

IFN-β and *IP10* mRNA. 28S and 18S ribosomal RNA bands on ethidium bromide-stained gel were used to control the RNA loading (lower panel).

(c) WT, RIG-I^{-/-} and MDA5^{-/-} MEFs were infected with moi=10 of SeV V(-) for the indicated periods. Total RNA was extracted and subjected to the Northern blot analysis for the expression of *IFN-β* and *IP10* mRNA. 28S and 18S ribosomal RNA bands on ethidium bromide-stained gel were used to control the RNA loading (lower panel).

(d) WT and MDA5^{-/-} PECs were exposed to moi=10 of EMCV for the indicated periods. Total RNA was extracted and subjected to the Northern blot analysis for the expression of *IFN-β*, *IP10*, *IL-6* and *β-actin* mRNA.

Supplementary Fig. 4: Role of MDA5 and RIG-I in the IFN-α responses against various viruses.

(a) WT, RIG-I^{-/-} and MDA5^{-/-} MEFs were exposed to negative-sense ssRNA viruses, including NDV, VSV NCP, SeV Cm, SeV V- and influenza ΔNS1. IFN-α production in the culture supernatants was measured by ELISA.

(b and c) GMCSF-DCs from RIG-I^{-/-} and MDA5^{-/-} mice and their littermate WT mice were infected with indicated moi of EMCV for 24 h. The production of IFN-α (b) and IL-6 (c) in the culture supernatants was measured by ELISA.

(d) GMCSF-DCs from wild-type and MDA5^{-/-} mice were infected with indicated moi of Theiler's virus or Mengovirus for 24 h. IFN-α production in the culture supernatants was measured by ELISA. The data are means ± S.D. of triplicates.

Supplementary Fig. 5: EMCV-mediated protein synthesis shutoff was not altered between wild-type and MDA5^{-/-} MEFs.

Cultures of wild-type and MDA5^{-/-} MEFs were infected with EMCV and labeled by incorporation of [³⁵S]Met-Cys for 1 h at various times after infection. Total cell extracts

were separated by polyacrylamide gel electrophoresis, and the proteins were visualized by autoradiography.

Supplementary Fig. 6: Differential involvement of MDA5 and MyD88 in EMCV-mediated IFN production in cDCs and pDCs.

DCs were induced from bone marrow cells obtained from MyD88^{+/-}, MyD88^{-/-}, MDA5^{+/-} and MDA5^{-/-} mice by cultivating in the presence of Flt3L. At day 7, B220⁺CD11c⁺ pDCs and B220⁺CD11c⁺ cDCs were purified by MACS, and infected with EMCV for 24h. IFN- α production in the culture supernatants was measured by ELISA. Error bars indicate \pm S.D. of triplicates.

Supplementary Fig. 7: The survival of MDA5^{-/-}, RIG-I^{-/-} or IFN α / β R^{-/-} mice in response to VSV infection.

The survival of the mice (3-weeks old) infected with 4×10^6 pfu VSV intranasally was monitored for 9 days ($p < 0.01$ by the generalized wilcoxon test between RIG-I^{-/-} mice and their littermate controls).

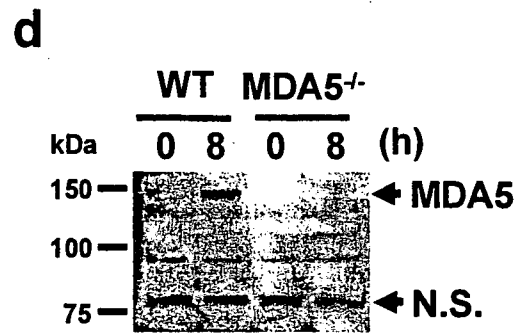
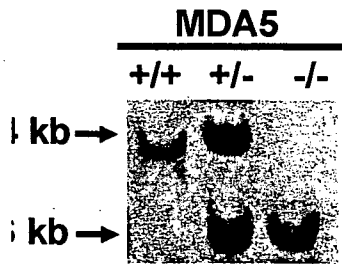
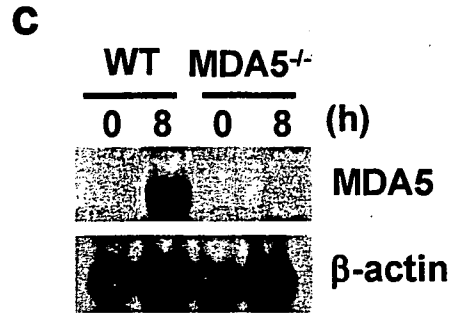
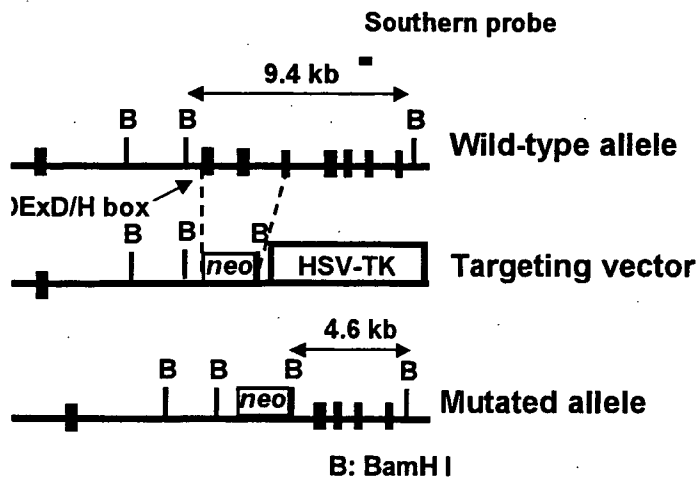
Supplementary Fig. 8: Responses of MDA5^{-/-} mice against EMCV infection

(a) MDA5^{+/-} and MDA5^{-/-} mice (n=5) were intravenously inoculated with 1×10^7 pfu EMCV. Sera were taken at 4 h after injection, and IFN- β , Rantes and IL-6 production levels were determined by ELISA. *, $P < 0.05$ versus controls by the student's t-test.

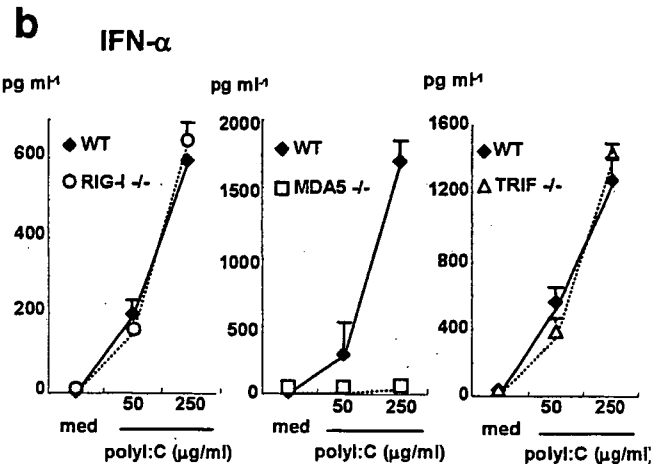
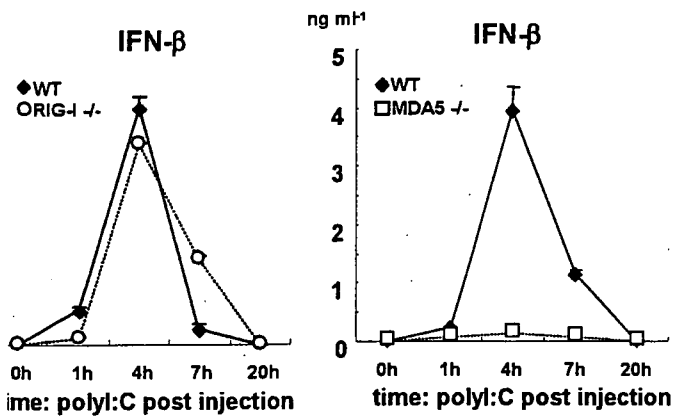
(b) Cardiac function of the mice 48 h after EMCV infection was assessed by echocardiography. Transthoracic M-mode echocardiographic tracings from MDA5^{+/-} and MDA5^{-/-} mice 48 h after EMCV infection.

Supplementary Table I: Genotypes of mice derived from RIG-I^{+/-} intercrosses or crosses between RIG-I^{+/-} and RIG-I^{-/-} mice.

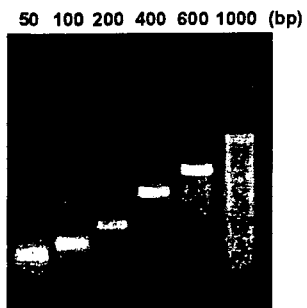
Supplemental Figure 1.



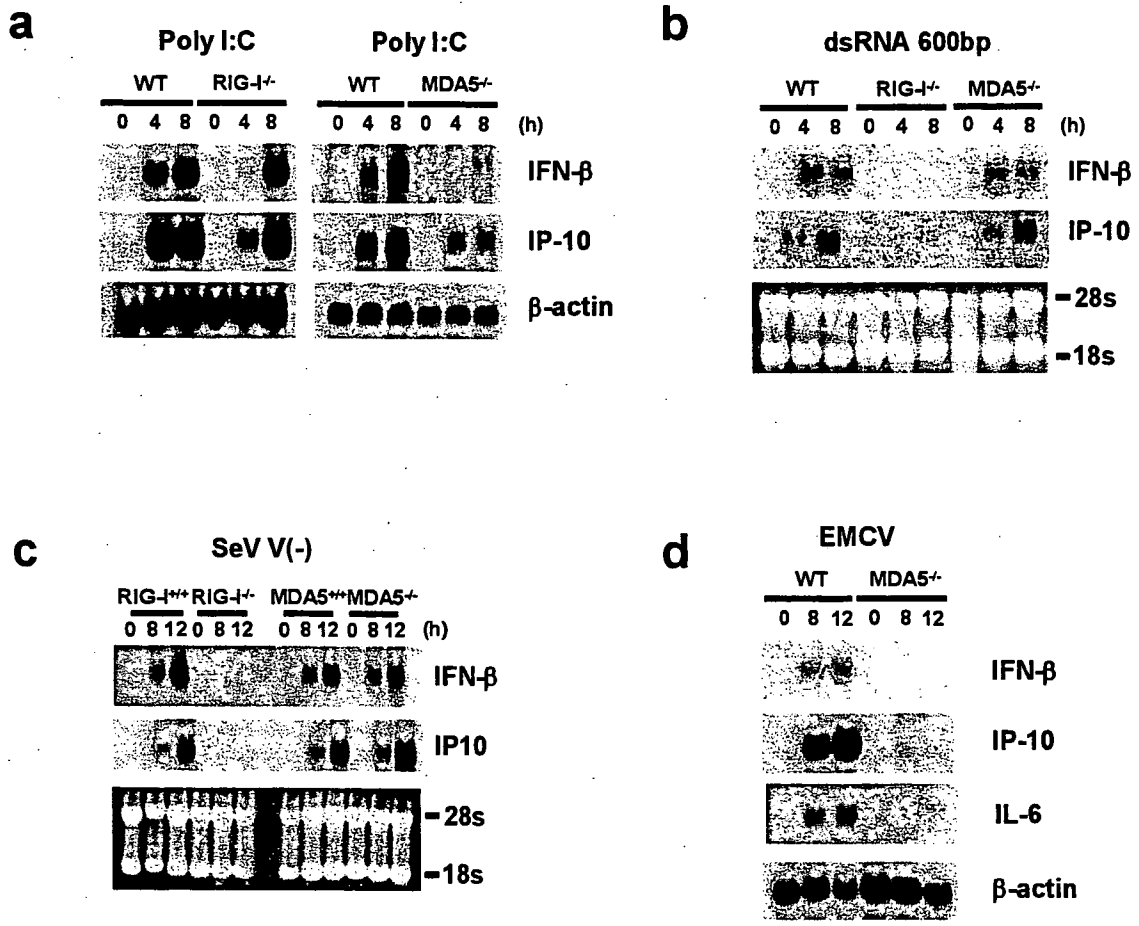
Experimental Figure 2.



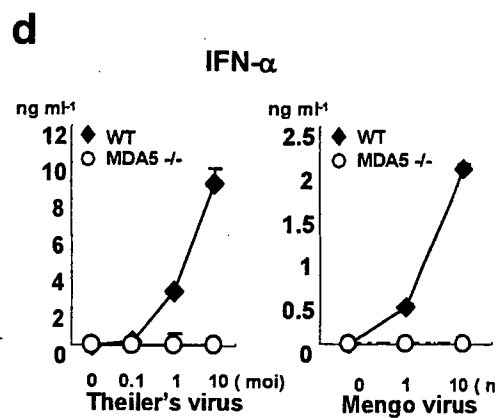
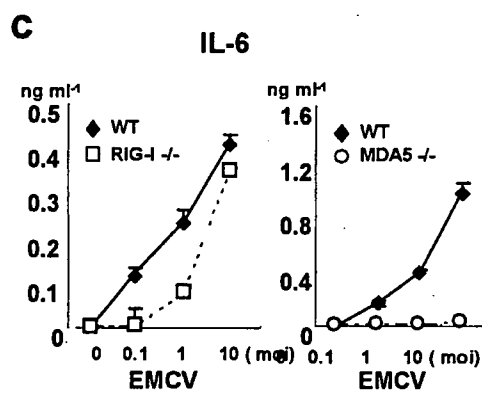
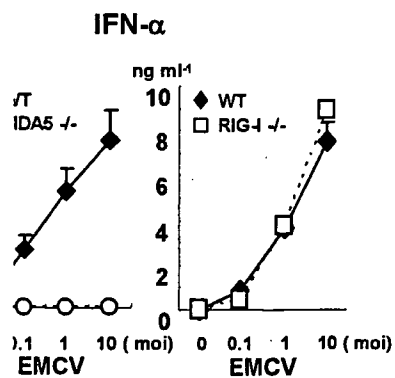
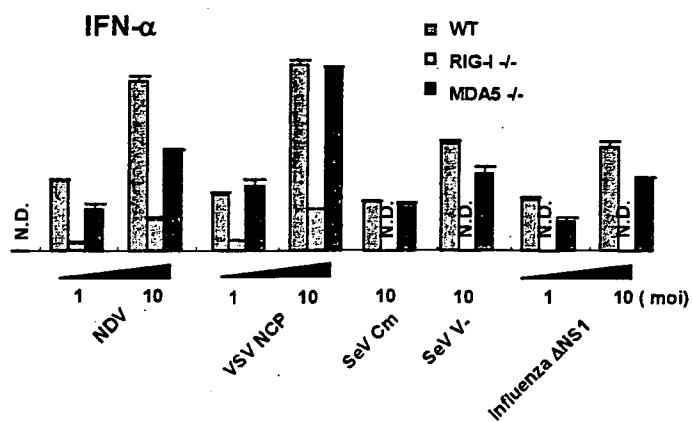
in vitro transcribed dsRNA



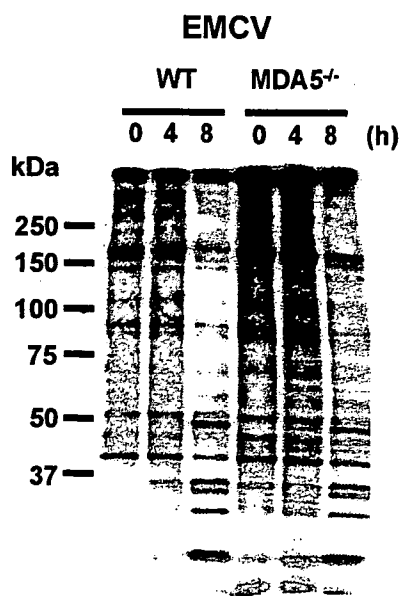
Supplemental Figure 3.



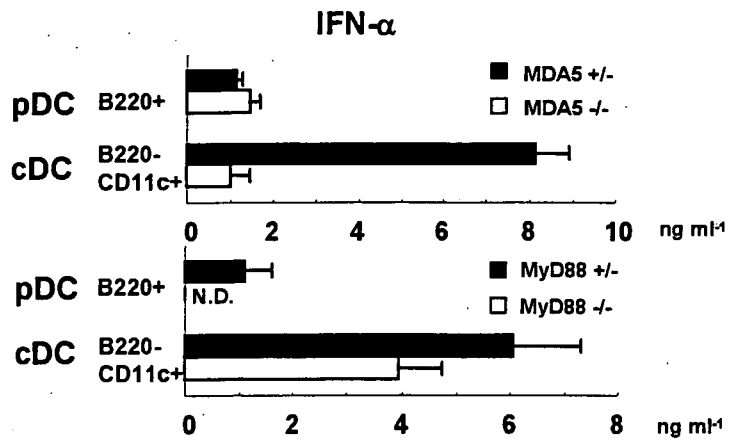
Supplemental Figure 4.



Supplemental Figure 5.

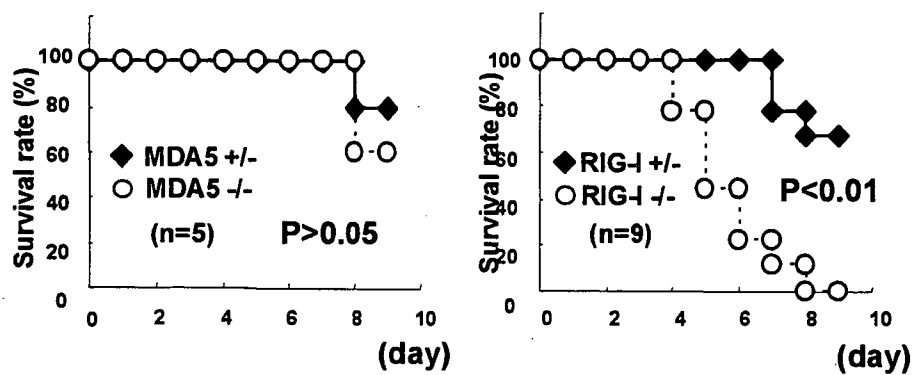


Supplemental Figure 6.

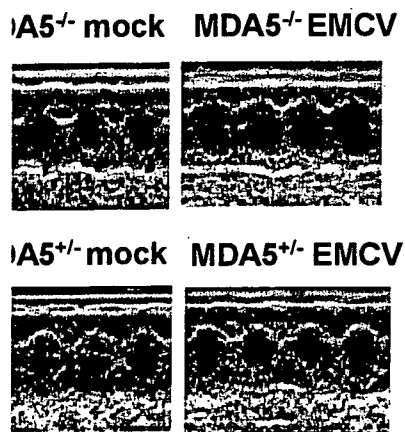
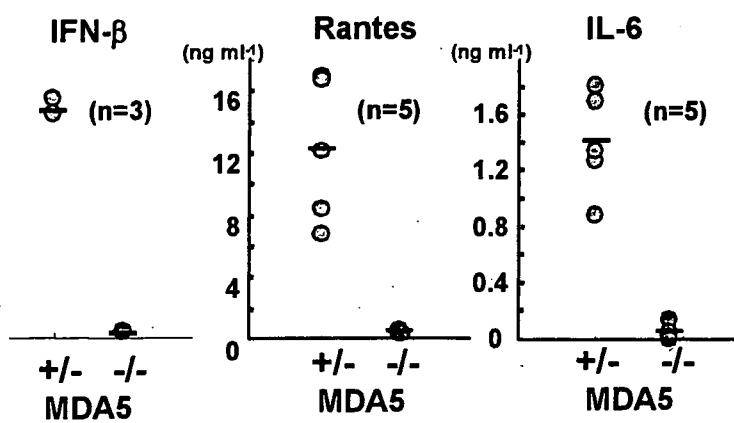


Supplemental Figure 7.

VSV infection



Supplemental Figure 8.



Supplemental Table 1.

a

	RIG-I^{+/+}	RIG-I^{+/-}	RIG-I^{-/-}	Total (%)
Postnatal (6w~)	16 (34)	28 (60)	3 (6)	47 (100)

b

	RIG-I^{+/-}	RIG-I^{-/-}	Total (%)
Postnatal (6w~)	108 (83)	22 (17)	130 (100)

研究成果—平成 19 年度

Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon

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Abstract We developed a reverse genetics system of hepatitis C virus (HCV) genotypes 1a and 2a using infectious clones and human hepatocyte chimeric mice. We inoculated cell culture-produced genotype 2a (JFH-1) HCV intravenously. We also injected genotype 1a CV-H77C clone RNA intrahepatically. Mice inoculated with HCV by both procedures developed measurable and transmissible viremia. Interferon (IFN) alpha treatment resulted in greater reduction of genotype 2a HCV levels than genotype 1a, as seen in clinical practice. Genetically engineered HCV infection system should be useful for analysis of the mechanisms of resistance of HCV to IFN and other drugs.

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Keywords: Human hepatocyte chimeric mouse; Human serum albumin; HCV RNA; Interferon

1. Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1]. HCV causes persistent infection in adults leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. The most effective therapy for viral clearance is a 48-week combination therapy of pegylated interferon (IFN)-alpha and ribavirin. However, the success rate of this

combination therapy is only about 50% [4]. Development of new anti-HCV drug had been severely restricted by the absence of a cell culture system that supports the efficient replication of HCV, as well as the lack of a small animal model. A cell culture system has been developed recently using a unique genotype 2a HCV genome (JFH-1), which does not require adaptive mutations for efficient replication [5–7]. Chimpanzee was the only useful animal for the study of HCV until recently, although the availability of this model is severely restricted [8]. Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into chimeric urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice with engrafted human hepatocytes [9]. This HCV-infected mouse model has been reported to be useful for evaluating anti-HCV drugs such as IFN-alpha and anti-NS3 protease [10]. We have generated a human hepatocyte chimeric mouse where mouse hepatocytes were extensively replaced by human hepatocytes [11], and established a genetically engineered hepatitis B virus (HBV) system [12]. Using this mouse, we show in this paper the development of reverse genetics system of genotypes 1a and 2a after intrahepatic injection of transcribed RNA and intravenous injection of cell culture-produced virus, respectively. We also show here that HCV in these mice can be transmitted to naïve mice. Interferon treatment of these mice resulted in a greater reduction of HCV titer in genotype 2a clone infected mice than in genotype 1a infected mice. As these results are consistent with our clinical experience, we consider this model suitable for the study of resistance of HCV against IFN and other drugs.

2. Materials and methods

2.1. Generation of human hepatocyte chimeric mice and quantification of human serum albumin

Generation of the uPA^{+/+}/SCID^{+/+} mice and transplantation of human hepatocytes were performed as described recently by our group [11,12]. All mice used in this study were transplanted with frozen

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Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator

human hepatocytes obtained from one donor. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index [11], and were measured as described previously [12]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University.

2.2. HCV RNA transcription and inoculation into chimeric mice

A plasmid containing the full-length genotype 1a HCV cDNA clone, pCV-H77C, was kindly provided by Dr. Robert H. Purcell (National Institutes of Health). Ten micrograms of plasmid DNA, linearized by *Xba*I (Promega, Madison, WI) digestion, was transcribed in a 100- μ l reaction volume with T7 RNA polymerase (Promega) at 37 °C for 2 h [13], and analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μ l of phosphate-buffered saline (PBS) and injected into the liver of chimeric mice. Transcripts of plasmid pJFH-1 containing the full-length HCV genotype 2a were transfected into Huh7 cells as described previously [6]. Seventy-two hours after transfection, 200 μ l of the culture medium was injected intravenously into the chimeric mice. IFN-treatment was also performed by intramuscular injection of diluted IFN solutions. IFN-alpha was a kind gift from Hayashibara Biochemical Labs, Inc. (Okayama, Japan). Serum samples collected every 2 weeks after inoculation were frozen at -80 °C until further analysis.

2.3. Human serum samples

For control infection experiments, human serum containing a high titer of genotype 1b HCV (2.2×10^6 copies/ml) was obtained from a patient with chronic hepatitis after obtaining a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use.

2.4. RNA extraction and amplification

RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyak, Tokyo), dissolved in 8.8 μ l RNase-free H₂O, and reverse transcribed by using a random primer (Takara Bio, Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20 μ l reaction mixture according to the instructions provided by the manufacturer. One microliter of cDNA solution was amplified by Light Cycler (Roche Diagnostic, Japan, Tokyo) for quantitation of HCV. The primers used for amplification were 5'-TTTATCCAAGAAAGGACCC-3' and 5'-TTCACGCAGAAAGCGTCTAGC-3'. The amplification conditions included initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 5 s, and extension at 72 °C for 6 s. The lower detection limit of this assay is 10^3 copies/ml. Nested PCR was used with the outer primers NC1 (5'-CAACTACTCGGCTAGCAGT-3') and NC2 (5'-CCTGTGAGGAACTACTGTC-3') and inner primers cc6 (5'-TTTATCCAAGAAAGGACCC-3') and cc7 (5'-TTCACGCAGAAAGCGTCTAGC-3'). The amplification condition included 35 cycles of 94 °C for 30 s, 58 °C for 1 min 30 s, and 72 °C for 1 min after 5 min of initial denaturation at 94 °C followed by 7 min of final extension using Gene Taq (Wako Pure Chemicals, Tokyo) with anti-Taq high according to the instructions provided by the manufacturer (TOYOBO).

2.5. Histochemical analysis of mouse liver

Histopathological analysis and immunohistochemical staining using an antibody against HSA (Bethyl Laboratories Inc.) were performed as described previously [12].

3. Results

3.1. High serum HCV RNA titer in human hepatocyte chimeric mice after inoculation of serum samples obtained from HCV-infected patient

We inoculated 50 μ l of genotype 1b serum samples into five chimeric mice intravenously to test their susceptibility to HCV infection. All mice became positive for HCV RNA by nested

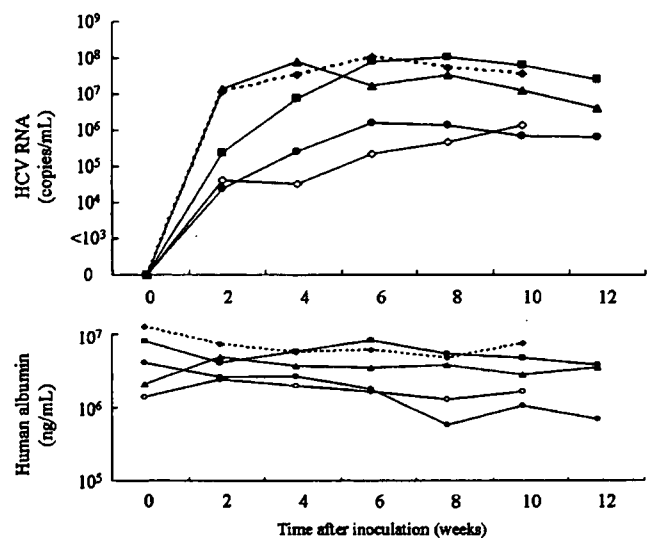


Fig. 1. Serial changes in HCV RNA and human serum albumin in sera of mice inoculated with human serum samples positive for genotype 1b HCV. Fifty microliter serum samples were injected intravenously into each mouse. Mice serum samples were obtained every 2 weeks after injection, and HCV RNA titer was analyzed.

PCR at 2 weeks after inoculation (Fig. 1). The viremia reached a plateau level at 6–8 weeks after infection, and persisted for more than 12 weeks.

3.2. Infection with *in vitro*-transcribed genotype 1a HCV RNA and cell culture generated genotype 2a HCV

In the next step, we tried to establish infection of cloned HCV using infectious genotype 1a and genotype 2a clones. In these experiments, we used two different strategies to establish infection using these two clones because genotype 1a has not been confirmed to replicate in cell culture system. We used genotype 1a HCV RNA (CV-H77C), which has been reported to be infectious to chimpanzee [13]. *In vitro*-transcribed HCV RNA was directly injected intrahepatically in three chimeric mice. We also infected three chimeric mice by intravenous injection of Huh7 cell-produced genotype 2a HCV after transfection of *in vitro* transcribed RNA from an infectious clone JFH-1. This clone has been shown to be infectious to a chimpanzee [6] and a chimeric mouse [7]. All mice developed measurable viremia 2 weeks after inoculation. At 6 weeks after inoculation, HCV RNA titer was 2.4×10^7 copies/ml (range: 8.8×10^6 – 2.9×10^7 copies/ml) in genotype 1a HCV-infected mice, and 2.5×10^5 copies/ml (range: 1.4×10^5 – 3.7×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 2).

3.3. Passage experiment of HCV to naïve chimeric mice

We then performed passage experiments using naïve mice. Each of three mice was inoculated intravenously with 10 μ l serum samples obtained from the above genotype 1a and genotype 2a HCV-infected mice at week 6. Two weeks after injection, all mice developed measurable viremia, and the titer was 8.5×10^6 copies/ml (range: 1.4×10^6 – 2.4×10^7 copies/ml) in genotype 1a, and 1.7×10^5 copies/ml (range: 1.5×10^5 – 2.5×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 3).

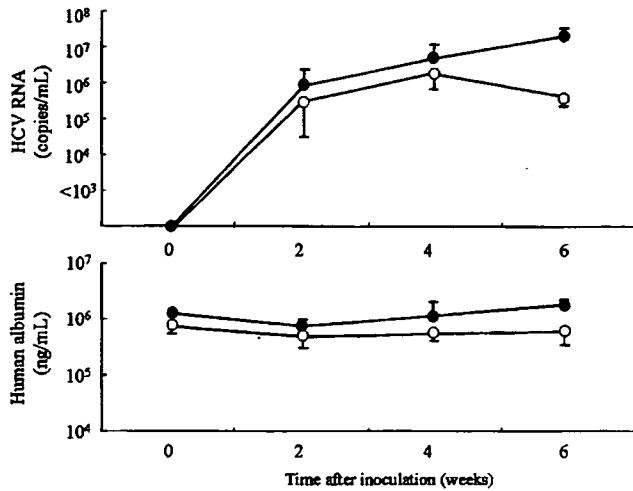


Fig. 2. Changes in HCV RNA and human albumin concentrations in serum of mice infected with clonal HCV. Each of three mice were inoculated intrahepatically with in vitro transcribed genotype 1a HCV RNA (closed circles) or intravenously with a culture medium collected from Huh7 cells transfected with JFH-1 genome intravenously (open circles). Data are mean \pm S.D.

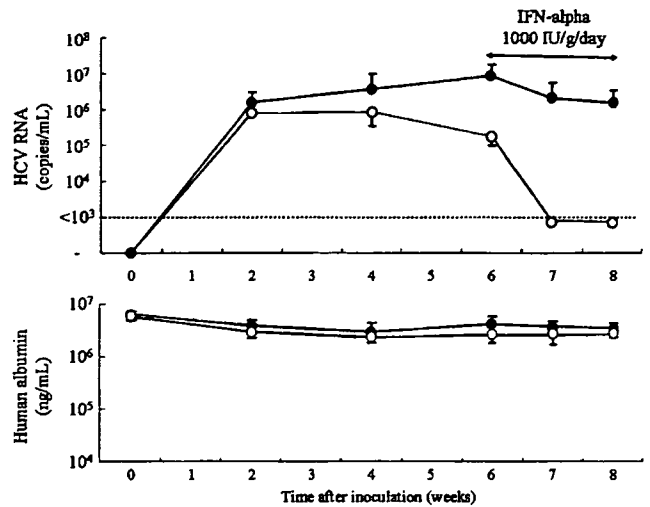


Fig. 3. Passage experiment and response to IFN-alpha therapy in mice infected with HCV genotypes 1a and 2a clones. Serum samples (10 μ l) obtained from genotype 1a and 2a clonal HCV-infected mice sera (see Fig. 2) were inoculated intravenously into each of three naïve chimeric mice. Six weeks after infection, all six mice were injected intramuscularly with 1000 IU/g/day of IFN-alpha daily for 2 weeks. Closed circles: genotype 1a HCV-infected mice, open circles: genotype 2a HCV-infected mice. Data are mean \pm S.D.

3.4. Variable susceptibility of HCV clones to IFN therapy

We treated each of the three mice infected with genotype 1a and 2a clones by passage experiments with 1000 IU/g of IFN-alpha daily for 2 weeks. Such treatment induced only a slight decrease in HCV in genotype 1a-infected mice; the viral load decreased only 0.6 and 0.7 log after 1 and 2 weeks of treatment, respectively (Fig. 3). In contrast, the same treatment re-

duced HCV genotype 2a RNA to undetectable levels after 1 and 2 weeks of IFN therapy. During IFN-treatment, serum HSA levels did not decrease in mice infected with genotype 1a or 2a HCV. Histopathological examination showed no morphological changes or apoptotic hepatocytes in replaced

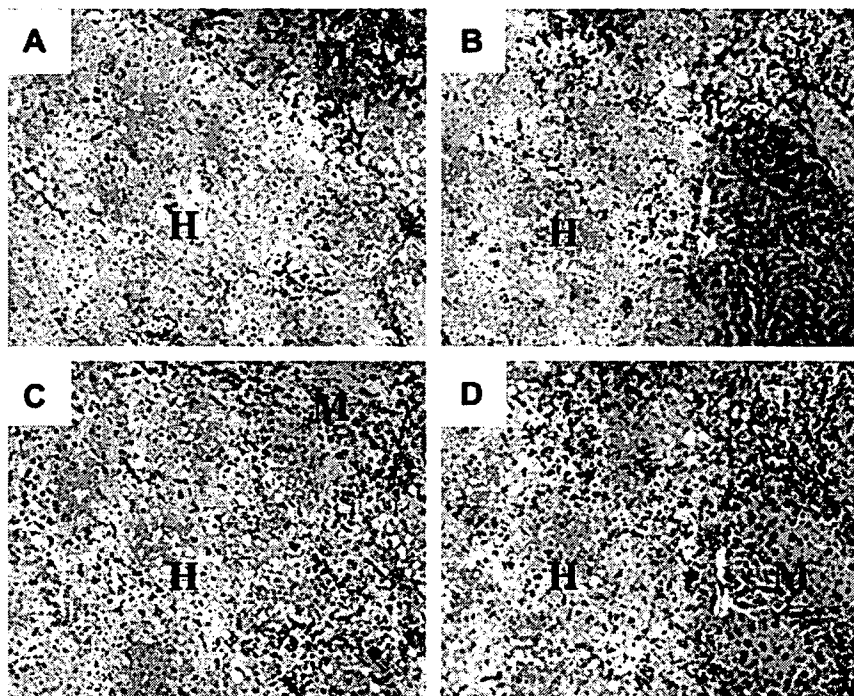


Fig. 4. Histochemical analysis of the tissues of infected chimeric mice. Liver samples obtained from mice infected with genotype 1a (A, C) and genotype 2a (B, D) stained with hematoxylin–eosin staining (A, B) or by immunohistochemical staining with anti-human serum albumin antibody (C, D). Regions are shown as human (H) and mouse (M) hepatocytes, respectively. (Original magnification, $\times 100$.)

human hepatocytes in mice infected with each genotype after 2-week IFN-treatment (Fig. 4). These results suggest that the decrease in HCV is due to the direct anti-viral effect of IFN and not induced by liver cell damage. The difference in the virus titer and susceptibility to IFN are considered to be due to the characteristics of the genotypes.

4. Discussion

In this study, we established a reverse genetics system of HCV genotype 1a and 2a clones using human hepatocyte chimeric mice. The HCV genotype 2a clone, JFH-1, has remarkable features, i.e., infects cultured Huh7 cell line as well as establish infection in chimeric mouse [7]. It has been reported that HCV genotype 1a clone, H77-S, also infects Huh7 cell line and produces infectious virion [14]. In the present study, we intrahepatically inoculated genotype 1a infectious clone, CV-H77C. As reported in chimpanzee [13,15–17], we were able to establish genotype 1a infection using human hepatocyte chimeric mice. Using this technique, it is hoped that we can conduct further experiments in the future using genetically engineered HCV clones. Experiments using chimeric clone described by Lindenbach et al. [7] should also provide further information regarding the variable replication property of HCV genomes. Modifying genomes with nucleotide substitutions allowed examination of the functions of HCV peptides as we showed with HBV [12].

As reported recently by Kneteman et al. [10], the mouse model system is useful for evaluating the effect of anti-HCV drugs such as IFN, protease inhibitors and polymerase inhibitors. As we showed in this study, the response to IFN therapy varied according to HCV genotype. Further experiments are necessary to determine whether differences in response to IFN are due to the different replication ability (replication level of genotype 2a clone was slightly lower than that of genotype 1b, see Figs. 2 and 3) or differences in genotypes, as has been reported in clinical studies [18]. As we showed in this study (Fig. 4), there is no hepatocyte damage or inflammation in the liver of the infected chimeric mouse. Thus, this model is suitable for the study of mechanisms involved in HCV replication and IFN resistance.

The intrahepatic injection method used in this study simplified our experiments using genetically engineered virus. This is particularly important in studies of protease inhibitors and polymerase inhibitors because HCV will easily develop resistance against these small molecule agents.

Previous studies identified amino acid sequences that correlate with different susceptibilities of genotype 1b HCV against IFN therapy, namely, interferon sensitivity determining region [19] and the PKR-eIF2 phosphorylation homology domain [20,21]. To elucidate such issues, we are currently trying to establish genotype 1b infection system using the method described in this paper.

In summary, we showed in the present study the successful application of a genetically engineered HCV in human hepatocyte chimeric mice. Using this mouse model, we showed that genotypes 1a and 2a HCV clones exhibit different susceptibilities to IFN- α therapy. Our mouse model seems useful for the study of HCV virology and resistance of HCV against IFN and for the development of new anti-HCV therapy.

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References

- [1] WHO. (1999) Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. *J. Viral Hepat.* 6, 35–47.
- [2] Kiyosawa, K., Sodeyama, T., Tanaka, E., Gibo, Y., Yoshizawa, K., Nakano, Y., Furuta, S., Akahane, Y., Nishioka, K. and Purcell, R.H. (1990) Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 12, 671–675.
- [3] Niederau, C., Lange, S., Heintges, T., Erhardt, A., Buschkamp, M., Hurter, D., Nawrocki, M., Kruska, L., Hensel, F., Petry, W. and Haussinger, D. (1998) Prognosis of chronic hepatitis C: results of a large, prospective cohort study. *Hepatology* 28, 1687–1695.
- [4] Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Goncalves Jr., F.L., Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J. and Yu, J. (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med.* 347, 975–982.
- [5] Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D.R., Wieland, S.F., Uprichard, S.L., Wakita, T. and Chisari, F.V. (2005) Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. USA* 102, 9294–9299.
- [6] Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R. and Liang, T.J. (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 11, 791–796.
- [7] Lindenbach, B.D., Meuleman, P., Ploss, A., Vanwolleghem, T., Syder, A.J., McKeating, J.A., Lanford, R.E., Feinstone, S.M., Major, M.E., Leroux-Roels, G. and Rice, C.M. (2006) Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. *Proc. Natl. Acad. Sci. USA* 103, 3805–3809.
- [8] Shimizu, Y.K., Weiner, A.J., Rosenblatt, J., Wong, D.C., Shapiro, M., Popkin, T., Houghton, M., Alter, H.J. and Purcell, R.H. (1990) Early events in hepatitis C virus infection of chimpanzees. *Proc. Natl. Acad. Sci. USA* 87, 6441–6444.
- [9] Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R., Tyrrell, D.L. and Kneteman, N.M. (2001) Hepatitis C virus replication in mice with chimeric human livers. *Nat. Med.* 7, 927–933.
- [10] Kneteman, N.M., Weiner, A.J., O'Connell, J., Collett, M., Gao, T., Aukerman, L., Kovelsky, R., Ni, Z.J., Zhu, Q., Hashash, A., Kline, J., His, B., Schiller, D., Douglas, D., Tyrrell, D.L. and Mercer, D.F. (2006) Anti-HCV therapies in chimeric scid-Alb/uPA mice parallel outcomes in human clinical application. *Hepatology* 43, 1346–1353.
- [11] Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K., Hino, H., Asahara, T., Yokoi, T., Furukawa, T. and Yoshizato, K. (2004) Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am. J. Pathol.* 165, 901–912.
- [12] Tsuge, M., Hiraga, N., Takaishi, H., Noguchi, C., Oga, H., Imamura, M., Takahashi, S., Iwao, E., Fujimoto, Y., Ochi, H., Chayama, K., Tateno, C. and Yoshizato, K. (2005) Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* 42, 1046–1054.
- [13] Yanagi, M., Purcell, R.H., Emerson, S.U. and Bukh, J. (1997) Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc. Natl. Acad. Sci. USA* 94, 8738–8743.

- [14] Yi, M., Villanueva, R.A., Thomas, D.L., Wakita, T. and Lemon, S.M. (2006) Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc. Natl. Acad. Sci. USA* 103, 2310–2315.
- [15] Kolykhalov, A.A., Agapov, E.V., Blight, K.J., Mihalik, K., Feinstone, S.M. and Rice, C.M. (1997) Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* 277, 570–574.
- [16] Yanagi, M., StClaire, M., Emerson, S.U., Purcell, R.H. and Bukh, J. (1999) In vivo analysis of the 3' untranslated region of the hepatitis C virus after in vitro mutagenesis of an infectious cDNA clone. *Proc. Natl. Acad. Sci. USA* 96, 2291–2295.
- [17] Beard, M.R., Abell, G., Honda, M., Carroll, A., Gartland, M., Clarke, B., Suzuki, K., Lanford, R., Sangar, D.V. and Lemon, S.M. (1999) An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. *Hepatology* 30, 316–324.
- [18] McHutchison, J.G., Gordon, S.C., Schiff, E.R., Shiffman, M.L., Lee, W.M., Rustgi, V.K., Goodman, Z.D., Ling, M.H., Cort, S. and Albrecht, J.K. (1998) Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N. Engl. J. Med.* 339, 1485–1492.
- [19] Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Ogura, Y., Izumi, N., Marumo, F. and Sato, C. (1996) Mutations in the non-structural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N. Engl. J. Med.* 334, 77–81.
- [20] Taylor, D.R., Shi, S.T., Romano, P.R., Barber, G.N. and Lai, M.M. (1999) Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science* 285, 107–110.
- [21] Chayama, K., Suzuki, F., Tsubota, A., Kobayashi, M., Arase, Y., Saitoh, S., Suzuki, Y., Murashima, N., Ikeda, K., Takahashi, N., Kinoshita, M. and Kumada, H. (2000) Association of amino acid sequence in the PKR-eIF2 phosphorylation homology domain and response to interferon therapy. *Hepatology* 32, 1138–1144.

Dual effect of APOBEC3G on *Hepatitis B virus*

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G to A hypermutation of *Hepatitis B virus* (HBV) and retroviruses appears as a result of deamination activities of host APOBEC proteins and is thought to play a role in innate antiviral immunity. Alpha and gamma interferons (IFN- α and - γ) have been reported to upregulate the transcription of APOBEC3G, which is known to reduce the replication of HBV. We investigated the number of hypermutated genomes under various conditions by developing a quantitative measurement. The level of hypermutated HBV in a HepG2 cell line, which is semi-permissive for retrovirus, was 2.3 in 10^4 HBV genomes, but only 0.5 in 10^4 in permissive Huh7 cells. The level of APOBEC3G mRNA was about ten times greater in HepG2 cells than in Huh7 cells. Treatment of HepG2 cells with either IFN- α or - γ increased the transcription of APOBEC3G and hypermutation of HBV. These mRNAs and hypermutation of HBV genomes were induced more prominently by IFN- γ than by IFN- α . Both IFNs decreased the number of replicative intermediate of HBV. Overexpression of APOBEC3G reduced the number of replicative intermediate of HBV and increased hypermutated genomes 334 times, reaching 968 in 10^4 genomes. Deamination-inactive APOBEC3G did not induce hypermutation, but reduced the virus equally. Our results suggest that APOBEC3G, upregulated by IFNs, has a dual effect on HBV: induction of hypermutation and reduction of virus synthesis. The effect of hypermutation on infectivity should be investigated further.

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INTRODUCTION

Hepatitis B virus (HBV) is a small, enveloped DNA virus with partially double-stranded DNA as a genome (Ganem & Schneider, 2001; Seeger & Mason, 2000). The virus replicates through transcription of pregenome RNA and reverse transcription, like retroviruses (Skalka & Goff, 1993; Summers & Mason, 1982). Infection with HBV causes chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma (Wright & Lau, 1993; Bruix & Llovet, 2003; Ganem & Prince, 2004).

Recent reports have shown that a cytidine deaminase, APOBEC3G, which is packaged in human immunodeficiency virus (HIV) virions in non-permissive cells, induces G to A hypermutation to a nascent reverse transcript of HIV and serves as part of the innate antiviral activity (Mangeat *et al.*, 2003; Zhang *et al.*, 2003; Lecossier *et al.*, 2003; Harris

et al., 2003). Recent studies have demonstrated that a small number of HBV DNA in serum samples of patients with chronic HBV infection contains hypermutated genomes (Gunther *et al.*, 1997; Suspene *et al.*, 2005a; Noguchi *et al.*, 2005). We reported previously that there are small numbers of hypermutated genomes in serum samples of the majority of patients with chronic HBV infection and that G to A hypermutation could be induced in cultured liver cells derived from HepG2 cell lines (Noguchi *et al.*, 2005) using a peptide nucleic acid-mediated PCR clamping method. Suspene *et al.* (2005a) developed the more sensitive differential DNA denaturation (3D)-PCR method to detect hypermutated genomes and found that some APOBEC proteins induce G to A, and in some cases C to T, hypermutations in HBV DNA (Suspene *et al.*, 2005a). Why only a very small proportion of the HBV genome is hypermutated is unknown at present. Furthermore, the

mechanism that controls the level of APOBEC protein expression and degree of hypermutation has not been fully investigated. Recently, Tanaka *et al.* (2006) identified an interferon (IFN)-stimulated response element (ISRE) in the promoter region of APOBEC3G and showed that IFN- α upregulates transcription of APOBEC3G. Peng *et al.* (2006) also reported that IFN- α and - γ upregulate mRNA transcription of APOBEC proteins. However, these reports did not analyse whether increased numbers of APOBEC proteins actually increase hypermutation. More recently, Bonvin *et al.* (2006) demonstrated that IFN induces transcription of APOBEC proteins and increases hypermutation of HBV.

IFNs are cytokines that play a major role against many pathogens (Samuel, 2001; Colonna *et al.*, 2002; Grandvaux *et al.*, 2002). We also reported in a previous study that both IFN- α and - γ reduce virus replication in stably HBV-transfected cell lines without inducing a remarkable increase in G to A hypermutation (Noguchi *et al.*, 2005). However, the method used in previous experiments for detection of hypermutation was not as sensitive as the method of Suspene *et al.* (2005a, b) and not quantitative. To assess the level of hypermutation, a reliable measurement of hypermutated genome is needed. In the present study, we developed a new and sensitive method for the measurement of hypermutated genome levels. Using this method, we show here that both IFN- α and - γ increased the levels of hypermutated genomes in cultured cell lines. Furthermore, both IFNs increased the mRNA level of APOBEC3G. We also performed overexpression experiments to examine whether APOBEC3G and its inactive mutants increase the levels of hypermutation and reduce HBV replication.

METHODS

Plasmid constructs. The expression vector for haemagglutinin (HA)-tagged human APOBEC3G, pcDNA3/HA-A3G, was constructed as described previously (Kobayashi *et al.*, 2004). APOBEC3F cDNA was obtained by modifying APOBEC3F like (IMAGE clones from Open Biosystems) to have the same sequence as human APOBEC3F transcript variant 1 (GenBank NM_145298) and cloned into pcDNA3/HA (Invitrogen). APOBEC3G mutants were constructed using a QuikChange mutagenesis kit (Stratagene). The construction of wild-type HBV 1.4 genome length, pTRE-HB-wt, has been described previously (GenBank accession no. AB206816) (Tsuge *et al.*, 2005).

Cell culture and transfection. Huh7 and HepG2 cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum at 37 °C in 5% CO₂. Cells were seeded to semi-confluence in six-well tissue culture plates. Transient transfection of the plasmids into HepG2 and Huh7 cell lines was performed using *TransIT-LT1* (Mirus) according to the instructions provided by the supplier. A plasmid encoding a secreted form of human placental alkaline phosphatase (SEAP) was co-transfected to adjust the transfection efficiency. The SEAP assay in the culture medium was performed using the Great EscAPE SEAP Reporter System 3 (BD Bioscience).

T23 cells are HepG2 cells stably transfected with the plasmid pTRE-HB-wt. They were cultured using a method described previously

(Tsuge *et al.*, 2005). Cells were seeded to semi-confluence in six-well tissue culture plates and then treated with medium containing either IFN- α (Hayashibara Biochemical Laboratories) or IFN- γ (Shionogi & Co.). The cells were harvested 12–72 h after IFN treatment. Core-associated HBV DNA was extracted from the cells for HBV DNA quantification and quantitative analysis of G to A hypermutated genomes (Noguchi *et al.*, 2005).

Analysis of core-associated HBV DNA. The cells were harvested 4 days after transfection and lysed with 250 μ l lysis buffer [10 mM Tris/HCl pH 7.4, 140 mM NaCl and 0.5% (v/v) NP-40] followed by centrifugation for 2 min at 15000 g. The core-associated HBV genome was immunoprecipitated from the supernatant by mouse anti-core monoclonal antibody anti-HBc determinant α (Institute of Immunology, Tokyo, Japan) and subjected to quantitative analysis after SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR using the 7300 Real-Time PCR system (Applied Biosystems). The primers used for amplification were #1, 5'-ACTTCAACCCCAACAMRRATCA-3' (nt 2978–2999) [numbers are those of HBV subtype C reported by Norder *et al.* (1994)] and #2, 5'-AGAGYTTGKTGGAATGKTGGA-3' (nt 24–1), where M is A/C, R is G/A, Y is T/C and K is G/T. The probe was a 6-carboxy-fluorescein (FAM)-labelled minor-groove binder (MGB) probe, 5'-(FAM)-TTAGAGGTGGAGAGATGG-(MGB)-3' (nt 3184–3167). Real-time PCRs were set up in 25 μ l TaqMan Universal Master Mix with 1 μ l DNA solution, 0.9 μ M each primer and 0.25 μ M probe. The amplification conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and extension at 62 °C for 90 s).

Amplification and analysis of hypermutated HBV genomes by 3D-PCR. HBV DNA was extracted from 100 μ l serum obtained from a chronic HBV carrier (genotype C) by SMITEST (MBL International) and was dissolved in 20 μ l H₂O. Hypermutated genomes were detected by modified 3D-PCR using primers #1 and #2 and DNA solution from serum containing 8.0×10^7 or 2.3×10^5 copies of core-associated HBV DNA in 25 μ l of 100 mM Tris/HCl pH 8.3, 50 mM KCl, 15 mM MgCl₂, 0.2 mM each dNTP, 10 pmol each primer and 1.25 U Taq DNA polymerase (Gene Taq, Nippon Gene Co.), together with 0.25 μ g anti-Taq high (TOYOBO Co.). The amplification conditions included an initial denaturation step at 83–95 °C for 5 min, followed by 45 cycles of denaturation at 83–95 °C for 1 min, annealing at 50 °C for 30 s, extension at 72 °C for 30 s followed by 10 min of final extension. Amplicons were separated by electrophoresis on 2% (w/v) agarose gel, cloned and sequenced in an ABI PRISM 3130 Genetic Analyzer with a BigDye Terminator version 3.1 cycle sequencing ready reaction kit (Applied Biosystems). The PCR products were also analysed on Hanse Analytik (HA)-yellow gel as described previously (Suspene *et al.*, 2005b; Tsuge *et al.*, 2005; Abu-Daya *et al.*, 1995).

Quantitative analysis of hypermutated genomes by real-time PCR. Hypermutated genomes were quantified by real-time PCR using the 7300 Real-Time PCR system (Applied Biosystems) and the above primers and probes. The amplification conditions included activation at 95 °C for 10 min followed by initial denaturation at 88 °C for 20 min and 45 cycles of amplification (denaturation at 88 °C for 15 s, annealing at 50 °C for 30 s and extension at 62 °C for 90 s). We chose 88 °C as this temperature is appropriate for detection of about 20% hypermutated genomes. There are 200–300 such hypermutated genomes in 10^4 genomes present in HepG2 cells transiently transfected with APOBEC3G. The buffer comprised 10 mM Tris/HCl pH 8.3, 50 mM KCl, 3 mM MgCl₂, 10 mM EDTA, 60 nM Passive Reference 1 (Applied Biosystems), 0.2 mM each dNTP, 0.9 μ M each primer, 0.25 μ M probe, 5×10^6 copies of HBV DNA

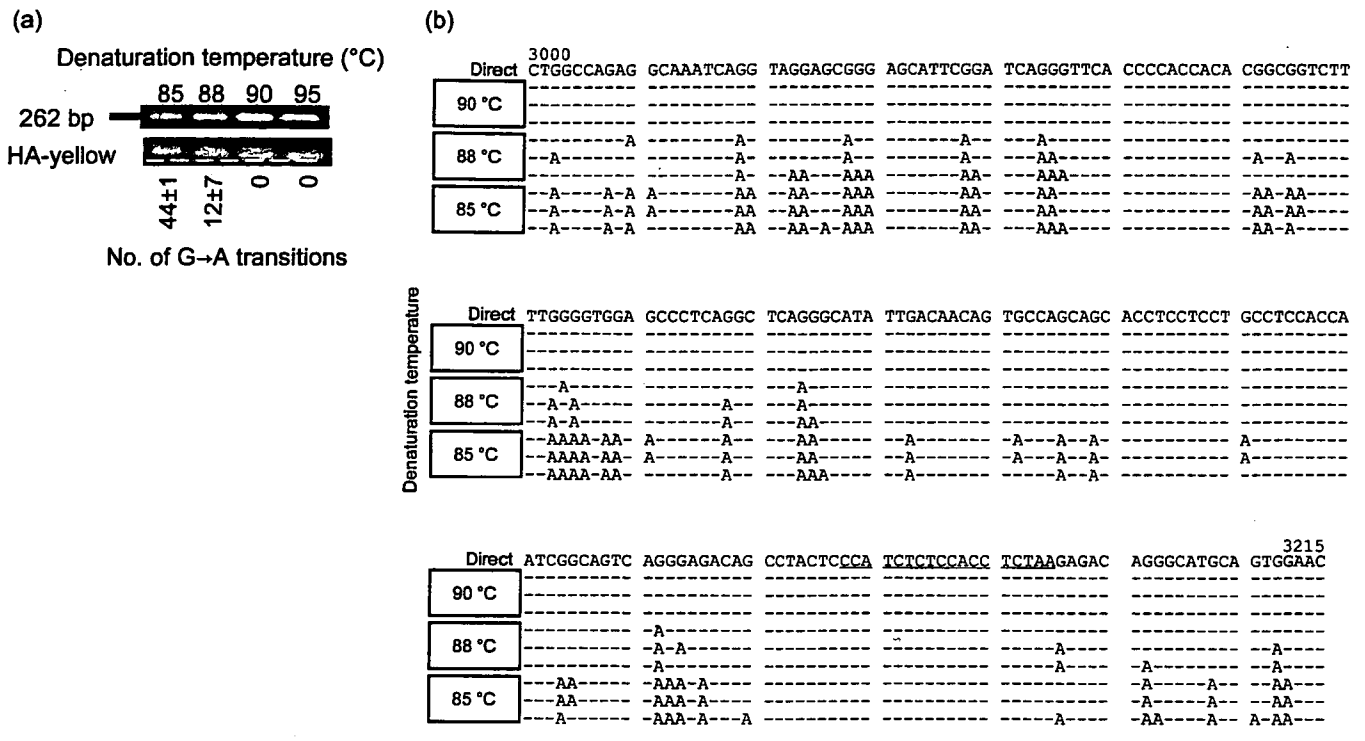


Fig. 1. Amplification of HBV DNA by 3D-PCR. (a) Detection of hypermutated genomes by HA-yellow agarose gel electrophoresis. The numbers of G to A transitions are expressed as means \pm SD generated from the sequence analysis of five independent clones from PCR products. The white dotted line was added to help visualize the retardation of AT-rich DNA in HA-yellow agarose gel. (b) Nucleotide sequences of HBV amplified by 3D-PCR. The nucleotide sequences obtained by direct sequencing are used as a reference sequence. The nucleotide sequences where the probe hybridizes are underlined. Note that the number of G to A mutations correlates with denaturation temperature.

and 0.625 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in a final volume of 25 μ l. A standard curve was constructed by the simultaneous amplification of serial dilutions of the 3D-PCR products.

Western blot analysis. Cell lysates were prepared as described above, resolved on 10% (w/v) SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Whatman) via electro-blotting. The membranes were incubated with anti-haemagglutinin fusion epitope monoclonal antibody (Roche) or with anti- β -actin monoclonal antibody (Sigma-Aldrich) followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit antibody or sheep anti-mouse immunoglobulin (Amersham Biosciences). Proteins were visualized via the ECL system (Amersham Biosciences).

Quantification of mRNA of APOBEC3G or APOBEC3F by reverse transcription and real-time PCR. Total RNA was extracted from HepG2 cell lines by using an RNeasy Mini kit (Qiagen). The RNA was reverse transcribed with random primers and Moloney murine leukemia virus reverse transcriptase (ReverTra Ace, TOYOBO Co.) at 42 °C for 60 min according to the instructions provided by the manufacturer. Quantitative analysis of APOBEC3G and APOBEC3F cDNA was performed by real-time PCR using TaqMan Gene Expression assays (Applied Biosystems). To confirm that the APOBEC3G and -3F PCR primers specifically amplify the target genes, quantitative PCR on the expression plasmids encoding human APOBEC3G and -3F, used as templates, was performed. No cross amplification was observed, even when we used 10^7 copies of APOBEC3G plasmid in the amplification reaction of

APOBEC3F and vice versa. A standard curve was constructed by the amplification of serial dilutions of the known number of plasmids containing human APOBEC3G and APOBEC3F. The target cDNA was normalized to the endogenous RNA level of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers and FAM-labelled probe used to quantify GAPDH were purchased from Applied Biosystems.

Infectivity of luciferase reporter viruses produced from HepG2 and Huh7 cell lines. Luciferase reporter viruses with or without viral infectivity factor (Vif) were prepared by co-transfection of pNL43/ Δ Env-Luc (wild-type) or pNL43/ Δ Env Δ vif-Luc (Δ Vif) plus pVSV-G together with a mock vector or expression vectors for A3G by Lipofectamine (Invitrogen) as described previously (Janini *et al.*, 2001; Shindo *et al.*, 2003). Productive infection was measured by luciferase activity. Values were presented as percentage of infectivity relative to the value of each virus without expression of APOBEC3G proteins.

RESULTS

Quantitative analysis of hypermutated genome by real-time PCR

Using serum samples from a patient with a high viral load, we amplified a large number of hypermutated genomes by 3D-PCR and detected them by HA-yellow agarose gel electrophoresis (Fig. 1a). Nucleotide sequence analysis