

FIG. 5. Panels a and b, HBV DNA (panels a) and HCV RNA (panels b) in noncancerous (non-Ca) (A) and cancerous (Ca) (B) liver tissue obtained from a patient coinfecting with HBV and HCV were detected by PCR-ISH (55 cycles of PCR) and RT-PCR-ISH (45 cycles of PCR), respectively. Panels c, serial sections were stained with HE. Magnification, $\times 160$.

intensity of HBV protein expression. HBV has four overlapping open reading frames regulated by two enhancer elements and four promoters (14).

We had reported previously that HCV RNA and HCV core protein levels are relatively stable irrespective of fibrosis (33, 35). In the present study, HCV RNA was detected in almost all hepatocytes in all sections. Furthermore, we emphasize the similar staining pattern of almost all hepatocytes at the cellular level. HCV RNA was detected in the cytoplasm but not in the nucleus. The detection of an intense signal at the perinuclear site may reflect the replication process of HCV (10). HCV RNA was also detected in the epithelium of the large bile duct but not in the endothelial cells, portal tracts, or sinusoidal lymphocytes, which is consistent with the results of previous studies (5, 8). Thus, the epithelium of the large bile duct, but not that of the small duct, can support HCV replication.

We confirmed the specificity for detecting the HCV RNA copy number by quantification in a small area of an LCM section (Fig. 4C); similar amounts of HCV RNA were detected in each area. The detection of HCV RNA in each area by LCM and RTD-PCR excluded the possibility of diffusion of the PCR product by RT-PCR-ISH.

Recent molecular biological techniques have demonstrated low-level HBV viremia in some patients with chronic hepatitis C who were negative for all serological HBV markers. In these cases, HBV DNA not only is integrated in the human chromosomes but also replicates in hepatocytes (34). In the previous study, we measured the levels of HBV DNA and HCV RNA using RTD-PCR with singly infected or coinfecting noncancerous and cancerous liver tissues (34). In the case of coinfection, HCV replication was dominant in the noncancerous

tissue whereas HBV replication was dominant in the cancerous tissue. Some studies have shown that HCV inhibits HBV gene expression and replication (7, 15).

Using this novel, highly specific and sensitive PCR-ISH method, we could visualize the tissue staining patterns of HBV and HCV, which were consistent with those seen by RTD-PCR. This revealed the novel finding that almost all hepatocytes are infected with HBV or HCV in patients with chronic liver disease, suggesting that the viruses spread throughout the liver in the chronic stage. However, further study with a large number of samples from each stage of infection is needed to clarify the mechanism of persistent infection via our assay method.

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Persistent expression of the full genome of hepatitis C virus in B cells induces spontaneous development of B-cell lymphomas in vivo

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Extrahepatic manifestations of hepatitis C virus (HCV) infection occur in 40%-70% of HCV-infected patients. B-cell non-Hodgkin lymphoma is a typical extrahepatic manifestation frequently associated with HCV infection. The mechanism by which HCV infection of B cells leads to lymphoma remains unclear. Here we established HCV transgenic mice that express the full HCV genome in B cells (RzCD19Cre mice) and observed a 25.0% incidence of diffuse large B-cell non-Hodgkin lymphomas

(22.2% in males and 29.6% in females) within 600 days after birth. Expression levels of aspartate aminotransferase and alanine aminotransferase, as well as 32 different cytokines, chemokines and growth factors, were examined. The incidence of B-cell lymphoma was significantly correlated with only the level of soluble interleukin-2 receptor α subunit (sIL-2R α) in RzCD19Cre mouse serum. All RzCD19Cre mice with substantially elevated serum sIL-2R α levels (> 1000 pg/

mL) developed B-cell lymphomas. Moreover, compared with tissues from control animals, the B-cell lymphoma tissues of RzCD19Cre mice expressed significantly higher levels of IL-2R α . We show that the expression of HCV in B cells promotes non-Hodgkin-type diffuse B-cell lymphoma, and therefore, the RzCD19Cre mouse is a powerful model to study the mechanisms related to the development of HCV-associated B-cell lymphoma. (*Blood*. 2010;116(23):4926-4933)

Introduction

More than 175 million people worldwide are infected with hepatitis C virus (HCV), a positive-strand RNA virus that infects both hepatocytes and peripheral blood mononuclear cells.¹ Chronic HCV infection may lead to hepatitis, liver cirrhosis, hepatocellular carcinomas^{2,3} and lymphoproliferative diseases such as B-cell non-Hodgkin lymphoma and mixed-cryoglobulinemia.^{1,4-6} B-cell non-Hodgkin lymphoma is a typical extrahepatic manifestation frequently associated with HCV infection⁷ with geographic and ethnic variability.^{8,9} Based on a meta-analysis, the prevalence of HCV infection in patients with B-cell non-Hodgkin lymphoma is approximately 15%.⁸ The HCV envelope protein E2 binds human CD81,¹⁰ a tetraspanin expressed on various cell types including lymphocytes, and activates B-cell proliferation¹¹; however, the precise mechanism of disease onset remains unclear. We previously developed a transgenic mouse model that conditionally expresses HCV cDNA (nucleotides 294-3435), including the viral genes that encode the core, E1, E2, and NS2 proteins, using the *Cre/loxP* system (in core~NS2 [CN2] mice).^{12,13} The conditional transgene activation of the HCV cDNA (core, E1, E2, and NS2) protects mice from Fas-mediated lethal acute liver failure by inhibiting cytochrome c release from mitochondria.¹³ In HCV-infected mice, persistent HCV protein expression is established by targeted disruption of *irf-1*, and high incidences of lymphoproliferative disorders are found in CN2 *irf-1*^{-/-} mice.¹⁴ Infection and replication of HCV also occur in B cells,^{15,16} although the direct effects,

particularly in vivo, of HCV infection on B cells have not been clarified.

To define the direct effect of HCV infection on B cells in vivo, we crossed transgenic mice with an integrated full-length HCV genome (Rz) under the conditional *Cre/loxP* expression system with mice expressing the Cre enzyme under transcriptional control of the B lineage-restricted gene *CD19*,¹⁷ we addressed the effects of HCV transgene expression in this study.

Methods

Animal experiments

Wild-type (WT), Rz, CD19Cre, RzCD19Cre mice (129/sv, BALB/c, and C57BL/6J mixed background), and MxCre/CN2-29 mice (C57BL/6J background) were maintained in conventional animal housing under specific pathogen-free conditions. All animal experiments were performed according to the guidelines of the Tokyo Metropolitan Institute of Medical Science or the Kumamoto University Subcommittee for Laboratory Animal Care. The protocol was approved by the Institutional Review Boards of both facilities.

Measurements of HCV protein and RNA

Mice were anesthetized and bled, and tissues (spleen, lymph nodes, liver, and tumors) were homogenized in lysis buffer (1% sodium dodecyl sulfate; 0.5% (wt/vol) nonyl phenoxypolyethoxyethanol; 0.15M NaCl; 10 mM

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tris(hydroxymethyl)aminomethane, pH 7.4) using a Dounce homogenizer. The concentration of HCV core protein in tissue lysates was measured using an HCV antigen enzyme-linked immunosorbent assay (ELISA; Ortho).¹⁸ HCV mRNA was isolated by a guanidine thiocyanate protocol using ISOGEN (Nippon Gene) and was detected by reverse transcription polymerase chain reaction (RT-PCR) amplification using primers specific for the 5' untranslated region of the *HCR6* sequence.^{19,20} Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen) with random primers. PCR primers NCR-F (5'-TTCACGCA-GAAAGCGTCTAGCCAT-3') and NCR-R (5'-TCGTCTGGCAATTCCG-GTGTACT-3') were used for the first round of HCV cDNA amplification, and the resulting product was used as a template for a second round of amplification using primers NCR-F INNER (5'-TTCCGACAGACCCTATGGCT-3') and NCR-R INNER (5'-TTCCGACAGACCCTATGGCT-3').

Collection of serum for chemokine ELISA

Blood samples were collected from the supraorbital veins or by heart puncture of killed mice. Blood samples were centrifuged at 10 000g for 15 minutes at 4°C to isolate the serum.²¹ Serum concentrations of interleukin (IL)-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, Eotaxin, granulocyte colony-stimulating factor (CSF), granulocyte-macrophage-CSF, interferon (IFN)- γ , keratinocyte-derived chemokine (KC), monocyte chemoattractant protein-1, macrophage inflammatory protein (MIP)-1 α , MIP-1 β , Regulated upon Activation, Normal T-cell Expressed, and Secreted, tumor necrosis factor- α , IL-15, fibroblast growth factor-basic, leukemia inhibitory factor, macrophage-CSF, human monokine induced by gamma interferon, MIP-2, platelet-derived growth factor β , and vascular endothelial growth factor were measured using the Bio-Plex Pro assay (Bio-Rad). Serum soluble IL-2 receptor α (sIL-2R α) concentrations were determined by ELISA (DuoSet ELISA Development System; R&D Systems). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined using a commercially available kit (Transaminase CII test; Wako Pure Chemical Industries).

Histology and immunohistochemical staining

Mouse tissues were fixed with 4% formaldehyde (Mildform 10 N; Wako Pure Chemical Industries), dehydrated with an ethanol series, embedded in paraffin, sectioned (10- μ m thick) and stained with hematoxylin and eosin. For tissue immunostaining, paraffin was removed from the sections using xylene following the standard method.¹⁴ and sections were incubated with anti-CD3 or anti-CD45R (Santa Cruz Biotechnology) in phosphate-buffered saline without Ca²⁺ and Mg²⁺ (pH 7.4) but with 5% skim milk. Next, the sections were incubated with biotinylated anti-rat immunoglobulin (Ig)G (1:500), followed by incubation with horseradish peroxidase-conjugated avidin-biotin complex (Dako Corp), and the color reaction was developed using 3,3'-diaminobenzidine. Sections were observed under an optical microscope (Carl Zeiss).

Detection of immunoglobulin gene rearrangements by PCR

Genomic DNA was isolated from tumor tissues, and PCR was performed as described.²² In brief, PCR reaction conditions were 98°C for 3 minutes; 30 cycles at 98°C for 30 seconds, 60°C for 30 seconds, 72°C for 1.5 minutes, and 72°C for 10 minutes. Mouse V κ genes were amplified using previously described primers.²³ Amplification of mouse V λ genes was performed using V κ con (5'-GGCTGCAGSTTCAGTGGCAGTGGRTC-WGGRAC-3'; R, purine; W, A or T) and J κ 5 (5'-TGCCACGTCACCT-GATAATGAGCCCTCTC-3') as described.²⁴

Results

Establishment of transgenic mice with B lineage-restricted HCV gene expression

We defined the direct effect of HCV infection on B cells *in vivo* by crossing transgenic mice that had an integrated full-length HCV

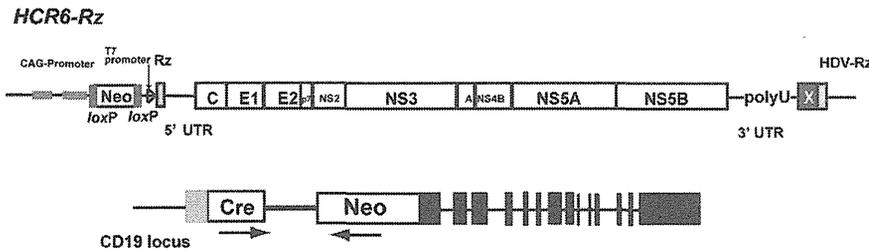
genome (Rz) under the conditional Cre/*loxP* expression system (Figure 1A upper schematic)^{12,19,25} with mice that expressed the Cre enzyme under transcriptional control of the B lineage-restricted gene *CD19*¹⁷ (RzCD19Cre; Figure 1A lower schematic). Expression of the HCV transgene in RzCD19Cre mice was confirmed by ELISA (Figure 1B); a substantial level of HCV core protein was detected in the spleen (370.9 \pm 10.2 pg/mg total protein), but levels were lower in the liver (0.32 \pm 0.03 pg/mg) and plasma (not detectable). RT-PCR analysis of peripheral blood lymphocytes (PBLs) from RzCD19Cre mice indicated the presence of HCV transcripts (Figure 1C). The weights of RzCD19Cre, Rz (with the full HCV genome transgene alone), CD19Cre (with the Cre gene knock-in at the CD19 gene locus) and WT mice were measured weekly for more than 600 days post birth; there were no significant differences between these groups (data not shown); the total number of transgenic and WT mice was approximately 200. The survival rate in each group was also measured for > 600 days (Figure 1D); survival in the female RzCD19Cre group was lower than that of the other groups.

The spontaneous development of B-cell lymphomas in the RzCD19Cre mouse

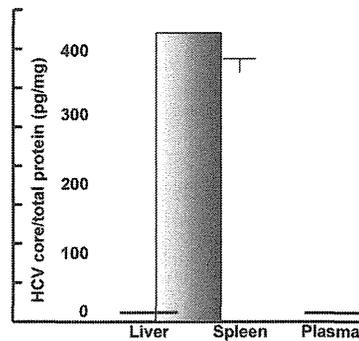
At 600 days post birth, mice (n = 140) were killed by bleeding under anesthesia, and tissues (spleen, lymph node, liver, and tumors) were excised and examined by hematoxylin and eosin staining (Figure 2A; supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). The incidence of B-cell lymphoma in RzCD19Cre mice was 25.0% (22.2% in males and 29.6% in females) and was significantly higher than the incidence in the HCV-negative groups (Table 1). This incidence is significantly higher than those of the other cell-type tumors developed spontaneously in all mouse groups (supplemental Table 1). Because nodular proliferation of CD45R-positive atypical lymphocytes was observed, lymphomas were diagnosed as typical diffuse B-cell non-Hodgkin lymphomas (Figure 2Aiv,vi-vii; supplemental Figure 1B,E,H,M). Mitotic cells were also positive for CD45R (Figure 2Avi arrowheads). CD3-positive T-lymphocytes were small and had a scattered distribution. Intrahepatic lymphomas had the same immunophenotypic characteristics as B-cell lymphomas (supplemental Figure 1K arrowheads, inset; 1L-N, ID No. 24-4, RzCD19Cre mouse); lymphoma tissues were markedly different compared with the control lymph node (Figure 2Ai,iii,v; ID No. 47-4, CD19Cre mouse) and liver (supplemental Figure 1J; ID No. 24-2, Rz mouse; tissues were from a littermate of the mice used to generate the data in supplemental Figure 1D-I,K-N). All samples were reviewed by at least 2 expert pathologists and classified according to World Health Organization classification.²⁶ Lymphomas were mostly CD45R positive and located in the mesenteric lymph nodes (Figure 2A; supplemental Figure 1), and some were identified as intrahepatic lymphomas (incidence, 4.2%; supplemental Figure 1K-N). HCV gene expression was detected in all B-cell lymphomas of RzCD19Cre mice (Figure 2B).

To examine the Ig gene configuration in the B-cell lymphomas of the RzCD19Cre mice, genomic DNA was isolated and analyzed by PCR. Ig gene rearrangements were identified in each case (Figure 2C). Genomic DNA isolated from the tumors of a germinal center-associated nuclear protein (GANP) transgenic mouse (GANP Tg#3) yielded a predominant J κ 5 PCR product (Figure 2C, V κ -J κ); a predominant JH1 product and a minor JH2 product (supplemental Figure 2, DH-JH) were also identified, as previously reported,²² indicating that the lymphoma cells proliferated from the transformation of an oligo B-cell clone. The B-cell lymphomas of

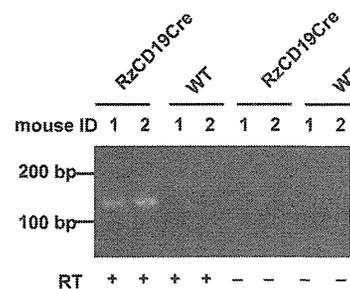
A



B



C



D

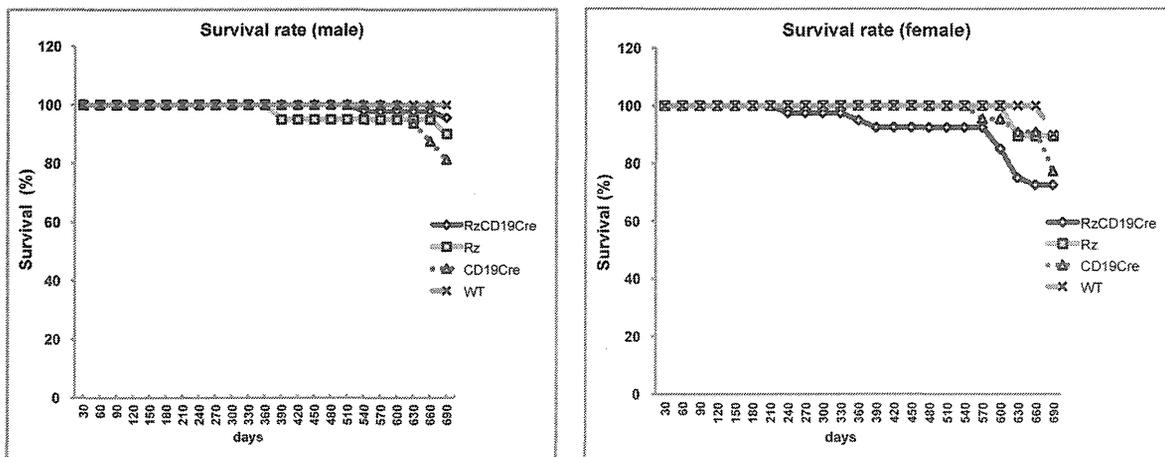


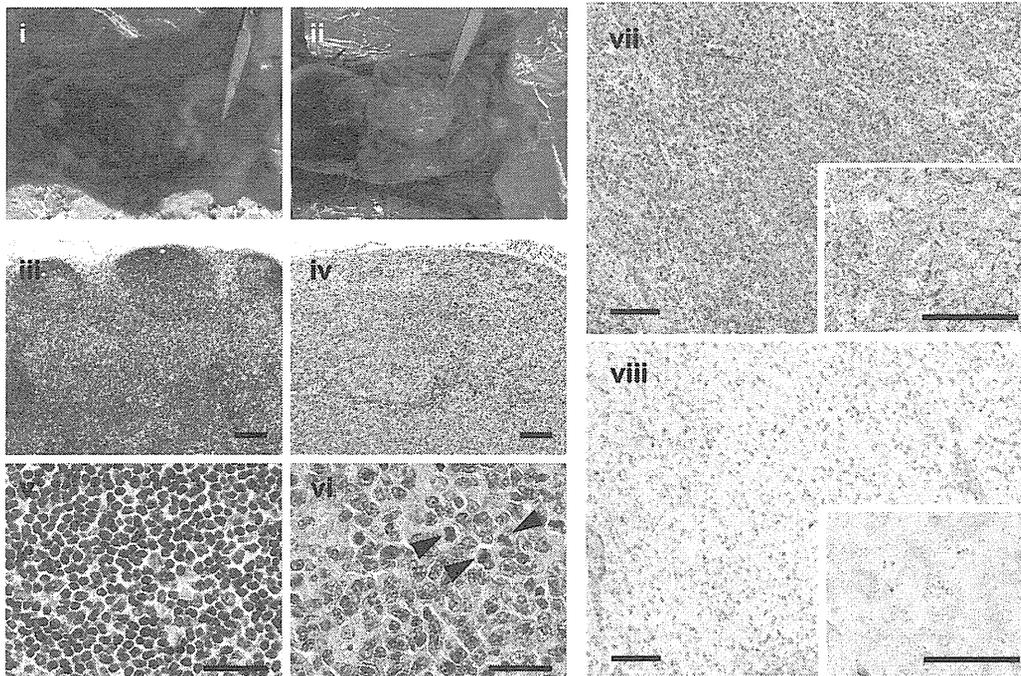
Figure 1. Establishment of RzCD19Cre mice. (A) Schematic diagram of the transgene structure comprising the complete HCV genome (*HCR6-Rz*). HCV genome expression was regulated by the *Cre/loxP* expression cassette (top diagram). The *Cre* transgene was located in the *CD19* locus (bottom diagram). (B) Expression of HCV core protein in the liver, spleen, and plasma of RzCD19Cre mice was quantified by core ELISA. Data represent the mean \pm SD (n = 3). (C) Detection of HCV RNA in PBLs by RT-PCR. Samples that included the RT reaction are indicated by +, and those that did not include the RT reaction are indicated by -. (D) Survival rates of male and female RzCD19Cre mice (males, n = 45; females, n = 40), Rz mice (males, n = 20; females, n = 19), CD19Cre mice (males, n = 16; females, n = 22), and WT mice (males, n = 5; females, n = 10).

8 RzCD19Cre mice (mouse ID Nos. 24-1, 54-1, 56-5, 69-5, 42-4, 43-4, 36-3 [data not shown] and 62-2 [data not shown]) yielded a $\text{J}\kappa$ -5 gene amplification product, and the lymphomas from 3 other mice had the alternative gene configurations $\text{J}\kappa$ -1 (mouse ID No. 31-4), $\text{J}\kappa$ -2 (mouse ID No. 24-4) and $\text{J}\kappa$ -3 (mouse ID No. 42-4; Figure 2C). PCR amplification products from the genes JH4 (mouse ID Nos. 24-1, 24-4, 54-1, 43-4, 56-5, 69-5, 62-2 [data not shown], 36-3 [data not shown]), JH1 (mouse ID Nos. 31-4, 42-4) and JH3 (mouse ID Nos. 31-4, 42-4, 56-5, 43-4, 36-3 [data not shown]) were also detected (supplemental Figure 2). The mutation frequencies in the $\text{J}\kappa$ -1, -3 and -5 genes were the same as the

mutation frequency in the genomic V-region gene.²² Few or no sequence differences in the variable region were identified among clones from which DNA was amplified. These results indicate the possibility that tumors judged as B-cell lymphomas based on pathology criteria were derived from the transformation of a single germinal center of B-cell origin.

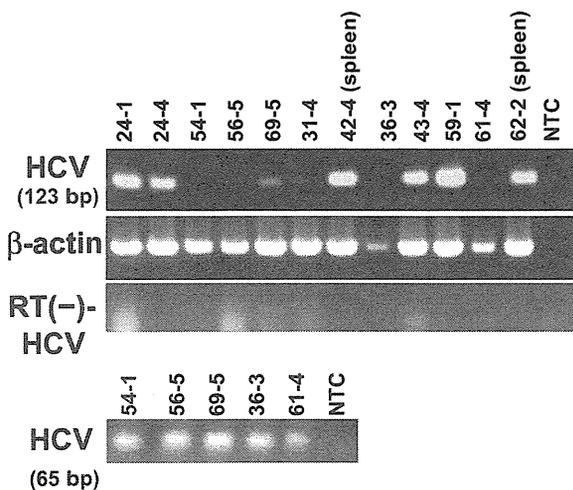
To rule out the oncogenic effect caused by a transgenic integration into a specific genomic locus, we examined if HCV transgene inserted into another genomic site also causes B-cell lymphomas using another HCV transgenic mouse strain, *MxCre/CN2-29* (supplemental Figure 3). Expression of the HCV CN2

A



B

HCV-RNAs in B-lymphomas



C

Vκ-Jκ

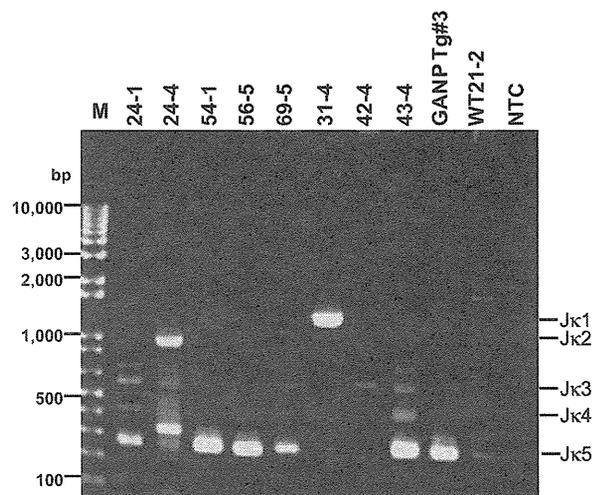


Figure 2. Histopathologic analysis of B-cell lymphomas in RzCD19Cre mouse tissues. (A) Histologic analysis of tissues from a normal mouse (i, iii, v; CD19Cre mouse, ID No. 47-4, male) and a B-cell lymphoma from a RzCD19Cre mouse (ii, iv, vi; ID No. 69-5, male). Paraformaldehyde-fixed and paraffin-embedded tumor tissues were stained with hematoxylin and eosin (iii-vi) or immunostained using anti-CD45R (vii; bottom right, inset) and anti-CD3 (viii; bottom right, inset). Also shown is a macroscopic view of the lymphoma from a mesenchymal lymph node (ii, indicated by forceps), which is not visible in the normal mouse (i). Mitotic cells are indicated with arrowheads (vi). Scale bars: 100 μ m (iii-iv, vii-viii) and 20 μ m (v-vi, insets in vii-viii). (B) Expression of HCV RNA in B-cell lymphomas from RzCD19Cre mice was examined by RT-PCR. The first round of PCR amplification yielded a 123-base pair fragment of HCV cDNA (upper panel), and a second round of PCR amplification yielded a 65-base pair fragment (lower panel). The β -actin mRNA was a control. As an additional control, the first and second rounds of amplification were performed using samples that had not been subjected to reverse transcription. NTC, no-template control. (C) Ig gene rearrangements in the tumors of RzCD19Cre mice. Genomic DNA isolated from B-cell lymphoma tissues of RzCD19Cre mice (ID Nos. 24-1, 24-4, 54-1, 56-5, 69-5, 31-4, 42-4, 43-4) and spleen tissues of a WT mouse (ID No. 21-2) was PCR amplified using primers specific for V κ -J κ genes. Amplification of controls was performed using genomic DNA isolated from a GANP transgenic mouse (GANP Tg#3) and in the absence of template DNA (no-template control, NTC). M, DNA ladder marker.

gene (nucleotides 294-3435)¹² was induced by the Mx promoter-driven cre recombinase with poly(I:C) induction¹⁴ (supplemental

Figure 3A). HCV core proteins were detected in both normal spleen (mouse ID Nos. 2, 3, 4) and intra-splenic B-cell lymphoma tissues

Table 1. Lymphoma incidence in HCV-expressing and control mice

HCV expression	Mouse genotype	No.	Incident B lymphoma, number (%)	Incident T lymphoma, number (%)
+	RzCD19Cre	72	18 (25.0)	3 (4.1)
-	Rz	34	1 (2.9)	1 (2.9)
-	CD19Cre	22	2 (9.1)	1 (4.5)
-	WT	12	1 (8.3)	1 (8.3)

(mouse ID Nos. 5, 6, 7) of MxCre/CN2-29 mice but not in spleens of the CN2-29 mouse (mouse ID No. 1, Figure 3B). After 12 months, the MxCre/CN2-29 mice developed B-cell lymphomas in the spleen at a high incidence (33.3%: 3/9), whereas the CN2-29 mice did not (0/13; supplemental Figure 3C), indicating that the

development of B-cell lymphomas in HCV transgenic mice occurred similarly to RzCD19Cre mice. MxCre/CN2-29 mice also developed hepatocellular carcinomas (10%, 360 days, 17%, 480 days, 50%, 600 days after onset of HCV expression; Sekiguchi et al, submitted).

The results obtained in 2 HCV transgenic mouse strains indicate that the expression of the HCV gene or the proteins indeed induces the spontaneous development of B-cell lymphomas irrespective of the integrated site in the mouse genome.

The levels of cytokines and chemokines in B-cell lymphomas and other tumors and in tumor-free control mice

Abnormal induction of cytokine production occurs in HCV-associated non-Hodgkin lymphomas^{27,28} and in patients with

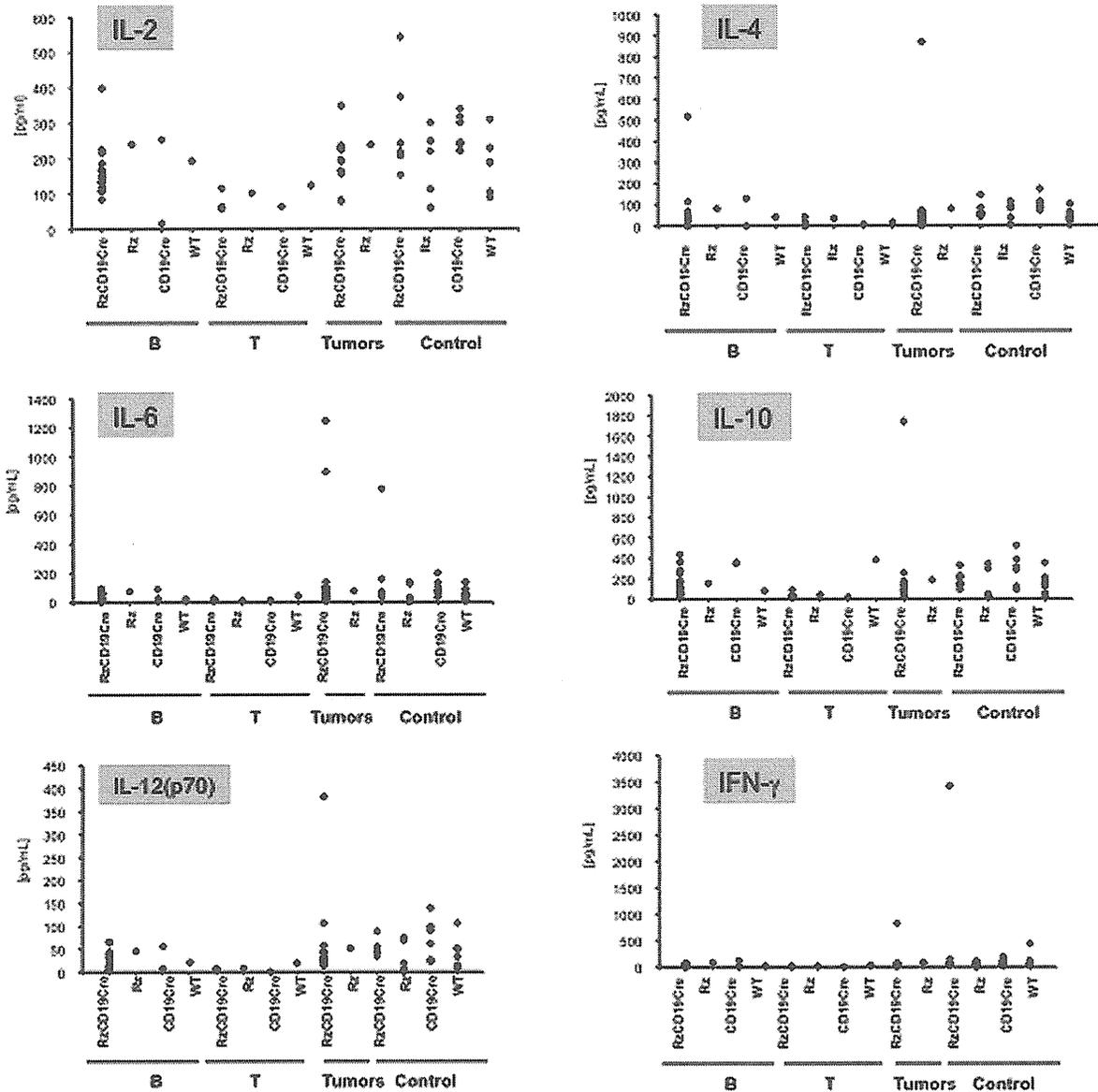


Figure 3. Analysis of serum cytokine levels using a multisuspension array system. The serum concentration levels of IL-2, IL-4, IL-6, IL-10, IL-12(p70), and IFN-γ were measured in RzCD19Cre mice with B-cell lymphomas (B), T-cell lymphomas (T), and other tumors (mammary tumor, sarcoma, and hepatocellular carcinoma) and in tumor-free RzCD19Cre, Rz, CD19Cre, and WT mice.

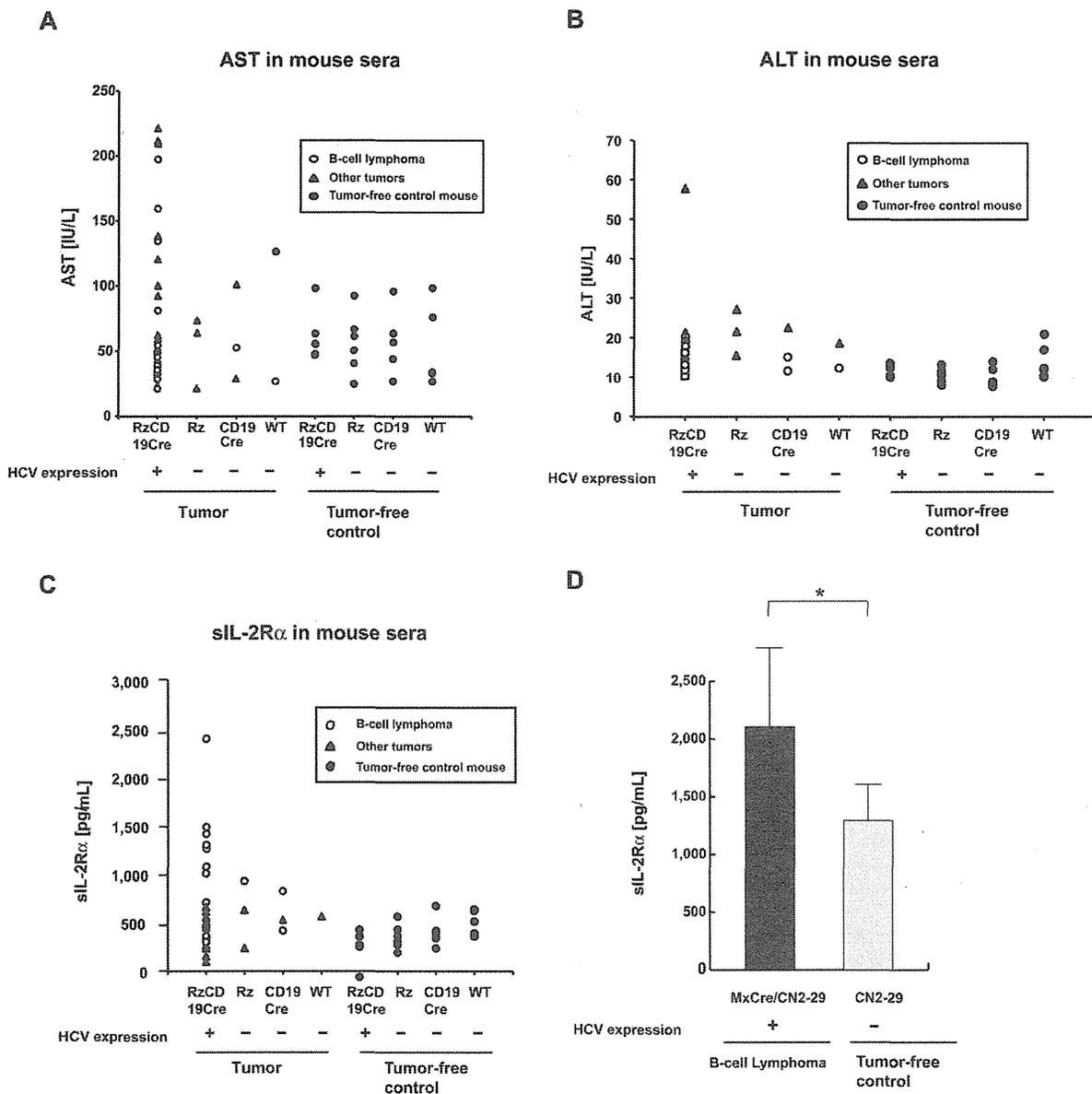


Figure 4. Serum titers of AST, ALT and soluble IL-2R α in transgenic and control mice lacking or harboring B-cell lymphomas. (A-B) The AST (A) and ALT (B) assays were performed on serum samples from tumor-free control mice and the RzCD19Cre, Rz, CD19Cre and WT mice with or without B-cell lymphomas or other tumors. (C) ELISA analysis was performed to determine the sIL-2R α concentration in serum samples from tumor-free control mice and the RzCD19Cre, Rz, CD19Cre, and WT mice with or without B-cell lymphomas or other tumors. (D) Concentration of soluble IL-2R α in sera from transgenic (MxCre/CN2-29 or CN2-29) mice with or without B-cell lymphomas (* $P < .05$).

chronic hepatitis.^{29,30} Therefore, we examined tumor cytokine and chemokine levels using a multisuspension array system. The levels of IL-2, IL-4, IL-6, IL-10, IL-12(p70), and IFN- γ (Figure 3), which may have a link with lymphoproliferation¹⁴ or lymphoma^{28,31} induced by HCV, and IL-1 α , IL-1 β , IL-3, IL-5, IL-9, IL-12(p40), IL-13, IL-17, Eotaxin, granulocyte-CSF, granulocyte-macrophage-CSF, KC, monocyte chemoattractant protein-1, MIP-1 α , MIP-1 β , Regulated upon Activation, Normal T-cell Expressed, and Secreted, tumor necrosis factor- α , IL-15, fibroblast growth factor-basic, leukemia inhibitory factor, macrophage-CSF, human monokine induced by gamma interferon, MIP-2, platelet-derived growth factor β and vascular endothelial growth factor (supplemental Figure 4) were measured in sera from mice with B-cell lymphomas, T-cell lymphomas, and other tumors and in sera from tumor-free

RzCD19Cre, Rz, CD19Cre, and WT control mice. The levels of these cytokines and chemokines in sera from tumor-bearing RzCD19Cre mice with B-cell lymphomas were not significantly different from those of the control groups, and thus, changes in the expression of these cytokines and chemokines were not strictly correlated with the occurrence of B-cell lymphoma in RzCD19Cre mice.

The levels of amino transferases and sIL-2R α in mice lacking or harboring B-cell lymphomas

We also examined the levels of AST and ALT in the RzCD19Cre, Rz, CD19Cre, and WT mice. There were no significant differences in the levels of AST and ALT in the sera of mice lacking or harboring B-cell lymphomas ($P > .05$; Figure 4A-B; AST:

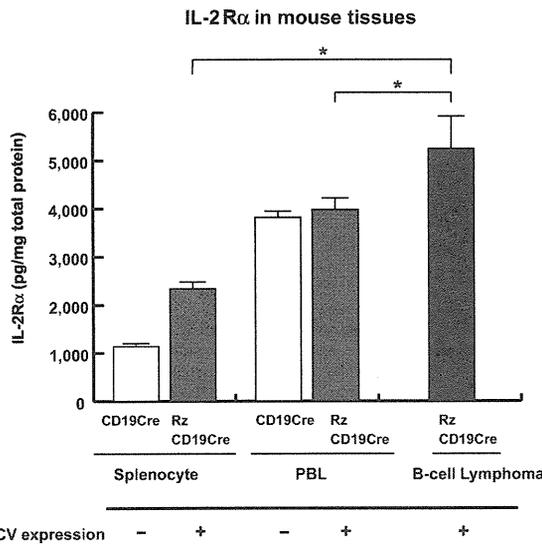


Figure 5. Levels of IL-2R α in transgenic and control mice lacking or harboring B-cell lymphomas. The expression level of IL-2R α in splenocytes and PBLs from CD19Cre and RzCD19Cre mice and in B-cell lymphomas from RzCD19Cre mice was measured by ELISA. IL-2R α levels per total protein are indicated (picograms per milligram). Data from quadruplicate samples are shown as the mean \pm SD (* P < .05).

RzCD19Cre mice with B-cell lymphomas, 72.2 ± 60.5 IU/L; normal controls, 55.2 ± 23.0 IU/L and ALT: RzCD19Cre mice with B-cell lymphomas, 14.2 ± 3.1 IU/L; normal controls, 11.5 ± 3.0 IU/L).

Finally, we examined the level of sIL-2R α in the sera of the RzCD19Cre mice with B-cell lymphomas; sIL-2R α is generated by proteolytic cleavage of IL-2R α (CD25) residing on the surface of activated T and natural killer cells, monocytes, and certain tumor cells.^{24,32} The average sIL-2R α level in the RzCD19Cre mice with B-cell lymphomas (830.3 ± 533.0 pg/mL) was significantly higher than that in the tumor-free control groups, including the RzCD19Cre, Rz. CD19Cre and WT mice (499.9 ± 110.2 pg/mL; $P < .0057$; Figure 4C). The average sIL-2R α levels in other tumor-containing groups (430.46 ± 141.15 pg/mL) were not significantly different from those in the tumor-free control groups ($P > .05$; Figure 4C). Moreover, all RzCD19Cre mice with a relatively high level of sIL-2R α (> 1000 pg/mL) presented with B-cell lymphomas (Figure 4C).

We also examined the level of sIL-2R α in MxCre/CN2-29 mice and observed a significant increase in sIL-2R α in mice that expressed HCV and that had B-cell lymphomas compared with tumor-free control (CN2-29) mice (Figure 4D).

Expression of IL-2R α in B-cell lymphomas of the RzCD19Cre mice

To examine whether sIL-2R α was derived from lymphoma tissues, we quantified IL-2R α concentrations in splenocytes, PBLs and B-cell lymphoma tissues (Figure 5). The concentration of IL-2R α was significantly higher in splenocytes from RzCD19Cre mice compared with those from CD19Cre mice; the concentration was even higher in B-cell lymphoma tissues than in splenocytes from RzCD19Cre mice (Figure 5). These results strongly suggest that B-cell lymphomas directly contribute to the elevated serum concentrations of sIL-2R α in RzCD19Cre mice.

Discussion

We have established HCV transgenic mice that have a high incidence of spontaneous B-cell lymphomas. In this animal model,

the HCV transgene is expressed during the embryonic stage, and these RzCD19Cre mice are expected to be immunotolerant to the HCV transgene product. Thus, the results from this study reveal the potential for the HCV gene to induce B-cell lymphomas without inducing host immune responses against the HCV gene product. A retrospective study indicated that viral elimination reduced the incidence of malignant lymphoma in patients infected with HCV.³³ The results in our study may be consistent with this retrospective observation, indicating the significance of the direct effect of HCV infection on B-cell lymphoma development. Another HCV transgenic mouse strain (MxCre/CN2-29) showed the similarly high incidence of B-cell lymphoma, which strongly supported that development of B-cell lymphomas occurred by the expression of HCV transgene.

Recent findings have revealed the significance of B lymphocytes in HCV infection of liver-derived hepatoma cells.³⁴ In 4.2% of the RzCD19Cre mice, CD45R-positive intrahepatic lymphomas were identified, and infiltration of B cells into the hepatocytes was frequently observed (data not shown). These phenomena suggest that HCV could modify the in vivo tropism of B cells. The RzCD19Cre mouse is a powerful model system to address these mechanisms in vivo.

As a circulating membrane receptor, sIL-2R α is localized in lymphoid cells and some other types of cancer cells and is highly expressed in several cancers³⁵⁻⁴⁰ and autoimmune diseases.⁴¹ Recent findings indicate a link between sIL-2R α levels and hepatocellular carcinoma in Egyptian patients.⁴² Appearing on the surface of leukemic cells derived from B and pre-B lymphocytes and other leukemic cells, IL-2R α is one of the subunits of the IL-2 receptor, which is composed of an α chain (CD25), a β chain (CD122), and a γ chain (CD132).⁴³ IL-2R ectodomains are thought to be proteolytically cleaved from the cell surface^{34,44,45} instead of produced as a result of posttranscriptional splicing.²⁴ In RzCD19Cre splenocytes, the level of IL-2R α was higher than that in splenocytes from CD19Cre mice; however, serum concentrations of sIL-2R α in RzCD19Cre mice without B-cell lymphomas did not show significant differences compared with other control groups (Rz. CD19Cre, and WT). These results indicate the possibility that HCV may increase IL-2R α expression on B-cells; proteolytic cleavage of IL-2R α was increased after B-cell lymphoma development in the RzCD19Cre mouse. The detailed mechanism that induces IL-2R α as a result of HCV expression is still unclear at present, but we have found previously that the HCV core protein induces IL-10 expression in mouse splenocytes.¹⁴ IL-10 up-regulates the expression of IL-2R α (Tac/CD25) on normal and leukemic B lymphocytes,⁴⁶ and therefore, through IL-10, the HCV core protein might induce IL-2R α in B cells of the RzCD19Cre mouse.

In conclusion, this study established an animal model that will likely provide critical information for the elucidation of molecular mechanism(s) underlying the spontaneous development of B-cell non-Hodgkin lymphoma after HCV infection. This knowledge should lead to therapeutic strategies to prevent the onset and/or progression of B-cell lymphomas.

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Authorship

Contribution: K.T.-K. conceived of the project; K.K., M.K., and K.T.-K. designed the studies; Y.K., S.S., M. Saito, K.T., M. Satoh, M.T., and K.T.-K. performed experiments and analyses; N.S. and Y.H. provided scientific advice; and K.T.-K. wrote the manuscript.

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Sphingomyelin Activates Hepatitis C Virus RNA Polymerase in a Genotype-Specific Manner^{∇†}

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Hepatitis C virus (HCV) replication and infection depend on the lipid components of the cell, and replication is inhibited by inhibitors of sphingomyelin biosynthesis. We found that sphingomyelin bound to and activated genotype 1b RNA-dependent RNA polymerase (RdRp) by enhancing its template binding activity. Sphingomyelin also bound to 1a and JFH1 (genotype 2a) RdRps but did not activate them. Sphingomyelin did not bind to or activate J6CF (2a) RdRp. The sphingomyelin binding domain (SBD) of HCV RdRp was mapped to the helix-turn-helix structure (residues 231 to 260), which was essential for sphingomyelin binding and activation. Helix structures (residues 231 to 241 and 247 to 260) are important for RdRp activation, and 238S and 248E are important for maintaining the helix structures for template binding and RdRp activation by sphingomyelin. 241Q in helix 1 and the negatively charged 244D at the apex of the turn are important for sphingomyelin binding. Both amino acids are on the surface of the RdRp molecule. The polarity of the phosphocholine of sphingomyelin is important for HCV RdRp activation. However, phosphocholine did not activate RdRp. Twenty sphingomyelin molecules activated one RdRp molecule. The biochemical effect of sphingomyelin on HCV RdRp activity was virologically confirmed by the HCV replicon system. We also found that the SBD was the lipid raft membrane localization domain of HCV NS5B because JFH1 (2a) replicon cells harboring NS5B with the mutation A242C/S244D moved to the lipid raft while the wild type did not localize there. This agreed with the myriocin sensitivity of the mutant replicon. This sphingomyelin interaction is a target for HCV infection because most HCV RdRps have 241Q.

Hepatitis C virus (HCV) has a positive-stranded RNA genome and belongs to the family *Flaviviridae* (21). HCV chronically infects more than 130 million people worldwide (34), and HCV infection often induces liver cirrhosis and hepatocellular carcinoma (19, 28). To date, pegylated interferon (PEG-IFN) and ribavirin are the standard treatments for HCV infection. However, many patients cannot tolerate their serious side effects. Therefore, the development of new and safer therapeutic methods with better efficacy is urgently needed.

Lipids play important roles in HCV infection and replication. For example, the HCV core associates with lipid droplets and recruits nonstructural proteins and replication complexes to lipid droplet-associated membranes which are involved in the production of infectious virus particles (24). HCV RNA replication depends on viral protein association with raft membranes (2, 30). The association of cholesterol and sphingolipid with HCV particles is also important for virion maturation and infectivity (3). The inhibitors of the sphingolipid biosynthetic

pathway, ISP-1 and HPA-12, which specifically inhibit serine palmitoyltransferase (SPT) (23) and ceramide trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus (37), suppress HCV virus production in cell culture but not viral RNA replication by the JFH1 replicon (3). Other serine SPT inhibitors (myriocin and NA255) inhibit genotype 1b replication (4, 29, 33). Very-low-density lipoprotein (VLDL) also interacts with the HCV virion (15).

Sakamoto et al. reported that sphingomyelin bound to HCV RNA-dependent polymerase (RdRp) at the sphingomyelin binding domain (SBD; amino acids 230 to 263 of RdRp) to recruit HCV RdRp on the lipid rafts, where the HCV complex assembles, and that NA255 suppressed HCV replication by releasing HCV RdRp from the lipid rafts (29). In the present study, we analyzed the effect of sphingomyelin on HCV RdRp activity *in vitro* and found that sphingomyelin activated HCV RdRp activity in a genotype-specific manner. We also determined the sphingomyelin activation domain and the activation mechanism. Finally, we confirmed our biochemical data by a HCV replicon system.

MATERIALS AND METHODS

HCV RNA polymerase. A C-terminal 21-amino-acid deletion was made to the HCV RdRps of strains HCR6 (genotype 1b) (36), NN (1b) (35), Con1 (1b) (5), JFH1 (2a) (36), J6CF (2a) (25), H77 (1a) (7), and RMT (1a), and the mutants

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† Supplemental material for this article may be found at <http://jvi.asm.org>.

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were purified from bacteria as described previously (36). HCR6 (1b) RdRp with the mutation L245A [RdRp(L245A)] or I253A [RdRp(I253A)] or the double mutation L245A and I253A [RdRp(L245A/I253A)]; JFH1 (2a) RdRp with the mutation(s) A242C/S244D, A242, S244D, or T251Q; J6CF (2a) RdRp with the mutation(s) R241Q, S244D, or R241Q/S244D; and H77 (1a) RdRp(A238S/Q248E) were introduced using an *in vitro* mutagenesis kit (Stratagene) and the oligonucleotides listed in Table S1 in the supplemental material. HCR6 (1b) His₆-tagged RdRp(L245A/I253A) was removed from pET21b/KM (36) and cloned into the BamHI/XhoI site of pGEX-6P-3 (GE), resulting in pGEXHCVHCR6RdRp(L245A/I253A).

***In vitro* HCV transcription.** *In vitro* HCV transcription was performed as described previously (36). Briefly, following 30 min of preincubation without ATP, CTP, or UTP, 100 nM HCV RdRp was incubated in 50 mM Tris-HCl (pH 8.0), 200 mM monopotassium glutamate, 3.5 mM MnCl₂, 1 mM dithiothreitol (DTT), 0.5 mM GTP, 50 μM ATP, 50 μM CTP, 5 μM [α-³²P]UTP, 200 nM RNA template (SL12-1S), 100 U/ml human placental RNase inhibitor, and the lipid (amount indicated below) at 29°C for 90 min. ³²P-labeled RNA products were subjected to 6% polyacrylamide gel electrophoresis (PAGE) containing 8 M urea. The resulting autoradiograph was analyzed with a Typhoon Trio plus image analyzer (GE).

RNA filter binding assay. An RNA filter binding assay was performed as described previously (36). Briefly, 100 nM HCV RdRp and 100 nM ³²P-labeled RNA template (SL12-1S) were incubated with or without 0.01 mg/ml egg yolk sphingomyelin in 25 μl of 50 mM Tris-HCl (pH 7.5), 200 mM monopotassium glutamate, 3.5 mM MnCl₂, and 1 mM DTT at 29°C for 30 min. After incubation, the solutions were diluted with 0.5 ml of TE (50 mM Tris-HCl [pH 7.5], 1 mM EDTA) buffer and filtered through nitrocellulose membranes (0.45-μm pore size; Millipore). The filter was washed five times with TE buffer, and the bound radioisotope was analyzed by Typhoon Trio plus after being dried.

Enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtiter plates (Corning) were coated with 250 ng of egg yolk sphingomyelin in ethanol by evaporation at room temperature. After the wells were blocked with phosphate-buffered saline (PBS) and 3% bovine serum albumin (BSA), they were incubated with 1 pmol of the HCV RdRp of HCR6 (1b) wild type (wt) or L245A, I253A, or L245A/I253A mutant; NN (1b); H77 (1a); RMT (1a); J6CF (2a); or JFH1 (2a) wt or A242C/S244D, A242, S244D, or T251Q mutant in Tris-buffered saline (50 mM Tris-HCl [pH 7.5] and 150 mM NaCl) for 1.5 h at room temperature. After being blocked with 3% BSA, the bound HCV RdRp was detected by adding rabbit anti-HCV RdRp serum (1:5,000) (see Fig. S1 in the supplemental material) (17) before incubation with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:5,000; Southern Biotech). The optical density at 450 nm (OD₄₅₀) was measured with a Spectra Max 190 spectrophotometer (Molecular Devices) using a TMB (3,3',5,5'-tetramethylbenzidine) Liquid Substrate System (Sigma).

HCV subgenomic replicon. A D244S mutation was introduced into the HCV strain NN (1b) subgenomic replicon pLMH14 (35), resulting in pLMH(NN)5B(D244S) [where 5B(D244S) is the NS5B protein with the mutation D244S]. The A242C/S244D mutation was introduced into the HCV JFH1 (2a) replicon, pSGR-JFH1/luc (25), resulting in pSGR-JFH1/luc5B(A242C/S244D). The HpaI and XbaI fragment of pSGR-JFH1 (18) was replaced with that of pSGR-JFH1/luc5B(A242C/S244D), resulting in pSGR-JFH15B(A242C/S244D). The A238S/Q248E mutation was introduced into HCV H77 (1a) replicon pHCVrep13(S2204I)/Neo (7) after the neomycin gene was replaced by the firefly luciferase gene [pH77(I)/luc] by insertion of AflII and AscI sites (see Table S1 in the supplemental material), resulting in pH77(I)/luc5B(A238S/Q248E). Subgenomic replicon RNA was transcribed *in vitro* by T7 RNA polymerase using MegaScript (Ambion) after the replicon plasmids were linearized by XbaI (strain NN and JFH1 replicons) or HpaI (strain H77 replicon). Subgenomic replicon RNA was stored at -80°C after being purified by phenol-chloroform extraction and ethanol precipitation.

Replicon assay with myriocin. Huh7.5.1 cells were kindly provided by F. Chisari and were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Gibco) (38). HCV replicon RNA (10 μg) was transfected into 4 × 10⁶ Huh7.5.1 cells (1 × 10⁷/ml) in OptiMEM 1 (Gibco) by electroporation (GenePulser Xcell; Bio-Rad) at 270 V, 100 Ω, and 950 μF. After transfection, the cells were plated in 12-well plates incubated in DMEM-10% FBS. At 6 h after transfection, cells were treated with 0, 5, and 50 nM myriocin. At 4, 54, and 78 h after transfection (48 and 72 h after myriocin treatment), the cells were harvested, and luciferase activity was measured using a Dual-Glo luciferase assay kit and a GloMax 96 Microplate Luminometer (Promega). Luciferase activity was normalized against the activity at 4 h after transfection (26).

HCV JFH1 wt and NS5B(A242C/S244D) replicon cells. Huh7/scr cells were kindly provided by F. Chisari of the Scripps Research Institute and were maintained in Dulbecco's modified Eagle's medium (Gibco) with 10% fetal bovine serum (Gibco). RNA (10 μg each) from SGR-JFH1 and SGR-JFH1 with the mutations A242C/S244D in NS5B [NS5B(A242C/S244D)] was transfected into 4 × 10⁶ Huh7/scr cells (1 × 10⁷/ml) in OptiMEM 1 (GIBCO) by electroporation (GenePulser Xcell; Bio-Rad) at 270 V, 100 Ω, and 950 μF. After transfection, the cells were plated in 10-cm dishes and incubated in DMEM-10% FBS with 1.0 and 0.5 mg/ml G418 (Gibco). JFH1 wt and NS5B(A242C/S244D) replicon cells were maintained in DMEM-10% FBS and 0.5 mg/ml G418.

Membrane floating assay. JFH1 wt and NS5B(A242C/S244D) replicon cells were suspended in two packed cell volumes of hypotonic buffer (10 mM HEPES-NaOH [pH 7.6], 10 mM KCl, 1.5 mM MgCl₂, 2 mM DTT, and 1 tablet/25 ml of EDTA-free protease inhibitor cocktail tablets [Roche]) and disrupted by 30 strokes of homogenization in a Dounce homogenizer using a tight-fitting pestle at 4°C. After nuclei were removed by centrifugation at 2,000 rpm for 10 min at 4°C, the supernatant (postnuclear supernatant [PNS]) was treated with 1% Triton X-100 in TNE buffer (25 mM Tris-HCl [pH 7.6] 150 mM NaCl, 1 mM EDTA) for 30 min on ice. The lysates were supplemented with 40% sucrose and centrifuged at 38,000 rpm in a Beckman SW41 Ti rotor (Beckman Coulter) overlaid with 30% and 10% sucrose in TNE buffer at 4°C for 14 h.

Western blotting. Western blotting using anti-HCV RdRp (17), rabbit anti-NS3 (32), anti-NS5A (16) and anti-caveolin-2 was performed as previously published (17).

Reagent. Egg yolk sphingomyelin, cholesterol phosphocholine, myriocin, and rabbit anti-caveolin-2 antibodies were purchased from Sigma. Hexanoyl sphingomyelin, C₆-ceramide, C₆-β-D-glucosyl ceramide, and C₆-β-D-lactosyl ceramide were purchased from Avanti Polar Lipids. [α-³²P]UTP was purchased from New England Nuclear.

Statistical analysis. Significant differences were evaluated using *P* values calculated from a Student's *t* test.

Nucleotide sequence accession number. The sequence of HCV RMT has been deposited in the GenBank under accession number AB520610.

RESULTS

Sphingomyelin activation of HCV RNA polymerases of various genotypes. There are several sequence variations in the sphingomyelin binding domain (SBD; amino acids 231 to 260 of HCV RdRp) among HCV genotypes (see Fig. 7A). In order to compare the RdRps of different genotypes of HCV, we purified RdRp from genotypes 1b (strains HCR6, NN, and Con1), 1a (H77 and MRT), and 2a (JFH1 and J6CF) (see Fig. S2 in the supplemental material). First, the effect of ethanol on HCV HCR6 (1b) RdRp transcription was examined because lipids were suspended in ethanol before they were added to the HCV transcription reaction mixture. We found that 2% ethanol did not inhibit HCV transcription (see Fig. S3 in the supplemental material); therefore, all subsequent experiments were performed using less than 2% ethanol.

The kinetics of sphingomyelin activation were analyzed using egg yolk sphingomyelin for HCR6 (1b) RdRp wt (Fig. 1A) and subtype 2a (JFH1 and J6CF) RdRps (Fig. 1B), and *N*-hexanoyl-D-erythro-sphingosylphosphorylcholine (hexanoyl sphingomyelin) was used for HCR6 (1b) RdRp wt (Fig. 1C) and subtype 1a (H77 and RMT) RdRps (Fig. 1D). The egg yolk sphingomyelin activation curve of HCR6 (1b) RdRp wt at low concentrations (<0.01 mg/ml) was sigmoid. The transcription activity of HCR6 (1b) RdRp wt increased in a dose-dependent manner. It was activated 11-fold at 0.01 mg/ml and then plateaued (14-fold activation) at 0.1 mg/ml. However, JFH1 (2a) and J6CF (2a) RdRps were activated 2.5-fold and 2.2-fold, respectively, at 0.01 mg/ml sphingomyelin, at which point they plateaued.

Egg yolk sphingomyelin is a mixture. In order to obtain the optimal molar ratio for sphingomyelin activation of HCR6 (1b)

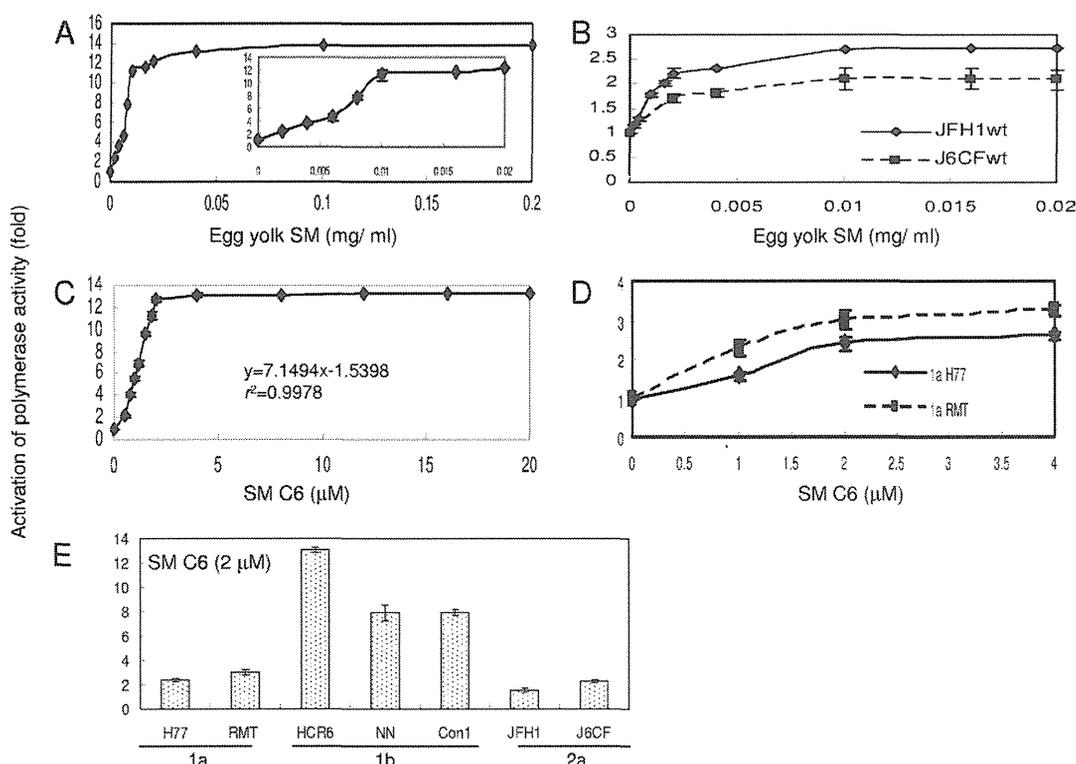


FIG. 1. Spingomyelin activation of HCV RNA polymerases. (A) Activation kinetics of HCV HCR6 (1b) RdRp wt by egg yolk spingomyelin (SM). The inset shows activation produced by 0 to 0.02 mg/ml egg yolk spingomyelin. Activation kinetics of HCV 2a (JFH1 and J6CF) RdRps by egg yolk spingomyelin (B) and of HCV HCR6 (1b) RdRp wt by hexanoyl spingomyelin (SM C6) (C). In panel C, the first order of the graph was fitted by linear regression; the calculated equation is indicated in the graph. (D) Activation kinetics of HCV 1a (H77 and RMT) RdRps by hexanoyl spingomyelin. (E) Activation effect of hexanoyl spingomyelin on HCV RdRp of various genotypes. HCV RdRp (100 nM) was incubated with or without 2 μM SM C6. The names of the RdRps are indicated below the graph. Mean \pm standard deviation of the activation ratio was calculated from three independent experiments.

RdRp wt, its activation kinetics were calculated using hexanoyl spingomyelin (Fig. 1C, SM C6). The equation for the first-order ratio of hexanoyl spingomyelin activation according to linear regression fitting was as follows: $y = 7.1494x - 1.5398$, where y is the activation ratio and x is the spingomyelin concentration ($r^2 = 0.9978$). RdRp activation had almost plateaued at 2 μM hexanoyl spingomyelin. The activation kinetics of JFH1 (2a) and J6CF (2a) RdRps in egg yolk spingomyelin were biphasic and plateaued at 0.01 mg/ml. Those of RMT (1a) and H77 (1a) RdRps in hexanoyl spingomyelin were also biphasic and plateaued at 2 μM. The curve of the first order was fitted by linear regression. The molar ratio of RdRp to hexanoyl spingomyelin at its plateau was calculated as 1:20.

Because RdRp activation had almost plateaued at 2 μM hexanoyl spingomyelin, we compared the effect of spingomyelin on 100 nM concentrations of RNA polymerases of the HCV 1a, 1b, and 2a genotypes using 2 μM hexanoyl spingomyelin (Fig. 1E and Table 1).

Helix-turn-helix structure for spingomyelin binding and activation. Spingomyelin binds to the SBD peptide (see HCV SBD in Fig. 7) (29). Initially, we tested whether SBD was the spingomyelin binding site in HCV RdRp by ELISA (Fig. 2A and Table 1). When the L245 and I253 residues of the SBD

peptide were mutated to A, spingomyelin binding activity was lost (29). We introduced the same mutations in HCV HCR6 (1b) RdRp and purified HCR6 (1b) RdRp with mutations L245A, I253A, and L245A/I253A. Because the C-terminal Histagged HCR6 RdRp(L245A/I253A) was not soluble, it was solubilized by tagging of glutathione *S*-transferase (GST) sequence at the N terminus but lost polymerase activity. As the L245A/I253A mutant had lost its polymerase activity, polymerase activation was tested only for L245A and I253A (Fig. 2B and Table 1). These results confirmed that SBD located in the finger domain (residues 230E to 263G) successfully achieved spingomyelin binding in HCV RdRp and that spingomyelin did not bind to the SBD when the helix-turn-helix structure had been destroyed by the L245A or I253A mutation (29).

The spingomyelin binding activities of genotype 1a and 2a RdRps were also tested (Fig. 2 and Table 1). Both JFH1 and J6CF were tested for genotype 2a because J6CF (2a) RdRp had an additional amino acid difference at position 241 in the SBD, and its spingomyelin binding activity was very low (Fig. 2A and 7A; Table 1). J6CF (2a) RdRp(R241Q) showed the same spingomyelin binding activity as HCR6 (1b) RdRp wt, indicating that 241Q was the critical amino acid for spingomyelin binding. J6CF (2a) RdRp(S244D) and RdRp(R241Q/S244D) also showed higher spingomyelin binding activity

TABLE 1. Summary of sphingomyelin activation of HCV RNA polymerase activities

Parameter	Value for the parameter by RdRp genotype, strain, and variant ^a																		
	1b							1a							2a				
	HCR6		NN		Con1		RMT		H77		J6CF		JFH1						
	wt	L245A	I253A	L245A/I253A	D244S	wt	wt	wt	wt	A238S/Q248E	wt	R241Q	S244D	R241Q/S244D	wt	A242C	S244D	A242C/S244D	T251Q
SM binding (%) ^b	100	24.3	30.8	15.5	78.7	93.4	117	144	86.7	82.5	19.3	118	53.1	80.2	70.4	75.5	93.1	92.4	80.7
Activation of polymerase (n-fold) ^c	13.0	(2.8) ^d	(2.5) ^d	ND	3.6	7.9	7.9	3.0	2.0	8.1	2.3	4.3	5.6	3.4	1.6	1.0	3.1	4.4	1.8
Activation of RNA binding (n-fold) ^c	4.5	2.6	1.7	ND	1.9	ND	ND	ND	1.4	3.3	1.5	3.6	3.2	1.7	1.3	ND	ND	1.4	ND

^a Numbers were averaged from three independent experiments. ND, not done.

^b Egg yolk sphingomyelin (SM; 250 ng) was used.

^c Hexanoyl sphingomyelin (2 μM) was used.

^d Egg yolk sphingomyelin (0.01 mg/ml) was used.

than the wt ($P < 0.001$) but lower binding than the R241Q mutant. However, S244D showed higher RdRp activation than R241Q ($P < 0.005$), while the RdRp activation ratio of the double mutant (R241Q/S244D) was lower than that of S244D or R241Q, although all of them activated RdRp with sphingomyelin ($P < 0.005$) (Fig. 2A and C and Table 1). For JFH1, when the JFH1 RdRp SBD was modified (A242C/S244D) to allow it to bind with more sphingomyelin than the wt ($P < 0.005$), the mutant JFH1 RdRp(A242C/S244D) was activated more than the wt by sphingomyelin ($P < 0.005$) (Fig. 2A and C; Table 1). The sphingomyelin binding activity of JFH1 RdRp(T251Q) was 80.7% of that of HCR6 (1b), and its activation ratio was 1.8-fold. These results agree that SBD is both the sphingomyelin activation and binding domain and that the domains for these two activities are somehow different.

We determined which amino acid, 242C or 244D, enhanced sphingomyelin binding by comparing HCR6 (1b) and JFH1 (2a) RdRps. Sphingomyelin binding of HCR6 (1b) RdRp(D244S) was 79% of that of the wt ($P < 0.005$) (Fig. 2A and Table 1), and its activation by sphingomyelin was only 3.6-fold (Fig. 2C and Table 1). The sphingomyelin binding of JFH1 (2a) RdRp(A242C) and RdRp(S244D) increased to 75.5% and 93.1%, respectively, of HCR6 (1b) RdRp wt (Fig. 2A and Table 1). This was significantly higher than that of JFH1 (2a) RdRp wt ($P < 0.005$), and the sphingomyelin activation of JFH1 (2a) RdRp(A242C) and RdRp(S244D) was increased 1.0-fold and 3.1-fold, respectively ($P < 0.005$) (Fig. 2C and Table 1). From these mutation analyses of the J6CF and JFH1 RdRps, we concluded that 244D enhanced sphingomyelin binding and RdRp activation.

HCV 1a RdRps were not activated even though sphingomyelin bound to them (Fig. 1E and 2A and Table 1). We then tried to elucidate the domains responsible for sphingomyelin activation. There are 14 amino acids (residues 19, 25, 81, 111, 120, 131, 184, 270, 272, 329, 436, 464, 487, and 540) unique to genotype 1a RdRp in the region of residues 1 to 570 and two amino acid differences unique to 1a RdRp in SBD, i.e., 238A and 248Q (see Fig. 6A). Initially, we focused on the SBD and introduced the A238S and Q248E mutations into the H77 (2a) RdRp SBD (Fig. 2A and D and Table 1). The sphingomyelin binding activity of H77 (2a) RdRp(A238S/Q248E) was similar to that of H77 (2a) RdRp wt. The sphingomyelin activation ratio of H77 (2a) RdRp(A238S/Q248E) was increased 8.1-fold, leading us to conclude that these mutations are essential to sphingomyelin activation.

Effect of lipids on HCV RNA polymerase activity. In order to elucidate the structure of the lipids involved in activation of HCV RdRp, D-lactosyl-β-1,1'-N-octanoyl-D-erythro-sphingosine [C₈-lactosyl(β) ceramide], D-glucosyl-β-1-17-N-octanoyl-D-erythro-sphingosine (C₈-β-D-glucosyl ceramide), N-hexanol-D-erythro-sphingosine (C₆-ceramide), and cholesterol were tested for their abilities to activate RdRp. The relative polymerase activities of 100 nM HCV HCR6 (1b) RdRp activated with 0.01 mg/ml egg yolk sphingomyelin, 2 μM hexanoyl sphingomyelin, 8 μM C₈-lactosyl(β) ceramide, 12 μM C₈-β-D-glucosyl ceramide, 12 μM C₆-ceramide, and 0.02 mg/ml cholesterol were 11.2, 13.0, 5.66, 4.19, 1.12, and 2.25 of that without lipids, respectively (Fig. 3A). The amount of lipids that gave the maximum activation was calculated from the kinetics of the lipids bound to HCR6 (1b) and JFH1 (2a) RdRps (Fig. 3B and

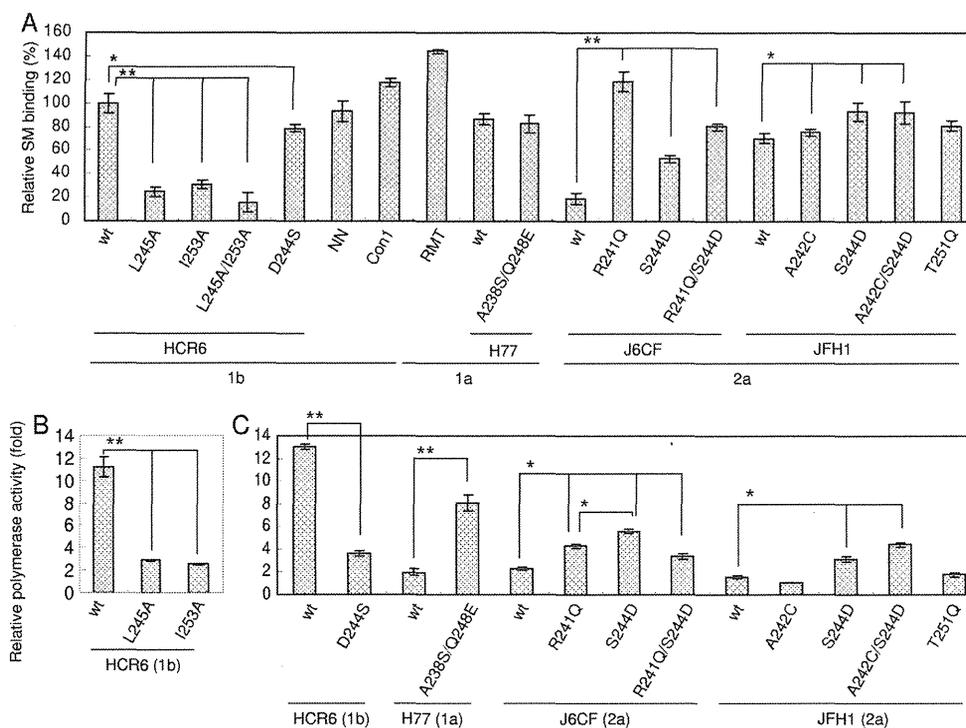


FIG. 2. Spingomyelin binding and activation of HCV RNA polymerase sphingomyelin binding domain mutants. Names of RdRps are indicated below the graphs. (A) Egg yolk spingomyelin (SM) binding activity relative to that of HCR6 (1b) RdRp wt. Mean \pm standard deviation of the binding was calculated from three independent experiments. (B) Egg yolk spingomyelin activation of HCR6 (1b) RdRps. RdRps (100 nM) were incubated with or without 0.01 mg/ml egg yolk spingomyelin. (C) Hexanoyl spingomyelin activation of the RdRps (RdRp names are indicated below the graphs). HCV RdRps (100 nM) were incubated with or without 2 μ M hexanoyl spingomyelin. The mean \pm standard deviation of the activation ratio was calculated from three independent experiments. *, $P < 0.005$; **, $P < 0.001$.

C). C_8 -lactosyl(β) ceramide and C_8 - β -D-glucosyl ceramide activated HCR6 (1b) RdRp compared with the linear regression kinetics of the reaction with hexanoyl spingomyelin as it plateaued (Fig. 1C and 3B). Cholesterol activated HCR6 (1b) RdRp slightly but did not activate JFH1 (2a) RdRp (Fig. 3C). We therefore concluded that the phosphocholine of spingomyelin bound to the SBD of HCV RdRp because the order of HCV RdRp activation was hexanoyl spingomyelin > C_8 -lactosyl(β) ceramide > C_8 - β -D-glucosyl ceramide, and C_6 -ceramide did not activate HCV HCR6 (1b) RdRp. The polarity of the phosphocholine of spingomyelin is important for HCV RdRp activation (see Fig. S5 in the supplemental material).

In order to test whether phosphocholine activated HCV RdRp (Fig. 3D), HCR6 (1b) RdRp was incubated with 0.4, 2, 20, 100, and 400 μ g and 2, 4, 11, 54, and 100 mg of phosphocholine. Up to 400 μ g of phosphocholine did not affect RdRp activity, but more than 2 mg of phosphocholine inhibited RdRp activity.

Effect of spingomyelin on the template RNA binding of HCV RNA polymerase. The mechanism of HCV RdRp activation was analyzed. RNA polymerase changes its conformation throughout the different transcription steps, and template binding is the first step of transcription (9). Therefore, the effect of spingomyelin on template RNA binding activity was tested (Fig. 4A and Table 1). Spingomyelin enhanced the template RNA binding of HCR6 (1b) RdRp wt but not that of JFH1 (2a), H6CF (2a), or H77 (1a) wt RdRp. When the

A238S/Q248E mutation was introduced into H77 (1a) RdRp, the RNA binding was enhanced. J6CF (2a) RdRp R241Q and S244D mutants showed similar enhancement of RNA binding, but the R241Q/S244D double mutant did not. The activation effect of RNA binding of HCR6 (1b) RdRp wt and RdRp(L245A, I253A, and D244S) was lower than that of RdRp wt. JFH1 (2a) RdRp wt and RdRp(A242C/S244D) showed similar RNA binding activation levels. Based on a comparison of the spingomyelin activation of HCR6 (1b) RdRp wt and its mutants which lost spingomyelin binding with J6CF (2a) RdRp wt and the R241Q and S244D mutants and H77 (1a) RdRp wt and the A238S/Q248E mutant, we concluded that polymerase activation by spingomyelin was induced mainly via activation of the template RNA binding of RdRp. RNA binding activity of JFH1 (2a) RdRp wt and RdRp(A242C/S244D) was almost saturated because RNA binding of these RdRps was not activated by spingomyelin (see Fig. S4 in the supplemental material).

HCV RdRp has to be bound with spingomyelin before or at the same time as it binds to template RNA. After RdRp had bound to the template RNA, spingomyelin did not enhance template RNA binding strongly (Fig. 4B).

Effect of the spingomyelin binding domain mutations for HCV replicon activity with myriocin. In order to confirm spingomyelin activation of HCV polymerase activity in a viral replication system, HCV replicon activity of the loss-of-function mutant HCV NN (1b) NS5B(D244S) and the gain-of-

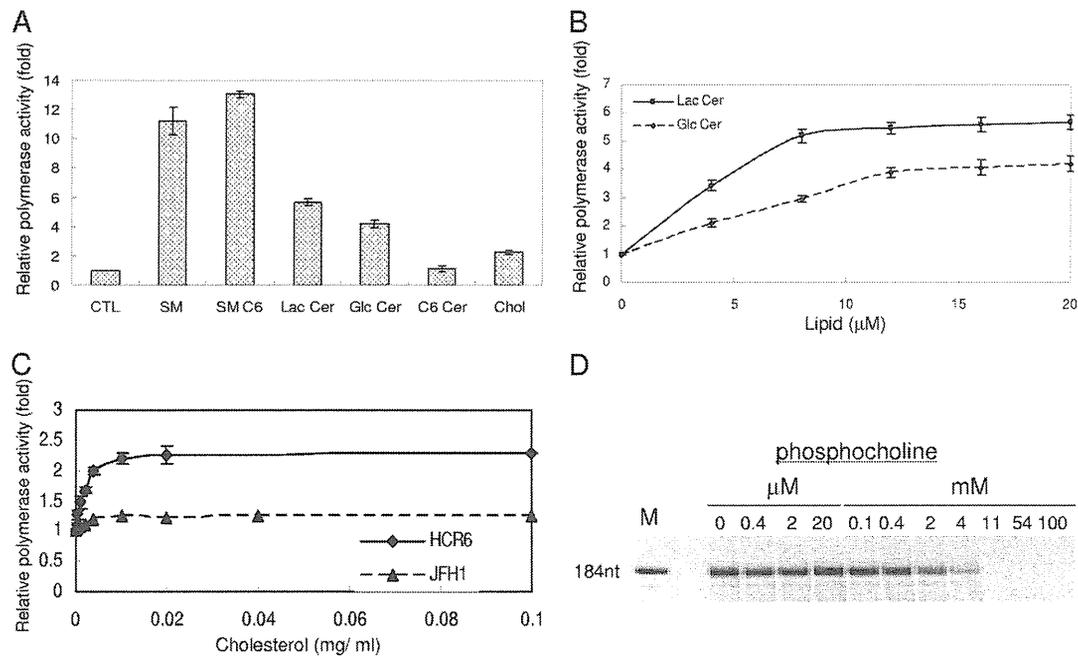


FIG. 3. HCV RNA polymerase activation effect of lipids. (A) Lipid activation of HCR6 (1b) RdRp wt. HCV HCR6 (1b) RdRp wt (100 nM) was incubated with or without (control [CTL]) 0.01 mg/ml egg yolk sphingomyelin (SM), 2 μM hexanoyl sphingomyelin (SM C6), 8 μM C₈-lactosyl(β) ceramide (Lac Cer), 12 μM C₈-β-D-glucosyl ceramide (Glc Cer), 12 μM C₆-ceramide (C6 Cer), or 0.02 mg/ml cholesterol (chol). (B) Activation kinetics of C₈-lactosyl(β) ceramide (Lac Cer) and C₈-β-D-glucosyl ceramide (Glc Cer) on HCR6 (1) RdRp. (C) Activation kinetics of cholesterol on HCR6 (1b) and JFH1 (12a) RdRps. (D) The effect of phosphocholine on HCR6 (1b) RdRp. The mean ± standard deviation of the activation ratio was calculated from three independent experiments.

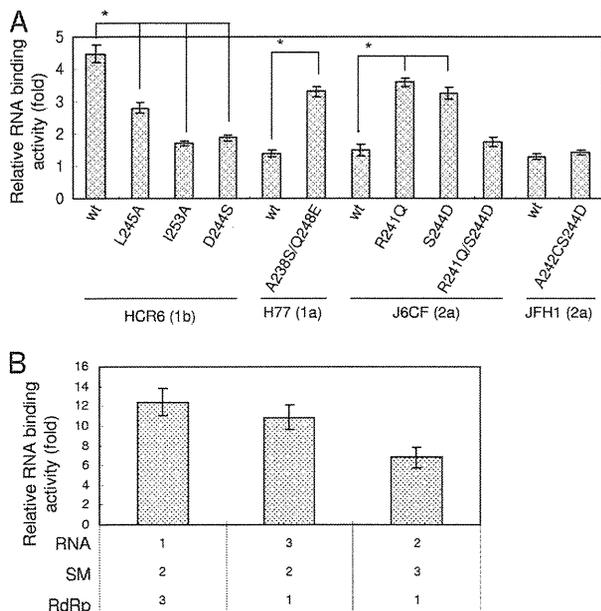


FIG. 4. Sphingomyelin activation of the RNA binding activity of HCV RNA polymerase. (A) Sphingomyelin activation of RNA filter binding of HCV RdRps (RdRp names are indicated below the graph). RdRps and ³²P-labeled RNA template (SL12-1S) were incubated with or without egg yolk sphingomyelin (SM), before filtration. (B) Effect of the order of sphingomyelin treatment. Numbers below the graph indicate the order in which the reagents were added. The graph represents the ratio to RNA binding without sphingomyelin. The mean ± standard deviation of the activation ratio was calculated from three independent experiments. *, *P* < 0.01.

function mutants H77 (1a) NS5B(A238S/Q248E) and JFH1 (2a) NS5B(A242C/S244D) were compared with 5 and 50 nM myriocin treatment for 72 h (Fig. 5).

First, HCV replicon activity was compared as the relative luciferase activity (Fig. 5A). Both JFH1 (2a) wt and NS5B(A242C/S244D) replicons showed similar and strong replicon activity ($133 \times 10^3 \pm 12 \times 10^3$ and $138 \times 10^3 \pm 8.5 \times 10^3$, respectively). JFH1 (2a) wt replicon was resistant to myriocin treatment, as reported by Aizaki et al. using other SPT inhibitors (3). The JFH1 (2a) NS5B(A242C/S244D) replicon became sensitive to myriocin but still showed higher replicon activity than NN (1b) or H77 (1a) replicons even at 50 nM myriocin.

To analyze the effect of mutations precisely, the replicon activity relative to each wt strain was compared (Fig. 5B). The JFH1 (2a) wt replicon with 50 nM myriocin showed the same luciferase activity as the wt without myriocin ($102\% \pm 9.6\%$). JFH1 (2a) NS5B(A242C/S244D) replicon activity was the same as that of the wt without myriocin ($103\% \pm 12\%$); with 5 nM myriocin it was $84.1\% \pm 6.6\%$ of the wt level, but with 50 nM myriocin it was $70.3\% \pm 5.3\%$ of the wt level, which was significantly lower (*P* < 0.01). NN (1b) wt replicon activity was $45.3\% \pm 6.6\%$ with 5 nM myriocin and $21.7\% \pm 2.9\%$ with 50 nM myriocin relative to the wt level without myriocin. NN (1b) NS5B(D244S) replicon activity was $72.2\% \pm 12\%$ without myriocin (*P* < 0.05), $44.0\% \pm 7.4\%$ with 5 nM myriocin, and $38.1\% \pm 4.2\%$ with 50 nM myriocin relative to wt level without myriocin, which was significantly higher (*P* < 0.01). Thus, NN (1b) NS5B(D244S) showed lower replicon activity than the wt

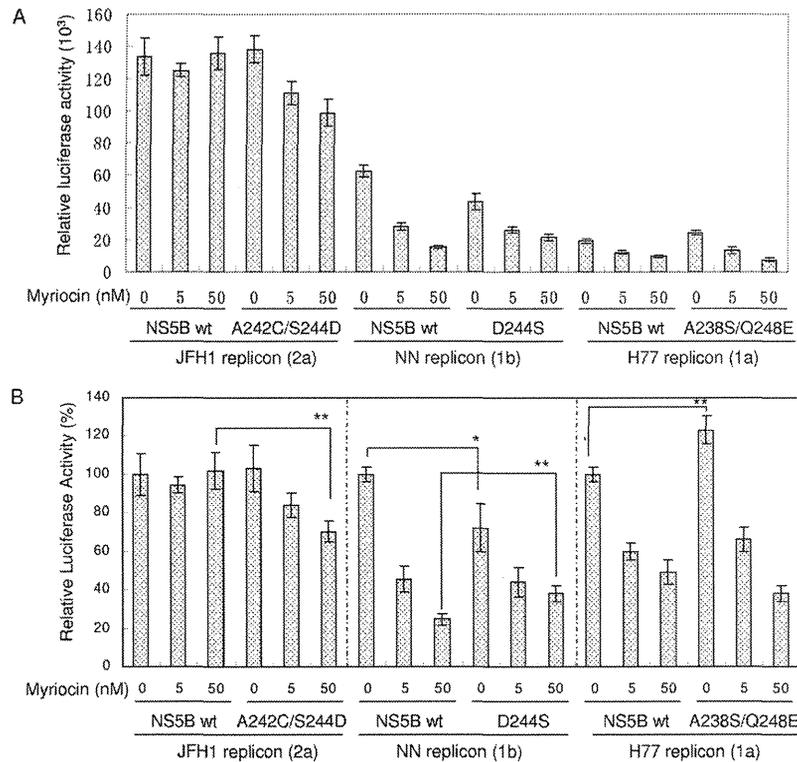


FIG. 5. Myriocin inhibition of HCV replicon activity. Huh7.5.1 cells were incubated with myriocin after transfection with the HCV replicons indicated below the graphs. Means ± standard deviations of the relative luciferase activity at 72 h after myriocin treatment compared to activity at 4 h after transfection (A) and to that of each wt without myriocin (B) were calculated from three independent measurements. *, $P < 0.05$; ** $P < 0.01$.

and was less sensitive to myriocin than the wt. H77 (1a) wt replicon activity was $59.9\% \pm 4.2\%$ with 5 nM myriocin and $49.2\% \pm 6.4\%$ with 50 nM myriocin relative to the wt level without myriocin. H77 (1a) NS5B(A238S/Q248E) replicon activity was $123\% \pm 7.1\%$ without myriocin ($P < 0.01$), $66.1\% \pm 6.3\%$ with 5 nM myriocin, and $38.0\% \pm 4.1\%$ with 50 nM myriocin relative to wt level without myriocin. Both H77 (1a) wt and NS5B(A238S/Q248E) replicons were sensitive to myriocin, and the replicon activity of NS5B(A238S/Q248E) was higher than that of the wt.

JFH1 (2a) RdRp(A242C/S244D) localized in the DRM fractions. Myriocin sensitivity of JFH1 (2a) NS5B(A242C/S244D) replicon indicates the importance of 244D in JFH1 NS5B for sphingomyelin binding. To further confirm the role of 244D for recruitment of HCV RdRp to the detergent-resistant membrane (DRM), where the HCV replication complex exists, we compared the distribution of NS5A and NS5B of JFH1 (2a) wt and NS5B(A242C/S244D) in their replicon cells by sucrose density gradient centrifugation of the DRM (Fig. 6). NS5A proteins of both JFH1 (2a) wt and NS5B(A242C/S244D) replicons localized in the DRM fraction where caveolin-2 was present (11, 27), but most of NS5B wt localized in the Triton-soluble fractions. NS5B of JFH1 (2a) NS5B(A242C/S244D) replicon was shifted to the DRM fraction from the soluble fraction. The shift of NS5B(A242C/S244D) localization into the DRM demonstrated that SBD was the DRM localization domain of NS5B and that residue 244D was important for this localization.

DISCUSSION

Hepatitis C virus is an envelope virus, and the lipid components of the virion play important roles in HCV infectivity and virion assembly (3, 15, 20, 24). HCV replication complexes localize in lipid raft structures/DRMs in the membrane frac-

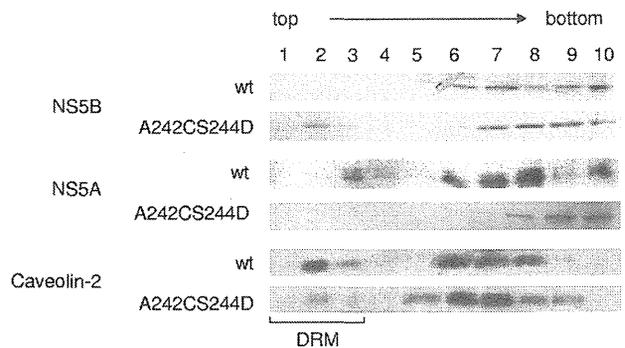


FIG. 6. Membrane floating assay of JFH1 wt and NS5B(A242C/S244D) replicon cells. The PNS fractions of HCV JFH1 (2a) wt and NS5B(A242C/S244D) replicon cells were treated with 1% Triton X-100 in TNE buffer for 30 min at 4°C and subjected to 10 to 40% sucrose gradient centrifugation in TNE buffer. Each fraction was subjected to 10% SDS-PAGE, followed by Western blotting with anti-NS5A, -NS5B, and -caveolin-2 antibodies. Fractions are numbered as indicated at the top of the panel. The DRM fractions (fractions 1 to 3) are indicated.

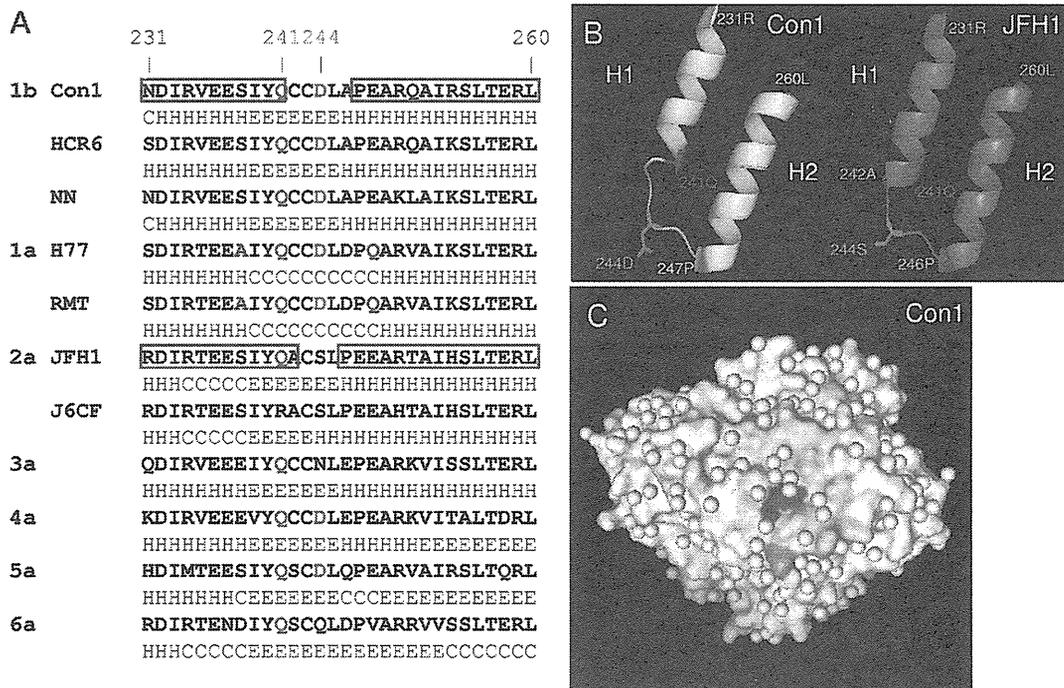


FIG. 7. Sphingomyelin binding domain (SBD) of HCV RNA polymerase. (A) The SBDs (231N to 260L) of HCV RdRps are aligned together with their secondary structure predicted by the Chou-Fasman program (10). The predicted secondary structure is indicated below the sequence as follows: H, α -helix; E, β -sheet; and C, coil. The α -helix structures of HCV Con1 (1b) RdRp and JFH1 (2a) RdRp are boxed in red. Residues 241Q and 244D are indicated in red and green, respectively. The 238A and 248E of the H77 and RMT (1a) RdRps are indicated in purple. GenBank accession numbers of HCV genotypes 3a, 4a, 5a, and 6a are GU814263 (12), GU814265 (12), Y13184 (8), and Y12083 (1), respectively. (B) Comparison of the SBDs of HCV Con1 (1b) (yellow) and JFH1 (2a) RdRps (magenta). The starting and ending amino acids of H1 and H2 are indicated. The sphingomyelin binding site, 241Q, is indicated in red, and 244D of Con1 (1b) and 244S of JFH1 (2a) RdRp are indicated in green. (C) Surface model of HCV Con1 (1b) RdRp. SBD is indicated in yellow, and 241Q and 244D are indicated in red and green, respectively. The structures of the Con1 and JFH1 RdRps were constructed by PyMOL, version 1.1.1 (<http://www.pymol.org/>). PDB numbers of Con1 (1b) RdRp and JFH1 (2a) RdRp are 3FQL (14) and 3I5K (31), respectively.

tions of subgenomic replicon cells (30). Lipid rafts are composed mainly of sphingomyelin, cholesterol, and glycosphingolipids. Most reports regarding the relationship between lipids and HCV have examined virion assembly, infectivity, and the localization of HCV, but their biochemical interactions have not been reported. Our findings clearly demonstrate that sphingomyelin plays an important role not only in HCV replication complex formation and its localization but also in HCV RdRp activity.

The helix-turn-helix structure of the SBD (residues 230 to 263), which is located between RNA polymerase motifs A and B, has been proposed as the sphingomyelin binding domain of HCV RdRp (29). We compared the SBD of Con1 (1b) (Protein Data Bank [PDB] 3FQL) (14) and JFH1 (2a) (PDB 3I5K) (31) and the secondary structure of the amino acids (201 to 290) in the SBD predicted by the Chou-Fasman program (10) (Fig. 7; see also Fig. S5 in the supplemental material) because the helix structures of the SBD of Con1 (helix 1 [H1], 231N to 241Q; helix 2 [H2], 247A to 260L) and JFH1 (H1, 231R to 242A; H2, 246P to 260L) RdRp fit with those predicted by the Chou-Fasman program. The structures contributing to sphingomyelin binding and activation are H1 and H2 and the junction (turn) between the two helix structures that are similar to the human immunodeficiency virus (HIV) gp120 V3 domain,

prion protein (PrP), and β -amyloid peptide (13, 22). Although Con1 (1b) RdRp has a shorter helix structure than JFH1 (2a) RdRp (Fig. 6B), the structures of their SBDs are very similar (Fig. 7; see also Fig. S5). When the helix-turn-helix structure of the SBD was destroyed (HCR6 genotype 1b RdRp mutants L245A and I253A), the RdRp lost sphingomyelin binding activity and lost its activation (Fig. 2).

In order to study the structure-function relationship of the SBD and sphingomyelin, we compared the SBD of genotype 1a, 1b and 2a RdRps and particularly focused on residue 244D in the turn and residues 241Q and 238S/248E in the helix domains. The polar amino acid 241Q and the negatively charged 244D of Con1 (1b) RdRp located on the surface of the RdRp molecule bind and interact with the positively charged choline residue of sphingomyelin (Fig. 7C; see also Fig. S5 in the supplemental material). The positively charged 241R repels the choline residue of sphingomyelin, and as a result, J6CF (a) RdRp wt did not bind to sphingomyelin. J6CF (2a) RdRp(R241Q) showed almost the same sphingomyelin binding activity as HCR6 (1b) RdRp wt. This ionic interaction between SBD and sphingomyelin agrees with the activation of lipids with different sphingosine structures and fatty acid chains (Fig. 3A). JFH1 (2a) RdRp does not interact well with sphingomyelin because it does not have the negatively charged

amino acids at the tip of its turn structure. Once its 244S was changed to D, more sphingomyelin bound to JFH1 (2a) RdRp and activated the RdRp (Fig. 2A and C). The reason for the low activation of J6CF (2a) RdRp(R241Q/S244D) is not clear. Sometimes mutations affect the entire conformation of the molecule. In conclusion, from the comparison of sphingomyelin binding and activation of HCR6 (1b), J6CF (2a), and JFH1 (2a) RdRp SBD mutants, 241Q is the essential amino acid for sphingomyelin binding in the SBD. Amino acid 244D enhanced both binding and RdRp activation.

The *in vitro* sphingomyelin binding and RdRp activation experiments indicate that sphingomyelin binding and its RdRp activation are different biochemical reactions because we found controversial activation rates for sphingomyelin binding and RdRp activation among J6CF (2a) RdRp mutants (Fig. 2). The relationship between sphingomyelin binding and the activation of polymerase activity was studied by comparing genotype 1b and 1a RdRps, both of which bind to sphingomyelin (Fig. 2). However, 1a RdRp is not activated by sphingomyelin because both of the helix structures of 1a RdRp are probably terminated at 238A and 248Q, making its helix structures shorter than those of 1b RdRp (Fig. 6A). The length of the helix structure may be essential for sphingomyelin activation because RdRp changes its structure to bind to template RNA when sphingomyelin binds to SBD (Fig. 4).

HCV RdRp changes its conformations at the early stages of transcription initiation, including the template RNA binding step (6, 9). Sphingomyelin binding is likely to change the conformation of 1b RdRp to recruit template RNA and initiate transcription efficiently. Comparison of the activation ratio of RNA binding and polymerase activity of 1b RdRp, J6CF (2a) RdRp wt and R241Q and S244D mutants, and JFH1 (2a) RdRp wt and mutant A242C/S244D suggests that steps other than RNA binding are also likely to be activated by sphingomyelin.

From a kinetic analysis of sphingomyelin activation (Fig. 1C and D), 20 sphingomyelin molecules are estimated to interact with the SBD of RdRp and activate it because sphingomyelin activation plateaued at 20 sphingomyelin molecules per HCV RdRp molecule. It is not clear whether 20 sphingomyelin molecules form a micelle or a layer structure. However, the structure of sphingomyelin is important for the activation of HCV RdRp because phosphocholine did not activate the RdRp (Fig. 3D).

To confirm these biochemical findings in HCV replication, we tested the effect of SBD mutations in HCV replicon systems with the SPT inhibitor myriocin (Fig. 5) (4, 33) because NA255 was not available. The loss-of-function mutant, HCV NN (1b) NS5B(D244S), showed lower replicon activity than NN (1b) wt and more resistance to 50 nM myriocin, which did not affect the viability of cells (4, 33), than the wt. The gain-of-function mutant, H77 (1a) NS5B(A238S/Q248E), showed higher replicon activity than H77 wt and retained myriocin sensitivity because it had the sphingomyelin binding sites 241Q and 244D. At 50 nM myriocin, another gain-of-function mutant, JFH1 (2a) NS5B(A242C/S244D), was inhibited although its activity was the same as that of JFH1 (2a) wt without myriocin because the JFH1 wt replicon had high replicon activity without myriocin (Fig. 5A). The JFH1 replicon activity may be maximal in the system; therefore, the JFH1 (2a) NS5B(A242C/S244D) replicon did not show higher activity than JFH1 (2a) wt with-

out myriocin while H77 (1a) NS5B(A238S/Q248E) showed higher replicon activity than H77 wt.

The binding and RdRp activation activity of the amino acid 244 mutants by sphingomyelin did not differ greatly from the wt *in vitro*. However, the myriocin sensitivity of JFH1 (2a) NS5B(S244D) was demonstrated clearly. That of H77 (1a) NS5B(A238S/Q248E) indicated that sphingomyelin binding was the target of myriocin inhibition, not the sphingomyelin activation of RdRp. These data confirm the importance of 241Q, 244D, and the helix structure in SBD for HCV replication in the cells.

Sphingomyelin is the major component of the lipid raft structure/DRM where the HCV genome replicates. To confirm that the SBD is the membrane binding site of HCV RdRp, we analyzed the localization of NS5B of JFH1 (2a) wt and NS5B(A242C/S244D) replicons by membrane floating assay (Fig. 6). JFH1 (2a) NS5B wt did not localize in the DRM. However, the localization of NS5B of the JFH1 (2a) NS5B(A242C/S244D) replicon shifted to the DRM from the soluble fractions. Previously, HCV NS5B was believed to localize in the DRM by its C-terminal hydrophobic sequences (21). However, our data demonstrate that the SBD is the membrane localization domain of HCV NS5B, which agrees with the myriocin sensitivity of JFH1 (2a) NS5B(A242C/S244D) replicons (Fig. 5) and the release of HCV 1b NS5B from the DRM by another SPT inhibitor, NA255 (29).

This is the first report of RNA polymerase activation by lipids. Twenty sphingomyelin molecules interact with SBD, particularly with residues 241Q and 244D of HCV (1b) RdRp, and change the conformation of the RdRp in order to recruit RNA templates. At the same time, HCV RdRp molecules may be aligned on the sphingomyelin layer formed via interactions between the hydrocarbon chains of sphingosine and fatty acids via placement of their SBD into the layer (Fig. 7C). Consistent with previous research (3, 23, 37), our findings explain why the inhibitors of the sphingolipid biosynthetic pathway influence subgenomic replicons derived from HCV genotypes 1a and 1b but not those derived from JFH1 (2a) (Fig. 5). Most HCV isolates have 241Q in NS5B, and some of them also have 244D (Fig. 7A). These sphingomyelin interactions are new targets for the treatment of HCV.

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