2.6% (25/976). The antibody-positive rate was 1.2% in Hokkaido, 2.2% in Miyazaki, 3.1% in Iwate, 3.1% in Hyogo, and 3.6% in Miyagi. The difference among these prevalence was not statistically significant ($P>0.05$).

Eighty-eight paired stool and serum samples from deer captured in Hokkaido (10), Iwate (23), Tochigi (7), Chiba (2), Nagano (5), Mie (4), Hyogo (8), Hiroshima (3), Oita (4), Fukuoka (1), Kumamoto (9), Miyazaki (6), and Kagoshima (6), 166 deer serum samples obtained in Aichi (18), Hyogo (28), Nagano (10), and Miyagi (110), and 159 deer liver tissue samples collected in Hyogo (50), Iwate (11) and Hokkaido (98) were tested by RT-PCR for HEV RNA. Total RNA was extracted with RNAzol-LS reagent (Invitrogen, Inc., Carlsbad, CA) using 200 µl of the deer serum, and 10% stool suspension Reverse transcription (RT) was performed at 42 °C for 50 min followed by 70 °C for 15 min in 20 µl reaction mixture containing 1 µl of Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen, Inc., Carlsbad, CA), 1 µl of the oligo (dT) primer, 1 µl of RNaseOUT™, 2 µl of 0.1 M dithiothreitol, 4 µl of 5×RT buffer, 1 µl of 10 mM deoxynucleoside triphosphates, 5 µl of RNA, and 5 µl of distilled water. Two microliters of the resulting cDNA was amplified in a 50 µl reaction mixture containing ExTaq DNA polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan) with an external sense primer HEV-F1 (5′-GGBGTBGCNGAGGAGGAGGC-3′, nucleotide (nt) residues 5903–5922 of G1 Myanmar strain, D10330) and an external antisense primer HEV-R2 (5′-TGYTGGTTRTCRTARTCCTG-3′, nt residues 6486–6467 of G1 Myanmar strain, GenBank D10330), using the GeneAmp PCR System 9700 (PE Biosystems, Foster City, CA). Each cycle consisted of denaturation at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec, and an extension reaction at 72 °C for 60 sec followed by a final extension at 72 °C for 7 min. The nested PCR was done by using 2 µl of the first PCR product with an internal sense primer HEV-F2 (5′-TAYCGHAA YCAAGGHTGGCG-3′; nt residues 5939–5958) and an internal antisense primer HEV-R1 (5′-CGACGAAATYAATTCTG TCG-3′, nt residues 6316–6297) under the same conditions [9, 10]. Total RNA in deer liver was extracted from 100 mg of the tissue using an RNeasy Mini Kit (Qiagen, Inc.) and dissolved in 50 µl nuclease-free distilled water. The nested RT-PCR was carried out as described by Takahashi et al. [20]. However, we were not able to amplify any HEV sequences in these samples.

An ELISA with recombinant VLPs was used to detect anti-HEV IgG in sika deer in Japan. This assay was previously shown to be capable of detecting anti-HEV antibodies in human and mongoose sera with high sensitivity and specificity [8, 10]. To establish a system for detecting anti-HEV IgG in deer, we first prepared a positive control serum by immunizing deer with the G1 VLPs. After two doses of immunization, an antibody whose titer was as high as 1:3,276,800 was obtained. The specificity of this antibody was confirmed by Western blot assay, indicating that G1 VLPs was an excellent antigen to induce a strong immune response in deer.

In the present study, we tested a total of 976 deer serum samples for the presence of anti-HEV IgG antibody and made the following observations. First, the antibody prevalence was low in sika deer in Japan. Only 25 of 976 (2.6%) samples were positive for anti-HEV IgG by ELISA, which is lower than the prevalence in pigs (58%) and wild boars (44%), both of which are thought to be reservoirs of HEV in Japan [10, 21]. Second, the OD value and titer of anti-HEV IgG were low in deer. The highest OD value was 0.486 and the highest titer was 1:400. This observation is also different from that in pigs and wild boars, where the highest OD values were greater than 3.000 and the titers were greater than 1:51200. Third, the distribution of OD values indicated that only one peak was less than 0.486. The bimodal distribution observed in pigs and wild boars was not seen in deer, indicating that the rate of infection by HEV under natural conditions is extremely low in deer, and suggesting that deer do not play an important role as a reservoir of HEV in Japan.

This study included 254 serum samples from deer captured in Hyogo, where the first deer positive for HEV RNA was found [24]. The prevalence of the antibody-positive rate was 3.1% (5/132) in female and 2.5% (3/122) in male deer, and the difference

between the sexes was not statistically significant ($P>0.05$). These antibody-positive rates are also not significantly different from those in other areas, including Hokkaido, Iwate, and Miyazaki prefecture. The age of anti-HEV IgG-positive deer was 0–8 years, and no significant correlation between age and prevalence was observed. We also tested HEV RNA in 36 serum samples from deer captured in the same area in Hyogo prefecture where the deer that was positive for HEV RNA was reported. However, we were not able to amplify any HEV sequences in these samples.

Since wild boars are prevalent throughout Japan, with the exception of Hokkaido, and they seem to be eventually infected with HEV, the virus is spread throughout their habitat via their stools. Because wild deer and wild boars share this environment, wild deer might be exposed to HEV. Only low-titer anti-HEV IgG was detected in deer serum in this study, suggesting that either the antibody detected in this study was not induced by HEV infection or that deer have low sensitivity to HEV. If deer were to occasionally come into contact with a small amount of HEV, but were not susceptible to HEV, then a strong immune response to HEV might not be induced.

In summary, the prevalence of anti-HEV IgG in sika deer was lower than the prevalence in two possible reservoirs, pigs and wild boars, and no HEV RNA was detected in 254 sera, 88 stool and 159 liver tissue samples, indicating that wild deer may not be a reservoir of HEV in Japan.

Acknowledgments

The authors thank Tomoko Mizoguchi for her secretarial work. This study was supported in part by grants-in-aid for Research on Emerging and Re-emerging Infectious Diseases, Research on Hepatitis, and Research on Food Safety from the Ministry of Health, Labor and Welfare, Japan.

We also would like to thank the Hyogo Hunting Association, the Okoppe, Shari, and Kushiro branches of the Hokkaido Hunting Association, the Shiretoko Nature Center, the Deer Research group in Lake Toya, and Envision for their extensive help in collecting the samples. This work was supported by a grant from JSPS (no. KAKENHI16380202) and by a grant from the 21st Century COE Program, "Program of Excellence for Zoonosis Control," from the Ministry of Education, Science, Sports and Culture of Japan.

References

1. Balayan MS (1997) Epidemiology of hepatitis E virus infection. J Viral Hepat 4: 155–165
2. Buisson Y, Grandadam M, Nicand E, Cheval P, van Cuyck-Gandre H, Innis B, Rehel P, Coursaget P, Teyssou R, Tsarev S (2000) Identification of a novel hepatitis E virus in Nigeria. J Gen Virol 81: 903–909
3. Emerson SU AD, Arankalle A, Meng XJ, Purdy M, Schlauder GG, Tsarev SA (2005) Hepevirus. In: Fauquet CM, Maniloff J, Desselberger U, Ball LA (eds) Virus taxonomy, VIIIth Report of the ICTV. Elsevier/Academic Press, London, pp 853–857
4. Hijikata M, Hayashi S, Trinh NT, Ha le D, Ohara H, Shimizu YK, Keicho N, Yoshikura H (2002) Genotyping of hepatitis E virus from Vietnam. Intervirology 45: 101–104
5. Jameel S (1999) Molecular biology and pathogenesis of hepatitis E virus. Expert Rev Mol Med 1999: 1–16
6. Koike H, Ohtaishi N (1985) Prehistoric hunting pressure estimated by the age composition of excavated sika deer (*Cervus nippon*) using the annual layer of tooth cement. J Archaeol Sci 12: 443–456
7. Li TC, Yamakawa Y, Suzuki K, Tatsumi M, Razak MA, Uchida T, Takeda N, Miyamura T (1997) Expression and self-assembly of empty virus-like particles of hepatitis E virus. J Virol 71: 7207–7213
8. Li TC, Zhang J, Shinzawa H, Ishibashi M, Sata M, Mast EE, Kim K, Miyamura T, Takeda N (2000) Empty virus-like particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. J Med Virol 62: 327–333
9. Li TC, Chijiwa K, Sera N, Ishibashi T, Etoh Y, Shinohara Y, Kurata Y, Ishida M, Sakamoto S, Takeda N, Miyamura T (2005) Hepatitis E virus transmission from wild boar meat. Emerg Infect Dis 11: 1958–1960
10. Li TC, Saito M, Ogura G, Ishibashi O, Miyamura T, Takeda N (2006) Serologic evidence for hepatitis E virus infection in mongoose. Am J Trop Med Hyg 74: 932–936
11. Magden J, Takeda N, Li T, Auvinen P, Ahola T, Miyamura T, Merits A, Kaariainen L (2001) Virus-specific mRNA capping enzyme encoded by hepatitis E virus. J Virol 75: 6249–6255
12. Maila HT, Bowyer SM, Swanepoel R (2004) Identification of a new strain of hepatitis E virus from an outbreak in Namibia in 1995. J Gen Virol 85: 89–95
13. Meng XJ, Purcell RH, Halbur PG, Lehman JR, Webb DM, Tsareva TS, Haynes JS, Thacker BJ, Emerson SU (1997) A novel virus in swine is closely related to the human hepatitis E virus. Proc Natl Acad Sci USA 94: 9860–9865
14. Meng XJ, Halbur PG, Shapiro MS, Govindarajan S, Bruna JD, Mushahwar IK, Purcell RH, Emerson SU (1998) Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. J Virol 72: 9714–9721

15. Meng XJ (2003) Swine hepatitis E virus: cross-species infection and risk in xenotransplantation. Curr Top Microbiol Immunol 278: 185–216
16. Purcell RH, Emerson SU (2001) Hepatitis E virus. In: Knipe DM, Howley PM (eds) Fields virology, vol 1. Lippincott Williams & Wilkins, Philadelphia, pp 3051–3061
17. Schlauder GG, Dawson GJ, Erker JC, Kwo PY, Knigge MF, Smalley DL, Rosenblatt JE, Desai SM, Mushahwar IK (1998) The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States. J Gen Virol 79 (Pt 3): 447–456
18. Schlauder GG, Mushahwar IK (2001) Genetic heterogeneity of hepatitis E virus. J Med Virol 65: 282–292
19. Takahashi K, Iwata K, Watanabe N, Hatahara T, Ohta Y, Baba K, Mishiro S (2001) Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. Virology 287: 9–12
20. Takahashi K, Kang JH, Ohnishi S, Hino K, Miyakawa H, Miyakawa Y, Maekubo H, Mishiro S (2003) Full-length sequences of six hepatitis E virus isolates of genotypes III and IV from patients with sporadic acute or fulminant hepatitis in Japan. Intervirology 46: 308–318
21. Takahashi M, Nishizawa T, Miyajima H, Gotanda Y, Iita T, Tsuda F, Okamoto H (2003) Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. J Gen Virol 84: 851–862
22. Takahashi M, Nishizawa T, Okamoto H (2003) Identification of a genotype III swine hepatitis E virus that was isolated from a Japanese pig born in 1990 and that is most closely related to Japanese isolates of human hepatitis E virus. J Clin Microbiol 41: 1342–1343
23. Tam AW, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry KE, Reyes GR (1991) Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology 185: 120–131
24. Tei S, Kitajima N, Takahashi K, Mishiro S (2003) Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 362: 371–373
25. Tei S, Kitajima N, Ohara S, Inoue Y, Miki M, Yamatani T, Yamabe H, Mishiro S, Kinoshita Y (2004) Consumption of uncooked deer meat as a risk factor for hepatitis E virus infection: an age- and sex-matched case-control study. J Med Virol 74: 67–70
26. Velazquez O, Stetler HC, Avila C, Ornelas G, Alvarez C, Hadler SC, Bradley DW, Sepulveda J (1990) Epidemic transmission of enterically transmitted non-A, non-B hepatitis in Mexico, 1986–1987. JAMA 263: 3281–3285
27. Wang Y, Ling R, Erker JC, Zhang H, Li H, Desai S, Mushahwar IK, Harrison TJ (1999) A divergent genotype of hepatitis E virus in Chinese patients with acute hepatitis. J Gen Virol 80 (Pt 1): 169–177
28. Wang Y, Zhang H, Ling R, Li H, Harrison TJ (2000) The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3. J Gen Virol 81: 1675–1686
29. Wu JC, Chen CM, Chiang TY, Tsai WH, Jeng WJ, Sheen IJ, Lin CC, Meng XJ (2002) Spread of hepatitis E virus among different-aged pigs: two-year survey in Taiwan. J Med Virol 66: 488–492
30. Yazaki Y, Mizuo H, Takahashi M, Nishizawa T, Sasaki N, Gotanda Y, Okamoto H (2003) Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be foodborne, as suggested by the presence of hepatitis E virus in pig liver as food. J Gen Virol 84: 2351–2357

Epidemiological study of hepatitis E virus infection of dogs and cats in Japan

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Veterinary Record (2006) 159, 853-854

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HEPATITIS E virus (HEV) is the causative agent of human hepatitis E in many developing and some industrialised countries (Aggarwal and Krawczynski 2000, Meng 2000a, Schlauder and Mushahwar 2001, Huang and others 2002, Mizuo and others 2002, Takahashi and others 2003). It is widely known that in humans HEV is primarily transmitted by the faecal-oral route through contaminated water, and occasionally causes large epidemics in endemic areas (Arankalle and others 1994, Aggarwal and Krawczynski 2000). However, in non-endemic areas, the reservoir for sporadic human cases with no history of travel to HEV-endemic areas has remained unknown for many years (Harrison 1999, Schlauder and Mushahwar 2001, Mizuo and others 2002, Takahasi and others 2002). In 1997, the first animal HEV strain, swine HEV, was discovered in a pig in the USA (Meng and others 1997). Subsequent epidemiological studies indicated that, for example, in Japan, most farmed pigs had been exposed to swine HEV (Takahashi and others 2003). There is a growing consensus that HEV is a potential zoonotic agent and that pigs can act as a reservoir for humans.

HEV has also been detected in chickens (Haqshenas and others 2001) and wild rodents (Kabrane-Lazizi and others 1999, Favorov and others 2000, Arankalle and others 2001, He and others 2002, Hirano and others 2003a), and anti-HEV antibodies have been found in cattle, water buffaloes, sheep, goats, non-human primates, dogs and cats (Tsarev and others 1993, Meng 2000b, Arankalle and others 2001, Hirano and others 2003b, Usui and others 2004). In an unusual case, an incident was reported in which a cat was suspected to be a reservoir for human infection (Kuno and others 2003). This short communication describes the examination of blood samples and rectal swabs from dogs and cats for evidence of HEV infection.

An ELISA using the purified, empty virus-like particles (VLPs) of HEV (Li and others 1997) was performed with 424 canine and 202 feline serum samples. Samples were collected from animals at animal hospitals over a five-year period, from 2000 to 2004, in over 30 prefectures covering northern Hokkaido and south-western Okinawa, Japan, and stored at –20°C. The ELISA method used was the same as that described by Li and others (2000). The sera were examined simultaneously using VLP-coated and VLP-uncoated (blank) plates, and the antibody titre was expressed as an optical density at 492 nm (OD_{492}) by subtracting the OD_{492} value of the blank plate from that of the VLP-coated plate.

OD_{492} values of greater than 0·1 were obtained from 10 canine and eight feline serum samples (Table 1). These samples were subsequently absorbed with the same VLPs used as the antigen in the ELISA to confirm the specificity of the reaction. The serum sample (100 µl at a dilution of 1:200) was mixed with 1 µg of VLPs, and the mixture was incubated at 37°C for one hour, and then re-examined. A reduction of the OD_{492} value of the sample by greater than 50 per cent after the absorption was considered to indicate an anti-HEV antibody-positive result, as described by Arankalle and others (2001). On this basis all the canine serum samples were considered to be anti-HEV antibody-negative, and four feline serum samples (1·98 per cent) were considered to be anti-HEV antibody-positive (Table 2).

A reverse transcriptase-PCR (RT-PCR) assay, described for the detection of swine HEV (Huang and others 2002), was used to test 100 canine and 66 feline rectal swabs from animal hospitals located in Tokyo and its environs obtained over a six-year period from 1999 to 2004. In addition, the 18 sera showing an OD_{492} value of greater than 0·1 (Table 1) were also examined by RT-PCR. When a questionable PCR product was obtained, it was analysed by sequencing. No specific PCR products were amplified from any of the canine or feline samples examined.

Only a small number of cases of HEV infection in dogs and cats have been reported (Tien and others 1997, Arankalle and others 2001, Usui and others 2004). In the present study, the samples were collected nationwide in Japan and anti-HEV antibody was found in a very small percentage of the cats and in none of the dogs tested by the ELISA. These results were in contrast to a previous report. Among 135 cats visiting an animal hospital in a provincial capital in Japan, 44 (33 per cent) possessed anti-HEV antibody but no HEV RNA was recovered from the 135 sera (Usui and others 2004). No convincing explanation for the discrepancy in the seroprevalence rates between the previous and the present reports was possible. It should be noted that there is a possibility that the recombinant antigen itself could have non-specific, cross-reacting epitopes among antibodies in animal sera. According to a report describing HEV infection in dogs from India (Arankalle and others 2001) and Vietnam (Tien and others 1997), 22·7 per cent to 27 per cent of the dogs were anti-HEV antibody-positive. It may be true that dogs and cats in such HEV-endemic areas have been exposed to HEV more frequently than animals in non-endemic areas.

In non-endemic areas, there may be some common infectious source affecting both human beings and their companion animals, as they share mostly the same living environment. One probable reservoir, especially for companion animals, is rodents, and it has recently been reported in Japan that wild rats have been found to be infected with HEV (Hirano and others 2003a). Cats are rodent hunters, which is inherent to their nature, but this is not so much the case in dogs. This causal relationship between disease prevalence and a behavioural characteristic has already been seen in other viral diseases in cats, such as Borna disease virus (Berg and

TABLE 1: Results of an ELISA for antibody to hepatitis E virus in dog and cat sera from Japan

Animal	Number of samples tested	Optical density (mean) at 492 nm ≤0	0·001-0·099	0·1-0·499	≥0·5
Dog	424	254	160 (0·039)	10 (0·113)	0
Cat	202	83	111 (0·044)	7 (0·178)	1 (1·189)

TABLE 2: Details of cats suspected or confirmed as hepatitis E virus (HEV) antibody-positive by ELISA

Cat	Place of residence	Age (years)	Sex	Clinical signs	Reduction rate of ELISA optical density at 492 nm after absorption (%)	HEV antibody*
1	Niigata	Unknown	MN	None	60	+
2	Tokyo	11	MN	Kidney dysfunction	49·6	Suspected
3	Tokyo	6	F	None	58·6	+
4	Kanagawa	7	M	Stomatitis	32·3	Questionable
5	Aichi	15	FN	None	82·1	+
6	Okinawa	2-3†	M	None	96·2	+

* Greater than 50 per cent reduction was regarded as HEV antibody-positive
† Estimated
MN Male neutered, F Female, M Male, FN Female neutered, + Positive

others 1998) and cowpox virus infections (Nowotny and others 1994).

In conclusion, natural HEV infections in both dogs and cats in Japan appear to be rare and these animal species may be accidental hosts for human beings.

References

AGGARWAL, R. & KRAWCZYNSKI, K. (2000) Hepatitis E: an overview and recent advances in clinical and laboratory research. *Journal of Gastroenterology and Hepatology* 15, 9-20

ARANKALLE, V. A., CHADHA, M. S., TSAREV, S. A., EMERSON, S. U., RISBUD, A. R., BANERJEE, K. & PURCELL, R. H. (1994) Seroepidemiology of water-borne hepatitis in India and evidence for a third enterically-transmitted hepatitis agent. *Proceedings of the National Academy of Sciences of the United States of America* 91, 3428-3432

ARANKALLE, V. A., JOSHI, M. V., KULKARNI, A. M., GANDHE, S. S., CHOBE, L. P., RAUTMARE, S. S., MISHARA, A. C. & PADBIDRI, V. S. (2001) Prevalence of anti-hepatitis E virus antibodies in different Indian animal species. *Journal of Viral Hepatitis* 8, 223-227

BERG, A-L., REID-SMITH, R., LARSSON, M. & BONNETT, B. (1998) Case control study of feline Borna disease in Sweden. *Veterinary Record* 142, 715-717

FAVOROV, M. O., KOSOY, M. Y., TSAREV, S. A., CHILDS, J. E. & MARGOLIS, H. S. (2000) Prevalence of antibody to hepatitis E virus among rodents in the United States. *Journal of Infectious Diseases* 184, 449-455

HAQSHENAS, G., SHIVAPRASAD, H. L., WOOLCOCK, P. R., READ, D. H. & MENG, X. J. (2001) Genetic identification and characterization of a novel virus related to human hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States. *Journal of General Virology* 82, 2449-2462

HARRISON, T. J. (1999) Hepatitis E virus – an update. *Liver* 19, 171-176

HE, J., INNIS, B. L., SHRESTHA, M. P., CLAYSON, E. T., SCOTT, R. M., LINTHICUM, K. J., MUSSER, G. G., GIGLIOTTI, S. C., BINN, L. N., KUSCHNER, R. A. & VAUGHN, D. W. (2002) Evidence that rodents are a reservoir of hepatitis E virus for humans in Nepal. *Journal of Clinical Microbiology* 40, 4493-4498

HIRANO, M., DING, H., LI, T. C., TAKEDA, N., KAWABATA, H., KOIZUMI, N., KADOSAKA, T., GOTO, I., MASUZAWA, T., NAKAMURA, M., TAIRA, K., KUROKI, T., TANIKAWA, T., WATANABE, H. & ABE, K. (2003a) Evidence for widespread infection of hepatitis E virus among wild rats in Japan. *Hepatology Research* 27, 1-5

HIRANO, M., DING, H., TRAN, H. T. T., LI, T. C., TAKEDA, N., SATA, T., NAKAMURA, S. & ABE, K. (2003b) Prevalence of antibody against hepatitis E virus in various species of non-human primates: evidence of widespread infection in Japanese monkeys (*Macaca fuscata*). *Japanese Journal of Infectious Diseases* 56, 8-11

HUANG, F. F., HAQSHENAS, G., GUENETTE, D. K., HALBUR, P. G., SCHOMMER, S. K., PIERSON, F. W., TOTH, T. E. & MENG, X. J. (2002) Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. *Journal of Clinical Microbiology* 40, 1326-1332

KABRANE-LAZIZI, Y., FINE, J. B., ELM, J., GLASS, G. E., HIGA, H., DIWAN, A., GIBBS, C. J., Jr., MENG, X. J., EMERSON, S. U. & PURCELL, R. H. (1999) Evidence for wide-spread infection of wild rats with hepatitis E virus in the United States. *American Journal of Tropical Medicine and Hygiene* 61, 331-335

KUNO, A., IDO, K., ISODA, N., SATOH, Y., ONO, K., SATOH, S., INAMORI, H., SUGANO, K., KANAI, N., NISHIZAWA, T. & OKAMOTO, H. (2003) Sporadic acute hepatitis E of a 47-year-old man whose pet cat was positive for antibody to hepatitis E virus. *Hepatology Research* 26, 237-242

LI, T-C., YAMAKAWA, Y., SUZUKI, K., TATSUMI, M., AZEEZ RAZAK, M. A., UCHIDA, T., TAKEDA, N. & MIYAMURA, T. (1997) Expression and self-assembly of empty virus-like particles of hepatitis E virus. *Journal of Virology* 71, 7207-7213

LI, T-C., ZHANG, J., SHINZAWA, H., ISHIBASHI, M., SATA, M., MAST, E. E., KIM, K., MIYAMURA, T. & TAKEDA, N. (2000) Empty virus-like particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. *Journal of Medical Virology* 62, 327-333

MENG, X. J. (2000a) Novel strains of hepatitis E virus identified from humans and other animal species: is hepatitis E a zoonosis? *Journal of Hepatology* 33, 842-845

MENG, X. J. (2000b) Zoonotic and xenozoonotic risks of the hepatitis E virus. *Infectious Disease Review* 2, 35-41

MENG, X. J., PURCELL, R. H., HALBUR, P. G., LEHMAN, J. R., WEBB, D. M., TSAREVA, T. S., HAYNES, J. S., THACKER, B. J. & EMERSON, S. U. (1997) A novel virus in swine is closely related to the human hepatitis E virus. *Proceedings of the National Academy of Sciences of the United States of America* 94, 9860-9865

MIZUO, H., SUZUKI, K., TAKIKAWA, Y., SUGAI, Y., TOKITA, H., AKAHANE, Y., ITOH, K., GOTANDA, Y., TAKAHASHI, M., NISHIZAWA, T. & OKAMOTO, H. (2002) Polyphyletic strains of hepatitis E virus are responsible for sporadic cases of acute hepatitis in Japan. *Journal of Clinical Microbiology* 40, 3209-3218

NOWOTNY, N., FISCHER, O. W., SCHILCHER, F., SCHWENDENWEIN, I., LOUPAL, G., SCHWARZMANN, T., MEYER, J. & HERMANNS, W. (1994) Poxvirus infection in the domestic cat: clinical, histopathological, virological and epidemiological studies. *Wiener Tierärztliche Monatsschrift* 81, 362-369

SCHLAUDER, G. G. & MUSHAHWAR, I. K. (2001) Genetic heterogeneity of hepatitis E virus. *Journal of Medical Virology* 65, 282-292

TAKAHASHI, M., NISHIZAWA, T., MIYAJIMA, H., GOTANDA, Y., IITA, T., TSUDA, F. & OKAMOTO, H. (2003) Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. *Journal of General Virology* 84, 851-862

TAKAHASHI, M., NISHIZAWA, T., YOSHIKAWA, A., SATO, S., ISODA, N., IDO, K., SUGANO, K. & OKAMOTO, H. (2002) Identification of two distinct genotypes of hepatitis E virus in a Japanese patient with acute hepatitis who had not travelled abroad. *Journal of General Virology* 83, 1931-1940

TIEN, N. T., CLAYSON, H. T., KHIEM, H. B., SAC, P. K., CORWIN, A. L., MYINT, K. S. & VAUGHN, D. W. (1997) Detection of immunoglobulin G to the hepatitis E virus among several animal species in Vietnam. *American Journal of Tropical Medicine and Hygiene* 57, 211

TSAREV, S. A., TSAREVA, T. S., EMERSON, S. U., KAPIKIAN, A. Z., TICEHURST, J., LONDON, W. & PURCELL, R. H. (1993) ELISA for antibody to hepatitis E virus (HEV) based on complete open reading frame-2 protein expressed in insect cells: identification of HEV infection in primates. *Journal of Infections Diseases* 168, 369-378

USUI, R., KOBAYASHI, E., TAKAHASHI, M., NISHIZAWA, T. & OKAMOTO, H. (2004) Presence of antibodies to hepatitis E virus in Japanese pet cats. *Infection* 32, 57-58

Influence of food intake on the clinical response to cyclosporin A in canine atopic dermatitis

Veterinary Record (2006) 159, 854-856

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CYCLOSPORIN A (CsA) (Atopica, Atoplus soft capsules; Novartis Animal Health) was introduced recently as an oral microemulsion formulation for the treatment of dogs with atopic dermatitis. Several randomised, controlled and open clinical studies have proven its clinical efficacy (Olivry and others 2002, Steffan and others 2003, 2004). Based on pharmacokinetic data, administration of CsA is recommended two hours before or after feeding, as its bioavailability decreases and the variability of individual blood concentrations increases when the drug is given with food (Steffan and others 2004). Dosing fasted dogs is therefore recommended to optimise the drug's bioavailability. However, the clinical response of dogs after dosing with or without food has never been evaluated. This short communication describes a study to determine whether the administration of CsA with or without food could influence the clinical response in dogs with atopic dermatitis.

Twenty-five dogs with non-seasonal atopic dermatitis, diagnosed by Prélaud's criteria (Prélaud and others 1998) and by exclusion of differential diagnoses, were included in a multicentre, randomised study involving seven investigators

Am. J. Trop. Med. Hyg., 76(1), 2007, pp. 170–172
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SHORT REPORT: DETECTION OF HEPATITIS E VIRUS RNA FROM THE BIVALVE YAMATO-SHIJIMI (*CORBICULA JAPONICA*) IN JAPAN

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Abstract. To evaluate whether bivalves are contaminated with hepatitis E virus (HEV), samples of a bivalve called Yamato-Shijimi (*Corbicula japonica*) were examined for HEV by reverse transcription–polymerase chain reaction. Genotype 3 HEV was detected from 2 of 32 packages of Yamato-Shijimi obtained from Japanese rivers, which indicated that HEV contaminates river water in Japan.

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus recently classified as the sole member of the genus *Hepevirus* in the family Hepeviridae.[1,2] This virus causes human hepatitis E and is transmitted primarily by the fecal-oral route through contaminated drinking water.[3,4] However, recent studies have demonstrated that various animal species have serum antibodies to HEV, and its viral genome has been detected in swine, wild deer, wild boar, and mongoose, which suggests that hepatitis E is a zoonotic disease.[5–10] Because HEV is excreted into feces,[11,12] there is a risk of HEV contamination in environmental water. In fact, HEV has been detected in sewage from industrialized countries, including Spain, the United States, and France.[13–15] However, HEV contamination of river water has not been examined. In the present study we detected the HEV genome from a bivalve called Yamato-Shijimi (*Corbicula japonica*), which suggested that river water in Japan is contaminated with HEV.

A total of 32 packages of Yamato-Shijimi were obtained at a fish market in December 2005 and March 2006. All samples were harvested at areas A, B, C, D, E, F, G, and H in western Japan. The package numbers and collection days are shown in Table 1. The samples were shucked, and the digestive diverticulum were removed by dissection and weighed. One gram of digestive diverticulum obtained from 10–15 Yamato-Shijimi was homogenized with an Omni-mixer (OCI Instruments, Waterbury, CT) in 10 mL of phosphate-buffered saline, pH 7.4, for two 30-second intervals at a maximum speed of 18,000 rpm. After centrifugation at 10,000 × *g* for 30 minutes at 4°C, the supernatant was centrifuged at 100,000 × *g* for 2 hours in an SW41 rotor (Beckman Instruments, Inc., Fullerton, CA). The pellet was resuspended in 140 μL of distilled water and stored at -80°C until use.

Total RNA was extracted with the QIAmp viral RNA mini kit (Qiagen, Hilden, Germany) and resuspended in 20 μL of DNase-, RNase-, and proteinase-free water. Reverse transcription (RT) was performed at 42°C for 50 minutes, followed by 70°C for 15 minutes in a 20-μL reaction mixture containing 1 μL of Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA), 1 μL of oligo (dT) primer, 1 μL of RNaseOUT™ (Invitrogen), 2 μL of 0.1 M dithiothreitol, 4 μL of 5× RT buffer, 1 μL of 10 mM deoxynucleoside triphosphates, 5 μL of RNA, and 5 μL of distilled water. An RT–polymerase chain reaction was performed to amplify part of the open reading frame 2 (ORF2) as described previously.[8,10] Two microliters of the cDNA was used for the first PCR in a 50-μL reaction mixture with external forward primer HEV-F1 (5′-GGBGTBGCNGAGGAGGAGGC-3′) and external reverse primer HEV-R2 (5′-TGYTGGTTRT-CRTARTCCTG-3′), which corresponded to nucleotide residues 5903–5922 and 6486–6467, respectively, of the G1 Myanmar strain (D10330). Each cycle consisted of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, followed by final extension at 72°C for 7 minutes. Two microliters of the first PCR product was used for a nested PCR with internal forward primer HEV-F2 (5′-TAYCGHAAYCAAGGHTG-GCG-3′; nucleotide residues 5939–5958) and internal reverse primer HEV-R1 (5′-CGACGAAATYAATTCTGTCG-3′, nucleotide residues 6316–6297) under the same conditions.

Two packages, B4 and B6, collected in area B on February 7, 2006, and March 1, 2006, were positive for HEV RNA by

TABLE 1
Detection of hepatitis E virus (HEV) in *Corbicula japonica*

Package no.	Collection day	HEV RNA
A1	12/08/05	–
A2	12/22/05	–
A3	1/14/06	–
A4	1/22/06	–
A5	1/22/06	–
A6	2/05/06	–
A7	2/17/06	–
A8	3/02/06	–
A9	3/14/06	–
A10	3/15/06	–
B1	12/10/05	–
B2	12/17/05	–
B3	1/24/06	–
B4	2/07/06	+
B5	2/19/06	–
B6	3/01/06	+
B7	3/17/06	–
B8	3/18/06	–
C1	1/10/06	–
D1	1/16/06	–
D2	1/20/06	–
D3	3/14/06	–
E1	1/21/06	–
E2	1/26/06	–
E3	2/25/06	–
E4	3/10/06	–
F1	3/13/06	–
F2	3/14/06	–
F3	3/18/06	–
F4	3/18/06	–
G	3/18/06	–
H	3/18/06	–

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RT-PCR. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and cloned into TA cloning vector pCR2.1 (Invitrogen). The nucleotide sequence of each of 10 clones was determined. Most of the sequences formed a single genotype 3 cluster. The exceptions (B4-13) formed a different cluster along with Sakai-9 detected from a wild boar in 2004 in Japan (Figure 1). We found large numbers of small different nucleotide sequences among the clones with 88.9–100% identity, even when they were derived from the same package, which indicated that multiple HEV strains were accumulated in the digestive diverticulum of Yamato-Shijimi.

To further analyze the HEV RNA detected in the Yamato-Shijimi, the entire ORF2 of B4 RNA was amplified as overlapping segments, and the nucleotide sequences were determined. The full-length ORF2 consisted of 1,980 basepairs and were phylogenetically classified into genotype 3. High amino acid identities (97.57–98.87%) were observed with HEV strains detected from hepatitis E patients, swine, wild boar, and wild deer in Japan. This is the first report on the detection of HEV from a bivalve.

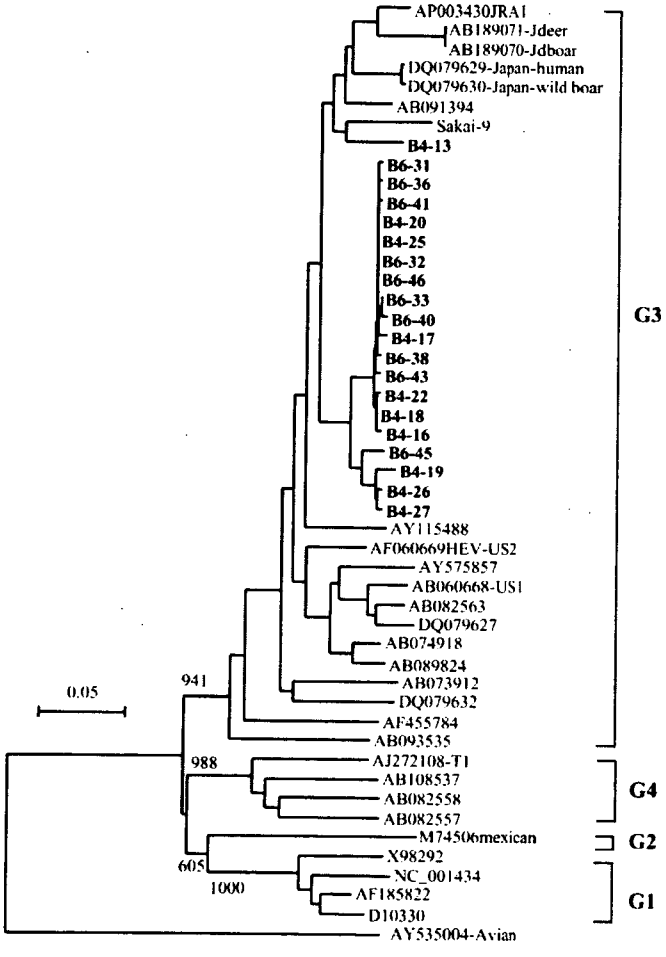

FIGURE 1. Phylogenetic trees of hepatitis E virus (HEV) constructed with avian HEV as an outgroup. A partial open reading frame 2 ORF2 (338 basepairs) of each of 10 clones of B4 (B4-13, B4-16, B4-17, B4-18, B4-19, B4-20, B4-22, B4-25, B4-26, and B4-27) and B6 (B6-31, B6-32, B6-33, B6-36, B6-38, B6-40, B6-41, B6-43, B6-45, and B6-46) were analyzed by the neighbor-joining method. The bootstrap values correspond to 1,000 replications. All nucleotide sequences determined in this study are shown in bold. Other HEV sequences were obtained from GenBank.

The HEV sequences were detected from Yamato-Shijimi (*Corbicula japonica*) harvested on February 7, 2006, and March 1, 2006, in western Japan. The Yamato-Shijimi, a brackish-water bivalve, grows in sandy mud in or near rivers, ponds, and lakes. During breathing and feeding, this bivalve filters a large amount of water. When the water is contaminated with HEV, the virus is ultimately concentrated in the digestive diverticula of the bivalves.

The source of HEV in this organism is not known. Since Japan was considered not to be endemic for this virus, and disposal of sewage is in this country is efficient, the risk of HEV contamination from human stool was believed to be low. There have been no outbreaks of hepatitis E in Japan from drinking water. However, HEV has been detected in wild deer, wild boar, and mongoose in Japan, and HEV shed in the feces of these animals may pollute environmental water. Wild deer and wild boar are controlled in Japan to eliminate their ability to damage agriculture and forestry; hunting is the main control strategy. Hunters usually wash killed animals in river water and this would increase the risk of HEV contamination in river water. These wild animals presumably play an important role in the contamination of environmental water.

In Japan, many outbreaks caused by bivalves contaminated with hepatitis A virus and noroviruses have been reported. Fortunately, Yamato-Shijimi is generally eaten as an ingredient in hot miso soup in Japan, and the heat, usually at 100°C for nearly 10 minutes, decreases the risk of HEV transmission from Yamato-Shijimi to humans. However, more efforts are needed to determine the infectivity and stability of HEV in the natural environment, including that in Yamato-Shijimi.

Received June 19, 2006. Accepted for publication September 10, 2006.

Acknowledgments: We thank Tomoko Mizoguchi and Satoko Ogawa for secretarial work.

Financial support: This study was supported in part by grants for Research on Emerging and Re-emerging Infectious Diseases, Research on Hepatitis, and Research on Food Safety from the Ministry of Health, Labor and Welfare, Japan.

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REFERENCES

1. Tam AW, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry KE, Reyes GR, 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology 185:* 120–131.
2. Emerson SU, Anderson D, Arankalle A, Meng XJ, Purdy M, Schlauder GG, Tsarev SA, 2005. Hepevirus. Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, eds. *Virus Taxonomy, VIIIth Report of the ICTV.* London: Elsevier/ Academic Press, 851–855.
3. Purcell RH, Emerson SU, 2001. Hepatitis E virus. Knipe DM, Howley PM, eds. *Fields Virology.* Philadelphia: Lippincott Williams and Wilkins, 3051–3061.
4. Balayan MS, 1997. Epidemiology of hepatitis E virus infection. *J Viral Hepat 4:* 155–165.
5. Meng XJ, 2000. Novel strains of hepatitis E virus identified from humans and other animal species: is hepatitis E a zoonosis? *J Hepatol 33:* 842–845.

6. Tei S, Kitajima N, Takahashi K, Mishiro S, 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. *Lancet 362:* 371–373.
7. Yazaki Y, Mizuo H, Takahashi M, Nishizawa T, Sasaki N, Gotanda Y, Okamoto H, 2003. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. *J Gen Virol 84:* 2351–2357.
8. Li TC, Chijiwa K, Sera N, Ishibashi T, Etoh Y, Shinohara Y, Kurata Y, Ishida M, Sakamoto S, Takeda N, Miyamura T, 2005. Hepatitis E virus transmission from wild boar meat. *Emerg Infect Dis 11:* 1958–1960.
9. Nakamura M, Takahashi K, Taira K, Taira M, Ohno A, Sakugawa H, Arai M, Mishiro S, 2006. Hepatitis E virus infection in wild mongooses of Okinawa, Japan: demonstration of anti-HEV antibodies and a full-genome nucleotide sequence. *Hepatol Res 34:* 137–140.
10. Li TC, Saito M, Ogura G, Ishibashi O, Miyamura T, Takeda N, 2006. Serologic evidence for hepatitis E virus infection in mongoose. *Am J Trop Med Hyg 74:* 932–936.
11. Chauhan A, Jameel S, Dilawari JB, Chawla YK, Kaur U, Ganguly NK, 1993. Hepatitis E virus transmission to a volunteer. *Lancet 341:* 149–150.
12. Meng XJ, Halbur PG, Shapiro MS, Govindarajan S, Bruna JD, Mushahwar IK, Purcell RH, Emerson SU, 1998. Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. *J Virol 72:* 9714–9721.
13. Clemente-Casares P, Pina S, Buti M, Jardi R, Martin M, Bofill-Mas S, Girones R, 2003. Hepatitis E virus epidemiology in industrialized countries. *Emerg Infect Dis 9:* 448–454.
14. Pina S, Buti M, Cotrina M, Piella J, Girones R, 2000. HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. *J Hepatol 33:* 826–833.
15. Pina S, Jofre J, Emerson SU, Purcell RH, Girones R, 1998. Characterization of a strain of infectious hepatitis E virus isolated from sewage in an area where hepatitis E is not endemic. *Appl Environ Microbiol 64:* 4485–4488.

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Efficient regulation of viral replication by siRNA in a non-human primate surrogate model for hepatitis C

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Received 20 June 2007
Available online 16 July 2007

Abstract

RNA interference (RNAi) represents a new technology which could offer potential applications for the therapeutics of human diseases. RNAi-mediated therapy has recently been shown to be effective toward infectious diseases in *in vitro* and rodent models, however, it remains unclear whether RNAi therapy with systemic application could be effective in primates. In this study, we examined if RNAi therapy could be effective toward infectious diseases by using a non-human primate surrogate model for hepatitis C. Administration into marmosets of cationic liposome-encapsulated siRNA (CL-siRNA) for GB virus B (GBV-B), which is most closely related to hepatitis C virus, repressed GBV-B replication in a dose-dependent manner. Especially, 5 mg/kg of the CL-siRNA completely inhibited the viral replication. Since the serum interferons (IFNs) were induced by CL-siRNA *in vivo*, inhibition of viral regulation by anti-GBV-B CL-siRNA may include an antiviral effect of IFN. However, contribution of induced IFN may be partial, since the control CL-siRNA which induced a stronger IFN response than GBV-B CL-siRNA could only delay the viral replication. Our results suggest the feasibility of systemic administration of CL-siRNA as an antiviral strategy.
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Keywords: siRNA; Hepatitis C; Marmoset; Interferon; GB virus B

RNA interference is a powerful tool for silencing gene expression and has spurred considerable interest in its experimental and therapeutic potential. RNAi has been characterized as a cellular process of post-transcriptional gene silencing. An RNaseIII-like enzyme, called Dicer, cleaves double stranded RNA (dsRNA) in to 21–23 nucleotide RNA duplex, termed small interfering RNAs (siRNAs). siRNAs are unwound in the RNA-induced-silencing-complex (RISC), and single-stranded siRNAs then act as a guide to substrate selection, leading to the cleavage of a homologous target RNA molecule [1].

Hepatitis C virus (HCV) infection contributes significantly to human morbidity and mortality worldwide. It is estimated that 40–60% of infected individuals progress to chronic liver disease, and many of these patients develop liver cirrhosis and hepatocellular carcinoma [2]. Currently,

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0006-291X/$ - see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.bbrc.2007.06.182

the only treatment available for patients with chronic HCV infections is combinational therapy with interferon (IFN) and ribavirin. The standard therapy is only effective for approximately 50% of patients with chronic HCV hepatitis [3]. Therefore, there is a great need for less complicated and more generally efficient therapeutics for HCV infection.

We and others reported that the synthetic siRNA and the siRNA-expressing adenovirus targeting 5′-UTR of HCV genome efficiently and specifically inhibited the HCV replication *in vitro* [4–6]. Other than humans, only chimpanzees can be productively infected by HCV. Together with ethical issues it has becomes increasingly difficult to access chimpanzees for experimental studies. The new world monkeys, tamarins and marmosets, undergo hepatitis upon infection with the GBV-B, which is most closely related to HCV. The significant similarity between HCV and GBV-B at the genomic and biochemical levels led to the proposal of the GBV-B/monkey system as a good surrogate model for hepatitis C [7,8]. Taking advantage of this non-human primate surrogate model, we investigated the feasibility of siRNA-mediated therapy against infectious diseases caused by pathogenic viruses.

Materials and methods

Preparation of siRNA. The sequence of siRNA for GBV-B was cucguagaccguagcacau dTdT in the sense strand and auugugcuacggucuacgagdTdT in the antisense strand which was designed to target the GBV-B RNA (Fig. 1). The sequence of control siRNA for experiments of Fig. 2A and B was uua ugc cga ucg cgu cac a dTdT in the sense strand and ugu gac gcg auc ggc aua a dTdT in the antisense strand which was designed to target beta-galactosidase RNA, and that for experiments of Figs. 3 and 4 was gct atg aaa cga tat ggg c dTdT in the sense strand and g ccc aua ucg uuu cau ugc dTdT in the antisense strand which was designed to target *firefly*-luciferase RNA. siRNA oligonucleotides were chemically synthesized and purified by reverse-phase high-performance liquid chromatography, while the unconjugated RNA oligonucleotides were purified by anion-exchange high-performance liquid chromatography. The sense and antisense strands were annealed at 95 °C for 1 min followed by slow cooling in RNAse free water. Positively charged liposomes containing cationic lipid analogue were synthesized at Nippon Shinyaku Co., as described previously [9]. To prepare CL-siRNA,

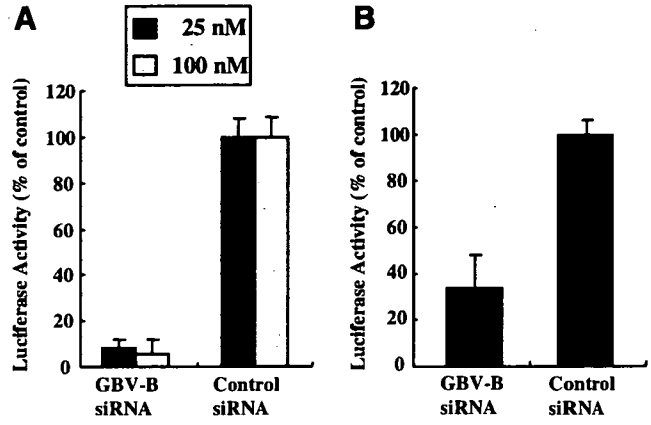
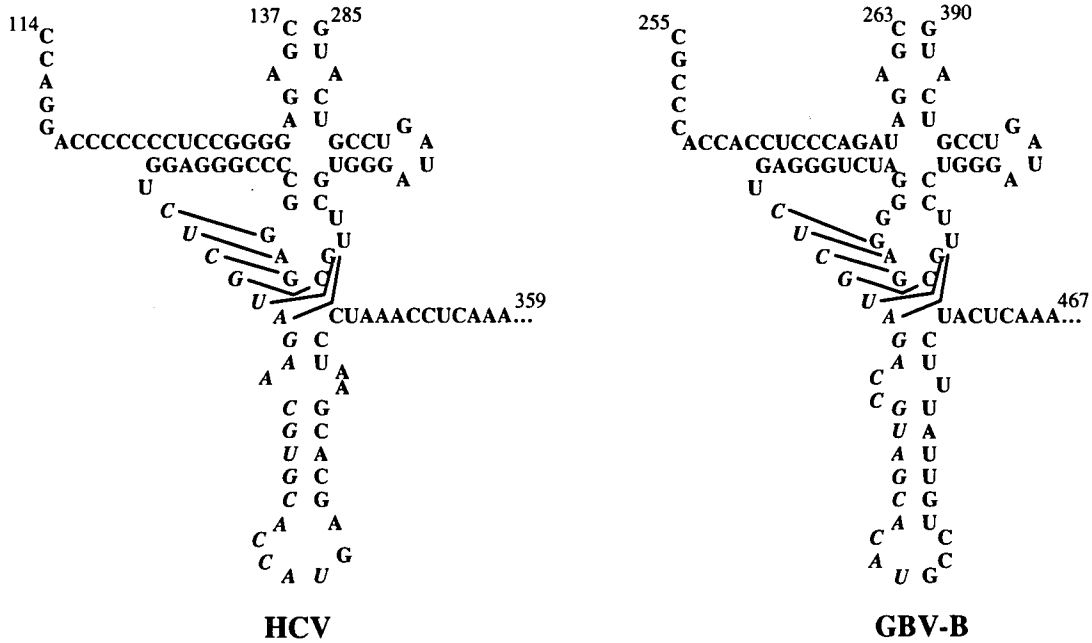


Fig. 2. Effects of the siRNA oligonucleotides on expression of GBV-B-reporter gene in culture cells. (A) and liver of mice (B).



Sense sequence of siRNA

HCV : CUCGUAGA*A*CGU*(A)*GCACAU

GBV-B : CUCGUAGA*C*CGU *A* GCACAU

Fig. 1. Predicted secondary structure of the 5′-untranslated region around the target site (italic) in the HCV and GBV-B genome (nucleotide 114–137 and 285–359 of HCV, and 263–255 and 390–467 of GBV-B), and the sense sequences of siRNA.

annealed siRNA was added to the same volume of liposome solution with sonication. The ratio of oligonucleotide to LIC-101 was 1:16 (w/w).

Cells culture and transfection. The human embryonic kidney cell line, 293 T, was maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, Missouri) supplemented with 10% fetal calf serum at 37 °C under 5% CO_2. Transfections of the siRNA oligonucleotides and the plasmids were performed in 24-well plates using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. GBV-B-RNA-reporter gene vector, pGBV-B-Rluc, was used as a target, which expressed mRNA consisting of GBV-B 5′-untranslated region and upstream part of the core region (nucleotide 1–377) connected with upstream of *renilla* luciferase (RLuc) gene. Fifty nanograms of the pGBV-B-Rluc and 2 and 25 nM of siRNA were transiently transfected with 20 ng of *firefly* luciferase (FLuc)-expressing plasmid (pRL-RSV, Promega). The RLuc activity was adjusted by the FLuc activity, to normalize the transfection efficiency.

Luciferase assays. Luciferase activities were quantified with a luminometer (Lumat LB9501, Promega) using the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and the results expressed as means ± SD as percentages of controls.

Animals. Male BALB/c or ICR mice, 9 weeks of age, were obtained from CLEA Japan and subject to a 2-week quarantine and acclimation period before use. Male juvenile common marmosets (*Callithrix jacchus*) were housed in individual cages at the Tsukuba Primate Medical Center. All animal studies were conducted in accordance with the protocols of experimental procedures which were approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation and Tokyo Medical and Dental University.

In vivo efficacy experiments in mice. For the *in vivo* delivery of the siRNA to the liver of mice and monkeys, we used a novel cationic liposome that was synthesized by Nippon Shinyaku Co., Ltd. This cationic liposome was reported to be a good vehicle for the delivery of nucleic acid polymers and siRNAs to the liver when it was administered intravenously [9,10] or to the bladder by intravesical administration [11]. For the delivery of plasmid DNA to the liver of mice we used the hydrodynamic injection method in which a large volume of nucleotides solution was rapidly injected from tail vein [12]. *Three mice for each group were examined.* 5.0 mg/kg GBV-B or control CL-siRNA was administered as a regular intravenous injection from the tail vein in 0.2 ml 10% maltose over a period of 1–3 s. Three minutes later, the 50 μg of the pGBV-B-Rluc and 20 μg of pRL-RSV plasmids in a volume equivalent to 5% of the body weight were rapidly injected in 3–5 s into the mouse tail vein according to the hydrodynamic injection method. Phosphate buffer saline (PBS) was used as a carrier solution for injection. Successful injection was monitored when the conjunctiva of mouse became transiently anemic and confirmed by the luciferase activity in the liver.

In vivo efficacy experiments in monkeys. Negative control (n = 2; with or without control siRNA) and treatment group (n = 3; 1.0, 2.5 and 5.0 mg/kg of anti-GBV-B siRNA) were employed in this study. GBV-B-infectious serum obtained from a tamarin [8] was intrahepatically inoculated with the GBV-B RNA. The siRNA to GBV-B and control siRNA formulated by the cationic liposome, or just 10% maltose (sham) was administered by standard intravenous injection via the saphenous vein of the marmosets for three days. On the second day, the GBV-B infectious serum (1.3×10^9 viral RNA copies/inoculum) was directly injected to the liver of five marmosets. Blood samples were periodically collected from the femoral vein of the monkeys under anesthetization. GBV-B RNA in plasma from the monkeys was quantified by a real-time, 5′ exonuclease PCR (TaqMan) assay using a primer-probe combination that recognized a portion of the GBV-B capsid gene as previously described [8]. The Platelet cell counts were performed at FALCO Biosystems, Co., Ltd.

Measurement of IFNs in mice and monkeys. The siRNA/cationic liposome was injected from tail vein of ICR mice or saphenous vein of the marmosets. Bood samples were taken 3 h after the injection. Mouse IFN-α levels were quantified by using sandwich ELISA kits for mouse IFNs (PBL Biomedical Laboratories, Biosource). Marmoset IFN-α and -γ levels were by using sandwich ELISA kits for human and rhesus macaque IFN, respectively (U-CyTech bioscience) according to the manufacture's instructions. Assays were performed in duplicate and the results expressed as means ± SD as percentages of controls.

Results

We selected the siRNA-targeting site to the GBV-B genome from its 5′-UTR, the most conservative portion in both GBV-B and HCV genomes [13], to protect the siRNA from escape mutations of the virus [4]. The secondary structures of virus genome RNAs of HCV and GBV-B around the target site are very similar to each other, and the designed siRNA was different from the corresponding sequence of HCV by only two nucleotides (Fig. 1).

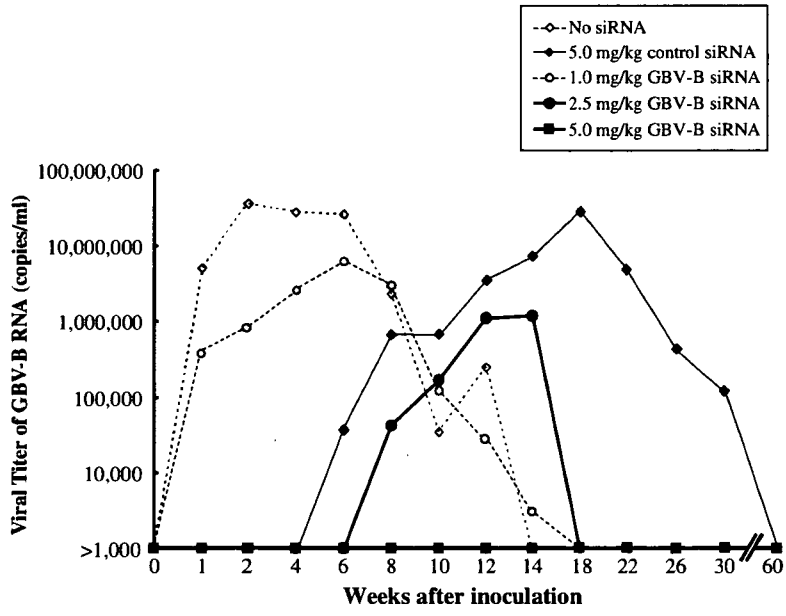


Fig. 3. Effect of the GBV-B siRNA/cationic liposome complex on replication of GBV-B in marmosets.

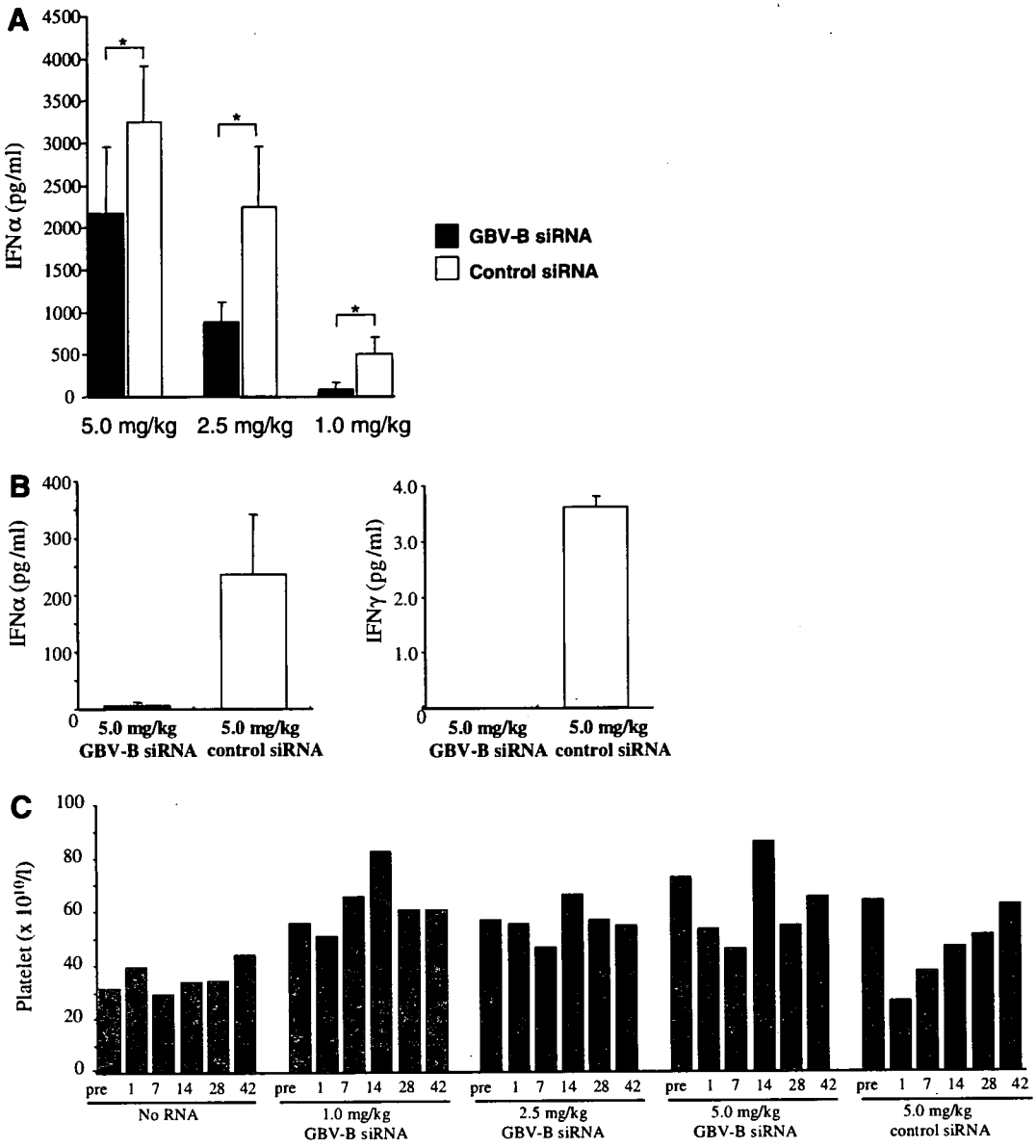


Fig. 4. Side effects of siRNA/cationic liposome complex. (A) Induction of IFN-α was evaluated by measuring mouse serum 3 h after intravenous injection of 1–5 mg/kg GVB-B or control siRNA/cationic liposome complex ($n = 3$). *<0.05 (Student t test). (B) Induction of IFN-α and γ was evaluated by measuring marmoset serum 3 h after intravenous injection of 5 mg/kg GVB-B or control siRNA/cationic liposome complex), respectively ($n = 3$). (C) Peripheral blood platelet was counted in the five marmosets examined in the same experiment shown in Fig. 3.

Effect of siRNA in vitro and in mice

First, we confirmed the efficient cleavage of GBV-B RNA by the siRNA in 293 T cells. The cells were harvested at 24 h of transfection with pGBV-B-*R*luc, pRL-RSV and siRNA oligonucleotides, and internal luciferase activities were measured and ratio of RLuc versus FLuc value was calculated. More than 90% the *R*Luc activities were inhibited by expressing co-transfected siRNA (Fig. 2A). This result clearly indicated that GBV-B siRNA efficiently inhibited the expression of GBV-B RNA in culture cells.

Next, we investigated the *in vivo* effect of siRNA formulated in the cationic liposome on silencing the viral gene expression in the liver of mice. BALB/c mice were injected intravenously from the tail vein with GVB-B CL-siRNA followed by hydrodynamically injection of pGBV-B-*R*luc and pRL-RSV. We found that intravenously administered GBV-B CL-siRNA efficiently suppressed the expression of GBV-B genome in the liver of mice (Fig. 2B).

Effect of siRNA on GBV-B replication in marmosets

The 1.0, 2.5 and 5.0 mg/kg/day of siRNA to GBV-B, 5.0 mg/kg/day of control siRNA formulated by the cationic liposome, or just 10% maltose (sham) were administered by standard intravenous injections via the saphenous vein of the marmosets for three consecutive days. On the second day, GBV-B infectious serum

(1.3×10^9 viral RNA copies/inoculum) was directly injected to the liver. Before and after the inoculation, GBV-B RNA in the serum was quantified by a real-time, 5' exonuclease PCR. In a sham-administered marmoset, the viral RNA was transiently increased in plasma after infection and the viral load reached to the peak level (3.6×10^7 copies/ml) (Fig. 3). It has been reported that GBV-B infection in marmosets as well as tamarins causes semi-acute viremia which generally ceases within 10–12 weeks post-infection [8,14,15]. This viral kinetics is consistent with the cases of HCV-infected human or chimpanzee, thus it appears to be in vivo characteristics of genus hepativirus where HCV and GBV-B belong to. Virological or immunological implication for the transient viremia is not fully addressed.

In contrast to sham-administered marmoset, we could find that the administration of CL-siRNA significantly delayed or suppressed the replication of GBV-B in a dose-dependent manner; the 5.0 mg/kg CL-siRNA completely suppressed the replication of GBV-B for more than 6 months after the infection (Fig. 3), even though the siRNA was administered only for the initial 3 days. Unexpectedly, the 5.0 mg/kg of control CL-siRNA was also able to delay the virus replication, while the peak level was comparable with that of the untreated monkey (Fig. 3).

Induction of interferons

We evaluated the induction of serum IFN-α by intravenous administration of the siRNA with cationic liposome in mice. IFN-α was induced by CL-siRNA but not by the cationic liposome nor siRNA oligonucleotide alone (data not shown). Induced IFN levels in the sera were dose-dependent and were significantly higher in mice with the *control CL-siRNA* than those with the *GBV-B CL-siRNA* (Fig. 4A).

An independent experiment using marmosets showed that single injection of 5.0 mg/kg control CL-siRNA substantially induced the serum interferon (IFN)-α and -γ, whereas the same dose of CL-siRNA induced a minimal level of IFN-α and no detectable level of IFN-γ (Fig. 4B).

In addition, a transient and mild decrease in peripheral blood platelets was more clearly observed in the marmoset treated with 5.0 mg/kg of control than 5.0 mg/kg of GBV-B CL-siRNA (Fig. 4C). There was no other remarkable abnormality related to siRNA administration in biochemical parameters indicating liver dysfunction which include alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase and albumin.

Discussion

Many viruses produce some dsRNA as a byproduct of their replication [16], and RNAi serves as an important defense against viruses in plants [17]. Therefore, mammalian viruses have been expected to be a good therapeutic target of RNAi, and indeed, several animal viruses have been successfully inhibited to replicate *in vitro* [18]. Locally delivered siRNA have proven effective in abrogating infection from respiratory [19–22] and vaginal [23] viruses. Recently, systemically-delivered siRNA in mice has been successfully suppressed the expression of endogeous gene of the liver [24–26]. However, it remains to be ascertained if the RNAi-mediated gene therapy with systemically-delivered siRNA would be applicable to hepatitis virus in non-human primates. In this study, we examined if RNAi therapy could be effective toward infectious diseases by using a non-human primate surrogate model for hepatitis C. Administration into marmosets of CL-siRNA for GBV-B, which is most closely related to hepatitis C virus, repressed GBV-B replication in a dose-dependent manner. Our results suggest the feasibility of systemic administration of CL-siRNA as an antiviral strategy.

The 5.0 mg/kg GBV-B CL-siRNA dramatically inhibited the replication of GBV-B. However, control CL-siRNA also delayed the virus replication. Intravenous injection of siRNA formulated with liposomes was reported to stimulate mammalian immune system [26,27]. In relation to antiviral effect of IFNs, we therefore measured the serum IFN levels. Since the GBV-B siRNA/cationic liposome had less effect in IFN induction than the control but better antiviral effect than the control, it is possible that inhibition of the viral replication by the GBV-B siRNA/cationic liposome complex was at least in part caused by RNA interference. On the other side, it is also likely that IFN locally induced in the marmoset liver contributed the suppression of the viral replication. Because the induced level of mice serum IFN-α by GBV-B CL-siRNA was significant, although it was less than that by control CL-siRNA. Moreover, estimated IFNs level in marmoset serum was minimal but their actual levels might have been more, because the standard IFN in the ELISA was human or rhesus macaque IFN. Therefore, we considered that the antiviral effect of CL-siRNA was made by both RNA interference and induced IFNs.

In therapeutic application of siRNA to humans, general safety is a most important problem. The side effect of CL-siRNA to the liver is thought to consist of direct liver toxicity which is probably caused by its hydrophobic nature and its immuno-stimulatory effect [26–28]. Recently, Zimmermann et al. has reported that siRNA delivered systemically in a cationic liposome, stable nucleic acid lipid particles (SNALP), inhibited endogenous gene expression in the liver of the cynomolgus monkeys, which supports our notion concerning the therapeutic potential of systemically injected siRNA in primates. Although they made excellent chemical modifications to siRNA oligonucleotides to reduce IFN induction, their siRNA complex produced a considerable liver damage with a marked increase of transaminases at the dose (2.5 mg/kg) of maximal suppression effect. This indicated that the therapeutic window of their siRNA complex is overlapped with its toxic window. In contrast, our CL-siRNA induced much less liver damage, since even the 5.0 mg/kg of our CL-siRNA did not show a marked liver damage, but induced a sub-

stantial immune responses. A number of recent studies revealed that siRNA/cationic liposome complex has an immunological effects of siRNAs including the induction of proinflammatory cytokines and type I IFNs (IFN-α and IFN-β) through activation of RNA-sensing immunoreceptors including three members of the Toll-like receptor (TLR) family (TLR3, TLR7 and TLR8) [29]. Detection of siRNA molecules could trigger antiviral innate defense mechanisms including the induction of type I IFNs. In fact, double strand RNA molecule, poly I/C, was reported to eliminate the virus in GBV-B-infected tamarin hepatocytes by activating TLR3 [30,31]. These knowledges lead us to postulate that it is one of sophisticated strategy for siRNA to inhibit hepatitis virus to use this immuno-stimulatory side effect as an antiviral innate defense, only if the systemic side effects are tolerable.

Acknowledgments

We thank members of Corporation for Production and Research of Laboratory Primates for their technical support, N. Enomoto (Yamanashi University) for the invaluable suggestions and encouragements, and S. Arold (CNRS, France) for critical reviewing of the manuscript. RNAs and cationic liposome were supplied by Nippon Shinyaku Co., Ltd. This work was supported by Grants from the Ministry of Education, Science and Culture, Japan (#18390215, T.Y. and H.A.) and from the Ministry of Health, Labor and Welfare, Japan (#2212065, T.Y; #17200101, H.A.).

References

[1] G.J. Hannon, RNA interference, Nature 418 (2002) 244–251.
[2] W.R. Kim, The burden of hepatitis C in the United States, Hepatology 36 (2002) S30–S34.
[3] J.M. Mchutchison, Hepatitis C advances in antiviral therapy: what is accepted treatment now? J. Gastroenterol. Hepatol. 17 (2002) 431–441.
[4] T. Yokota, N. Sakamoto, N. Enomto, H. Tanabe, M. Maekawa, M. Miyagishi, K. Taira, M. Watanabe, H. Mizusawa, Inhibition of intracellular hepatitis C Virus replication by synthetic and vector-derived small interfering RNAs, EMBO Rep. 4 (2003) 602–608.
[5] M.Y. Seo, S. Abrignani, M. Houghton, J.H. Han, Small interfering RNA-mediated inhibition of hepatitis C virus replication in the human hepatoma cell line Huh-7, J. Virol. 77 (2003) 810–812.
[6] M. Korf, D. Jarczak, C. Beger, M.P. Manns, M. Kruger, Inhibition of hepatitis C virus translation and subgenomic replication by siRNAs directed against highly conserved HCV sequence and cellular HCV cofactors, J. Hepatol. 43 (2005) 225–234.
[7] A. Martin, F. Bodola, D.V. Sangar, K. Goettge, V. Popov, R. Rijnbrand, R.E. Lanford, S.M. Lemon, Chronic hepatitis associated with GB virus B persistence in a tamarin after intrahepatic inoculation of synthetic viral RNA, Proc. Natl. Acad. Sci. USA 100 (2003) 9962–9967.
[8] K. Ishii, S. Iijima, N. Kimura, Y.-J. Lee, N. Ageyama, S. Yagi, K. Yamaguchi, N. Maki, K. Mori, S. Yoshizaki, S. Machida, T. Suzuki, N. Iwata, T. Sata, K. Terao, T. Miyamura, H. Akari, GBV-B as a pleiotropic virus: distribution of GBV-B in extrahepatic tissues in vivo, Microb. Infect. 9 (2007) 515–521.
[9] K. Hirabayashi, J. Yano, T. Inoue, T. Yamaguchi, K. Tanigawara, G.E. Smyth, K. Ishiyama, T. Ohgi, K. Kimura, T. Irimura, Inhibition of cancer cell growth by polyinosinic-polycytidylic acid/cationic liposome complex: a new biological activity, Cancer Res. 59 (1999) 4325–4333.
[10] J. Yano, K. Hirabayashi, S. Nakagawa, T. Yamaguchi, M. Nogawa, I. Kashimori, H. Naito, H. Kitagawa, K. Ishiyama, T. Ohgi, T. Irimura, Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer, Clin. Cancer Res. 10 (2004) 7721–7726.
[11] M. Nogawa, T. Yuasa, S. Kimura, M. Tanaka, J. Kuroda, K. Sato, A. Yokota, M. Koizumi, T. Maekawa, Intravesical administration of small interfering RNA targeting PLK-1 successfully prevents the growth of bladder cancer, J. Clin. Invest. 115 (2005) 978–985.
[12] F. Liu, Y.K. Song, D. Liu, Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA, Gene Ther. 6 (1999) 1258–1266.
[13] A.A. Kolylhalov, K. Mihalik, S.M. Feinstone, C.M. Rice, Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication in vivo, J. Virol. 74 (2000) 2046–2051.
[14] J.R. Jacob, K.C. Lin, B.C. Tennant, K.G. Masnfield, GB virus B infection of the common marmoset (*Callithrix jacchus*) and associated liver pathology, J. Gen. Virol. 85 (2004) 2525–2533.
[15] R.E. Lanford, D. Chavez, L. Notvall, K.M. Brasky, Comparison of tamarins and marmosets as hosts for GBV-B infections and the effect of immunosuppression on duration of viremia, Virology 311 (2003) 72–80.
[16] B.L. Jacobs, J.O. Langland, When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA, Virology 219 (1996) 339–349.
[17] M.T. Mcmanus, Small RNAs and immunity, Immunity 21 (2004) 747–756.
[18] J.N. Leonard, D.V. Shaffer, Antiviral RNAi therapy: emerging approaches for hitting a moving target, Gene Ther. 13 (2006) 532–540.
[19] V. Bitko, A. Musiyenko, O. Shulyayeva, S. Barik, Inhibition of respiratory viruses by nasally administered siRNA, Nat. Med. 11 (2005) 50–55.
[20] W. Zhang, H. Yang, X. Kong, S. Mohapatra, H. San Juan-Vergara, G. Hellermann, S. Behera, R. Singam, R.F. Lockey, S.S. Mohapatra, Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene, Nat. Med. 11 (2005) 56–62.
[21] B.J. Li, Q. Tang, D. Cheng, C. Qin, F.Y. Xie, Q. Wei, J. Xu, Y. Liu, B.J. Zheng, M.C. Woodle, N. Zhong, P.Y. Lu, Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque, Nat. Med. 11 (2005) 944–9451.
[22] S.M. Tompkins, C.Y. Lo, T.M. Tumpey, S.L. Epstein, Protection against lethal influenza virus challenge by RNA interference in vivo, Proc. Natl. Acad. Sci. USA 101 (2004) 8682–8686.
[23] D. Palliser, D. Chowdhury, Q.Y. Wang, S.J. Lee, R.T. Bronson, D.M. Knipe, J. Lieberman, An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection, Nature 439 (2006) 89–94.
[24] J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R.K. Pandey, T. Racie, K.G. Rajeev, I. Rohl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan, H.P. Vornlocher, M. Manoharan, H.P. Vornlocher, Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs, Nature 432 (2004) 173–178.
[25] E. Song, P. Zhu, S.K. Lee, D. Chowdhury, D.M. Dykxhoorn, Y. Feng, D. Palliser, D.B. Weiner, P. Shankar, W.A. Marasco, J. Lieberman, Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors, Nat. Biotechnol. 23 (2005) 709–717.

[26] D.V. Morrissey, J.A. Lockridge, L. Shaw, K. Blanchard, K. Jensen, W. Breen, K. Hartsough, L. Machemer, S. Radka, V. Jadhav, N. Vaish, S. Zinnen, C. Vargeese, K. Bowman, C.S. Shaffer, L.B. Jeffs, A. Judge, I. Maclachlan, B. Polisky, Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs, Nat. Biotechnol. 23 (2005) 1002–1007.

[27] A.D. Judge, V. Sood, J.R. Shaw, D. Fang, K. Mcclintock, I. Maclachlan, Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA, Nat. Biotechnol. 23 (2005) 457–462.

[28] T.S. Zimmeemamm, A.C. Lee, A. Akinc, B. Bramlage, D. Bumcrot, M.N. Fedoruk, J. Harborth, J.A. Heyes, L.B. Jeffs, M. John, A.D. Judge, K. Lam, K. McClintock, L.V. Nechev, L.R. Palmer, T. Racie, I. Rohl, S. Seiffert, S. Shanmugam, V. Sood, J. Soutschek, I. Toudjarska, A.J. Wheat, E. Yaworski, W. Zedalis, V. Koteliansky, M. Manoharan, H.P. Vornloche, I. MacLachlan, RNAi-mediated gene silencing in non-human primates, Nature 441 (2006) 111–114.

[29] M. Schlee, V. Hornung, G. Hartmann, siRNA and isRNA: two edges of one sword, Mol. Ther. 14 (2006) 463–470.

[30] R.E. Lanford, D. Chavez, B. Guerra, J.Y.N. Lau, Z. Hong, K.M. Brasky, B. Beames, Ribavirin induces error-prone replication of GB virus B in primary tamarin hepatocytes, J. Virol. 75 (2001) 8074–8081.

[31] K. Li, Z. Chen, N. Kato, M. Gale Jr., S.M. Lemon, Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes, J. Biol. Chem. 280 (2005) 16739–16747.

JOURNAL OF VIROLOGY, Aug. 2007, p. 8030–8040 Vol. 81, No. 15
0022-538X/07/$08.00+0 doi:10.1128/JVI.02088-06
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The NS3 Helicase and NS5B-to-3′X Regions Are Important for Efficient Hepatitis C Virus Strain JFH-1 Replication in Huh7 Cells[▽]

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Received 23 September 2006/Accepted 8 May 2007

The JFH-1 strain of hepatitis C virus (HCV) is a genotype 2a strain that can replicate autonomously in Huh7 cells. The J6 strain is also a genotype 2a strain, but its full genomic RNA does not replicate in Huh7 cells. However, chimeric J6/JFH-1 RNA that has J6 structural-protein-coding regions and JFH-1 nonstructural-protein-coding regions can replicate autonomously and produce infectious HCV particles. In order to determine the mechanisms underlying JFH-1 RNA replication, we constructed various J6/JFH-1 chimeras and tested their RNA replication and virus particle production abilities in Huh7 cells. Via subgenomic-RNA-replication assays, we found that both the JFH-1 NS5B-to-3′X (N5BX) and the NS3 helicase (N3H) regions are important for the replication of the J6CF replicon. We applied these results to full-length genomic RNA replication and analyzed replication using Northern blotting. We found that a chimeric J6 clone with JFH-1 N3H and N5BX could replicate autonomously but that a chimeric J6 clone with only JFH-1 N5BX had no replication ability. Finally, we tested the virus production abilities of these clones and found that a chimeric J6 clone with JFH-1 N3H and N5BX could produce infectious HCV particles. In conclusion, the JFH-1 NS3 helicase and NS5B-to-3′X regions are important for efficient replication and virus particle formation of HCV genotype 2a strains.

Hepatitis C virus (HCV) is a major cause of chronic liver disease (7, 22). The lack of a robust cell culture system for producing virus particles has hampered the development of HCV research (2). Although the development of a subgenomic-replicon system enabled research into HCV RNA replication (32), infectious-virus-particle production remained impossible. Recently, an HCV cell culture system was developed using a JFH-1 genotype 2a strain of HCV cloned from a fulminant hepatitis patient (30, 48, 54), allowing investigation of the virus life cycle.

HCV is a positive-strand RNA virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family. The HCV genome comprises about 9,600 nucleotides that encode a single polyprotein of around 3,000 amino acids (8, 18, 44), which is processed by cellular and viral encoded proteases into at least 10 different structural and nonstructural proteins (11, 13, 14, 33).

The JFH-1 strain of HCV is a genotype 2a strain, and it is the first HCV strain that can produce HCV particles in Huh7 cells (48). Subgenomic replicons of JFH-1 replicate efficiently in Huh7 cells and do not require cell culture-adaptive mutations (19). The J6CF strain of HCV is also a genotype 2a strain and is known to be infectious in chimpanzees (49), but its entire genomic RNA does not replicate in Huh7 cells, despite the ~90% nucleotide sequence homology between JFH-1 and J6CF. However, J6/JFH-1 chimeric RNA that has J6 structural-protein-coding regions and JFH-1 nonstructural-protein-coding regions can replicate autonomously and produce infectious HCV particles (30, 39). Why only the JFH-1 clone can replicate efficiently in Huh7 cells remains unclear.

In this study, to investigate the mechanisms underlying efficient JFH-1 replication, we focused on the differences in replication between JFH-1 and J6CF strains by using intragenotypic JFH-1 and J6CF chimeras and compared their respective abilities to replicate RNA and produce virus particles in Huh7 cells.

MATERIALS AND METHODS

Cell culture. Huh7 cells (36) were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under 5% CO_2 conditions.

Subgenomic-replicon constructs. pSGR-JCH1 and pSGR-JCH4 were constructed based on pSGR-JFH1 (19, 21). pSGR-J6CF was also constructed from pJ6CF (a kind gift from Jens Bukh) (49), using the same method used to construct pSGR-JFH1. Plasmids used in luciferase assays were constructed based on pSGR-JFH1/Luc (20). Chimeric replicons were constructed by substitution of the corresponding regions. For convenience, several restriction enzyme recognition sites (ClaI [2275], EcoT22I [3639], and BsrGI [6127]) were introduced into the pSGR-J6CF sequence via nucleotide substitutions. The substitutions of the corresponding regions were achieved as follows, with the 5′ untranslated region (5′UTR) inserted between NotI and AgeI: NS3, PmeI-EcoT22I; NS3 protease, PmeI-ClaI; NS3 helicase, ClaI-EcoT22I; NS4, EcoT22I-MunI; NS5A, MunI-BsrGI; NS5B, BsrGI-StuI; and 3′UTR, StuI-XbaI (see Fig. 2A and 3A). pSGR-JCH1/Luc and pSGR-JCH4/Luc were also constructed using the same procedure as that for pSGR-JFH1/Luc (20, 21). The Con1 replicon (pSGR-Con1/Luc) was

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▽ Published ahead of print on 23 May 2007.

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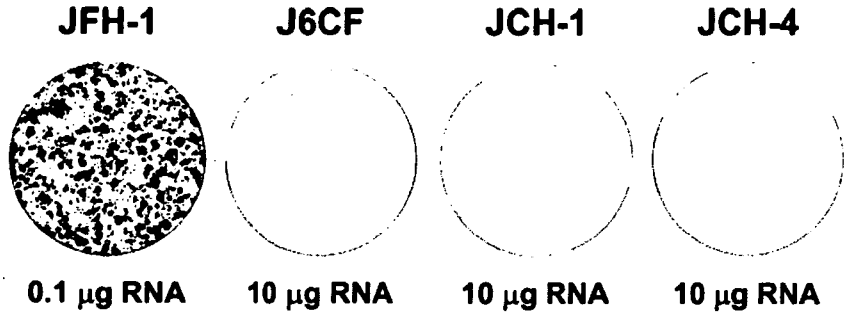

FIG. 1. G418-resistant colony formation of JFH-1, J6CF, JCH-1, and JCH-4. Subgenomic RNAs were synthesized in vitro, using pSGR-JFH1, pSGR-J6CF, pSGR-JCH1, and pSGR-JCH4 as templates. Transcribed subgenomic RNAs were electroporated into Huh7 cells, and cells were cultured with G418 for 3 weeks before staining with crystal violet as described in Materials and Methods. JFH-1 subgenomic RNA (0.1 μg) and 10 μg of J6CF, JCH-1, and JCH-4 subgenomic RNAs were transfected into Huh7 cells. Experiments were performed in triplicate, and representative staining examples are shown.

constructed from pFK-I389/neo/NS3-3′/wt (a kind gift from Ralf Bartenschlager) (32), and the H77c replicon (pSGR-H77c/Luc) was constructed from pCV-H77c (a kind gift from Robert H. Purcell) (50). For convenience, ClaI (2275) and BsrGI (6127) recognition sites were introduced into the pSGR-Con1/Luc and pSGR-H77c/Luc sequences via nucleotide substitutions. Substitutions of the NS3 helicase region and N5BX regions were performed as described above.

Full-length genomic HCV constructs. Plasmids used in the analysis of genomic RNA replication were constructed based on pJFH1 (48) and pJ6CF (49). For convenience, several restriction enzyme recognition sites (ClaI [3929], EcoT22I [5293], and BsrGI [7781]) were introduced into the J6CF sequence via nucleotide substitutions. Substitutions of the NS3 helicase regions were performed by replacement of the ClaI-EcoT22I fragment, substitutions of the N5BX regions were performed by replacement of the BsrGI-XbaI fragment, substitutions of the N5B regions were performed by replacement of the BsrGI-StuI fragment, and a substitution of the 3′UTR was performed by replacement of the StuI-XbaI fragment (see Fig. 5A).

RNA synthesis and transfection. RNA synthesis and transfection were performed as described previously (48). In brief, plasmids were linearized with XbaI, treated with mung bean nuclease (New England Biolabs, Ipswich, MA), and purified. Linearized, purified DNAs were used as templates for in vitro RNA synthesis using a MEGAscript T7 kit (Ambion, Austin, TX) in accordance with the manufacturer's instructions. Synthesized RNA was treated with DNase I (Ambion), followed by purification using ISOGEN-LS (Nippon Gene, Tokyo, Japan). The quality of synthesized RNA was examined by agarose gel electrophoresis. Ten micrograms of in vitro-synthesized RNA was used for each electroporation. Trypsinized Huh7 cells (3×10^6 cells) were washed with Opti-MEM I (Invitrogen, Carlsbad, CA) and resuspended in Cytomix buffer (47). RNA was mixed with 400 μl of cell suspension, and the mixture was then transferred to an electroporation cuvette (Precision Universal Cuvettes, Thermo Hybaid, Middlesex, United Kingdom). The cells were then pulsed at 260 V and 950 μF using a Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were immediately transferred to 10-cm culture dishes or six-well plates, each containing culture medium, and incubated at 37°C under 5% CO_2. Luciferase mRNA was synthesized from luciferase T7 control DNA (Promega, Madison, WI) by using a mMESSAGE mMACHINE T7 kit (Ambion). To monitor transfection efficiency, in vitro-synthesized luciferase RNA was cotransfected with HCV RNA and luciferase activity measured at 4 h after transfection.

G418-resistant colony formation assay. The G418-resistant colony formation assay was performed as described previously (19). In brief, 0.1 μg or 10 μg of transcribed RNAs was transfected into 3×10^6 Huh7 cells by electroporation. Transfected cells were immediately transferred to 10-cm culture dishes containing 10 ml of culture medium. G418 (1.0 mg/ml) (Nakalai Tesque, Kyoto, Japan) was added to the culture medium at 16 to 24 h after transfection. Culture medium supplemented with G418 was replaced every 3 days. Three weeks after transfection, cells were fixed with buffered formalin and stained with crystal violet.

Luciferase reporter assay. The luciferase activities of the JFH-1 subgenomic replicon and chimeras in Huh7 cells were measured as described previously (20). Briefly, 5 μg of transcribed RNAs was transfected into 3×10^6 Huh7 cells by electroporation. Transfected cells were immediately resuspended in culture medium and seeded into six-well culture plates. Cells were harvested serially at 4, 24, and 48 h after transfection and lysed with 200 μl of cell culture lysis reagent (Promega). Debris was then removed by centrifugation. Luciferase activity was quantified using a Lumat LB9507 luminometer (EG & G Berthold, Bad Wildbad, Germany) and a luciferase assay system (Promega). Assays were performed three times independently, with each value corrected for transfection efficiency as determined by measuring luciferase activity 4 h after transfection. The data are expressed as relative luciferase units (RLU).

Quantification of HCV core protein. To estimate the concentration of HCV core protein in the culture medium, we performed an HCV core enzyme-linked immunosorbent assay (Ortho-Clinical Diagnostics, Tokyo, Japan) in accordance with the manufacturer's instructions.

Northern blot analysis. Northern blot analysis was performed as described previously (48). In brief, total cellular RNA from HCV RNA-transfected cells was extracted using ISOGEN (Nippon Gene) in accordance with the manufacturer's instructions. Isolated RNA (2 μg) was separated on a 1% agarose gel containing formaldehyde, transferred to a Hybond N+ positively charged nylon membrane (GE Healthcare, Piscataway, NJ), and immobilized using a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). Hybridization was performed with [α-^{32}P]dCTP-labeled DNA by using Rapid-Hyb buffer (GE Healthcare). The DNA probe was synthesized using the NS5B-to-3′X fragment of JFH1 excised from pJFH1 by BsrGI and XbaI and labeled using the Megaprime DNA labeling system (GE Healthcare).

Infection of cells with secreted HCV and determination of infectivity. Culture medium from RNA-transfected cells was collected at 72 h posttransfection. Huh7 cells were seeded at a density of 1×10^4 cells per well in poly-D-lysine-coated 96-well plates (CORNING, Corning, NY). On the following day, the collected culture media were serially diluted and used for inoculation of the seeded cells, and the plates were incubated for another 3 days at 37°C. The cells were fixed in methanol for 15 min at −20°C, and the infected foci were visualized by immunofluorescence as described below.

Cells were blocked for 1 h with BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) supplemented with 0.3% Triton X-100 and then washed with phosphate-buffered saline, followed by incubation with anti-core antibody at 50 μg/ml in BlockAce. After incubation for 1 h at room temperature, the cells were washed and incubated with a 1:400 dilution of AlexaFluor 488-conjugated anti-mouse immunoglobulin G (Molecular Probes, Eugene, OR) in BlockAce. The cells were then washed and examined using fluorescence microscopy (Olympus, Tokyo, Japan). Infectivity was quantified by counting the infected foci and expressed as numbers of focus-forming units per milliliter (FFU/ml).

RESULTS

G418-resistant colony formation of JFH-1, J6CF, and other genotype 2a subgenomic replicons. First, to compare the replication efficiencies of the JFH-1 and J6CF strains, we performed a G418-resistant colony formation assay with JFH-1 and J6CF RNAs by using subgenomic replicons. The JFH-1 subgenomic replicon formed many colonies with transfection of only 0.1 μg RNA, but the J6CF subgenomic replicon formed no colonies, even with transfection of 10 μg RNA (Fig. 1). We also tested

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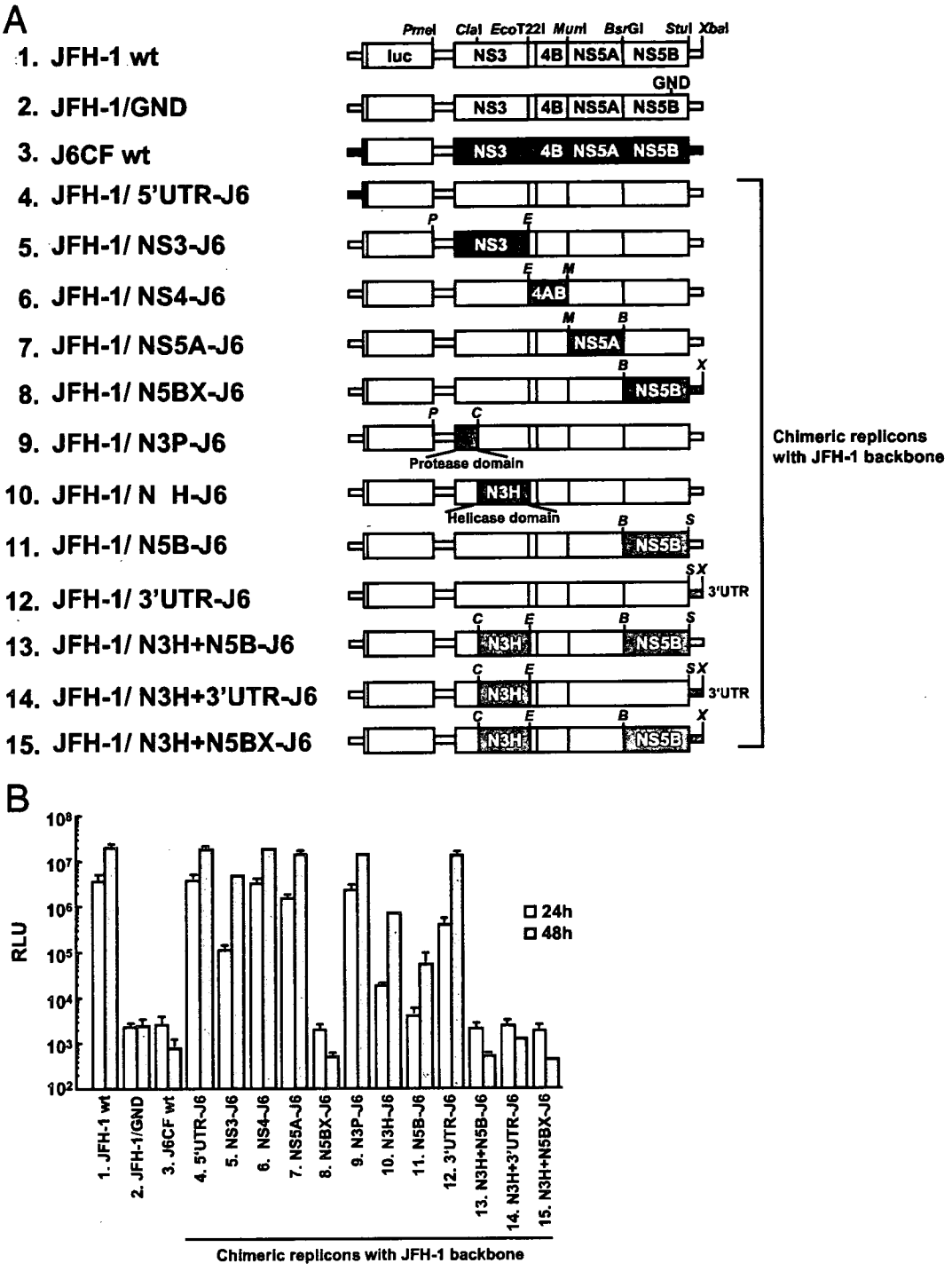

FIG. 2. Luciferase activities of chimeric replicons with a JFH-1 backbone. (A) Structures of chimeric subgenomic replicons with a JFH-1 backbone. The restriction enzyme recognition sites used for the construction of plasmids are indicated. *P*, PmeI; *C*, ClaI; *E*, EcoT22I; *M*, MunI; *B*, BsrGI; *S*, StuI; *X*, XbaI; wt, wild type. (B) Subgenomic RNAs were synthesized in vitro from wild-type or chimeric replicon constructs. Transcribed subgenomic RNAs (5 μg) were electroporated into Huh7 cells, and cells were harvested serially at 4, 24, and 48 h after transfection. The harvested cells were lysed, and then luciferase activities in the cell lysates were measured. The assays were performed three times independently and the results expressed as luciferase activities (RLU). Each value was corrected for transfection efficiency as determined by measuring the luciferase activity 4 h after transfection. Data are presented as means and standard deviations for luciferase activity at 24 h (white bars) and 48 h (gray bars) after transfection.

other genotype 2a clones (the JCH-1 and JCH-4 strains), which were isolated from patients with chronic hepatitis C (21). Their subgenomic replicons did not form colonies either. Given that chimeric J6/JFH-1 RNA that has the J6 structural-protein-coding regions and JFH-1 nonstructural-protein-coding regions reportedly replicates autonomously and produces infectious HCV particles (30, 39), we hypothesized that some of the JFH-1 nonstructural-protein-coding regions are important for JFH-1 replication.

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Regions of JFH-1 essential for replication. In order to determine which regions of JFH-1 are important for JFH-1 RNA replication, we constructed a series of chimeric JFH-1 subgenomic replicons replacing the 5′UTR, NS3, NS4AB, NS5A, and NS5B-to-3′X (N5BX) regions from the J6CF strain and tested their replication abilities. For this analysis, we adopted luciferase replicon systems (20) because colony formation assays are time-consuming to perform and it is difficult to evaluate precise replication levels using this method. Furthermore, efficient JFH-1 RNA replication may reduce cellular growth, thus affecting colony formation efficiency (34). We constructed JFH-1 chimeric subgenomic luciferase replicons with the J6CF clone because this clone was reportedly infectious in a chimpanzee (49). However, the JCH-1 and JCH-4 clones were not tested for infectivity. The 5′UTR, NS3, NS4AB, NS5A, or N5BX sequences of the JFH-1 replicon were replaced by J6CF sequences (5′UTR-J6, NS3-J6, NS4-J6, NS5A-J6, or N5BX-J6, respectively [Fig. 2A]). The luciferase activities of these replicons are shown in Fig. 2B. The JFH-1 subgenomic replicon replicated efficiently and had a luciferase activity of approximately 10^7 RLU (Fig. 2B, JFH-1 wt). GND, which was replication incompetent because of a mutation at the GDD motif in the NS5B region, had a luciferase activity of only 10^3 RLU (Fig. 2B, JFH-1/GND), which was taken as the background level. The J6CF subgenomic replicon did not replicate and had the same luciferase activity as GND (Fig. 2B, J6CF wt). Replacement of the 5′UTR, NS4AB, and NS5A sequences of JFH-1 by J6CF sequences (5′UTR-J6, NS4-J6, and NS5A-J6, respectively) did not reduce replication (Fig. 2B, 5′UTR-J6 and NS4-J6) or reduced it only slightly (Fig. 2B, NS5A-J6). However, there was no replication for the JFH-1 chimera with J6 N5BX (Fig. 2B, N5BX-J6). In addition, the JFH-1 chimera with the J6 NS3 region (NS3-J6) had a replication level that was more than 10-fold lower at 24 h and around 10-fold lower at 48 h than that of the wild-type JFH-1 replicon (Fig. 2B, JFH-1 wt and NS3-J6). These data show that the JFH-1 NS5B-to-3′X region is essential for JFH-1 RNA replication and indicate that the JFH-1 NS3 region is also important for JFH-1 RNA replication.

Involvement of the NS3 helicase region in efficient JFH-1 replication. The JFH-1 chimera with the J6 NS3 region (NS3-J6) reduced the replication level (Fig. 2B, NS3-J6). The NS3 protein is known to have two domains: a protease domain at the amino terminal one-third and a helicase domain at the carboxyl terminal two-thirds. To determine which region is important for replication, we compared the replication activity of a JFH-1 chimera with that of the NS3 protease-coding region of J6CF (N3P-J6) and that of a JFH-1 chimera with that of the NS3 helicase-coding region of J6CF (N3H-J6) (Fig. 2A, JFH-1/N3P-J6 and JFH-1/N3H-J6). Although N3P-J6 had the same luciferase activity as JFH-1, N3H-J6 had lower activity than JFH-1 (Fig. 2B, N3P-J6 and N3H-J6). These data show that the JFH-1 NS3 helicase-coding region has an important role in JFH-1 replication.

Importance of the JFH-1 NS5B-coding region and 3′UTR in replication. The JFH-1 chimera with J6 N5BX completely abolished replicon replication (Fig. 2B, N5BX-J6). The N5BX region contains two regions, the NS5B protein-coding region and the 3′UTR. The NS5B protein-coding region encodes RNA-dependent RNA polymerase. To analyze which region of N5BX is important for replication, we separated N5BX into two regions, that is, the NS5B-coding region and the 3′UTR. JFH-1 replicons with NS5B or with the 3′UTR of J6 were constructed (Fig. 2A, JFH-1/N5B-J6 and JFH-1/3′UTR-J6) and their replication abilities analyzed. The replication level of JFH-1/N5B-J6 was reduced more than 100-fold compared with that of the wild-type JFH-1 replicon at 48 h (Fig. 2B, N5B-J6). JFH-1/3′UTR-J6 replicated similarly to JFH-1 at 48 h, but the replication activity at 24 h was reduced more than 10-fold compared with that of the original JFH-1 replicon (Fig. 2B, 3′UTR-J6). These data indicate that the NS5B-coding region and the 3′UTR of JFH-1 are both involved in efficient JFH-1 replication.

Rescue of J6CF replicon replication by incorporation of the JFH-1 sequences. Because the JFH-1 N5BX region appeared to be essential for JFH-1 replication (Fig. 2B, N5BX-J6), we tested whether JFH-1 N5BX could restore the replication of J6CF RNA. We constructed a chimeric J6CF subgenomic replicon containing the JFH-1 N5BX region (Fig. 3A, J6/N5BX-JFH1) and tested its replication abilities. The luciferase activity of J6CF subgenomic RNA was recovered by inclusion of JFH-1 N5BX (Fig. 3B, N5BX-JFH1), but this chimeric replicon showed lower replication activity than the original JFH-1 replicon (Fig. 3B, JFH-1 wt). Furthermore, J6CF replication was not restored by only JFH-1 NS5B (J6/N5B-JFH1) or only the 3′UTR (J6/3′UTR-JFH1) (Fig. 3B, N5B-JFH1 or 3′UTR-JFH1, respectively). These observations clearly indicate that the JFH-1 NS5B-to-3′X region is essential, and the NS5B-coding region and 3′UTR are both important for efficient RNA replication in Huh7 cells. However, other JFH-1 regions are also involved in efficient replication.

The JFH-1 NS3 helicase-coding region was also important for efficient replication, and we thus tested whether the JFH-1 NS3 helicase region by itself could restore J6CF replication (as occurred for the JFH-1 N5BX region). Insertion of only the NS3 helicase region of JFH-1 into J6CF (Fig. 3A, J6/N3H-JFH1) did not restore replication (Fig. 3B, N3H-JFH1). However, replication of the J6 chimeric replicon seemed considerably restored by insertion of JFH-1 NS5B or the 3′UTR in addition to the NS3 helicase-coding region (Fig. 3B, N3H+N5B-JFH-1 or N3H+3′UTR-JFH-1, respectively) and fully restored by insertion of the JFH-1 NS3 helicase region and JFH-1 N5BX region (Fig. 3B, N3H+N5BX-JFH1). These results indicate that the JFH-1 N5BX region is essential for subgenomic-replicon replication and that the JFH-1 NS3 helicase-coding region has an additional role in replication. This was also confirmed by analysis of the replication abilities of JFH-1 replicons with double substitutions of J6CF (Fig. 2A, JFH-1/N3H+N5B-J6, JFH-1/N3H+3′UTR-J6, and JFH-1/N3H+N5BX-J6). Neither of these chimeric JFH-1 replicons replicated (Fig. 2B, N3H+N5B-J6, N3H+3′UTR-J6, and N3H+N5BX-J6).

The NS3 helicase and NS5B-3′X regions of JFH-1 can restore the replication of other genotype 2a replicons but not of genotype 1 replicons. To test whether the JFH-1 NS3 helicase and N5BX regions could restore other HCV replicon replication, chimeric replicon constructs N3H-JFH1, N5BX-JFH1, and N3H+N5BX-JFH1 were constructed using two genotype 2a replicons (JCH-1 and JCH-4), a genotype 1a replicon (H77c), and a genotype 1b replicon (Con1), respectively. The

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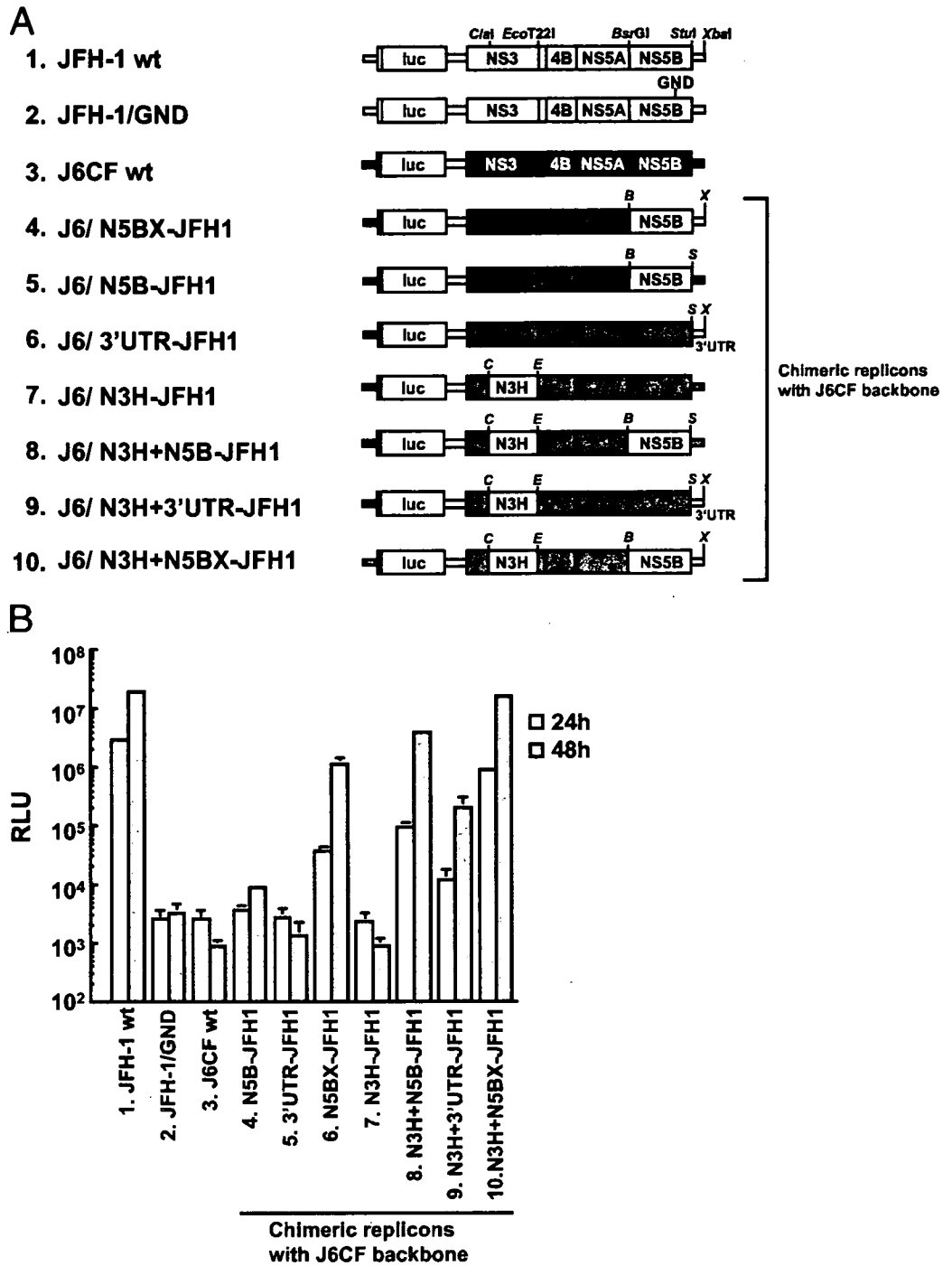

FIG. 3. Luciferase activities of chimeric replicons with a J6CF backbone. (A) Structures of chimeric subgenomic replicons with a J6CF backbone. The restriction enzyme recognition sites used for the construction of plasmids are indicated. *C*, ClaI; *E*, EcoT22I; *B*, BsrGI; *S*, StuI; *X*, XbaI; wt, wild type. (B) Wild-type or chimeric subgenomic RNAs were transfected into Huh7 cells, and the luciferase activities of the transfected cells were examined as described in the legend to Fig. 2B. Assays were performed three times independently, and data are presented as means and standard deviations for luciferase activity (RLU) at 24 h (white bars) and 48 h (gray bars) after transfection.

replication level of each wild-type and chimeric replicon was evaluated by luciferase activity measurement after transient transfection of replicon RNA. No replication of any of the wild-type replicons (Fig. 4, JCH-1 wt, JCH-4 wt, H77c wt, and Con1 wt) or of any of the replicons with insertion of the JFH-1 NS3 helicase region (Fig. 4, JCH-1/N3H-JFH1, JCH-4/N3H-JFH1, H77c/N3H-JFH1, and Con1/N3H-JFH1) was detected. However, genotype 2a replicons with insertion of the JFH-1 N5BX region increased their replication levels severalfold at 48 h (Fig. 4, JCH-1/N5BX-JFH1 and JCH-4/N5BX-JFH1). Furthermore, insertion of both the N3H and the N5BX regions increased the JCH-1 replication over 10-fold compared to that

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