SUMMARY: Hepatitis C virus (HCV) core and envelope proteins are suggested to be responsible for the pathogenesis of hepatic and extrahepatic manifestations in chronic hepatitis C. Moreover, the core protein is implicated in the regulation of the transcription of cellular genes including c-myc, RB and p53. Determining the subcellular localization of the core and envelope proteins is therefore necessary to elucidate their behaviors, particularly in vivo ones, regarding the interaction with transcriptional regulatory proteins or gene elements. We defined the subcellular localization of HCV envelope and core proteins which were expressed in substantial levels in the liver of transgenic mice. Subcellular fractionation by ultra-centrifugation revealed that the envelope proteins were present principally in the microsomes of the liver, while a small amount of the protein was detected also in the nuclei. Immunohistochemistry confirmed the localization of envelope proteins in the nuclei. In contrast, the core protein was detected principally in the cytoplasmic fraction, where it was closely associated with lipids. A low level of the core protein was detected also in the nuclei and microsomal fraction. These
results suggest possible interaction of the HCV structural proteins with some factors in hepatocytes thereby perturbing intracellular circumstances.

Hepatitis C virus (HCV) (1) is an enveloped positive-strand RNA virus belonging to the family Flaviviridae (2). The HCV genome RNA is approximately 9,600 bases long and is composed of a 5' nontranslated region (NTR) that functions as an internal ribosome entry site, a long open reading frame encoding a polyprotein of approximately 3,000 amino acids, and a 3' NTR. The HCV polyprotein is processed by host signal peptidases and viral proteinases to yield at least 10 different structural and nonstructural proteins (3). To date, several strategies have been used to elucidate the processing of the polyprotein which is translated from the HCV genome and to characterize individual proteins. Studies have also been conducted on the expression of HCV structural proteins (4-8), including in vivo studies in transgenic animals (9-11).

Recently, the HCV envelope proteins have been shown to be responsible for the pathogenesis of sialadenitis resembling Sjögren syndrome (12) which has been reported to be observed in patients with chronic hepatitis C (13). On the other hand, the HCV core protein has been implicated in the regulation of the transcription of cellular genes including c-myc (14) and RB (15). The core gene has also been suggested to be involved in the transformation of fibroblasts in collaboration with the H-ras oncogene (16) and in the sensitization of cells to apoptotic signals (17). A recent work using transgenic mice has revealed that the core protein induces hepatic steatosis (18), which is one of the histopathological features of chronic hepatitis C (19,20). Studying the localization of these HCV structural proteins is therefore important for understanding their roles, particularly in vivo ones in modifying gene transcription or protein interaction. In this study, we determined the subcellular localization of the HCV envelope and core proteins in the liver of transgenic mice.

The production of HCV envelope gene- and HCV core gene-transgenic mice has already been described (9,18). In mice of the envelope gene transgenic mouse lines, E101 and E139, the envelope proteins, E1 and E2, were expressed in the liver, and the core protein in the mouse liver of the core gene transgenic mouse lines, C21 and C49, as detected by Western blotting. In the present study, mice from the envelope and core transgenic mouse lines were analyzed.
Fig. 1. Subcellular localization of the hepatitis C virus envelope proteins, E1 and E2. Liver tissues from the envelope gene transgenic mice were separated into subcellular fractions by ultracentrifugation, and analyzed by Western blotting with anti-E1 and anti-E2 monoclonal antibodies. Both the E1 and E2 proteins were detected in the nuclear fraction as well as in the microsomal fraction. Lanes Pc, positive control pBEP825-transfected HepG2 cells; lanes nT, liver from non-transgenic mouse; lane n, nuclear fraction; lanes cs, cytosol fraction; lane m, microsomal fraction; T, liver from transgenic mouse. E1 and E2 denote the positions of the E1 and E2 proteins, respectively. The numbers in the middle are the positions of the size markers in kDa.

For microscopical analysis, tissue sections (5-μm thick) fixed in 10% neutral-buffered formalin or frozen were used for hematoxylin and eosin staining or immunostaining. The HCV proteins were stained with anti-core (18), anti-E1, anti-E2 (9) or normal rabbit serum. For protein detection, biotinylated anti-rabbit IgG followed by avidin-biotin peroxidase (Vector Lab., Inc., Burlingame, CA) was used. Specificity control of immunostaining was carried out as follows: liver tissues and other organs from a normal littermate mouse were tested with immune serum and transgenic mouse liver with normal rabbit serum.

Subcellular fractionation was conducted by using a modification of a previously described technique (21). Liver tissue was homogenized in solution A
Fig. 2. Immunohistochemistry of the hepatitis C virus E2 protein. A: Frozen tissue from an envelope gene transgenic mouse was stained with anti-E2 rabbit serum. Numerous nuclei of hepatocytes are stained darker (arrows) than those of other hepatocytes (arrowheads). Note that the cytoplasms of hepatocytes around the central vein are also stained dark. B: Staining of a normal mouse liver with anti-E2 rabbit serum. C: Staining of an envelope gene transgenic mouse liver with normal rabbit serum. Counterstained with hematoxylin (×120).

[250 mM sucrose, 3 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA] in a tight-fitting Dounce homogenizer (20 gentle strokes). The homogenate was centrifuged at 50 × g after being passed through a mesh. The pellet (P1) was used for the preparation of the nuclear fraction. The supernatant (S1) was then layered on solution B [340 mM sucrose, 3 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA] and centrifuged at 700 × g for 10 min. The P2 pellet was combined with the P1 one, which was re-homogenized in solution C (2.4 M sucrose and 3 mM CaCl₂) and centrifuged at 40,000 × g for 60 min. The upper phase was re-centrifuged at 54,000 × g for 60 min to yield microsomal and cytosolic fractions. All fractions were adjusted to 1× in Western sample buffer [62.5 mM Tris-HCl (pH 6.8), 1% SDS, 5% 2-mercaptoethanol]. Fractionated tissue homogenates were subjected to 10.0% or 12.5% SDS/polyacrylamide gel electrophoresis and electro-transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) as described.
previously (9). The filter was then allowed to react with anti-core, anti-E1 or anti-E2 monoclonal antibody, followed by anti-mouse IgG conjugated with horse radish peroxidase (Vector Labs.) and proteins were visualized with an ECL kit (Amersham Intl., Buckinghamshire, UK).

In the liver of the envelope gene transgenic mice, both the E1 and E2 proteins were detected chiefly in the microsomal fraction, while a small amounts of both proteins were detected also in the nuclear fraction as 35 and 58 kDa proteins, respectively (Fig. 1). Localization of the envelope proteins in the nuclei was confirmed also by immunohistochemistry. Figure 2A shows a representative micrograph of the localization of the E2 protein, in which the nuclei of hepatocytes around the central vein exhibited darker staining than those in other areas, demonstrating the localization of the E2 protein in the nuclei. Control mouse liver was not stained with anti-E2 rabbit serum, nor was transgenic mouse liver with normal rabbit serum (Fig. 2B and C).

We then carried out Western blot analysis on the samples that were processed for subcellular fractionation. Because the HCV core protein had been shown to be associated with lipid droplets in cells by immunohistochemistry (18,22), the lipid layer in the cytosol fraction was analyzed separately. As we expected, a substantial level of the core protein was detected in the lipid layer which was separated as an upper phase of the cytosol fraction (Fig. 3). A low level of expression of the core protein was detected also in the nuclear and microsomal fraction. The localization of the core protein in the nuclei was confirmed also by immunoelectron microscopy (data not shown).

In our analysis of the structural proteins of HCV that are expressed in the liver of living animals, both the core and envelope proteins (E1 and E2) were detected in the hepatocytic nuclei, while the majority of the core protein in the lipid layer in the cytosol fraction and the envelope proteins in the microsomal fraction. These results are partly consistent with the findings in HCV transgenic mouse lines reported by other researchers. In one HCV core gene transgenic mouse line, when liver tissue was separated into the nuclear and cytoplasmic fractions by simple ultracentrifugation, the core protein was detected in the latter fraction (10). However, it is not clear whether the core protein is associated with lipids since the authors did not mention the occurrence of lipid accumulation in their transgenic mouse liver. A possible problem with their study is that the existence of the core protein was demonstrated by Western blotting only with human sera from chronic hepatitis C patients, whereas the HCV core protein has been reported to be readily detectable with monoclonal antibodies (11,18,23,24). Splicing
Fig. 3. Subcellular localization of the hepatitis C virus core protein. A: Liver tissues from the core gene-transgenic mice were separated into fractions by ultracentrifugation, and analyzed by Western blotting with anti-core monoclonal antibody. The core protein is detected chiefly in the lipid layer of the cytosol fraction. B: A longer exposure of A. A low level of expression is observed also in the nuclear and microsomal fractions. Lanes n, nuclear fraction; lanes cs, cytosol fraction; lanes m, microsomal fraction; lane cs.l, lipid layer in cytosol fraction. nT, nontransgenic mouse; T, transgenic mouse; Ac316, baculovirus expressing the core gene as a positive control. Core denotes the position of the core protein.

of RNA transcribed from the cDNA of the HCV genome might be a cause of an obstacle in the expression of the viral proteins in transgenic mice. In another study, Kawamura et al. (11) using immunohistochemistry reported that the core protein was present chiefly in the cytoplasm but was detected also in the nuclei of some hepatocytes. However, their result is not conclusive because further study including subcellular localization analysis was not carried out. Kawamura et al. (11) reported also that the E2 protein was detected in the cytoplasm by
immunohistochemical analysis although the staining was equivocal. In both re-
ports, low levels of expression of the proteins may have hampered the localization
analysis of these proteins. This may also account for the absence of steatosis in
the liver of their core gene transgenic mice, although the observation periods
were short, which phenotype has been described in mice exhibiting unambiguous
expression of the core protein (18). It is notable that the levels of the core protein
in our transgenic mice are comparable to those in the liver from patients with
chronic hepatitis C (Fujie et al., unpublished data), indicating that the phenotype
in our transgenic mice, hepatic steatosis, is not the consequence of an
artifactually high-level expression of the core protein.

Our results showing the localization of the structural proteins of HCV in the
nuclei of hepatocytes support the hypothesis that these proteins are involved in
regulating the expression of cellular genes and are thereby associated with
hepatocarcinogenesis or the development of other manifestations of HCV infec-
tion including sialadenitis-resembling Sjögren syndrome.

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