EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 38, No. 3, 241-246, June 2006

Expression of hepatitis C virus nonstructural 4B in transgenic mice

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Accepted 12 May 2006

Abbreviations: HCV, hepatitis C virus; NS4B, nonstructural 4B

Abstract

Hepatitis C virus (HCV) is a pathogen that is of great medical significance in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide. Although the HCV proteins have been intensively investigated over the past decade, the biochemical functions of the NS4B protein are still largely unknown. To investigate NS4B as a potential causative agent of liver disease, transgenic mice expressing the NS4B protein in liver tissue were produced. The transgenic animals were phenotypically similar to their normal littermates for up to 18 months of age. Our results suggest that the HCV NS4B protein is not directly cytopathic or oncogenic in our transgenic mice model.

Keywords: hepacivirus; liver; mice, transgenic; NS4 protein, hepatitis C virus; viral nonstructural proteins

Introduction

The hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide (1997, 1999, Moriwaki, 2002), and chronic hepatitis C has become a serious global medical problem and a considerable burden on health care systems (Tan *et al.*, 2001). Unfortunately, neither a vaccine nor any other means of effective chemotherapy is available to control HCV infection at this time. Furthermore, research efforts have been hampered by the lack of both a cell culture system that allows for efficient HCV replication and small animal models that either support replication of the HCV or mimic the pathologic features of chronic hepatitis C in humans (Lemon *et al.*, 1998). Transgenic animal models have contributed pivotal information on hepatitis virus-mediated pathogenesis (Hanahan, 1988; Chisari, 1995; Milich, 2000). Studies involving HCV Core transgenic mice have suggested that core protein may play an important role in HCV induced steatosis and HCC (Moriya *et al.*, 1997; Moriya *et al.*, 1998). Recently, Lerat *et al.* reported that transgenic mice expressing complete HCV viral polyprotein developed steatosis and liver cancer as early as 13 months of age (Lerat *et al.*, 2002). The findings suggested that the expression of structural proteins enhances a low background of steatosis in C57BL/6 mice, while the additional low-level expression of nonstructural proteins increases the risk of cancer.

The functions of the NS4B protein, which is one of the nonstructural proteins of the HCV, are largely unknown. The locations of the NS4B protein have been identified. It is mainly associated with the endoplasmic reticulum (ER) and is partly contained in the cytoplasm (Lundin *et al.*, 2003). However, the translocation of NS4B to the nucleus has also been suggested (Westaway *et al.*, 1997). One function of NS4B related to HCV replication was reported (Piccininni *et al.*, 2002; Hanley *et al.*, 2003). In addition, the inhibitory function of NS4B on host protein synthesis during translation (Florese *et al.*, 2002, Kato *et al.*, 2002) may contribute to the support of HCV survival in host cells. In addition, NS4B inhibited $p21^{Waf1/Cip1}$ and p53 (Florese *et al.*, 2002), suggesting its potential function in HCV-induced pathological changes in the liver. The mutation of NS4B has also been found to significantly reduce pathological injury induced by bovine viral diarrhea virus (BVDV) (closely related to HCV) in cells (Qu *et al.*, 2001). Moreover, NS4B in association with Ha-ras plays an important role in the

malignant transformation of cells (Park *et al.*, 2000). However, *in vivo* experiments have not been performed to contribute to these *in vitro* studies.

In the present study, we report the generation of transgenic mice, the expression of HCV NS4B, and the histological findings of the liver. Our results indicate that HCV NS4B is not cytopathic to cells *in vivo*.

Materials and Methods

Generation and identification of transgenic mice

To generate the p*HEX/NS4B* vector, a 0.78-kb fragment containing HCV genotype 1b *NS4B* (Korea strains) was subcloned into the *Nco* I/*Bgl* II sites of p*HEX* to replace the X-gene (Figure 1A). The plasmid p*HEX* is a previously reported vector prepared by us that expresses the *HBX* gene under authentic promoter control (Yu *et al.*, 1999). Injectable DNA containing *HBV* enhancer, *NS4B,* and *SV40* poly A sequences was obtained by the removal of phagemid sequences from pUC118 at *Kpn* I/*Hind* III sites. DNA was microinjected into fertilized mouse eggs of hybrid BCF1 (C57BL/6J♀ x CBA/J), and the injected eggs were transferred into pseudopregnant recipients as previously described (Yu *et al.*, 1999). Potential founders were analyzed by polymerase chain reaction (PCR) and Southern blot analysis of tail DNA. Transgenic lineages were transmitted to the C57BL/6J inbred line by mating with C57BL/6J mice. The mice were housed in a controlled, specific pathogen-free environment and were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, South Korea).

RT-PCR analysis

We performed RT-PCR to detect the expression of the transgene in transgenic mouse tissues. The mice were killed, and the tissue samples were immediately frozen in liquid nitrogen. Total RNA was isolated from mouse tissues using the TRIzol reagent (Invitrogen Life Technologies Inc., Carlsbad, CA). RT-PCR was performed using a reverse transcription system (Promega Corp., Madison, WI) according to the manufacturer's instructions. The primers used to detect the expression of the transgene were the sense primer, 5'-GCCTCACACCTTCCTTAC-3', and the anti-sense primer, 5'-CAGGATGTCCACAAGCAC-3'. A separate RT-PCR primer was used to detect GAPDH as a loading control.

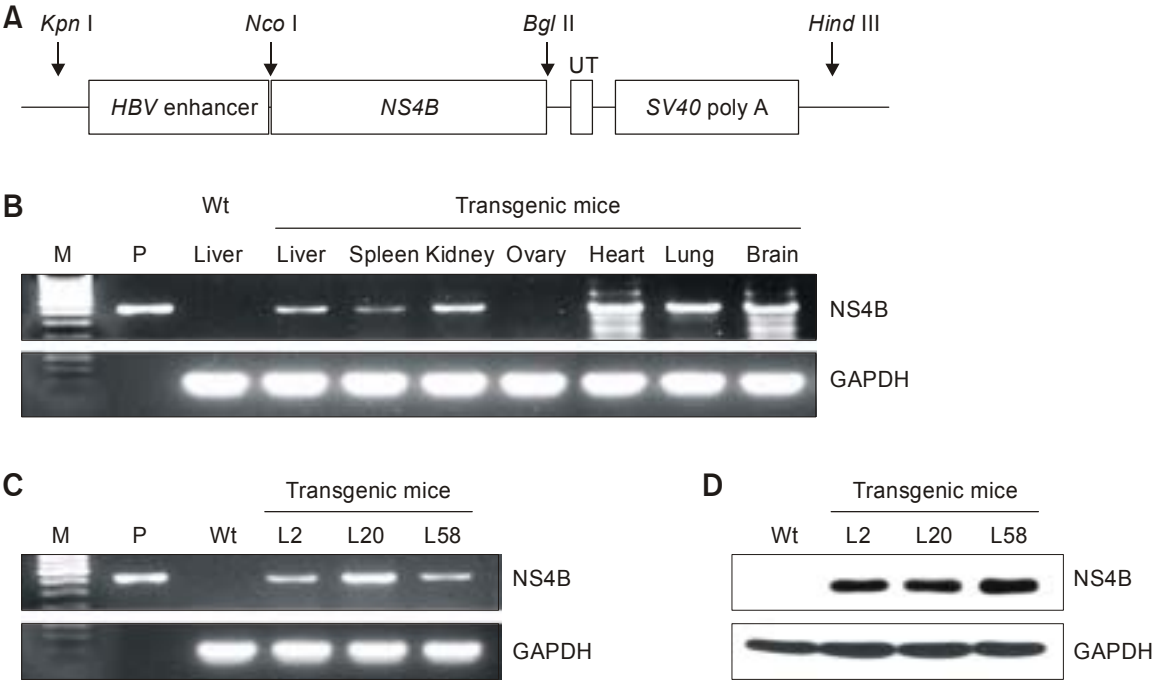

Figure 1. Vector construction and transgene expression. (A) Schematic of the expression vector *pHEX/NS4B*. (B) The expression of *NS4B* in the tissues of lineage 2 was analyzed by RT-PCR. (C) The expression of *NS4B* in liver tissues of transgenic lineages was analyzed by RT-PCR. (D) The expression of HCV NS4B protein level was measured performed in liver tissues of transgenic lineages by Western blot analysis. GAPDH was used as a loading control. M, size marker; P, positive control; Wt, normal littermate; L2, lineage 2; L20, lineage 20; L58, lineage 58.

Western blot analysis

Tissue lysates were prepared by homogenizing tissues that had been freshly retrieved or flash-frozen in lysis buffer. For Western blot analysis, equal amount (20 μg) of proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membranes were probed with the primary and secondary antibodies: mouse monoclonal anti-NS4B antibody (Advanced Immunochemical Inc.); anti-GAPDH (Trevigen, Gaithersburg, MD); horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); and HRP-conjugated goat anti-mouse IgG (Amersham Biosciences, Buckinghamshire, UK). Positive bands were detected using an enhanced chemiluminescence system (Pierce, Rockford, IL).

Histopathological diagnoses and immunohistochemical staining

Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) according to standard methods. The histopathological diagnoses were based on the criteria described by Frith and Ward (Frith *et al.*, 1979). The immunohistochemical studies were performed on formalin-fixed, paraffin-embedded, 4 μm-thick tissue sections using the streptavidin-biotin peroxidase complex method, as previously described (Yu *et al.*, 1999). The primary antibody used was the mouse monoclonal anti-NS4B antibody (Advanced Immunochemical Inc.).

Results

Generation of NS4B transgenic mice

To generate the transgenic mice, the HCV NS4B genomic region under the control of the HBV enhancer was microinjected into fertilized oocytes from BCF1 hybrid mice. Four founders containing the HBV enhancer-HCV NS4B sequence were identified. Among them, three were selected for the establishment of the transgenic lineage by backcrossing against the C57BL/6J parental strain.

Expression of the NS4B transgene

In order to determine the expression of the HCV NS4B transgene at the mRNA level in transgenic mouse tissues, RT-PCR was performed. The results showed that NS4B was expressed widely in the tissues of transgenic mice, including the liver, but was not expressed in normal control littermates (Figure 1B and C). This expression pattern was similar to the pattern that appeared in the transgenic mice expressing the HBV X protein and HCV core protein under the direction of the HBV enhancer sequence in our previous study (Yu *et al.*, 1999; Wang *et al.*, 2004). To find out if the mRNA was translated to the protein in the liver, Western blot analysis and immunohistostaining were performed. The Western blot data showed that HCV NS4B protein was substantially expressed in liver tissues (Figure 1D). In addition, the immunohistostaining showed that the expression of HCV NS4B protein was present throughout the cytoplasmic of hepatocytes in transgenic mice (Figure 2).

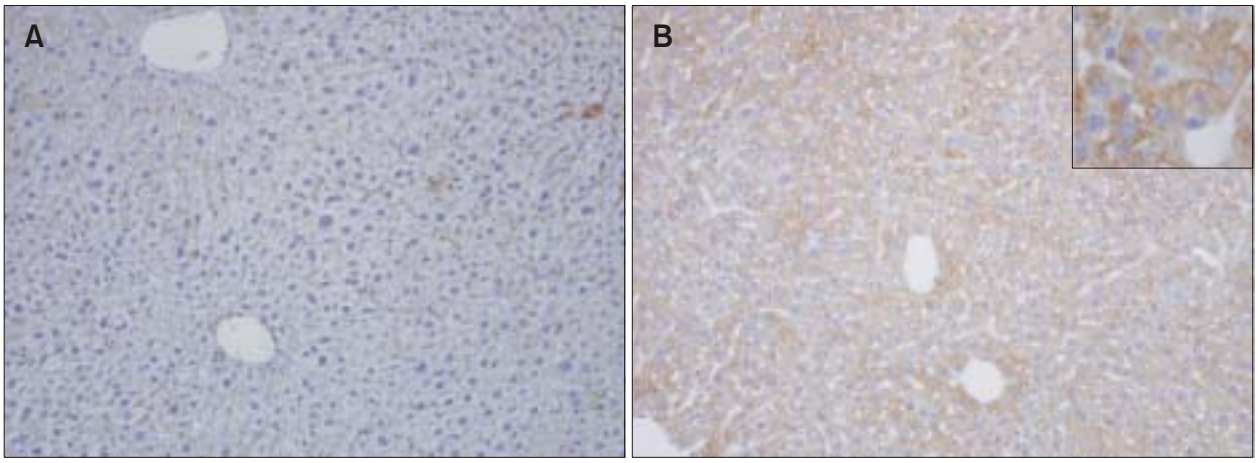

Figure 2. Immunohistochemical analysis of HCV NS4B in the livers of normal littermates (A) and transgenic mice (B) using a specific monoclonal antibody. NS4B antibody staining is predominantly in the cytoplasm of transgenic hepatocytes showed in *B* at magnification 20 × and 200 × (upper-right corner). Magnification for (A) is 20 ×.

Table 1. Histological summary of HCV NS4B transgenic mouse lineages.

Transgenic line	Number of mice examined	Age (months)	Liver abnormalities	Other organs abnormalities
Line 2	14	9-18	None	None
Line 20	10	9-18	None	None
Line 58	11	9-18	None	None
Normal	15	9-18	None	None

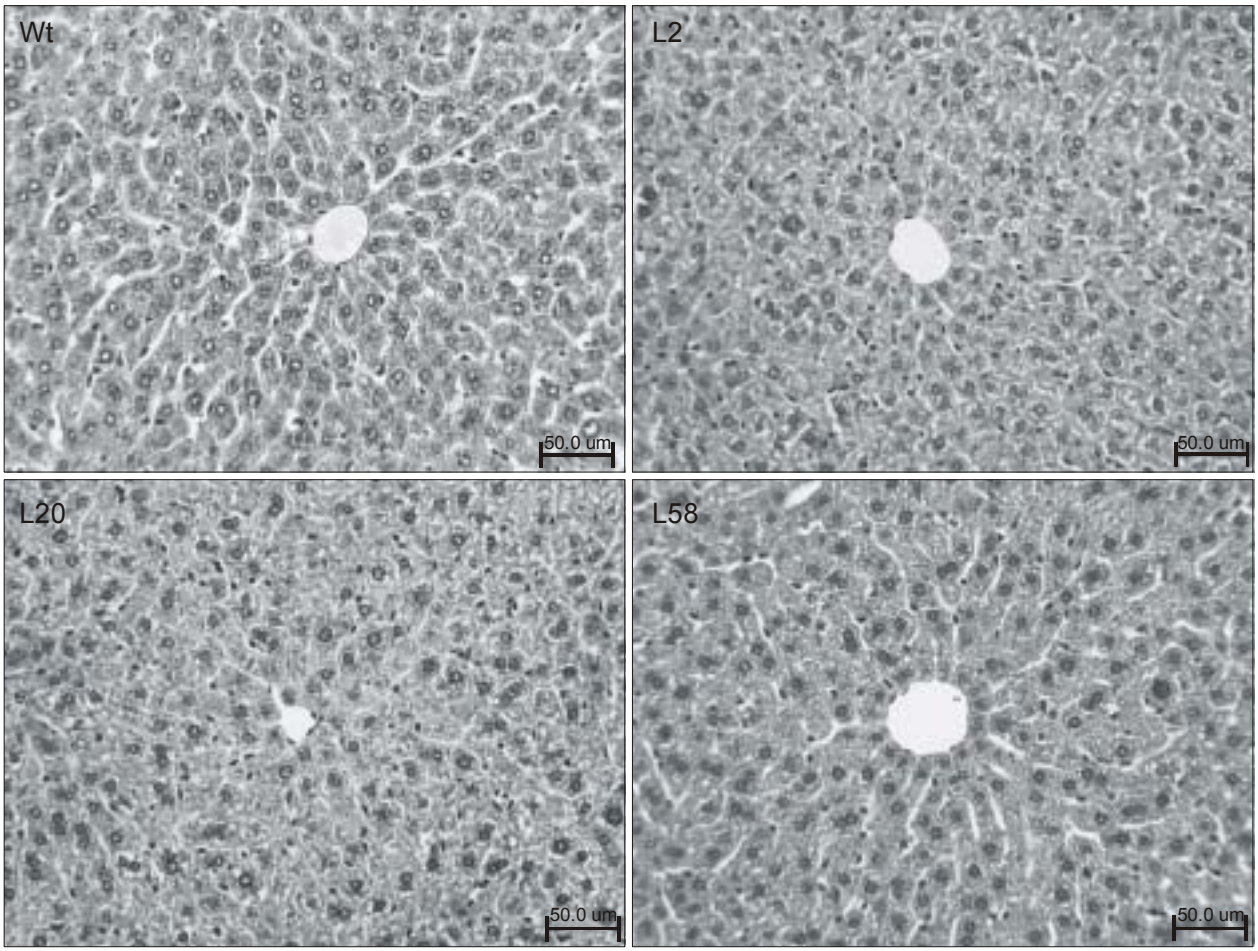

Figure 3. Histological analysis of livers from normal and transgenic mice. Hematoxylin and eosin-stained paraffin sections of liver histology from normal littermates (Wt) and from transgenic lineages 2 (L2), 20 (L20), and 58 (L58) are shown at 40 ×.

Histological examinations

To detect whether NS4B was cytopathic to the transgenic mice, the mice were sampled between 3 and 18 months of age. As the NS4B transgene was found to be expressed in the transgenic mice tissues, the organ tissues including the liver were examined by histopathological analysis using H&E-stained sections in this study. A summary of the pathological assays is shown in Table 1. No pathological changes were detected in the spleen, kidney, heart, lung, brain, testis, or ovary. Inflammation and steatosis were rarely detected in the livers of transgenic mice or in normal control littermates during the experimental period. The histological assays of the three lineages are shown in Figure 3.

Discussion

The expression of transgenic NS4B at the mRNA

and protein levels was confirmed in the three established transgenic lineages. However, the expression of NS4B was not cytopathic to liver tissue and to other cell types in these mice. Compared with the tumorigenesis and cell dysplasia in liver tissues of our previously reported HBX and S99Q transgenic mice, which were produced using the same HBV enhancer to induce the expression of transgenes (Yu *et al.*, 1999; Wang *et al.*, 2004), the non-pathologic changes detected in our NS4B transgenic lineages indicated a non-cytopathic function of NS4B in most cell types, even though the *in vitro* investigation showed the possibility of functions relating to the cell cycle (Florese *et al.*, 2002) or transformation (Park *et al.*, 2000, Qu *et al.*, 2001).

An understanding of HCV-mediated pathogenesis and the control of HCV infection have been impaired in part because of the unavailability of an efficient cell culture system for virus growth and a convenient small animal model. Advances in the field of transgenic technology have offered us unique opportunities to develop valuable rodent models for human diseases (Majumder *et al.*, 2003). Currently published data suggest that the expression of the HCV virus gene from the HCV genotype 1b (Japanese strains) in the genetic background of the C57BL/6J mouse can induce hepatic alterations (Moriya *et al.*, 1997; Moriya *et al.*, 1998; Lerat *et al.*, 2002). However, transgenic mice expressing the HCV gene that were established using another genotype of *HCV* or another genetic background of mouse strain (Kawamura *et al.*, 1997; Pasquinelli *et al.*, 1997; Majumder *et al.*, 2003; Wang *et al.*, 2004) failed to generate hepatic alterations. In Japan, approximately 15, 90, and 80% of patients with sporadic acute hepatitis, post-blood transfusion chronic hepatitis, and HCC, respectively, are positive for HCV infection (Watashi *et al.*, 2003). The HCV genotype 1b (Japanese strains) is the main causative agent for liver diseases. The collective data suggest that the genotype of the HCV and the mouse strain should be carefully selected when investigating the possible mechanisms related to HCV proteins. If the genetic background is proven to be an important factor in determining the disease expression in HCV-transgenic mice, it may provide useful clues to the mechanisms underlying the variable risk of disease in persons with chronic HCV infection (Lerat *et al.*, 2002). In this study, even the genotype HCV 1b NS4B (Korean strains) did not show cytopathic effects on cells *in vivo* in the C57BL/6J genetic background, but other HCV genotypes of NS4B remain to be elucidated.

The HCV Core transgenic mice suggested that core protein may play an important role in HCV-induced steatosis and HCC (Moriya *et al.*, 1997; Moriya *et al.*, 1998; Shin *et al.*, 2005). Recently, Lerat *et al.* suggested that the expression of the structural proteins enhances a low background of steatosis in C57BL/6 mice, while the additional low-level expression of the nonstructural proteins increases the risk of cancer (Lerat *et al.*, 2002). Among the nonstructural proteins, NS5A and NS4B are the primary candidates, but their functions are largely unknown. It has been reported that transgenic mice expressing NS5A protein did not show any pathological changes (Majumder *et al.*, 2003), which indicates that NS5A protein alone is not cytopathic or oncogenic to hepatocytes. In this study, we first screened for possible pathological functions, which we inferred, of the NS4B protein *in vivo*. Mice in the three established transgenic lineages that expressed the NS4B transgene constitutively in transgenic tissues did not show any pathological changes. However, the occurrence of cooperative function with other virus proteins in relation to liver disease remains to be elucidated.

In conclusion, our investigation indicated that the HCV NS4B protein is not a causative agent of liver disease in our transgenic model. However, the possibility of cooperative functions with other viral proteins or causative agents remains to be elucidated.

Acknowledgement

This work was supported by the 21st Century Frontier Program in the Functional Human Genome Project of Korea (grant no. HGC0300324).

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