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Figure Legends

Figure. 1. Schematic representation of the constructs of HCV subgenomic and genome-length replicon RNA. On the left, the constructs of each replicon RNA are shown. HCV strains as well as genotypes from which the replicon RNA sequences are derived are indicated in the second column. The names of replicon cell clones established with each replicon RNA are in the third column. The sensitivity to CsA of each replicon RNA revealed in this study is summarized in the right column. The replicon RNAs comprise the HCV 5'-UTR including HCV IRES, the neomycin phosphotransferase gene (Neo^r), EMCV IRES or HCV IRES, the coding region for HCV proteins NS3 to NS5B (subgenomic) or Core to NS5B (genome-length or full-genome), and HCV 3'-UTR. MH-14 (NN/1b/SG), #50-1 (NN/1b/SG), MH14#W31 (NN/1b/SG), SN1 (Con1/1b/SG), sO (O/1b/SG), JFH1#4-1 (JFH1/2a/SG), and JFH1#2-3 cells (JFH1/2a/SG) carry subgenomic replicons while NNC#2 (NN/1b/FL), SN1A#2 (Con1/1b/FL), and SNC#7 cells (Con1/1b/FL) have genome-length replicons. NNC#2 (NN/1b/FL) and SNC#7 cells (Con1/1b/FL) contain the replicon RNA without EMCV IRES.

Figure. 2. CsA suppressed the replication of HCV genome irrespective of the presence of the structural proteins. (A) Detection of HCV proteins from NNC#2 (NN/1b/FL) genome-length replicon. Core (panel a), E2 (panel b), NS3 (panel c), NS5A (panel d), NS5B (panel e), and tubulin (panel f) in Huh-7, NNC#2 (NN/1b/FL), and MH-14 cells (NN/1b/SG) analyzed by immunoblot analysis. (B) HCV RNA in Huh-7, NNC#2 (NN/1b/FL), and MH-14 cells

(NN/1b/SG) quantified by real time RT-PCR analysis. The data represent the mean of three independent experiments. (C) CsA decreased the production of HCV proteins in NNC#2 (NN/1b/FL) as well as MH-14 cells (NN/1b/SG). After treatment with 1 μg/ml CsA ("+") for 5 days or without treatment ("-"), total cell lysate of NNC#2 (NN/1b/FL) and MH-14 cells (NN/1b/SG), together with Huh-7 cells as a negative control, was recovered to examine the production of HCV NS5A (upper panel), NS5B (middle panel), and tubulin as an internal control (lower panel) by immunoblot analysis. The same result was obtained at day 7 after the treatment. (D) The sensitivity to CsA of HCV genome-length replicon was almost the same as that of subgenomic replicon. HCV RNA was quantified by real time RT-PCR analysis using total RNA from NNC#2 (NN/1b/FL), SN1A#2 (Con1/1b/FL), and SNC#7 cells (Con1/1b/FL) treated with various concentrations of CsA for 7 days. The relative amount of HCV RNA was plotted against the concentration of CsA (μg/ml). (E) Effect of CsA on cell proliferation. NNC#2 cells (NN/1b/FL) were treated with various amount of CsA for 7 days. Cell numbers were counted and relative cell numbers to that of cells without treatment were plotted against the concentration of CsA.

Figure. 3. Replication of a genotype 2a strain, JFH1, was less sensitive to CsA. (A) Sensitivity to CsA of HCV genotype 1b and JFH1 replicons. SN1 (Con1/1b/SG), MH-14 (NN/1b/SG), sO (O/1b/SG), #50-1 (NN/1b/SG), JFH1#4-1 (JFH1/2a/SG), and JFH1#2-3 cells (JFH1/2a/SG), carrying HCV subgenomic replicon, were treated with 1 μg/ml CsA for 7 days. HCV RNA titers were quantified by real time RT-PCR analysis and the relative amount is shown. The bars represent the mean of three independent experiments. White bars, no treatment; black bars, 1

μg/ml CsA. The numbers above the black bars indicate fold difference of the titer under the treatment of 1 μg/ml CsA compared with that with no treatment. (B) Levels of NS3 and tubulin as an internal control in MH14#W31 (NN/1b/SG) and JFH1#4-1 cells (JFH1/2a/SG) without ("-") or with ("+") 1 μg/ml CsA treatment for 5 days were detected by immunoblot analysis. (C) HCV RNA was quantified and plotted as described in Fig. 2D in genotype 1b replicon cells such as MH-14 (NN/1b/SG), #50-1 (NN/1b/SG), MH14#W31 (NN/1b/SG), SN1 (Con1/1b/SG), and sO (O/1b/SG), and JFH1-carrying replicon cells, JFH1#4-1 (JFH1/2a/SG) and JFH1#2-3 cells (JFH1/2a/SG). (D) Effect of CsA on cell proliferation. The cell growth of MH-14 (NN/1b/SG) and JFH1#4-1 cells (JFH1/2a/SG) were examined as described in the legend for Fig. 2E.

Figure. 4. JFH1 replication was less sensitive to a CsA derivative, NIM811. (A) MH14#W31 (NN/1b/SG) and JFH1#4-1 cells (JFH1/2a/SG) were treated with 0.5 μg/ml NIM811 for 7 days. HCV RNA titers were quantified as described in Fig. 3A. White bars, no treatment; black bars, 0.5 μg/ml NIM811. (B) (C) HCV RNA in replicon cells treated with various concentrations of NIM811 (B) or PSC833 (C) for 7 days was quantified and plotted against the concentration of NIM811 (B) or PSC833 (C) (μg/ml) as described in Fig. 3C.

Figure. 5. Interaction of HCV NS5B with CyPB in the JFH1 replicon. (A) Co-immunoprecipitation of endogenous CyPB with NS5B. Lysates from MH14#W31 (NN/1b/SG), JFH1#4-1 (JFH1/2a/SG), and Huh-7 cells as a negative control were used for immunoprecipitation with normal mouse IgG ("IgG") or anti-NS5B antibody ("NS5B"), followed by immunoblot analysis with either anti-CyPB (upper panel) or anti-NS5B antibodies

(lower panel). "IP" indicates the antibodies used for immunoprecipitation. (B) The interaction of CyPB with NS5B in JFH1 replicon was disrupted by CsA treatment. Co-immunoprecipitation between CyPB and NS5B in MH14#W31 (NN/1b/SG) or JFH1#4-1 cells (JFH1/2a/SG) treated without (lanes 1 and 5) or with CsA (0.3 μg/ml in lanes 2 and 6, 1 μg/ml in lanes 3 and 7, and 3 μg/ml in lanes 4 and 8) was analyzed.

Figure. 6. CyPB in HCV replication of genotype 1b and JFH1. (A) Expression level of endogenous CyPB protein (upper panel) and tubulin as an internal control (lower panel) in MH14#W31 (NN/1b/SG), SN1 (Con1/1b/SG), sO (O/1b/SG), JFH1#4-1 (JFH1/2a/SG), and Huh-7 cells. (B) Knock-down of endogenous CyP proteins. MH14#W31 (NN/1b/SG) and JFH1#4-1 cells (JFH1/2a/SG) were transfected with siRNA specific for CyPA ("si-CyPA"), CyPB ("si-CyPB"), a broad range of CyP subtypes ("si-CyP(broad)") or with a randomized siRNA ("si-control"). At 72 h posttransfection, CyPA (upper panels), CyPB (middle panels) and tubulin as an internal control (lower panels) were detected in total cell lysates of MH14#W31 (NN/1b/SG) (left panels) and JFH1#4-1 cells (JFH1/2a/SG) (right panels) by immunoblot analysis. (C) Depletion of CyPB did not affect HCV replication of JFH1 replicon. At 5 days posttransfection, HCV RNA titers in MH14#W31 (NN/1b/SG) (left panel) and JFH1#4-1 cells (JFH1/2a/SG) (right panel) were quantified by real time RT-PCR analysis. No treatment, treatment with only the transfection reagent in the absence of siRNA. (D) Effect of siRNA on cell proliferation. Cell numbers of MH14W#31 (NN/1b/SG) and JFH1#4-1 cells (JFH1/2a/SG) treated with siRNA for 5 days were counted. Relative cell numbers were indicated.

Figure. 7. RNA binding capacity of JFH1 NS5B was independent of CyPB. (A) RNA-protein binding precipitation assay was performed using MH14#W31 (NN/1b/SG) (lanes 1-6) and JFH1#4-1 cells (JFH1/2a/SG) (lanes 7-12) as described in Materials and Methods. MH14#W31 (NN/1b/SG) and JFH1#4-1 cells (JFH1/2a/SG) preincubated without (lanes 1, 3, 5, 7, 9, and 11) or with (lanes 2, 4, 6, 8, 10, and 12) CsA were treated with digitonin, followed by digestion with proteinase K to isolate the replication complex. This fraction was then incubated with poly-U RNA-sepharose (lanes 5, 6, 11, and 12) or protein G-sepharose as a negative control (lanes 3, 4, 9, and 10). Precipitates were detected by immunoblot analysis with anti-NS5B antibody. "INP" indicates the 1/6 amount of cell lysate used in the precipitation assay. "G" and "pU" designate the samples using protein G-sepharose and poly-U-sepharose, respectively. (B) In vitro RNA binding assay was performed as described in Materials and Methods. In vitro synthesized NS5B of MH-14 (lanes 1-6) or JFH1 (lanes 7-12) using the rabbit reticulocyte lysate in the presence of [^{35}S]methionine were incubated with protein G-sepharose (lanes 2 and 8) or poly-U-sepharose in the absence (lanes 3 and 9) or presence of varying amount of purified recombinant GST-CyPB (2 ng in panels 4 and 10, 10 ng in panels 5 and 11, and 50 ng in panels 6 and 12). The resultant precipitates were fractionated by SDS-PAGE followed by the detection of radiolabeled protein. (C) The density of the bands of NS5B in the RNA binding fraction was quantified and plotted against the amount of the recombinant GST-CyPB (ng). Solid line, NS5B of MH-14; faint line, NS5B of JFH1.

Figure. 8. Amino acid sequence alignment of NS5B encoded by HCV strains, NN, Con1, O, and JFH1. The numbers above the sequence indicate the amino acid number. Conserved

residues are shown by dashes. The region of 521-591 aa, which is involved in the interaction with CyPB, is boxed.

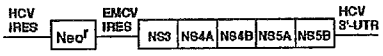
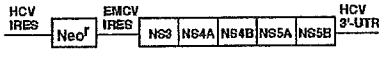
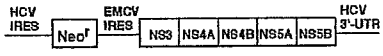
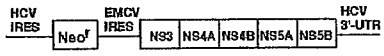
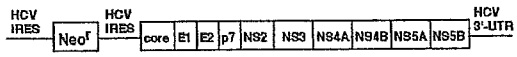
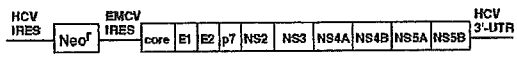
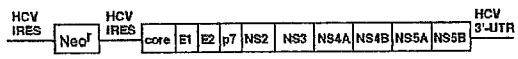
	HCV strain (genotype)	replicon cell clone	sensitivity to CsA
	NN (1b)	MH-14, #50-1 MH14#W31	highly sensitive
	Con1 (1b)	SN1	highly sensitive
	O (1b)	sO	highly sensitive
	JFH1 (2a)	JFH1#4-1, JFH1#2-3	less sensitive
	NN (1b)	NNC#2	highly sensitive
	Con1 (1b)	SN1A#2	highly sensitive
	Con1 (1b)	SNC#7	highly sensitive

Fig. 1

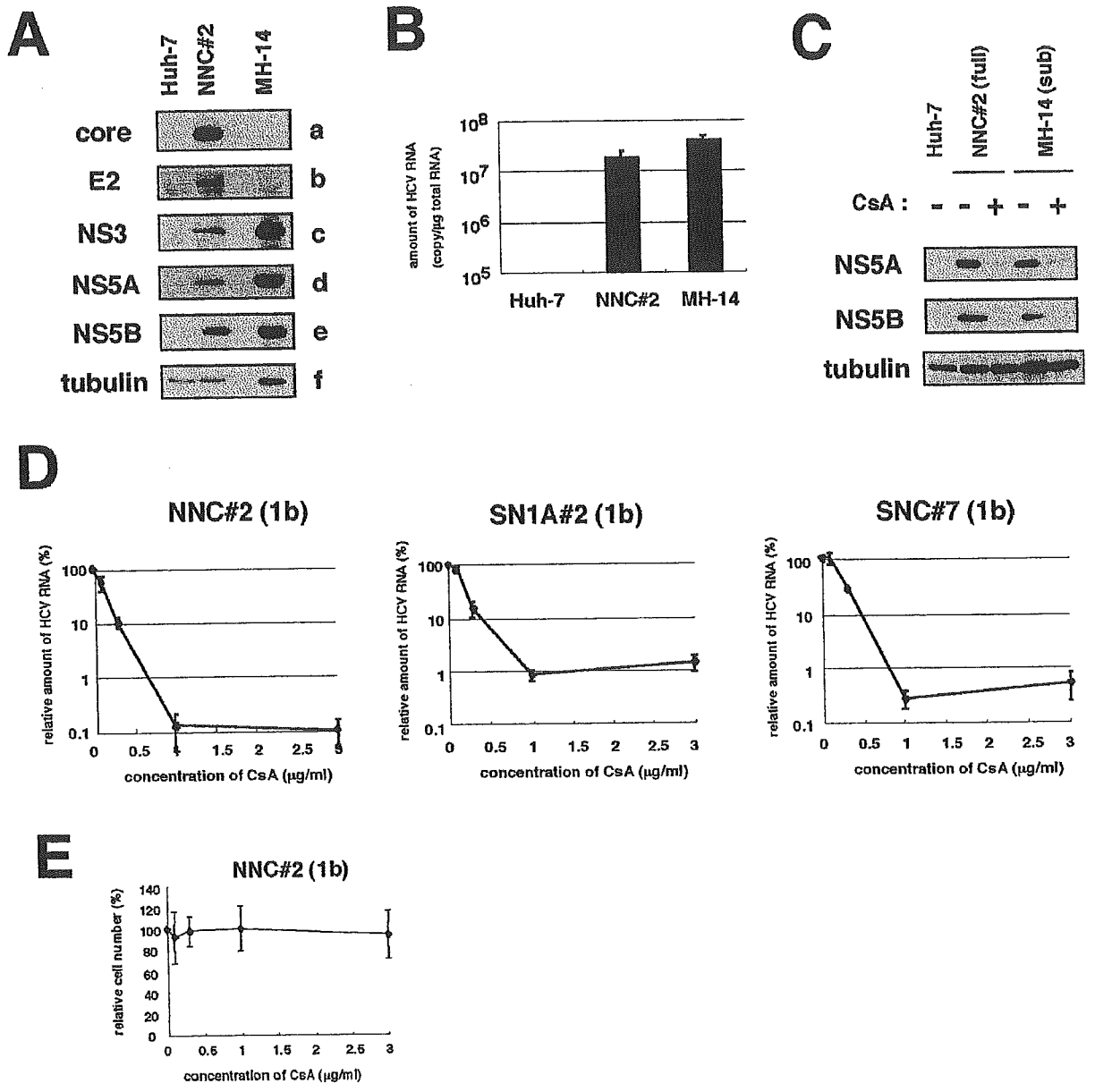

A
Huh-7
NNC#2
MH-14
core
E2
NS3
NS5A
NS5B
tubulin
a
b
c
d
e
f
B
amount of HCV RNA (copy/μg total RNA)
Huh-7
NNC#2
MH-14
C
Huh-7
NNC#2 (full)
MH-14 (sub)
CsA :
NS5A
NS5B
tubulin
D
NNC#2 (1b)
SN1A#2 (1b)
SNC#7 (1b)
relative amount of HCV RNA (%)
concentration of CsA (μg/ml)
E
NNC#2 (1b)
relative cell number (%)
concentration of CsA (μg/ml)

Fig. 2

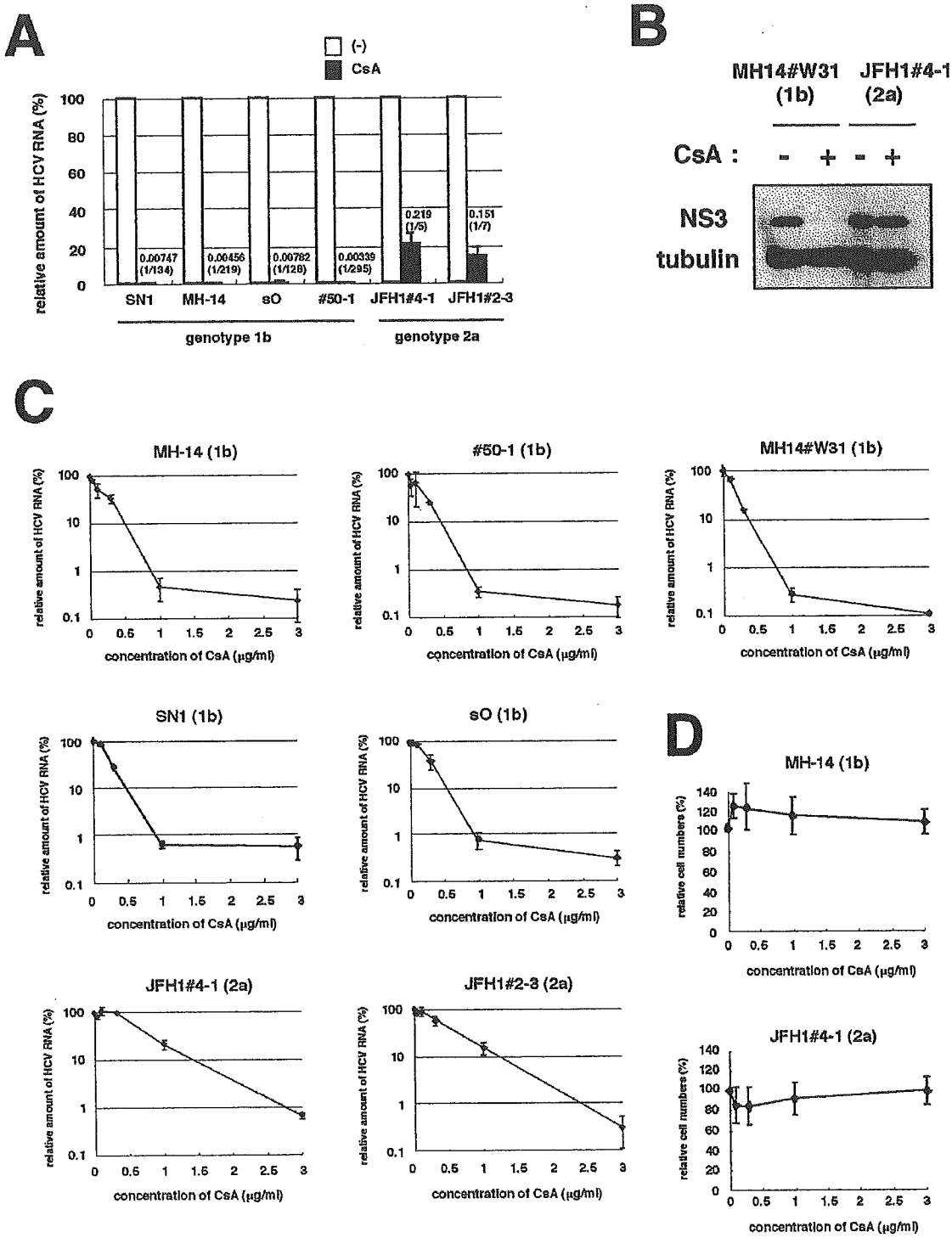

A
(-)
CsA
relative amount of HCV RNA (%)
100
80
60
40
20
0
0.00747 (1/134)
0.00456 (1/219)
0.00782 (1/128)
0.00339 (1/295)
0.219 (1/5)
0.151 (1/7)
SN1
MH-14
sO
#50-1
JFH1#4-1
JFH1#2-3
genotype 1b
genotype 2a
B
MH14#W31 (1b)
JFH1#4-1 (2a)
CsA : - + - +
NS3
tubulin
C
MH-14 (1b)
#50-1 (1b)
MH14#W31 (1b)
SN1 (1b)
sO (1b)
JFH1#4-1 (2a)
JFH1#2-3 (2a)
relative amount of HCV RNA (%)
concentration of CsA (μg/ml)
D
MH-14 (1b)
JFH1#4-1 (2a)
relative cell numbers (%)
concentration of CsA (μg/ml)

Fig. 3

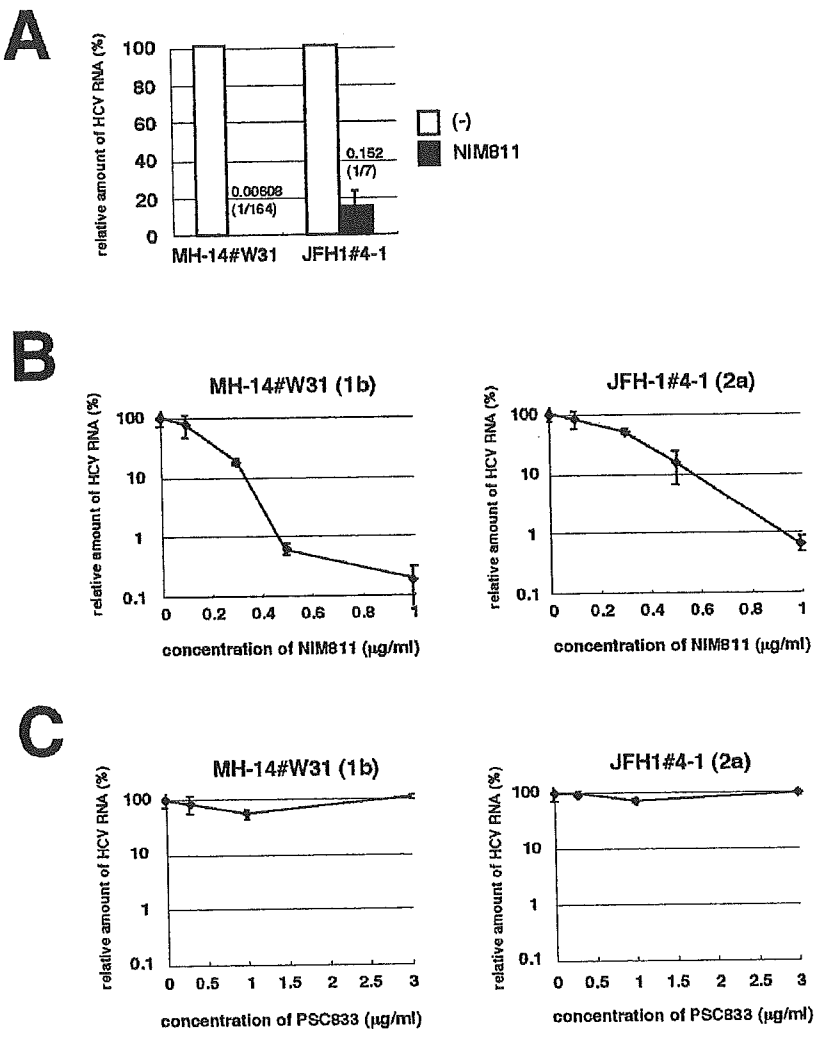

A
relative amount of HCV RNA (%)
100
80
60
40
20
0
0.00608
(1/164)
0.152
(1/7)
(-)
NIM811
MH-14#W31
JFH1#4-1
B
MH-14#W31 (1b)
JFH-1#4-1 (2a)
relative amount of HCV RNA (%)
100
10
1
0.1
0
0.2
0.4
0.6
0.8
1
concentration of NIM811 (μg/ml)
C
MH-14#W31 (1b)
JFH1#4-1 (2a)
relative amount of HCV RNA (%)
100
10
1
0.1
0
0.5
1
1.5
2
2.5
3
concentration of PSC833 (μg/ml)

Fig. 4

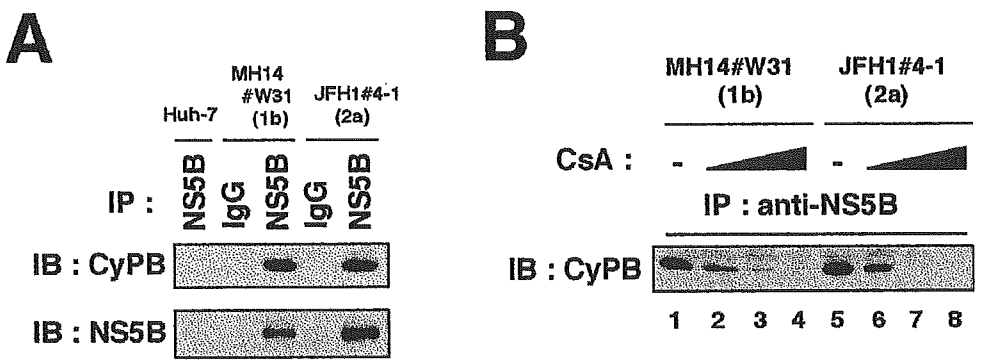

A
Huh-7
MH14 #W31 (1b)
JFH1#4-1 (2a)
IP :
NS5B
IgG
NS5B
IgG
NS5B
IB : CyPB
IB : NS5B
B
MH14#W31 (1b)
JFH1#4-1 (2a)
CsA :
-
-
IP : anti-NS5B
IB : CyPB
1 2 3 4 5 6 7 8

Fig. 5

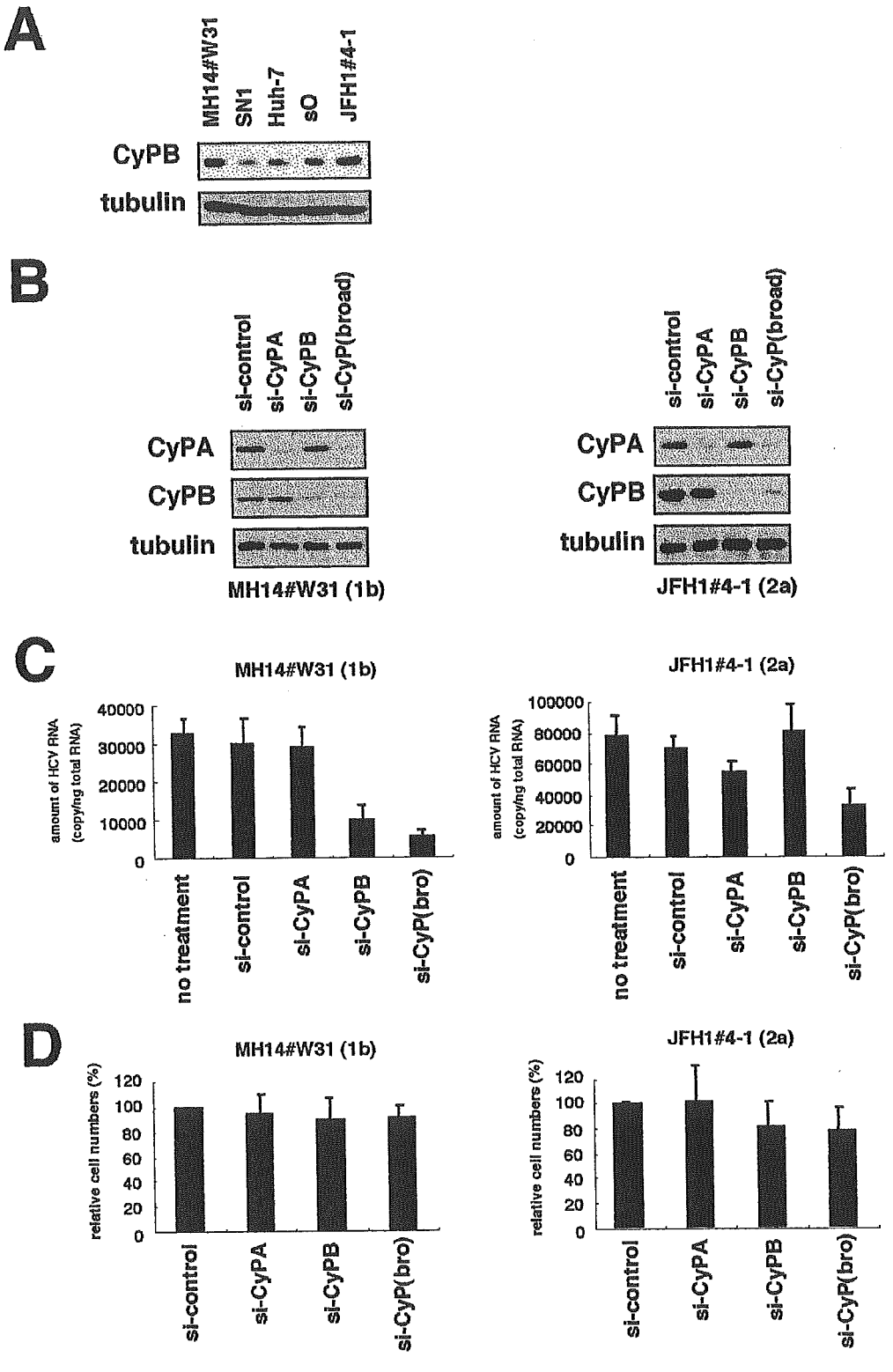

A
MH14#W31
SN1
Huh-7
sO
JFH1#4-1
CyPB
tubulin
B
si-control
si-CyPA
si-CyPB
si-CyP(broad)
CyPA
CyPB
tubulin
MH14#W31 (1b)
JFH1#4-1 (2a)
C
MH14#W31 (1b)
JFH1#4-1 (2a)
amount of HCV RNA (copy/ng total RNA)
40000
30000
20000
10000
0
100000
80000
60000
40000
20000
0
no treatment
si-control
si-CyPA
si-CyPB
si-CyP(bro)
D
MH14#W31 (1b)
JFH1#4-1 (2a)
relative cell numbers (%)
120
100
80
60
40
20
0
si-control
si-CyPA
si-CyPB
si-CyP(bro)

Fig. 6

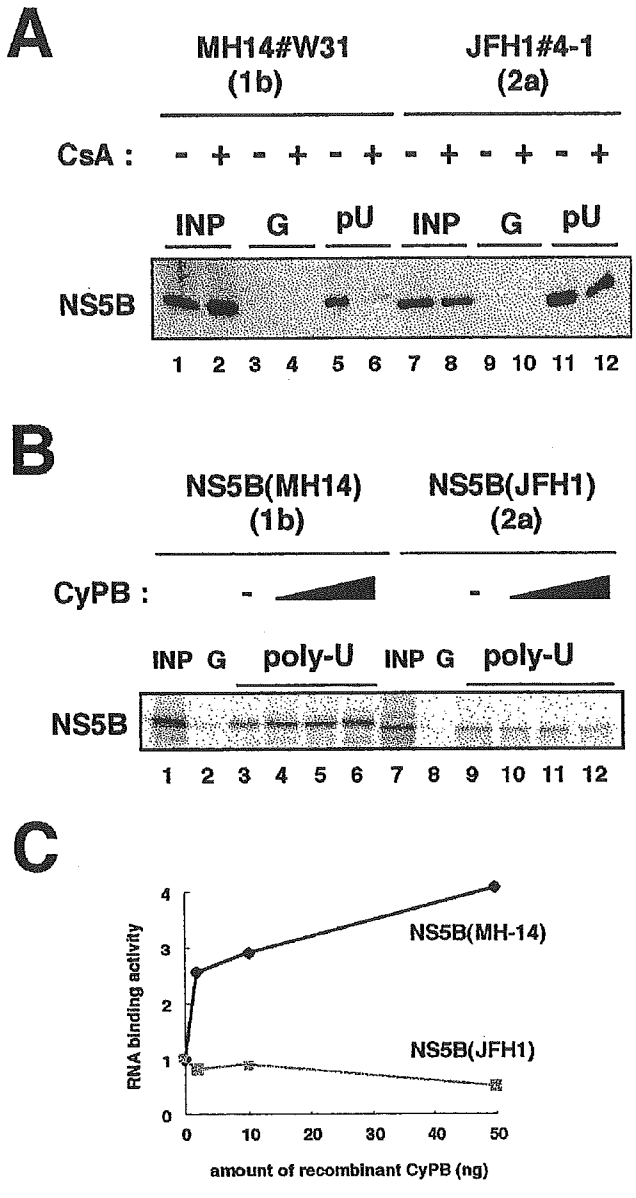

A
MH14#W31
(1b)
JFH1#4-1
(2a)
CsA : - + - + - + - + - + - +
INP G pU INP G pU
NS5B
1 2 3 4 5 6 7 8 9 10 11 12
B
NS5B(MH14)
(1b)
NS5B(JFH1)
(2a)
CyPB : -
INP G poly-U INP G poly-U
NS5B
1 2 3 4 5 6 7 8 9 10 11 12
C
RNA binding activity
NS5B(MH-14)
NS5B(JFH1)
0 1 2 3 4
0 10 20 30 40 50
amount of recombinant CyPB (ng)

Fig. 7

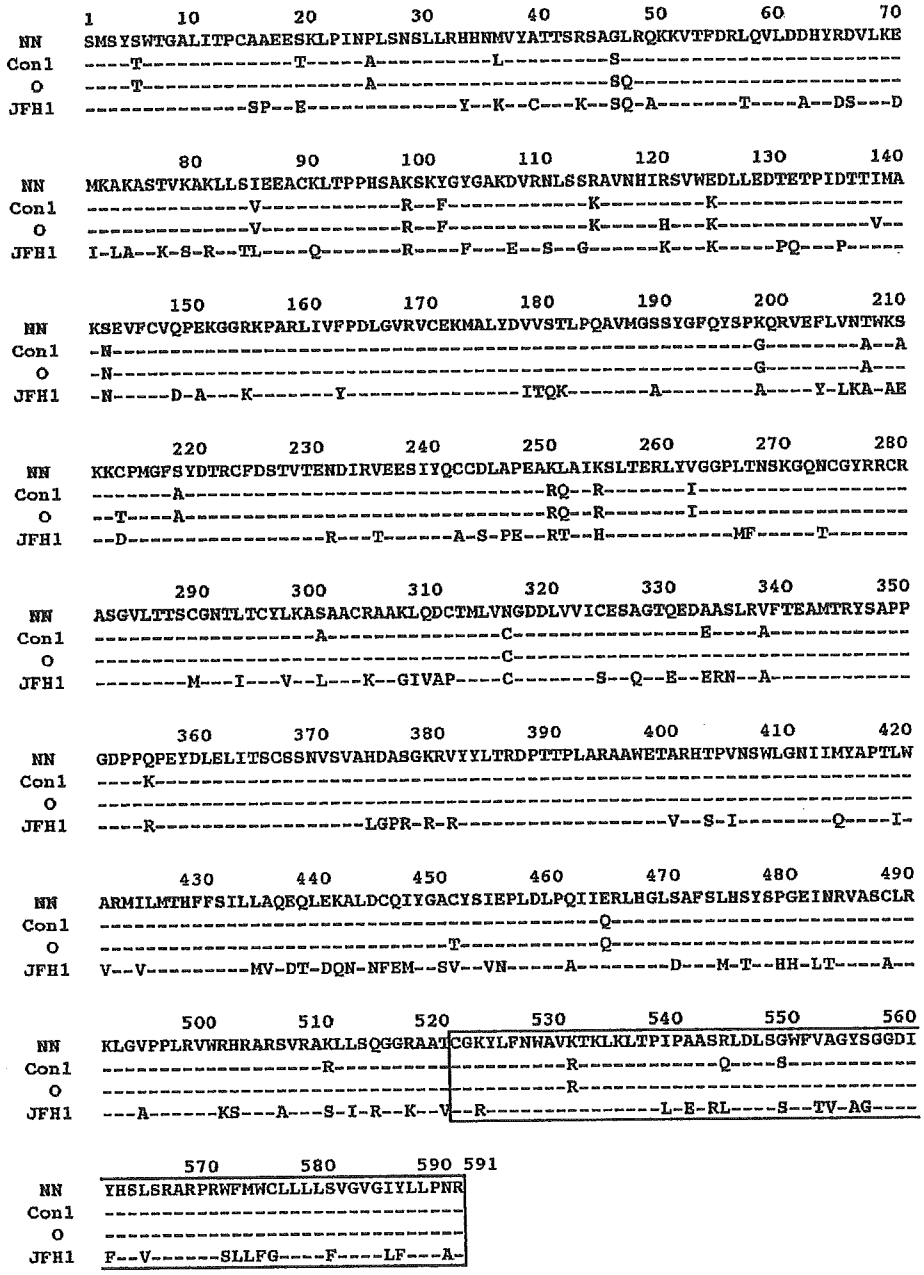

1 10 20 30 40 50 60 70
NN SMSYSWTGALITPCAAEESKLPINPLSNSLLRHHNMVYATTSRSAGLRQKKVTFDRLQVLDDHYRDVLKE
Con1 ----T----------T-----A----------L---------S----------
O ----T----------A----------SQ----------
JFH1 ----------SP--E----------Y--K--C---K--SQ-A-------T----A--DS---D
80 90 100 110 120 130 140
NN MKAKASTVKAKLLSIEEACKLTPPHSAKSKYGYGAKDVRNLSSRAVNHIRSVWEDLLEDTETPIDTTIMA
Con1 ----------V----------R--F----------K---------K----------
O ----------V----------R--F----------K-----H---K----------V--
JFH1 I-LA--K-S-R--TL----Q-------R----F---E--S--G------K---K-----PQ---P-----
150 160 170 180 190 200 210
NN KSEVFCVQPEKGGRKPARLIVFPDLGVRVCEKMALYDVVSTLPQAVMGSSYGFQYSPKQRVEFLVNTWKS
Con1 -N----------G---------A--A
O -N----------G---------A---
JFH1 -N-----D-A---K-------Y----------ITQK-------A--------A-----Y-LKA-AE
220 230 240 250 260 270 280
NN KKCPMGFSYDTRCFDSTVTENDIRVEESIYQCCDLAPEAKLAIKSLTERLYVGGPLTNSKGQNCGYRRCR
Con1 -------A----------RQ--R--------I----------
O --T----A----------RQ--R--------I----------
JFH1 --D----------R---T-------A-S-PE--RT--H----------MF------T--------
290 300 310 320 330 340 350
NN ASGVLTTSCGNTLTCYLKASAACRAAKLQDCTMLVNGDDLVVICESAGTQEDAASLRVFTEAMTRYSAPP
Con1 ----------A----------C----------E----A----------
O ----------C----------
JFH1 --------M---I---V--L---K--GIVAP----C--------S--Q--E--ERN--A----------
360 370 380 390 400 410 420
NN GDPPQPEYDLELITSCSSNVSVAHDASGKRVYYLTRDPTTPLARAAWETARHTPVNSWLGNIIMYAPTLW
Con1 ----K----------
O ----------
JFH1 ----R----------LGPR-R-R----------V--S-I--------Q----I-
430 440 450 460 470 480 490
NN ARMILMTHFFSILLAQEQLEKALDCQIYGACYSIEPLDLPQIIERLHGLSAFSLHSYSPGEINRVASCLR
Con1 ----------Q----------
O ----------T----------Q----------
JFH1 V--V---------MV-DT-DQN-NFEM--SV--VN-----A--------D---M-T--HH-LT-----A--
500 510 520 530 540 550 560
NN KLGVPPLRVWRHRARSVRAKLLSQGGRAATCGKYLFNWAVKTKLKLTPIPAASRLDLSGWFVAGYSGGDI
Con1 ----------R----------R----------Q----S----------
O ----------R----------
JFH1 ---A------KS---A---S-I-R--K--V--R----------L-E-RL----S--TV-AG----
570 580 590 591
NN YHSLSRARPRWFMWCLLLLSVGVGIYLLPNR
Con1 ----------
O ----------
JFH1 F--V------SLLFG----F----LF---A-

Fig. 8

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Subcellular Localization of Hepatitis C Virus Structural Proteins in a Cell Culture System That Efficiently Replicates the Virus

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Due to the recent development of a cell culture model, hepatitis C virus (HCV) can be efficiently propagated in cell culture. This allowed us to reinvestigate the subcellular localization of HCV structural proteins in the context of an infectious cycle. In agreement with previous reports, confocal immunofluorescence analysis of the subcellular localization of HCV structural proteins indicated that, in infected cells, the glycoprotein heterodimer is retained in the endoplasmic reticulum. However, in contrast to other studies, the glycoprotein heterodimer did not accumulate in other intracellular compartments or at the plasma membrane. As previously reported, an association between the capsid protein and lipid droplets was also observed. In addition, a fraction of labeling was consistent with the capsid protein being localized in a membranous compartment that is associated with the lipid droplets. However, in contrast to previous reports, the capsid protein was not found in the nucleus or in association with mitochondria or other well-defined intracellular compartments. Surprisingly, no colocalization was observed between the glycoprotein heterodimer and the capsid protein in infected cells. Electron microscopy analyses allowed us to identify a membrane alteration similar to the previously reported "membranous web." However, no virus-like particles were found in this type of structure. In addition, dense elements compatible with the size and shape of a viral particle were seldom observed in infected cells. In conclusion, the cell culture system for HCV allowed us for the first time to characterize the subcellular localization of HCV structural proteins in the context an infectious cycle.

Hepatitis C virus (HCV) is a small enveloped virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family (27). Its genome encodes a single polyprotein precursor of ~3,010 amino acid residues, which is synthesized on endoplasmic reticulum (ER)-associated ribosomes. The polyprotein is cleaved co- and posttranslationally by cellular and viral proteases to yield at least 10 mature products. HCV genome encodes three structural proteins: a capsid protein (C) and two envelope glycoproteins (E1 and E2). These proteins are released from the N-terminal region of the polyprotein by signal peptidase cleavages (15). In addition, processing in the C-terminal region of the capsid protein by a signal peptide peptidase leads to the generation of a mature capsid protein (32).

In the absence of a robust cell culture model for HCV, the analyses of the subcellular localization of HCV proteins have been performed with heterologous expression systems or in the context of HCV replicons (reviewed in references 15 and 33). Transient expression of HCV envelope glycoproteins with heterologous expression systems has shown that HCV envelope glycoproteins E1 and E2 assemble as a noncovalent heterodimer (11). Due to the presence of retention signals in the transmembrane domains of HCV envelope glycoproteins (8, 9), the glycoprotein heterodimer is mainly retained in the ER (17). However, in some expression systems, a fraction of HCV envelope glycoproteins has also been found to be located in the intermediate compartment and the *cis*-Golgi apparatus (12, 29, 37) and at the plasma membrane (3, 13, 24).

When expressed with heterologous expression systems or in the context of HCV replicons, the subcellular distribution of the capsid protein seems to be complex. Most of the protein is cytoplasmic where it is found both attached to the ER and at the surface of lipid droplets (for a review, see reference 31). The different extents to which the capsid protein is attached either to lipid droplets or membranes may be dependent on the amount of lipid droplets present in various cell types (22). In some conditions, a minor proportion of the capsid protein has also been found to be located in the nucleus (43). More recently, the capsid protein has also been found to colocalize with mitochondrial markers in Huh-7 cells containing a full-length HCV replicon (39).

Very recently, a cell culture model has been developed for HCV (26, 42, 44). This system is based on the transfection of the human hepatoma cell line Huh-7 with genomic RNA derived from a cloned viral genome. This culture system allows the production of virus that can be efficiently propagated in cell culture. Although a large amount of data has been accumulated on most HCV proteins during the past 15 years, the development of a cell culture system for HCV allows reinvestigation of the biological and biochemical properties of HCV proteins in a more relevant context. Here, we analyzed the

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