Efficient Conditional Transgene Expression in Hepatitis C Virus cDNA Transgenic Mice Mediated by the Cre/loxP System*

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Conditional gene expression has greatly facilitated the examination of the functions of particular gene products. Using the Cre/loxP system, we developed efficient conditional transgene activation of hepatitis C virus (HCV) cDNA (nucleotides 294–3435) in transgenic mice. Efficient recombination was observed in transgenic mouse liver upon intravenous administration of adenovirus that expresses Cre DNA recombinase. After transgene activation, most hepatocytes were stained with anti-core polyclonal antibody, and 21-, 37-, and 64-kDa proteins were detected by Western blot analysis in liver lysates using anti-core, E1, and E2 monoclonal antibodies, respectively. Serum core protein was detected in transgenic mice 7 days after transgene activation with concurrent increases in serum alanine aminotransferase levels. Subsequently, an anti-core antibody response was detected 14 days after infection. Furthermore, a CD4 and CD8 positive cell depletion assay normalized both the serum alanine aminotransferase increases and pathological changes in the liver. These results suggest that HCV proteins are not directly cytopathic and that the host immune response plays a pivotal role in HCV infection. Thus, this HCV cDNA transgenic mouse provides a powerful tool with which to investigate the immune responses and pathogenesis of HCV infection.

One desired goal of transgenic technology is temporal control of target gene expression in a particular stage of development or in a specific organ. To this end, heavy metal or hormone-inducible or organ specific promoters have been used to generate transgenic animals (1). However, the extent and timing of expression of the target gene can only rarely be experimentally manipulated in such animals. Recently, such precise conditional gene manipulation has been possible through the use of a DNA recombination event mediated by Cre recombinase, which catalyzes DNA cleavage between two loxP sequences (2). This Cre/loxP system has been used in combination with recombinant adenovirus vector expressing Cre to alter gene expression in the liver of transgenic mice (3). One suitable application of this transgene activation system is the investigation of the functions of viral gene products. When a particular virus of interest has a narrow host range and exhibits limited tissue tropism, it is often difficult to develop a suitable animal model. Temporal control of viral gene expression using a conditional transgene activation system would enable detailed analysis of the immune responses of the host and cytopathic effects of the viral proteins.

Hepatitis C virus (HCV) contains a single-stranded, positive-sense RNA genome of approximately 9,500 nucleotides encoding a single large polyprotein of ~3,010 amino acids (4). The coding region encodes structural proteins in its N-terminal portion, which includes a nonglycosylated core protein (p21) that associates with genomic RNA to form the nucleocapsid (5). Immediately downstream from the core are two proteins designated E1 and E2, which correspond to the putative envelope glycoprotein (6). HCV infection is the major cause of post-transfusion non-A non-B hepatitis (7, 8), and widespread screening for antibodies in blood products intended for transfusion has reduced the risk of fresh infection. Nonetheless, persistent HCV infection often progresses to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, usually more than a decade after initial infection (9). Thus, the development of adequate treatment and prophylactics for HCV infection has been important. HCV is not infectious in vivo except in primates, a phenomenon that has resulted in the lack of a proper HCV culture system and inbred animal model, which has in turn hampered detailed analysis of viral life cycle and pathogenesis of HCV infection.

In the present study we used the Cre/loxP conditional expression system to construct an HCV cDNA transgenic mouse. We confirmed the strong expression of HCV structural proteins in the mouse liver only after transgene activation and subsequent antibody response directed against the transgene product. In addition, over the short, 1-week observation period, HCV structural proteins appeared to have no cytopathic effect on the mice. Thus, this HCV transgenic line should serve as a useful animal model of HCV infection.

EXPERIMENTAL PROCEDURES

Transgene Construction and CN2 Transgenic Animal Production—R6CN2 HCV cDNA (nucleotides 294–3435, numbered according to Chen et al. (10)) was cloned from the plasma of patient R6, which has chronic active hepatitis. The infectious titer of R6 plasma has been previously determined in chimpanzee and Mol8 cells and has been denoted as plasma K by Dr. Shimizu (11). R6CN2 was determined to be

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‡‡The abbreviations used are: HCV, hepatitis C virus; ALT, alanine aminotransferase; pfu, plaque-forming unit; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
genotype 1b HCV cDNA by sequence homology analysis. pCALNCN2/59–2 is a plasmid that contains the expression-switching transgene unit CALNCN2 (see Fig. 1, Upper), which is similar in design to CALNLZ (12). CALNCN2 consists of the CAG promoter, a loxP sequence, the neo-resistance gene, the SV40 poly(A) signal, a second loxP sequence, and R6CN2 HCV cDNA, and is then linearized by HindIII digestion and purified by gel electrophoresis and ultracentrifugation. Purified CALNCN2 was microinjected into fertilized BALB/cSltc mouse eggs (Japan SLC, Inc., Hamamatsu, Japan). Potential founders were analyzed for the presence of the transgene by polymerase chain reaction of mouse genomic DNA isolated from ear punch specimens (12). A concentrated and purified virus stock was then used as the positive and negative controls, respectively. For protein analysis, 100 mg of liver, thymus, heart, lung, spleen, kidney, stomach, intestines, and muscle samples were homogenized in radioimmunoprecipitation assay buffer (1% SDS, 0.5% Nonidet P-40, 10 mM Tris, pH 7.4, 150 mM NaCl, and 1 mM phenylmethanesulfonyl fluoride) and rocked for 30 min at 4 °C. After the lysis, 0.5-ml aliquots (5 × 106 pfu) diluted in a final volume of 100 μl of phosphate-buffered saline was injected into the tail veins of 8–10-week-old male mice. The animals (2 mice/dose) were sacrificed 3 or 7 days after intravenous injection of adenovirus. Genomic DNA from mouse liver, lung, spleen, and kidney was extracted using the Qiagen blood and cell culture DNA kit (Qiagen, Chatsworth, CA) for Southern blot analysis. For protein analysis, 100 mg of liver, thymus, heart, lung, spleen, kidney, stomach, intestines, and muscle samples were homogenized in radioimmunoprecipitation assay buffer (1% SDS, 0.5% Nonidet P-40, 10 mM Tris, pH 7.4, 150 mM NaCl, and 1 mM phenylmethanesulfonyl fluoride). CD4+ and CD8+ T cell depletion was performed by inculating mice intraperitoneally with 0.5-ml aliquots of a 1:10 dilution of mouse ascites fluid containing both the GKL.5 (anti-CD4) and 53–67.2 (anti-CD8), both hybridomas were kindly provided by Dr. S. Kayasu, Keio University, Tokyo, Japan) monoclonal antibodies (14, 15). The monoclonal antibodies were injected 6 days before adenovirus administration and then every 3 days until completion of the experiment. Lymphocytes were prepared from spleen cell suspensions by density separation and stained with fluorescein isothiocyanate or phycoerythrin-conjugated monoclonal antibodies (PharMingen, San Diego, CA). Stained cells were analyzed using a FACs calibur flow cytometer (Becton Dickinson, Bedford, MA).

Quantification of HCV Core Protein and Detection of Antibodies to HCV Core Protein in Mouse Serum—Transgenic mice serum (200 μl) was precipitated using polyethylene glycol 4000 and then assayed as described previously (16). Anti-core antibody production in mouse sera was determined using the Immuchek HCV Ab kit (International Reagents Corp., Kobe, Japan). Horseradish peroxidase-labeled anti-mouse IgG, A, M (Zymbex Laboratories Inc., South San Francisco, CA) was used as the detection antibody. Sera from mice immunized with recombinant core protein and normal mouse sera were used as the positive and negative controls, respectively.

Production of Transgenic Mice—Nine animals containing the transgene were identified by polymerase chain reaction screening. To identify the animals capable of expressing HCV protein, spleen cells and fibroblasts were surgically isolated and cultured in vitro. The cultured cells were then infected with AxCANCre at a multiplicity of infection of 100. Two founder lines, CN2-8 and CN2-29, were used as the positive and negative controls, respectively.

RESULTS

Production of Transgenic Mice—Nine animals containing the transgene were identified by polymerase chain reaction screening. To identify the animals capable of expressing HCV protein, spleen cells and fibroblasts were surgically isolated and cultured in vitro. The cultured cells were then infected with AxCANCre at a multiplicity of infection of 100. Two founder lines, CN2-8 and CN2-29, were used as the positive and negative controls, respectively.

Cre-mediated Transgene Recombination in Liver—AxCANCre (0.9, 1.8, 2.7, 3.6, or 5.4 × 109 pfu) was infused into 2 mice of each of the CN2-8 and CN2-29 lines/dose by tail vein injection to examine whether Cre-mediated transgene recombination (Fig. 1) had occurred in mouse liver. Three days after the injection of adenovirus, genomic DNA was prepared from liver and analyzed by Southern blot analysis. Transgene recombination occurred in a dose-dependent manner in the livers of the transgenic mice (Fig. 2). Efficient recombination was observed by injection of more than 1.8 × 109 pfu for the CN2-8 mice (Fig. 2A) and 3.6 × 109 pfu for the CN2-29 mice (Fig. 2B). When whole CALNCN2 unit was used as the probe, an additional signal at 2.8 kilobase pairs was detected in the CN2-8 genome (Fig. 2A). This signal disappeared with the CN2 HCV cDNA probe (Fig. 5B). Thus, this additional signal was a result of the CALNCN2 probe binding to a mouse genomic DNA fragment adjacent to the CAG promoter of the transgene.

Induction of HCV Protein Expression in the Liver of CN2 Transgenic Mice—As shown in Fig. 2, CN2-8 mice expressed more than six times as much HCV core protein than the CN2-29 mice, as determined in the total liver lysate. In both
lines, the amount of core protein in the liver increased in accordance with the dose of injected adenovirus (0.9 ~ 5.4 × 10^9 pfu/mouse). However, similar levels of transgene recombining were observed by Southern blot among the CN2-8 mice that received 1.8 × 10^9 and more than 1.8 × 10^9 pfu and between CN2-29 mice that received 3.6 × 10^9 pfu and more than 3.6 × 10^9 pfu.

Western blot analysis was performed to determine whether HCV protein production was induced by transgene activation and whether these proteins were correctly processed. Liver samples were obtained from CN2-8 and CN2-29 transgenics with or without AxCANCre injection (3.6 × 10^9 pfu/mouse) or from AxCANCre-injected littermates 3 days after injection. The representative results of one mouse from each mouse group are shown in Fig. 3. Core, E1, and E2 proteins were identified in the total tissue lysates in addition to nonspecific signals (data not shown); however, in the membrane fraction these nonspecific signals were reduced, and the HCV protein signals were clearly visible (Fig. 3). When the 5E3 anti-core monoclonal antibody was used as the probe, core protein (21 kDa) was identified only in the samples from adenovirus-injected transgenic mice and not in the transgenics without adenovirus injection or littermates with adenovirus injection (Fig. 3A). The HCV envelope glycoproteins E1 (37 kDa) and E2 (64 kDa) were identified only in the samples in which core protein was detected (Fig. 3, B and C). All three HCV proteins were the same sizes in the transgenics and positive controls. The relative production of HCV core E1 and E2 proteins in the liver membrane fractions of CN2-29 mice was 55.7 and 16.9% and 16.8% of CN2-8, respectively, as determined by densitometric scanning analysis of the films obtained in Western blot (Fig. 3).

Immunofluorescence staining confirmed HCV core protein expression in AxCANCre-injected CN2 transgenic mouse liver (Fig. 4). Core protein staining was negative in the liver sections from transgenics without AxCANCre injection or AxCANCre-injected littermates (Fig. 4, A, D, and G). On the other hand, core protein staining was clearly evident in most of the hepatocytes in the lobule of the liver section of AxCANCre-infused CN2-8 mice (Fig. 4, B and C). In the case of CN2-29 mice with adenovirus injection, approximately 20% of hepatocytes stained clearly, whereas the remaining 80% stained only faintly or uncertainly (Fig. 4, E and F). In both lines, core protein staining in the cytoplasm was diffuse and punctate, whereas no staining in the nuclei was observed (Fig. 4, C and F).

**Fig. 1. Structure of CALNCN2, the Cre-mediated activation transgene unit.** R6CN2 HCV cDNA (nucleotides 294–3435) was cloned downstream of the CAG promoter and neomycin-resistant gene (neo) and poly(A) signal that was flanked by two loxP sequences. R6CN2 HCV cDNA contains the core, E1, E2, and NS2 regions. This construct does not lead to HCV mRNA transcription before recombination. Upon recognition of the loxP site, Cre recombinase deletes the neo gene and poly(A) signal along with one of the loxP sequences and then joins the CAG promoter and HCV cDNA. This genomic structure alteration enables production of HCV mRNA.

**Fig. 2. Cre-mediated genomic DNA recombination and HCV core protein production in transgenic mouse liver.** A, CN2-8 transgenic mice received 0, 0.9, 1.8, 2.7, or 3.6 × 10^9 pfu AxCANCre by injection and were sacrificed 3 days later. XbaI-digested genomic liver DNA was probed with a digoxigenin-labeled CALNCN2 probe. HCV core protein production was also determined in the same liver sample as described under “Experimental Procedures.” The result for each sample is shown below each lane. B, 0, 0.9, 1.8, 3.6, or 5.4 × 10^9 pfu of AxCANCre was intravenously infused into CN2-29 transgenic mice. Liver samples were harvested as described above. kb, kilobase pairs.

**Fig. 3. HCV structural protein expression after AxCANCre injection into CN2 transgenic mice.** Western blot analysis of liver samples obtained from CN2-8 and CN2-29 transgenic mice and nontransgenic littermates before and 3 days after injection of 3.6 × 10^9 pfu AxCANCre. SDS-polyacrylamide gel electrophoresis and Western blots were performed as described under “Experimental Procedures.” A, 5E3, an anti-HCV core monoclonal antibody was used. PC, positive control core protein expressed in RK13 cells by full-length R6HCV cDNA transfection; LM, nontransgenic littermate; −, liver sample obtained from mouse without AxCANCre injection; +, liver sample obtained from mouse 3 days after AxCANCre injection. B and C, 384 (anti-E1) and 1864 (anti-E2) monoclonal antibodies were used. The same abbreviations as in A apply.
analysis. Fig. 5A illustrates that core protein expression was predominant in the liver compared with other tissues, particularly in the CN2-8 lineage. The level of core protein production in the CN2-8 and CN2-29 lineages was essentially identical in the lung and spleen (2.6 ± 0.5 versus 2.9 ± 0.5 ng/mg and 6.0 ± 0.7 versus 5.5 ± 1.4 ng/mg, respectively), whereas it differed significantly in the liver (71.9 ± 4.9 ng/mg versus 6.6 ± 5.6 ng/mg, p < 0.0005). HCV core protein was barely detected in the thymus, kidney, stomach, intestines, and muscle. Southern blot analysis using genomic DNA from the same tissue samples (Fig. 5B) revealed that, as expected (3), transgene recombination occurred only in the liver in both lines.

**Serum Core Protein and Anti-core Antibody Production in CN2 Transgenic Mice**—Quantification of HCV core protein in 200 μl of mouse serum was performed according to the method used for HCV-infected human patient sera (16). Seven days after injection of 3.6 × 10⁹ pfu AxCANCre into three CN2-8 transgenics, the serum HCV core protein level was determined to be 152.9 ± 129.5 pg/ml (Table I). Recombinant defective adenovirus itself induces considerable liver injury that is dependent on the presence of both CD4⁺ and CD8⁺ cells (15, 19). The serum ALT level of these three CN2-8 mice was 2605.4 ± 704.9 Karmen units/liter. At the same time, CD4⁺ and CD8⁺ cells were depleted in three CN2-8 mice, which were then injected with 3.6 × 10⁹ pfu AxCANCre. Seven days after adenovirus injection, the serum ALT levels and the presence of circulating HCV core protein were determined, and splenic lymphocyte subpopulations were analyzed. As shown in Table I, the levels of circulating core protein in CN2-8 mice was primarily dependent on hepatocyte destruction.

Fourteen days after AxCANCre injection, serum samples were collected from CN2-8 and CN2-29 transgenics and littermates, and anti-core antibody titers were determined by the enzyme immunoassay method. CN2-8 mice had significantly higher anti-core antibody titers than the CN2-8 mice or the littermates (Table II). One of the four other CN2-8 mice also infected with AxCANCre developed a positive antibody titer at a 1 in 10,000 serum dilution 28 days after adenovirus injection, and two of four other CN2-29 mice also seroconverted 28 days after AxCANCre injection.

**Histological Findings in Transgenic Mouse Liver**—Analysis of liver tissue 7 days after adenovirus administration revealed...
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the development of substantial hepatic pathology in CN2-8 mice (Fig. 6C) that was not present in naive CN2-8 mice (Fig. 6A) or CD4⁺ and CD8⁺ cell-depleted CN2-8 mice (Fig. 6B).

DISCUSSION

Recent advances in molecular biology have enabled examination of the function of genes of interest by raising stable cell lines or transgenic animals with consistent gene expression. However, if the transgene is harmful or disadvantageous for cell growth or embryogenesis, the resultant cell lines or animals may be already genetically changed to tolerate the effects of the transgene products. These problems may be avoided using the Cre/loxP-mediated conditional expression system, which has already been successfully applied to mammalian cells, transgenic animals, and gene targeting. Another advantage of this system is that the transgenic animal is immunocompetent for the transgene product, which is extremely useful for examining immunological reactions against transgene products such as infectious agents. Since HCV infects immunocompetent adults and causes persistent infection, this system may allow for the development of a disease model.

We obtained two founder transgenic lines, CN2-8 and CN2-29, using the CALNCN2 unit. In the absence of Cre recombinase, no HCV gene expression was observed in either line. In addition, their growth and liver histology did not differ from normal BALB/c mice (data not shown). Injection of the recombinant adenovirus AxCANCre, which express Cre recombinase in the cells or tissues it infects, resulted in HCV gene expression activation predominantly in the mouse liver in a dose-dependent manner. Efficient transgene recombination was confirmed in the both lines by Southern blot analysis (Fig. 2). Normal-sized HCV structural proteins were expressed as determined by Western blot (Fig. 3). Immunostaining revealed that most of the hepatocytes were expressing HCV core protein in CN2-8 mouse liver (Fig. 4). Interestingly, the level of core protein expression in the liver and spleen of the CN2-29 line was almost identical, whereas transgene recombination was complete in the liver but hardly detected in the spleen (Fig. 5). This suggests that the tissue distribution of HCV proteins in CN2-29 transgenic mice is dependent not only on the tissue tropism of recombinant adenovirus infection (3, 19) but also on the site of transgene integration into the mouse genome. These results also indicate that transgene recombination is well controlled by AxCANCre infection in these mice and that the level and sites of induced HCV protein expression depend on lineage differences. The difference in HCV expression in the liver between the CN2-8 and CN2-29 mice may be the result of the transgene being under the control of tissue-specific negative control elements in CN2-29 mice.

The diffuse and punctate cytoplasmic staining pattern of core protein observed in the CN2-8 and CN2-29 transgenic mice is similar to that seen in a mammalian cell culture expression system and primate liver specimens (20, 21). Punctate and vesicular staining has been reported in HCV cDNA-transfected mammalian cells, and immunoelectron microscopy has revealed that core protein surrounds lipid droplets in the cytoplasm (17, 20). In the present study, core protein staining was observed only in the cytoplasm and not in the nucleus. On the other hand, the nuclear localization of HCV core protein and its suppression of HBV replication has been reported (22). Thus, future research will need to clarify whether HCV core protein exists in the nucleus and plays a role in the natural HCV life cycle.

In the present study, the E1 and E2 proteins, which are considered to be glycoproteins (6), migrated at 37 and 64 kDa, respectively. However, the signals of both proteins were broad in Western blot analysis; in particular, that of the E2 protein (62 – 66 kDa, Fig. 3). This is most likely because the E2 protein displays variations in glycosylation.

The mechanism of HCV secretion into circulation in the natural viral life cycle is incompletely understood. Core protein is considered to be processed from viral precursor polypeptide outside the endoplasmic reticulum, whereas envelope proteins are processed inside endoplasmic reticulum. Viral-like particles have been observed in the endoplasmic reticulum during electron microscopic analysis of viral maturation using a full-length HCV cDNA clone.² In the present study, the amount of circulating core protein in transgenic sera corresponded with serum ALT levels, which indicates hepatocyte damage (Table I). However, whether the release of HCV proteins by hepatocyte destruction is important in the natural viral life cycle remains unclear.

Koike et al. (23) reported that HCV core gene transgenic mice developed hepatic steatosis and that HCV E1, E2 gene transgenic mice displayed no hepatitis phenotype in their liver, although they did have sialadenitis. Pasquinelli et al. (24) reported that neither core nor E2 transgenic mice displayed any pathological changes. Furthermore, Kawamura et al. (25) established HCV transgenic mice using HCV cDNA that contained HCV core to part of NS2 genome and also found no histological changes. In the present study, the CN2 transgenic mice developed acute hepatitis after AxCANCre infection (Fig. 6C), which was comparable to the reported mouse hepatitis induced by recombinant adenovirus infection (14, 15, 19). However, we observed no significant histopathological changes between infected and uninfected CN2 transgenic mice when CD4- and CD8-positive cells were depleted in the infected mice (Fig.

Table II

Serum anti-HCV core protein production 14 days after AxCANCre administration

| Type of mouse (n) | Anti-HCV core (A490) |
|------------------|---------------------|
| CN2-8 (6)        | 0.182 ± 0.127^      |
| CN2-29 (6)       | 0.024 ± 0.054       |
| Littermate (6)   | 0.028 ± 0.029       |

^ Significantly different compared with CN2-29 and littermates (P < 0.05).

Fig. 6. Histopathology of liver of recipient animals. A, uninfected CN2-8 mouse. B, CD4⁺ and CD8⁺ cell-depleted CN2-8 mouse infected with AxCANCre and analyzed at day 7. C, CN2-8 mouse infected with AxCANCre and analyzed at day 7. (× 250).

* M. Kohara, M. Kaito, and S. Watanabe, unpublished observation.
6, A and B). Having said that, these mice were only observed for 1 week. Taken together, these results suggest that HCV structural proteins are not strongly cytopathic to hepatocytes in the absence of immune responses.

The Cre/loxP system enabled us to express an HCV transgene under temporal control. HCV proteins were transiently expressed and detected mainly in the liver. Importantly, an anti-core antibody response was observed in mouse sera after core protein production. Thus, this system allows investigation of the role of immune responses to transgene products. In addition, adoptive transfer of helper or killer T cells specific for HCV proteins to HCV-expressing transgenic nude mice should lead to the construction of an HCV hepatitis model. Therefore, this transgenic mouse system can be used to understand immunological phenomena in HCV infections. Furthermore, the delivery of Cre DNA recombinase is not limited to the use of recombinant adeno-virus. Transgenics in which Cre is under control of the interferon-inducible Mx promoter or the tetracycline-responsive promoter have already been successfully constructed (26, 27). Thus, future research will focus on this line of investigation.

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