Jpn. J. Cancer Res. **89**, 150–158, February 1998

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 precusor, followed by two glycoproteins (E1, E2), both of which represent potential components of the viral envelope.[5–7)] 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.[7–10)]

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,[11–19)] human fibroblasts,[20)] human peripheral blood mononuclear cells,[21)] non-neoplastic human hepatocytes,[12, 22)] human hepatoblastoma cells,[23)] chimpanzee primary hepatocytes[24)] 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 HCV[12, 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) mice[28)] 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 *Eco*RI site of pSG-1. Before injection into fertilized mouse eggs, the plasmid was digested with *Hin*dIII and *Xho*I. 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 pronuclei 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 m*M* Tris-HCl; pH 8.0/1 m*M* EDTA). Southern blot analysis was used to determine the presence of transgenes. Ten micrograms aliquots of sample DNA were digested with *Eco*RI, 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 conditions with a random primed digoxigenin-labeled (DIG DNA Labeling Kit, Boehringer Mannheim, Mannheim, Germany) HCV core or NS5 regional DNA probe corresponding to the HCV genome (Fig. 1B). Signals were identified by chemiluminescent (CSPD, Boehringer Mannheim) or colorimetric (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NTB/BCIP), Boehringer 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 complementary 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 thickness of 4 μm. Probes were labeled with digoxigenin by *in vitro* transcription.[44] For ISH, antisense or sense RNA

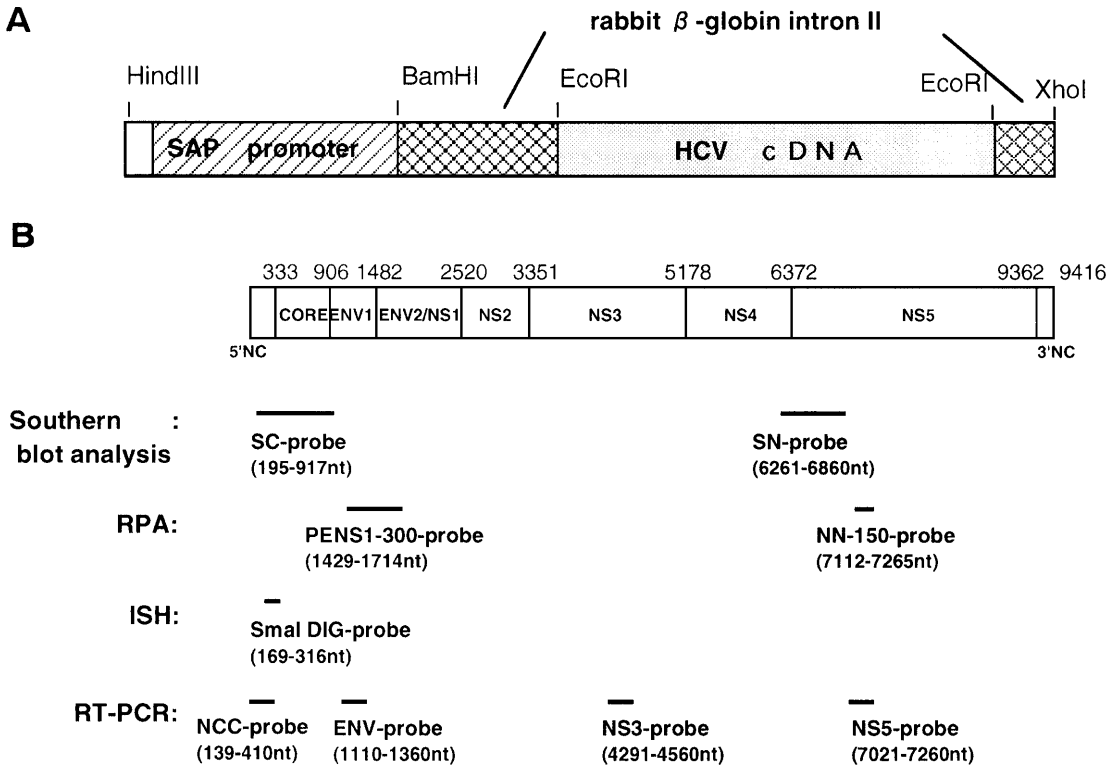

Fig. 1. Structure of the transgene construct. A, The transgene consisted of hSAP, rabbit β globin intron II and full-length HCV cDNA. Restriction enzyme sites (*Bam*HI, *Eco*RI, *Hin*dIII, *Xho*I) are shown. B, The target regions of probes for Southern blot analysis, RPA, ISH and RT-PCR.

probes (*Sma*I DIG probe) complementary to the nontranslated region (169 nt to 346 nt) of the HCV genome were used (Fig. 1B). Before hybridization, sectioned samples were fixed with 4% paraformaldehyde solution, and treated with 1μg/ml proteinase K in 0.1 *M* Tris-HCl (pH 8.0) containing 10 m*M* EDTA for 7 min at room temperature. To prevent further digestion, sections were refixed in 4% paraformaldehyde solution for 5 min. To reduce nonspecific signals, sections were treated with 0.2 *N* HCl for 10 min at room temperature. They were dipped in phosphate-buffered saline (PBS) and acetylated in 0.1 *M* triethanolamine with 0.25% acetic anhydride for 20 min. They were then rinsed in PBS, dehydrated through a series of ethanol solutions (70% to 100%), and allowed to dry before hybridization. The probes were denatured for 5 min at 95°C, and applied to slides. Hybridization was performed at 42°C for 16 h in a moist chamber. The hybridization solution contained 1 ng/μl probe. Hybridization solution consisted of 10 m*M* Tris-HCl; pH 7.6, 200 μg/ml tRNA, 1× Denhardt's solution, 10% dextran sulfate, 600 m*M* NaCl, 0.25% SDS, 1 m*M* EDTA (pH 8.0), and 50% formamide. After hybridization, the slides were rinsed in 2× standard saline citrate (SSC), and unhybridized probe was digested with 100 μg/ml ribonuclease A in 2× SSC for 30 min at 37°C. After having been rinsed in 2× SSC for 15 min at room temperature, slides were incubated for 30 min with alkaline phosphatase-conjugated anti-digoxigenin antibody and the bound antibody was detected by means of the standard immuno-alkaline phosphatase reaction, with NTB/BCIP as a substrate.

Assay of serum 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, Glostrup, Denmark) was used. As a control, mouse anti-HCV core monoclonal antibody JCC4-143-3 produced in our laboratory was used. Plasma glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were assayed by the POP·POD method (Nesucort GOT·GPT kit-K, Nihon Shouji, Osaka).

Histology Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at a thickness of 4 μm. The sections were routinely stained with hematoxylin and eosin.

Immunohistochemical analysis The livers were immediately frozen in embedding medium (O.C.T. compound, Miles) and sectioned at a thickness of 4 μm. Sections were treated with PBS and blocked with normal goat serum. Mouse anti-HCV core monoclonal antibody JCC4-143-3 (Kaketsuken) was applied at 15 μg/ml for 60 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)-H_2O_2 as a substrate and counterstained with methyl green.

RESULTS

Establishment of the transgenic line Several hundred copies of the HCV transgene 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 F_1 or F_2 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

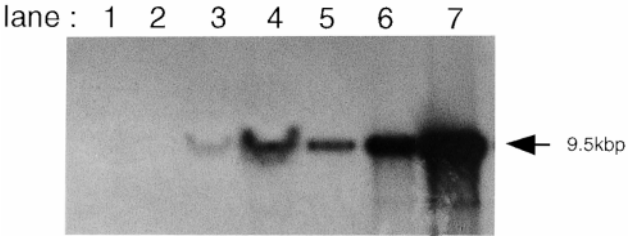

Fig. 2. Southern blot analysis of F_0 mouse tail DNA. Samples were digested with *Eco*RI and hybridized with SN-probe (Fig. 1). Lane 1: normal mouse as a negative control. Lanes 2–4: F_0 mouse (lane 2: non transgenic, lanes 3–4: transgenic). Lanes 5–7: HCV cDNA as a positive control (lane 5: 1 pg, lane 6: 10 pg, lane 7: 100 pg).

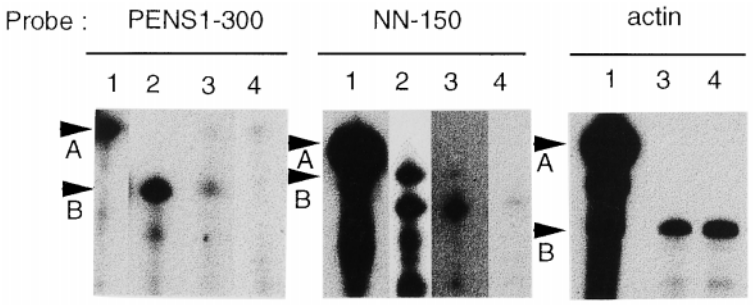

Fig. 3. RNase protection assay. An aliquot of 10 μg of total RNA from liver was run in each lane, except for probe lanes. PENS1-300 and NN-150 probe (Fig. 1) were used here. Actin probe was used for the control. Lane 1: probe RNA. Lane 2: full-length HCV RNA synthesized by *in vitro* transcription. Lane 3: total RNA from transgenic mouse liver. Lane 4: total RNA from negative littermate liver. Arrow A: probe size. Arrow B: digested probe size.

Table I. Establishment of HCV Transgenic Mouse Lines

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.

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.

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

Age	Titer (ng/ml)		
4 weeks	1.6	0	0
8 weeks	0	0	0
12 weeks	0.6	0	0
1 year	6.3	0	6.1

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 (*Sma*I 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).

HCV-related antibody and plasma transaminase in serum of transgenic mice To examine the production of HCV-related antibodies and the presence of liver cell injury in transgenic mice, serum samples were taken from three mice 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).

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

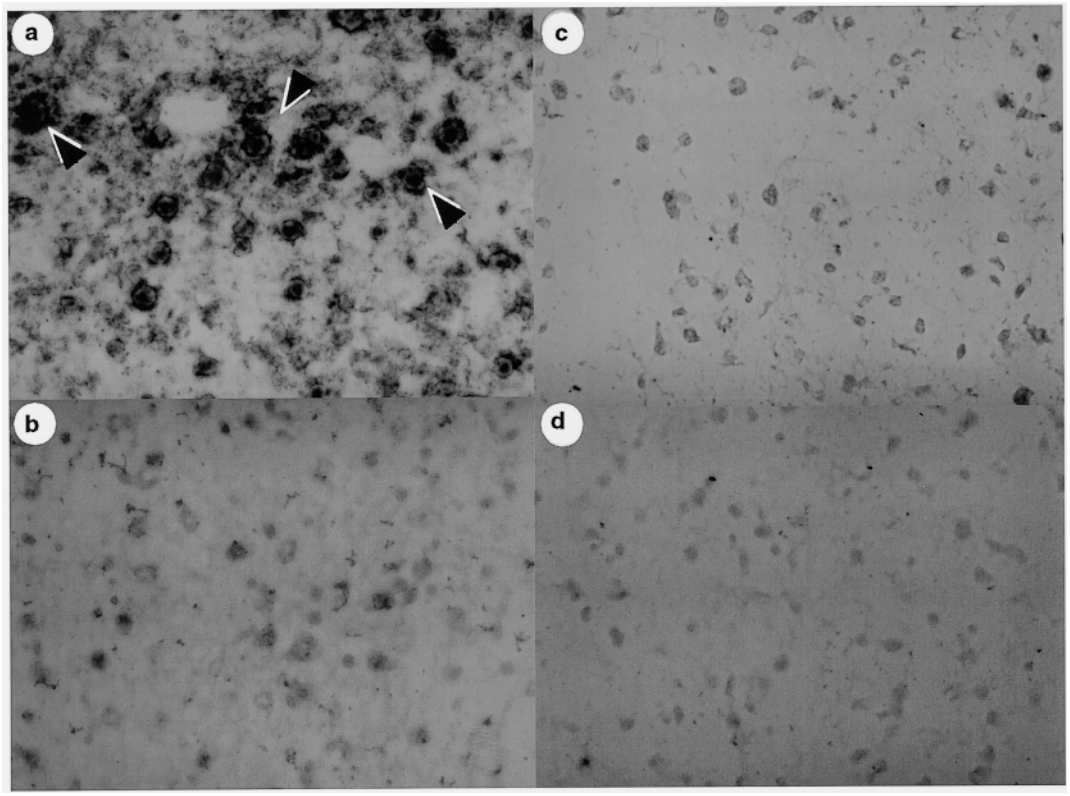

Fig. 4. ISH of HCV transgenic and negative littermates. The distribution of HCV mRNA in HCV transgenic mouse liver was observed by means of *in situ* hybridization. The target region of *Sma*I antisense and sense probes was shown in Fig. 1. a, Antisense probe on HCV transgenic mouse. The arrow shows the localization of HCV RNA signals. b, Sense probe on HCV transgenic mouse. c, Antisense probe on negative littermate. d, Sense probe on negative littermate.

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			
	Animal No.	Cont.[a)]	1	2	3	Cont.	1	2	3	Cont.	1	2	3	Cont.	1	2	3
Cellular infiltration of parenchyma		±[b)]	±	±	±	–	–	±	–	–	±	–	±	+	±	++	±
Degeneration and necrosis of hepatocytes		–	–	–	–	–	–	–	–	–	–	–	±	±	–	+	±
Cellular infiltration of portal tracts		–	–	±	–	–	–	–	–	–	–	–	–	+	++	–	–
Proliferation of polymorphic cells in sinusoids		–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–

a) Cont., negative littermates.
b) Grade: – , nagative; ± , slight; + , mild; ++ , moderate.

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

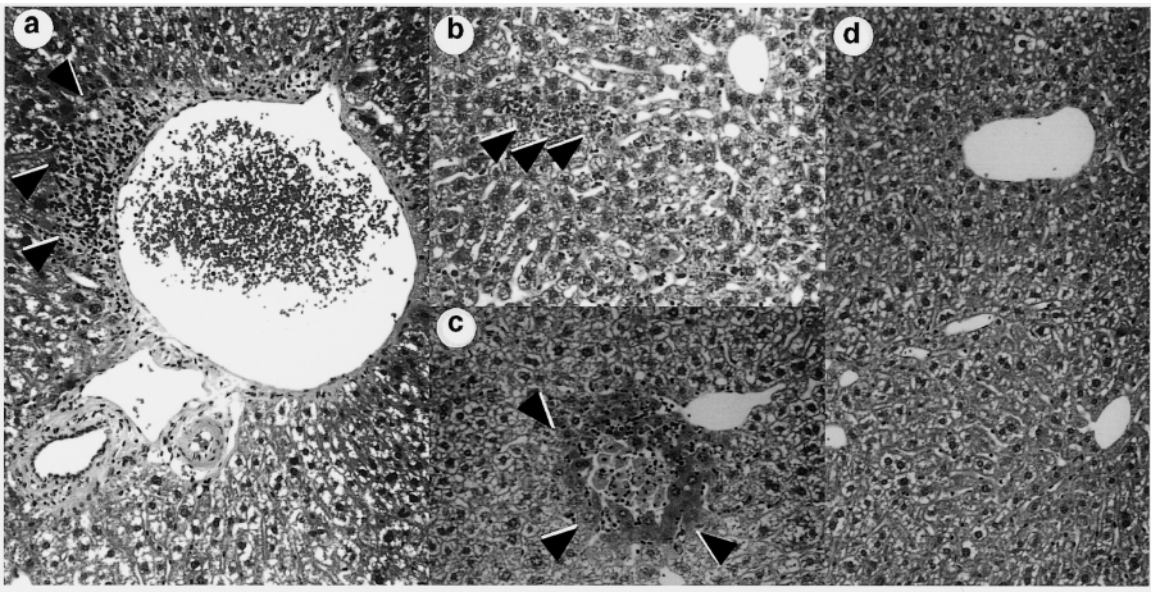

Fig. 5. Histopathological findings in HCV transgenic mouse liver. a–c, One-year-old HCV transgenic mouse (animal No.1). a, Cellular infiltration of portal tracts; b, proliferation of polymorphic cells in sinusoid; c, degeneration and necrosis of hepatocytes; d, 1 year-old negative littermate. Arrows show histopathological changes. HE stain.

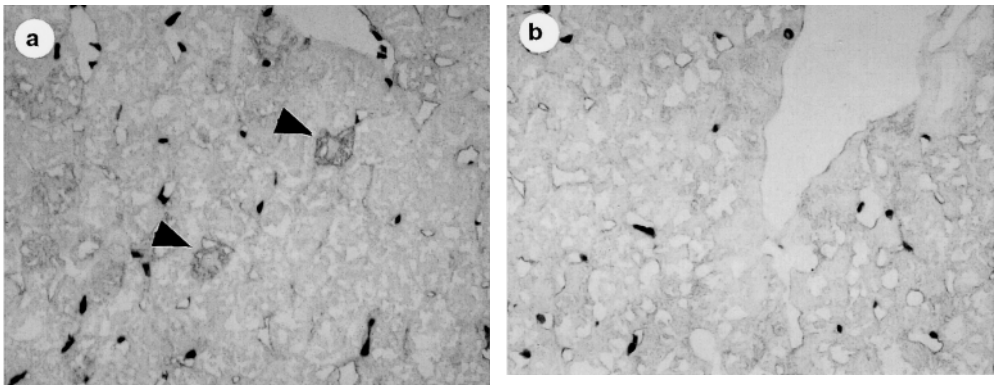

Fig. 6. Localization of HCV core protein in transgenic mouse liver. a, Transgenic mouse. The arrow shows the localization of HCV core protein. b, Negative littermate.

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.[47] HCV capsid protein was observed within the cytoplasm of hepatocytes, but not in their nuclei, by means of immunohistochemical analysis.[47] 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 consisted with findings in some other transgenic mice.[33–36] 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.[37] 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.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Science, Sports and Culture, by a grant from the Osaka Foundation for Promotion of Clinical Immunology, and a grant from the Science and Technology Agency.

(Received September 6, 1997 / Revised November 19, 1997 / Accepted November 25, 1997)

REFERENCES

1) Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q.-L., Houghton, M. and Kuo, G. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA*, **87**, 6547–6549 (1990).
2) Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T. and Shimotohno, K. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc. Natl. Acad. Sci. USA*, **87**, 9524–9528 (1990).
3) Choo, Q.-L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, A., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G. and Houghton, M. Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. USA*, **88**, 2451–2455 (1991).
4) Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami,

S., Fujita, J., Onishi, E., Andoh, T., Yoshida, I. and Okayama, H. Structure and organization of hepatitis C virus genome isolated from human carriers. *J. Virol*., **65**, 1105–1113 (1991).
5) Spaete, R. R., Alexander, D., Rugroden, M. E., Choo, Q.-L., Berger, K., Crawford, K., Kuo, C., Leng, S., Lee, C., Ralston, R., Thudium, K., Tung, J. W., Kuo, G. and Houghton, M. Characterization of the hepatitis C virus E2/NS1 gene product expressed in mammalian cells. *Virology*, **188**, 819–830 (1992).
6) Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M. and Shimotohno, K. Gene mapping of the putative structural region of the hepatitis C virus genome by *in vitro* processing analysis. *Proc. Natl. Acad. Sci. USA*, **88**, 5547–5551 (1991).
7) Selby, M. J., Choo, Q.-L., Berger, K., Kuo, G., Glazer, E., Eckart, M., Lee, C., Chien, D., Kuo, C. and Houghton, M. Expression, identification and subcellular localization of the proteins encoded by the hepatitis C viral genome. *J. Gen. Virol*., **74**, 1103–1113 (1993).
8) Eckart, M. R., Selby, M., Masiarz, F., Lee, C., Berger, K., Crawford, K., Kuo, C., Kuo, G., Houghton, M. and Choo, Q.-L. The hepatitis C virus encodes a serine protease involved in processing of the putative nonstructural proteins from the viral polyprotein precursor. *Biochem. Biophys. Res. Commun*., **192**, 399–406 (1993).
9) Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M. and Rice, C. M. Expression and identification of hepatitis C virus polyprotein. *J. Virol*., **67**, 1385–1395 (1993).
10) Grakoui, A., McCourt, D. W., Wychowski, C., Feinstone, S. M. and Rice, C. M. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage site. *J. Virol*., **67**, 2832–2843 (1993).
11) Shimizu, Y. K., Purcell, R. H. and Yoshikura, H. Correlation between the infectivity of hepatitis C virus *in vivo* and its infectivity *in vitro*. *Proc. Natl. Acad. Sci. USA*, **90**, 6037–6041 (1993).
12) Ikeda, M., Kato, N., Mizutani, T., Sugiyama, K., Tanaka, K. and Shimotohno, K. Analysis of the cell tropism of HCV by using *in vitro* HCV-infected human lymphocytes and hepatocytes. *J. Hepatol*., **27**, 445–454 (1997).
13) Hijikata, M., Mizuno, K., Rikihisa, T., Shimizu, Y. K., Iwamoto, A., Nakajima, N. and Yoshikura, H. Selective transmission of hepatitis C virus *in vivo* and *in vitro*. *Jpn. Arch. Virol*., **140**, 1623–1628 (1995).
14) Kato, N., Nakazawa, T., Mizutani, T. and Shimotohno, K. Susceptibility of human T-lymphotropic virus type I infected cell line MT-2 to hepatitis C virus infection. *Biochem. Biophys. Res. Commun*., **206**, 863–869 (1995).
15) Mizutani, T., Kato, N., Hirota, M., Sugiyama, K., Murakami, A. and Shimotohno, K. Inhibition of hepatitis C virus replication by antisense oligonucleotide in culture cells. *Biochem. Biophys. Res. Commun*., **212**, 906–911 (1995).
16) Mizutani, T., Kato, N., Ikeda, M., Sugiyama, K. and Shimotohno, K. Long-term human T-cell culture system supporting hepatitis C virus replication. *Biochem. Biophys. Res. Commun*., **227**, 822–826 (1996).
17) Serafino, A., Valli, M. B., Alessandrini, A., Ponzetto, A., Carloni, G. and Bertolini, L. Ultrastructural observations of viral particles within hepatitis C virus-infected human B lymphoblastoid cell line. *Res. Virol*., **148**, 153–159 (1997).
18) Shimizu, Y. K., Feinstone, S. M., Kohara, M., Purcell, R. H. and Yoshikura, H. Hepatitis C virus: detection of intracellular virus particles by electron microscopy. *Hepatology*, **23**, 205–209 (1996).
19) Sugiyama, K., Kato, N., Mizutani, T., Ikeda, M., Tanaka, T. and Shimotohno, K. Genetic analysis of the hepatitis C virus (HCV) genome from HCV-infected human T cells. *J. Gen. Virol*., **78**, 329–336 (1997).
20) Zibert, A., Schreier, E. and Roggendorf, M. Antibodies in human sera specific to hypervariable region 1 of hepatitis C virus can block viral attachment. *Virology*, **208**, 653–661 (1995).
21) Crbier, B., Schmitt, C., Bingen, A., Kirn, A. and Keller, F. *In vitro* infection of peripheral blood mononuclear cells by hepatitis C virus. *J. Gen. Virol*., **76**, 2485–2491 (1995).
22) Kato, N., Ikeda, M., Mizutani, T., Sugiyama, K., Noguchi, M., Hirohashi, S. and Shimotohno, K. Replication of hepatitis C virus in cultured non-neoplastic human hepatocytes. *Jpn. J. Cancer Res*., **87**, 787–792 (1996).
23) Dash, S., Halim, A. B., Tsuji, H., Hiramatsu, N. and Gerber, M. A. Transfection of HepG2 cells with infectious hepatitis C virus genome. *Am. J. Pathol*., **151**, 363–373 (1997).
24) Lanford, R. E., Sureau, C., Jacob, J. R., White, R. and Fuerst, T. R. Demonstration of *in vitro* infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT/PCR. *Virology*, **202**, 606–614 (1994).
25) Liu, Q., Tackey, C., Bhat, R. A., Prince, A. M. and Zhang, P. Regulated processing of hepatitis C virus core protein is linked to subcellular localization. *J. Virol*., **71**, 657–662 (1997).
26) Barba, G., Haraper, F., Harada, T., Kohara, M., Goulinet, S., Matsuura, Y., Eder, G., Schaff, Zs., Chapman, M. J., Miyamura, T. and Brechot, C. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc. Natl. Acad. Sci. USA*, **94**, 1200–1205 (1997).
27) Ide, Y., Zhang, L., Chen, M., Inchauspe, G., Bahl, C., Sasaguri, Y. and Padmanabhan, R. Characterization of the nuclear localization signal and subcellular distribution of hepatitis C virus nonstructural protein NS5A. *Gene*, **182**, 203–211 (1996).
28) Eithan, G., Tatjana, B., Mali, K., Ido, L., Elias, S., Yuval, K., Ahmed, E., Yaron, I., Avraham, R., Galina, P., Daniel, S. and Yair, R. Hepatitis C virus viremia in SCID-BNX mouse chimera. *J. Infect. Dis*., **172**, 25–30 (1995).
29) Yamamoto, M., Hayashi, N., Miyamoto, Y., Takehara, T., Mita, E., Seki, M., Fusamoto, H. and Kamada, T. *In vivo* transfection of hepatitis C virus complementary DNA into

rodent liver by asialoglycoprotein receptor mediated gene delivery. *Hepatology*, **22**, 847–855 (1995).
30) Takehara, T., Hayashi, N., Miyamoto, Y., Yamamoto, M., Mita, E., Fusamoto, H. and Kamada, T. Expression of the hepatitis C virus genome in rat liver after cationic liposome-mediated *in vivo* gene transfer. *Hepatology*, **21**, 746–751 (1995).
31) Chisari, F. V., Pinkert, C. A., Milich, D. P., Filippi, P., McLachlan, A., Palmiter, R. D. and Brinster, R. L. A transgenic mouse model of the chronic hepatitis B surface antigen carrier state. *Science*, **230**, 1157–1160 (1985).
32) Araki, K., Miyazaki, J., Hino, O., Tomita, N., Chisaka, O., Matsubara, K. and Yamamura, K. Expression and replication of hepatitis B virus genome in transgenic mice. *Proc. Natl. Acad. Sci. USA*, **86**, 207–211 (1989).
33) Koike, K., Moriya, K., Ishibashi, K., Matsuura, Y., Suzuki, T., Saito, I., Iino, S., Kurokawa, K. and Miyamura, T. Expression of hepatitis C virus envelope proteins in transgenic mice. *J. Gen. Virol.*, **76**, 3031–3038 (1995).
34) Koike, K., Moriya, K., Ishibashi, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Kurokawa, K., Matsuura, Y. and Miyamura, T. Sialadenitis histologically resembling Sjögren syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc. Natl. Acad. Sci. USA*, **94**, 233–236 (1997).
35) Pasquinelli, C., Shoenberger, J. M., Chung, J., Chang, K., Guidotti, L. G., Selby, M., Berger, K., Lesniewski, R., Hougton, M. and Chisari, F. V. Hepatitis C virus core and E2 protein expression in transgenic mice. *Hepatology*, **25**, 719–727 (1997).
36) Kawamura, T., Furusaka, A., Koziel, M. J., Chung, R. T., Wang, T. C., Schmida, E. V. and Liang, T. J. Transgenic expression of hepatitis C virus structural proteins in the mouse. *Hepatology*, **25**, 1014–1021 (1997).
37) Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Ishibashi, K., Matsuura, Y., Miyamura, T. and Koike, K. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J. Gen. Virol.*, **78**, 1527–1531 (1997).
38) Zhao, X., Araki, K., Jun-ichi, M. and Yamamura, K. Developmental and liver-specific expression directed by the serum amyloid P component promoter in transgenic mice. *J. Biochem.*, **111**, 736–738 (1992).
39) Hayashi, N., Higashi, H., Kaminaka, K., Sugimoto, H., Esumi, M., Komatsu, K., Hayashi, K., Sugitani, M., Suzuki, K., Okano, T., Nozaki, C., Mizuno, K. and Shikata, T. Molecular cloning and heterogeneity of the human hepatitis C virus genome. *J. Hepatol.*, **17**, 94–107 (1993).
40) Ohnishi, S., Maeda, S., Shimada, K. and Arao, T. Isolation and characterization of the complete complementary and genomic DNA sequences of human serum amyloid P component. *J. Biochem.*, **100**, 849–858 (1986).
41) Gordon, J. W., Scangos, G. A., Plotkin, D. J., Barbosa, J. A. and Ruddle, F. H. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl. Acad. Sci. USA*, **77**, 7380–7384 (1980).
42) Yamamura, K., Kikutani, H., Takahashi, N., Taga, T., Araki, S., Kawai, K., Fukuchi, K., Kumahara, Y., Honjo, T. and Kishimoto, T. Introduction of human gamma 1 immunoglobulin genes into fertilized mouse eggs. *J. Biochem.*, **96**, 357–363 (1984).
43) Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular cloning. *In* "A Laboratory Manual," 2nd Ed., Vol. 7, pp. 71–78 (1989). Cold Spring Harbor Laboratory Press, New York.
44) Kayhan, T., Aria, N., Sallie, R., Sanger, D., Graeme, J., Alexander, M., Smith, H., Byrne, J., Portmann, B., Adrian, L. W., Eddleston, F. and Williams, R. Detection of genomic and intermediate replicative strands of hepatitis C virus in liver tissue by *in situ* hybridization. *J. Clin. Invest.*, **91**, 2226–2234 (1993).
45) Takashima, H., Araki, K., Miyazaki, J., Yamamura, K. and Kimoto, M. Characterization of T-cell tolerance to hepatitis B virus (HBV) antigen in transgenic mice. *Immunology*, **75**, 398–405 (1992).
46) Araki, K., Hino, O., Miyazaki, J. and Yamamura, K. Development of two types of hepatocellular carcinoma in transgenic mice carrying the SV40 large T-antigen gene. *Carcinogenesis*, **12**, 2059–2062 (1991).
47) Nakane, P. K., Yamada, G., Nishimoto, H., Endou, H., Doi, T., Takahashi, M., Tsuji, T., Yoshizawa, H., Nozawa, M. and Koji, T. Localization of hepatitis C viral RNA and capsid protein in human liver. *Dig. Dis. Sci.*, **38**, 882–887 (1993).
48) Araki, K., Akagi, K., Miyazaki, J., Matsubara, K. and Yamamura, K. Correlation of tissue-specific methylation with gene inactivity in hepatitis B virus transgenic mice. *Jpn. J. Cancer Res.*, **81**, 1265–1271 (1990).
49) Kato, T., Ahmed, M., Yamamoto, T., Takahashi, H., Dohara, M., Ikeda, T., Aida, Y., Katsuki, M., Arakawa, Y., Shikata, T. and Esumi, M. Inactivation of hepatitis C virus cDNA transgene by hypermethylation in transgenic mice. *Arch. Virol.*, **141**, 951–958 (1996).
50) Di Biscceglie, A. M., Goodman, Z. D., Ishak, K. G., Hoofnagle, J. H., Melpolder, J. J. and Alter, H. Long-term clinical and histopathological follow-up of chronic post-transfusion hepatitis. *Hepatology*, **14**, 969–974 (1991).