Transgenic Mouse Expressing a Full-length Hepatitis C Virus cDNA

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Hepatitis C virus (HCV), a major causative agent of post transfusion non-A, non-B hepatitis (NANBH), can only infect humans and chimpanzees. We produced nine transgenic mouse lines carrying a full-length HCV cDNA with the human serum amyloid P component (hSAP) promoter that can direct liver-specific expression. In one of these lines HCV mRNA and HCV core protein were detected in the liver of the transgenic mouse, although the levels of expression were very low. In addition, HCV-related antibody was detected in the serum.

Key words: Hepatitis C virus — Transgenic mouse — Human serum amyloid P component (hSAP) promoter

Hepatitis C virus (HCV) is the most important etiological agent of post-transfusion non-A, non-B hepatitis (NANBH)1 and is a major cause of chronic liver disease and hepatocellular carcinoma. HCV is distantly related genetically to both the pestiviruses and flaviviruses. The HCV genome is about 9.4 kb in length and consists of a highly conserved 5' untranslated region followed by a single open reading frame that encodes a polyprotein of 3010 to 3033 amino acids.2,4 The host signal peptidase mediates the cleavage of a basic, putative nucleocapsid protein (C) from the N terminal of the polyprotein precursor, followed by two glycoproteins (E1, E2), both of which represent potential components of the viral envelope.2,7 Various putative nonstructural proteins (NS2–5) are processed from the downstream region of the polyprotein, mediated in part by a viral protease encoded within the NS3 domain.5–10

There is a need to develop vaccines and other antiviral therapies against HCV. A major reason for the lack of rapid progress is the absence of a convenient or inexpensive animal model for propagating HCV. As host animals are limited to humans and chimpanzees, there have been several attempts to develop in vitro HCV replication systems and in vivo infection or expression models. The former include in vitro replication systems using human T cells,21–19 human fibroblasts,20 human peripheral blood mononuclear cells,21 non-neoplastic human hepatocytes,12, 22 human hepatoblastoma cells,23 chimpanzee primary hepatocytes24 and other cells.25–27 Such HCV-infected culture systems will be useful for various biological studies, including investigations into the mechanisms of HCV replication of the viral genome and cellular tropism of HCV12, 15, 16, 19, 25–27 and into the structure of viral particles.17, 18, 24 Advances in in vivo infection models for reproducible propagation of HCV and hepatitis C viremia have been achieved either by transplantation of HCV-infected liver fragments from patients into BNX (beige/nude/X-linked immunodeficient) mice28 or intravenous injection of HCV DNA using gene delivery systems.29, 30 However, there is not enough information about the mechanism of viral persistence, the cellular tropism of HCV, or its pathogenicity in vivo. Another approach to elucidate these questions, including in vivo behavior, would be to establish a transgenic model. One example of such a model of viral disease is a transgenic line that can express and replicate hepatitis B virus genome.31, 32 Concerning HCV, several laboratories have reported the establishment of transgenic mice that carry the HCV core or envelope genes.33–37 In the present study, a transgenic line was established by introducing a full-length HCV cDNA with the human serum amyloid P component (hSAP) promoter that can direct liver-specific expression of the transgene after birth.38

MATERIALS AND METHODS

DNA HCV cDNA was derived from pHCV7 carrying a full-length HCV cDNA isolated from a Japanese patient with HCV.39 The hSAP promoter was derived from the genomic DNA clone of the human SAP gene.40 A full-length HCV cDNA was inserted into the EcoRI site of pSG-1. Before injection into fertilized mouse eggs, the plasmid was digested with HindIII and XhoI. The resulting 12 kb fragment carrying the expression unit of the HCV cDNA (Fig. 1A) was then isolated and used for microinjection.

DNA injection C57BL/6 mice were used for the production of transgenic mice. Several hundred molecules of
the transgene fragment were microinjected into the pro-
nuclei of fertilized eggs according to the conventional
method.41, 42)

**Isolation and analysis of nucleic acids** Genomic DNA
was extracted from the tails of offspring. Tails were lysed
with 1% SDS and 1 mg/ml proteinase K (Sigma, St.
Louis, MO). Lysates were treated twice with phenol and
phenol/chloroform 1:1 (vol/vol). DNA was precipitated
with ethanol and dissolved in TE (10 mM Tris-HCl; pH
8.0/1 mM EDTA). Southern blot analysis was used to
determine the presence of transgenes. Ten micrograms ali-
quots of sample DNA were digested with EcoRI,
subjected to electrophoresis in 0.75% agarose gels, and
transferred to nylon membranes (Hybond-N+, Amersham,
UK) with NaCl/0.1 M NaOH by the alkaline transfer
method. Hybridization was performed under stringent con-
ditions with a random primed digoxigenin-labeled (DIG
DNA Labeling Kit, Boehringer Mannheim, Mannheim,
Germany) HCV core or NS5 regional DNA probe corre-
spanding to the HCV genome (Fig. 1B). Signals were
identified by chemiluminescent (CSPD, Boehringer Mann-
heim) or colorimetric (nitroblue tetrazolium chloride/5-
bromo-4-chloro-3-indolyl phosphate (NTB/BCIP), Boeh-
ringer Mannheim) detection.

**Ribonuclease protection assay** Expression of HCV
cDNA was examined by ribonuclease protection assay
(RPA).43) Total RNAs were prepared from the livers of
transgenic mice using TRIZOL reagent (Gibco BRL,
Grand Island, NY). RPA were performed with an RPAII
kit (Ambion, Danvers, MA). Two different labeled RNA
probes were used, of which one (PENS1-300) was com-
plementary to the envelope-NS1 region (1208 nt to 1714
nt), and the other (NN-150) was complementary to NS5
region (7112 nt to 7265 nt) of the HCV genome. As a
control, a mouse β actin RNA probe (540 nt to 833 nt)
was also used.

**In situ hybridization (ISH)** The distribution of HCV
mRNAs in the livers was analyzed by ISH. Livers were
immediately frozen in an embedding medium (O.C.T.
compound, Miles, Elkhart, IN), and sectioned at a thick-
ness of 4 µm. Probes were labeled with digoxigenin by in
vitro transcription.44) For ISH, antisense or sense RNA

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![Fig. 1. Structure of the transgene construct. A, The transgene consisted of hSAP, rabbit β-globin intron II and full-length HCV cDNA. Restriction enzyme sites (BamHI, EcoRI, HindIII, XhoI) are shown. B, The target regions of probes for Southern blot analysis, RPA, ISH and RT-PCR.](image-url)
transaminase (GOT) and glutamic-pyruvic transaminase in our laboratory was used. Plasma glutamic-oxaloacetic transaminase was assayed by enzyme-linked immunosorbent assay (ELISA). Anti-HCV core antibodies and concentrations of plasma transaminase were analyzed. Blood was obtained from the vena cava of three mice and sera were separated by centrifugation. Antibodies against HCV core were assayed by enzyme-linked immunosorbent assay using a JCC-2 kit (Kaketsuken, Kumamoto). To detect mouse antibodies, goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (Dako, Code No. P0447) was applied at a 1:100 dilution for 30 min at room temperature, then the sections were washed with PBS, and secondary antiserum (horseradish peroxidase-conjugated goat anti-mouse immunoglobulin; Dako, Code No. P0447) was applied at a 1:100 dilution for 30 min at room temperature. Then, the sections were stained using 3,3′-diaminobenzidine (DAB)-H2O2 as a substrate and counterstained with methyl green.

RESULTS

Establishment of the transgenic line Several hundred copies of the HCV gene were microinjected into fertilized eggs and 500 surviving eggs were transferred into the oviducts of foster mothers. In total, 91 mice were born. The tail DNAs from these mice were screened for the presence of HCV DNA by Southern blot analysis. Sixteen mice were found to carry the HCV transgene, and they all had the full-length HCV DNA (Fig. 2). The copy number of the integrated transgene ranged from 1 to more than 100, as shown in Table I. Nine lines were established from the founder mice. Four founders died during breeding and three founders were sterile (Table I).

Analysis of RNA We examined the presence of HCV RNA in the livers of F1 or F2 mice by northern blot analysis. However, no signals of HCV RNA were detected by this method. Therefore, we performed reverse transcription polymerase chain reaction (RT-PCR) on RNA from transgenic lines using primers corresponding to the 5′ region of the HCV genome. Positive signals were detected only in two lines, No. 4 and No. 10 (data not shown). We used No.4 line and his offspring in the following analysis.

We first analyzed the tissue-specificity of HCV RNA expression by RT-PCR. HCV RNA was detected only in the liver, but not in other tissues, including heart, lung, kidney, spleen, skeletal muscle, and stomach (data not shown).

Then, we performed RPA to determine the amount of HCV RNA. We detected signals in the liver with both the
probe for the 5' region (PENS1-300) of the HCV genome and that for the 3' region (NN-150) (Fig. 3). However, the level was low in comparison with that of endogenous actin mRNA. To examine whether each part of the HCV RNA was expressed, RT-PCR analyses were carried out using four additional probes (Fig. 1), i.e., the NCC probe, which covers the 5' untranslated region and core region (139 nt to 410 nt), ENV probe which covers the envelope region (1110 nt to 1360 nt), NS3 probe which covers the NS3 region (4291 nt to 4560 nt), and the NS5 probe which covers the NS5 region (7021 nt to 7260 nt). We detected RNA with all these probes. These results, together with those of the RPA assay, suggest the expression of full-length HCV RNA.

To observe the distribution of HCV RNA in the transgenic liver, ISH was performed (Fig. 4). Frozen sections from transgenic and negative littermate livers were hybridized with a digoxigenin-labeled probe (Smal DIG antisense or sense probe) corresponding to the 5' region of HCV RNA. With the antisense probe, positive signals were observed in transgenic hepatocytes on the outer border of the nuclear membrane and a diffuse signal was observed in the cytoplasm (Fig. 4a), while no signal was observed with the sense probe (Fig. 4b). In a negative littermate, no signals were detected using either the antisense probe or sense probe (Fig. 4, c and d).

**Histology**
To examine whether pathologic changes developed in the transgenic livers, tissue samples were taken from the same mice as used for serum analysis at 4, 8 and 12 weeks and 1 year of age. Low titers of anti-HCV antibody, 1.6 and 0.6 ng/ml, were detected in one of three transgenic mice at 4 and 12 weeks of age, respectively. High titers of antibody, 6.3 and 6.1 ng/ml, were detected at 1 year of age in two out of three transgenic mice (Table II). However, serum levels of GOT and GPT were not significantly elevated in these transgenic mice (data not shown).

| Transgenic line | Sex  | Copy number | Progeny |
|-----------------|------|-------------|---------|
| 2               | male | 10          | death\(a) |
| 4               | male | 10          | +       |
| 5               | male | 10–25       | sterile |
| 10              | male | 5           | +       |
| 12              | male | >100        | +       |
| 32              | male | 1           | death   |
| 63              | male | >50         | +       |
| 70              | male | 10–20       | +       |
| 73              | male | 5           | death   |
| 89              | female | 20–40   | +       |
| 91              | female | 1–5     | +       |
| 92              | female | >200    | death   |
| 93              | female | 5–10    | +       |
| 94              | female | 1        | sterile |
| 100             | female | 1       | sterile |
| 104             | female | 20–40   | +       |

\(a\) The founder mouse died.

**Table II.** Anti-HCV Antibody in the Serum of HCV Transgenic Mice

| Age     | Titer (ng/ml) |
|---------|---------------|
| 4 weeks | 1.6           |
| 8 weeks | 0             |
| 12 weeks| 0.6           |
| 1 year  | 6.3           |

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Fig. 3. RNase protection assay. An aliquot of 10 µg of total RNA from liver was run in each lane, except for probe lanes. PENS1-300 and NN-150 probe (Fig. 1) were used here. Actin probe was used for the control. Lane 1: probe RNA. Lane 2: full-length HCV RNA synthesized by *in vitro* transcription. Lane 3: total RNA from transgenic mouse liver. Lane 4: total RNA from negative littermate liver. Arrow A: probe size. Arrow B: digested probe size.
12 weeks and 1 year of age. Transgenic mice were phenotypically normal on gross observation and no pathologic changes in the liver at 4, 8, and 12 weeks of age were detected. However, in one transgenic mouse at 1 year of age, infiltration of mononuclear cells and neutrophils was found in almost all portal tracts (Table III, Fig. 5), and polymorphic cells with rod-shaped or round, large, pale nuclei proliferated in sinusoids. Although similar changes can be observed in the liver infected with mouse hepatitis virus, we could not detect antibody to mouse hepatitis virus.

Table III. Histopathological Findings in Liver of HCV Transgenic Mice (No.4 line) at Various Ages

| Findings in liver                                                                 | Age   | 4 weeks | 8 weeks | 12 weeks | 1 year |
|----------------------------------------------------------------------------------|-------|---------|---------|----------|--------|
|                                                                                  |       | Cont.a  | Cont.    | Cont.    | Cont.  |
|                                                                                  |       | 1 2 3   | 1 2 3   | 1 2 3    | 1 2 3  |
| Cellular infiltration of parenchyma                                             | ±±±±  | − ± ± ± | − ± ± ± | − ± ± ± | ± ± ± ± |
| Degeneration and necrosis of hepatocytes                                         | − − − −| − − − − | − − − − | − − − − | ± − + ± |
| Cellular infiltration of portal tracts                                           | − − ± ±| − − − − | − − − − | − − − − | + ++ − −|
| Proliferation of polymorphic cells in sinusoids                                  | − − − −| − − − − | − − − − | − − − − | − + − − |

a) Cont., negative littermates.
b) Grade: −, negative; ±, slight; +, mild; ++, moderate.
virus in this case. Thus, these changes may be due to the direct effect of HCV or the result of immune response to HCV.

**Immunohistochemical analysis** To determine the location of HCV-related protein (core) in the transgenic liver, immunohistochemical analysis was performed. Three samples were taken from transgenic mice that produced anti-HCV antibody at 4 weeks, 12 weeks and 1 year of age.

Two samples were taken from transgenic mice that did not produce anti-HCV antibody at 4 weeks and at 8 weeks of age. Two control samples were also taken from the negative littermates at 4 weeks and 1 year of age. HCV core protein was observed in the liver of one transgenic mouse at 12 weeks of age, but not in any other mice. The HCV core protein was detected in the cytoplasm of some hepatocytes (Fig. 6).
DISCUSSION

In the present study, we established one transgenic line which is considered to express the full-length HCV RNA under the hSAP promoter. As we could not detect mRNA by northern blot analysis because of the low level of expression, we do not have clear-cut evidence for the expression of the full-length HCV. However, there has been no report indicating the existence of alternative splicing products from HCV genome, or transcription initiation from a different site. We detected HCV RNA by RPA as well as RT-PCR using 6 probes that correspond to parts of all the proteins except for NS2. Taken together, these results suggest the expression of full-length HCV RNA. The reason why the hSAP promoter was used is that the hSAP promoter can direct liver-specific, post-natal expression of a heterologous gene.38, 45, 46) Thus, we expected an immune response to HCV proteins and resultant hepatitis in transgenic mice. As expected, we detected the full-length HCV mRNA and core protein, and anti-core antibody appeared in transgenic mice.

In HCV patients, HCV RNAs were detected diffusely in the cytoplasm of hepatocytes. Some RNAs were localized in the perinuclear area or focally in the cytoplasm. The number of HCV RNA-positive hepatocytes was small and these hepatocytes were localized sporadically or diffusely within hepatic lobulus by RNA in situ hybridization.55) HCV capsid protein was observed within the cytoplasm of hepatocytes, but not in their nuclei, by means of immunohistochemical analysis.56) These hepatocytes were distributed diffusely or sporadically in hepatic lobuli. The localization of HCV RNAs and core proteins in the liver of our transgenic mice are similar to those in HCV patients. The only difference between human patients and transgenic mice is the expression level, which is lower in transgenic mice.

Among nine transgenic lines, only two expressed HCV RNA. As the hSAP promoter can express the heterologous genes quite efficiently, this could be due to the nature of HCV DNA. In addition, the level of HCV RNA is very low. The level of expressed core protein in our transgenic mice seemed to be lower than has been reported by others.33–37) However, they used a part of the HCV gene, that is the core or envelope, while we used the whole genome. Although the reason for the low level of expression of HCV is unclear, one possibility may be methylation of the introduced HCV genome. Viral genomes such as HBV or HCV are reported to be heavily methylated upon integration.48, 49) Methylation of the HCV gene was also observed in our transgenic mice (data not shown). In any case, this is consistent with the fact that HCV is a low-titer virus in humans and clinical symptoms are generally mild with minimal biochemical changes. However, HCV infection in patients is generally persistent and results in chronic hepatitis at a high rate (up to 50%), leading to liver cirrhosis and hepatocellular carcinoma. In chronic hepatitis C, spontaneous decreases in HCV RNA and improvement in serum aminotransferase levels are rare.

The mechanism of liver cell damage in HCV infection is not clear. It is possible that induced liver cell damage is due to an immune response to viral antigens expressed on the hepatocyte surface.50) In this study, we detected a relatively high titer of anti-HCV core antibody at 1 year of age and some pathological changes were also recognized. However, plasma transaminases did not increase. This result is consistent with findings in some other transgenic mice.55–58) HCV core protein seems not to be directly toxic at this level of expression. Interestingly, transgenic mice carrying HCV core gene under the control of a regulatory region of the HBV showed steatosis, which is characteristic of HCV-induced pathological changes in the liver.59) This suggests that the HCV core is toxic if expressed at a high level. Whether liver cell damage is also induced via an immune response remains to be elucidated.

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