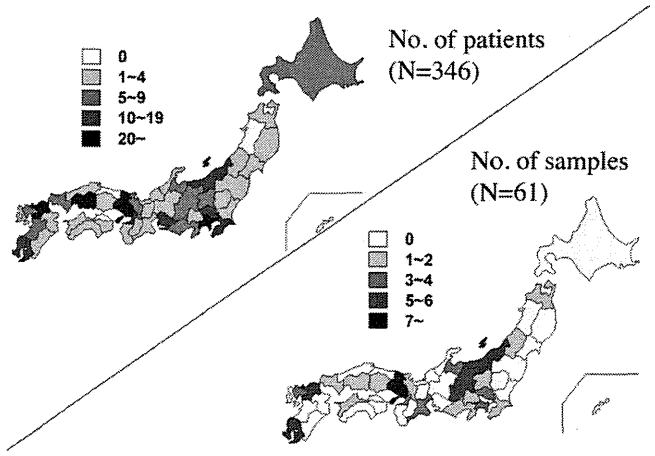

Fig. 1. Geographical distribution of acute hepatitis A patients and collected samples in Japan in 2010.

2. Objectives

The primary objective of this study was to investigate the causes of the 2010 HAV resurgence in Japan by using molecular epidemiological and genetic analyses. This study, performed in collaboration with local institutes of public health, is expected to provide insights useful for setting appropriate public health guidelines for HAV control.

3. Study design

3.1. Data collection

We collected stool and plasma specimens from 98 acute hepatitis A patients in collaboration with 28 local institutes of health in Japan. The collection sites were located at 22 different prefectures (regions in Japan) (Fig. 1).

3.2. RNA extraction, RT-PCR and phylogenetic analysis

A 10% fecal suspension (wt/vol) was prepared with phosphate-buffered saline (PBS; pH 7.2) and centrifuged at 10,000 × *g* for 10 min. Viral RNA was extracted from the fecal suspension or sera by using a QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription (RT) was performed with the SuperScript III cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Seven microliters of the purified RNA was added to a reaction mixture (final volume, 20 μl) containing 50 pmol of random hexamer, 25 mM $MgCl_2$ buffer, 10 mM deoxynucleotide triphosphates, 10× RT buffer, 0.1 M dithiothreitol, and 200 U SuperScript III RT. The mixture was incubated at 42 °C for 1 h, after which 10 U of RNase H was added at 37 °C for 20 min.

Four degenerate primers (P1 to P4) were used in PCR to amplify the VP1/2A region of the HAV genome.[1] The sequences of these primers were:

HAV-2799 (5′-ATTCAGATTAGACTGCCTTGGTA-3′)
HAV-2907 (5′-GCAAATTACAATCATTCTGATGA-3′)
HAV-3162 (5′-CTTCYTGAGCATACTTKARTCTTTG-3′)
HAV-3273 (5′-CCAAGAAACCTTCATTATTTCATG-3′)

PCR was carried out using the HAV-2799 and HAV-3273 primer pair, followed by nested PCR with the HAV-2907 and HAV-3162 primer pair. PCR was performed with EX-taq (Takara, Shiga, Japan) according to the manufacturer's instructions. Amplification was performed for 40 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 15 min. Three microliters of the PCR product was used as the template for a second round of PCR amplification under the same conditions. The PCR product was purified with the QIAquick PCR Purification Kit (Qiagen) and used as a template for direct sequencing.

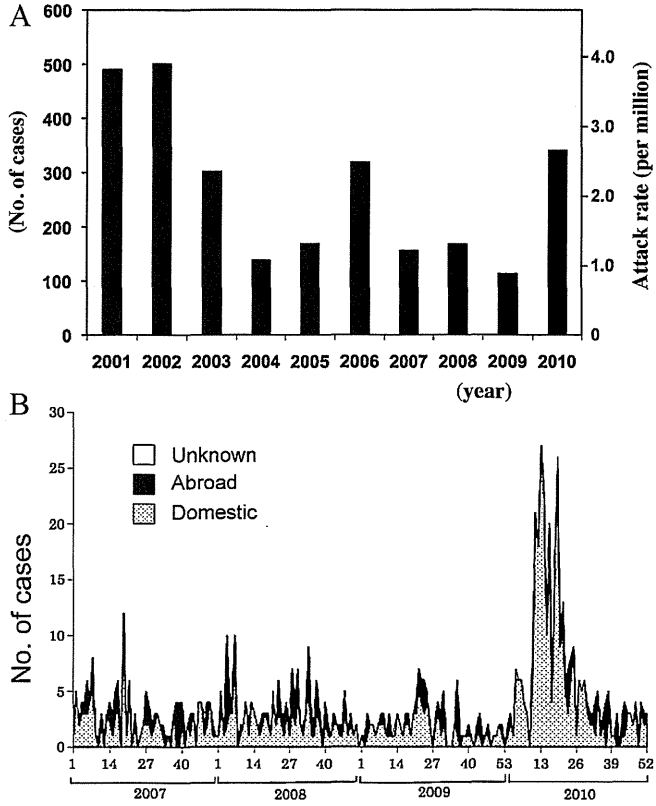

Fig. 2. (A) Reported number of acute hepatitis A patients in Japan from 2001 through 2010. The increase of the number in 2010 was statistically significant compared with the number in 2007 ($t = 5.4 \times 10^{-}$), 2008 ($t = 5.6 \times 10^{-5}$) and 2009 ($t = 1.8 \times 10^{-5}$). (B) Weekly acute hepatitis A cases from week 1 of 2007 to week 52 of 2010.

Phylogenetic trees were constructed with the MEGA software (DNA DATA Bank of Japan) by the neighbor-joining method from a Kimura two-parameter distance matrix, and bootstrap values were determined from 1000 bootstrap re-samplings of the original data.[19–22] All reference sequences used in this study were obtained from GenBank.

4. Results

In 2010, the number of acute hepatitis A cases increased to 346 (2.71 per million) (Fig. 2A) because of a diffuse outbreak that occurred from March through May (Fig. 2B). Most of the patients in this outbreak reflected domestic infection events (Fig. 2B). Clinical descriptions of these patients are summarized in Table 1.

Sera and fecal samples from 98 patients were available for PCR. Of these, 61 yielded a PCR product that could be used for sequencing. Among these 61 isolates, 44 were of genotype IA, one was of genotype IB and 16 were of genotype IIIA by phylogenetic analysis (Fig. 3). The genotype IA isolates could be sorted into two sub-lineages. One sub-lineage (referred to as IA-1 in this paper) grouped with several isolates found in 2006,[23–25] suggesting that the isolates in this lineage were endemic to Japan. In contrast, the sequences of most of the genotype IA isolates belonged to a second sub-lineage (referred to as IA-2 in this paper) with sequences almost identical to one another. Among the IA-2-infected patients, two had developed acute hepatitis shortly after returning from Philippines,

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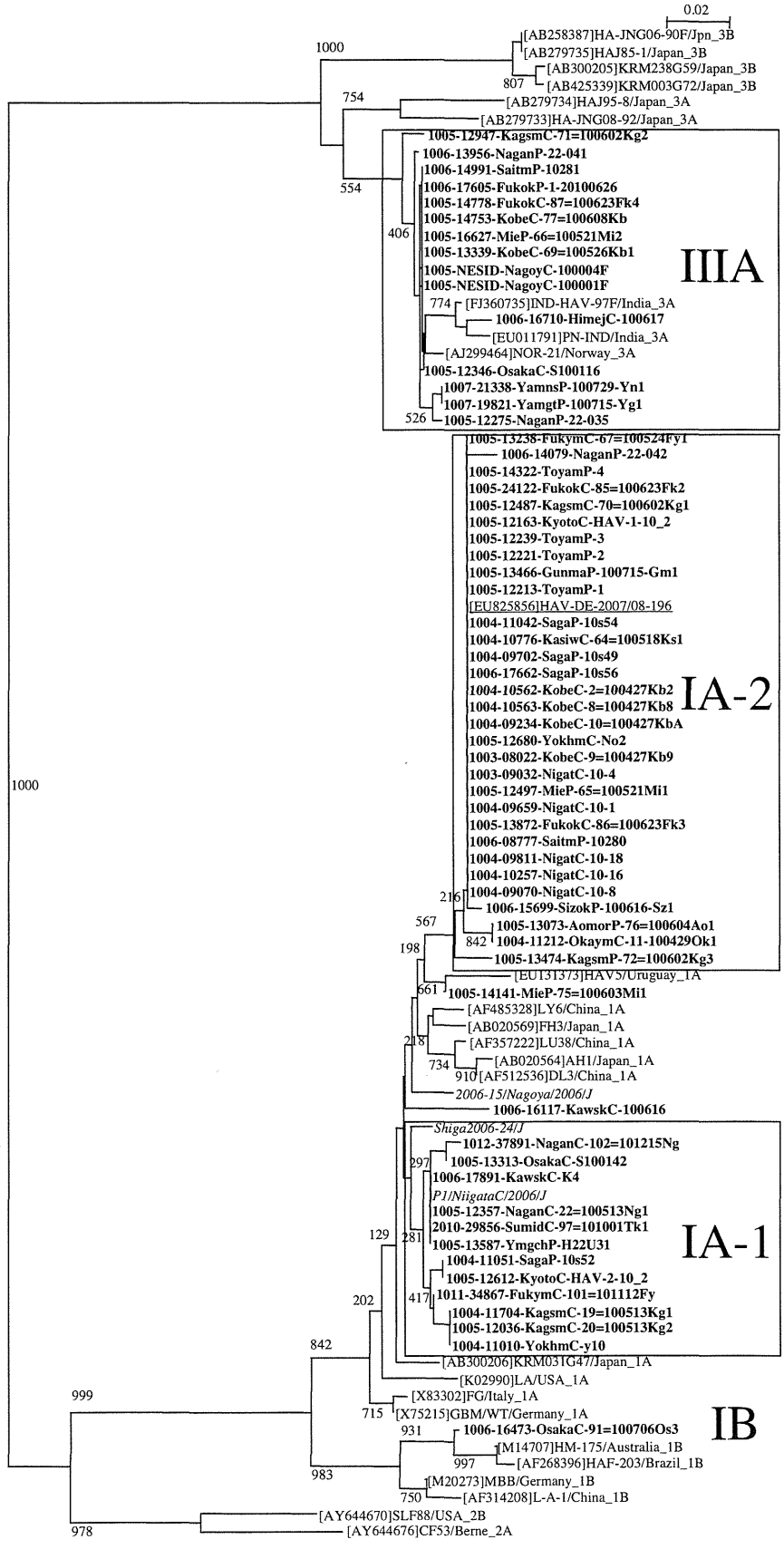

Fig. 3. Neighbor-joining phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from hepatitis A virus isolates. Reference strains are used in this study and indicated as GenBank accession numbers. Sequences of 61 isolates from this study are shown as YYMM-NESID-KKKKKKKKK (YYMM represents the reported year (YY) and month (MM); NESID (National Epidemiological Surveillance of Infectious Diseases) is the ID number of the patient; KKKKKKKKK is the name of the isolate given by local institute). The scale bar at the bottom indicates nucleotide distance. Numbers at the branches show boostrap percentages obtained after 1000 replications of bootstrap sampling.

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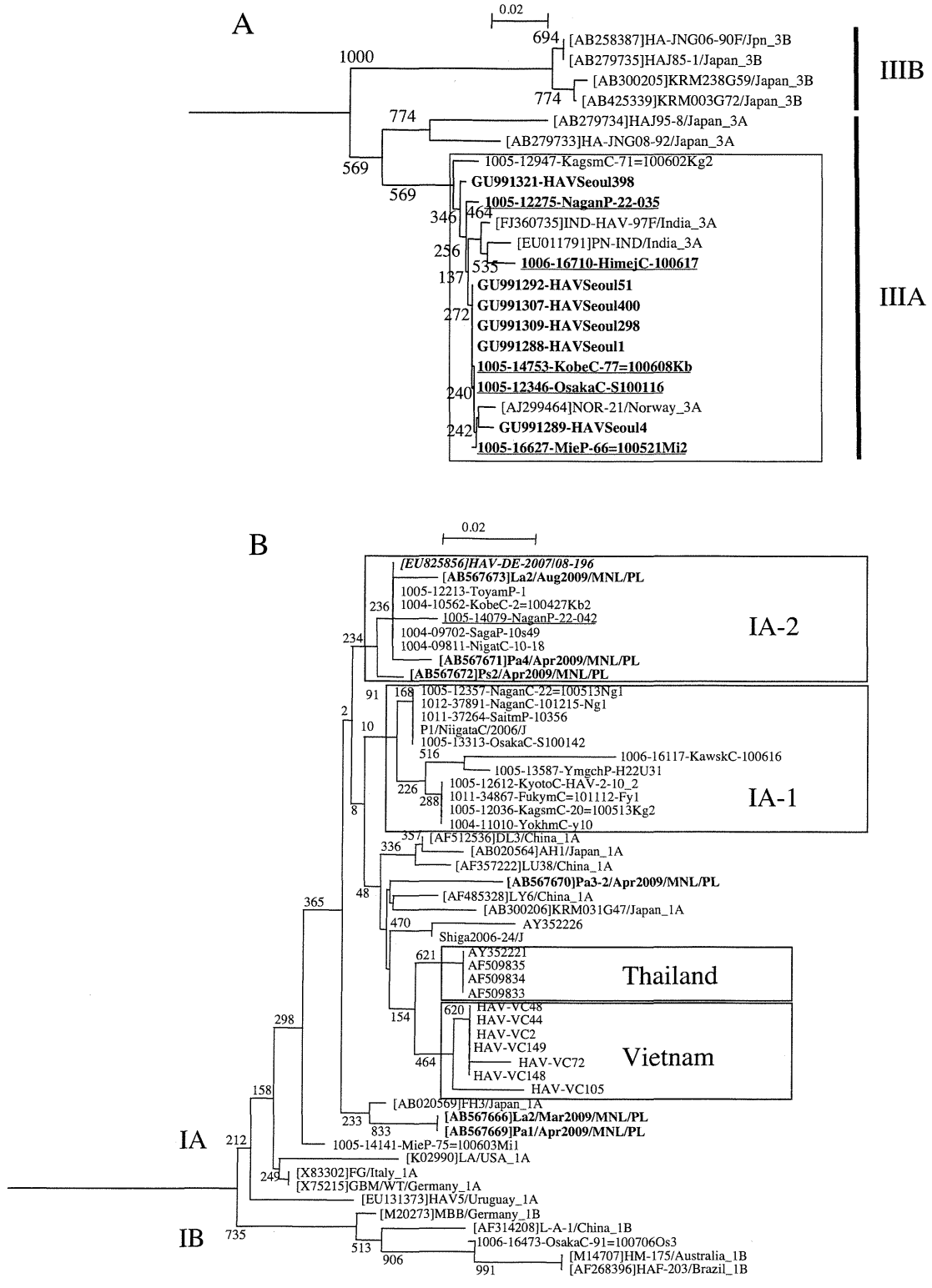

Fig. 4. (A) Phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from HAV strains (genotype IIIA) isolated from Japan (bold underline) and Korea (bold). Numbers at the branches show boostrap percentages obtained after 1000 replications of bootstrap sampling. (B) Phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from hepatitis A virus strains (genotype IA) isolated from Japan, Thailand, Vietnam and river and sewage from Philippines (shown in bold). HAV sequences of Japanese patients who developed acute hepatitis shortly after travel to Philippines are underlined. HAV-DE-2007/08-196 is shown in italics. In IA-2 sub-lineage, 26 identical sequences are represented by four sequences (1005-12213, 1004-10562, 1004-09702, 1004-09811). Numbers at the branches show boostrap percentages obtained after 1000 replications of bootstrap sampling.

suggesting the relationship of this lineage with HAV viruses from that geographical source.

A slightly different region of VP1-2A (nt: 2975–3364) was used for phylogenetic analysis in South Korea (Yoo et al., unpublished, available on GenBank) compared to the region of VP1-2A (nt: 2930–3161) used in the present study. Unfortunately, the overlap between these sequences was not long enough for comparison between the two studies. To permit such a comparison, we

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Table 1
Clinical descriptions of hepatitis A cases during diffuse outbreak period (from 10th to 28th week of 2010, 236 cases).

Items	Data
Age (median)	5–88 yr (48 yr)
Sex	Male 138 (58%), female 98 (42%)
Suspected infection route	Fecal–oral 199 (84%), others/unknown 37 (16%)
Suspected food vehicle	Oyster 58 (29%), fishery product 27 (14%), well water/tap water in foreign country 4 (2%), others/unknown 46 (23%), unnoted 64 (32%)
Icteric	171 (72%)
Fulminant (severe) hepatitis	6 (3%)
Diagnosed by	IgM 223 (94%), PCR 2 (1%), IgM and PCR 11 (5%)

sequenced VP1-2A fragments (nt: 2822–3272) generated by the first PCR reaction on some of the Japanese genotype IIIA strains. These sequences were compared with Korean genotype IIIA strains. Phylogenetic analysis revealed that the Japanese and Korean genotype IIIA isolates could be classified into a single cluster (Fig. 4A). This observation suggests a close relationship between the Japanese genotype IIIA strains and those derived from the recent Korean outbreak.

5. Discussion

In recent years, the incidence of hepatitis A in developed countries has decreased dramatically. Changes in the genotypes or subtypes of HAV strains, including the emergence of HAV strains that are new to the area, have been observed in patients with acute hepatitis A in developed countries,[26] probably due to the transport of HAV strains via international transport of foods and agricultural products. HAV strains also could be imported by unvaccinated human carriers who have traveled to endemic countries. National surveillance of HAV in Japan has shown that more than 90% of people over 65 years of age, but fewer than 10% of people under 34 years of age, are seropositive for HAV.[27] Most of the infections that have occurred in Japan represent sporadic events, with exceptional occurrences of small-scale outbreaks. In 2010, however, there was a spike of hepatitis A infections in Japan, with 346 cases reported by the Infectious Disease Surveillance Center, NIID.

One of the genotype IA sub-lineages (referred to as IA-1 in this paper) was related to an isolate found in small outbreaks in Shiga and Niigata prefectures in 2006.[23,24] The isolates belonging to this sub-lineage have been detected in Japan since at least 2001 (Tamada and Yano, personal communication), suggesting that the isolates of this sub-lineage were locally endemic strains of Japan. On the other hand, more than half of genotype IA isolates displayed identical or virtually identical sequences across a 230-nt interval of the VP1-2A segment of the genome. Among the isolates in this sub-lineage (IA-2 in this paper), two (Fig. 4B, underlined) were from patients who had recently visited the Philippines, suggesting a relationship between IA-2 sub-lineage and this geographical site. This sequence also was found to be identical to HAV-DE-2007/08-196 (Fig. 4B, italics), which was identified in Germany in 2007.[28] The patient of HAV-DE-2007/08-196 was an 11-year old female who developed acute hepatitis shortly after traveling to the Philippines (Faber et al., personal communication). To assess this proposal, we also obtained sequence data for HAV derived from river and sewage of Manila and included these sequences in our phylogenetic analysis (Fig. 4B; HAV from river and sewage of Manila are shown in bold). Some sequences classified with the IA-2 sub-lineage, supporting the hypothesized Philippine connection. Genotype IA isolates of HAV from other Southeast Asian countries, such as Vietnam[29] and Thailand,[30] formed distinct clusters (Fig. 4B). However, caution is necessary with this result, because

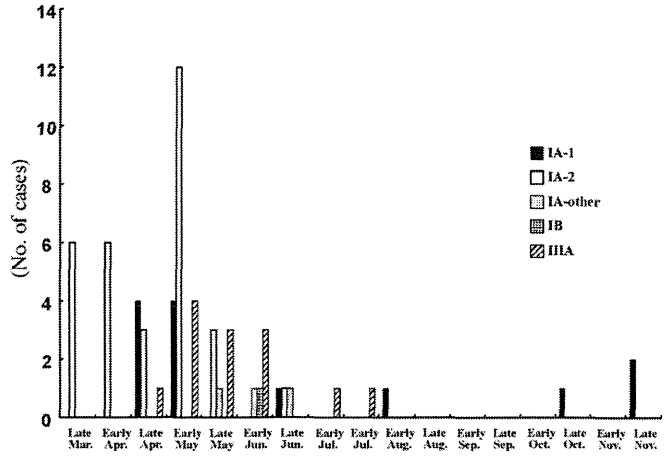

Fig. 5. Temporal distribution of HAV genotypes from late March to late November in 2010.

the sequences of HAV from these countries were determined 4–5 years before the Japanese diffuse outbreak in 2010, and a shorter 168-bp fragment (nt: 3024–3191, corresponding to the sequence data of Thai isolates) was used for the analysis. The isolates belonging to the IA-2 sub-lineage were detected mainly from late March through May, and could not be detected after June (Fig. 5). On the other hand, a regional imbalance of hepatitis A cases associated with this strain was not observed. Together with the uniformity of this cluster, we propose that this strain expanded from a single infection source (possibly an imported food product) that caused diffuse outbreak without a secondary expansion. Unfortunately the source(s) of HAV isolates belonging to the IA-2 remain unidentified.

Until recently, Japanese isolates of genotype IIIA were detected only on rare occasion, with the exception of some imported cases.[31–33] However, in 2010, approximately 26% of HAV isolates were classified as genotype IIIA. In South Korea, the incidence of reported HAV cases were increased dramatically since 2005, and most of the HAV isolates from this period clustered within genotype IIIA lineage. These results suggest genotype IIIA as the major epidemic strain for this outbreak, despite the fact that the predominant genotype in Korea, until 2005, was genotype IA.[12,34] Since the VP1-2A region of HAV genome amplified by nested RT-PCR for phylogenetic analysis in Korea differed from that in our study, we could compare only those Japanese IIIA isolates for which we obtained sequences of the region amplified by the first PCR reaction. Phylogenetic analysis revealed that the Japanese and Korean IIIA isolates clustered together (Fig. 4A), suggesting a correlation between the Japanese IIIA strain in 2010 and the recent Korean outbreak.

In conclusion, our data revealed that the diffuse outbreak of hepatitis A in Japan in the spring of 2010 was derived not only from locally circulating strains, but also from two other newly emerged HAV strains, possibly imported from the Philippines (IA-2) and Korea (IIIA). More detailed and extensive epidemiological analyses, ideally in collaboration with these countries, are needed to determine the source of the imported strains. However, in order to provide a better phylogeny, the use of a longer fragment, such as the entire VP1 gene and/or VP3 gene, is highly desirable. Together with the changing epidemiology of HAV infection, our findings may help the authorities in formulating public guidelines, including HAV vaccination policies targeted at susceptible populations.

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Competing interests

None.

Ethical approval

Not required.

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Hepatitis E Virus Outbreak in Monkey Facility, Japan

Hiroshi Yamamoto, Juri Suzuki, Atsushi Matsuda, Takafumi Ishida, Yasushi Ami, Yuriko Suzaki, Isao Adachi, Takaji Wakita, Naokazu Takeda, and Tian-Cheng Li

An outbreak of hepatitis E virus occurred in an outdoor monkey breeding facility in Japan during 2004–2006. Phylogenetic analysis indicated that this virus was genotype 3. This virus was experimentally transmitted to a cynomolgus monkey. Precautions should be taken by facility personnel who work with monkeys to prevent infection.

Wild or reared monkeys have been used as disease models in animal facilities worldwide. Because disease caused by hepatitis E virus (HEV) is a zoonosis (*1–4*), monkeys might be infected. We examined the prevalence of antibodies against HEV in serum and fecal samples collected from monkeys in animal facilities at the Primate Research Institute of Kyoto University in Japan for 6 years (2004–2009). We found that spontaneous infection and transmission of HEV occurred in a monkey facility.

The Study

There are 9 monkey colonies (A–I) at the Primate Research Institute of Kyoto University. Colonies A–G contained Japanese monkeys (*Macaca fuscata*), and colonies H and I contained rhesus monkeys (*Macaca mulatta*). Each colony was bred in a separate outdoor breeding facility. A total of 588 monkey serum samples were collected during September–November 2004–2009 and tested for IgG and IgM against HEV and for HEV RNA by ELISA or reverse transcription PCR (RT-PCR) as described (*5–7*). Samples from colonies G and F were collected during 2004–2006, whereas in 2009 samples were collected from colonies A, C, D, and I.

The prevalence of IgG against HEV was 0% in 2004, 20.0% in 2005, and 78.5% in 2006, followed by a gradual decrease to 35.9% in 2009 (Table 1). The prevalence of IgM against HEV increased from 0% in 2004 to 2.5% in 2005 and to 6.6% in 2006, and then decreased to 1.1% in 2007 and 0% in 2008 and 2009.

Table 1. Prevalence of IgG and IgM against hepatitis E virus in monkeys at monkey facility, Japan, 2004–2009

Year	No. positive/no. tested (%)	
	IgG	IgM
2004	0/110	0/110
2005	24/120 (20.0)	3/120 (2.5)
2006	96/121 (78.5)	8/121 (6.6)
2007	73/96 (76.0)	1/96 (1.1)
2008	47/90 (52.2)	0/90
2009	18/51 (35.3)	0/51

IgG against HEV was not detected in any of the 9 colonies in 2004, indicating that HEV infection did not occur before October 2004. However, in 2005, the prevalence of IgG reached 100% in colony D and 20% in colony G (Figure 1). ELISA titers were high, ranging from 0.293 to 1.641 in colony D and from 0.230 to 0.845 in colony G. These results suggested that HEV infection occurred after October 2004 in the monkey facility. The prevalence of IgG was higher in colony D than in colony G, and IgM was not detected in colony D, suggesting that HEV infection occurred earlier in colony D than in colony G. These colonies adjoined each other, indicating that the first HEV infection occurred in colony D and was then transmitted to colony G. Colonies A, C, D, E, and H each had an IgG prevalence of 90%–100%, and colonies B and G had an IgG prevalence >80% in 2006 (Figure 1). These results demonstrated that infection spread over a large area, except for colony F, during 2005 and 2006.

To compare the kinetics of IgG formation during 2004–2009, serum samples from 25 monkeys awhose peak ELISA optical density (OD) values for IgG against HEV were each higher than 1.0 were selected. In most monkeys, OD values for IgG increased rapidly and then decreased gradually year by year. The kinetic pattern of monkey M1543 was different from those of other monkeys that had high OD values (2.568–2.738). IgM was detected exclusively in this monkey in 2006 (OD value 0.620).

Serum samples from the 25 monkeys were used to detect HEV RNA by RT-PCR. Four serum samples were positive for HEV RNA; all were from the same monkey (M1543) from which samples were collected in 2006, 2007, 2008, and 2009. Nucleotide sequences of 348 bp coding the partial open reading frame 2 showed 100% identity. This result indicated that monkey M1543 was infected persistently with HEV and produced virus continuously.

To examine whether HEV was present in feces, 2 fecal samples were collected from monkey M1543 in September and November 2009 for detection of HEV RNA. Both samples were positive for HEV RNA. Nucleotide sequences of these samples were identical to those detected from serum samples.

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Primers were designed on the basis of sequences of swine HEV (GenBank accession no. AB248522), and RT-PCR was performed to amplify the viral genome except for the N terminus noncoding region. This strain was designated the monkey HEV Inuyama strain (JQ026407). Phylogenetic analysis of its genome indicated that this strain belongs to HEV genotype 3 (Figure 2). Infectivity of the monkey HEV strain was examined ex vivo with a human hepatocarcinoma cell line (PLC/PRF/5), and in vivo with

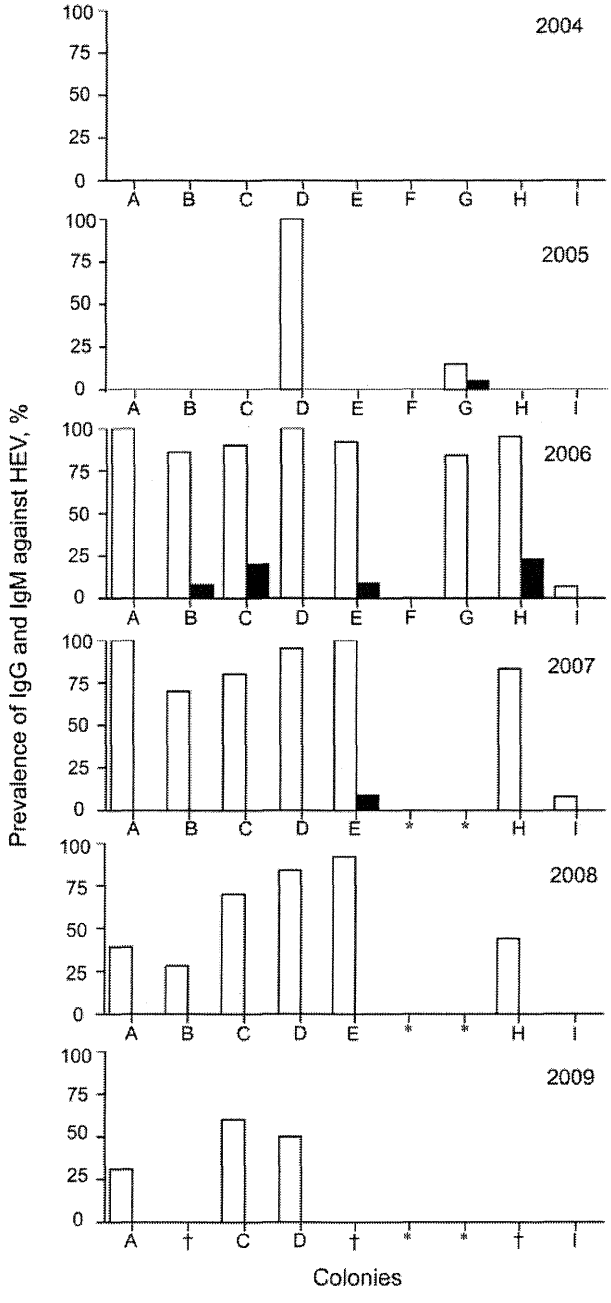

Figure 1. Prevalence of IgG (white bars) and IgM (black bars) against hepatitis E virus (HEV) in monkey facility, Japan, 2004–2009. *Monkeys were moved to another animal facility; †specimen not available.

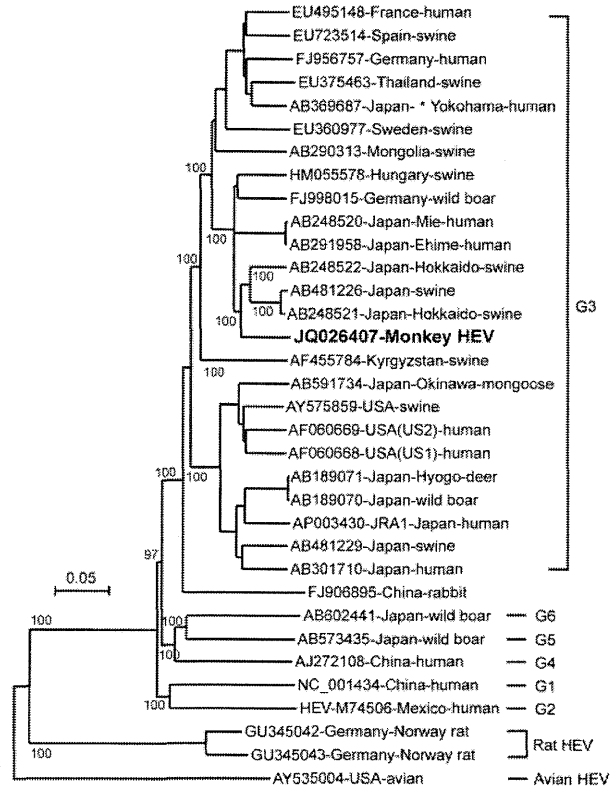

Figure 2. Phylogenetic analysis of monkey hepatitis E virus (HEV) Inuyama strain on the basis of nucleotide sequence of the HEV genome except for a 5′ noncoding region (7,206 nt) by using avian HEV as an outgroup. Values along the branches are bootstrap values determined on the basis of 1,000 resamplings of datasets. **Boldface** indicates strain isolated in this study. Genotypes are indicated on the right. Scale bar indicates nucleotide substitutions per site.

2 HEV-negative cynomolgus monkeys. Both experiments showed that the virus was infectious (online Technical Appendix Figures 1 and 2, wwwnc.cdc.gov/EID/pdfs/12-0884-Techapp.pdf).

A total of 94 human serum samples were collected from staff of the Primate Research Institute and subjected to ELISA for detection of IgG and IgM against HEV. All serum samples were negative for IgM against HEV, but the prevalence of IgG was 6.9% in 2007, 9.7% in 2008, and 11.8% in 2009, although differences among these years were not significant ($p>0.05$) (Table 2). No HEV RNA was detected in serum samples, and none of the staff had symptomatic hepatitis E during the 6-year study.

Conclusions

We conducted long-term monitoring of HEV infection in monkeys and report natural infection and transmission of HEV in a monkey facility. We sought to determine the source of the HEV outbreak and where HEV was intro-

Table 2. Prevalence of IgM against hepatitis E virus in serum samples from animal handlers at monkey facility, Japan, 2007–2009*

Year	No. positive/no. tested (%)
2007	2/29 (6.9)
2008	3/31 (9.7)
2009	4/34 (11.8)

*All samples were negative for IgG against hepatitis E virus and for virus RNA.

duced to colony D. At our research institute, each monkey colony is bred in a separate outdoor breeding facility built on a mountain, and the monkeys live in an environment similar to their natural habitat. Because each outdoor feeding facility is isolated by a double fence, natural reservoirs of HEV (wild boars and deer) cannot enter it. Phylogenetic analysis of monkey HEV strains indicated that this virus was genotype 3, and BLAST analysis showed that the monkey isolate is closest to HEV strains isolated from pigs in Japan. Nucleotide identities were 92%–93% (AB248521, AB248522, and AB481226). However, no evidence indicates that HEV is transmitted from pigs or wild boars to monkeys.

A notable finding in this study was the persistence of HEV infection. Generally, HEV infection is self-limiting and symptoms are transient. Persistent HEV infection occurs in solid-organ transplant recipients who have received immunosuppressive drugs (*8*) or in patients with other conditions associated with immunosuppression, such as HIV infection (*9*) and hematologic malignancies (*10,11*). However, there is no evidence of immunosuppression in monkey M1543, and the cause of the persistent HEV infection in this monkey is unknown.

The fact that the infectious HEV strain was detected in a monkey facility and caused an HEV outbreak cast doubt and apprehension on the safety of handling monkeys. Although no staff member showed development of symptomatic hepatitis E, precautions should be taken by facility workers who work with monkeys to prevent infection with HEV.

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Characterization of Full Genome of Rat Hepatitis E Virus Strain from Vietnam

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We amplified the complete genome of the rat hepatitis E virus (HEV) Vietnam strain (V-105) and analyzed the nucleotide and amino acid sequences. The entire genome of V-105 shared only 76.8%–76.9% nucleotide sequence identities with rat HEV strains from Germany, which suggests that V-105 is a new genotype of rat HEV.

Hepatitis E virus (HEV) is a positive-sense single-stranded RNA virus (*1*), classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (*2*). Hepatitis E, caused by HEV infection, is a serious public health concern in developing countries and is recognized as sporadic and endemic acute hepatitis (*3*). To date, at least 4 genotypes of HEV have been isolated from humans (*4*). In addition, HEV has been isolated from other mammals, including pigs, wild boars, wild deer, rabbits, ferrets, bats, chickens, and wild rats (*5–9*). Much direct evidence indicates that HEV is transmitted from pigs or wild boars to humans, and therefore hepatitis E caused by genotypes 3 and 4 is recognized as a zoonotic disease (*6,8,10*).

Rat HEV was first isolated from Norway rats in Germany (*7,11*). Since then, rat HEV strains have been isolated from wild rats in other areas of Germany and detected in wild rats in the United States and Vietnam (*12–14*). Those results suggest that rat HEV infection is not restricted to Germany but is broadly distributed in wild rats throughout the world. The nucleotide sequences of the rat HEV isolated in Germany and the United States are similar; however, the partial sequences of the Vietnam rat HEV strain (V-105, JN040433) have been found to have 78.18%–79.43% identities with isolates from Germany, R63 and R68 (*14*). To confirm whether new genotypes of rat HEV exist, we amplified the entire genome of the rat HEV V-105 strain and analyzed the sequences. We confirmed that the rat HEV strain isolated in Vietnam belongs to a new genotype of rat HEV.

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The Study

The rat HEV used in this study was isolated from a 10% lung homogenate of a wild rat from Vietnam, which was positive for rat HEV RNA by reverse transcription PCR (RT-PCR) (*14*). Because of the limited availability of rat specimens that are positive for HEV RNA, we first transmitted the rat HEV to a laboratory rat (Wistar) to produce a large amount of virus for RNA extraction and genome amplification. After intravenous inoculation of the rat, fecal specimens positive for HEV RNA were collected, and a 10% suspension was prepared as described (*15*). RT-PCR was performed by using Superscript II RNase H^- (Invitrogen, Carlsbad, CA, USA) and primer TX30SXN (*14*). The full-length genome of the V-105 strain was amplified by RT-PCR with primers based on the nucleotide sequences of GU345042 and JN040433 (Table 1). All PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and cloned into TA cloning vector pCR2.1 (Invitrogen). The nucleotide sequencing was carried out by using an ABI 3130 Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA).

Because 901 nt of V-105, corresponding to nt 4108–5008 of the R63 genome, were already known (*14*), primers F13 and open reading frame (ORF) 1–R12 were designed. An ≈2,100-nt fragment of the C-terminus of the rat HEV V-105, nt 4923-poly (A) tail, was amplified with a pair of primers, F13 and TX30SXN, by the first RT-PCR. The ORF1 region was amplified with primers ORF1-F1 and ORF1-R12. Two fragments, 440 nt (nt 11–450) and 1,182 nt (nt 2990–4171), were amplified by nested PCR with 2 sets of primers, ORF1-F2/ORF1-R1 and ORF1-F7/ORF1-R12, respectively. On the basis of the nucleotide sequences of those amplified fragments, ORF1-F9, ORF1-F16, ORF1-R16, ORF1-F18, and ORF2-R21 were designed, and 3 fragments, 1,830 nt (nt 388–2217), 996 nt (nt 2080–3075), and 1,110 nt (nt 3991–5100), were amplified with 3 sets of primers, ORF1-F9/ORF1-R10, ORF1-F16/ORF1-R16, and ORF1-F18/ORF2-R21, respectively.

To amplify the N-terminus nonstructural region of V-105, we synthesized cDNA with primer ORF1-R14, and a DNA anchor (P-CACGAATTCACTATCGATTCTGG AACCTTCAGAGG-NH_3) was linked to the N-terminus of the cDNA by T4 RNA Ligase I (BioLabs, Tokyo, Japan). By using this anchor-cDNA as the template, the first and the nested PCRs were carried out with 2 sets of primers, anchor-1/ORF1-R14 and anchor-2/ORF1-R13, respectively.

Table 1. Oligonucleotides used in amplifying the complete genome of the rat HEV Vietnam strain, V-105*

Primers	Product length,
Forward ORF1-F1 (1-21)† 5'-GCAACCCCCGATGGAGACCCA-3'‡ Reverse ORF1-R12 (4149-4171) 5'-GGCGGCCTCGAACTTCTCCTGAA-3'	§
Forward ORF1-F2 (11-30) 5'-ATGGAGACCCATCAGTATGT-3'† Reverse ORF1-R1 (431-450) 5'-GTGCAAAAGGAAAGATCAGT-3'	440
Forward ORF1-F9 (388-408) 5'-AGCTAACAACATCCGCCGTTG-3' Reverse ORF1-R10 (2197-2217) 5'-TGGGTTCGGTCGAAGGCCTCT-3'†	1,830
Forward ORF1-F16 (2080-2100) 5'-TGCAGCCGTTTATGAGGGAGA-3' Reverse ORF1-R16 (3055-3075) 5'-CGCCATTCTGTGGGTTCTAGA-3'	996
Forward ORF1-F7 (2990-3009) 5'-GACCCAAGGCAGATCCCTGC-3'† Reverse ORF1-R12 (4149-4171) 5'-GGCGGCCTCGAACTTCTCCTGAA-3'	1,182
Forward ORF1-F18 (3991-4011) 5'-ATTCACCACAGACGAGCCAGT-3' Reverse ORF2-R21 (5079-5100) 5'-GGTGATAGCCAATTGGTAAGCT-3'	1,110
Forward F13 (4896-4915) 5'-AATAACACTCTGGGCTGTAG-3' ReverseTX30SXN 5'-GACTAGTTCTAGATCGCGAGCGGCCGCCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3'	2,092
Forward primer Anchor-1: 5'-CCTCTGAAGGTTCCAGAATCGATAG-3' Reverse primer ORF1-R14 (276-296) 5'-TAGACCTAGGGTGCGCACCGA-3'	§
Forward primer Anchor-2: 5'-GAATCGATAGTGAATTCGTG-3' Reverse primer ORF1-R13 (200-220) 5'-AACACGCTGTACCGGATGCGA-3'	240

*HEV, hepatitis E virus; ORF, open reading frame.
†Numbers in a parentheses show the positions of primers corresponding to the entire genome of rat HEV V-105.
‡Primer designed based on rat HEV (GU345042).
§The PCR product was not detected.

The V-105 genome consisted of 6,927 nt plus a poly (A) tail of a still-undetermined length (GenBank accession no. JX120573). The genomic structure of V-105 was, from the N-terminus toward the C-terminus, the N 5′–untranslated region (UTR) at nt 1–10, ORF1at nt 11–4900, ORF3 at nt 4917–5225, ORF2 at nt 4928–6862, the 3′-UTR at nt 6863–6927, and the poly (A) tail starting at nt 6928. ORF2 and ORF3 encode 644 aa and 102 aa, respectively, as do R63 and R68. However, ORF1 of V-105 encodes 1,629 aa, which is 7 aa shorter than either R63 or R68. The V-105 genome possessed 2 aa insertions (Ser-Pro) between the aa residues 591 and 592 and 9 aa deletions (Ser-Pro-Pro-Gly-Pro-Pro-Pro-Ala-Gly) between aa residues 852 and 853, corresponding to those of R63. The 3′-UTR was 65 nt as were R63 and R68. Unlike R63 and R68, only 1 additional putative ORF, corresponding to ORF4 (nt residues 27–578), was found in V-105, suggesting that other putative ORFs, ORF5 and ORF6 found in R63 and R68, are not common in rat HEV.

When the V-105 genome was compared with reported HEV genomes, the V-105 genome shared identities of only 50.5% with avian HEV, 53.6% with rabbit HEV, 53.7%–54.0% with wild boar HEV, and 53.1%–53.5% with HEV genotypes 1–4. In contrast, V-105 shared relatively high nucleotide sequence identities (76.8%–76.9%) with rat HEV strains (R63 and R68) (Table 2). The nucleotide and amino acid sequences of ORF1, ORF2, and ORF3 of V-105 were compared with those of other HEV genotypes, and the identities among them are shown in Table 2. Together, these results suggest that V-105 is more similar to rat HEV than to other HEV genotypes.

Phylogenetic trees were generated on the basis of the nucleotide sequences derived from the entire genome and ORF3 of the genotypes 1–4, wild boar, rabbit, chicken, and rat HEV isolates. These trees demonstrated that V-105 does not belong to any known genotype and should probably be classified into a new genotype (Figure).

Table 2. Nucleotide and deduced amino acid sequence identities between human, wild boar, rabbit, rat, and avian HEV strains, compared with Vietnam rat HEV V-105 strain*

HEV strain (GenBank accession no.)	Vietnam rat HEV strain						
	Entire genome	Nucleotides, %			Amino acids, %		
		ORF1	ORF2	ORF3	ORF1	ORF2	ORF3
Genotype 1 (NC_001434)	53.5	50.7	60.8	51.0	54.1	55.5	33.3
Genotype 2 (M74506)	53.3	51.2	59.1	51.4	53.1	54.6	30.6
Genotype 3 (AF060668)	53.3	50.8	59.2	53.8	51.6	57.1	26.5
Genotype 4 (AJ272108)	53.1	50.9	58.9	52.6	50.7	55.4	24.5
Wild boar HEV (AB573435)	53.7	51.5	59.6	53.4	50.3	56.3	28.2
Wild boar HEV (AB602441)	54.0	51.5	59.6	53.4	50.3	56.3	28.2
Rabbit HEV (FJ906895)	53.6	51.3	59.3	51.5	52.1	56.2	26.2
Rat HEV (GU345042)/R63	76.9	75.7	79.6	80.6	87.0	91.6	66.7
Rat HEV (GU345043)/R68	76.8	75.5	79.8	80.9	86.4	92.1	66.7
Avian (chicken) HEV (AY535004)	50.5	49.7	54.2	47.0	44.7	47.4	33.9

*HEV, hepatitis E virus; ORF, open reading frame.

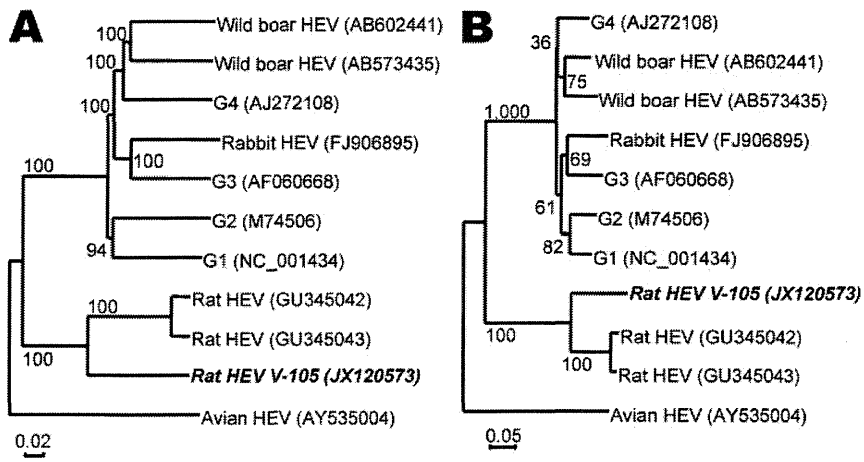

Figure. Phylogenetic relationships among genotypes 1–4, wild boar, rabbit, avian (chicken), and rat hepatitis E virus (HEV) isolates. The nucleic acid sequence alignment was performed by using ClustalX 1.81 (www.clustal.org). The genetic distance was calculated by the Kimura 2-parameter method. A phylogenetic tree with 1,000 bootstrap replicates was generated by the neighbor-joining method, based on the entire genome (A) and open reading frame 3 (B) of the genotypes 1–4, wild boar, rabbit, avian (chicken), and rat HEV isolates. Scale bar indicates nucleotide substitutions per site. **Boldface** indicates isolate used in this study.

Conclusions

In this study we successfully amplified the entire genome of an HEV strain isolated from a wild rat in Vietnam. Phylogenetic analyses and nucleotide and amino acid sequence comparisons demonstrated that the complete rat HEV genome sequences were consistently well separated from those of mammalian genotypes 1–4, wild boar, rabbit, and chicken HEV and close to those of the rat HEV strains. Although the entire genome of V-105 shared nucleotide sequence identities of only 76.8%–76.9% with the isolates from Germany (R63 and R68), the ORF1 and ORF3 amino acid identities between V-105 and these isolates were 86.4%–87.0% and 66.7%, respectively, which suggests that V-105 can be classified into a new genotype of rat HEV. However, ORF2 has relatively high amino acid identities with R63 and R68 (91.6%–92.1%), indicating that the V-105 and rat HEV isolates from Germany share similar antigenicity. In fact, rat HEV–like particles derived from R63 are cross-reactive to serum from V-105–infected wild rats (*14*).

In conclusion, we isolated and identified rat HEV strain V-105 from a wild rat in Vietnam, and this strain was highly divergent from known rat HEV isolates. We propose that the strain from Vietnam, V-105, is a new member of the rat HEV genotype.

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Hepatitis C Virus Infection Suppresses GLUT2 Gene Expression via Downregulation of Hepatocyte Nuclear Factor 1α

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Hepatitis C virus (HCV) infection causes not only intrahepatic diseases but also extrahepatic manifestations, including type 2 diabetes. We previously reported that HCV replication suppresses cellular glucose uptake by downregulation of cell surface expression of glucose transporter 2 (GLUT2) (D. Kasai et al., J. Hepatol. 50:883–894, 2009). GLUT2 mRNA levels were decreased in both HCV RNA replicon cells and HCV J6/JFH1-infected cells. To elucidate molecular mechanisms of HCV-induced suppression of GLUT2 gene expression, we analyzed transcriptional regulation of the GLUT2 promoter using a series of GLUT2 promoter-luciferase reporter plasmids. HCV-induced suppression of GLUT2 promoter activity was abrogated when the hepatocyte nuclear factor 1α (HNF-1α)-binding motif was deleted from the GLUT2 promoter. HNF-1α mRNA levels were significantly reduced in HCV J6/JFH1-infected cells. Furthermore, HCV infection remarkably decreased HNF-1α protein levels. We assessed the effects of proteasome inhibitor or lysosomal protease inhibitors on the HCV-induced reduction of HNF-1α protein levels. Treatment of HCV-infected cells with a lysosomal protease inhibitor, but not with a proteasome inhibitor, restored HNF-1α protein levels, suggesting that HCV infection promotes lysosomal degradation of HNF-1α protein. Overexpression of NS5A protein enhanced lysosomal degradation of HNF-1α protein and suppressed GLUT2 promoter activity. Immunoprecipitation analyses revealed that the region from amino acids 1 to 126 of the NS5A domain I physically interacts with HNF-1α protein. Taken together, our results suggest that HCV infection suppresses GLUT2 gene expression via downregulation of HNF-1α expression at transcriptional and posttranslational levels. HCV-induced downregulation of HNF-1α expression may play a crucial role in glucose metabolic disorders caused by HCV.

Hepatitis C virus (HCV) is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. HCV is a single-stranded, positive-sense RNA virus that is classified into the *Flaviviridae* family, *Hepacivirus* genus (21). More than 170 million people worldwide are chronically infected with HCV. The 9.6-kb HCV genome encodes a polyprotein of approximately 3,010 amino acids (aa). The polyprotein is cleaved co- and posttranslationally into at least 10 proteins by viral proteases and cellular signalases: the structural proteins core, E1, E2, and p7 and the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (21).

Persistent HCV infection causes not only intrahepatic diseases but also extrahepatic manifestations, such as type 2 diabetes. Clinical and experimental data suggest that HCV infection is an additional risk factor for the development of diabetes (26, 29, 30). HCV-related glucose metabolic changes and insulin resistance have significant clinical consequences, such as accelerated fibrogenesis, reduced virological response to alpha interferon (IFN-α)-based therapy, and increased incidence of hepatocellular carcinoma (29). Therefore, the molecular mechanism of HCV-related diabetes needs to be clarified.

We have sought to identify a novel mechanism of HCV-induced diabetes. We previously demonstrated that HCV suppresses hepatocytic glucose uptake through downregulation of cell surface expression of glucose transporter 2 (GLUT2) in a human hepatoma cell line (19). The uptake of glucose into cells is conducted by facilitative glucose carriers, i.e., glucose transporters (GLUTs). GLUTs are integral membrane proteins that contain 12 membrane-spanning helices. To date, a total of 14 isoforms have been identified in the GLUT family (24). GLUT2 is expressed in the liver, pancreatic β-cells, hypothalamic glial cells, retina, and enterocytes. Glucose is transported into hepatocytes by GLUT2 (34). We previously reported that GLUT2 expression was reduced in hepatocytes obtained from HCV-infected patients (19). We also demonstrated that GLUT2 mRNA levels were lower in HCV replicon cells and in HCV J6/JFH1-infected cells than in the control cells. GLUT2 promoter activity was suppressed in HCV-replicating cells. However, the molecular mechanism of HCV-induced suppression of GLUT2 gene expression remains to be elucidated.

In the present study, we aimed to clarify molecular mechanisms of HCV-induced suppression of GLUT2 gene expression. We analyzed transcriptional regulation of the GLUT2 promoter in HCV replicon cells. We demonstrate that HCV infection downregulates hepatocyte nuclear factor 1α (HNF-1α) expression at both transcriptional and posttranslational levels, resulting in suppression of GLUT2 promoter. We propose that HCV-induced downregulation of HNF-1α may play a crucial role in glucose metabolic disorders caused by HCV.

MATERIALS AND METHODS

Cell culture. The human hepatoma cell line Huh-7.5 (4) was kindly provided by Charles M. Rice (The Rockefeller University, New York, NY).

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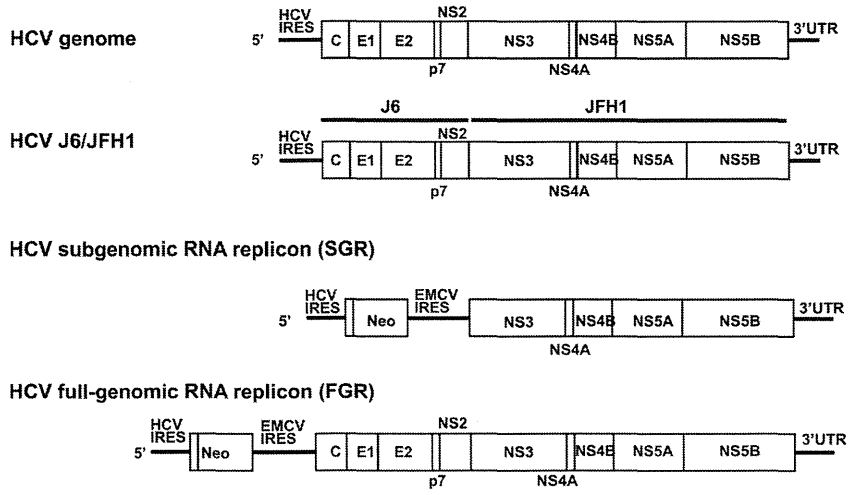

FIG 1 The HCV genome, chimeric HCV J6/JFH1, and the HCV RNA replicons. Schematic diagrams of the HCV genome, the chimeric HCV J6/JFH1 genome, SGR, and FGR are shown. IRES, internal ribosome entry site; EMCV, encephalomyocarditis virus; Neo, neomycin resistance gene.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (high glucose) with L-glutamine (Wako, Osaka, Japan) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin (Gibco, NY), 10% heat-inactivated fetal bovine serum (Biowest, France), and 0.1 mM nonessential amino acids (Invitrogen, NY) at 37°C in a 5% CO_2 incubator. Cells were transfected with plasmid DNA using FuGENE 6 transfection reagents (Promega, Madison, WI).

Huh-7.5 cells stably harboring an HCV-1b subgenomic RNA replicon (SGR) were prepared as described previously (18), using pFK5B/2884Gly (a kind gift from R. Bartenschlager, University of Heidelberg, Heidelberg, Germany). The SGR cells express the genomic region from NS3 to NS5B of the HCV Con1 strain (19) (Fig. 1). Cells harboring a full-genome HCV-1b RNA replicon (FGR) derived from Con1 (27) or pON/C-5B (17, 19) (a kind gift from N. Kato, Okayama University, Okayama, Japan) were also used. The FGR cells express all of the HCV proteins (the region ranging from the core protein to NS5B).

The pFL-J6/JFH1 plasmid that encodes the entire viral genome of a chimeric strain of HCV-2a, J6/JFH1 (23), was kindly provided by Charles M. Rice. The HCV genome RNA was synthesized *in vitro* using pFL-J6/JFH1 as a template and was transfected into Huh-7.5 cells by electroporation (6, 9, 23, 37). The virus produced in the culture supernatant was used for infection experiments (6).

Cells were treated with 1,000 IU/ml of IFN-α (Sigma, St. Louis, MO) for 10 days to eliminate HCV replication (19).

Luciferase reporter assay. We constructed the human GLUT2 promoter-luciferase reporter plasmid by cloning a 1.6-kb genomic fragment that encompasses the human GLUT2 promoter region from −1291 to +308, yielding pGLUT2(−1291/+308)-Luc (2, 19), into the pGL4 vector plasmid (Promega). The pGLUT2(−1291/+308)-Luc construct contains a 1,291-bp fragment of the human GLUT2 promoter upstream of the minimal promoter and the coding sequence of the *Photinus pyralis* (firefly) luciferase. We also used seven different GLUT2 promoter-luciferase reporter plasmids, i.e., pGLUT2(−1193/+308)-Luc, pGLUT2 (−1155/+308)-Luc, pGLUT2(−1100/+308)-Luc, pGLUT2(−1030/+308)-Luc, pGLUT2(−206/+308)-Luc, pGLUT2(+29/+308)-Luc, and pGLUT2(+126/+308)-Luc, which lack the binding sequence of the CCAAT/enhancer binding site (C/EBP), cyclic AMP (cAMP) response element (CRE), AP-1 binding site, HNF-1α binding site, CAAT box, TATA-like motif, and transcriptional initiation, respectively (Fig. 2A). The reporter plasmid pRL-CMV-*Renilla* (where CMV is cytomegalovirus) (Promega) was used as an internal control. Cells were transfected with each pGLUT2-Luc construct together with pRL-CMV-*Renilla*. At 48 h after transfection, samples were harvested and assayed for luciferase activity. The luciferase assays were performed using a dual-luciferase reporter assay system (Promega). Luciferase activity was measured by a Lumat LB 9501 instrument (Berthold Technologies GmbH & Co., Bad Wildbad, Germany). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample. The number of relative light units (RLU) of the SGR cells or FGR cells transfected with each reporter plasmid is expressed as a ratio of the number of Huh-7.5 cells transfected with each reporter plasmid.

Expression plasmids. Expression plasmids for core protein, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B were described previously (9, 10, 18). To express E1 and E2 (E1/E2), the cDNA fragment of nucleotides (nt) 825 to 2676 derived from the HCV Con1 strain was amplified by PCR using the plasmid pFKI389neo/core-3′/Con1 (a kind gift from R. Bartenschlager) as a template. Specific primers used for PCR were as follows: sense primer, 5′-CCAGTGTGGTGAATTCAC CATGGTGAACTATGCAACAGGGAA-3′; antisense primer, 5′-CGAAG GGCCCTCTAGAGATGTACCAGGCAGCACAGA-3′. To express NS3 and NS4A (NS3/4A), the cDNA fragment of nt 3420 to 5474 derived from the HCV Con1 strain was amplified by PCR. Specific primers were as follows: sense primer, 5′-CCAGTGTGGTGAATTCACCATGGCGCCTA TTACGGCCTACTC-3′; antisense primer, 5′-CGAAGGGCCCTCTAGA GCACTCTTCCATCTCATCGAA-3′. These amplified PCR products were purified, and each of them was inserted into the EcoRI-XbaI site of pEF1/myc-His A (Invitrogen) using an In-Fusion HD-Cloning kit (Clontech, Mountain View, CA). To express a series of NS5A deletion mutants as hemagglutinin (HA)-tagged proteins, each fragment was amplified by PCR and cloned into the NotI site of pCAG-HA. pEF1A-NS5A (Con1)-myc-His was used as a template (18). The primer sequences used in this study are available from the authors upon request. The sequences of the inserts were extensively verified by sequencing (Operon biotechnology, Tokyo, Japan). The plasmids pEF1A-NS5A(1–126)-myc-His, consisting of residues 1 to 126 in NS5A, and pEF1A-NS5A(1–147)-myc-His were described previously (18).

Antibodies. The mouse monoclonal antibodies (MAbs) used in this study were anti-FLAG (M2) MAb (F-3165; Sigma), anti-NS5A MAb (MAB8694; Millipore), anti-core protein MAb (2H9) (37), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) MAb (MAB374; Millipore). Polyclonal antibodies (PAbs) used in this study were anti-HNF-1α rabbit PAb (sc-8986; Santa Cruz Biotechnology), anti-HNF-1α goat PAb (sc-6548; Santa Cruz Biotechnology), anti-NS5B goat PAb (sc-17532; Santa Cruz Biotechnology), anti-NS3 rabbit PAb (described elsewhere), and anti-actin goat PAb (C-11; Santa Cruz Biotechnology). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody

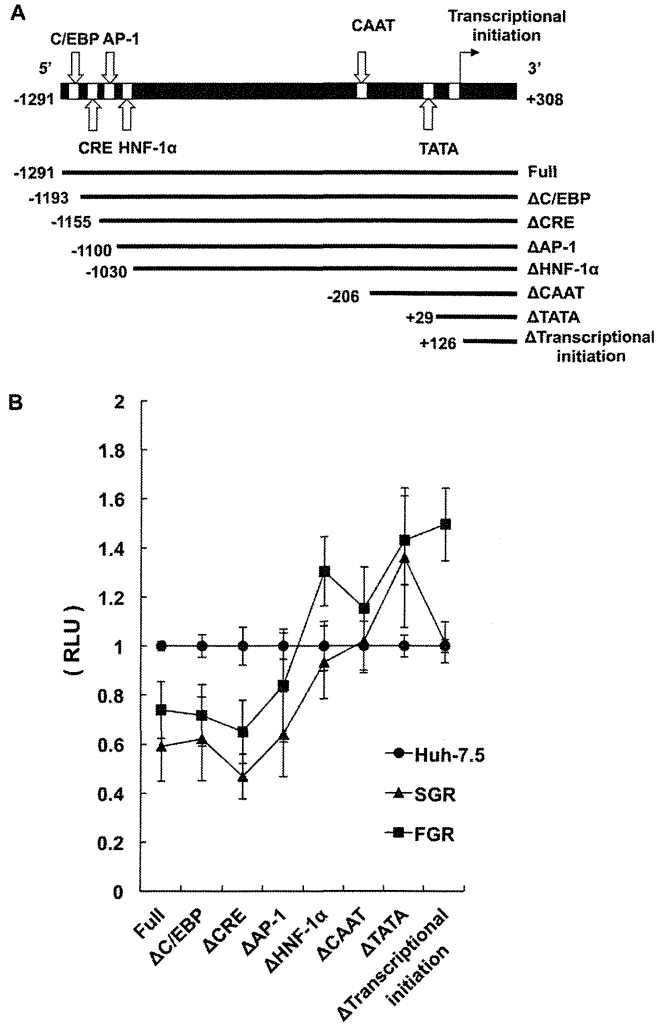

FIG 2 HNF-1α-binding site is important for HCV-induced suppression of GLUT2 promoter. (A) A series of constructs in which genomic GLUT2 promoter DNA fragments were fused to a promoterless firefly luciferase gene of the pGL4 vector were generated with the 3′ end always terminating at bases +308 from transcriptional start site. The 5′ ends began at bases −1291, −1193, −1155, −1100, −1030, −206, +29, and +126 The regions that represent potential binding sites for transcription factors are shown, including a CCAAT/enhancer binding site (C/EBP), cAMP response element (CRE), AP-1 binding site, HNF-1α binding site, CAAT box, and TATA-like motif. The nucleotide at the beginning of the construct is indicated. (B) Huh-7.5 cells, SGR cells, and FGR cells (2.5×10^5 cells/six-well plate) were transfected with each GLUT2 plasmid (0.5 μg) together with pRL-CMV-*Renilla* (25 ng). pRL-CMV-*Renilla* was used as an internal control. At 48 h posttransfection, cells were harvested and assayed for luciferase activities using a dual-luciferase reporter assay system. RLU is expressed as a ratio of the Huh-7.5 cells transfected with each reporter plasmid.

(Cell signaling), HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology), and HRP-conjugated anti-rabbit IgG (Cell signaling) were used as secondary antibodies.

Real-time quantitative reverse transcription-PCR (RT-PCR). Total cellular RNA was isolated using RNAiso reagent (TaKaRa Bio, Kyoto, Japan), and cDNA was generated using a QuantiTect Reverse Transcription system (Qiagen, Valencia, CA). Real-time quantitative PCR was performed using SYBR Premix *Ex Taq* (TaKaRa Bio) with SYBR green chemistry on an ABI Prism 7000 system (Applied Biosystems, Foster, CA), as described previously (11, 19). The β-glucronidase (GUS) gene was used as an internal control. The primers used for real-time PCR are as follows: for HNF-1α (NM_000545), 5′-AGCTACCAACCAAGAAGGGGC-3′ (nt 601 to 621) and 5′-TGACGAGGTTGGAGCCCAGCC-3′ (nt 801 to 781); HNF-1β (NM_000458), 5′-GTTACATGCAGCAACACAACA-3′ (nt 600 to 620) and 5′-TCATATTTCCAGAACTCTGGA-3′ (nt 801 to 782); GUS (NM_000181), 5′-ATCAAAAACGCAGAAAATACG-3′ (nt 1797 to 1817) and 5′-ACGCAGGTGGTATCAGTCTTG-3′ (nt 2034 to 2014).

Immunoblot analysis. Immunoblot analysis was performed essentially as described previously (9, 33). The cell lysates were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA). The membranes were incubated with primary antibody, followed by incubation with peroxidase-conjugated secondary antibody. The positive bands were visualized using ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, United Kingdom). To detect endogenous HNF-1α protein, ECL Plus Western blotting detection reagents were used (GE Healthcare).

Immunoprecipitation. Cultured cells were lysed with a buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 0.1% SDS, 1% NP-40, and Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The lysate was centrifuged at 12,000 × *g* for 20 min at 4°C, and the supernatant was immunoprecipitated with appropriate antibodies. Immunoprecipitation was performed as described previously (10). Briefly, the cell lysates were immunoprecipitated with control IgG and Dynabeads protein A (Invitrogen) and incubated with appropriate antibodies at 4°C overnight. After being washed with the washing buffer (0.1 M Na-phosphate buffer, pH 7.4) five times, the immunoprecipitates were analyzed by immunoblotting.

Statistical analysis. Results were expressed as means ± standard errors of the means (SEM). Statistical significance was evaluated by analysis of variance (ANOVA), and statistical significance was defined as a P value of <0.05.

RESULTS

HNF-1α-binding site is important for HCV-induced suppression of GLUT2 promoter. To gain an insight into potential regulatory sequences involved in HCV-induced suppression of GLUT2 gene transcription, a 1.6-kb genomic fragment that encompasses the human GLUT2 promoter (−1291 to +308) and a series of deletion mutants were analyzed (Fig. 2A). The ability of the upstream region of the GLUT2 gene to function as a promoter was assessed by its capacity to drive the expression of a luciferase reporter gene. GLUT2 promoter activity was assessed by measuring luciferase activity of the cell extracts derived from transiently transfected Huh-7.5 cells, SGR cells, and FGR cells. As shown in Fig. 2B, a deletion of the promoter sequence to −1100 [pGLUT2(−1100/+308)-Luc [ΔAP-1]] showed lower luciferase activities in HCV replicon cells than in the control cells. Successive removal of nucleotides from −1100 to −1030 completely or almost completely abolished the suppression of the luciferase activity in both FGR and SGR cells, suggesting that the HNF-1α-binding site is important for HCV-induced suppression of GLUT2 promoter.

HCV infection reduces HNF-1α mRNA levels. It is worth noting that HNF-1α is known to play a crucial role in diabetes. Mutations in the HNF-1α gene have been reported to cause a monogenic form of diabetes mellitus with autosomal dominant inheritance, termed maturity onset diabetes of the young 3 (MODY3) (25, 40). Cha et al. (7) reported that HNF-1α functions as a transcriptional transactivator in human GLUT2 gene expression in a human hepatoma cell line. These findings motivated us to further investigate a role of HNF-1α in HCV-induced glucose metabolic disorders in a human hepatoma cell line. To determine

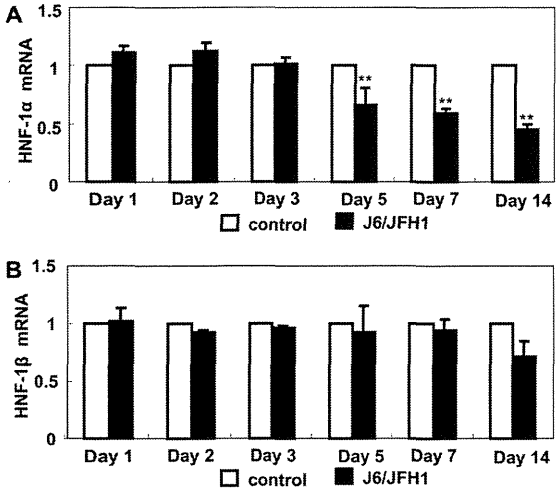

FIG 3 Quantitative RT-PCR analysis of mRNA for HNF-1α and HNF-1β in HCV J6/JFH1-infected cells. Huh-7.5 cells (2.5×10^5 cells/six-well plate) were infected with HCV J6/JFH1 at a multiplicity of infection of 2. Cells were cultured and harvested at the indicated times. Total RNA was extracted, and the levels of HNF-1α mRNA and HNF-1β mRNA were determined by quantitative RT-PCR. Mock-infected cells served as negative controls. **, $P < 0.01$, compared with mock-infected cells.

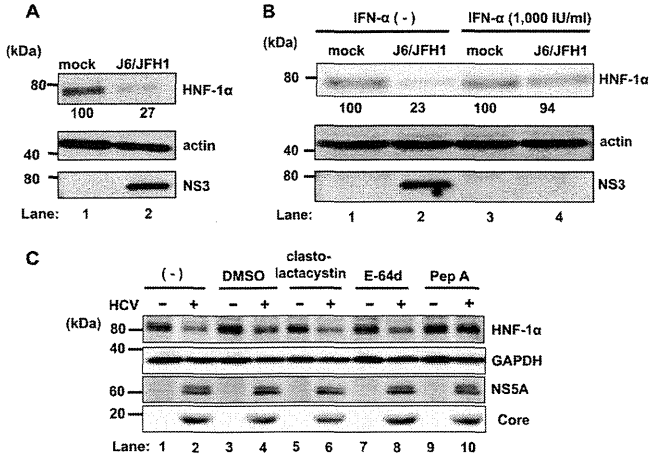

FIG 4 HCV infection induces lysosomal degradation of HNF-1α protein. (A) HCV infection decreased the levels of HNF-1α protein in Huh-7.5 cells. Huh-7.5 cells (2.5×10^5 cells/six-well plate) were infected with HCV J6/JFH1 at a multiplicity of infection of 2. Cells were cultured and harvested at 5 days postinfection. Cells were analyzed by immunoblotting with anti-HNF-1α, anti-NS3, and anti-actin antibodies. The level of actin served as a loading control. The relative levels of protein expression were quantitated by densitometry and are indicated below the respective lanes. (B) HCV-induced downregulation of HNF-1α protein was restored by treatment of the cells with IFN-α. Huh-7.5 cells were plated at 2.5×10^5 cells/six-well plate and cultured for 12 h. The cells were infected with HCV J6/JFH1 at a multiplicity of infection of 2 and cultured for 5 days. The cells were replated at 2.5×10^5 cells/six-well plate and cultured in complete DMEM with or without 1,000 IU/ml IFN-α for 10 days to eliminate HCV. The cells cultured in DMEM without IFN-α served as negative controls. (C) HCV-induced reduction of HNF-1α protein was restored by treatment of the cells with lysosomal protease inhibitor. Huh-7.5 cells were plated at 2.0×10^5 cells/six-well plate and cultured for 12 h. At 5 days postinfection, proteasome inhibitor (30 μM clasto-lactacystin β-lactone) or lysosomal protease inhibitors (40 μM E-64d and 20 μM pepstatin A) were administered to the cells. Cells were cultured for 12 h, harvested, and analyzed by immunoblotting as indicated. The level of GAPDH served as a loading control. DMSO, dimethyl sulfoxide; PepA, pepstatin A.

whether HCV infection suppresses HNF-1α mRNA expression, we quantified mRNA levels of HNF-1α and HNF-1β in HCV J6/JFH1-infected cells and in mock-infected cells by real-time RT-PCR. HNF-1α mRNA levels were significantly reduced in HCV J6/JFH1-infected cells from 5 days postinfection (dpi) to 14 dpi (Fig. 3A). On the other hand, HNF-1β mRNA levels remained unchanged until 14 dpi (Fig. 3B). These results suggest that HCV infection specifically downregulates HNF-1α mRNA expression.

HCV infection reduces HNF-1α protein levels. To determine whether HCV infection reduces HNF-1α protein levels, endogenous HNF-1α protein levels were examined by immunoblot analysis. The HNF-1α protein level was much lower in J6/JFH1-infected cells than in the mock-infected control (Fig. 4A, upper panel, lane 2). To determine whether HCV infection is specifically involved in reduction of HNF-1α protein, we eliminated HCV by treatment of the cells with IFN-α (Fig. 4B, lower panel, compare lane 2 with lane 4). Upon elimination of HCV, the HNF-1α protein expression level recovered to the level of the mock-infected control (Fig. 4B, upper panel, compare lane 2 with lane 4). These results suggest that HCV infection specifically reduces HNF-1α protein levels.

HCV-induced reduction of HNF-1α protein is restored by treatment of the cells with a lysosomal protease inhibitor. As shown in Fig. 3A, HNF-1α mRNA levels in HCV J6/JFH1-infected cells decreased slowly at day 5 postinfection. One possible explanation is that suppression of HNF-1α mRNA is an indirect effect caused by HCV infection. The degree of the reduction of the HNF-1α protein was larger than that of HNF-1α mRNA (Fig. 4A), suggesting the involvement of protein degradation in reduction of HNF-1α protein levels. To determine whether protein degradation is involved in HCV-induced reduction of HNF-1α protein, we assessed the role of proteasome or lysosome proteases in the reduction of HNF-1α protein. We treated the cells with a proteasome inhibitor, clasto-lactacystin β-lactone, or lysosome protease inhibitors E-64d and pepstatin A. Clasto-lactacystin β-lactone had no effect on the levels of HNF-1α protein (Fig. 4C, upper panel, lane 6). This result suggests that proteasome is not involved in the reduction of HNF-1α protein. E-64d is a cysteine protease inhibitor, and pepstatin A is an aspartic protease inhibitor. Pepstatin A, but not E-64d, restored the levels of HNF-1α protein (Fig. 4C, upper panel, lanes 10 and 8). These results suggest that a lysosomal protease, such as an aspartic protease, is involved in HCV-induced reduction of HNF-1α protein.

Overexpression of NS5A protein suppresses GLUT2 promoter activity. To determine which HCV protein is involved in the suppression of GLUT2 promoter, we examined the effects of transient expression of HCV proteins on GLUT2 promoter activity. Huh-7.5 cells were cotransfected with each HCV protein expression plasmid together with the GLUT2 promoter-luciferase plasmid. The pRL-CMV-*Renilla* plasmid was cotransfected as an internal control. At 48 h posttransfection, cells were harvested and assayed for luciferase activity. As shown in Fig. 5A, overexpression of the NS5A expression plasmid significantly reduced GLUT2 promoter activity. On the other hand, other HCV protein expression plasmids failed to suppress GLUT2 promoter activity (Fig. 5A, left and right panels). These results suggest that NS5A protein is involved in the suppression of GLUT2 promoter activity.

Overexpression of NS5A protein reduces the levels of endogenous HNF-1α protein. To investigate a role of NS5A in the sup-

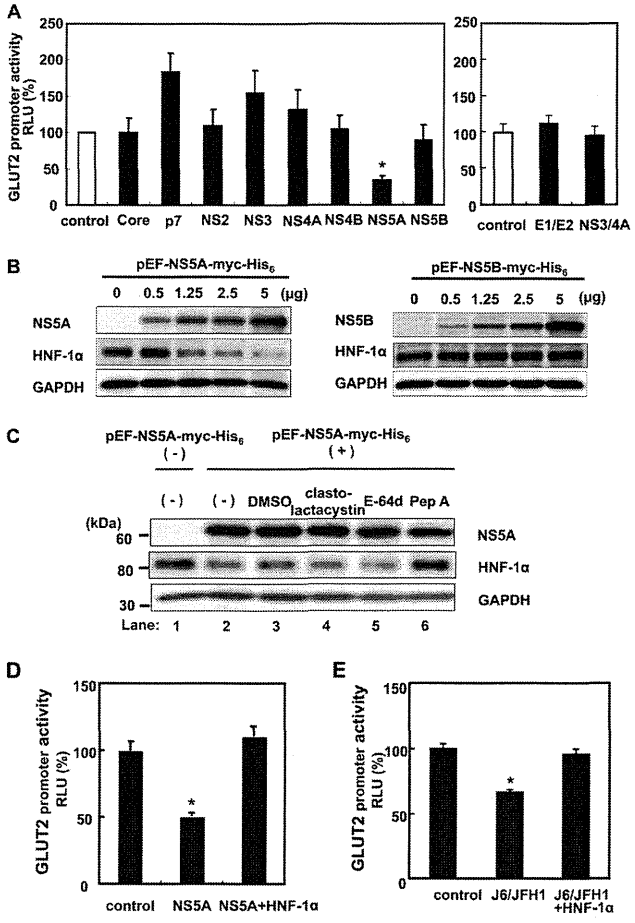

FIG 5 HCV NS5A protein is involved in suppression of GLUT2 promoter activity and lysosomal degradation of HNF-1α protein. (A) Huh-7.5 cells were plated at 1×10^5 cells/12-well plate. After cells were cultured for 12 h, cells were cotransfected with each HCV protein plasmid (0.5 μg), the human GLUT2 promoter reporter plasmid (0.5 μg), and pRL-CMV-*Renilla* (25 ng). pRL-CMV-*Renilla* was used as an internal control. At 48 h posttransfection, cells were harvested. Luciferase assays were performed by using a dual-luciferase reporter assay system. (B) Huh-7.5 cells were plated at 4×10^5 cells/six-well plate and cultured for 12 h. Cells were transfected with increasing amounts of either NS5A plasmid or NS5B plasmid as indicated. At 48 h posttransfection, cells were harvested. Whole-cell lysates were analyzed by immunoblotting with anti-HNF-1α, anti-NS5A, and anti-NS5B antibodies. The level of GAPDH served as a loading control. (C) Huh-7.5 cells (2.5×10^5 cells/six-well plate) were transfected with pEF1A-NS5A-myc-His_6. At 2 days posttransfection, proteasome inhibitor (30 μM clasto-lactacystin β-lactone) or lysosomal enzyme inhibitors (40 μM E-64d and 20 μM pepstatin A) were administered to the cells. Cells were cultured for 12 h and harvested, and the levels of endogenous HNF-1α protein were analyzed by immunoblotting with anti-HNF-1α goat PAb. The level of GAPDH served as a loading control. (D) Huh-7.5 cells (1.0×10^5 cells/12-well plate) were transfected with the human GLUT2 promoter reporter plasmid (0.5 μg) and pRL-CMV-*Renilla* (25 ng). The plasmid pEF1A/myc-His (0.5 μg) was cotransfected to the control cells. Cells were transfected with the plasmid pEF1A-NS5A-myc-His (0.5 μg) together with either empty plasmid pCMV4 (10 ng) or pCMV-HNF-1α (10 ng). At 48 h posttransfection, cells were harvested. Luciferase assays were performed by using a dual-luciferase reporter assay system. *, $P < 0.05$, compared with control. (E) Huh-7.5 cells (1.2×10^6 cells /10 cm-dish) were infected with HCV J6/JFH1 at a multiplicity of infection of 2 and cultured for 5 days. At day 5 postinfection, cells were plated at 1.0×10^5 cells/12-well plate and cultured for 12 h. Mock-infected cells were plated similarly. Cells were transfected with the human GLUT2 promoter reporter plasmid (0.5 μg) and pRL-CMV-*Renilla* (25 ng) together with either empty plasmid pCMV4 or pCMV-HNF-1α, cultured for 48 h, and harvested. Luciferase assays were performed by using a dual-luciferase reporter assay system. *, $P < 0.05$, compared with control.

pression of the GLUT2 promoter, we examined the effect of NS5A protein on the levels of endogenous HNF-1α protein. Huh-7.5 cells were transfected with increasing amounts of either an NS5A expression plasmid or NS5B expression plasmid. At 48 h posttransfection, cells were harvested, and the levels of endogenous HNF-1α protein were analyzed by immunoblot analysis. To detect endogenous HNF-1α protein, highly sensitive Western blotting detection reagents (ECL Plus Western blotting detection reagents) were used. Overexpression of NS5A (Fig. 5B, left panel) but not NS5B (Fig. 5B, right panel) significantly reduced endogenous HNF-1α protein. These results suggest that NS5A protein specifically reduces endogenous HNF-1α protein levels.

To determine if NS5A-dependent reduction of HNF-1α protein is due to lysosomal degradation, we treated the cells with lysosome protease inhibitors. Pepstatin A, but not E-64d, recovered the levels of HNF-1α protein (Fig. 5C, middle panel, lanes 5 and 6), which is consistent with the results found in HCV-infected cells. These results suggest that NS5A is responsible for HCV-induced lysosomal degradation of HNF-1α protein. Taken together, our results suggest that HCV infection suppresses GLUT2 promoter activity via NS5A-dependent lysosomal degradation of HNF-1α protein.

To verify a role of HNF-1α in the HCV-induced suppression of GLUT2 promoter activity, we examined the effects of ectopic expression of HNF-1α on GLUT2 promoter activity in NS5A-transfected cells as well as in HCV J6/JFH1-infected cells. As shown in Fig. 5D, overexpression of NS5A decreased GLUT2 promoter activity, and ectopic expression of HNF-1α restored GLUT2 promoter activity (Fig. 5D). Moreover, HCV J6/JFH1 infection significantly decreased GLUT2 promoter activity, and ectopic expression of HNF-1α restored GLUT2 promoter activity (Fig. 5E). These results are consistent with the notion that HNF-1α protein is a key regulator for HCV-induced suppression of GLUT2 promoter activity.

NS5A protein interacts with HNF-1α protein in Huh-7.5 cells and in FGR Con1 cells. It was previously reported that *in vitro* translated HNF-1 protein was pulled down with glutathione *S*-transferase (GST)-NS5A protein (32). To determine whether NS5A physically interacts with HNF-1α protein in cultured cells, Huh-7.5 cells were cotransfected with each FLAG-tagged NS5A expression plasmid together with the HNF-1α expression plasmid. Immunoprecipitation analysis revealed that HNF-1α protein was coimmunoprecipitated with FLAG-NS5A protein using anti-FLAG MAb (Fig. 6A, third blot, lane 8). No band was detected using control IgG for immunoprecipitation (Fig. 6A, third blot, lane 7). Conversely, immunoprecipitation analysis revealed that NS5A protein was coimmunoprecipitated with HNF-1α protein using anti-HNF-1α rabbit PAb (Fig. 6B, fourth blot, lane 8). Moreover, NS5A protein was coimmunoprecipitated with endogenous HNF-1α protein (Fig. 6B, fourth blot, lane 6), suggesting that NS5A protein indeed interacts with HNF-1α protein.

To confirm that HCV NS5A protein can interact with HNF-1α protein in HCV-replicating cells, we performed immunoprecipitation analysis using FGR Con1 (RCYM1) cells. NS5A protein was coimmunoprecipitated with endogenous HNF-1α protein (Fig. 6C, fourth blot, lane 2). Transfection of HNF-1α protein increased the level of coimmunoprecipitated NS5A protein (Fig. 6C, fourth blot, lane 4), suggesting that HCV NS5A protein indeed interacts with HNF-1α protein in HCV-replicating cells.

HNF-1α binds domain I of NS5A protein. To map the HNF-

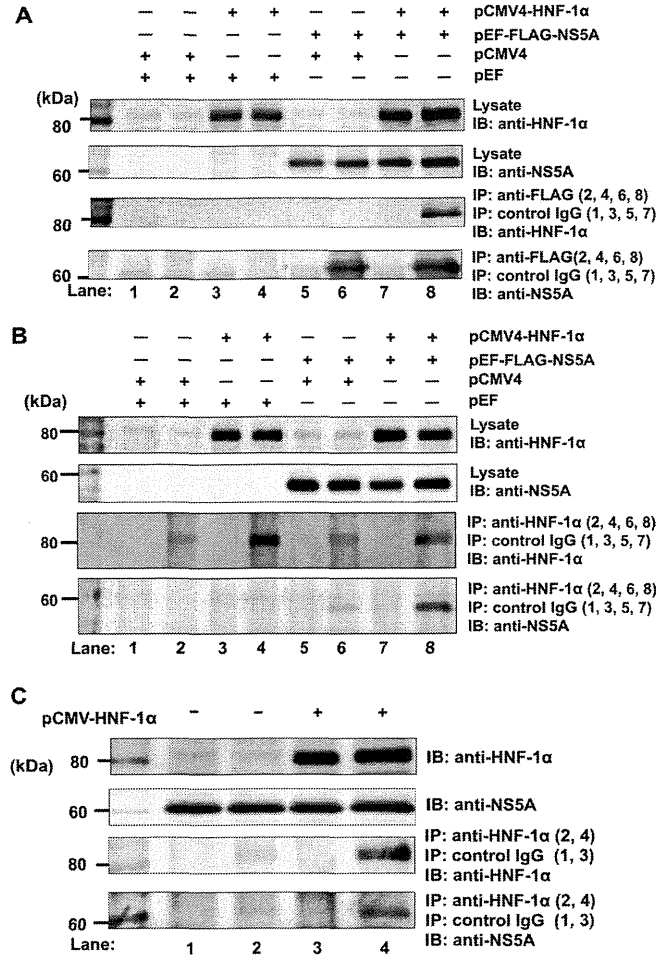

FIG 6 NS5A protein interacts with HNF-1α protein. (A) Huh-7.5 cells were plated at 1.2×10^6 cells/10-cm dish and cultured for 12 h. Cells were transfected with plasmids as indicated. At 48 h after transfection, cells were harvested. Cell lysates were immunoprecipitated with either anti-FLAG mouse MAb (lanes 2, 4, 6, and 8) or control IgG (lanes 1, 3, 5, and 7), and bound proteins were immunoblotted with anti-HNF-1α rabbit PAb (third blot) or anti-NS5A mouse MAb (fourth blot). Protein expression of HNF-1α or FLAG-NS5A was confirmed using the same cell lysates by immunoblotting with either anti-HNF-1α rabbit PAb (first blot) or anti-NS5A mouse MAb (second blot). (B) Cell lysates were immunoprecipitated with either anti-HNF-1α rabbit PAb (lanes 2, 4, 6, and 8) or control IgG (lanes 1, 3, 5, and 7), and bound proteins were immunoblotted with either anti-HNF-1α rabbit PAb (third blot) and anti-NS5A mouse MAb (fourth blot). (C) Full-genome replicon Con1 (RCYM1) cells were plated at 1.2×10^6 cells/10-cm plate and transfected with or without pCMV-HNF-1α plasmid and cultured for 48 h. Cells were harvested and assayed for immunoprecipitation with anti-HNF-1α rabbit PAb (lanes 2 and 4) or control IgG (lanes 1 and 3). Bound proteins were immunoblotted with anti-HNF-1α goat PAb (third blot) or anti-NS5A mouse MAb (fourth blot). Input samples were immunoblotted with either anti-HNF-1α PAb (first blot) or anti-NS5A MAb (second blot). IP, immunoprecipitation; IB, immunoblotting.

1α-binding site on NS5A protein, coimmunoprecipitation analyses were performed. By use of a panel of NS5A deletion mutants (Fig. 7A), FLAG-HNF-1α protein was found to coimmunoprecipitate with all of the HA-NS5A proteins except HA-NS5A consisting of aa 357 to 447 [HA-NS5A(357–447), HA-NS5A(250–447), or HA-NS5A(214–447) (Fig. 7B, lower left panel). These results suggest that domain I of NS5A consisting of aa 1 to 213 is important for HNF-1α binding. FLAG-HNF-1α protein was also found to coimmunoprecipitate with NS5A(1–126)-myc-His_6 and NS5A(1–147)-myc-His_6. These data led to the conclusion that the HNF-1α-binding domain of NS5A protein was aa 1 to 126.

DISCUSSION

In this study, we aimed to clarify molecular mechanisms of HCV-induced suppression of GLUT2 gene expression. The reporter assays of the human GLUT2 promoter suggest that the HNF-1α-binding site is crucial for HCV-induced suppression of GLUT2 promoter activity (Fig. 2). HCV infection significantly reduced the levels of HNF-1α mRNA (Fig. 3A). Moreover, HCV infection remarkably decreased HNF-1α protein levels (Fig. 4A). Our results suggest that HCV infection suppresses GLUT2 gene expression via NS5A-mediated lysosomal degradation of HNF-1α protein (Fig. 5). Immunoprecipitation analyses revealed that NS5A protein physically interacts with HNF-1α protein (Fig. 6) and that domain I of NS5A is important for HNF-1α binding (Fig. 7). Taken together, our results suggest that HCV infection suppresses GLUT2 transcription via downregulation of HNF-1α expression at both transcriptional and translational levels (Fig. 8).

We demonstrated that HNF-1α protein levels were greatly reduced compared to the reduced levels of HNF-1α mRNA. We demonstrated that pepstatin A, but not E64-d, restored the levels of HNF-1α protein, suggesting that an aspartic protease is involved in the degradation of HNF-1α protein. Pepstatin A is widely used for investigation of autophagy and lysosomal degradation. Further studies are needed to elucidate how HCV induces lysosomal degradation of HNF-1α protein and how HNF-1α protein is selectively downregulated by HCV infection. Our data suggest that the HCV NS5A protein is responsible for the HCV-induced degradation of HNF-1α protein. Using a panel of NS5A deletion mutants, we demonstrated that domain I of NS5A is important for association with HNF-1α protein. NS5A domain I is relatively conserved among HCV genotypes compared to domains II and III, suggesting that NS5A–HNF-1α interaction is common to all the HCV genotypes. Domain I coordinates a single zinc atom per protein molecule and is essential for HCV RNA replication (35). The crystal structure of NS5A domain I revealed the presence of a zinc coordination motif and a C-terminal disulfide bond (36). NS5A domain I was found to bind many host proteins, RNA, and membranes (16). It is possible that physical interaction between NS5A protein and HNF-1α protein is important for selective degradation of HNF-1α protein. One possible mechanism is that NS5A protein may recruit HNF-1α protein to the lysosome. Further study is necessary to test this possibility.

We observed that deletion of the GLUT2 transcriptional start site enhances expression of the GLUT2 reporter in FGR cells (Fig. 2B). Cha et al. (7) previously reported that deletion down to nucleotide +73 of the GLUT2 promoter resulted in a marked increase and that further deletion to nucleotide +188 caused a drastic decrease in luciferase activity, indicating the presence of negative- and positive-regulator elements in the 5′ untranslated region. The role of these elements in HCV-infected cells remains to be elucidated.

We demonstrated that HCV J6/JFH1 infection reduced the HNF-1α mRNA level and HNF-1α protein level. Our results contradict an earlier report (32) demonstrating that expression of HNF-1 mRNA was increased in subgenomic replicon Huh.8 cells (3). We observed downregulation of HNF-1α mRNA and

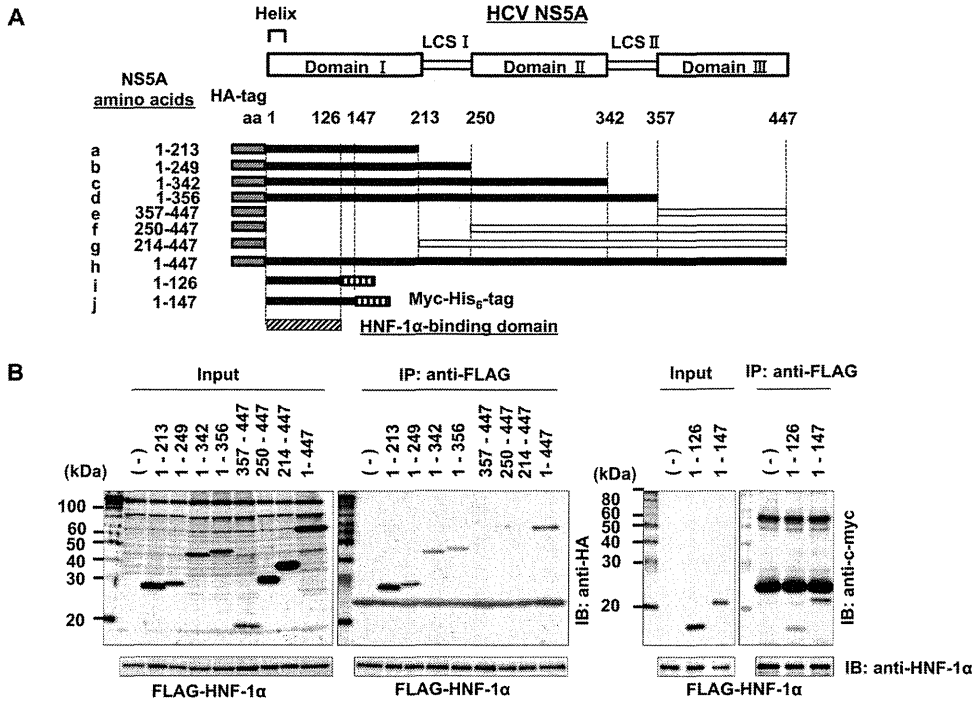

FIG 7 Mapping of the HNF-1α-binding domain for NS5A protein. (A) Schematic representation of the hepatitis C virus NS5A protein. NS5A consists of three domains (domains I, II, and III) with domains separated by low-complexity sequences (LCS I and II). The position of the amino-terminal amphipathic helix membrane anchor is shown (labeled helix). The NS5A deletion mutants (a to j) contain the NS5A amino acids indicated to the left. Each NS5A deletion mutant contains either HA tag in the N terminus (a to h) or myc-His_6 tag in the C terminus (i and j). The gray region of each represents the HA tag sequence. The lattice region of each represents the myc-His_6 tag (i and j). Closed boxes represent proteins that are bound specifically to HNF-1α protein, and open boxes represent those that are not bound. (B) Huh-7.5 cells were transfected with each NS5A mutant plasmid together with a FLAG-HNF-1α expression plasmid. At 48 h posttransfection, cells were harvested, and cell lysates were immunoprecipitated with anti-FLAG beads. Input samples and immunoprecipitated samples were immunoblotted with anti-HA MAb (two left panels, top), anti-c-myc MAb (two right panels, top), or anti-HNF-1α PAb (all panels, bottom).

HNF-1α protein in SGR cells as well as in FGR cells (data not shown). We also demonstrated that the ectopic expression of NS5A protein decreased the endogenous HNF-1α protein level. The reasons for these discrepancies remain to be elucidated.

We along with other groups previously reported that HCV NS5A protein is involved in mitochondrial reactive oxygen species (ROS) production (11, 13, 38). Mitochondrial ROS generation is known to induce the autophagy pathway (22) and lysosomal membrane permeabilization (8). Therefore, it is necessary to determine whether NS5A-induced ROS production enhances autophagic degradation or lysosomal membrane permeabilization. Several groups have reported that autophagy vesicles accumulate in HCV-infected cells and that autophagy proteins can function as proviral factors required for HCV replication (14). Autophagy degrades macromolecules and organelles. Based on the means by which cargo is delivered to the lysosomes, three different autophagy pathways are described: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). At first, autophagy was considered a nonselective bulk degradation process. CMA, however, results in specific degradation of the cytosolic proteins in a molecule-by-molecule fashion. Most known substrates for CMA contain a peptide sequence biochemically related to KFERQ (12). Although the typical KFERQ peptide motif is not found in HNF-1α protein, it is possible that KFERQ-like sequences can be generated by post-translational modifications. It is also possible that HNF-1α protein possesses other degradation motifs. The molecular mechanism underlying NS5A-dependent lysosomal degradation of HNF-1α protein needs to be elucidated.

HNF-1α is a homeodomain-containing transcription factor, which is expressed in the liver, pancreatic β cells, and other tissues (1). Intriguingly, HNF-1α is known to play a crucial role in diabetes. Heterozygous germ line mutations in the gene encoding HNF-1α are responsible for an autosomal dominant form of non-insulin-dependent diabetes, MODY3 (40). Mutations in the HNF-1α gene disrupt GLUT2 function as a glucose sensor in pancreatic β cells, resulting in severe insulin secretory defects (39). It is unclear whether HNF-1α mutations in the liver affect glucose homeostasis in MODY3 patients. Two strains of HNF-1α-deficient mice have been reported. The mice of the first strain, created using standard methods for making knockout mice, are born normally, but most die postnatally around the weaning period after a progressive wasting syndrome (31). Mice of the second strain, created using the Cre-loxP recombination method, had a normal life span (20). The knockout mice of the second strain were dwarfed, diabetic, and infertile. Moreover, the knockout mice had enlarged livers and exhibited progressive liver damage.

HNF-1α was also identified as a tumor suppressor gene involved in human liver tumorigenesis since biallelic inactivating mutations of the HNF-1α gene were found in 50% of hepatocellular adenomas and, in rare cases, of well-differentiated hepatocellular carcinomas developed in the absence of cirrhosis (5).

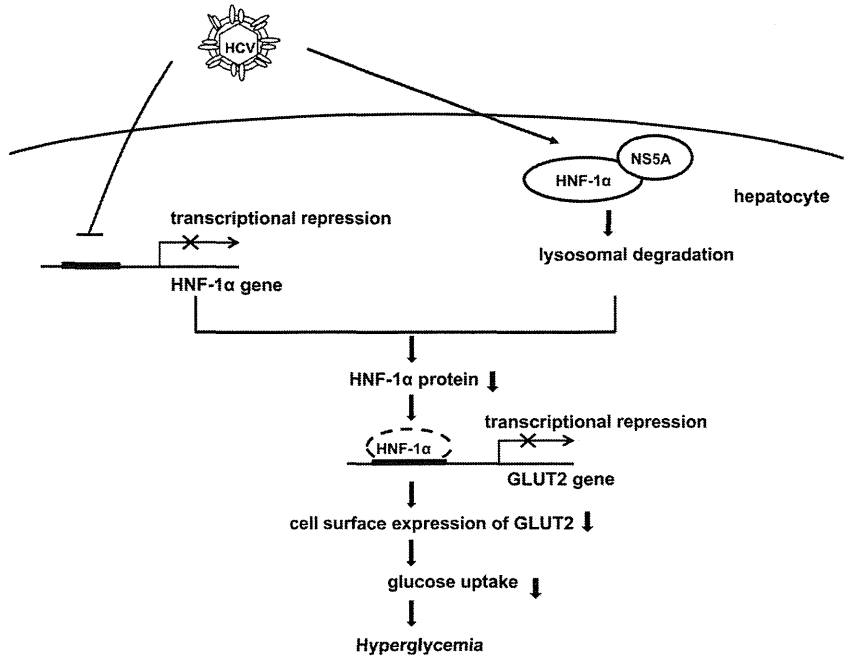

FIG 8 A proposed mechanism of the HCV-induced suppression of GLUT2 via downregulation of HNF-1α. HCV infection downregulates HNF-1α at transcriptional and posttranslational levels, resulting in suppression of GLUT2 gene transcription. HCV NS5A protein physically interacts with HNF-1α protein and enhances lysosomal degradation of HNF-1α protein.

Moreover, HNF-1α has been shown to regulate a large number of genes related to glucose, fatty acid, bile acid, cholesterol, and lipoprotein metabolisms as well as inflammation (1). Therefore, it is possible that HCV-induced downregulation of HNF-1α may play a crucial role in metabolic disorders as well as tumorigenesis.

To determine which HCV protein is involved in the suppression of the GLUT2 promoter, we examined the effects of transient expression of HCV proteins on GLUT2 promoter activity. Overexpression of NS5A suppressed GLUT2 promoter activity, whereas overexpression of p7 enhanced GLUT2 promoter activity (Fig. 5A). SGR cells express NS5A protein but lack p7 protein. FGR cells express both NS5A protein and p7 protein. However, GLUT2 promoter activity was suppressed in both SGR and FGR cells (Fig. 2B). This discrepancy between transient expression system and replicon cells may result from the differences in trafficking of p7 because it is a complex process potentially regulated by both the cleavage from its upstream signal peptides and targeting signals within the protein sequence (15).

We previously reported that HCV infection promotes hepatic gluconeogenesis in HCV J6/JFH1-infected Huh-7.5 cells (11). HCV infection transcriptionally upregulates the genes for phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), the rate-limiting enzymes for hepatic gluconeogenesis. We demonstrated that gene expression of PEPCK and G6Pase was regulated by the transcription factor forkhead box O1 (FoxO1) in HCV-infected cells. Phosphorylation of the FoxO1 at Ser319 was markedly diminished in HCV-infected cells, resulting in increased nuclear accumulation of FoxO1. HCV NS5A protein was directly linked with FoxO1-dependent increased gluconeogenesis. HCV-induced downregulation of GLUT2 expression and upregulation of gluconeogenesis may cooperatively contribute to development of type 2 diabetes in HCV-infected patients at least to some extent. HCV-induced downregulation of GLUT2 expression and upregulation of gluconeogenesis may result in high concentrations of glucose in HCV-infected hepatocytes. As suggested in a recent study, low glucose concentrations in the hepatocytes inhibit HCV replication (28). Therefore, high glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

In conclusion, we provided evidence suggesting that HCV infection downregulates HNF-1α expression at both transcriptional and posttranslational levels. HCV-induced downregulation of HNF-1α may play a crucial role in glucose metabolic disorders caused by HCV infection. Strategies aimed at HCV-induced downregulation of HNF-1α protein may lead to the development of new therapeutic agents for HCV-induced diabetes.

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