The Efficiency of E2-p7 Processing Modulates the Production of Infectious Hepatitis C Virus

Running title: E2-p7 processing on HCV production

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Abstract

Previous studies indicate that the processing of HCV E2-p7-NS2 precursor mediated by host signal peptidase is relatively inefficient, resulting in the accumulation of E2-p7-NS2 and E2-p7 precursors in addition to E2 in mammalian cells. In this study, we discovered that a significant inhibition of the processing at an E2-p7 junction site is detrimental for HCV production, whether it was caused by the mutations in p7 or by the strategic introduction of a mutation at a terminal residue of E2 to block the signal peptidase-mediated cleavage of this junction site. However, complete separation of E2 and p7 by inserting an EMCV IRES between these two proteins also moderately inhibited virus production. These results indicate that optimal processing of the E2-p7 junction site is critical for efficient HCV production. We further demonstrated that disrupting E2-p7 processing inhibits both NS2 localization to the putative virus assembly sites near lipid droplets (LD) and NS2 interaction with NS3 and E2. However, the impact of the p7-NS2 processing efficiency on HCV production seems relatively minor, if at all. In conclusion, these results imply that effective release of E2 and p7 from its precursor E2-p7 promotes HCV production by enhancing NS2-associated virus assembly complex formation near LD.
Introduction

Hepatitis C virus (HCV) is one of the major agents responsible for causing severe liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1, 2). It is an enveloped, positive-stranded RNA virus belonging to the Hepacivirus genus in the Flaviviridae family of viruses (3, 4). HCV encodes a single, large open-reading frame encoding a ~3000 amino acid polyprotein flanked by 5'- and 3'-noncoding sequences. The internal ribosome entry site (IRES) located at the 5'-noncoding region mediates translation of a polypeptide that is subsequently processed by both host- and virus-encoded proteases to yield 10 different proteins. The N-terminal one-third of the polyprotein, encoding the structural proteins including core and envelope proteins (E1 and E2), followed by p7 and NS2, is processed by host signal peptidase (5-7). Core is further processed by signal peptide peptidase into a mature form (8). The junction site between NS2 and NS3 is cleaved by an NS2-NS3 autoprotease (9, 10), and those of the remaining nonstructural proteins (NS3-NS4A-NS4B-NS5A-NS5B) are processed by NS3 protease with its cofactor NS4A (11-13). NS3 to NS5B along with the 5'- and 3'-terminal noncoding region were shown to be sufficient for the autonomous replication of HCV RNA (14).

Recent advances in the infectious HCV cell culture model allowed the investigation of HCV virus particle assembly processes (15-18). We have learned that the association of HCV core with lipid droplets (LD) is critical for virus assembly, since disrupting the core localization to LD inhibited infectious virus production (19, 20). We also learned that the association of the C-terminal domain of NS5A with core on LD is important for virus production (19, 21-24). It was suggested that NS5A is responsible for recruiting a replication complex to the vicinity of LD, since the localization of other nonstructural proteins to a close proximity of LD depended on the localization of NSSA to LD (19). Several independent studies, including ours, showed that NS2 is involved in virus particle assembly by interacting with both the structural proteins E1 and E2.
and also the nonstructural proteins NS3 and NS5A (25-28). Interestingly, these NS2-mediated interactions are associated with co-localization of E1, E2, NS2, NS3 and NS5A at dot-like structures near LD, and the relative percentages of HCV-replicating cells exhibiting these dot-like structures positively correlated with the infectious virus titer (25, 26, 28). Thus, it is likely that these dot-like structures in the vicinity of LD represent virus particle assembly sites.

P7 is a small hydrophobic protein with two transmembrane domains connected with short stretch of basic residues in the cytoplasm (29, 30). Previous studies showed that p7 forms a cation-selective ion channel as a hexameric- or heptameric-complex (31, 32). The ion channeling function of p7 is important for infectious HCV production, since the mutations that inhibited the ion channel function of p7 also inhibited infectious virus production. Furthermore, the ectopic expression of influenza virus M2 ion channel protein or treatment with Bafilomycin partially rescued the virus production defect caused by some p7 mutations (33, 34). However, Brohm and colleagues showed that p7 or its precursors, but not M2, was capable of rescuing the virus production defect of the Δp7-half deletion mutant (35). These results suggest that p7 is involved in HCV production in both ion channel-dependent and -independent manners (34-36).

Several previous studies demonstrated that the processing by signal peptidase between the junction sites of E2 and p7, and also p7 and NS2, is relatively inefficient, as evidenced by the detection of E2-p7 and E2-p7-NS2 precursors, despite the fact that the signal peptides of p7 and NS2 can function efficiently when they are fused to reporters (6, 37, 38). Subsequently, Carrère-Kremer and colleagues demonstrated that the incomplete cleavage at these junction sites is due to the structural determinants at the p7 junctions (37). The influence of the p7 sequence on the processing of p7 precursors was additionally supported by recent studies, in which investigators showed that some mutations introduced to p7 affected the processing at these two junction sites (33-35). The negative impact of p7 mutations on the processing of the
p7 precursors makes it difficult to interpret the p7 mutation-mediated viral phenotypes, since it is unclear whether the effects of some p7 mutations on HCV replication are due to a functional defect of p7 or an aberrant processing of p7 precursors. Importantly, the significance of the delayed processing of p7 junction sites on HCV replication is currently unknown.

In this report we describe results suggesting that the optimal processing of the E2-p7 junction site is critical for efficient virus production.

**Materials and Methods**

**Cells.** Clonal derivatives of Huh-7, Huh-7.5 (39) and FT3-7 (40) were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ environment.

**Plasmids.** HJ3-5, HJ3-5/p7(KRAA), HJ3-5/p7HA/IRES, HJ3-5/p7HA/IRES/p7(KRAA) and HJ3-5/p7/IRES were described previously (25, 41). The HA epitope tag (YPYDVPDYA) followed by triple Glycine was fused in-frame to the first residue of p7 within pHJ3-5, HJ3-5/p7(KRAA) and HJ3-5/p7/IRES by using overlapping primers and the QuikChange® II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) to make HJ3-5/HAp7, HJ3-5/HAp7(KRAA) and HJ3-5/HAp7/IRES. The mutation at the last residue of E2 A384R was introduced by using the QuikChange® II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). EMCV IRES was introduced to HJ3-5 to make HJ3-5/E2/IRES and HJ3-5/E2/IRES/p7(KRAA) and HJ3-5/E2/IRES/p7(IRES). The following steps. First, we introduced a unique restriction site *PmeI* between E2 and the p7 region of HJ3-5. Second, overlapping PCR was carried out to generate an EMCV IRES-NS2 fragment with an N-terminal *PmeI* site. Third, this fragment was digested with *PmeI* and *BglII* (located within NS2) restriction enzymes and ligated to *PmeI/BglII* fragments derived from pHJ3-5 with *PmeI* site indicated above to make HJ3-5/E2/IRES. P7(KRAA) mutations were introduced.
to HJ3-5/E2/IRES to make HJ3-5/E2/IRES/p7(KRAA) by the PCR-based mutagenesis methods described above. The sequence of the regions manipulated within each plasmid was verified by DNA sequencing.

**In vitro HCV RNA synthesis and transfection.** HCV cDNA (1µg) was linearized with XbaI (NEB, Hitchin, UK), followed by transcription to RNA by using a T7 Megascript kit (Ambion, Austin, TX). DNA-free RNA was purified with an RNeasy RNA isolation kit (Qiagen, Valencia, CA). RNA integrity and concentration were determined by agarose gel electrophoresis and absorbance at 260nm, respectively. *In vitro*-transcribed HCV RNA was transfected by electroporation into FT3-7 cells. In brief, 5 x 10⁶ cells were mixed with 10 µg of *in vitro*-transcribed HCV RNA in a 4-mm cuvette and pulsed once at 270V and 950 µF in a Gene Pulser System (Biorad, Hercules, CA). Electroporated cells were seeded into 12-well plates for HCV RNA analysis and 6-well plates for virus titration and HCV protein analysis.

**HCV infectivity assays.** For virus titration, serial 10-fold dilutions of clarified cell culture supernatant or freeze-thawed cell lysates in 100-µl aliquots were inoculated onto naive Huh-7.5 cells seeded at 1 x 10⁵ cells/well in 48-well plates. Cells were incubated at 37°C in a 5% CO₂ environment and fed with 200 µl medium 24 hr later. At 3 days post-inoculation, the cells were fixed with methanol : Acetone (1:1) for 10 minutes at room temperature, and then immunostained for core expression by using monoclonal antibody C7-50 (Thermo Scientific, Rockford, IL, 1:600 dilution), followed by AlexaFluorR 488-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA, 1:1000 dilution). Infectivity was determined by counting the clusters of infected cells staining for core, which were considered to be single, infectious focus-forming unit (FFU).

**Western blot analysis.** At day 2 post-electroporation, cells were lysed with ice-cold lysis buffer [PBS supplemented with 1% CHAPS and complete protease inhibitor cocktail (Roche, 6]
Indianapolis, IN)]. Aliquots of cell lysates containing 20 µg of proteins were separated by performing SDS-PAGE and transferred onto PVDF membranes. Membranes were probed with monoclonal antibodies to core (C7-50, Thermo Scientific, Rockford, IL, 1:2000 dilution), polyclonal rabbit anti-NS2 antibody (25) (1:10,000 dilution), NS3 (9-G2, Virogen, Watertown, MA, 1:1000 dilution), polyclonal Goat anti-E2 antibody (Virostat Inc, Portland, ME, 1:2000 dilution) and monoclonal anti-HA antibody (Sigma, St. Louis, MO, 1:1000 dilution). Proteins were visualized by subsequently probing the membranes with IRdye 800CW goat anti-mouse, IRdye 680 goat anti-rabbit, and IRdye 680 donkey anti-goat secondary antibodies (Li-COR Biosciences, Lincoln, NE), followed by imaging with an Odyssey infrared imaging system (Li-COR Biosciences, Lincoln, NE).

Co-Immunoprecipitation. Cell lysates were incubated with anti-NS2 antibody overnight at 4°C, followed by incubation for an additional 30 min on ice after adding Protein G MicroBeads (Miltenyi Biotech, Auburn, CA). These solutions were applied to µ columns that had been set up in the magnetic field of the µMACS separator (Miltenyi Biotech, Auburn, CA). The columns were washed four times by using wash buffer I [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0)], followed by one wash with buffer 2 (20 mM Tris-HCl, pH 7.5). Immune complexes were eluted by applying a pre-heated elution solution supplied by the manufacturer (Miltenyi Biotech, Auburn, CA).

Quantitative real-time RT-PCR. Viral RNA was detected by a quantitative TaqMan RT-PCR assay (25). Total RNA was isolated from cell lysates by using an RNeasy kit (Qiagen, Valencia, CA) in accordance with the manufacturer’s instructions. Quantitative real-time TaqMan RT-PCR analysis was carried out in a Bio-Rad iQ5 Real-time PCR Detection System by using primer pairs and a probe targeting a conserved 221-base sequence within the 5’non-translated RNA segment of the genome: HCV84FP, GCCATGGCGTTAGTATGAGTGT, HCV JFH_303RP,
CGCCCTATCA GGCAGTACCACAA and HCV146BHQ, FAM-
TCTGCGGAACCGGTGAGTACACC-DBH1. Reactions were incubated at 50º C for 2 min, 60º C
45 min, 95º C 2 min, followed by 40 cycles of 95º C for 20 sec and 60º C for 1 min.

Deglycosylation with Endoglycosidase H. Endoglycosidase-H (Endo-H, NEB, Hitchin, UK)
treatment was performed according to the manufacturer’s recommendations. Briefly, 20 µg of
protein was added to 4x denaturing SDS sample buffer and heated at 100º C for 10 min.
Subsequently, 2,000 units of Endo-H (2 µl) and 2 µl of 50 mM sodium citrate (pH5.5) were
added to samples in a final volume of 20 µl, and then these reaction mixtures were incubated
for 90 min at 37º C, followed by inactivation of Endo-H by at 75º C for 10 min.

Confocal microscopy. Electroporated cells were plated on 8-well chamber slides (BD
Biosciences, Bedford, MA) at a density of 1 x 10⁴ cells per well. Two days later, slides were
washed with PBS, fixed with 4% formaldehyde for 20 min at room temperature, and
permeabilized with 0.2% Triton X-100 in PBS for 10 min. Fixed and permeabilized cells were
incubated with Huh-7 cell lysate cleared NS2 antibody (1:1000 dilution) for 2 hr at room
temperature, followed by incubation with Alexa-488-conjugated, goat anti-rabbit antibody
(Invitrogen, Carlsbad, CA, 1:1000). Lipid droplets were stained with HCS LipidTOX™-deep red
neutral lipid stain (1:1000) (Molecular Probes Inc, Eugene, OR) for 30 minutes at room
temperature. Nuclei were labeled with Hoechst stain (Anaspec Inc, Fremont, CA). Slides were
examined with an Olympus Confocal Laser Scanning Biological Microscope FV1000-Fluoview.

Statistical analyses. Student’s t-test (unpaired, with Welch’s correction) was performed by
using GraphPad Prism 6 software (GraphPad, La Jolla, CA) to determine the significance in
differences between two paired values.
HA-epitope tag introduced to the N-terminus of p7 rescues the virus production defect of a p7 mutant. In a previous study, we introduced either a yellow fluorescence protein (YFP) or an HA-epitope tag in-frame to the C-terminus of p7 in an effort to detect p7, since p7 antibody was not readily available (25). However, while YFP tagging of p7 allowed the detection of YFP-fused p7, this modification prevented the production of infectious HCV (25). On the other hand, while the introduction of the HA epitope tag to the C-terminus of p7 (previously described as HJ3-5/p7HA/IRES) only slightly reduced virus production, we failed to detect HA-tagged p7, other than E2-p7HA precursor (25). In this study, we introduced an HA-epitope tag at the N-terminus of p7 followed by a three amino-acid linker sequence (gly-gly-gly) to genotype 1a/2a chimeric HCV HJ3-5 (HJ3-5/HAp7) (Fig. 1A). This modification not only allowed the detection of HA-tagged p7 (HAp7) (Fig. 1C, lane 3), but also permitted the production of infectious virus, although at a moderately reduced level (~1 log) compared to that from HJ3-5 at day 2 post-electroporation of this RNA (Fig. 1B, compare lanes 1 and 3). These results confirmed the recent report by Vieyres and colleagues who showed that introducing an N-terminal double HA-tag to the p7 of gt2a/2a chimera Jc1 allowed the production of virus, albeit at a reduced level compared to that from Jc1 (42). Since p7 in HJ3-5 is derived from gt1a, these results also indicate that p7 with an N-terminal epitope tag was functional in virus production in both a gt1a and gt2a genetic background.

We reported that double mutations located at the cytoplasmic loop region of p7 (K33A+R35A, a.k.a. “KRAA”) disrupted the ion channel activity of p7 and completely blocked infectious HJ3-5 virus production (Fig. 1B, lane 2) (25, 34). Corresponding p7 mutations were also lethal for gt2a JFH1 and gt1b/2a chimera Con1/C3 virus production (33). Also, these mutations severely decreased the virus production from gt2a/2a chimeras, including J6 and
J6/JFH1 (33, 43). Thus we were surprised to find that HJ3-5/HA\_p7 with KRAA mutations \{HJ3-5/HA\_p7 (KRAA)\} is capable of infectious virus production at a significant level (Fig. 1B, lane 4).

These results suggested that the N-terminal HA tag in p7 compensated for the virus assembly defect caused by KRAA mutations within p7. Thus we further investigated the mechanisms of this unexpected compensation by the N-terminal HA tag at p7 for the virus assembly defect caused by p7 mutations.

\textbf{HA\_p7-NS2 precursor is not responsible for the promotion of virus production from a p7 mutant.} One of our first findings upon introducing the N-terminal HA epitope tag to p7 in HJ3-5 to make HJ3-5/HA\_p7 was the marked appearance of an HA\_p7-NS2 precursor detectable by both anti-NS2 and anti-HA antibodies (Fig. 1C, lane 3). We also detected the corresponding HA\_p7-NS2 precursor from HJ3-5/HA\_p7(KRAA), although in reduced quantity compared to that from HJ3-5/HA\_p7 (Fig. 1C, compare lanes 3 and 4). This could be due to a lower stability of p7 caused by p7(KRAA) mutations, since the level of HA\_p7(KRAA) from HJ3-5/HA\_p7(KRAA) was significantly lower than that of HA\_p7 from HJ3-5/HA\_p7 (Fig. 1C, compare lanes 3 and 4). It is also possible that the processing of the HA\_p7(KRAA)-NS2 precursor is more efficient than that of the wt counterpart, resulting in a lesser accumulation of this precursor from the KRAA mutant (Fig. 1C, compare lanes 3 and 4). The third possibility is the preferential accumulation of the E2-\textsuperscript{HA\_p7(KRAA)-NS2} precursor from the KRAA mutant, as described below, which would consequently reduce the level of HA\_p7(KRAA)-NS2 precursor (Fig. 2A, see arrowhead in lane 4).

To investigate whether the increased level of HA\_p7(KRAA)-NS2 precursor could have accounted for the significant level of virus production observed from the HJ3-5/HA\_p7(KRAA) mutant, we inserted the stop codon followed by EMCV (encephalomyocarditis virus) IRES (internal ribosome entry site) at the junction site between p7 and NS2 to eliminate this precursor formation (Fig. 1A). This modification effectively eliminated the protein band detectable by both
anti-NS2 and anti-HA antibodies, as expected, thus confirming that this protein band was indeed the p7-NS2 precursor (Fig. 1C, compare lanes 3 and 4 with 5 and 6).

The RNA replication levels of the bi-cistronic genomes [HJ3-5/HA/p7/IRES and HJ3-5/HA/p7(KRAA)/IRES] were comparable to those of their monocistronic counterparts [HJ3-5/HA/p7 and HJ3-5/HA/p7(KRAA)] (Fig. 4A, compare lanes 6 and 8, and 7 and 9). However, both the intracellular- and extracellular-virus titers were moderately decreased from HJ3-5/HA/p7/IRES compared to those from HJ3-5/HA/p7 by about one log (Fig. 1B, compare lanes 3 and 5). Also, the virus titer from HJ3-5/HA/p7(KRAA)/IRES was increased, rather than decreased, compared to that from HJ3-5/HA/p7(KRAA) (Fig. 1B, compare lanes 4 and 6). Based on these results, it is unclear whether the delayed processing of p7-NS2 leading to the accumulation of p7-NS2 may play a role during HCV replication or not (see discussion). Importantly, these results suggested that aberrant processing between p7 and NS2 caused by the N-terminal HA-tagging of p7, which led to the accumulation of the p7-NS2 precursor, is not responsible for the restoration of the virus production defect caused by p7 KRAA mutations.

**HA epitope at the N-terminal region of p7 restores the E2-p7 processing defect caused by the p7 KRAA mutations.** During our previous study, we detected significantly higher levels of E2-p7 precursor from HJ3-5/p7(KRAA) compared to that from HJ3-5 (25). It was described previously by Carrère-Kremer and colleagues that modifying the sequence within the N-terminal region of p7, including the insertion of an HA-epitope sequence, enhanced the processing between E2 and p7 when they were expressed by using a vaccinia virus expression system (37). The location of HA-epitope insertion was slightly different between previous and our current studies, being between the P3’ and P4’ positions within p7 and at the N-terminus of p7, respectively. However, we considered the possibility that E2-p7 processing might have been enhanced in our case as well following HA insertion to p7 N-terminus. To investigate this, we
electroporated HCV RNAs to Huh-7 cells and detected E2 and E2-p7 proteins by performing western blot analysis 48 hr later. To better distinguish the protein bands corresponding to the E2-p7 precursors (E2-p7, E2-^{HA}p7 or E2-p7^{HA}) and E2, we also analyzed the cell lysates following treatment with Endo H (Endoglycosidase H) to remove the glycans from the E2 (Fig. 2A, bottom panel). The protein band intensities corresponding to the E2-p7 precursors and E2 from three-to-five independent experiments were quantified by using an Odyssey Infrared Imaging System (see Materials and Methods for detail), and the mean percentages (%) of E2 in E2-p7 precursor forms from such analyses along with standard deviations are shown in Fig. 2B.

First, we confirmed our previous finding that p7(KRAA) mutations in HJ3-5 significantly inhibit processing between E2 and p7 compared to the wt (25), as shown in the western blot analysis of the cell lysates from HJ3-5 and HJ3-5/p7(KRAA) by using an anti-E2 antibody (Fig. 2A and B, compare lanes 1 and 2). We detected ~60% of total E2 from p7(KRAA) mutant in the E2-p7 precursor form compared to ~15% of that from HJ3-5 (Fig. 2B, lanes 2 and 1). Second, the HA tag at the C-terminus of p7 did not significantly affect the E2-p7 processing in either HJ3-5/p7^{HA}/IRES or HJ3-5/p7^{HA}(KRAA)/IRES compared to that in HJ3-5 or HJ3-5/p7(KRAA) (Fig. 2A and B, compare lanes 1 and 7, and 2 and 8). Third, these analyses clearly indicate that the N-terminal insertion of an HA tag to p7 significantly enhanced the processing of E2-p7 derived from both HJ3-5^{HA}p7 and HJ3-5^{HA}p7(KRAA), decreasing the detectable level of E2-^{HA}p7 precursor from HJ3-5^{HA}p7 to ~4% and that from HJ3-5^{HA}p7(KRAA) to ~9%, compared to that from HJ3-5 and HJ3-5/p7(KRAA) (Fig. 2B, see lanes 3 and 4, and compare them to lanes 1 and 2). Thus the E2-p7 processing defect caused by the p7(KRAA) mutations was corrected by the HA tag at the N-terminus of p7.

However, although the processing of the E2-p7 junction site from HJ3-5^{HA}p7(KRAA), as judged by the relative level of this precursor, was more augmented than that from parental HJ3-
5 (~9% vs. ~15%) (Fig. 2B, compare lanes 4 and lane 1), it was still relatively low in this mutant compared to that in HJ3-5/HAp7 (~9% vs. ~4%) (Fig. 2B, compare lanes 4 and lane 3). These data may indicate that p7(KRAA) mutations still exert a negative influence on E2-p7 processing, even when the processing of this junction site was augmented by the HA tag at the p7 N-terminus. We also detected an E2-HAp7-NS2 precursor that was detectable by anti-E2, anti-HA and anti-NS2 antibodies from an HJ3-5/HAp7(KRAA) mutant (Fig. 2A, arrowhead in lane 4, data not shown) that is likely generated, in part, due to the relatively inefficient processing of E2-p7 and p7-NS2 junction sites in this mutant, as described above (Figs. 1C and 2A, lane 4). This E2-HAp7-NS2 precursor was hardly detectable from HJ3-5/HAp7, probably due to the efficient processing between E2 and p7 in this HCV clone (Fig. 2A, lane 3). Interestingly, we observed a small, but significant, increase in the level of E2-HAp7 precursor from HJ3-5/HAp7/IRES compared to that from HJ3-5/HAp7 (Fig. 2B, compare lanes 5 and lane 3). This result suggests the possibility that efficient processing of the p7-NS2 junction site may have a negative impact on E2-p7 processing. However, we did not observe any significant difference in the relative amounts of this precursor between HJ3-5/HAp7(KRAA)/IRES and HJ3-5/HAp7(KRAA) (Fig. 2B, compare lanes 6 and 4), probably due to the inherent adverse effect of KRAA mutations on E2-p7 cleavage.

Our attempt to directly measure the E2-p7 processing kinetics by using a pulse-chase experiment did not yield conclusive data due to the difficulty in separating the E2-p7 and E2 from each other and/or from host proteins that co-separated with them (data not shown). Therefore we could not completely eliminate the possibility that differences in the relative abundance of E2 in unprocessed E2-p7 precursor forms that we detected in this study (Fig. 2B) may have been derived from the relative difference in E2-p7 and/or E2 stabilities, rather than from the difference in protein processing efficiency. However, the former possibility is low, based on the following information. First, the average level of total E2 (E2 plus E2-p7) from the
different HCV derivatives used in this study was similar between them (Fig. 2, data not shown). Second, most of the HCV constructs encode exactly the same E2 sequence.

Optimal processing of E2-p7 precursor plays a critical role in HCV production. To formally demonstrate that enhanced processing of E2-p7 junction in the HJ3-5/KRAA is responsible for the production of infectious virus from this mutant and also to determine the importance of E2-p7 processing on HCV production, we performed the following experiments. First, we introduced a termination codon, followed by EMCV IRES, at the junction site of E2 and p7 to separate these two proteins independent of the signal peptidase-mediated cleavage (Fig. 3A). This modification caused a more than 1 log reduction in virus production, for both intracellular- and extracellular-virus, from the resulting HJ3-5/E2/IRES compared to that from HJ3-5 (Figs. 3B, compare lanes 1 and 5), although viral RNA replication was not affected by this change (Fig. 4A, compare lanes 1 and 4). However, the same modification led to infectious virus production from the resulting HJ3-5/E2/IRES/p7(KRAA), despite the fact that this clone lacked an HA tag at the N-terminus of p7 (Fig. 3B, lane 6). These results imply that the HA tag at the N-terminus of p7 per se was not responsible for causing the HJ3-5/KRAA to produce infectious virus. Rather, these results support the notion that improved processing between E2 and p7 caused by the HA insertion at the N-terminus of p7 in HJ3-5/KRAA led to the production of infectious virus from this mutant. To further understand the importance of E2-p7 processing on virus production, we introduced a mutation at the last residue of E2 (A384R) in HJ3-5 [HJ3-5/E2(AR)] to block the signal peptidase-mediated cleavage of the E2-p7 junction site (44). As shown in Fig. 3C, lane 3, the processing of E2-p7 precursor was severely impaired in HJ3-5/E2(AR), and nearly ~86% of E2 was detected as an E2-p7 precursor form (Fig. 2B, lane 9). Importantly, similar to the case of HJ3-5/KRAA, this mutation completely blocked virus production (Fig. 3B, lane 3), despite the fact that the p7 sequence in this mutant is wt.
To verify that the defect in E2-p7 processing, and not an AR mutation-mediated functional defect in E2, was responsible for the defective virus production from HJ3-5/E2(AR), we also generated HJ3-5/E2(AR)/IRES, in which we introduced a stop codon followed by an IRES sequence between E2 and p7 of HJ3-5/E2(AR) to separate these two proteins. As expected, E2(AR)-p7 precursor was no longer detected in cells replicating HJ3-5/E2(AR)/IRES (Fig. 3C, lane 4). Importantly, we detected virus production from HJ3-5/E2(AR)/IRES at a level similar to that from HJ3-5/E2/IRES, despite the fact that the former encoded E2(AR) and the latter encoded wt E2 (Fig. 3B, lanes 4 and 5). These results indicate that the AR mutation in E2 did not affect E2 functions involved in virus production.

In aggregate, these results indicate that complete separation of E2-p7, as in the case of HJ3-5/E2/IRES, and significantly reduced processing of this site, as in the case of HJ3-5/E2(AR) and HJ3-5/p7(KRAA), are both detrimental for HCV production. These data support the notion that optimal processing of E2-p7 is critical for efficient production of infectious HCV. It is also worth mentioning that the defective processing of E2-p7 in HJ3-5/p7HA(KRAA)/IRES, as shown in Fig. 2 (lane 8), due to the lack of an apparent positive effect from C-terminal HA tag at p7 on E2-p7 processing, correlates nicely with the virus production null phenotype of this mutant that we demonstrated before (25).

P7(KRAA) mutations delay the kinetics of virus assembly independent of its effect on viral protein processing. We observed a delay in virus production kinetics from p7(KRAA) mutants, including HJ3-5/E2/IRES/p7(KRAA), HJ3-5/HA-p7(KRAA) and HJ3-5/HA-p7(KRAA)/IRES, compared to their p7 wt counterparts (Fig. 4B, compare lanes 4 and 5, 6 and 7, and 8 and 9). It is unlikely that the difference in RNA replication efficiency difference between wt and p7(KRAA) mutants resulted in this phenotype, since the HCV RNA replication level was comparable between HJ3-5/HA-p7(KRAA) or HJ3-5/HA-p7(KRAA)/IRES and their p7 wt counterparts (Fig. 4A, 15
compare 6 and 7, and 8 and 9). Also, although we detected a reduction in HCV RNA levels from
the HJ3-5/E2/IRES/p7(KRAA) mutant, when compared to its p7 wt counterpart, a significant
difference in HCV RNA level was detected at and after the 48 hr time point and not at the 24 hr
time point when we detected a virus production delay from this mutant (Fig. 4A and B, compare
lanes 4 and 5). The inherent virus production efficiency (as judged by steady-state virus titer)
alone also could not explain the delayed kinetics of virus production observed from all of these
virus-producing KRAA mutants that we investigated, since the maximum virus titer from HJ3-
5/HAp7(KRAA)/IRES was comparable to that from its p7 wt counterpart (Fig. 4B, compare lanes
4 and 5), although the virus titers from HJ3-5/E2/IRES/p7(KRAA) and HJ3-5/HAp7(KRAA) were
reduced compared to those from their p7 wt counterparts (Fig. 4B, compare 6 and 7, and 8 and
9).

The delayed kinetics of virus production from KRAA mutants is also independent from
the processing efficiency of the junction site between E2 and p7 or p7 and NS2, since we
observed this particular phenotype in the backgrounds of both HJ3-5/E2/IRES and HJ3-
5/HAp7/IRES, in which the processing efficiency of these two junction sites is no longer an issue.
We observed similarly delayed kinetics of intracellular virus production from HJ3-
5/E2/IRES/p7(KRAA) and HJ3-5/HAp7(KRAA)/IRES compared to their p7 wt counterparts (Fig.
4C, compare lanes 1 and 2, and 3 and 4). Thus the delayed virus production from these p7
mutants is due to the delay in the intracellular virus assembly rather than the secretion of virus
to the extracellular medium. According to the study by StGelais and colleagues, KRAA
mutations in p7 impaired not only its ion channel function but also its insertion into membranes,
although they did not substantially affect the folding and oligomerizing properties of p7 (45). It is
likely that some of the disrupted functions of p7 in the p7(KRAA) mutant may be responsible for
the delay in virus production. Alternatively, the potentially decreased stability of p7 due to the
KRAA mutations, as evidenced by the lower level of HAp7 detected from HJ3-5/HAp7(KRAA) and
HJ3-5/HAp7(KRAA)/IRES compared to that from HJ3-5/HAp7 and HJ3-5/HAp7/IRES (Fig. 1C, compare lanes 3 and 4, and 5 and 6), may have played a role in delaying the kinetics of HCV assembly by reducing the level of p7 involved in virus assembly.

Both E2-p7 processing and functional p7 are required for NS2 localization to the dot-like complexes adjacent to LD. In a previous study, we showed that p7(KRAA) mutations disrupted the localization of NS2 to the putative virus assembly site near LD and inhibited NS2-mediated viral protein interaction (25). However, since p7(KRAA) mutations disrupted both the p7 function and E2-p7 processing, it was unclear which of these defects led to these phenotypes. To understand the determinants involved in NS2 subcellular localization and NS2-mediated viral protein complex formation, we electroporated HCV RNAs, including HJ3-5/E2(AR), which is defective in E2-p7 processing but encoding wt p7, and HJ3-5/E2/IRES/p7(KRAA), which is defective in p7 function but guarantees the complete separation of E2-p7 precursor, to Huh-7 cells. Next, we determined the localization of NS2 and the interaction between NS2 and NS3 or E2 by carrying out immunofluorescence analyses and co-immunoprecipitation assays, respectively.

First, confirming our previous findings (25), NS2 from HJ3-5 was localized to the distinct dot-like complexes near LD (punctate-LD distribution) at 48h post-electroporation of this RNA in the majority of NS2-positive cells that we analyzed (~ 80%, Fig. 5A and B, lane a). NS2 from HJ3-5/p7(KRAA) showed ER (endoplasmic reticulum)-like diffused distribution in the absence of strong co-localization with LD (ER-like distribution) in most of the cells replicating this RNA (~90%) and, in the remaining cells, less distinct, occasional distribution near LD (intermediate distribution, see Fig. 5A, lane e, insert #2, for an example) (Fig. 5A and B, lane b). Second, the NS2 from HJ3-5/E2(AR), which is defective in E2-p7 processing, displayed exactly the same NS2-distribution phenotype as that from HJ3-5/p7(KRAA) (Fig. 5, compare lanes b and c).
These results suggest that NS2 localization to virus assembly sites near LD depends on the processing between E2 and p7. Third, we also detected punctate-LD localization of NS2 from HJ3-5/E2/IRES in about ~50% of cells replicating this RNA and that from HJ3-5/Δp7 in about ~60% of cells, which are lesser % than that observed from HJ3-5 replicating cells (Fig. 5A and B, compare lanes a with d and f). These results suggest that the enhanced separation between E2 and p7 also may have a negative impact on NS2 localization to virus assembly sites near LD. It is likely that this reduced localization of NS2 to the virus assembly sites is at least partly responsible for reduced virus production from HJ3-5/E2/IRES and HJ3-5/Δp7 compared to that from HJ3-5 (see Fig. 4B, compare lanes 1 with 4 and 6). Fourth, about 25% of cells replicating HJ3-5/E2/IRES/p7(KRAA) or HJ3-5/Δp7(KRAA) displayed non-ER-like distribution of NS2, in comparison to about 10% of cells replicating HJ3-5/p7(KRAA) (Fig. 5A and B, compare lanes b with e and g). Interestingly, NS2 from HJ3-5/E2/IRES/p7(KRAA) in this non-ER-like distribution category showed 'intermediate' distribution and that from HJ3-5/Δp7(KRAA) showed punctate-LD localization (Fig. 5A and B, compare lanes e and g). Since E2 and p7 are completely separated in HJ3-5/E2/IRES/p7(KRAA) and incompletely in HJ3-5/Δp7(KRAA), these data provide additional support of our hypothesis that incomplete separation of E2-p7 is important for efficient localization of NS2 to the punctate virus assembly sites near LD. It is likely that the difference in NS2 distribution between these two HCVs may have caused a ~3 fold reduction in titer from HJ3-5/E2/IRES/p7(KRAA) than that from HJ3-5/Δp7(KRAA) (Fig. 4B, compare lanes 5 and 7). Importantly, these results suggest that separation of E2-p7 could enhance the localization of NS2 to the virus assembly sites even in the presence of p7(KRAA) mutations and explain the detectable levels of virus production from HJ3-5/E2/IRES/p7(KRAA) and HJ3-5/Δp7(KRAA) (see Fig. 4B, lanes 5 and 7). Fifth, p7(KRAA) mutations reduced NS2 localization to punctate-LD even when the E2-p7 processing defect caused by these mutations is no longer an issue, as in the case of HJ3-5/E2/IRES and its p7(KRAA) mutant pair and HJ3-5/Δp7 and its
HA\textsuperscript{p7}(KRAA) mutants pair (Fig. 5B, compare lanes d and e, and f and g). In summary, these results suggest that the disruption of normal E2-p7 processing (both the inhibition or enhancement) and the defect in p7 function caused by p7(KRAA) mutations negatively regulate NS2 localization to the punctate-LD, which is a putative virus assembly site.

**E2-p7 processing determines the efficiency of NS2 association with NS3 and E2.**

We have used NS2 antibody to pull-down the NS2 and NS2-associated viral proteins. Interestingly, NS2 from HJ3-5/p7(KRAA), HJ3-5/E2(AR) and HJ3-5/E2/IRES/p7(KRAA) (that we designated as A-group) was less efficiently immunoprecipitated by this antibody compared to that from HJ3-5 and HJ3-5/E2/IRES (B-group), although all of these HCVs encode exactly the same NS2 sequence (Fig. 6A, top panel and Fig. 6B). Since functional p7 availability is decreased in the A group (due to the disruption of E2-p7 processing and/or p7(KRAA) mutations), but not impaired in the B group, and our previous findings suggested that p7 affects NS2 conformation (25), we speculate that antibody binding epitope in NS2 from A-group is less accessible to NS2 antibody than that from the B group. Due to this finding, the degree of NS2 interactions with NS3 and E2 was calculated by three-step processes. First, the relative level of immunoprecipitated NS2 was normalized with input NS2 (Fig. 6B, IP-NS2). Second, the relative level of co-precipitated NS3 or E2 was normalized with input NS3 or E2 (Fig. 6 C or E, coIP-NS3 or coIP-E2). Third, the degree of interaction between NS2 and NS3 or E2 was calculated by dividing the coIP-NS3 or coIP-E2 values with the IP-NS2 values (Fig. 6D and F). The relative protein pulled-down values obtained from HJ3-5 was set at 100. The data were obtained from at least three different experiments, and the significance of difference in NS2-mediated NS3 or E2 pull-down efficiency between the different HCVs was calculated by performing the unpaired Student’s t-test (see materials and methods).
We detected a significant decrease in interactions between NS2 and NS3 or E2 from HJ3-5/p7(KRAA) compared to those from HJ3-5 (Fig. 6A, D and F, compare lanes a and b), which was similar to our previous findings when we used the NS2_YFP pull-down assay (25).

Importantly, NS2 interaction with NS3 and E2 was also significantly reduced in HJ3-5/E2(AR) (Fig. 6A, D and F, lane c). These results suggest that E2-p7 processing is critical for NS2-mediated complex formation with NS3 and E2.

Complete separation of E2 and p7 in HJ3-5/E2/IRES decreased the interaction between NS2 and E2, but not that between NS2 and NS3 (Fig. 6A, D and F, lane d). Similar phenotypes were described previously by using gt2a chimera Jc1 modified to encode EMCV IRES between E2 and p7 (27). These results suggest that the complete separation of E2 and p7 negatively regulates the interaction of NS2 with E2 in a genotype-independent manner. In addition, these findings provide mechanistic insight behind the lower level of virus production from HJ3-5/E2/IRES compared to that from HJ3-5 (Fig. 4B, compare lanes 1 and 4) and reinforce the notion that optimal separation of E2 and p7 is necessary for efficient NS2-mediated viral assembly complex formation. It is important to point out that separating NS2 and NS3 in HJ3-5 by inserting IRES between these two proteins (HJ3-5/NS2/IRES) also resulted in a ~5 fold reduction in virus production (data not shown), which is similar to the results from previous studies including that of Stapleford and Lindenbach (27, 43). Interestingly, according to their results, the interaction between NS2 and NS3, but not that between NS2 and E2, was defective in Jc1/NS2-IRES-NS3, which is a gt2a chimera equivalent of HJ3-5/NS2/IRES (27). These results suggest that complete separation of E2 and p7, and NS2 and NS3 due to IRES insertion between these proteins inhibited HCV production by specifically affecting the interaction between NS2 and E2, and NS2 and NS3, respectively. Furthermore, these results imply that the reduced virus production from HJ3-5/E2/IRES and HJ3-5/NS2/IRES is unlikely to be caused by the indirect impact of IRES insertion per se.
Interestingly, p7(KRAA) mutations did not affect the interaction between NS2 and NS3 or E2 in HJ3-5/E2/IRES background, in which E2-p7 processing is no longer an issue (Fig. 6A, D and F, compare lanes d and e). These results suggest that p7 may not be involved in the interaction between NS2 and other viral proteins or, at least, these particular mutations of p7 did not disrupt NS2-mediated protein interaction.

In aggregate, these results imply that inhibition of E2-p7 processing disrupts the interaction between NS2 with NS3 and E2, while the IRES insertion-mediated separation of E2 and p7 disrupts NS2 interaction with E2 but not with NS3. Therefore, optimal processing of E2-p7 is critical for NS2-mediated viral protein complex formation important for infectious virus production.

**Discussion**

This study revealed that impairment of E2-p7 processing is detrimental for infectious HCV production without affecting viral RNA replication. We also showed that introducing a stop codon, followed by EMCV IRES, between E2 and p7 to completely separate E2 and p7 in gt1a-gt2a chimera HJ3-5 (HJ3-5/E2/IRES) resulted in a moderate reduction in virus production, similar to that observed by Jones and colleagues who used a gt2a-gt2a chimera J6/JFH1 (43). Combined with the findings that E2-p7 processing is naturally delayed due to structural determinants at p7 (6, 37, 38) (Fig. 2), these data suggest that delayed, but effective, processing of the E2-p7 junction site is critical for efficient HCV production.

It is currently unknown how the delayed processing of E2-p7 junction may affect HCV production. On one hand, it is possible that E2-p7 precursor may play a role during infectious virus production. However, if this is the case, the putative role played by E2-p7 precursor is not essential for virus production, since removing this precursor completely, as in the case of HJ3-
5/E2/IRES, allowed a reduced, but significant, level of virus production (Fig. 3B and C, lane 5) (43). Also, the defective processing of E2-p7 leading to a significant accumulation of E2-p7 precursor, as in the case for HJ3-5/p7(KRAA) and HJ3-5/E2(AR), prevented virus production (Fig. 3B and C, lanes 2 and 3). Thus the E2-p7 precursor could not replace the functions of E2 and/or p7 during virus assembly. On the other hand, it is possible that the delayed processing of the E2-p7 precursor could ensure the delayed release of functional E2 and/or p7 until they are needed at late steps of HCV replication for virus particle assembly to prevent premature virus assembly. Our data support this possibility. First, the immediate availability of E2 and p7 without any delay during HCV replication, as in the case of HJ3-5/E2/IRES, reduced infectious virus production (Fig. 4B, compare lane 1 and 4). Second, functional p7 is required for efficient virus production even when E2 and p7 are efficiently separated (Fig. 4B, compare lanes 4 and 5, and 6 and 7 for the viral titers from HJ3-5/E2/IRES and HJ3-5/Δp7 in the presence and absence of p7(KRAA) mutations). Third, the release of functional E2 allowed limited levels of virus production even in the absence or reduced level of functional p7, since we observed the partial rescue of the virus production defect caused by p7(KRAA) mutations when E2 and p7 were efficiently separated, as in the case of HJ3-5/E2/IRES/p7(KRAA) and HJ3-5/Δp7(KRAA) (Fig. 4B, lanes 5 and 7). In aggregate, our data suggest that optimal availability of E2 and p7 is important for efficient HCV production.

The data in Figs. 5 and 6 revealed that E2-p7 processing is necessary for both NS2 localization to the putative virus assembly sites in close proximity of the LD and the interaction of NS2 with NS3 and E2 (Fig. 5 and 6, compare lanes a with b and c). However, the separation of E2 and p7 alone was not sufficient for the localization of functional NS2 complexes near LD, since p7(KRAA) mutations inhibited this localization even when the processing of E2-p7 precursor was no longer an issue (Fig. 5, compare lanes d and e, and f and g). These results suggest that, in addition to the E2-p7 processing, functional p7 is required for efficient
localization of NS2 to the virus assembly sites. Supporting this notion, p7 was shown, by Tedbury and colleagues, to affect NS2 subcellular localization in an HCV subgenomic replicon system (46). On the other hand, the same p7(KRAA) mutations did not significantly affect the interaction of NS2 with NS3 and E2, as long as E2 and p7 were separated (Fig. 6D and F, compare lanes d and e). These results suggest that p7 may not be involved in NS2-mediated viral protein complex formation. However, further study is necessary to prove this notion. We also confirmed the previous findings by Stapleford and Lindenbach, who showed that separating E2 and p7 by inserting an EMCV IRES between these two proteins in a gt2a chimera inhibited the interaction between NS2 and E2 without affecting that between NS2 and NS3 by using a gt1a chimera HJ3-5/E2/IRES (Fig. 6D and F, compare lanes a and d). These results suggest that optimal processing of E2 and p7 is necessary to ensure an efficient interaction between the NS2 and E2 involved in the virus assembly process. Based on these results and the literature mentioned above, we propose that the efficiency of E2-p7 processing regulates HCV particle assembly by controlling the timely release of p7. Released p7 will, then, target the NS2 to the site of virus assembly to form virus assembly complexes by interacting with both the structural and nonstructural proteins. HCV may be actively delaying the release of p7, by placing structural constraints that make the cleavage of p7 junction sites relatively inefficient, as shown by Carrère-Kremer and colleagues (37), to delay the NS2 targeting to virus assembly sites by p7 until the time of optimal virus assembly to ensure an efficient interaction between NS2 and E2.

The significance of p7-NS2 processing on HCV replication is less clear. The substantial inhibition of p7-NS2 processing caused by HA epitope tagging at the N-terminus of p7 in HJ3-5\textsuperscript{HA}p7 only moderately reduced infectious virus production (~ one log or so, Fig. 1, compare lanes 1 and 3). However, this modification also enhanced the processing between E2 and p7 (Fig. 2B, lane 3), and, thus, it is reasonable to expect that the enhanced E2-p7 processing could...
also have influenced the virus titer reduction in this HCV. Also, although the complete separation of HA*p7 and NS2 by inserting EMCV IRES in this HCV clone further decreased the virus titer (Fig. 1B and C, compare lanes 3 and 5), it is unclear whether this reduction is totally due to the absence of HA*p7-NS2 precursor, since this modification also affected E2-p7 processing (Fig. 2, compare lanes 3 and 5). In addition, we demonstrated previously that the insertion of EMCV IRES to separate the C-terminal HA tagged p7 and NS2 in HJ3-5/p7HA/EMCV only resulted in a 2- to 3-fold reduction in virus titer compared to that of the HJ3-5 (25). These data suggest that the impact of p7-NS2 processing efficiency on HCV production is relatively minor, if it occurs at all.

We observed that the efficient separation of E2 and p7, due to the insertion of either the EMCV IRES between them or the N-terminus HA tag at p7, allowed moderate levels of virus production from HCV with p7(KRAA) mutations (Fig. 4B, lanes 5, 7 and 9). Interestingly, these p7 mutations caused delays of up to 12 hr in the virus production (Fig. 4B, compare lanes 4 and 5, 6 and 7, and 8 and 9). One explanation for this phenotype could be the reduced virus production efficiency of the p7(KRAA) mutants compared to that of their wt counterparts, as in the cases of wt and p7(KRAA) mutant pairs of HJ3-5/E2/IRES and HJ3-5/HA*p7. However, the virus titers detected at different time points following the electroporation of HJ3-5/HA*p7/IRES without and with p7(KRAA) mutations were strikingly similar starting from 36 h, despite the fact that we were able to detect virus production in the former from 24 h, but not from the latter until 36 h (Fig. 4B, compare lanes 8 and 9; Fig. 4C, compare lanes 3 and 4). As the P7(KRAA) mutations significantly diminished the NS2 localization to the putative virus assembly sites (Fig. 5A and B, compare lanes a and b, d and e, and f and g), we speculate that the p7-dependent localization of NS2 to virus assembly sites signaled the timely initiation of the HCV assembly step and that, in the absence or reduced level of functional p7, the slower, p7-independent localization of NS2 to the virus assembly site delayed the initiation of virus assembly.
Introducing the mutations to p7 often led to varying degrees of E2-p7-NS2 processing defects, and, as a result, it had been difficult to understand the actual scope of p7-specific roles during HCV replication by p7 mutagenesis (33, 34). The p7(KRAA) mutations that we analyzed in this study were no exception, in that these mutations were shown to inhibit, not only the p7 ion channeling function (34), but also E2-p7 processing when introduced to HJ3-5 (25).

Interestingly, separating E2 and p7 in this p7-mutated HCV by introducing EMCV IRES between them partly restored virus production defects, despite the fact that p7 ion channel activity was still defective (Fig. 3B, compare lanes 2 and 6). These results suggest to us that although the ion channel activity of p7 is required for efficient HCV production, it is not absolutely essential for virus production. The controversial, mostly negative, clinical efficacy of currently available p7 ion channel inhibitors could be due to the modest impact of p7 ion channel activity on HCV production (47-50). However, it is likely that more potent p7 ion channel inhibitors in combination with other HCV inhibitors would benefit HCV therapy.

In conclusion, we showed that the efficiency of E2-p7 processing during HCV replication is the major determinant for optimal HCV assembly. Since the inhibition of this processing prevented HCV production, development of specific agents targeting this step may provide novel anti-HCV therapeutic options.

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Legends to Figures

Figure 1. The effect of the HA epitope tag at the N-terminus of p7 on infectious HCV production and p7-NS2 processing. (A) Organization of the gt1a/gt2a HCV chimera HJ3-5 modified to encode the HA epitope tag at the N-terminus of p7 with or without the insertion of a stop codon followed by the EMCV IRES at the junction site of p7 and NS2. The location of p7(KRAA) mutations was also indicated. The Gt1a-based sequence is shaded. (B) Intracellular- and extracellular- virus titers expressed as infectious focus-forming unit (FFU, see materials and methods). The dotted line indicates infectious virus detection limit. Mean titers ± standard deviations (S.D.) from 4 different experiments are shown. (C) The expression of NS2, NS3, \(^{\text{HA}}\)p7 and \(^{\text{HA}}\)p7-NS2 determined by western blot analysis by using anti-NS2 (\(\alpha\)-NS2), anti-NS3 (\(\alpha\)-NS3) and anti-HA (\(\alpha\)-HA) antibodies, at day 2 post-electroporation of indicated HCV RNAs.

Figure 2. The E2-p7 processing efficiency. (A) E2 and its precursors were detected by using western blot analyses of cell lysates collected at day 2 post-electroporation of the indicated HCV RNAs to Huh-7 cells by using anti-E2 (\(\alpha\)-E2) and anti-HA (\(\alpha\)-HA) antibodies. Arrowhead indicates the location of E2, \(^{\text{HA}}\)p7-NS2. The western blots of Endoglycosidase H (Endo H)-treated samples to better separate the E2 and p7 are shown in the bottom panel. E2 is detected in doublet bands upon Endo H treatment, similar to results from previous studies (47-49). GND represents replication-defective HCV RNA. (B) The percentage of E2 in unprocessed E2-p7 precursor forms, including E2-p7, \(^{\text{HA}}\)p7 and \(^{\text{HA}}\)p7, was calculated from western blot analyses of Endo H-treated samples following quantification of E2-related protein bands by using an Odyssey Infrared Imaging system (Li-Cor) after background correction. Mean percentage (%) of E2-p7 precursors and the standard deviations from three-to-five experiments are shown. Statistical analyses were performed by using Graphpad Prism 6 software (see Materials and Methods). Asterisks indicate statistically significant differences between two
paired values; *** (p<0.0005), ** (p<0.005) and * (P<0.05). The differences with P>0.05 were considered not significant (ns).

**Figure 3.** The effect of E2-p7 processing on HCV production. (A) The organization of HJ3-5 with and without E2 terminal residue mutation A384R (AR) to block E2-p7 processing by the signal peptidase and HJ3-5/E2/IRES, which is modified to encode a stop codon followed by EMCV IRES at the junction of E2 and p7 to separate E2 and p7. (B) Intracellular and extracellular virus titers determined at day 2 post-electroporation of indicated HCV RNAs. Mean titers ± S.D. from 4 different experiments are shown. The dotted line is the limit of virus titration. (C) Western blot analysis of cell lysates collected at the time point indicated above following Endo H treatment to detect E2 and E2-p7 by using an anti-E2 antibody.

**Figure 4.** Time course of HCV RNA replication and virus production. (A) The results of the quantitative Taqman RT-PCR assays for HCV RNAs in lysates of electroporated Huh-7 cells at indicated time points. The relative HCV RNA represents the copy number for each construct relative to the value present at 4 h after electroporation. (B) Extracellular- and (C) intracellular-virus titers determined at indicated time points. Mean titers ± S.D. from 4 different experiments are shown. The dotted line is the limit of virus titration assays.

**Figure 5.** The impact of E2-p7 processing on NS2-localization and its interaction with NS3 from a. HJ3-5, b. HJ3-5/p7(KRAA), c. HJ3-5/E2(AR), d. HJ3-5/E2/IRES, e. HJ3-5/E2/IRES/p7(KRAA), f. HJ3-5/Δp7 and g. HJ3-5/Δp7(KRAA). (A) Confocal image analysis by using an Olympus Fluoview® FV1000 laser scanning confocal microscope of cells at day 2 post-electroporation with HCV RNA encoding indicated genomes. Anti-NS2 antibody (green) and LipidTOX™ deep red neutral lipid stain (red) were used to detect NS2 and lipid droplets. At the bottom are the enlarged areas from the images at the top. (B) The relative percentage of NS2-positive cells displaying different NS2 localization patterns. NS2 localization patterns were examined...
analyzed from 50 NS2 immunostaining-positive cells from two independent (a, b, c, d and e) or single (f and g) experiments. ‘Punctate’ indicates NS2 localization similar to that detected from ‘a, d, f and g’ in Fig. 5A. ‘Intermediate’ indicates NS2 localization similar to that observed from ‘e’ in Fig. 5A. ‘Non-punctate’ indicates NS2 localization similar to that detected from ‘b and c’ in Fig. 5A.

Figure 6. The impact of E2-p7 processing on interaction of NS2 with NS3 and E2. (A) The interaction of NS2 with NS3 and E2 was determined by western blot analysis following NS2 pull-down assay. (B) The level of immunoprecipitated NS2 (IP-NS2) was normalized to that of NS2 present in input lysates (NS2-input). IP-NS2 (normalized with by NS2-input) from HJ3-5 was set to ‘100.’ The standard deviations from five different experiments are shown. Asterisks indicate statistically significant differences between ‘a’ and b, c, d or d paired values; *** (p<0.0005), ** (p<0.005) and * (P<0.05). The differences with P>0.05 were considered not significant (ns). (C) Same as description in (B), except that the level of immunoprecipitated NS3 (IP-NS3) was normalized to that of NS3 present in input lysates (NS3-input). Results from three different experiments. (D) The data shown in (C) were normalized to those shown in (B) from three different experiments to determine the efficiency of NS2 and NS3 interaction. (E) Same as the description in (B) except that the level of immunoprecipitated E2 (IP-E2) was normalized to that of E2 present in input lysates (E2-input). (F) The data shown in (E) were normalized to those shown in (B).
Fig. 1
Fig. 2
Fig. 3
Fig. 4
A.  a. HJ3-5  b. /p7(KRAA)  c. /E2(AR)  d. /E2/IRES  e. /E2/IRES/p7(KRAA)  f. /\mu\alpha p7  g. /\mu\alpha p7(KRAA)

B.  a. HJ3-5  b. /p7(KRAA)  c. /E2(AR)  d. /E2/IRES  e. /E2/IRES/p7(KRAA)  f. /\mu\alpha p7  g. /\mu\alpha p7(KRAA)

Fig. 5
Fig. 6