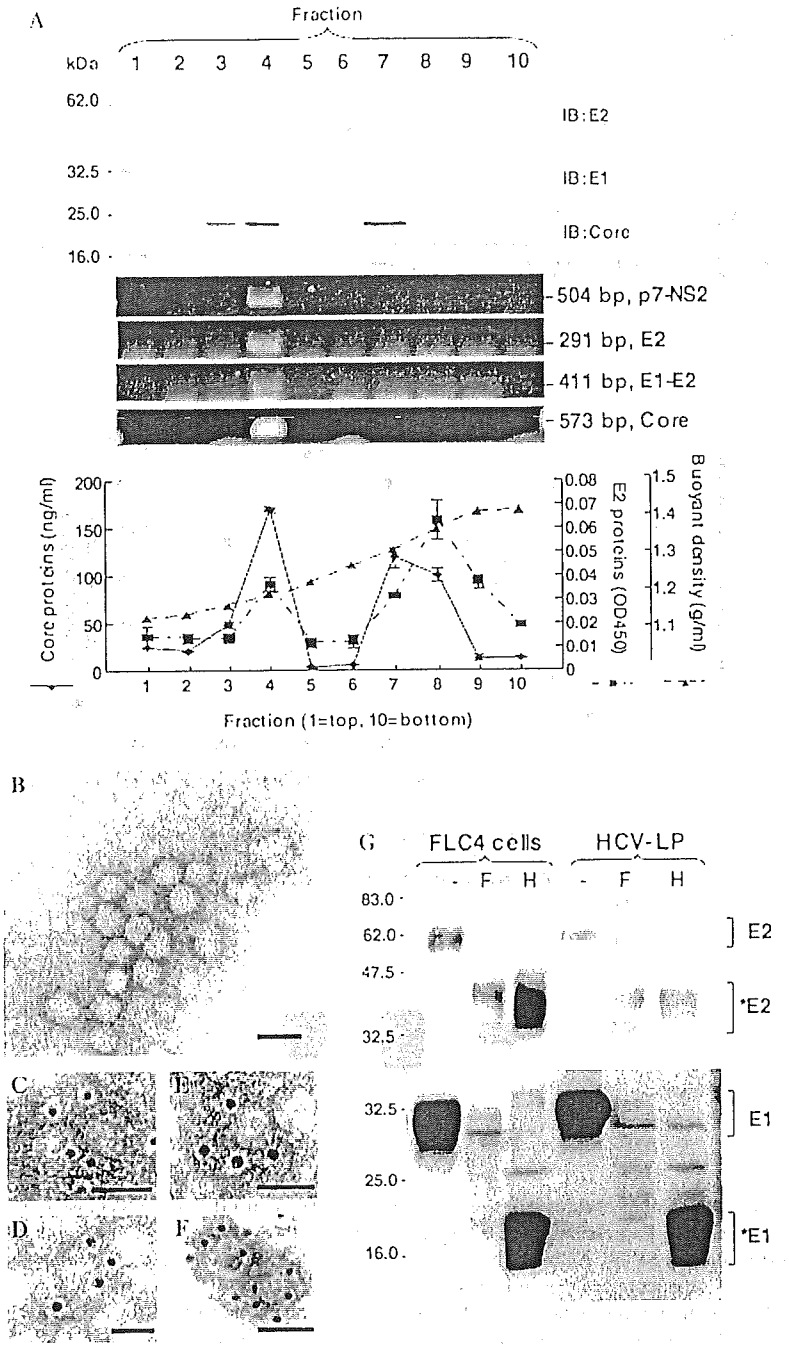

Fig. 3. (A) Purification of HCV-LP by CsCl equilibrium centrifugation. Supernatants of lysates of FLC4 cells infected with AcVSVG-CACN2 were spun down over a 30% (wt/vol) sucrose cushion and subjected to CsCl equilibrium centrifugation. Ten fractions were collected from the top and then analyzed by immunoblotting with anti-core (c-11-7, 10, 14), anti-E1 (0726cb1), and anti-E2 (187) monoclonal antibodies (top). Incorporation of HCV RNA into HCV-LP was examined by RT-PCR (middle). Core and E2 proteins in each fraction were determined by core ELISA (closed diamond) and E2 ELISA (closed square), respectively. Closed triangles indicate the buoyant density. (B–F) Electron microscopy of purified HCV-LP stained with 2% uranyl acetate (B). Immunogold labeling of HCV-LP with anti-E1 (C: 299; D: 0726cb1) and anti-E2 (E: 97; F: 187) mouse monoclonal antibodies. The bar represents 50 nm. (G) Deglycosylation of HCV envelope proteins. Cellular lysates and purified HCV-LP were treated with PNGase F (F), Endo H (H) or PBS (−) and subjected to immunoblotting with anti-E1 (299 and 384) and anti-E2 (187) monoclonal antibodies. Asterisks indicate deglycosylated envelope proteins.

Binding of HCV-LP to human hepatoma cell lines

The human hepatoma cell lines Huh7 and HepG2 are known to be target cells for the binding or infection of pseudotype viruses [15,16,42,43] or JFH-1 virus [39,40] as well as the HCV-LP derived from insect cells [8,11]. Therefore, the binding properties of HCV-LP generated in FLC4 cells were examined in Huh7 and HepG2 cells. The binding of HCV-LP occurred in a dose-dependent manner and was saturable to both Huh7 and HepG2 cells (Fig. 4A). Binding

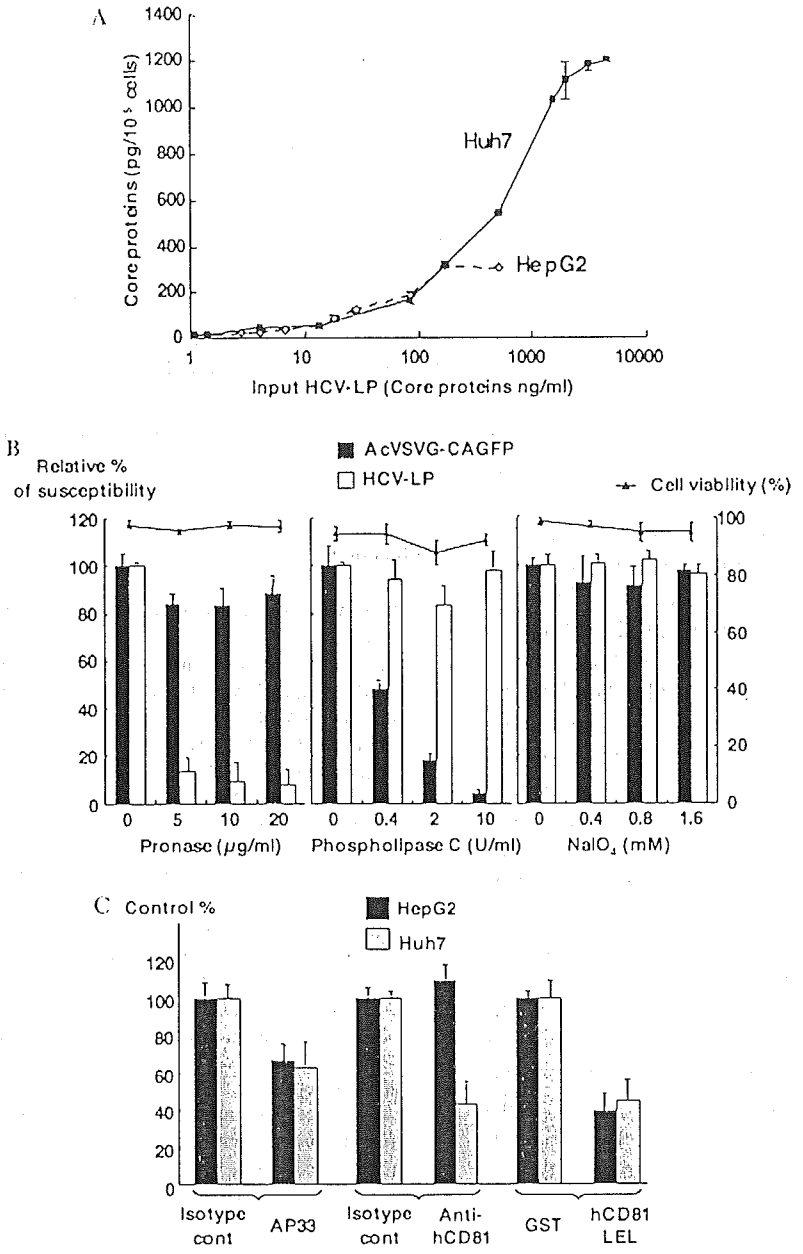

Fig. 4. (A) Binding of HCV-LP to HepG2 and Huh7 cells. Cells were incubated with increasing concentrations of HCV-LP, and bound HCV-LP were determined by core ELISA after extensive washing. (B) Effect of chemical modification of Huh7 on the binding of HCV-LP and the infection of AcVSVG-CAGFP. Huh7 cells were pre-incubated with various concentrations of Pronase, phospholipase C or sodium periodate. Binding of HCV-LP was determined by quantitative core ELISA. Infection of AcVSVG-CAGFP was examined by the expression of GFP at 24 h post-incubation. Cell viabilities were determined by trypan blue staining. (C) Effects of hCD81LEL or antibody to E2 or hCD81 on the binding of HCV-LP to HepG2 and Huh7 cells. HCV-LP were incubated with hCD81LEL (50 μg/ml) or anti-E2 (AP33, 50 μg/ml) overnight at 4 °C. HepG2 or Huh7 cells were incubated with anti-hCD81 antibody (20 μg/ml) for 1 h at 26 °C prior to inoculation of HCV-LP. The binding of HCV-LP to the target cells was determined by core ELISA. The results shown are the average of three independent assays with the error bars representing the SD.

of HCV-LP reached plateaus at concentrations of approximately 0.5 and 4.0 μg/ml of core protein for HepG2 and Huh7 cells, respectively. These results indicate that Huh7 cells have a greater capacity to bind HCV-LP produced in mammalian cells than HepG2 cells. HCV-LP produced in insect cells [8,10,11] and pseudotype VSV [15] exhibited higher affinity for HepG2 cells than Huh7 cells, in contrast to the pseudotype retrovirus, which exhibited infectivity to Huh7 cells but not to HepG2 cells [43]. To examine cell surface molecules involved in the binding of HCV-LP, Huh7 cells were pre-incubated with various concentrations of Pronase, phospholipase C or sodium periodate, and binding of HCV-LP was evaluated (Fig. 4B). Previously, it was demonstrated that infectivity of baculovirus [27] and binding of VLP of Norwalk virus [20] were decreased by the treatment with phospholipase C or Proteinase K. Treatment of Huh7 cells with Pronase, but not with phospholipase C or sodium periodate, decreased the binding of

HCV-LP, whereas infection of AcVSVG-CAGFP was inhibited by the treatment with phospholipase C, but not with Pronase or sodium periodate as reported previously [27]. These treatments exhibited no effect on cell viability. These results indicated that protein molecules on the cell surface play an important role for the binding of HCV-LP to Huh7 cells.

Involvement of hCD81 in the binding of HCV-LP

hCD81 has been suggested to be one of the major host factor candidates for the binding and infection of HCV [44]. Accumulating data from studies on binding or infection assays suggest that hCD81 is involved in the infection of HCV [8,10,16,18,33,40,42,43]. To determine the involvement of hCD81 in the binding of HCV-LP, target cells or HCV-LP were pre-treated with anti-hCD81 antibody or soluble hCD81LEL, and the effect on the HCV-LP binding was evaluated (Fig. 4C). The binding of the HCV-LP to both HepG2 and Huh7 cells was partially inhibited by the treatment with anti-E2 antibody (AP33) and hCD81LEL. The binding to Huh7 cells was also inhibited by the treatment with anti-hCD81 antibody, but no inhibition was observed in HepG2 cells due to the lack of hCD81 expression. The specific interaction of hCD81 with purified E2 protein has been reported [4,44,45]. Pseudotype retroviruses produced in 293T cells [18,42,43] and infectious HCV clones of the JFH-1 strain [39,40] were also shown to infect target cells through an hCD81-dependent pathway. However, HCV-LP derived from insect cells and pseudotype VSV produced in CHO cells exhibited an hCD81-independent binding [21] or infection [15]. In the case of HCV-LP derived from FLC4 cells, hCD81 did indeed play an important role in the binding to the target cells, but inhibition of binding by the anti-hCD81 and hCD81LEL was approximately 50%, suggesting that molecules other than hCD81 also participate in the binding. By contrast, in HCV-LP produced in insect cells, expression of hCD81 is not essential for binding [8,11,21]. Although we do not know the reason why the binding properties of HCV-LP derived from insect and human hepatoma cells are different, it may be that post-translational modifications, including glycosylation and maturation of envelope proteins, are different between insect and mammalian cells.

In conclusion, we could produce HCV-LP in a human hepatoma cell line using a recombinant baculovirus encoding HCV proteins and showed that the HCV-LP exhibited profiles different from those of HCV-LP derived from insect cells. The binding properties to the target cells were partially dependent on hCD81, but the involvement of other pathways was also suggested. The HCV-LP produced in FLC4 cells may help to clarify the differences in cell tropism of infection among HCV pseudotype viruses produced in different cell lines, and in binding profiles among the purified envelope proteins and HCV-LP produced in various cell lines. Further studies are needed to identify host factor(s) other than hCD81 involved in the binding of HCV-LP.

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References

[1] M.J. Alter, Epidemiology of hepatitis C, Hepatology 26 (1997) 62S–65S.
[2] M.E. Major, B. Rehermann, S.M. Feinstone, Hepatitis C Viruses, in: D.M. Knipe, P.M. Howley (Eds.), Fields Virology, 4th edition., Lippincott Williams & Wilkins, Philadelphia, 2001, pp. 1127–1161.
[3] A.M. Di Bisceglie, J.H. Hoofnagle, Optimal therapy of hepatitis C, Hepatology 36 (2002) S121–S127.
[4] M. Flint, C. Maidens, L.D. Loomis-Price, C. Shotton, J. Dubuisson, P. Monk, A. Higginbottom, S. Levy, J.A. McKeating, Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81, J. Virol. 73 (1999) 6235–6244.
[5] R.R. Spaete, D. Alexander, M.E. Rugroden, Q.L. Choo, K. Berger, K. Crawford, C. Kuo, S. Leng, C. Lee, R. Ralston, et al., Characterization of the hepatitis C virus E2/NS1 gene product expressed in mammalian cells, Virology 188 (1992) 819–830.
[6] T.F. Baumert, S. Ito, D.T. Wong, T.J. Liang, Hepatitis C virus structural proteins assemble into viruslike particles in insect cells, J. Virol. 72 (1998) 3827–3836.
[7] R.F. Clayton, A. Owsianka, J. Aitken, S. Graham, D. Bhella, A.H. Patel, Analysis of antigenicity and topology of E2 glycoprotein present on recombinant hepatitis C virus-like particles, J. Virol. 76 (2002) 7672–7682.
[8] S. Wellnitz, B. Klumpp, H. Barth, S. Ito, E. Depla, J. Dubuisson, H.E. Blum, T.F. Baumert, Binding of hepatitis C virus-like particles derived from infectious clone H77C to defined human cell lines, J. Virol. 76 (2002) 1181–1193.
[9] M. Triyatni, B. Saunier, P. Maruvada, A.R. Davis, L. Ulianich, T. Heller, A. Patel, L.D. Kohn, T.J. Liang, Interaction of hepatitis C virus-like particles and cells: a model system for studying viral binding and entry, J. Virol. 76 (2002) 9335–9344.
[10] A. Owsianka, R.F. Clayton, L.D. Loomis-Price, J.A. McKeating, A.H. Patel, Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2, J. Gen. Virol. 82 (2001) 1877–1883.
[11] D. Steinmann, H. Barth, B. Gissler, P. Schurmann, M.I. Adah, J.T. Gerlach, G.R. Pape, E. Depla, D. Jacobs, G. Maertens, A.H. Patel, G. Inchauspe, T.J. Liang, H.E. Blum, T.F. Baumert, Inhibition of hepatitis C virus-like particle binding to target cells by antiviral antibodies in acute and chronic hepatitis C, J. Virol. 78 (2004) 9030–9040.
[12] H.J. Ezelle, D. Markovic, G.N. Barber, Generation of hepatitis C virus-like particles by use of a recombinant vesicular stomatitis virus vector, J. Virol. 76 (2002) 12325–12334.
[13] E. Blanchard, D. Brand, S. Trassard, A. Goudeau, P. Roingeard, Hepatitis C virus-like particle morphogenesis, J. Virol. 76 (2002) 4073–4079.

[14] N. Acosta-Rivero, A. Rodriguez, A. Musacchio, V. Falcon, V.M. Suarez, G. Martinez, I. Guerra, D. Paz-Lago, Y. Morera, M.C. de la Rosa, J. Morales-Grillo, S. Duenas-Carrera, In vitro assembly into virus-like particles is an intrinsic quality of Pichia pastoris derived HCV core protein, Biochem. Biophys. Res. Commun. 325 (2004) 68–74.

[15] Y. Matsuura, H. Tani, K. Suzuki, T. Kimura-Someya, R. Suzuki, H. Aizaki, K. Ishii, K. Moriishi, C.S. Robison, M.A. Whitt, T. Miyamura, Characterization of pseudotype VSV possessing HCV envelope proteins, Virology 286 (2001) 263–275.

[16] K. Meyer, A. Basu, R. Ray, Functional features of hepatitis C virus glycoproteins for pseudotype virus entry into mammalian cells, Virology 276 (2000) 214–226.

[17] B. Bartosch, J. Bukh, J.C. Meunier, C. Granier, R.E. Engle, W.C. Blackwelder, S.U. Emerson, F.L. Cosset, R.H. Purcell, In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes, Proc. Natl. Acad. Sci. USA 100 (2003) 14199–14204.

[18] M. Hsu, J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C.M. Rice, J.A. McKeating, Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles, Proc. Natl. Acad. Sci. USA 100 (2003) 7271–7276.

[19] T.C. Li, Y. Yamakawa, K. Suzuki, M. Tatsumi, M.A. Razak, T. Uchida, N. Takeda, T. Miyamura, Expression and self-assembly of empty virus-like particles of hepatitis E virus, J. Virol. 71 (1997) 7207–7213.

[20] M. Tamura, K. Natori, M. Kobayashi, T. Miyamura, N. Takeda, Interaction of recombinant norwalk virus particles with the 105-kilodalton cellular binding protein, a candidate receptor molecule for virus attachment, J. Virol. 74 (2000) 11589–11597.

[21] M. Triyatni, J. Vergalla, A.R. Davis, K.G. Hadlock, S.K. Foung T.J. Liang, Structural features of envelope proteins on hepatitis C virus-like particles as determined by anti-envelope monoclonal antibodies and CD81 binding, Virology 298 (2002) 124–132.

[22] C. Hofmann, V. Sandig, G. Jennings, M. Rudolph, P. Schlag, M. Strauss, Efficient gene transfer into human hepatocytes by baculovirus vectors, Proc. Natl. Acad. Sci. USA 92 (1995) 10099–10103.

[23] I. Shoji, H. Aizaki, H. Tani, K. Ishii, T. Chiba, I. Saito, T. Miyamura, Y. Matsuura, Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors, J. Gen. Virol. 78 (1997) 2657–2664.

[24] F.M. Boyce, N.L. Bucher, Baculovirus-mediated gene transfer into mammalian cells, Proc. Natl. Acad. Sci. USA 93 (1996) 2348–2352.

[25] T. Shimoike, S. Mimori, H. Tani, Y. Matsuura, T. Miyamura, Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation, J. Virol. 73 (1999) 9718–9725.

[26] C. Fipaldini, B. Bellei, N. La Monica, Expression of hepatitis C virus cDNA in human hepatoma cell line mediated by a hybrid baculovirus-HCV vector, Virology 255 (1999) 302–311.

[27] H. Tani, M. Nishijima, H. Ushijima, T. Miyamura, Y. Matsuura, Characterization of cell-surface determinants important for baculovirus infection, Virology 279 (2001) 343–353.

[28] H. Tani, C.K. Limn, C.C. Yap, M. Onishi, M. Nozaki, Y. Nishimune, N. Okahashi, Y. Kitagawa, R. Watanabe, R. Mochizuki, K. Moriishi, Y. Matsuura, In vitro and in vivo gene delivery by recombinant baculoviruses, J. Virol. 77 (2003) 9799–9808.

[29] H. Aizaki, Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y. Matsuura, T. Miyamura, Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample, Hepatology 27 (1998) 621–627.

[30] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene 108 (1991) 193–199.

[31] Y. Kitagawa, H. Tani, C.K. Limn, T.M. Matsunaga, K. Moriishi, Y. Matsuura, Ligand-directed gene targeting to mammalian cells by pseudotype baculoviruses, J. Virol. 79 (2005) 3639–3652.

[32] K. Aoyagi, C. Ohue, K. Iida, T. Kimura, E. Tanaka, K. Kiyosawa, S. Yagi, Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen, J. Clin. Microbiol. 37 (1999) 1802–1808.

[33] A.H. Patel, J. Wood, F. Penin, J. Dubuisson, J.A. McKeating, Construction and characterization of chimeric hepatitis C virus E2 glycoproteins: analysis of regions critical for glycoprotein aggregation and CD81 binding, J. Gen. Virol. 81 (2000) 2873–2883.

[34] Y. Aoki, H. Aizaki, T. Shimoike, H. Tani, K. Ishii, I. Saito, Y. Matsuura, T. Miyamura, A human liver cell line exhibits efficient translation of HCV RNAs produced by a recombinant adenovirus expressing T7 RNA polymerase, Virology 250 (1998) 140–150.

[35] H. Aizaki, S. Nagamori, M. Matsuda, H. Kawakami, O. Hashimoto, H. Ishiko, M. Kawada, T. Matsuura, S. Hasumura, Y. Matsuura, T. Suzuki, T. Miyamura, Production and release of infectious hepatitis C virus from human liver cell cultures in the three-dimensional radial-flow bioreactor, Virology 314 (2003) 16–25.

[36] Y. Matsuura, T. Suzuki, R. Suzuki, M. Sato, H. Aizaki, I. Saito, T. Miyamura, Processing of E1 and E2 glycoproteins of hepatitis C virus expressed in mammalian and insect cells, Virology 205 (1994) 141–150.

[37] T. Kanto, N. Hayashi, T. Takehara, H. Hagiwara, E. Mita, M. Naito, A. Kasahara, H. Fusamoto, T. Kamada, Buoyant density of hepatitis C virus recovered from infected hosts: two different features in sucrose equilibrium density-gradient centrifugation related to degree of liver inflammation, Hepatology 19 (1994) 296–302.

[38] A.M. Prince, T. Huima-Byron, T.S. Parker, D.M. Levine, Visualization of hepatitis C virions and putative defective interfering particles isolated from low-density lipoproteins, J. Viral Hepat. 3 (1996) 11–17.

[39] B.D. Lindenbach, M.J. Evans, A.J. Syder, B. Wolk, T.L. Tellinghuisen, C.C. Liu, T. Maruyama, R.O. Hynes, D.R. Burton, J.A. McKeating, C.M. Rice, Complete replication of hepatitis C virus in cell culture, Science 309 (2005) 623–626.

[40] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H.G. Krausslich, M. Mizokami, R. Bartenschlager, T.J. Liang, Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (2005) 791–796.

[41] M. Kaito, S. Watanabe, K. Tsukiyama-Kohara, K. Yamaguchi, Y. Kobayashi, M. Konishi, M. Yokoi, S. Ishida, S. Suzuki, M. Kohara, Hepatitis C virus particle detected by immunoelectron microscopic study, J. Gen. Virol. 75 (Pt 7) (1994) 1755–1760.

[42] B. Bartosch, A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, F.L. Cosset, Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor, J. Biol. Chem. 278 (2003) 41624–41630.

[43] E.G. Cormier, F. Tsamis, F. Kajumo, R.J. Durso, J.P. Gardner, T. Dragic, CD81 is an entry coreceptor for hepatitis C virus, Proc. Natl. Acad. Sci. USA 101 (2004) 7270–7274.

[44] P. Pileri, Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A.J. Weiner, M. Houghton, D. Rosa, G. Grandi, S. Abrignani, Binding of hepatitis C virus to CD81, Science 282 (1998) 938–941.

[45] R. Petracca, F. Falugi, G. Galli, N. Norais, D. Rosa, S. Campagnoli, V. Burgio, E. Di Stasio, B. Giardina, M. Houghton, S. Abrignani, G. Grandi, Structure-function analysis of hepatitis C virus envelope-CD81 binding, J. Virol. 74 (2000) 4824–4830.

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Exploiting *cis*-Acting Replication Elements To Direct Hepatitis C Virus-Dependent Transgene Expression

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We describe here a novel targeting gene therapy strategy to direct gene expression responsive to hepatitis C virus (HCV). The goal was approached by engineering a construct containing the antisense sequence of the transgene and internal ribosome entry site of encephalomyocarditis virus flanked by 5′- and 3′-end sequences of HCV cDNA that contain *cis*-acting replication elements. Thus, expression of the transgene is only promoted when the minus-strand RNA has been synthesized by the functional replication machinery present in infected cells. Reporter assay and strand-specific reverse transcription-PCR showed selective transgene expression in Huh-7 cells harboring an autonomously replicating HCV subgenome but remaining silent in uninfected cells. Furthermore, using the cytosine deaminase suicide gene as a transgene coupled with recombinant adenovirus delivery, we demonstrated that cytosine deaminase was specifically expressed in replicon cells, resulting in marked chemosensitization of replicon cells to the cytotoxic effects of flucytosine. This new targeting strategy could be extended to other single-stranded RNA viruses encoding the unique RNA-dependent RNA polymerase that has no parallel in mammalian cells.

Chronic hepatitis C virus (HCV) infection, which frequently leads to liver cirrhosis and hepatocellular carcinoma (3, 34), remains a major public health problem worldwide. It has been estimated that more than 3% of the world population is infected with HCV. The plus-strand HCV RNA genome is approximately 9,600 nucleotides in length and encodes a polyprotein precursor of about 3,010 amino acids, which is cleaved co- and posttranslationally by cellular and viral proteases to produce structural and nonstructural (NS) proteins (8, 13, 15). One of the NS proteins, NS5B, is an RNA-dependent RNA polymerase (RdRp) that catalyzes the replication of HCV (5, 30).

Current treatment modalities available for HCV infection, including alpha interferon, has limited effectiveness. Only 20 to 30% of alpha interferon-treated patients develop a sustained remission, and increased or prolonged systemic administration is often associated with severe side effects or viral resistance. The combination of ribavirin and interferon is known to be significantly more effective than interferon monotherapy in naïve and relapser patients, but it induces a sustained response only in 41% of patients and in less than 30% of patients infected with genotype 1 (18). The therapeutic potential of small inhibitory molecules that target serine protease, helicase, or RdRp has proved to be promising (4, 6, 10), however, one unavoidable problem of this approach is selection of resistant mutants conferred by single or multiple mutations due to the error-prone nature of RdRp (24, 26, 29). Thus, an alternative approach for treating HCV patients that results in the death of infected cells, thereby limiting or eliminating virus production, while leaving uninfected cells unharmed would have an advantage over the HCV therapies now available.

Here we describe a targeting gene therapy approach that harnesses the viral replication machinery by constructing an HCV-like minigenome that consisted of the antisense sequence of cytosine deaminase (CD) suicide gene and internal ribosome entry site (IRES) element from encephalomyocarditis virus (EMCV) flanked by the 5′- and 3′-end regions of HCV, so that expression of CD is initiated only when the minus-strand RNA has been synthesized by the functional replication components present in infected cells. Using recombinant adenovirus delivery, we demonstrate that CD is specifically expressed in infected cells, resulting in marked chemosensitization of infected cells to the cytotoxic effects of flucytosine (5-FC).

MATERIALS AND METHODS

Cells. The cell lines Huh-7, HepG2, A549, and 293 were purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum and 50 u/ml penicillin and streptomycin in a 5% CO_2 humidified atmosphere. A Huh-7-derived cell line (Huh-NNRZ) stably replicating the HCV subgenomic replicon was grown in DMEM containing 300 μg/ml G418 (Geneticin, Invitrogen).

Plasmids. For construction of the reporter vectors pT7cRLs, a fragment containing HCV cDNA (1 to 377) with the T7 promoter directly coupled at the 5′ end was amplified by PCR with primers 5′-tataagcttTAATACGACTCACTATAGCCAGCCCCCGATTGGGGGC-3′ and 5′-tgctctagaTTTGGTTTTTCTTTGAGGTT-3′ (capital letters indicate the sequences originally contained in the target sequence, and lowercase letters indicate the attached sequences which were introduced for the convenience of molecular manipulation, such as restriction sites). The EMCV IRES sequence was amplified from pEMCVRL (42) with primers 5′-cgcggatccAACTAACTAACTAAGCTAGC-3′ and 5′-ctctctagaGTATTATCGTGTTTTTCAAA-3′. The PCR products were digested with HindIII and XbaI or BamHI and XbaI, respectively, and cloned into HindIII/BamHI-digested pUC18 to generate pTCE. The 3′ part of the NS5B coding region connected 3′ untranslated region (UTR) of HCV was amplified by PCR using primers 5′-cgcggatccGGAAACTTGGGGTCCCACCC-3′ and 5′-ataggcgc

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cagcgaggaggctgggaccatgccggccACATGATCTGCAGAGAGGCC-3', digested with BamHI and NarI and cloned, along with the annealed oligonucleotides containing the partial sequence of the HDV ribozyme, sense 5'-CGCCGGCTG GGCAACATTCCGAGGGGACCGTCCCCTCGGTAATGGCGAATGGGA CCg-3' and antisense 5'-aattcGGTCCCATTCGCCATTACCGAGGGGACGG TCCCCTCGGAATGTTGCCCAGCCGG-3', into BamHI/EcoRI-cut pTCE, generating pTCECD. The *Renilla* luciferase gene was amplified from pRL-TK (Promega) using primers 5'-ctctctagaATGACTTCGAAAGTTTATGA-3' and 5'-ctctctagaTTATTGTTCATTTTTGAGAA-3', digested with XbaI, and inserted into XbaI-digested pTCECD to generate pT7cRLNS5B1 or pT7RLNS5B1. pT7cRLNS5B2 or pT7cRLUTR, which contain the NS5B coding region from nucleotides 9307 to 9371 plus the 3'-UTR or 3'-UTR alone, was constructed similarly.

To construct pmCMVcRLmpA, the HCV minigenome was amplified from pTcRLNS5B1 by primers 5'-atagagctcTCTGGCTAACTGCCAGCCCCCGAT TGGGGGC-3' and 5'-ctcactagtACATGATCTGCAGAGAGGCC-3', digested with SacI and SpeI and cloned, together with the annealed minimal poly(A) oligonucleotides, sense 5'-CTAGAACTAGTAATAAAGGATCCTTTATTTTC ATTGGATCCGTGTGTTGGTTTTTTGTGTGCGGCCGCG-3' and antisense 5'-AATTCGCGGCCGCACACAAAAAACCAACACACGGATCCAATGAA AATAAAGGATCCTTTATTACTAGTT-3', into SacI/EcoRI-digested pShuttle (Clontech).

For construction of pmCMVcRLHD, a PCR product amplified from pT7cRLNS5B1 with primers 5'-ATGACTTCGAAAGTTTATGA-3' and 5'-ata cttaagGGTCCCATTCGCCATTACC-3' was digested with BstBI and AflII and cloned with the SacI-BstBI fragment from pmCMVcRLmpA into SacI/AflII-digested pShuttle. Using pmCMVcRLmpA as a template DNA, the 5' hammerhead ribozyme sequence was fused to the 5' UTR by PCR using the primers 5'-tatactagtGGGCTGGCCTGATGAGTCCGTGAGGACGAAACATGCATC TCCATGCATGTCGCCAGCCCCCGATTGGGGGC-3' and 5'-TTTCTCCGC ACCCGACATAG-3', after digestion with SpeI and EcoRI, the fragment was cloned into NheI/EcoRI-digested pShuttle generating pCMVHHcRLmpA. The CD gene was amplified by PCR using primers 5'-gtgtctagaAGGCTAACAAT GTCGAATAA−3' and 5'-atatctagaAGACAGCCGCTGCGAAGGCA−3', digested with XbaI, and ligated with the XbaI-digested pmCMVcRLmpA to generate pmCMVcCDmpA. The sequences of these constructs were confirmed by nucleotide sequencing.

Adenovirus. The expression cassette in pmCMVcCDmpA, which is flanked by I-CeuI and PI-SceI sites, was digested with these two restriction enzyme, and ligated to the E1- and E3-deleted Adeno-X viral DNA (I-CeuI and PI-SceI digested) (Adeno-X Expression System, Clontech). The resultant adenoviral DNA (AdmCMVcCDmpA) was digested with PacI and then transfected into low-passage 293 cells. Seven days following transfection, crude virus was prepared from the transfected cells by three cycles of freeze-thawing, and further amplified in 293 cells by several rounds of infection. The purified virus was aliquoted and stored at −80°C before use. The authenticity of recombinant adenoviral DNA was verified before preparing high-titer viral stocks. AdsiNS5B was constructed as described previously (42), the sense-strand sequence of short hairpin RNA is 5'-GAAGGTCACCTTTGACAGA-3'.

In vitro transcription. Plasmids were linearized at SalI site located immediately downstream of the HDV ribozyme, and these fragments were used as templates for runoff RNA synthesis with T7 RNA polymerase according to the protocol supplied by the manufacturer (Roche). For capped-RNA synthesis, T7 Cap-Scribe (Roche) was used. After transcription, 10 units of RQ DNaseI (Promega) were added to the reaction mixture to digest DNA templates. The mixture was extracted with phenol-chloroform and RNA was precipitated with ethanol-7.5 M ammonium acetate.

Transfection. Cells were seeded onto 35-mm-diameter tissue culture dishes 24 h before transfection. One microgram of each reporter vector, 1 μg of pAM8-1 (when expression from the T7 promoter was desired), and 0.1 μg of pGL3-Control vector were cotransfected into cells with TransFast Transfection Reagent (Promega). For RNA transfection, 1 μg of each reporter RNA and 0.5 μg of capped firefly luciferase RNA were cotransfected into cells with Lipofectin reagent (Invitrogen). The cells were harvested after 48 h, and cell lysates were assayed for luciferase activity as described below.

Luciferase assay. Cell lysates were prepared from transfected cells, centrifuged briefly, and 20 μl of the supernatants were used for luciferase assays with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase activities were measured using a TD-20/20 luminometer (Promega).

Strand-specific RT-PCR. RNAs were isolated from transfected cells with Trizol reagent (Invitrogen) and treated with RNase-free DNase (Promega). The DNA-free RNA was extracted with phenol-chloroform and precipitated with ethanol. The absence of DNA in the RNA templates was confirmed by a control PCR without reverse transcriptase. For reverse transcription (RT), 1 μg of RNA was denatured at 65°C for 2 min, and cDNA synthesis was performed in 20-μl reaction volume with Superscript II reverse transcriptase (Invitrogen) at 42°C for 1 h using a primer complementary to *Renilla* luciferase gene, 5'-CTTATCTTG ATGCTCATAGC-3'. The resulting cDNA was amplified for 35 cycles using primer 5'-ATGACTTCGAAAGTTTATGA-3' and the primer used in the RT reaction.

Northern blot analysis. Total RNAs were isolated and purified as described above, separated by denaturing agarose gel electrophoresis, and analyzed by Northern blot using Digoxigenin-labeled sense and antisense *Renilla* luciferase sequence to detect plus- and minus-strand transcripts.

Real-time RT-PCR. One microgram of DNase-treated total RNA was reverse-transcribed as described for strand-specific RT-PCR using a primer complementary to NS5B 5'-ACGGAGCGGATGTGGTTGAC-3'. After an incubation at 95°C for 5 min, the resulting cDNA was quantified with SYBR Green according to the protocol supplied by the manufacturer (Takara). PCR was performed with a primer 5'-TGGTCTACGCCACAACATCC-3' and the primer used in the RT reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level in each sample was simultaneously quantified to normalize the value of HCV replicon RNA.

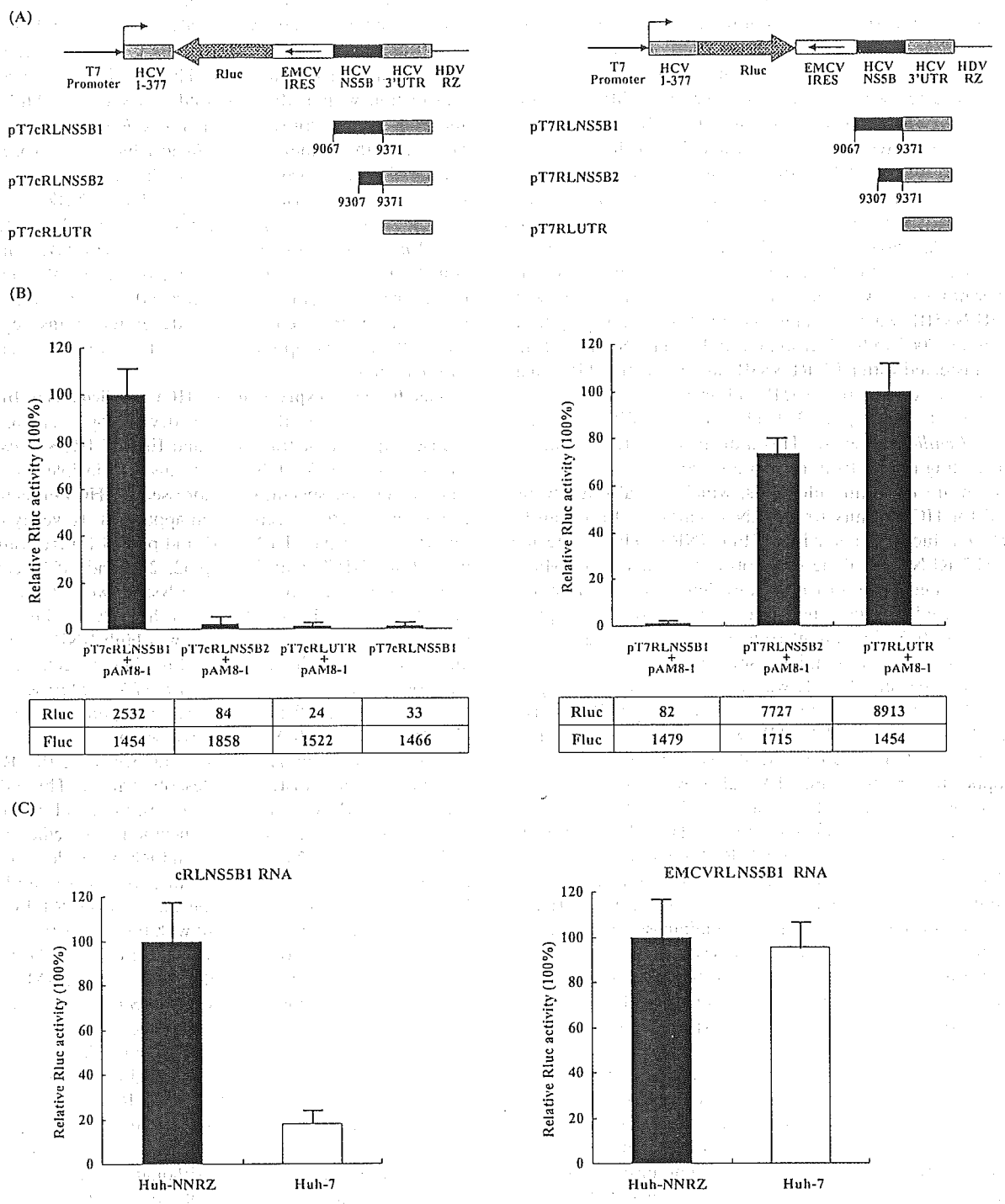
CD enzymatic assay. Huh-NNRZ or Huh-7 cells (1×10^6) were infected with AdmCMVcCDmpA at a multiplicity of infection (MOI) of 0, 10, 20, 50, 100, and 200. After 48 h, cells were harvested, washed once with phosphate-buffered saline, and resuspended in 0.5 ml 10 mM Tris-HCl. The cell suspension were sonicated, briefly centrifuged, and 100 μl supernatant was mixed with 170 μl PBS and 30 μl of 30 mM 5-FC solution. After incubation at 37°C for 24 h, 50 μl of reaction mixture was taken and quenched in 0.95 ml of 0.1 N HCl, the optical densities (ODs) were measured on a UV spectrophotometer at 255 and 290 nM. The amounts of 5-FC and 5-fluorouracil (5-FU) in the reaction mixture were calculated using the equations $(0.1191 \times OD_{290} - 0.02485 \times OD_{255}) \times 20$ = mM 5-FC and $(0.1849 \times OD_{255} - 0.04907 \times OD_{290}) \times 20$ = mM 5-FU; the conversion of 5-FC to 5-FU was then calculated as [mM 5-FC/(mM 5-FC + mM 5-FU)] × 100% (23).

Cytotoxic assay. Cells (Huh-NNRZ or Huh-7) were mock infected or infected with AdmCMVcCDmpA (MOI 80). Twenty-four hours later, the medium was changed with fresh DMEM containing 0 or 0.5 mM 5-FC. After an additional 4-day culture, cell viability was measured with cell proliferation reagent WST-1 (Roche) according to the manufacturer's instructions.

RESULTS

Determining *cis*-acting elements in the NS5B coding region essential for viral minus-strand RNA synthesis. Synthesis of HCV minus-strand RNA is initiated by recognition of the 3' end of RNA template by RdRp, which requires a membrane-associated replication complex of viral and cellular proteins and a viral RNA template containing *cis*-active replication elements. It was known that conserved sequences and structures in the 5' and 3' untranslated region (UTR) in HCV RNA function as *cis*-acting elements essential for viral replication (12, 21, 38). However, the precise mechanism of the initiation of RNA synthesis is not fully understood. Although Oh et al. (31) previously showed that RdRp can utilize the 3' UTR of HCV RNA as a minimal template in vitro, increasing evidence has supported that the 3' UTR may not be a good template by itself for efficient RdRp binding and subsequent RNA synthesis, and the 3' part of NS5B coding region with conserved stem-loop structure may also harbor functional *cis*-acting element required for viral replication (7, 39).

To further define the sequence of the NS5B coding region essential for HCV minus-strand RNA synthesis, we constructed HCV minigenome reporter vectors by inserting the antisense sequence of the *Renilla* luciferase gene and EMCV IRES between the 5' end (nucleotides 1 to 377) and differently truncated NS5B coding region-connected 3' UTR or 3' UTR alone (Fig. 1A). In view of the consideration that functional



Rluc	2532	84	24	33
Fluc	1454	1858	1522	1466

Rluc	82	7727	8913
Fluc	1479	1715	1454

FIG. 1. Determination of *cis*-acting replication elements in the NS5B coding region. (A) Schematic display of T7-based HCV minigenome reporter constructs. HCV minigenome containing the antisense (left) or sense (right) sequence of the *Renilla* luciferase gene and the antisense sequence of the EMCV IRES flanked by the 5′ end (1 to 377) and differently truncated NS5B coding region-connected 3′ UTR or 3′ UTR alone was juxtaposed precisely at the T7 transcription start site and followed by the HDV ribozyme gene. (B) The indicated reporter vectors were transfected into Huh-NNRZ cells with or without pAM8-1 expressing T7 RNA polymerase. Relative *Renilla* luciferase activities in the lysates were determined at 48 h posttransfection. The columns and bars represent the means and standard deviations of three independent triplicate transfections. (C) Huh-NNRZ cells were transfected with in vitro transcribed cRLNS5B1 RNA (left) or EMCVRLNS5B1 RNA (right) together with capped firefly luciferase RNA as an internal control. Relative *Renilla* luciferase activities in the lysates were determined at 24 h posttransfection. Absolute values of *Renilla* and firefly luciferase activity are listed below the corresponding bars.