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# Expression of hepatitis C virus non-structural 5A protein in the liver of transgenic mice

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**Abstract Hepatitis C virus (HCV) is a major etiologic agent for chronic hepatitis worldwide often leading to the development of cirrhosis and hepatocellular carcinoma. However, the mechanism for development of chronic hepatitis or hepatocarcinogenesis by HCV remains unclear. HCV NS5A protein possesses many intriguing properties, including sequestration of p53 in the cytoplasm, downregulation of p21 protein, activation of STAT3, and inhibition of tumor necrosis factor-α-mediated apoptosis. Thus, we investigated whether this viral protein has oncogenic property in vivo. In the absence of an efficient cell culture system for virus growth and a suitable small animal model for HCV infection, transgenic FVB mice were generated by targeting the HCV NS5A genomic region cloned under the control of a liver-specific apoE promoter or mouse major urinary promoter (MUP). The apoE promoter is constitutively expressed in liver, on the other hand, the MUP is developmentally regulated and expressed in the liver after birth. Reverse transcription polymerase chain reaction and Western blot analysis indicated establishment of HCV NS5A transgene expression in several lines from both groups of mice. Immunohistochemical studies suggested the presence of NS5A in the cytoplasm of hepatocytes. The transgenic animals were phenotypically similar to their normal littermates and did not exhibit a major histological change within the liver up to 24 months of age. Our results suggested HCV NS5A protein is not directly cytopathic or oncogenic in this FVB transgenic mouse model, although this viral protein promotes cell growth in vitro. These animals will be a valuable model of HCV immunopathology as well as for evaluation of siRNA, interferon and other cytokine therapies.**


*Key words:* Hepatitis C virus; NS5A protein; Transgenic mice; Liver specific expression

## 1. Introduction

The most prominent feature of persistent hepatitis C virus (HCV) infection is the development of chronic hepatitis in the majority of infected individuals and the potential for disease progression to hepatocellular carcinoma [1–4]. Unfortunately, a number of important issues related to HCV-mediated disease progression are unknown at this time. Furthermore, neither a vaccine nor any other means of effective chemotherapy is available to control HCV infection. The chimpanzee is the only available animal model to study HCV infection and has been used for characterizing the virus, analyzing the infectivity of clinical samples, and studying clinical and immunological aspects of viral infection [5]. It is, therefore, most difficult to study the biological properties and pathogenicity of HCV. The HCV genome contains a linear, positive-strand RNA molecule of ~9500 nucleotides [6]. A number of HCV genomes have been cloned, and the sequence divergence indicates several genotypes and a series of subtypes for this virus [7]. The HCV genome encodes a single polyprotein precursor of ~3000 amino acids [6] which is cleaved by both host and viral proteases [8–10] generating at least 10 individual proteins, Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B.

NS5A exists as two phosphoproteins, p56 and p58, which are phosphorylated at serine residues after the mature NS5A protein is released from the polyprotein [11,12]. Although phosphorylation of HCV NS5A occurs predominantly on serine, a low level of phosphorylation on threonine residues was also observed [13]. Sequence comparison of the regions surrounding the sites of phosphorylation indicates an extremely high level of conservation between different strains of the virus, but the biological significance of phosphorylation is still unclear. NS5A localizes in the cytoplasm. In recent years, NS5A has been intensively studied in vitro. We have shown that NS5A has a trans-regulatory property and transcriptionally modulates proliferating cell nuclear antigen and p21 cell cycle regulatory genes [14], physically associates and sequesters p53 in the cytoplasm, represses p53-mediated p21 transcription [15,16], and activates STAT3 [17]. Exogenous expression of NS5A in murine fibroblasts transformed cells to a tumorigenic phenotype [14,18]. NS5A also physically associates with several cellular proteins (SNARE, SRCAP and karyotype β), suggesting its role in cell growth regulation [19–21]. We have shown that NS5A protects against tumor necrosis factor-mediated apoptotic cell death [22,23]. Together these results suggest the possible involvement of NS5A in virus-mediated pathogenesis. Lerat et al. [24] reported transgenic mice expressing HCV proteins developed hepatocellular carcinoma (HCC), and suggested that non-structural proteins

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play a role for increasing the risk of HCC. However, the role of a specific non-structural protein has not been investigated. In this study, we have examined the role of NS5A protein-mediated pathogenesis in transgenic mice. Here, we report the generation of transgenic mice, expression of HCV NS5A protein, and histological findings of the liver.

## 2. Materials and methods

### 2.1. Microinjection and generation of NS5A transgenic mice

To generate a NS5A transgenic mouse model, the HCV NS5A genomic region from genotype 1a H77 strain was directed by the mouse major urinary promoter (MUP) for expression in mouse liver (Fig. 1A). The pSA11 vector [25] containing MUP for expression of the liver-specific gene (kindly provided by Dr. William Held, Roswell Park Cancer Institute, Buffalo, NY, USA) was used to clone the HCV NS5A genomic region. The *Nru*I/*Xba*I fragment of the cytomegalovirus NS5A plasmid was excised, filled in with Klenow and subcloned into blunt-ended pSA11 vector to position the NS5A genomic region downstream of the MUP. A polyadenylation sequence from respiratory syncytial virus chloramphenicol acetyltransferase was cloned at the carboxy-terminal site of NS5A. The orientation of the resulting transgene plasmid (pMUP-NS5A) was verified by restriction enzyme digestion and nucleotide sequence analysis of the junctions. Potential founders were analyzed by polymerase chain reaction (PCR) and Southern blot from tail DNA.

### 2.2. Analysis of transgene expression

At least two transgenic mice (one male and one female) and their normal littermates from each established line were killed at 8 weeks of age. Total RNA was extracted separately from liver, heart, kidney, lung, spleen and brain using the PUREscript kit (Gentra, Minneapolis, MN, USA). Reverse transcriptase (RT) PCR was performed using NS5A-specific sense (5′-GGACGATGAGGATCGTCGG-3′) and antisense (5′-TGGCTAGCCGAGGAGCTGG-3′) primers. A calculated size DNA band (469 bp) was visualized by electrophoretic analysis of the RT-PCR product on agarose gel. RT-PCR for GAPDH as an internal control was performed similarly using specific sense and antisense primers [23]. For the analysis of NS5A protein expression, livers from transgenic mice or non-transgenic control littermates were homogenized in RIPA buffer (0.15 M NaCl, 50 mM Tris pH 8.0, 0.5% sodium deoxycholate, 1% NP-40 and 1% sodium dodecyl sulfate (SDS)). Liver homogenate was clarified and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) for Western blot analysis. The blot was probed with a rabbit polyclonal antibody to HCV NS5A (kindly provided by C. Rice, Washington University, St. Louis, MO, USA) or monoclonal antibody to NS5A (Biogenesis, Kingston, NH, USA) and detected by chemiluminescence (ECL, Amersham, Piscataway, NY, USA).

### 2.3. Analysis of hepatocellular injury

Blood samples were collected from both transgenic mice and normal littermates. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in serum were measured using a clinical analyzer (Roche Cobad Mira plus). Mean values of both ALT and AST in serum of transgenic mice were compared with those of normal littermates. Both transgenic and normal mice at different age groups were killed and different tissues including liver, spleen and gall bladder were collected and fixed in 10% formaldehyde solution. Sections of 3–5 μm thick were obtained from paraffin-embedded tissues. The sections were then stained with hematoxylin and eosin and examined under a microscope.

## 3. Results and discussion

To generate transgenic mice, the HCV NS5A genomic region under the control of MUP was microinjected into fertil-

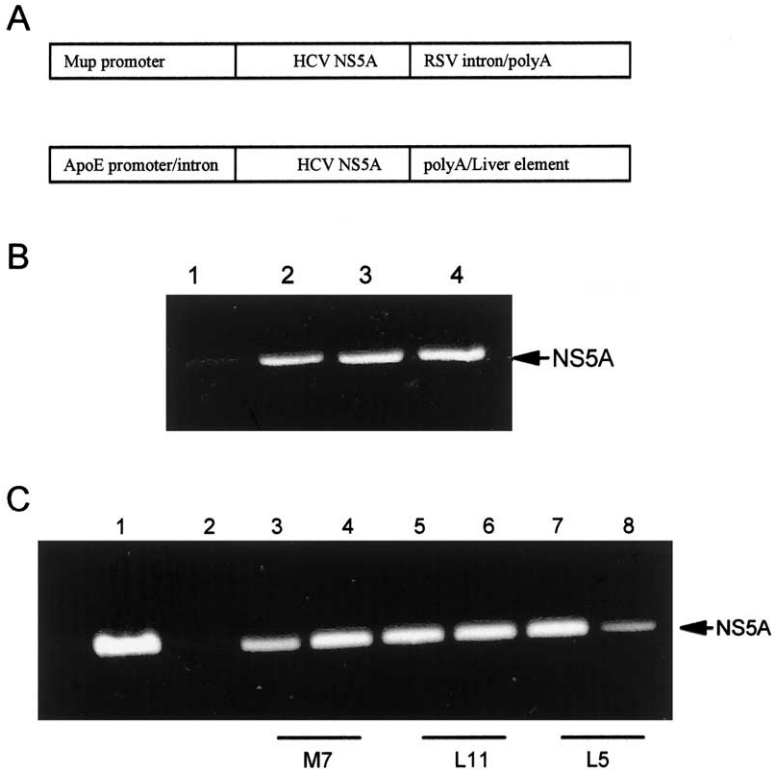


Fig. 1. A: Schematic presentation of MUP-NS5A and apoE-NS5A constructs. The transgenic vector contains the mouse MUP or apoE promoter elements followed by the HCV NS5A genomic region (1.3 kb) with intron and poly A signal. B: RT-PCR analysis for expression of NS5A in transgenic mouse liver from the M7 line at 8 weeks of age. PCR product from a negative control RNA of a normal littermate (lane 1), and RNA from three different transgenic mice (lanes 2–4), were analyzed by agarose gel electrophoresis. C: RT-PCR analysis showing liver-specific NS5A expression in the M7 line (lanes 3 and 4), L11 (lanes 5 and 6), and L5 (lanes 7 and 8) at 18 months of age. Lanes 1 and 2 represent positive and negative controls, respectively, for NS5A amplification.

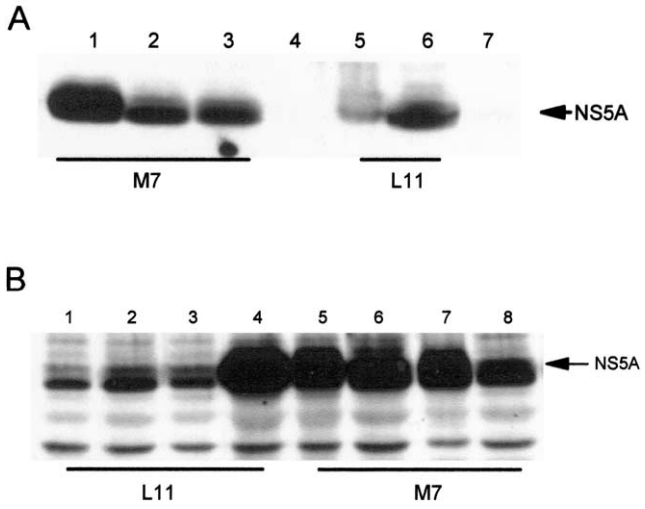


Fig. 2. A: Western blot analysis of liver homogenate from M7 (lanes 1–3), L11 (lanes 5 and 6) transgenic mice and normal littermates (lanes 4 and 7) at 8 weeks of age using a polyclonal antibody to NS5A. The molecular weight of the NS5A protein band (~56 kDa) was ascertained from the migration of standard protein molecular weight markers (Invitrogen). B: Liver-specific NS5A expression in transgenic mice at 18 months of age by Western blot analysis using specific antibody. The molecular weight of the NS5A band (~56 kDa) was ascertained from the migration of standard protein molecular weight markers (Invitrogen).

ized oocytes from FVB inbred mice. Two founders containing the MUP-HCV NS5A sequence were identified, however, upon subsequent breeding, only one line of transgenic mice (M7) was established, bearing approximately two copies of the transgene as determined by Southern blot analysis (data not shown). Generation of transgenic mouse lines expressing the liver-specific apoE promoter (kindly provided by John Taylor, Gladstone Institute of Cardiovascular Disease, San Francisco, CA, USA) was described previously [23]. Three lines were established and named L1, L5 and L11. However, the L1 mouse line became infertile; M7, L5 and L11 were fertile and were expanded by successive backcrossing against the FVB parental strain.

RNA isolated from control or transgenic mouse liver and other tissues at 8 weeks of age was analyzed for expression of the NS5A gene by RT-PCR. The results suggested expression of NS5A-specific sequence only in transgenic mouse lines, but not in control littermates (Fig. 1B). RNA isolated from kidney, spleen, brain, heart and lung of L5 and L11 lines did not exhibit NS5A expression by RT-PCR, suggesting that the transgene expression was restricted to the liver. Results from this study indicated the authenticity of the transgenic mouse line harboring the NS5A hybrid construct and expression of mRNA in mouse liver. RNA isolated from other tissues of transgenic mouse line M7.9 displayed NS5A expression at an early age (2–4 weeks), however, after 6 weeks of age, NS5A was expressed only in liver. A similar observation was reported in transgenic mice expressing SV40 T antigen (reviewed in [25]). This time period corresponds to the onset of sexual maturity and an increase in the level of hormones. These hormones affect MUP gene expression and may play a role in regulating MUP expression in the developmental pattern. RNA isolated from the liver of transgenic mice at 18 months of age displayed NS5A expression in all three transgenic mouse lines (Fig. 1C).

For analysis of NS5A protein expression, livers from transgenic mice or non-transgenic control littermates at 8 weeks of age were homogenized in RIPA buffer. Liver homogenate was clarified and subjected to SDS–PAGE for Western blot analysis. The blot was probed with a rabbit polyclonal antibody to HCV NS5A or monoclonal antibody to NS5A and detected by chemiluminescence. Expression of a ~56 kDa band from transgenic mouse liver was observed, while control mouse liver failed to exhibit a detectable signal by chemiluminescence (Fig. 2A). Expression of HCV NS5A protein from 18 month old mice was also confirmed by Western blot analysis using a monoclonal antibody to NS5A. Different levels of NS5A expression were observed from transgenic mouse liver (Fig. 2B) in both M7 and L11 lines. A similar result was also observed from L5 lines [23]. We have also performed immunohistological staining of NS5A in transgenic mouse liver (Fig. 3). Our results confirmed the expression of HCV NS5A protein in the hepatocytes of transgenic mice, while normal littermates did not display a detectable staining. HCV NS5A was expressed mostly in the zone 3 (peri-central vein) hepatocytes in transgenic mouse liver, and the expression pattern was similar in both transgenic mouse lines. NS5A staining was not associated with usual inflammatory infiltrates.

Both transgenic and normal mice of different age groups were killed and examined visually for any abnormalities. Different tissues including liver, spleen and gall bladder from

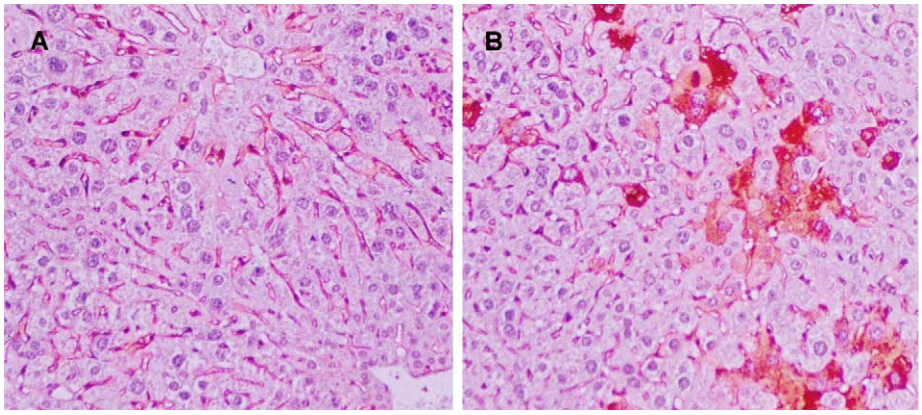


Fig. 3. Immunohistochemical analysis of HCV NS5A in liver of normal littermate (A) and transgenic mice (B) using a specific monoclonal antibody. NS5A antibody staining was predominantly in the cytoplasm of hepatocytes. HCV NS5A was expressed mostly in the zone 3 (peri-central vein) hepatocytes in transgenic mouse liver.

Table 1
Summary of HCV NS5A transgenic mouse lineages

| Transgenic line | Number of mice examined | Age group (months) | Liver abnormalities | Tumor in other organs | Urinary bladder inflammation |
|---|---|---|---|---|---|
| L11 | 32 | 6–24 | None | 3 | 4 |
| L5 | 40 | 6–23 | None | 5 | 6 |
| M7 | 40 | 6–24 | None | 3 | 2 |
| Normal | 30 | 6–24 | None | 4 | 1 |

both groups of mice were collected and fixed in 10% formaldehyde solution. Paraffin-embedded tissue sections (3–5 μm) were then stained with hematoxylin and eosin and examined under a microscope. Slides were examined by pathologists (N.J.P. and L.T.) who were blinded to the status of the animals. In the L11 line, 32 transgenic mice of age ranging from 6 to 24 months were examined (Table 1). Three out of 32 mice developed lung adenocarcinoma and splenic sarcoma between 16 and 24 months of age. Four mice from this transgenic line displayed prolapsed urinary bladder and seminal vesicle through their pelvic girdle starting from 9 to 24 months. On the other hand, only one normal mouse out of 30 showed urinary bladder inflammation. However, none of the mice examined in this transgenic line showed any abnormality in the liver by microscopic or histological staining of formalin-fixed tissue sections. In the L5 line, we examined 40 mice of different age groups. Like the L11 line, here also we observed solid tumor in thymus and lung adenocarcinoma in five mice, and urinary bladder inflammation in six mice between 9 and 23 months of age. A single 8 month old transgenic animal had macroscopically evident 0.5 mm white spots in the liver. However, no other significant abnormality was observed in histopathology. Mice of the M7 line were phenotypically normal and did not exhibit any gross liver abnormality even after 24 months of age. Three out of 40 mice examined developed tumor in thymus and lung adenocarcinoma, and showed ~20% steatosis in liver but did not develop nodularity or adenoma. Two mice of this line (8 months and 13 months) showed a moderate to high level of portal, interface and lobular inflammation, but no adenoma. None of the non-hepatic tumors from these mice displayed NS5A mRNA expression by RT-PCR. The clinical abnormality (urinary bladder inflammation) in mice was not statistically significant (Fisher's exact test, two-tailed $P$ value 0.1678). Interestingly, four out of 30 normal littermates developed lung adenocarcinoma, which could be a genetic disease in the FVB background at old age. Hematoxylin and eosin-stained liver sections were scored for necroinflammatory activity and fibrosis by the Scheuer [26] scale and evaluated for the presence of apoptosis, steatosis, adenoma, dysplasia, and hepatocellular carcinoma. No animal had hepatocellular carcinoma or hepatic adenoma. Cirrhosis or fibrosis was not observed. The Scheuer activity score was not significantly different between the individual transgenic lines and the pooled littermate controls (L5 transgenic, mean activity score 1.560, $n = 40$; L11 transgenic, mean activity score 1.318, $n = 32$; M7 transgenic, mean activity score 1.414, $n = 40$; normal littermate controls, mean activity 1.545, $n = 30$; Mann–Whitney test, two-tailed $P$ values 0.6022, 0.6824, and 0.8766). A representative histopathology of 18 month old mouse liver from each transgenic line and from normal littermate control is shown in Fig. 4. We also examined the serum ALT and AST activities. Blood samples were collected at 6 and 18 months of age from both transgenic and normal littermates. ALT/AST activities in serum were measured using a clinical analyzer (Roche Cobad Mira plus). Mean values of both ALT and AST in serum of transgenic

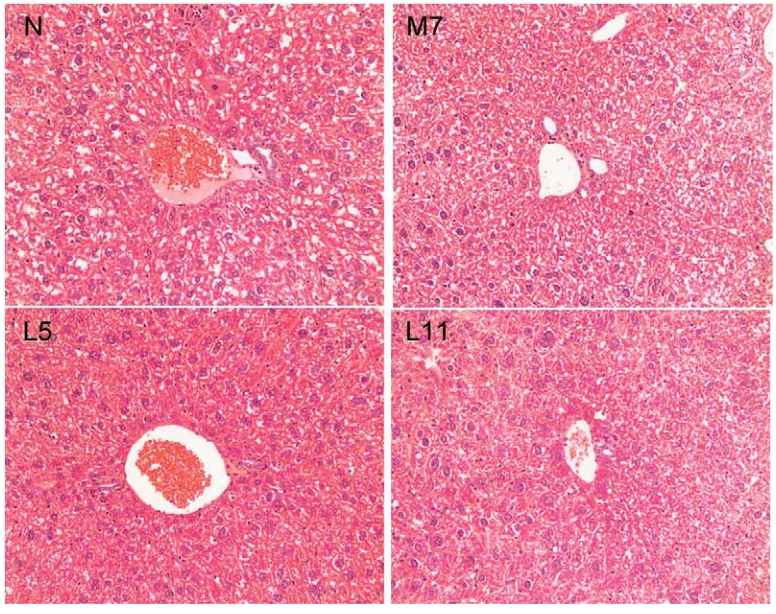


Fig. 4. Histological analysis of liver from normal and transgenic mice. Hematoxylin and eosin-stained paraffin sections of liver histology from normal littermates (N), transgenic mice M7, L5 and L11 are shown at 20×.

mice and normal littermates did not exhibit detectable differences.

Chronic HCV infection may eventually lead to HCC and viral protein(s) are likely to be one of the contributing factors. Success in understanding HCV-mediated pathogenesis and in controlling HCV infection has 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 offered us a unique opportunity to develop valuable rodent models for human diseases. We and others have shown that HCV NS5A plays an important role in transcriptional regulation of cell cycle regulatory genes as well as in the inhibition of apoptosis [14,15,18,23,27,28]. Functional p53 is a positive regulator of apoptosis. Viral proteins that bind p53 contribute to immortalization or are themselves immortalizing [29], and fibroblasts from mice that are deficient in p53 are immortalized readily [30]. Invalidation of the apoptosis mechanism is an important step in tumor development, and the same may be true for immortalization [31]. Therefore, we examined whether NS5A has a role in promoting tumor formation in transgenic mouse liver. Our results suggest that despite different expression levels of NS5A, transgenic mouse liver of FVB genetic background did not develop liver abnormalities. This may be due to a number of other contributing factors, including genetic background of the mouse strain used for generating transgenic animals [24,32–34].

We do not know at present whether NS5A promotes immortalization of primary murine hepatocytes as an initial event associated with the multi-step phenomenon of cancer. For example, expression of *cyto-MET* or transforming growth factor-α in transgenic mice does not develop tumor, but immortalizes hepatocytes [35–37]. Immortalized culture was also established from transgenic mice of hepatitis B virus [38]. Therefore, it is possible that expression of HCV NS5A protein may trigger the neoplastic events in the presence of other viral proteins as observed by Lerat et al. [24]. Although liver pathogenesis was not observed with NS5A transgenic mice, nonetheless, these animals will be a valuable model of HCV-related immunopathology, as well as for evaluation of antiviral therapies.

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