HAV genomes, the nucleotide sequence of 5′NTR is more conserved than those of other sites,[13,14] and 5′NTR is predicted to fold into a complex secondary/tertiary structure characterized by six major domains designated I–VI.[15] Domain VI contains the initiation codon. We previously showed that RNA interference targeting various domains of HAV IRES could suppress HAV translation and replication,[6] indicating that some HAV IRES domains might be used as a universal, effective target for specific inhibition of HAV infection.[6] HAV IRES could represent an appropriate target for antiviral drug development.

Amantadine is a tricyclic symmetric amine for use both as an antiviral and an anti-parkinsonian drug. Amantadine inhibits cell-culture-grown HAV IRES-mediated translation in human hepatoma cells,[7] supporting the observation that amantadine could suppress HAV replication in cell culture.[9,16–18] We do not know whether amantadine could suppress clinical isolates from hepatitis A patients.

Here, we examined the HAV IRES activities of clinical isolates from fulminant hepatitis and self-limited acute hepatitis patients in a number of cell lines and tested the effects of amantadine on their IRES-mediated translation by reporter assay. As translation of fulminant hepatitis-derived IRES varies, but it is still efficiently suppressed by amantadine, the approaches described here might open new strategies for useful therapeutic options in cases of fulminant hepatitis A.

METHODS

Cell lines and reagents

HUMAN HEPATOMA CELL lines Huh-7, HepG2 and HLE, the human cervical carcinoma cell line HeLa, and African green monkey kidney cell lines BSC-1 and CV-1 were purchased from Health Science Research Resources Bank (Japanese Collection of Research Bioresources, Osaka, Japan) and maintained in Dulbecco's minimum essential medium (Gibco BRL, Gaithersburg, MD, USA). Amantadine hydrochloride was purchased from Sigma-Aldrich (St Louis, MO, USA).

Bicistronic reporter plasmids

The simian virus (SV)40 promoter plasmid pSV40-HAV-HM175-IRES encodes in a bicistronic fashion the *Renilla reniformis* luciferase (Rluc), the HAV IRES derived from pHM175 (kindly provided by S. U. Emerson, National Institutes of Health, Bethesda, MD, USA),[19] followed by the firefly luciferase (Fluc). It was prepared by polymerase chain reaction (PCR)-based subcloning the IRES (nt. 139–854) of HAV strain HM175[19] and Rluc into pGL3-promoter Vector (Promega, Madison, WI, USA) (Fig. 1a, upper part). Plasmids pSV40-HAV-F1-IRES, pSV40-HAV-F2-IRES, pSV40-HAV-F3-IRES, pSV40-HAV-A1-IRES, pSV40-HAV-A2-IRES and pSV40-HAV-A3-IRES replaced HAV-F1-IRES, HAV-F2-IRES, HAV-F3-IRES, HAV-A1-IRES, HAV-A2-IRES and HAV-A3-IRES, respectively, into HAV-HM175 of the plasmid pSV40-HAV-HM175-IRES. F1–F3 and A1–A3 are derived from fulminant hepatitis and self-limited acute hepatitis, respectively.[20,21] The sequences of plasmids were confirmed by directly sequencing using ABI 377 (Applied Biosystems, Urayasu, Japan).

Transfection and *in vitro* reporter assays

Approximately 1.0×10^5 cells per well were placed in a six-well plate (Iwaki Glass, Tokyo, Japan) 24 h prior to transfection. Cells were transfected with 0.4 μg of pSV40-HAV-IRES using Effectene Transfection Reagent (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Six hours after transfection, the cells were washed once with phosphate buffered saline (PBS), and culture media with or without drugs were added. Forty-eight hours after transfection, cells were harvested using reporter lysis buffer (Toyo Ink, Tokyo, Japan), and luciferase activity was determined by luminometer (AB-2200-R; ATTO, Tokyo, Japan).[7] To control for variations in transcription, IRES activity was assessed by measuring the ratio of *Renilla* and firefly luciferases. All samples were run in triplicate.

Data analysis

The sequences reported in this study have been deposited in GenBank under accession numbers AB513790 to AB513795 for F1 to A3. Sequence analyses were performed using GENETYX ver. 9 (GENETYX, Tokyo, Japan). Data were expressed as mean ± standard deviation. Statistical analysis was done using Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Naturally occurring HAV IRES

HEPATITIS A VIRUS strains associated with human disease show genetic divergence.[22,23] First, we cloned the sequences of HAV derived from clinical isolates and made each bicistronic vector. PCR products were derived from serum samples of patients with fulminant hepatitis, in whom prothrombin time had

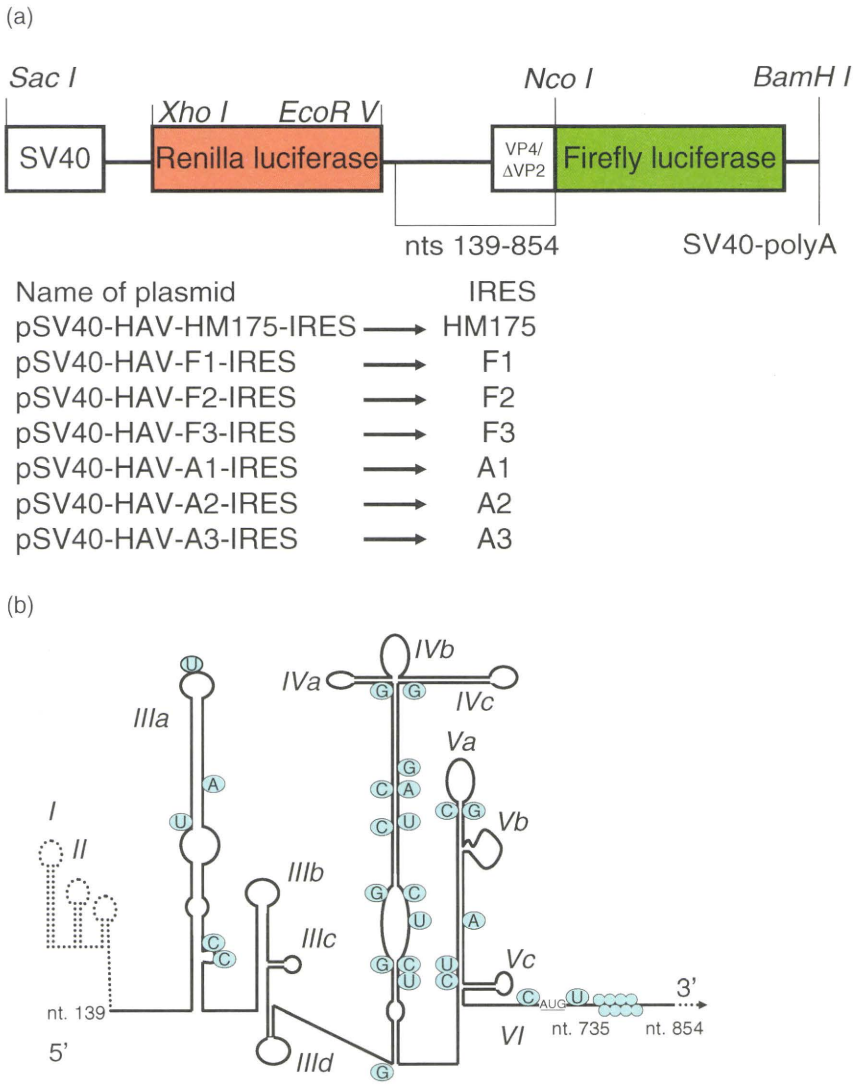
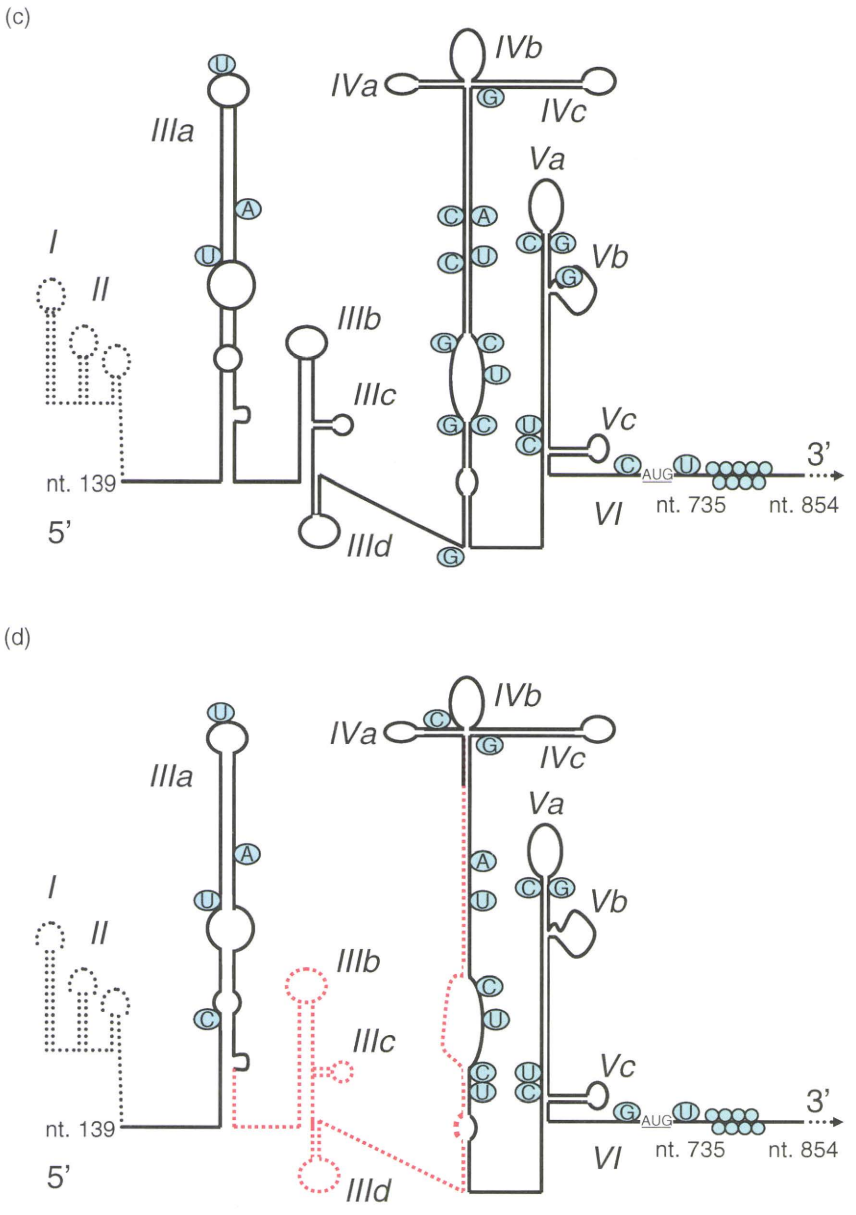


Figure 1 Bicistronic reporter constructs used in this study. (a) pSV40-HAV-HM175-IRES was described previously.[6,7] It encodes the *Renilla* luciferase genes, the internal ribosome entry site (IRES) of hepatitis A virus (HAV) HM175, and the firefly luciferase gene under the control of the simian virus 40 promoter (SV40). pSV40-HAV-F1-IRES, pSV40-HAV-F2-IRES, and pSV40-HAV-F3-IRES encode the IRES from fulminant hepatitis F1, F2, and F3, respectively, instead of the IRES of HM175. pSV40-HAV-A1-IRES, pSV40-HAV-A2-IRES and pSV40-HAV-A3-IRES encode the IRES from self-limited acute hepatitis A1, A2 and A3, respectively, instead of the IRES of HM175. (b–g) Secondary structure and mutations in the HAV IRES constructs used in this study.[6,7,11] Major structural domains are labeled I–VI; blue circles indicate mutations and red-dashed lines were deleted parts, compared with HM175 clone. HAV IRES constructs used in this study include the black line parts. F1 (b), F2 (c) and F3 (d) and A1 (e), A2 (f) and A3 (g) were derived from fulminant and acute self-limited hepatitis, respectively.

decreased to less than 40% with hepatic encephalopathy of grade II or more within 8 weeks after the onset of disease,[24] and with self-limited acute hepatitis in whom prothrombin time had not decreased below 40%. We sequenced these isolates, showing the differences of these sequences in Figure 1(b–g). Of note, one was derived from fulminant hepatitis F3 with a sequence deletion from nt. 233–380 in the HAV IRES region (Fig. 1d) corresponding to a portion of domain III to a part of domain IV. This F3 nucleotide sequence matched 75.60–77.93% of those of cell culture-derived clone HM175 or other clinical isolates in this study. The



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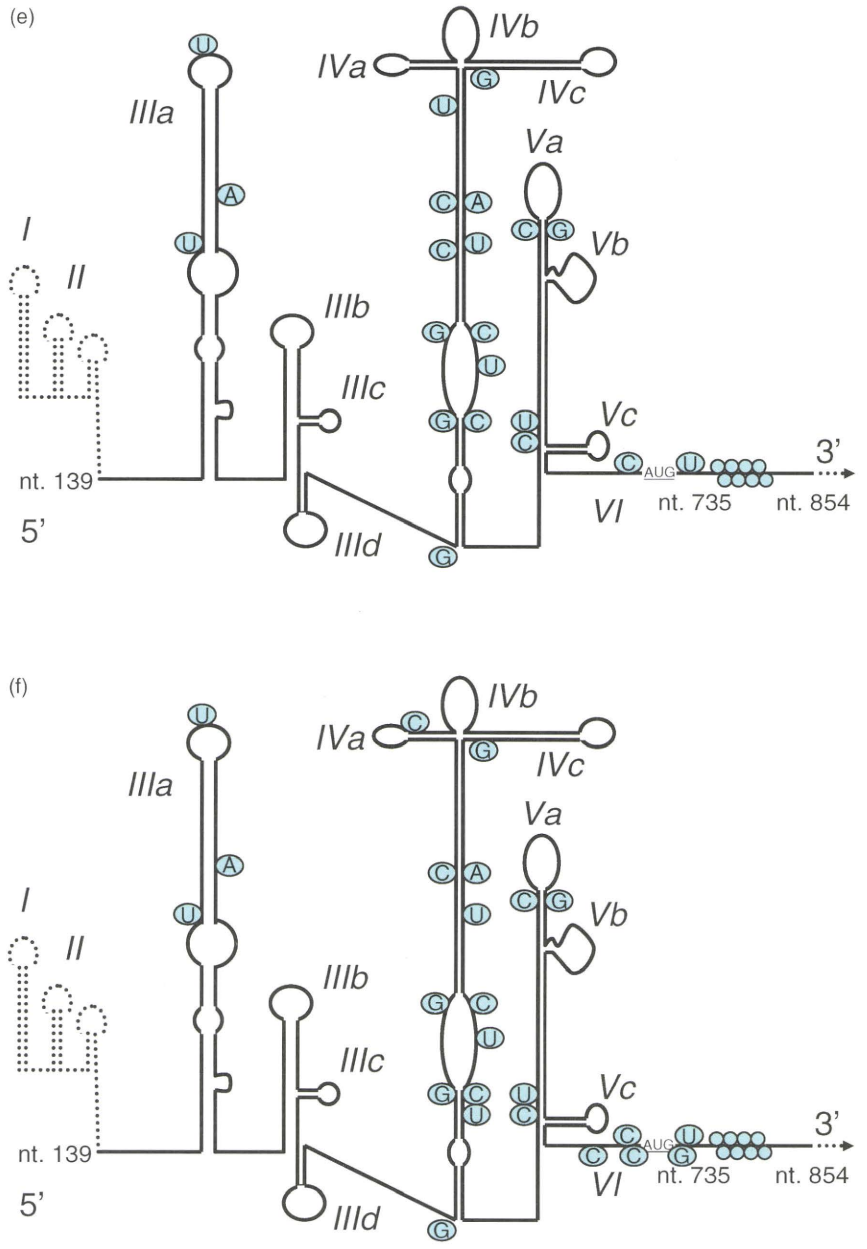
nucleotide sequences of other clinical isolates matched 95.25–95.81% of that of HM175. The ones other than F3 matched 98.18–99.30% with each other (data not shown).

HAV IRES activities from clinical isolates vary in human hepatocytes

Several mutational studies of HAV IRES were previously reported.[25,26] Our major concern before starting this study was whether HAV IRES activities were correlated to the severities of the clinical manifestations of hepatitis A, as HAV genome replication is directly dependent on IRES-mediated translation. Then, we examined how these IRES activities behaved in hepatocytes and non-hepatocytes. It is thought that HAV mainly replicates in the liver where it induces inflammation,[1,2] so we initially examined translation efficiencies of HAV IRES in the following human hepatoma cells: Huh-7, HepG2 and HLE cells (Fig. 2a–c). The IRES activities (firefly luciferase/*Renilla* luciferase: cap-independent/cap-



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dependent translation efficiencies) of HM175 were used as control. The HM175 strain of HAV was originally recovered from stool of a patient with hepatitis A in Melbourne, Australia. It is well-known that plasmid pHM175 was isolated directly from primary African green monkey kidney cells infected with this virus and *in vitro* transcribed RNA from this clone were cell-culture grown.[13,19] In Huh-7 cells, in which HAV can replicate and which are commonly used for HAV research,[27] the IRES activities of F1, F2, F3, A1, A2 and A3 were 1.05-, 0.39-, 3.76-, 0.048-, 2.19- and 0.6-fold, respectively, of that of HM175 (Fig. 2a). In HepG2, the IRES activities of F1, F2, F3, A1, A2 and A3 were 1.29-, 0.33-, 3.68-, 0.12-, 8.91- and 4.23-fold, respectively, of that of HM175 (Fig. 2b). In HLE, the IRES activities of F1, F2, F3, A1, A2 and A3 were 0.85-, 1.35-, 5.17-, 0.55-, 29.06- and 4.31-

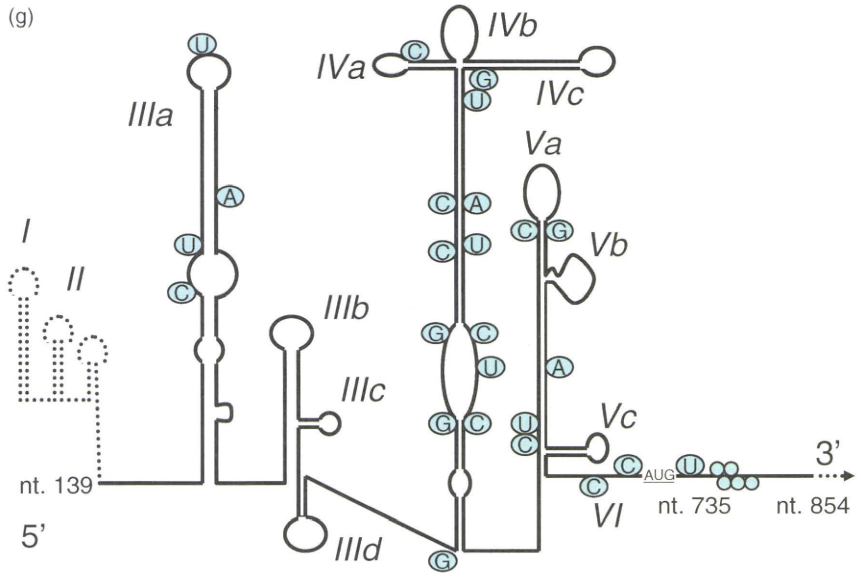

Figure 1 *Continued.*

fold, respectively, of that of HM175 (Fig. 2c). Compared with HM175, F3, A2 and A3 tended to have higher IRES activities in liver-derived cell lines, F1 had similar IRES activity to HM175, and F2 and A1 tended to have lower IRES activities than HM175.

F3 has a large deletion in domains III–IV (Fig. 1d). In A2, several nucleotide mutations around the AUG codon in domain VI of IRES were seen. These mutations possibly affected their IRES activities. A3 IRES activities in Huh-7, HepG2 and HLE were 0.6-, 4.23- and 4.31-fold, respectively. A2 showed very high activity in HLE compared to other IRES. These results may have been influenced by certain cellular factors.

HM175 HAV IRES activities not lower than those of clinical isolates in non-hepatocyte-derived cell lines

In HeLa, another permissive cell line[28] for HAV, the IRES activities of F1, F2, F3, A1, A2 and A3 were 0.33-, 0.31-, 1.0-, 0.11-, 0.87- and 0.32-fold, respectively, of that of HM175 (Fig. 2d).

Acute hepatitis A occasionally presents the manifestation of acute renal failure during the course of the disease.[10] We thus examined HAV IRES activities in African green monkey kidney cell lines BSC-1 and CV-1, as they were reported to be permissive for HAV replication,[29,30] and then we examined the HAV IRES activities in these two cell lines. In BSC-1, the IRES activities of F1, F2, F3, A1, A2 and A3 were 0.11-, 0.10-, 0.73-, 0.02-, 0.48- and 0.86-fold, respectively, of that of HM175 (Fig. 2e). In CV-1, the IRES activities of F1, F2, F3, A1, A2 and A3 were 0.04-, 0.20-, 0.46-, 0.05-, 0.76- and 0.62-fold, respectively, of that of HM175 (Fig. 2f). IRES activities of F3, A2 and A3, which tended to be higher in hepatocytes (Fig. 2a–c), did not differ much from that of HM175 in these non-hepatic cells (Fig. 2d–f). The IRES activities of HM175 in Huh-7, HepG2, HLE, HeLa, BSC-1 and CV-1 were 43.3, 469, 18.2, 618, 1814 and 1097, respectively.

Amantadine has inhibitory effect on clinical isolate-derived HAV-IRES-mediated translations

Amantadine has potential as an antiviral agent against HAV.[9,16–18] We previously reported that amantadine has an inhibitory effect on HAV HM175 IRES-mediated translation.[7] However, the effects of amantadine on clinical isolates from hepatitis A patients were still unknown. Concentrations of 1–100 μg/mL of amantadine were non-cytotoxic to hepatocytes.[7] Huh-7 cells were treated with 100 μg/mL of amantadine or PBS 24 h after transfection of reporter plasmids. Forty-eight hours after transfection, dual-reporter assay was performed for the evaluation of cap-dependent and cap-independent translation initiation (Fig. 3). In A2 isolates, IRES activity in the presence of amantadine was 0.93-fold that in its absence. However, amantadine at 100 μg/mL was effective against all fulminant hepatitis-derived IRES

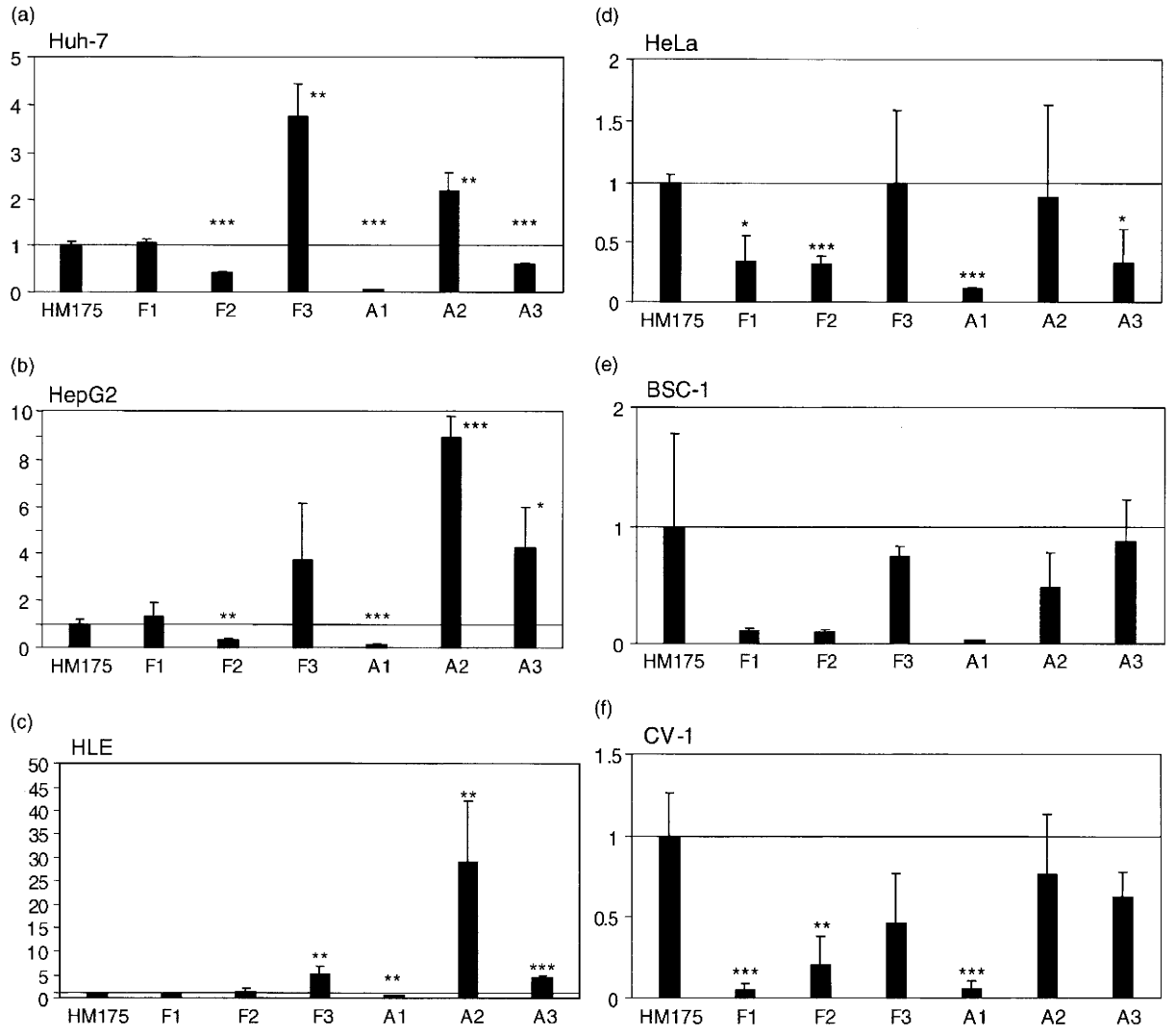

Figure 2 Clinical isolate-derived hepatitis A virus (HAV) internal ribosome entry site (IRES) activities in hepatocytes and non-hepatocytes. Plasmids of pSV40-HAV-IRES were transfected into hepatocytes: Huh-7 (a), HepG2 (b), HLE (c) and non-hepatocytes: HeLa (d), BSC-1 (e) and CV-1 (f). Forty-eight hours after transfection, dual-luciferase assays were performed. The IRES activities (firefly luciferase/*Renilla* luciferase) of pSV40-HAV-HM175-IRES were set at 1. F1, F2 and F3 and A1, A2 and A3 were derived from fulminant and acute self-limited hepatitis, respectively. $*P < 0.05$ vs HM-175; $**P < 0.01$ vs HM-175; $***P < 0.005$ vs HM-175.

activities (Fig. 3). Future studies will reveal whether fulminant hepatitis-derived HAV is more sensitive to amantadine than HAV from self-limited acute hepatitis.

Clinical manifestations among hepatitis A patients in the present study varied from self-limited acute hepatitis to severe fulminant hepatitis. It was reported that chronic liver diseases and older patients influenced the severity of hepatitis A, and the host immune response may vary at the cellular level in such patients. An absence of obvious correlation between genotypes and clinical status has been reported.[31] In this study, the fulminant hepatitis F3 clone had a relatively higher IRES activity in hepatocytes. We previously demonstrated that inhibition of IRES activities by small interfering RNA (siRNA) leads to the suppression of HAV viral replication in cell culture. Fujiwara *et al.*[32] reported that higher viral load in patients with fulminant and severe hepatitis A may be associated with the pathogenesis of disease severity. Further studies are needed.

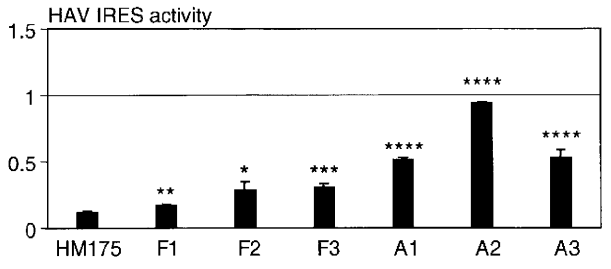

Figure 3 The effects of amantadine against clinical isolate-derived hepatitis A virus (HAV) internal ribosome entry site (IRES) activities. Twenty-four hours after transfection of reporter plasmids, Huh-7 cells were treated with 100 μg/mL of amantadine or phosphate buffered saline (PBS). The effects of amantadine against IRES activities (firefly luciferase/*Renilla* luciferase) are shown. Each value of IRES activity treated with PBS was set at 1. F1, F2 and F3 and A1, A2 and A3 were derived from fulminant and acute self-limited hepatitis, respectively. $*P < 0.05$ vs HM-175; $**P < 0.01$ vs HM-175; $***P < 0.005$ vs HM-175; $****P < 0.001$ vs HM-175.

It was reported that an antibiotic resistance titration assay (ARTA) is useful for HAV neutralization including virus-receptor interaction.[33,34] We also tested the effects of amantadine against HAV IRES of clinical isolates and confirmed the effects against fulminant hepatitis clones. Recently, it was reported that new acridines and hydrazones derived from cyclic β-diketone have stronger antiviral activities against HAV than amantadine.[9] Bicistronic reporter assay using clinical isolates may be another useful tool for testing antiviral activities like those of these drugs.

In conclusion, HAV IRES activities from clinical isolates vary in relation to different cell lines. For the development of antiviral agents against HAV, it seems important to investigate these effects on clinical isolates.

ACKNOWLEDGEMENTS

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Hepatitis A virus (HAV) proteinase 3C inhibits HAV IRES-dependent translation and cleaves the polypyrimidine tract-binding protein

T. Kanda,[1] V. Gauss-Müller,[2] S. Cordes,[2] R. Tamura,[1] K. Okitsu,[1] W. Shuang,[1] S. Nakamoto,[1] K. Fujiwara,[1] F. Imazeki[1] and O. Yokosuka[1] [1]*Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Inohana, Chuo-ku, Chiba, Japan; and* [2]*Institute of Medical Molecular Biology, University of Lübeck, Lübeck, Germany*

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SUMMARY. Hepatitis A virus (HAV) infection is still an important issue worldwide. A distinct set of viruses encode proteins that enhance viral cap-independent translation initiation driven by an internal ribosome entry site (IRES) and suppress cap-dependent host translation. Unlike cytolytic picornaviruses, replication of HAV does not cause host cell shut off, and it has been questioned whether HAV proteins interfere with its own and/or host translation. HAV proteins were coexpressed in Huh-7 cells with reporter genes whose translation was initiated by either cap-dependent or cap-independent mechanisms. Among the proteins tested, HAV proteinase 3C suppressed viral IRES-dependent translation. Furthermore, 3C cleaved the polypyrimidine tract-binding protein (PTB) whose interaction with the HAV IRES had been demonstrated previously. The combined results suggest that 3C-mediated cleavage of PTB might be involved in down-regulation of viral translation to give way to subsequent viral genome replication.

Keywords: 3C protease, hepatitis A virus, IRES, PTB, translation.

INTRODUCTION

The messenger-sense RNA genome of hepatitis A virus (HAV) is about 7500 nucleotides in length and contains a single large open-reading frame (ORF) encoding a polyprotein with the capsid proteins representing the amino-terminal third and the remainder comprising a series of nonstructural proteins required for viral RNA replication: 2B, 2C, 3A, 3B, $3C^{pro}$ (cysteine proteinase responsible for most post-translational cleavage events within the polyprotein) and $3D^{pol}$ (RNA-dependent RNA polymerase, see Fig. 1a, top panel) [1]. In a regulated cascade, the viral polyprotein is cleaved by $3C^{pro}$ into intermediate and mature products that fulfill distinct functions in the viral life cycle. At both ends of the picornaviral genome, the ORF is flanked by highly structured nontranslated regions (5′NTR and 3′NTR). The down-stream part of the 5′NTR presents an internal ribosome entry site (IRES) that allows translation by a cap-independent mechanism [1–3]. Several IRES trans-acting factors (ITAF) have been identified as mediating IRES binding to the ribosome [4]. Whereas glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and La autoantigen suppress HAV IRES activity, the poly(C)-binding protein (PCBP) and the polypyrimidine tract-binding protein (PTB) were found to enhance HAV translation [3,5–9]. PTB, a 57-kDa protein, is a member of the heterogeneous nuclear ribonucleoprotein family that shuttles between the nucleus and cytoplasm [10]. While experimental data have demonstrated PTB binding to polypyrimidine tracts (UCUUU or UCUUC) in picornaviral IRES, the exact cellular functions of PTB are as yet incompletely defined [3,10,11].

Proteolytic cleavage of host proteins is a common mechanism executed by picornaviruses to shut off host cell protein synthesis and to regulate viral protein and RNA synthesis. These two synthetic processes are central in the viral life cycle and mutually exclusive on the same RNA template. As HAV does not shut off host protein synthesis, it seems that HAV cap-independent translation constantly

Abbreviations: GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; HAV, Hepatitis A virus; IRES, internal ribosome entry site; ITAF, IRES trans-acting factors; ORF, open-reading frame; PABP, poly(A)-binding protein; PCBP, poly(C)-binding protein; PTB, polypyrimidine tract-binding protein.

Correspondence: Dr Tatsuo Kanda, Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, Japan. E-mail: kandat-cib@umin.ac.jp

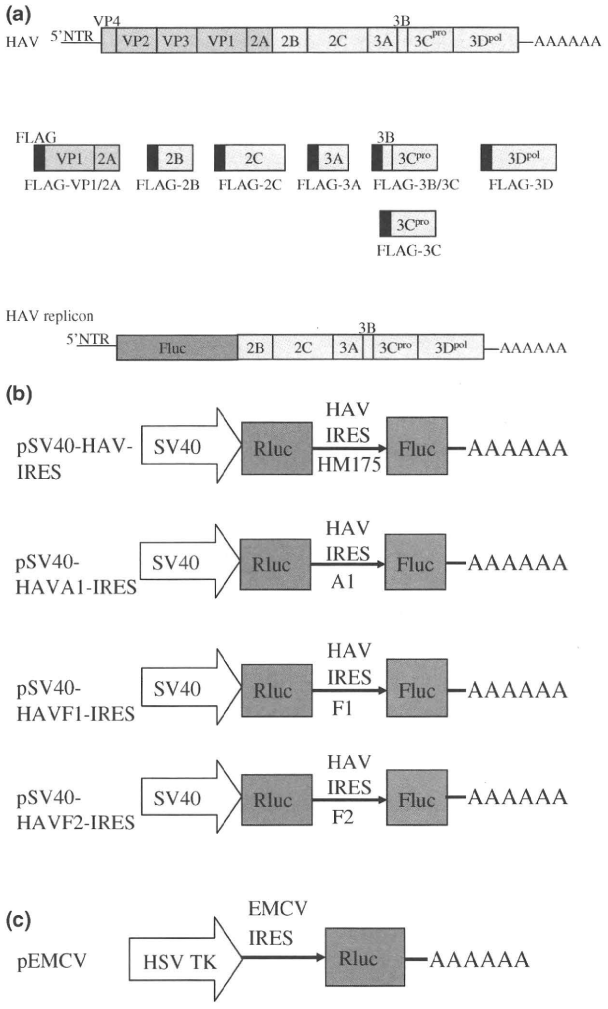

Fig. 1 Schematic representation of the hepatitis A virus (HAV) constructs used in this study. (a) Structures of the HAV genome (upper panel), FLAG-tagged HAV proteins (middle panel; ref. 17) and HAV replicon pT7-18f-luciferase (lower panel; ref. 13). AAAAAA, poly A tail. (b) Structure of the bicistronic plasmids used. pSV40-HAV-internal ribosome entry site (IRES) encodes the Renilla luciferase, the IRES of HAV strain HM175 and the firefly luciferase (Fluc) under the control of the simian virus 40 promoter (SV40) (ref. 16). pSV40-HAVA1-IRES, pSV40-HAVF1-IRES and pSV40-HAVF2-IRES encode IRES elements derived from an acute hepatitis and two fulminant hepatitis cases, respectively. (c) Structure of plasmid pEMCV.

competes with cap-dependent translation of host proteins [1,12]. In this study, we show that HAV $3C^{pro}$ cleaved PTB and suppressed cap-independent translation initiation. The data indicate that the viral proteinase might play an important role in the regulation of HAV IRES-mediated cap-independent translation by targeting noncanonical translation factors.

MATERIALS AND METHODS

Cell lines

Huh-7, a human hepatoma cell line, and its stably transformed derivative Huh-T7 that expresses the T7 RNA polymerase [3] were grown in Dulbecco's modified Eagle medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated foetal bovine serum with or without G418 sulfate (400 μg/mL; Promega, Madison, WI, USA), in addition to penicillin and streptomycin.

Plasmids

pT7-18f-luciferase (LUC), a replication-competent HAV replicon, containing an open-reading frame with the firefly luciferase (Fluc) flanked by the first four amino acids of the HAV polyprotein and by 12 C-terminal amino acids of VP1, followed by the P2 and P3 domains of the HAV polyprotein (HAV strain HM175 18f, GenBank Accession No. M59808), and pT7-18f-LUCmut, a replication-deficient replicon, were described previously [13] (Fig. 1a).

The constructs encoding the simian virus 40 (SV40) promoter-driven *Renilla reniformis* luciferase (Rluc), the IRES derived from the cell culture adapted HAV strain HM175 [14], and Fluc, named pSV40-HAV-IRES, was prepared as described previously [2,15] (Fig. 1b). To investigate the specific effect exerted by the HAV IRES sequences, bicistronic reporter constructs (pSV40-HAVA1-IRES, pSV40-HAVF1-IRES and pSV40-HAVF2-IRES; Kanda *et al.*, manuscript in preparation) were prepared, which included the IRES of clinical specimens. Construction of HAV protein expression plasmids was described previously [16]. Briefly, seven regions of the HAV genome were amplified by reverse transcription-polymerase chain reaction (PCR) with HAV region-specific primers [16]. These regions were HAV VP1-2A, 2B, 2C, 3A, 3BC, 3C and 3D. The amplified products were cloned into pCXN2 (kindly provided by Prof. J. Miyazaki, Osaka University, Japan), a mammalian expression vector with a beta-actin-based CAG promoter and a SV40 origin [17], to generate pCXN2-FLAG-VP1-2A, 2B, 2C, 3A, 3BC, 3C, 3D expressing FLAG-tagged proteins [16] (Fig. 1a). To control for the target specificity, pEMCV, which contains the encephalomyocarditis virus (EMCV) IRES upstream of Rluc, was generated (Fig. 1c). Transient expression of $3C^{pro}$ using vaccinia virus, pGEM-3C, and pEXT7-HAV3C was described before [12].

Transfection and protein analyses

Approximately 60% confluent Huh-7 cells, grown in 6-well culture plates, were transfected with 0.3 μg of the LUC reporter plasmid and 0.1 μg of each HAV protein-expressing plasmid using Effectene transfection reagent (Qiagen, Tokyo, Japan). Forty-eight hours after transfection, cell

extracts were prepared, and a LUC assay kit (Toyo Ink, Tokyo, Japan) was used according to the manufacturer's instructions. LUC activity was measured in relative light units with a luminometer (AB-2200-R; ATTO, Tokyo, Japan). The assays were adjusted to protein amount and were conducted, on average, in duplicate [18]. To determine cleavage of the host proteins PTB and poly(A)-binding protein (PABP), extracts of transfected cells were analysed for viral antigen and host proteins, as described previously [12]. Viral proteins were identified using anti-FLAG and anti-HAV 3C antibodies. PTB was recognized by the monoclonal antibody BB7 [19].

RESULTS

HAV proteinases 3BC and 3C suppress IRES-dependent translation

Translation of the HAV polyprotein is initiated cap-independently and is driven by an IRES. As a first approach to assess the role of HAV proteins, we examined cap-independent and cap-dependent translation using the bicistronic reporter constructs depicted in Fig. 1. pSV40-HAV-IRES, which contains the IRES of HAV strain HM175 (Fig. 1b), was transfected into Huh-7 together with various expression vectors encoding FLAG-tagged HAV protein (Fig. 1a). The expression of these proteins was confirmed by Western blotting with anti-FLAG antibodies (data not shown and ref. 16). Compared to the control (pCXN2) and to the other HAV proteins tested (VP1-2A, 2B, 2C, 3A and 3D), expression of HAV 3BC or 3C specifically inhibited cap-independent translation initiated by the HAV IRES as determined by the Fluc activity (Fig. 2).

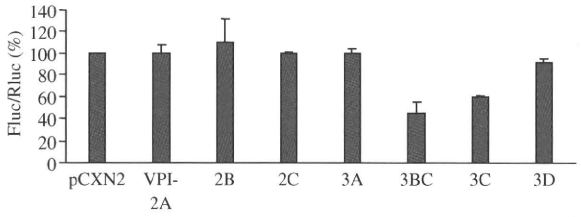

Fig. 2 Effects of hepatitis A virus (HAV) proteins on HAV internal ribosome entry site (IRES)-dependent translation. Relative luciferase activities are indicated (IRES/Cap; firefly luciferase/*Renilla reniformis* luciferase, %). Luciferase activities were determined in three independent experiments. Error bars represent standard errors of the mean.

To corroborate the observed suppression of HAV IRES-independent translation, we next examined the effect of $3C^{pro}$ on translation, which was dependent on HAV IRES elements derived from clinical isolates; IRES A1 was taken from an acute self-limited hepatitis (pSV40-HAVA1-IRES), and F1 and F2 were derived from fulminant HAV infections (pSV40-HAVF1-IRES and pSV40-HAVF2-IRES) (Fig. 3a–c). After coexpression of pSV40-HAVA1-IRES, pSV40-HAVF1-IRES and pSV40-HAVF2-IRES with 3BC or $3C^{pro}$, the Fluc activity was specifically suppressed when compared to the control (pCXN2, Fig. 3a–c). The results confirm our findings shown in Fig. 2 and demonstrate that HAV proteinases 3BC and $3C^{pro}$ suppress HAV IRES-dependent translation. For yet unknown reasons, the negative effect of 3BC was generally more pronounced than that exerted by $3C^{pro}$. However, as $3C^{pro}$ is the prevailing and stable form of the viral proteinase, only this form was used in the subsequent studies.

Translation of the viral polyprotein is the first metabolic step in the viral life cycle and a prerequisite for viral RNA synthesis. It can be assumed that a negative effect on

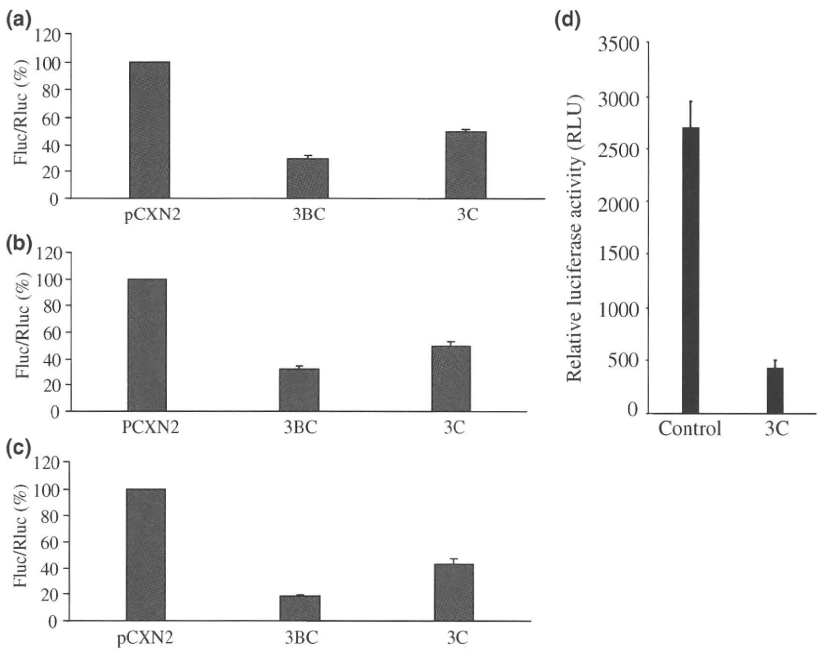

Fig. 3 Effects of hepatitis A virus (HAV) 3BC or $3C^{pro}$ on HAV internal ribosome entry site (IRES)-dependent translation (a–c) and on expression of the HAV replicon [pT7-18f-luciferase (LUC)] (d). HAV was derived from clinical isolates: (a) acute hepatitis; (b) and (c) two forms of fulminant hepatitis. Relative luciferase activities are indicated (IRES/Cap; firefly luciferase/*Renilla reniformis* luciferase, %) (a–c). LUC activities are presented as an average of three independent experiments. Error bars represent standard errors of the mean. RLU, relative light units.

translation might indirectly cause a reduction in viral genome production. HAV replication was efficiently studied using the viral replicon (see Fig. 1a, lower panel) with Fluc as reporter gene in place of the viral structural proteins [13]. The reporter gene activity is directly proportional to viral RNA synthesis. To investigate whether 3C-mediated suppression of translation affects genome replication, we cotransfected HAV replicon RNA with the 3C-expression or control vector into Huh-T7 cells (Fig. 3d). HAV replicon replication was monitored by reporter assay 72 h post-transfection. Compared to the replication-deficient replicon (pT7-18f-LUCmut), the reporter activity at this point was derived from newly synthesized viral genomes and therefore represents viral genome synthesis [13]. Compared to the control, HAV replication was significantly suppressed in the presence of excess 3C, indicating that 3C-mediated inhibition of translation restrained HAV genome replication in human hepatoma cells.

To assess the specificity of the inhibitory effect exerted by HAV proteinase $3C^{pro}$, translation initiated at the EMCV IRES was compared with the HAV IRES. For this, HAV $3C^{pro}$ was coexpressed with pEMCV (Fig. 1c), and the Rluc activity of the cell extracts collected 48 h post-transfection was determined. Compared to the HAV IRES tested in parallel experiments, the EMCV IRES activity was similar in the presence and absence of coexpressed HAV $3C^{pro}$ [118 ± 29 (%)]. Combined and in light of the results described in the following, these findings suggest that an essential ITAF was cleaved by HAV $3C^{pro}$. As shown in the following, HAV $3C^{pro}$ partially cleaved PTB, whose active role in picornaviral IRES-dependent translation has been demonstrated previously [6,8]. As EMCV IRES translation was unaffected by HAV $3C^{pro}$, PTB is not an essential ITAF for this IRES, confirming an earlier report [20]. Intriguingly, an excess of PTB even suppressed EMCV IRES-driven translation [21]. The abundance and distribution of PTB varies significantly among cell types [6,8]. Large amounts of PTB were found in the cytoplasmic fraction of Huh-7 cells that were used in our studies. Based on these observations, the results reported here suggest that HAV $3C^{pro}$ reduced the cytoplasmic levels of intact PTB to such a degree that only the activity of the HAV IRES was affected, but not that of the EMCV IRES.

$3C^{pro}$ cleaves PTB

It has been reported that the HAV IRES is associated with La autoantigen, GAPDH, PTB, PABP and PCBP [6,8,12–14]. The latter two proteins were cleaved by HAV $3C^{pro}$ [12,13]. Furthermore, it was shown that PTB is cleaved by polioviral $3C^{pro}$ and that PTB fragments inhibit polioviral IRES-dependent translation [22]. To assess whether the observed suppression of HAV IRES translation might be because of 3C-mediated cleavage of PTB, we tested the levels of endogenous PTB after transient expression of $3C^{pro}$ in Huh-7 cells. As GAPDH was found to suppress HAV IRES translation and to antagonize the enhancing effect of PTB [8], GAPDH levels were tested in parallel. As control for the proteolytic activity of $3C^{pro}$ *in vivo*, cleavage of the poly(A)-binding protein was also analysed. Recombinant $3C^{pro}$ was identified by immunoblot with anti-3C (Fig. 4, left panel) [12,13], and PABP was partially cleaved as demonstrated earlier (Fig. 4, middle panel). Whereas the levels of GAPDH were unchanged (data not shown), a PTB cleavage product of approximately 45 kDa and a slightly faster migrating polypeptide were clearly detectable when HAV $3C^{pro}$ was expressed (Fig. 4, right panel). The extent of host protein cleavage significantly depended on the amount of 3C expressed (compare lanes 1 and 3). Specific PTB cleavage was also observed when the extracts used in Fig. 2 were tested (not shown). Moreover, PTB of Huh-7 cells, the rabbit

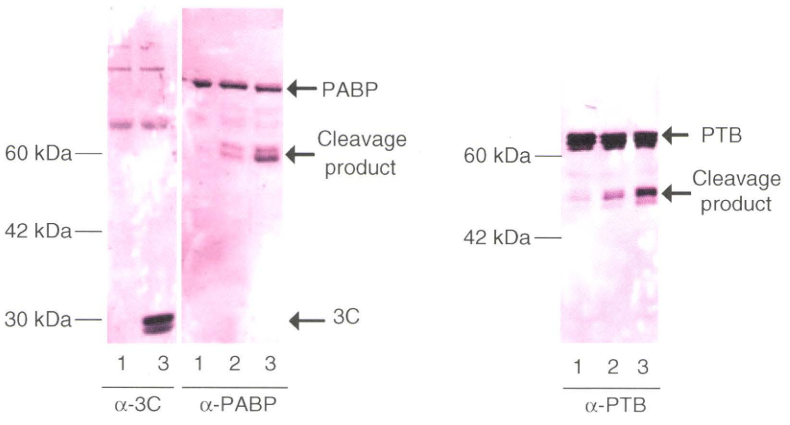

Fig. 4 Hepatitis A virus (HAV) $3C^{pro}$ cleaves the polypyrimidine tract-binding protein (PTB). Huh-7 cells were transfected with pGEM (lanes 1), pGEM-3C (lanes 2) and pEXT7-HAV3C (lanes 3) and infected with vaccinia virus T7. Cell lysates were collected 24 h post-transfection and subjected to immunoblot using anti-3C, anti-poly(A)-binding protein and anti- PTB. As less $3C^{pro}$ was produced by pGEM-3C in comparison with pEXT7-HAV3C, cleavage of host proteins was more pronounced in lanes 3 when compared to lanes2.

reticulocyte lysate, and recombinant PTB produced in *E. coli* was substrate to cleavage-mediated *in vitro* by purified recombinant HAV $3C^{pro}$ (data not shown). Combined with its well-documented translation enhancing effect and binding specificity to stem-loop IIIa of the HAV IRES [6–8], the results strongly suggest that the inhibitory effect of HAV $3C^{pro}$ on HAV IRES translation is because of proteolytic cleavage of PTB.

DISCUSSION

The expression level of the viral proteinase was found to substantially affect the detection of PTB cleavage products (see Fig. 4). Neither in HAV-infected cells nor in cells expressing the HAV replicon were PTB cleavage fragments detectable (not shown). A similar discrepancy was observed for PCBP, another ITAF that is essential for picornaviral translation and the molecular switching to RNA replication [9,23,24]. Whereas PCBP cleavage by recombinant HAV $3C^{pro}$ was clearly shown, PCBP-processing products were not apparent in extracts of HAV-infected cells [9]. Combined, our findings on HAV-3C-mediated cleavage of PCBP and PTB suggest that because of the protracted replication of HAV, very low quantities of $3C^{pro}$ are present in infected cells and cleavage of these host proteins is not discernible. This is in clear contrast to poliovirus whose highly efficient replication resulted in obvious cleavage of both PCBP and PTB [22,24].

The functional domains of PTB are four RNA recognition motifs that all bind short pyrimidine-rich sequences. By binding to different sites on the same RNA molecule, PTB can lead to distinctive RNA restructuring. Such conformational changes are thought to be critical in enabling the ribosomal recruitment in IRES-driven translation initiation. Our constructs do not include the 1–138 nt region of 5′NTR, in which a pyrimidine-rich-tract exists. PTB interacts with stem-loop IIIa of the HAV IRES that contains short polypyrimidine tracts [7]. These binding sites can be bridged by a single PTB molecule, which is an arrangement that favours a role for PTB as an RNA chaperone. It is likely that PTB stabilizes or alters the IRES structure to enable the recruitment of the ribosome and to position it correctly at the start codon.

For poliovirus, direct evidence was provided that PTB cleavage products inhibited IRES-dependent translation [22]. As outlined by the authors, it is possible that PTB fragments may interfere with the binding of intact PTB to poliovirus IRES or that cleaved PTB may no longer function as translational activator that facilitates the recruitment of translational machinery to the IRES element. Although not directly assessed here, it is assumed that suppression of HAV IRES translation is induced by similar mechanism(s). Moreover, in poliovirus-infected Hela cells, PTB cleavage fragments are redistributed to the cytoplasm [22]. As abundant quantities of PTB are present in the cytoplasm of Huh-7 cells used in our study [8], PTB redistribution might not be essential for the effect of PTB cleavage on HAV translation. Yet it is attractive to speculate that the PTB fragment(s) might have altered RNA-binding specificity. For poliovirus IRES translation, an attractive model was put forward for the participation of PTB and PCBP in the molecular switch from viral translation to RNA replication [22]. Supposedly, after viral 3C-mediated cleavage, PTB and PCBP lose their enhancing function. Once IRES translation is stalled, replication of the viral RNA consequently is turned on. Taken together with our earlier observations [9], HAV translation is inhibited indirectly by its own product, $3C^{pro}$, through the proteolytic cleavage of PCBP and PTB.

The HAV 3B and 3C proteins are 23 and 219 amino acids in length, respectively [25]. The 3B moiety was found to be essential for the 3AB interaction with 3CD [26]. It seems that 3BC was more suppressive than 3C in cap-independent translation. Further studies will reveal the 3B function in the interaction with PTB and 3BC. In conclusion, HAV proteinase 3C cleaved PTB and suppressed HAV IRES-dependent translation.

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STATEMENT OF PERSONAL INTERESTS

None of the authors have personal interests relevant to this research to declare.

DECLARATION OF FUNDING INTERESTS

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ORIGINAL PAPER

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A nationwide survey for prevalence of hepatitis E virus antibody in qualified blood donors in Japan

H. Takeda,[1] K. Matsubayashi,[1] H. Sakata,[1] S. Sato,[1] T. Kato,[1] S. Hino,[2] K. Tadokoro[2] & H. Ikeda[1]

[1] *Japanese Red Cross Hokkaido Blood Center, Sapporo, Japan*

[2] *Blood Service Headquarters, Japanese Red Cross Society, Tokyo, Japan*

Vox Sanguinis

Background and Objectives In previous studies, we reported the transmission of hepatitis E virus (HEV) by transfusion, and the frequent detection of HEV markers in Japanese blood donors with elevated ALT levels. For the current study, we carried out a nationwide survey of the prevalence of IgG anti-HEV in qualified blood donors throughout Japan.

Materials and Methods The 12 600 samples from qualified blood donors were collected from seven blood centres (1800 per centre) representing nearly all regions of Japan. Samples were from age- and sex-matched blood donors who tested negative for all the current blood screening tests. The samples were screened using the in-house IgG anti-HEV ELISA. Sequentially, the positive samples were tested by the commercial IgG anti-HEV ELISA.

Results Of 12 600 samples, 431 (3·4%) were regarded as positive for IgG anti-HEV. The prevalence of IgG anti-HEV was higher in eastern Japan (5·6%) than in western Japan (1·8%) ($P < 0·001$), and was also age-dependent and higher in men (3·9%) than in women (2·9%) ($P = 0·002$).

Conclusion The spread of the domestic infection of HEV was observed in qualified blood donors in Japan. A higher prevalence of IgG anti-HEV was observed in male donors, older donors and in donors residing in eastern Japan. Further studies are necessary to clarify the potential risk of transfusion-transmission of HEV in Japan.

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Introduction

Hepatitis E virus (HEV) is one of the major causative agents of acute hepatitis in many developing countries, such as in South-East Asia, Africa, Central and South America and in the Middle East [1]. HEV infection in endemic countries is reported to occur mainly via the faecal–oral route of transmission through contaminated water supplies. HEV-associated hepatitis also occurs among industrialized countries including Japan, where hepatitis E had previously been believed to be non-endemic [2–8]. Of 4 HEV genotypes, genotype 3 and genotype 4 are responsible for indigenous hepatitis E in industrialized countries, while genotype 1 is dominant in the endemic countries. Recent studies have documented that wild boars, wild deer or pigs may act as reservoirs for HEV infection in Japan [9–13], where the major route of infection is likely to be food-borne zoonosis through HEV-infected animal meat. A potential risk of blood-borne transmission exists, because HEV-infected individuals displayed long-term viremia [14–16]. Abe *et al.* [4] found that transmission routes remained obscure in most cases (approximately 60%), whereas about 30%, 8% and 2% were attributed to zoonotic food-borne transmission, imported infection and via blood transfusion, respectively. We previously reported two cases of transfusion-transmitted hepatitis E in Hokkaido, Japan [14,16]. HEV RNA and/or anti-HEV were detected in some Japanese blood donors with elevated ALT levels [14,17–19].

Correspondence: Hiromi Takeda, Japanese Red Cross Hokkaido Blood Center, 2-2 Yamanote, Nishi-ku, Sapporo 063-0002, Japan
E-mail: niwa@hokkaido.bc.jrc.or.jp

We reported the nationwide survey for HEV prevalence among Japanese blood donors with elevated ALT levels, where HEV markers (HEV RNA and anti-HEV) were frequently detected in such donor samples collected throughout Japan [19]. However, the real situation of HEV prevalence in blood donors was unknown in the previous report because donor samples with elevated ALT levels were unevenly distributed in men, and the sample size of age groups of 10s, 50s and 60s was small. Consequently, it is important to study HEV prevalence in the typical donor population in Japan. A few studies have been reported on the prevalence of IgG anti-HEV in Japanese healthy individuals in certain areas of Japan [17,20]. The results can be summarized as follows: seroprevalence is (1) highly variable (1·9–14·1%), (2) higher in men and (3) shows age-dependency. In those reports, samples were collected in limited areas and/or the sample sizes were rather small. There has been no report that tried to compare the prevalence of HEV antibodies in age- and sex-matched healthy population at the same period all over Japan so far. Accordingly, further systemic study with a larger number of those individuals from more different parts of Japan will be necessary to draw a definitive conclusion.

To verify the real situation of HEV prevalence in healthy blood donors, we carried out a nationwide survey for the prevalence of IgG anti-HEV in qualified blood donors who were collected separately by age group and sex. IgG anti-HEV are reported to remain long after the disappearance of the virus in the infected individuals and can serve as the marker of the HEV prevalence [14,16]. In this study, we collected 12 600 donor samples from seven Japanese Red Cross (JRC) Blood Centers representing most regions of Japan and tested for the presence of IgG anti-HEV.

Materials and methods

HEV specimens

For the preliminary study, we used serum samples of HEV patients (n = 55) that had been obtained for our investigations including trial HEV nucleic acid testing for transfusion in Hokkaido [19] and qualified random blood donor samples (n = 16) that were collected at JRC Hokkaido Blood Center in 2005. These samples were tested with the commercial IgG anti-HEV ELISA (Cosmic corporation, Ltd., Tokyo, Japan) and the in-house IgG anti-HEV ELISA. The tests were performed as described later. The samples were kept frozen below −20°C until testing.

Qualified blood donor samples

In JRC Blood Centers, donated blood is screened for infectious diseases (HBV, HCV, HIV-1/2, human T-lymphotropic virus type 1, syphilis, human parvovirus B19). In addition, ALT level has been used as a surrogate test for hepatitis caused by viruses other than HBV or HCV. A total of 12 600 serum samples were collected from qualified Japanese blood donors who had an ALT level of 60 IU/l or lower and tested negative for all the current blood screening tests in the seven blood centres at Hokkaido, Miyagi, Tokyo, Aichi, Osaka, Okayama and Fukuoka from November 2005 through February 2006. Donors were categorized into six age groups: 16–19 (10s), 20–29 (20s), 30–39 (30s), 40–49 (40s), 50–59 (50s) and 60–69 (60s). The samples at each blood centre were collected separately by age group and by sex, resulting in 150 male and 150 female samples in each age group (a total of 1800 samples).

ELISA for detecting IgG anti-HEV

In the absence of specific confirmatory assay that detects IgG anti-HEV, the samples were screened by the in-house IgG anti-HEV ELISA and the positive samples were tested by the commercial IgG anti-HEV ELISA (Fig. 1). An in-house ELISA was developed according to the methods of Li *et al.* [20], with some modifications [19]. In the in-house assay, virus-like particles (VLPs) consisting of recombinant ORF2 proteins of HEV genotype 1 expressed in cultured recombinant baculovirus-infected insect cells were used as antigens [21]. Wells of microplates (Number 2592, 96-well Stripwell, flat bottom; Corning Life Sciences, Corning, NY) were coated with 50 μl of the recombinant ORF2 protein [3 μg/ml in phosphate-buffered saline (PBS)], and the plates were incubated at room temperature (RT) for 2 h followed by incubation with 100 μl of blocking buffer containing 40% (v/v) calf serum (Gibco-BRL, Tokyo, Japan) at RT for 1 h. To test for IgG anti-HEV, 50 μl of each sample

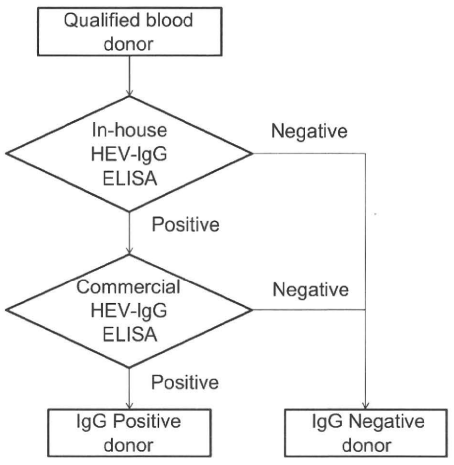

Fig. 1 The algorithm of IgG anti-hepatitis E virus test.

was added to each well at a dilution of 1:100 in saline containing 40% calf serum. The microplates were incubated at RT for 1 h and then washed five times with 0·05% Tween 20 in PBS (PBS-T). Fifty microlitre of horseradish peroxidase-conjugated mouse anti-human IgG (IGB22; Institute of Immunology Co., Ltd., Tokyo, Japan; 1:2000) in PBS-T containing 25% (v/v) foetal calf serum (PAA Laboratories GmbH, Pasching, Austria) was added to each well and incubated at RT for 1 h. The mouse anti-human IgG used in the in-house ELISA was not crossreactive to human IgM at the conditions in the test. The wells were washed five times with PBS-T. Fifty microlitre of tetramethylbenzidine-soluble reagent (Dako Co., Ltd., Carpinteria, CA) as a substrate was added to each well. The plate was incubated at RT for 10 min in the dark, and then 50 μl of 1 N sulphuric acid (Kanto Chemical Co., Inc., Tokyo, Japan) as tetramethylbenzidine stop buffer was added to each well. The optical density (OD) of each sample was read at 450 nm. Test samples with OD values equal to or greater than the cut-off value were considered positive for the presence of IgG anti-HEV in this ELISA. The cut-off value for the in-house ELISA was set tentatively at 7 SDs above the mean value of 81 HEV RNA-negative sera for IgG anti-HEV. The reactive samples were subsequently tested with a commercial IgG anti-HEV ELISA (Cosmic corporation, Ltd., Tokyo, Japan), which was developed according to the methods of Mizuo *et al.* [3] and utilizes VLPs consisting of recombinant ORF2 proteins of HEV genotype 4 expressed in recombinant baculovirus-infected silkworm pupae. The commercial ELISA was performed according to the manufacturer's protocol. Samples were determined to be positive, if they were reactive by both ELISA methods.

Statistical analysis

Statistical analysis was performed by the chi-square test. A difference with a P-value of $< 0·05$ was considered significant.

Results

The Comparison of the in-house IgG anti-HEV ELISA and the commercial IgG anti-HEV ELISA among the HEV specimens

In the preliminary study, the 71 HEV specimens were tested with the in-house and the commercial IgG anti-HEV ELISA (Fig. 2). Of the 55 sera from the HEV patients, 34 (62%) were reactive in the commercial assay and all of which were also reactive in the in-house assay. Twenty-one were from the patients with HEV RNA and/or IgM anti-HEV in very early stage of HEV infection. Of the 21 sera, 20 sera were not reactive with both ELISAs and one sera was borderline positive (index was 12·9 and cut-off index was 11) in the in-house assay and borderline negative (index was 12·9 and cut-off index was 13) in the commercial assay. In addition, 16 samples were not reactive for IgG anti-HEV by both ELISAs among 16 qualified random blood donor samples. The result of preliminary study with the HEV specimens indicated that the in-house IgG anti-HEV ELISA and the commercial IgG anti-HEV ELISA was 99% concordant and could discriminate IgG anti-HEV-positive samples from negative samples. Overall, the index of the in-house IgG anti-HEV ELISA appeared to be lower than that of the commercial IgG anti-HEV ELISA.

IgG anti-HEV in qualified Japanese blood donors

Of the 12 600 donor samples, 431 (3·4%) showed the presence of IgG anti-HEV (Figs 3–5). The positivity rate of IgG anti-HEV among each age group: 10s, 20s, 30s, 40s, 50s and 60s was 0·5% (10/2100), 1·0% (21/2100), 2·4% (50/2100), 4·3% (90/2100), 6·0% (126/2100) and 6·4% (134/2100), respectively (Fig. 3). The prevalence of IgG anti-HEV showed clear age-dependence and increased with age in both sexes. The peak of the positivity rate was in 60s age group in men (92/1050 = 8·8%) and in 50s age group

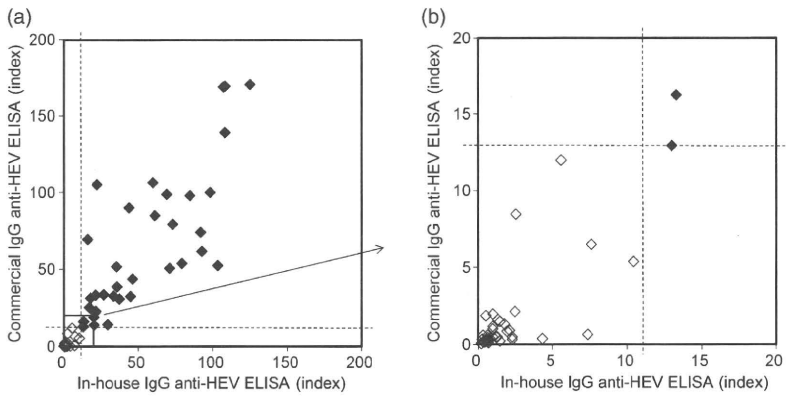

Fig. 2 Correlation of the two ELISAs for IgG anti-hepatitis E virus among the hepatitis E virus specimens (n = 71). The closed and open diamonds represent the hepatitis E virus patients samples (n = 55) and qualified random blood donor samples (n = 16), respectively. Horizontal and vertical broken lines represent cut-off index of the in-house ELISA for IgG anti-hepatitis E virus (cut-off index = 11) and the commercial kit for IgG anti-hepatitis E virus (cut-off index = 13), respectively. The box area in the (A) was magnified as shown in the (B).

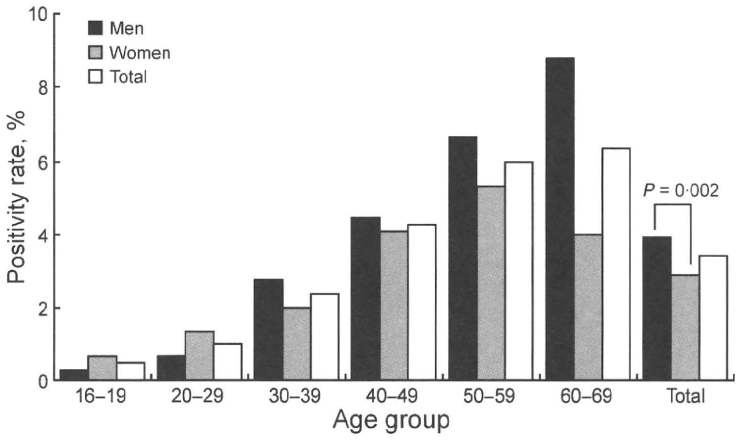

Fig. 3 IgG anti-hepatitis E virus prevalence by sex and age group in qualified blood donors. Each age group consisted of 1050 men (black bars) and 1050 women (grey bars).

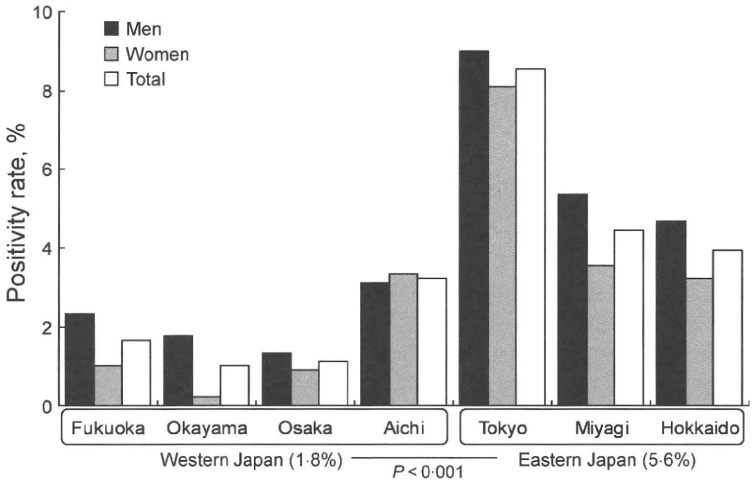

Fig. 4 IgG anti-hepatitis E virus prevalence by blood centre in qualified blood donors. Samples from each blood centre consisted of 900 men (black bars) and 900 women (grey bars).

in women (56/1050 = 5·3%), respectively. Overall, a significant difference in the positivity rate was found between male and female samples (248/6300 = 3·9% vs. 183/6300 = 2·9%; $P = 0·002$). In 60s age group, the positivity rate among men was significantly higher than women (92/1050 = 8·8% vs. 42/1050 = 4·0%; $P < 0·001$).

The positivity rate of IgG anti-HEV in Hokkaido, Miyagi, Tokyo, Aichi, Osaka, Okayama and Fukuoka was 3·9% (71/1800), 4·4% (80/1800), 8·6% (154/1800), 3·2% (58/1800), 1·1% (20/1800), 1·0% (18/1800) and 1·7% (30/1800), respectively (Fig. 4). There was a significant difference in the positivity rate between eastern Japan (Hokkaido, Miyagi and Tokyo) and western Japan (Aichi, Osaka, Okayama and Fukuoka) (305/5400 = 5·6% vs. 126/7200 = 1·8%; $P < 0·001$). Moreover, in most areas except Aichi, the positivity rate among men was higher than among women.

Figure 5 shows the geographic prevalence of IgG anti-HEV classified by sex and age in the seven regions. The age-dependence was observed more or less in each region, and it was most clear in Tokyo. In Hokkaido, the positivity rates among men in 30s–60s age groups were very similar (> 5%) and the age-dependence was less clear.

Discussion

Here, we have shown a nationwide survey for IgG anti-HEV among qualified Japanese blood donors in seven districts representing most regions of Japan for the first time. The 12 600 donors tested for HEV seroprevalence were qualified donors who were sex- and age-matched, and can be regarded as healthy individuals representing a well-balanced population living in various regions of the country. The HEV seroprevalence in Japanese blood donors have been reported [17,20]. However, in those reports, the samples were collected in some limited areas and/or sample sizes were small, which may not represent nationwide HEV seroprevalence.

In this study, the samples were screened first by the in-house IgG anti-HEV ELISA and the positive samples were tested by the commercial kit because it was not realistic to screen 12 600 samples with the expensive commercial kit.

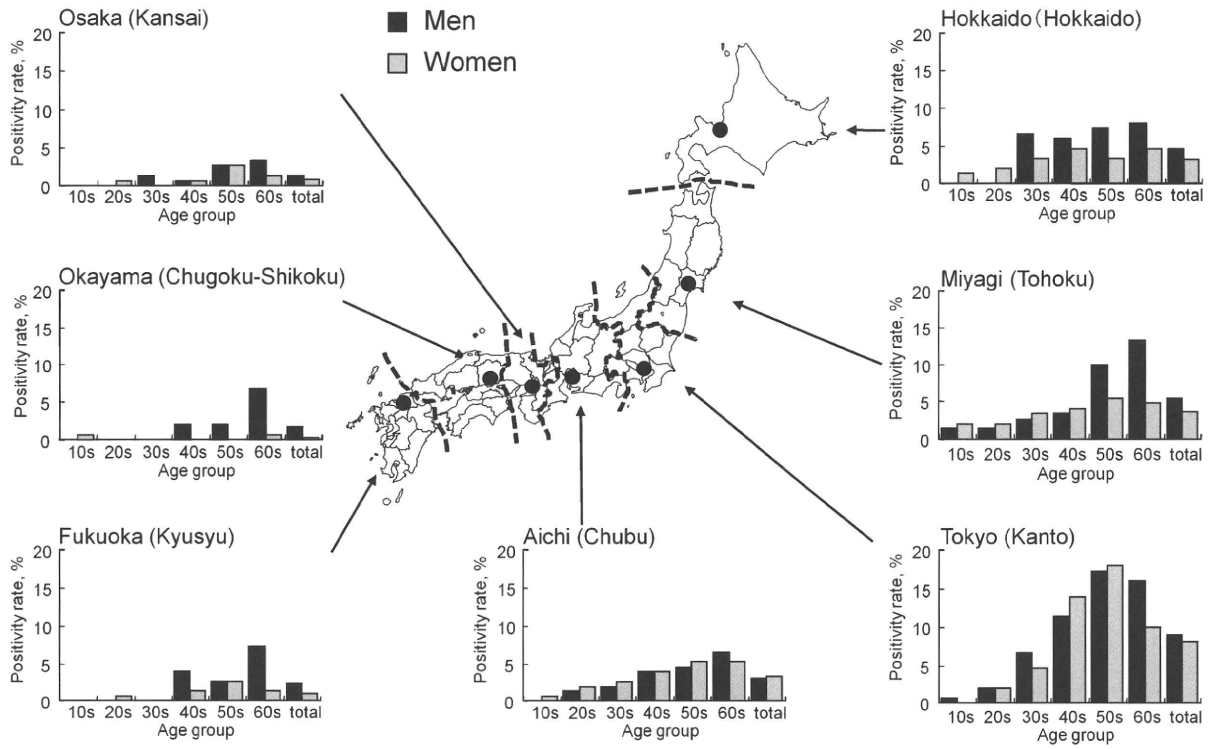

Fig. 5 Geographical prevalence of IgG anti-hepatitis E virus by sex and age group in the seven blood centres. The map of Japan shows the locations of seven geographic regions (every region has been divided in heavy broken line). JRC Blood Centers belong to eastern Japan (three regions: Hokkaido, Tohoku and Kanto) or western Japan (four regions: Chubu, Kansai, Chugoku-Shikoku and Kyusyu) from north to south. The closed circle shows the place of the main JRC Blood Center. Regional names of each JRC Blood Centers are written down in parentheses. Samples in each blood centre consisted of 900 men (black bars) and 900 women (grey bars).

In the preliminary study, the HEV specimens were tested with the commercial kit as well as the in-house IgG anti-HEV ELISA. Although the sensitivity of the two assays appeared comparable, we are not certain whether there may be false-negative samples for the in-house assay. The overall sensitivity of the algorithm is dependent on the in-house assay as samples reactive on the in-house assay are tested on the commercial kit. Thus, the study might underestimate the seroprevalence.

IgG anti-HEV appears to be present for a more prolonged period after the disappearance of HEV in blood [14,16]. Of the 12 600 qualified blood donors, 431 (3·4%) samples were positive for IgG anti-HEV. The positivity rate of IgG anti-HEV among male donors was significantly higher than that among female donors (3·9% vs. 2·9%; $P = 0·002$). Fukuda *et al.* [17] reported the prevalence of IgG anti-HEV among blood donors with normal ALT (ALT level of 60 IU/l or lower) at the two JRC Blood Centers, where the prevalence of IgG anti-HEV was higher among male donors than female donors. The prevalence of hepatitis E in Japan was 3·5 times higher among men than women [4], which may be relevant to the higher prevalence in IgG anti-HEV among male donors. The reasons for the higher prevalence in men are not very clear, although the dietary pattern of the Japanese male may be associated with the higher HEV seroprevalence.

In our results, there was a significant difference in the positivity rate of IgG anti-HEV between eastern Japan (Hokkaido, Miyagi and Tokyo) and western Japan (Aichi, Osaka, Okayama and Fukuoka) (5·6% vs. 1·8%, $P < 0·001$). Li *et al.* [20] reported that the positivity rate of IgG anti-HEV in healthy individuals was 1·9% in the southern (western) prefecture, 3·3% in the northern (eastern) prefecture and 14·1% in the central (eastern) prefecture, respectively, although the sample size was rather small in each age group. Also, Fukuda *et al.* [17] showed that the detection rate of IgG anti-HEV at one blood centre, 5·5% (located near Tokyo) was significantly higher than that at another blood centre, 2·1% (located near Fukuoka). The results were in agreement with our present report. We previously reported that among donors with ALT levels at or > 200 IU/l, the positivity rate of IgG anti-HEV in western Japan and eastern Japan was 2·1% and 5·0%, respectively [19]. Although the donor samples with elevated ALT levels were unbalanced in age and sex, the results corresponded to our present results using the large number of non-biased

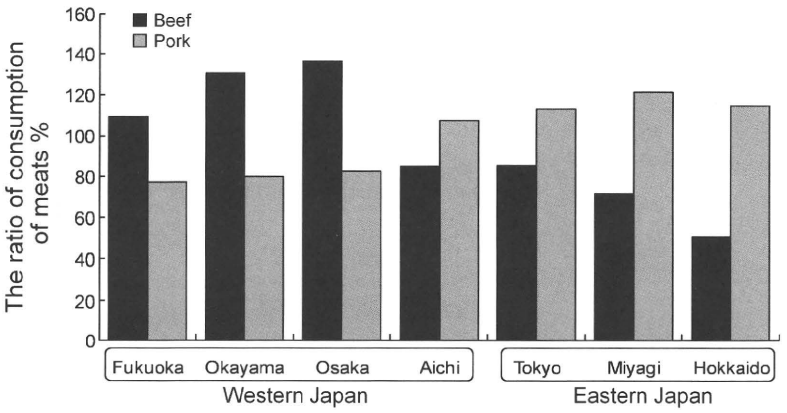

Fig. 6 Regional trend of consumption of beef and pork in Japan. Data was Annual Report of Family Income and Expenditure Survey in 2002 through 2005 Statistic Bureau, Ministry of Internal Affairs and Communications, Japan. The ratio of consumption of meats was the ratio to the national average of consumption of meats in weight.

samples. Depending on the region, the positivity rate varied from 1·0% in Okayama to 8·6% in Tokyo in this study. The difference in the dietary pattern of consumption of meat between eastern Japan and western Japan may be relevant for the higher seroprevalence in eastern Japan; people in eastern Japan are more likely to consume pork than beef (Fig. 6). A number of reports have pointed out that pigs are reservoirs for HEV [13,16,22–25]. IgG anti-HEV were detected in 90% of pigs between 5 and 6 months of age at commercial swine farms in Japan [23]. HEV RNA was detected in packages of pork purchased from local grocery stores in Japan and in the United States [13,24]. In addition, Abe *et al.* [4] described that the zoonotic food-borne route appeared to be the most common cause of HEV infection in Japan, 70% of which have a connection with consumption of pork. Another example is reported in Indonesia where differences in the gastronomic culture have induced a difference in the positivity rate of IgG anti-HEV. Among pregnant women in Bali, anti-HEV was significantly less frequent in Muslims, who were strictly prohibited from eating or touching pigs, than Hindus, who have no such restrictions [25]. Also in industrialized countries including Japan, the widespread distribution of HEV infection among humans and animals has been reported [2,5–8].

Overall, similar to the results found in other studies, the prevalence of IgG anti-HEV increased with age; however, the degree of the age-dependency was varied by region. The clear age-dependent increase of IgG anti-HEV observed in Tokyo may represent a past prevalence of HEV infection. On the other hand, the trend in Hokkaido was less clear, which may indicate that there may be a more recent or an on-going spread of HEV infection. Recently, hepatitis E has been frequently reported in Hokkaido [4].

In conclusion, the spread of the domestic infection of HEV was observed widely in qualified blood donors in Japan and older donors, male donors and donors living in eastern Japan were associated with a higher prevalence of IgG anti-HEV. Further studies are necessary to clarify the infectious routes and the potential risk of transfusion-transmission of HEV in Japan.

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Conflict of interest

The authors declare no conflict of interest.

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