nature
cell biology

The lipid droplet is an important organelle for hepatitis C virus production

Yusuke Miyanari[1,2], Kimie Atsuzawa[3], Nobuteru Usuda[3], Koichi Watashi[1,2], Takayuki Hishiki[1,2], Margarita Zayas[4], Ralf Bartenschlager[4], Takaji Wakita[5], Makoto Hijikata[1,2] and Kunitada Shimotohno[1,2,6]

The lipid droplet (LD) is an organelle that is used for the storage of neutral lipids. It dynamically moves through the cytoplasm, interacting with other organelles, including the endoplasmic reticulum (ER)[1-3]. These interactions are thought to facilitate the transport of lipids and proteins to other organelles. The hepatitis C virus (HCV) is a causative agent of chronic liver diseases[4]. HCV capsid protein (Core) associates with the LD[5], envelope proteins E1 and E2 reside in the ER lumen[6], and the viral replicase is assumed to localize on ER-derived membranes. How and where HCV particles are assembled, however, is poorly understood. Here, we show that the LD is involved in the production of infectious virus particles. We demonstrate that Core recruits nonstructural (NS) proteins and replication complexes to LD-associated membranes, and that this recruitment is critical for producing infectious viruses. Furthermore, virus particles were observed in close proximity to LDs, indicating that some steps of virus assembly take place around LDs. This study reveals a novel function of LDs in the assembly of infectious HCV and provides a new perspective on how viruses usurp cellular functions.

Hepatitis C virus (HCV) has a plus-strand RNA genome that encodes the viral structural proteins Core, E1 and E2, the p7, and the nonstructural (NS) proteins 2, 3, 4A, 4B, 5A and 5B (refs 7, 8). NS proteins are reported to localize on the cytoplasmic side of endoplasmic reticulum (ER) membranes[9]. To elucidate the mechanisms of virus production, we used a HCV strain, JFH1, which can produce infectious viruses[10-12]. We first investigated the subcellular localization of the HCV proteins in cells that had been transfected with JFH1^{E2FL} RNA, in which a part of the hypervariable region 1 of E2 was replaced by the FLAG epitope tag (see Supplementary Information, Fig. S1, S2a–d). Core localized to the lipid droplets (LDs; Fig. 1a), as previously reported[5]. Interestingly, NS proteins were also detected around LDs in 60–90% of JFH1^{E2FL}-replicating cells (Fig. 1a, c). Similar levels of colocalization of LDs with viral proteins were observed in cells that had been transfected with chimeric HCV genomes expressing structural proteins, p7 and part of NS2 of the genotype 1b (Con1) or the genotype 1a (H77) isolate (see Supplementary Information, Fig. S1, S2e)[13]. In contrast, there was no close association between the LDs and NS proteins in cells that had been transfected with JFH1^{dC3} RNA (Fig. 1b, c), which lacked the coding region of Core (Supplementary Information, Fig. S1). NS proteins were diffusely present on the ER, suggesting that NS proteins are translocated from the ER to LDs in JFH1^{E2FL}-replicating cells in a Core-dependent manner. Importantly, there was no association between LDs and PDI, an ER marker protein, indicating that either ER membranes were absent in close proximity to LDs or that PDI was excluded from such membranes (Fig. 1c). These results were supported by western blot analysis of the LD fraction (Fig. 1d). The LD fraction contained ADRP, an LD marker, but not the ER markers Calnexin and Grp78 (data not shown), indicating that there was no ER contamination in the LD fraction. However, the LD fraction from JFH1^{E2FL}-replicating cells contained high levels of viral proteins in contrast to the LD fraction from JFH1^{dC3}-replicating cells (in which HCV proteins were virtually absent (Fig. 1d, LD fraction)), even though the expression levels of the NS proteins in whole-cell extracts were similar (Fig. 1d, whole-cell extract). About 20–45% of the total HCV proteins associated with the LDs in JFH1^{E2FL}-replicating cells (Fig. 1e). Consistent with previous reports that Core enhances the formation of LDs[14], overproduction of LDs was observed in JFH1^{E2FL}-, but not JFH1^{dC3}-replicating cells (Supplementary Information, Fig. S3a–l). Treatment of the cells with oleic acid, which enhanced the formation of LDs, did not affect either HCV protein levels or the recruitment of viral proteins to LDs in JFH1^{dC3}-replicating cells (Supplementary Information, Fig. S3m–p). Thus, the overproduction of LDs is insufficient for the recruitment of HCV proteins to LDs. To examine the ability of Core to recruit NS proteins to LDs, JFH1^{dC3}-replicating cells were transfected with a plasmid-expressing Core (Core^{WT}) (Fig. 1f, g). NS5A accumulated around LDs (Fig. 1f, arrowheads and panel 2), as did NS3 and NS4AB (Fig. 1g), in cells expressing Core^{WT}. The translocation of NS proteins to LDs was, however, not observed in JFH1^{dC3}-replicating cells expressing Core$^{PP/AA}$ (Fig. 1g and Supplementary Information, Fig. S2f–h),

[1]Department of Viral Oncology, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan; [2]Graduate School of Biostudies, Kyoto University, Kyoto 606-8507, Japan; [3]Department of Anatomy, Fujita Health University School of Medicine, Toyoake 470-1192, Japan; [4]Department of Molecular Virology, University of Heidelberg, 69120 Heidelberg, Germany; [5]Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan
[6]Correspondence should be addressed to K.S. (e-mail: shimkuni@z8.keio.jp)

Received 16 March 2007; accepted 31 July 2007; published online 26 August 2007; DOI: 10.1038/ncb1631

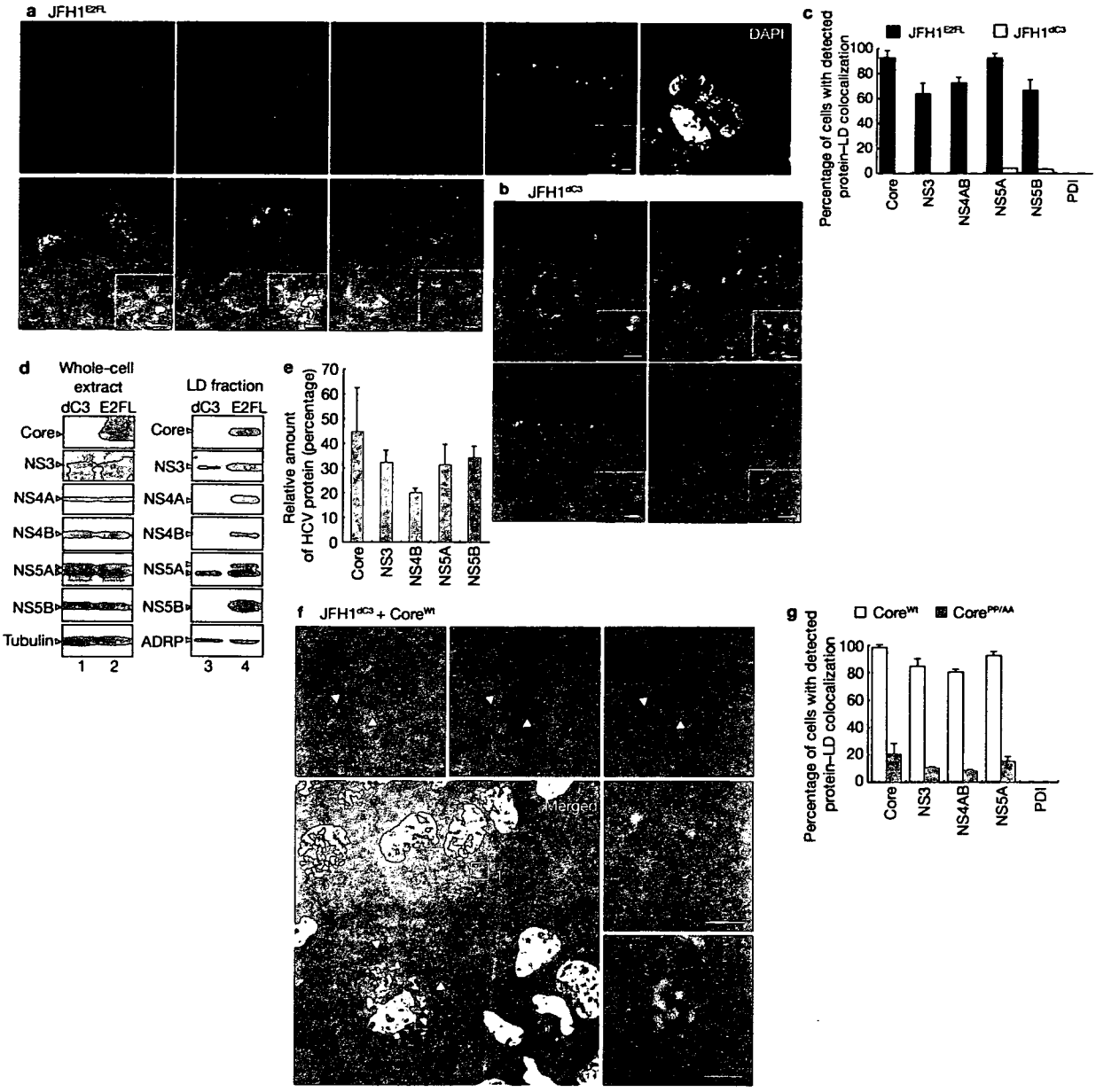

Figure 1 Core recruits NS proteins to LDs. (**a**) Huh-7 cells transfected with JFH1^{E2FL} RNA were labelled with antibodies against Core (red), NS5A (blue), NS3 (red), NS4AB (red) or NS5B (red). Lipid droplets (LDs) and nuclei were stained with BODYPI 493/503 (green) and DAPI (white in upper panel, blue in lower panels), respectively. Insets are high magnification images of areas in the respective panel. (**b**) JFH1^{dC3} replicon-bearing cells were labelled with DAPI (blue), BODIPY 493/503 (green) and indicated antibodies (red). The insets are high magnifications of the corresponding panel. (**c**) Percentages of JFH1^{E2FL}- or JFH1^{dC3}-bearing cells in which hepatitis C virus (HCV) proteins or PDI colocalize with LDs ($n > 200$). (**d**) Western blot analysis of HCV proteins and marker proteins in whole-cell extracts and the LD fractions from cells transfected with JFH1^{E2FL} (E2FL) or JFH1^{dC3} (dC3) RNA. (**e**) HCV proteins were quantified by using western blotting data of the purified LD fraction and whole-cell extracts of JFH1^{E2FL}-replicating cells. Results are shown as relative amounts of HCV proteins co-fractionated with LDs. This results correspond well with results obtained by quantitative immunofluorescence staining (data not shown). (**f**) Trans-complementation with Core^{Wt} relocates NS proteins to LDs. JFH1^{dC3} replicon-bearing cells were transfected with pcDNA3–Core^{Wt} and labelled with BODIPY 493/503 (green), DAPI (white) and antibodies against NS5A (red) and Core (blue). Arrowheads indicate Core^{Wt}-expressing cells. Higher-magnification images of area 1 and area 2 are shown in panels 1 and 2, respectively. Scale bars, 2 μm. (**g**) The percentages of cells in which HCV proteins colocalize with LDs in the presence of Core^{Wt} or Core $^{PP/AA}$ ($n > 200$). Uncropped images of gels are shown in Supplementary Information Fig. S6. All error bars are derived from s.d.

a variant of Core containing two alanine substitutions at amino-acid positions 138 and 143 that fails to associate with LDs[15]. These results show that LD-associated Core recruits NS proteins from the ER to LDs.

Next, we investigated whether Core also recruited HCV RNA to LDs. *In situ* hybridization analysis showed that in more than 80% of JFH1^{E2FL}-replicating cells, both plus- and minus-strand RNAs were diffusely

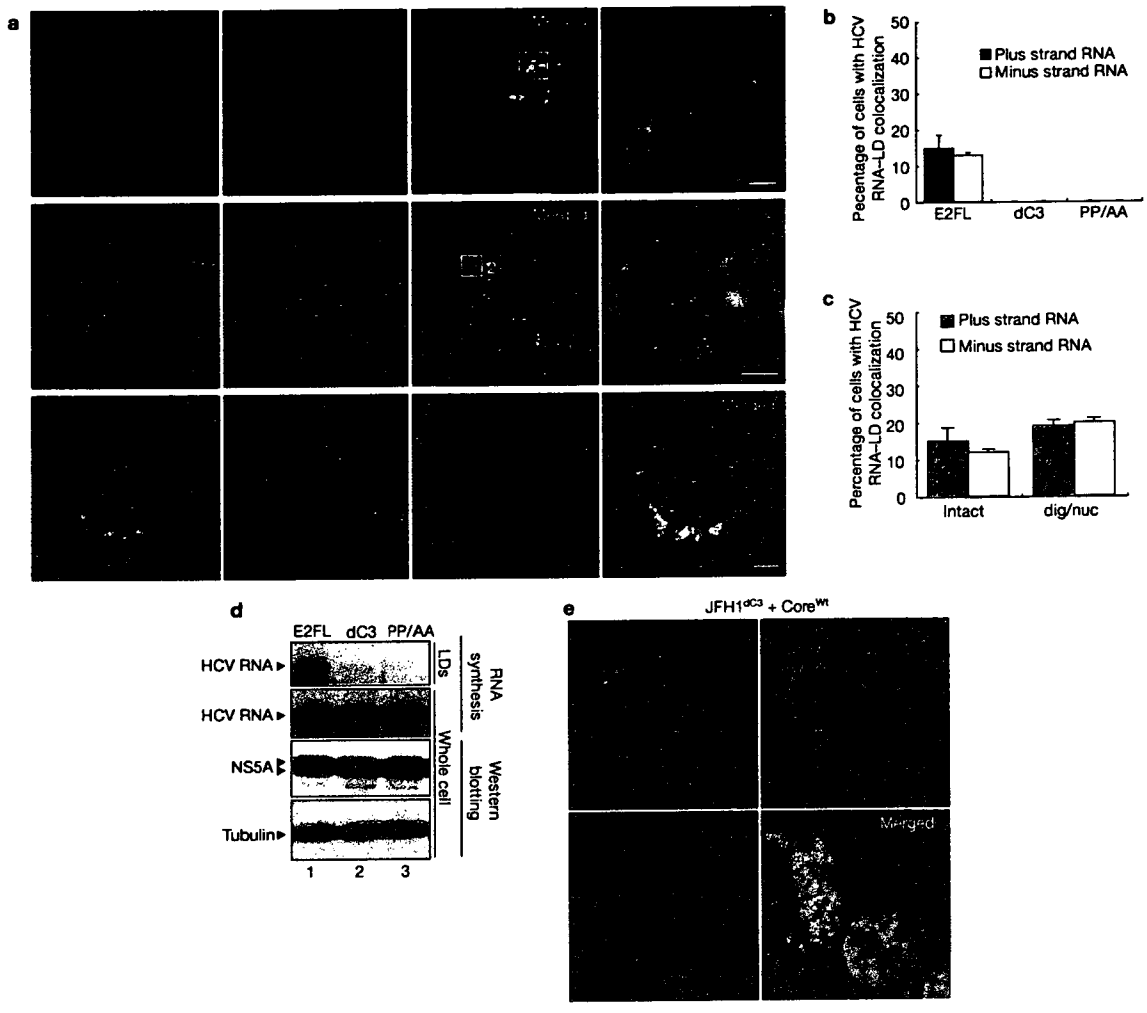

Figure 2 Core-dependent recruitment of active HCV replication complexes to the LD. (**a**) Huh-7 cells transfected with JFH1^{E2FL} RNA were analysed by *in situ* hybridization with strand-specific probes (plus or minus). The cells were labelled to simultaneously visualize lipid droplets (LDs), NS5A and Core (lower panels). Higher-magnification images of area 1 and area 2 are shown in the upper and middle right panels 1 and 2, respectively. Scale bars: 2 μm (panels 1, 2); 10 μm (lower right panel). (**b**) The percentages of JFH1^{E2FL}-, JFH1^{dC3}- and JFH1$^{PP/AA}$-expressing cells positive for overlapping signals for LDs and plus- or minus-strand hepatitis C virus (HCV) RNA ($n > 200$). (**c**) Intact or digitonin and nuclease-treated (dig/nuc) JFH1^{E2FL} replicon-bearing cells were analysed by *in situ* hybridization. The percentages of cells with overlapping signals for LD and plus- or minus-strand HCV RNA are shown ($n > 200$). (**d**) RNA-synthesizing activity in the LD fractions purified from cells transfected with JFH1^{E2FL}, JFH1^{dC3} or JFH1$^{PP/AA}$ RNA (top panel). As a control, HCV RNA synthesis activity in digitonin-permeabilized cells was analysed (second panel from the top). HCV protein levels represented by NS5A are shown, together with the level of tubulin (bottom two panels). (**e**) Localization of plus-strand HCV RNA and Core in JFH1^{dC3} replicon-bearing cells transfected with pcDNA3–Core^{Wt} (Scale bar, 10 μm). Uncropped images of gels are shown in Supplementary Information Fig. S6. All error bars are derived from s.d.

located in the perinuclear region (see Supplementary Information, Fig. S4a). More importantly, in about 20% of these cells, plus- and minus-strand RNAs accumulated around LDs (Fig. 2a, upper and middle panels; 2b) and colocalized with HCV proteins such as Core and NS5A (Fig. 2a, lower panels). No association between HCV RNA and LDs was detected in JFH1^{dC3}- or JFH1$^{PP/AA}$-replicating cells (Fig. 2b). Northern blot analysis revealed that 4.8% and 5.4% of total plus- and minus-strand HCV RNA, respectively, were detected in purified LD fractions of JFH^{E2FL}-replicating cells (data not shown). Induction of LD formation with oleic acid did not affect HCV RNA accumulation around LDs (data not shown). These results provide strong evidence that Core recruits HCV RNA as well as NS proteins to LDs.

The HCV replication complex is compartmentalized by lipid bilayer membranes[16–18]. Therefore, HCV RNA in the complex is resistant to nuclease treatment in digitonin-permeabilized cells[17] (Supplementary Information, Fig. S4b–d). *In situ* hybridization analysis did not reveal a significant difference in the number of cells containing LD-associated HCV RNA before and after nuclease treatment (Fig. 2c), indicating that HCV RNA around LDs is part of the replication complex. An RNA synthesis assay showed that the purified LD fraction from JFH1^{E2FL}-, but not JFH1^{dC3}- or JFH1$^{PP/AA}$-replicating cells, possessed HCV RNA synthesis activity, even though the expression levels of viral proteins and RNA-synthesizing activities in total cell lysates were similar (Fig. 2d). Moreover, the addition of Core^{Wt} rescued the localization of plus- and minus-strand

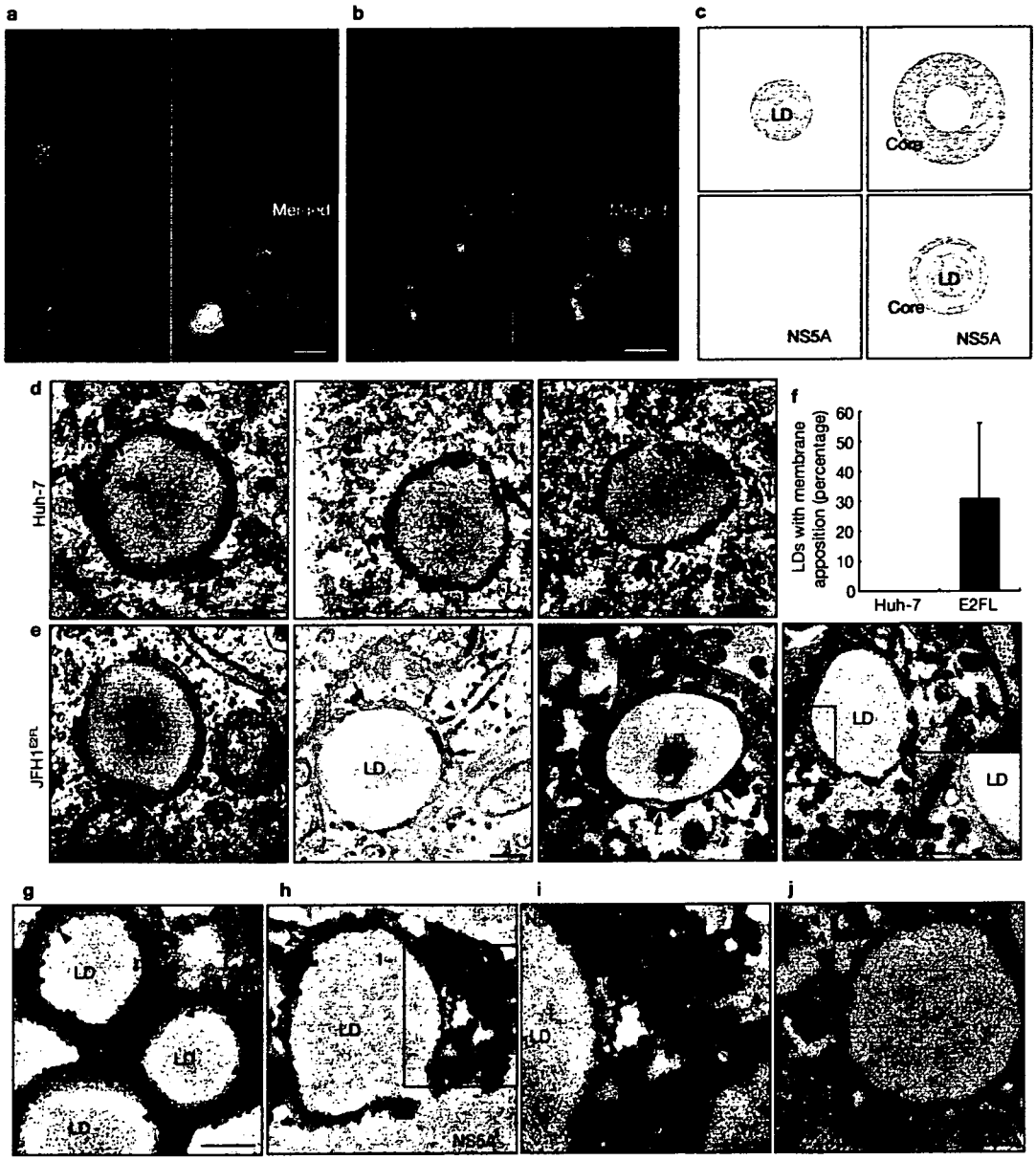

Figure 3 Spatial distribution of Core and NS5A relative to the LD. (**a**, **b**) The localizations of Core, NS5A and ADRP around the lipid droplets (LDs) in JFH1^{E2FL} replicon-bearing cells were analysed using immunofluorescence microscopy. Scale bars, 1 μm. (**c**) Typical images of the localization of LDs, Core, NS5A and merged images are shown with the relative scale of each image. (**d**, **e**) Transmission electron micrographs of LDs in naïve Huh-7 cells and JFH1^{E2FL}-expressing cells. Arrows and arrowheads indicate LD-associated membranes and rough ER membranes, respectively. (**f**) Frequency of LDs with close appositions of membrane cisternae. About 100 Huh-7 cells or JFH1^{E2FL}-expressing cells, respectively, were chosen randomly. LDs with apposed membrane cisternae, as exemplified in panel **e**, were counted as positive. The LDs judged as positive were divided by the total number of LDs. (**g**–**j**) Immunoelectron micrographs of LDs labelled with antibodies against Core (**g**), NS5A (**h**, **i**) or both (**j**) are shown. Panel **i** is a higher magnification of area 1 in panel **h**. In panel **j**, Core and NS5A are labelled with 15 nm and 10 nm gold particles, respectively. Scale bars, 200 nm. All error bars are derived from s.d.

HCV RNA around LDs in JFH^{dC3}-replicating cells (Fig. 2e and data not shown). Both plus- and minus-strand RNA associated with LDs were nuclease resistant (data not shown). These results demonstrate that Core recruits biologically active replication complexes to LDs.

The LD is surrounded by a phospholipid monolayer[19], whereas HCV replication complexes are likely to be surrounded by lipid bilayer membranes[16,17]. Therefore, the replication complexes might not be directly associated with the membranes of LDs. To characterize the colocalization of LDs, viral proteins and replication complexes more precisely, we analysed the localization of NS5A with high-resolution immunofluorescence microscopy. Core was completely colocalized with ADRP, residing on the surface of LDs[20] (Fig. 3a), thus indicating that Core also directly associates with the surface of LDs. More importantly, NS5A mainly localized around the Core-positive area, resulting in a doughnut-shaped signal with a diameter slightly

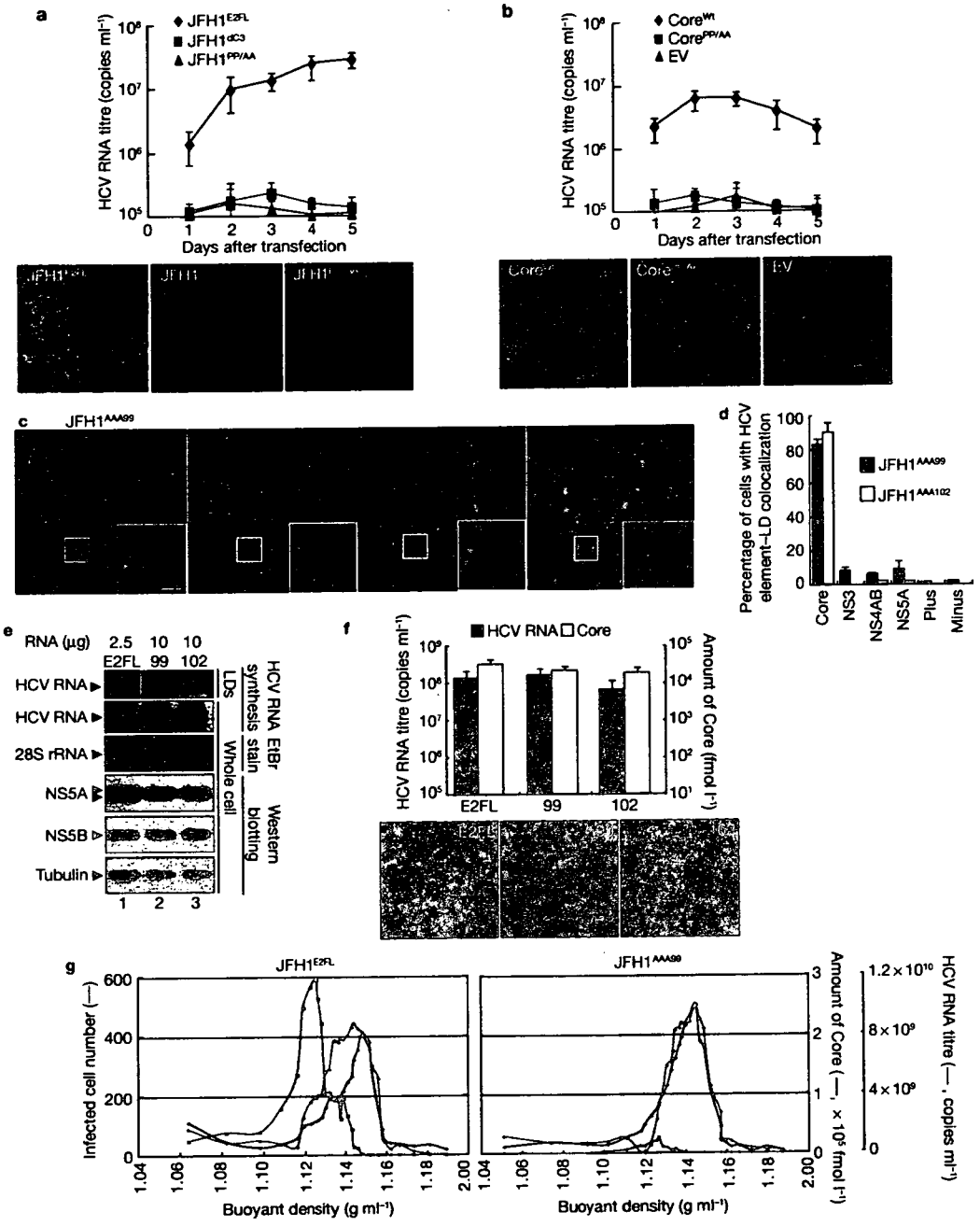

Figure 4 LD associations of Core and NS proteins are necessary for the production of infectious HCV particles. (**a**) The culture medium from JFH1^{E2FL}-, JFH1^{dC3}- or JFH1$^{PP/AA}$-replicating cells was collected at the indicated time points and the titre of hepatitis C virus (HCV) RNA was measured by real-time RT-PCR (upper panel, $n = 3$). The culture medium was added to naïve Huh7.5 cells and, 24 h after inoculation, and cells were labelled with anti-HCV antibodies (lower panels, red). (**b**) JFH1^{dC3} replicon-bearing cells were transfected with pcDNA3 (EV), pcDNA3–Core^{Wt} (Core^{Wt}) or pcDNA3–Core$^{PP/AA}$ (Core$^{PP/AA}$). The level of HCV RNA and the infectivity of the culture medium were examined as described above ($n = 3$). (**c**) Subcellular localization of NS5A and Core in cells expressing JFH1^{AAA99}. The insets are high magnifications of the area of the corresponding panel. Scale bar, 2 μm. (**d**) Percentages of cells in which the signals for given HCV proteins, and plus- and minus-strand HCV RNA, overlapped with those for LDs ($n > 200$). (**e**) Different amounts of JFH1^{E2FL} (E2FL), JFH1^{AAA99} (99) or JFH1^{AAA102} (102) RNAs, respectively, were transfected into the same number of Huh-7 cells. HCV RNA synthesis activity in purified LD fractions (LD) and whole-cell lysates (whole cell) was analysed (HCV RNA synthesis). 28S rRNA was used as a control. Western blot analysis of NS5A, NS5B and tubulin in cells is also shown. All the RNA samples in the top panel were run on the same gel. (**f**) Analysis of HCV released from cells expressing JFH1^{E2FL}, JFH1^{AAA99} or JFH1^{AAA102}. HCV RNA titres (black bars) and amounts of Core (white bars) accumulated in the culture medium at 5 d after RNA transfection were measured (upper panel, $n = 3$). Infectivity of the culture medium for naïve Huh-7.5 cells was analysed as described above (lower panels). (**g**) Concentrated culture medium from JFH1^{E2FL}- and JFH1^{AAA99}-replicating cells was fractionated using 20–50% sucrose density-gradient centrifugation at 100,000 *g* for 16 h. For each fraction, the amounts of Core (black line), HCV RNA (blue line) and infectivity (represented by infected cell numbers in a well; red line) are plotted against the buoyant density (*x*-axis) ($n = 3$). Uncropped images of gels are shown in Supplementary Information Fig. S6. All error bars are derived from s.d.

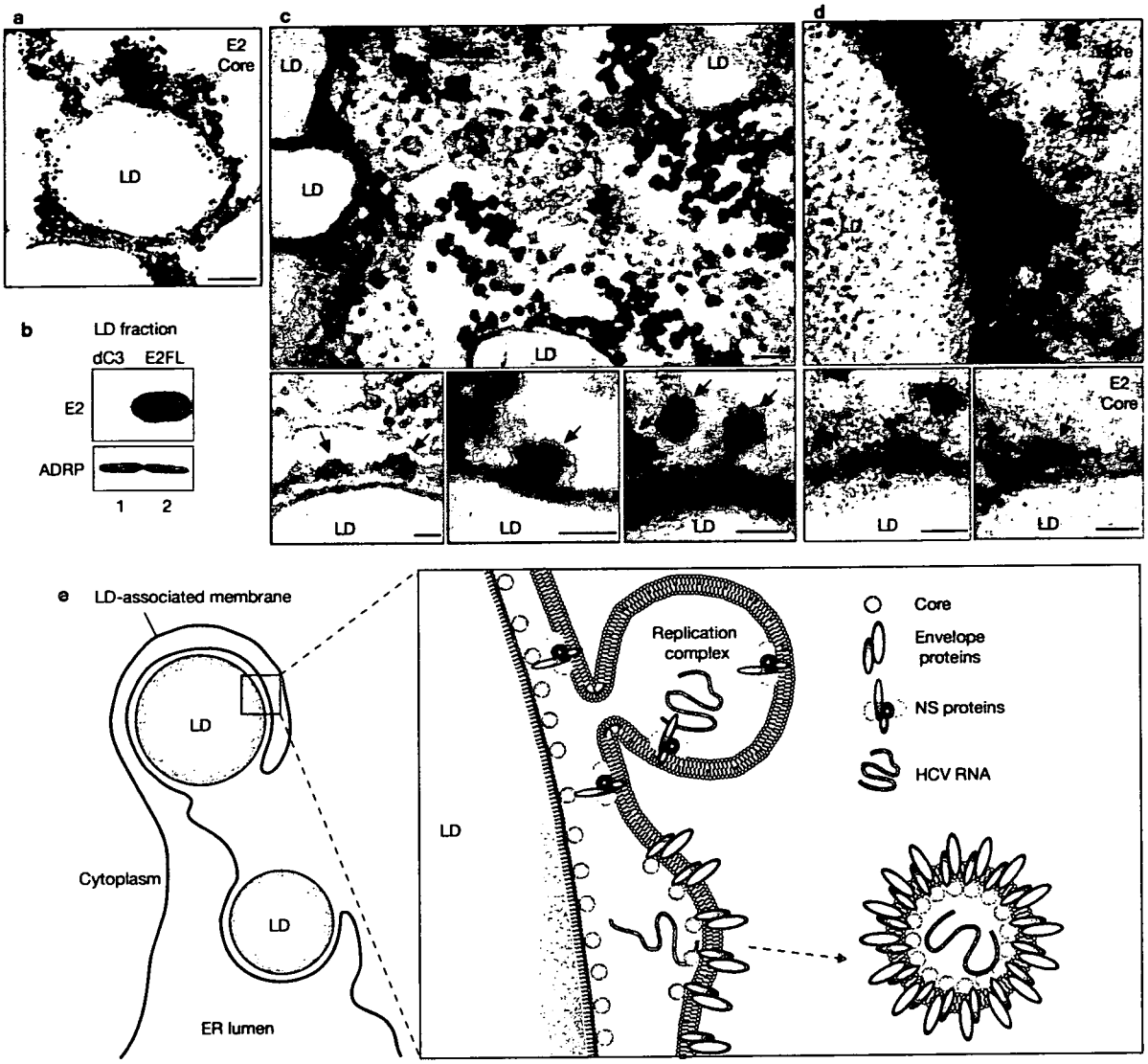

Figure 5 Virus-assembly takes place around the LDs. (**a**) Immunoelectron microscopic detection of E2 and Core in JFH1^{E2FL}-replicating cells. E2 and Core are labelled with 15 nm and 10 nm gold particles, respectively. (**b**) Western blot analysis of the lipid droplet (LD) fraction from JFH1^{E2FL} and JFH1^{dC3} replicon-bearing cells with anti-E2 and anti-ADRP antibodies. (**c**) Transmission electron micrographs of JFH1^{E2FL}-replicating cells. Arrows indicate virus-like particles. (**d**) Immunoelectron micrographs of LDs labelled with antibodies against Core (10 nm) and E2 (15 nm) are shown. Arrows show Core in electron-dense granules. Scale bar: **a** and upper panel of **c**: 100 nm; in **d** and lower panels of **c**: 50 nm. (**e**) A model for the production of infectious hepatitis C virus (HCV). Core mainly localizes on the monolayer membrane that surrounds the LD. HCV induces the apposition of the LD to the endoplasmic reticulum (ER)-derived bilayer membranes (LD-associated membrane). Core recruits NS proteins, as well as replication complexes, to the LD-associated membrane. NS proteins around the LD can then participate in infectious virus production. E2 also localizes around the LD. Through these associations, virion assembly proceeds in this local environment. Uncropped images of gels are shown in Supplementary Information Fig. S6.

larger than that of Core (Fig. 3b). The LD-proximal NS5A signal partially overlapped with the Core signal (Fig. 3b, c, grey). This concentric staining pattern was also observed with the other NS proteins (Supplementary Information, Fig. S5a), indicating that NS proteins associate with Core on the surface of LDs. Electron microscopic analysis only rarely revealed a close association of LDs with other organelles in naïve Huh-7 cells (Fig. 3d, f). However, in the case of JFH1^{E2FL}-replicating cells, about 30% of the LDs were in close proximity to membrane cisternae (Fig. 3e, arrows; 3f), arguing for a HCV-induced membrane rearrangement around LDs. Core was mainly located on the periphery of LDs, and occasionally signals were observed in more distal areas of the LDs (Fig. 3g, arrowheads and arrows, respectively). Although some NS5A signals were observed on the surface of the LD, the majority of NS5A signals were detected more distal of LDs (Fig. 3h, i). Furthermore, we often observed membrane cisternae as white lines in the same area as NS5A signals (Fig. 3i, arrows). When the same section was labelled with anti-Core and anti-NS5A antibodies, Core was detected on the surface of the LDs, whereas NS5A was mainly observed in the peripheral area of the LDs (Fig. 3j, arrowheads and arrows, respectively). In summary, these results show that Core recruits NS proteins, as well as HCV replication complexes, to the LD-associated membranes.

The above results prompted us to ask whether Core–LD colocalization is important for the production of infectious virus particles. JFH1^{E2FL}-replicating cells released virions into the culture medium and these viruses were highly infectious for naïve Huh-7.5 cells[11,21], although culture medium from JFH1$^{PP/AA}$- or JFH1^{dC3}-replicating cells did not contain significant levels of HCV RNA and infectious virus (Fig. 4a). However, following trans-complementation with Core^{Wt}, a high titre of HCV RNA and infectious virus could be rescued from JFH1^{dC3}-replicating cells (Fig. 4b; and see Supplementary Information, Fig. S5b, c). In contrast, the production of infectious viruses was not rescued by trans-complementation with Core$^{PP/AA}$ (Fig. 4b). RNA-binding properties and oligomerization of Core^{Wt} and Core$^{PP/AA}$, which are both necessary for virus assembly, were similar (Supplementary Information, Fig. S5d; ref. 22), arguing that the primary defect of this mutant in preventing infectious virus production is the inability to associate with LDs.

To investigate the contribution of NS proteins around LDs to infectious virus production, we used variants of NS5A, which were not recruited to LDs even in the presence of Core. We assumed that NS5A was crucial for recruiting other NS proteins to LDs, because the level of NS5A recruited to LDs via Core was higher than the levels of the other recruited NS proteins (Fig. 1c, JFH1^{E2FL}). Using alanine-scanning mutagenesis within the NS5A coding region of JFH1^{E2FL}, we generated two mutants, JFH1^{AAA99} and JFH1^{AAA102}, in which the amino-acid sequence APK (aa 99–101 of NS5A) or PPT (aa 102–104 of NS5A) was replaced by AAA (Supplementary Information, Fig. S1). In JFH1^{AAA99}- and JFH1^{AAA102}-replicating cells, NS5A was rarely detected around LDs, whereas Core was still localized to LDs (Fig. 4c, d). Importantly, these mutations impaired not only the NS5A association with LDs, but also the recruitment of other NS proteins and viral RNAs to LDs (Fig. 4d). These results indicate that NS5A is a key protein that recruits replication complexes to LDs. Importantly, HCV RNA synthesis activity in the LD fractions from these mutant JFH1-replicating cells was also severely impaired (Fig. 4e), corroborating the lack of association of HCV replication complexes with LDs.

To investigate the infectious virus production of these NS5A mutants, we prepared cells expressing similar levels of HCV proteins and RNA by adjusting the amount of transfected HCV RNA (Fig. 4e). This was necessary, because replication activities of these mutants were lower compared with JFH1^{E2FL}. Under these conditions, the amounts of Core and HCV RNA that were released into the culture medium from cells transfected with the mutants were comparable to JFH ^{E2FL} (Fig. 4f, upper graph). However, infectivity titres of the mutants were severely reduced (Fig. 4f, lower panels). In sucrose density-gradient centrifugation of culture medium from JFH1^{E2FL}-bearing cells, two types of HCV particles were detected: low-density particles (about 1.12 g ml^{-1}) with high infectivity (Fig. 4g, green area of JFH1^{E2FL}), and high-density particles (about 1.15 g ml^{-1}) without infectivity (yellow area). This result indicates that only a minor portion of released HCV particles is infectious, whereas the majority of released particles lack infectivity. In contrast, cells bearing the JFH1^{AAA99} mutant almost exclusively released non-infectious particles of around 1.15 g ml^{-1}, whereas infectious particles were barely detectable (Fig. 4g, JFH1^{AAA99}). Taken together, these results provide convincing evidence that the association of NS proteins and replication complexes around LDs is critical for producing infectious viruses, whereas production of non-infectious viruses seems to follow a different pathway.

The results described so far imply that some step(s) of HCV assembly take place around LDs. To explore this possibility, we analysed the distribution of the major envelope protein E2 around the LD. Electron microscopic analysis revealed that, in about 90% of JFH1^{E2FL}-replicating cells, E2 was localized in the peripheral area of the LDs (Fig. 5a, large grains). This labelling pattern was similar to the one observed for NS5A (Fig. 3j), indicating that E2 also localizes on the LD-associated membranes. Western blot analysis of the LD fraction supported this conclusion, because the LD fraction that was purified from JFH1^{E2FL}-replicating cells, but not from JFH1^{dC3}-replicating cells, contained E2 (Fig. 5b). Furthermore, spherical virus-like particles with an average diameter of about 50 nm were observed around LDs in JFH1^{E2FL}-replicating cells (Fig. 5c, upper panel). These particles were never observed in naïve Huh-7 cells. A more refined analysis indicates that these particles are closely associated with membranes in close proximity to LDs (Fig. 5c, lower panels, arrows). Finally, these particles around the LDs reacted with Core- and E2-specific antibodies, arguing that the particles represent true HCV virions (Fig. 5d). These results suggest that infectious HCV particles are generated from the LD-associated membranous environment.

In this study, we have demonstrated that Core recruits NS proteins, HCV RNAs and the replication complex to LD-associated membranes. Mutations of Core and NS5A (Fig. 4), which failed to associate with LDs, impaired the production of infectious virus. We note that the mutant Core retains the ability to interact with RNA (Supplementary Information, Fig. S5b) and to assemble into nucleocapside[22]. Similarly, the NS5A mutant still supports viral genome replication and the formation of capsids or virus-like particles, arguing that the introduced mutations in Core and NS5A do not affect overall protein folding, stability or function (Fig. 4). Taken together, the data show that the association of HCV proteins with LDs is important for the production of infectious viral particles (Fig. 5e).

Our results also indicate that NS proteins around the LDs participate in the assembly of infectious virus particles. In one scenario, NS proteins may indirectly contribute to the different steps of virus production — for example, by establishing the microenvironment around the LDs that is required for infectious virus production. Alternatively, NS proteins around the LDs may directly participate in virus production — for example, as components of the replication complex that provide the RNA genome to the assembling nucleocapsid.

In support of the role of LDs in virus formation, we observed that colocalization of HCV protein with LDs was low in cases of the chimera Jc1, supporting up to 1,000-fold higher infectivity titres compared with JFH1 (ref. 13). In a Jc1-infected cell, only about 20% of LDs demonstrated detectable colocalization with Core, but this value increased to 80% in the case of a Jc1 mutant lacking most of the envelope glycoprotein genes and thus being unable to produce infectious virus particles (data not shown). This inverse correlation between the efficiency of virus production and Core protein accumulation on LDs indicates that rapid assembly and virus release results in the rapid liberation of HCV proteins from the LDs.

Steatosis and abnormal lipid metabolism caused by chronic HCV infection may be linked to enhanced LD formation[14]. In fact, the overproduction of LDs is induced by Core (Supplementary Information, Fig. S3) and HCV also induces membrane rearrangements around LDs (Fig. 3d–f). Our findings suggest that excessive Core-dependent formation of LDs

and membrane rearrangements are required to supply the necessary microenvironment for virus production. NS proteins and HCV RNA seem to be translocated from the ER to the LD-associated membranes. Interestingly, the LD-associated membranes were occasionally found in continuity with ribosome-studded rough ER (Fig. 3e, arrowheads). Thus, at least parts of the LD-associated membranes are likely to be derived from ER membranes. ER marker proteins, however, were not detected in the LD fraction, suggesting that the LD-associated membrane is characteristically distinct from that of ER membranes.

To our knowledge, this is the first report showing that LDs are required for the formation of infectious virus particles. The fact that capsid protein of the hepatitis G virus also localizes to LDs[15] indicates that LDs might be important for the production of other viruses as well. Our findings demonstrate a novel function of LDs, provide an important step towards elucidating the mechanism of HCV virion production and open new avenues for novel antiviral intervention. □

METHODS

Antibodies. The antibodies used for immunoblotting and immunolabelling were specific for Core (#32-1 and RR8); E2 (AP-33 (ref. 23); 3/11, CBH5 and Flag M2 (Sigma-Aldrich, St Louis, MO); NS3 (R212)[17]; NS4A and 4B (PR12); NS5A (NS5ACL1); NS5B (NS5B-6 and JFH1-1)[24]; ADRP (Progen Biotechnik, Heidelberg, Germany); tubulin (Oncogene Research Products, MA, USA); Grp78 (StressGen, Victoria, Canada); PDI (StressGen); and Calnexin-NT (StressGen). Antibodies specific for Core (#32-1 and RR8), NS3 (R212) and NS4AB (PR12) were gifts from Dr Kohara (The Tokyo Metropolitan Institute of Medical Science, Japan). Anti-E2 antibody (AP-33) was provided by Dr Patel (MRC Virology Unit, UK). Anti-NS5B (NS5B-6) antibody was kindly provided by Dr Fukuya (Osaka University, Japan). Rabbit polyclonal antibodies specific for NS5A were raised against a bacterially expressed GST-NS5A (1–406 aa) fusion protein. In the case of the HCV chimeras Con1/C3 and H77/C3, immunofluorescence analyses were performed by using the following antibodies: Core (C7/50)[5], a JFH1 NS3-specific rabbit polyclonal antiserum; NS4B (#86)[25]; and NS5A (Austral Biologicals, San Ramon, CA).

Indirect immunofluorescence analysis. Indirect immunofluorescence analysis was performed essentially as described previously[17], with slight modifications. Cells transfected with JFH1 RNA were seeded onto a collagen-coated Labtech II 8-well chamber (Nunc, NY, USA). The coating with collagen was performed using rat-tail collagen type I (BD Bioscience, Palo Alto, CA) according to manufacturer's instructions. Three days after seeding, the cells were washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4) and fixed with fixation solution (4% paraformaldehyde and 0.15 M sodium cacodylate at pH 7.4) for 15 min at room temperature. After washing with PBS, the cells were permeabilized with 0.05% Triton X-100 in PBS for 15 min at room temperature. For the precise localization of the proteins, the cells were permeabilized with 50 μg ml^{-1} of digitonin in PBS for 5 min at room temperature[26]. After incubating the cells with blocking solution (10% fetal bovine serum and 5% bovine serum albumin (BSA) in PBS) for 30 min, the cells were incubated with the primary antibodies. The fluorescent secondary antibodies were Alexa 568- or Alexa 647-conjugated anti-mouse or anti-rabbit IgG antibodies (Invitrogen, Carlsbad, CA). Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI). LDs were visualized with BODIPY 493/503 (Invitrogen). Analyses of JFH1 were performed on a Leica SP2 confocal microscope (Leica, Heidelberg, Germany). Analysis of the Con1/C3 and the H77/C3 chimeras was performed in the same way, except that imaging was performed on a Nikon C1 confocal microscope (Nikon, Tokyo, Japan).

Electron microscopy. For conventional electron microscopy, cells cultured in plastic Petri dishes were processed *in situ*. The cells were fixed in 2.5% glutaraldehyde and 0.1 M sodium phosphate (pH 7.4), and then in OsO_4 and 0.1 M sodium phosphate (pH 7.4). The cells were then dehydrated in a graded ethanol series and embedded in an epoxy resin. Ultrathin sections were cut perpendicular to the base of the dish. For immuno-electron microscopy, cells were detached from the dish with a cell scraper after fixation in 4% paraformaldehyde, 0.1% glutaraldehyde and 0.1 M sodium phosphate (pH 7.4) for 24 h, and washed in 0.1 M lysine, 0.1 M sodium phosphate (pH 7.4) and 0.15 M sodium chloride. After dehydrating the cells in a graded series of cold ethanol, they were embedded in Lowicryl K4M at −20 °C. Ultrathin sections were labelled with primary antibodies and colloidal gold particles (15 nm) conjugated to anti-mouse IgG or anti-rabbit IgG antibodies. For double labelling, colloidal gold particles with different diameters (10 nm and 15 nm) conjugated to anti-mouse IgG or anti-rabbit antibodies were used. Samples were observed after staining with uranyl acetate and lead citrate with a JEM 1010 electron microscope at the accelerating voltage of 80 kV. Anti-Core (#32-1 and RR88), anti-NS5A (NS5ACL1) and anti-E2 (Flag M2) antibodies were used.

Preparation of the lipid droplets. Cells at a confluency of ~80% on a dish with a diameter of 14 cm were scraped in PBS. The cells were pelleted by centrifugation at 1,500 rpm. The pellet was resuspended in 500 μl of hypotonic buffer (50 mM HEPES, 1 mM EDTA and 2 mM $MgCl_2$ at pH 7.4) supplemented with protease inhibitors (Roche Diagnostics, Basel, Switzerland) and was incubated for 10 min at 4 °C. The suspension was homogenized with 30 strokes of a glass Dounce homogenizer using a tight-fitting pestle. Then, 50 μl of 10× sucrose buffer (0.2 M HEPES, 1.2 M KoAc, 40 mM $Mg(oAc)_2$ and 50 mM DTT at pH 7.4) was added to the homogenate. The nuclei were removed by centrifugation at 2,000 rpm for 10 min at 4 °C. The supernatant was collected and centrifuged at 16,000 *g* for 10 min at 4 °C. The supernatant (S16) was mixed with an equal volume of 1.04 M sucrose in isotonic buffer (50 mM HEPES, 100 mM KCl, 2 mM $MgCl_2$ and protease inhibitors). The solution was set at the bottom of 2.2-ml ultracentrifuge tube (Hitachi Koki, Tokyo, Japan). One milliliter of isotonic buffer was loaded onto the sucrose mixture. The tube was centrifuged at 100,000 *g* in an S55S rotor (Hitachi Koki) for 30 min at 4 °C. After the centrifugation, the LD fraction on the top of the gradient solution was recovered in isotonic buffer. The suspension was mixed with 1.04 M sucrose and centrifuged again at 100,000 *g*, as described above, to eliminate possible contamination with other organelles. The collected LD fraction was used for western blotting or the HCV RNA synthesis assay.

HCV RNA synthesis assay. An assay of HCV RNA synthesis using digitonin-permeabilized cells was performed as described previously[17]. For RNA synthesis assays using the LD fraction, the LD fraction collected by sucrose-gradient sedimentation was suspended in buffer B, which contained 2 mM manganese (II) chloride, 1 mg ml^{-1} acetylated BSA (Nacalai Tesque, Kyoto, Japan), 5 mM phosphocreatine (Sigma), 20 units/ml creatine phosphokinase (Sigma), 50 μg ml^{-1} actinomycin D, 500 μM ATP, 500 μM CTP, 500 μM GTP (Roche Diagnostics) and 1.85 MBq of [α-^{32}P] UTP (GE Healthcare, Little Chalfont, UK), and incubated at 27 °C for 4 h. The reaction products were analysed by gel electrophoresis followed by autoradiography.

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGEMENTS

We thank T. Fujimoto and Y. Ohsaki at Nagoya University for helpful discussions and technical assistance. Y.M. is a recipient of a JSPS fellowship. K.S. is supported by Grants-in-Aid for cancer research and for the second-term comprehensive 10-year strategy for cancer control from the Ministry of Health, Labour and Welfare, as well as by a Grant-in-Aid for Scientific Research on Priority Areas "Integrative Research Toward the Conquest of Cancer" from the Ministry of Education, Culture, Sports, Science and Technology of Japan. T.W. is also supported, in part, by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science; and by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation. R.B. is supported by the Sonderforschungsbereich 638 (Teilprojekt A5) and the Deutsche Forschungsgemeinschaft (BA1505/2-1). M.Z. and R.B. thank the Nikon Imaging Center at the University of Heidelberg for providing access to their confocal fluorescence microscopes and Ulrike Engel for the excellent support.

AUTHOR CONTRIBUTIONS

Y.M. and K.S. planned experiments and analyses. Y.M. was responsible for experiments for Figs 1, 2, 3a–c, 4a–e and 5b. K.A., N.U., electron microscopy; T.H., Fig. 1e; M.Z., R.B., Fig. S2e; and K.S. and K.W., Fig. 4f–g. T.W. provided JFH1 strain. Y.M. and K.S. wrote the manuscript. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Published online at http://www.nature.com/naturecellbiology/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Martin, S. & Parton, R. G. Lipid droplets: a unified view of a dynamic organelle. *Nature Rev. Mol. Cell Biol.* **7**, 373–378 (2006).
2. Blanchette-Mackie, E. J. *et al.* Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. *J. Lipid Res.* **36**, 1211–1226 (1995).
3. Vock, R. *et al.* Design of the oxygen and substrate pathways. VI. structural basis of intracellular substrate supply to mitochondria in muscle cells. *J. Exp. Biol.* **199**, 1689–1697 (1996).
4. Liang, T. J. *et al.* Viral pathogenesis of hepatocellular carcinoma in the United States. *Hepatology* **18**, 1326–1333 (1993).
5. Moradpour, D., Englert, C., Wakita, T. & Wands, J. R. Characterization of cell lines allowing tightly regulated expression of hepatitis C virus core protein. *Virology* **222**, 51–63 (1996).
6. Deleersnyder, V. *et al.* Formation of native hepatitis C virus glycoprotein complexes. *J. Virol.* **71**, 697–704 (1997).
7. Kato, N. *et al.* Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc. Natl Acad. Sci. USA* **87**, 9524–9528 (1990).
8. Hijikata, M. & Shimotohno, K. [Mechanisms of hepatitis C viral polyprotein processing]. *Uirusu* **43**, 293–298 (1993).
9. Dubuisson, J., Penin, F. & Moradpour, D. Interaction of hepatitis C virus proteins with host cell membranes and lipids. *Trends Cell Biol.* **12**, 517–523 (2002).
10. Wakita, T. *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nature Med.* **11**, 791–796 (2005).
11. Lindenbach, B. D. *et al.* Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623–626 (2005).
12. Zhong, J. *et al.* Robust hepatitis C virus infection in vitro. *Proc. Natl Acad. Sci. USA* **102**, 9294–9299 (2005).
13. Pietschmann, T. *et al.* Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc. Natl Acad. Sci. USA* **103**, 7408–7413 (2006).
14. Moriya, K. *et al.* Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J. Gen. Virol.* **78**, 1527–1531 (1997).
15. Hope, R. G., Murphy, D. J. & McLauchlan, J. The domains required to direct core proteins of hepatitis C virus and GB virus-B to lipid droplets share common features with plant oleosin proteins. *J. Biol. Chem.* **277**, 4261–4270 (2002).
16. Egger, D. *et al.* Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* **76**, 5974–5984 (2002).
17. Miyanari, Y. *et al.* Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral genome replication. *J. Biol. Chem.* **278**, 50301–50308 (2003).
18. Quinkert, D., Bartenschlager, R. & Lohmann, V. Quantitative analysis of the hepatitis C virus replication complex. *J. Virol.* **79**, 13594–13605 (2005).
19. Tauchi-Sato, K., Ozeki, S., Houjou, T., Taguchi, R. & Fujimoto, T. The surface of lipid droplets is a phospholipid monolayer with a unique fatty acid composition. *J. Biol. Chem.* **277**, 44507–44512 (2002).
20. Londos, C., Brasaemle, D. L., Schultz, C. J., Segrest, J. P. & Kimmel, A. R. Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Semin. Cell Dev. Biol.* **10**, 51–58 (1999).
21. Blight, K. J., McKeating, J. A. & Rice, C. M. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J. Virol.* **76**, 13001–13014 (2002).
22. Klein, K. C., Dellos, S. R. & Lingappa, J. R. Identification of residues in the hepatitis C virus core protein that are critical for capsid assembly in a cell-free system. *J. Virol.* **79**, 6814–6826 (2005).
23. Owsianka, A. *et al.* Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. *J Virol* **79**, 11095–11104 (2005).
24. Ishii, N. *et al.* Diverse effects of cyclosporine on hepatitis C virus strain replication. *J. Virol.* **80**, 4510–4520 (2006).
25. Lohmann, V., Korner, F., Herian, U. & Bartenschlager, R. Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J Virol* **71**, 8416–8428 (1997).
26. Ohsaki, Y., Maeda, T. & Fujimoto, T. Fixation and permeabilization protocol is critical for the immunolabeling of lipid droplet proteins. *Histochem. Cell Biol.* **124**, 445–452 (2005).

Journal of General Virology (2007), 88, 2495–2503 DOI 10.1099/vir.0.82872-0

Robust production of infectious viral particles in Huh-7 cells by introducing mutations in hepatitis C virus structural proteins

David Delgrange,[1] André Pillez,[1] Sandrine Castelain,[1,2] Laurence Cocquerel,[1] Yves Rouillé,[1] Jean Dubuisson,[1] Takaji Wakita,[3] Gilles Duverlie[1,2] and Czeslaw Wychowski[1]

Correspondence
Czeslaw Wychowski
czeslaw.wychowski@ibl.fr

[1]CNRS-UMR 8161, IBL, Université de Lille I et Lille II, Institut Pasteur de Lille, 59021 Lille cedex, France

[2]Laboratoire de Virologie, Centre Hospitalier Universitaire-Hôpital Sud, 80054 Amiens cedex, France

[3]Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan

Recently, the characterization of a cell culture system allowing the amplification of an authentic virus, named hepatitis C virus cell culture (HCVcc), has been reported by several groups. To obtain higher HCV particle productions, we investigated the potential effect of some amino acid changes on the infectivity of the JFH-1 isolate. As a first approach, successive infections of naïve Huh-7 cells were performed until high viral titres were obtained, and mutations that appeared during this selection were identified by sequencing. Only one major modification, N534K, located in the E2 glycoprotein sequence was found. Interestingly, this mutation prevented core glycosylation of E2 site 6. In addition, JFH-1 generated with this modification facilitated the infection of Huh-7 cells. In a second approach to identify mutations favouring HCVcc infectivity, we exploited the observation that a chimeric virus containing the genotype 1a core protein in the context of JFH-1 background was more infectious than wild-type JFH-1 isolate. Sequence alignment between JFH-1 and our chimera, led us to identify two major positions, 172 and 173, which were not occupied by similar amino acids in these two viruses. Importantly, higher viral titres were obtained by introducing these residues in the context of wild-type JFH-1. Altogether, our data indicate that a more robust production of HCVcc particles can be obtained by introducing a few specific mutations in JFH-1 structural proteins.

Received 23 January 2007
Accepted 1 May 2007

INTRODUCTION

The *Hepatitis C virus* (HCV) is the only member of the genus *Hepacivirus* of the family *Flaviviridae*. HCV is a major cause of chronic hepatitis, liver cirrhosis, hepatocellular carcinoma (Major *et al.*, 2001) as well as several extrahepatic diseases (Houghton, 1996). An estimation of about 170 million people infected with HCV worldwide has been reported (Poynard *et al.*, 2003; Thomas, 2000).

HCV is an enveloped single-strand, positive-sense RNA virus and its genome encodes a unique open reading frame that is flanked by two structured non-translated regions in 5′ and 3′ ends of HCV genome (5′NTR and 3′NTR). Mediated by an internal ribosome entry site (Tsukiyama-Kohara *et al.*, 1992), the translation of HCV RNA genome results in polyprotein synthesis that is processed by cellular and viral proteases into at least 10 structural and non-structural (NS) proteins (Grakoui *et al.*, 1993; Hijikata *et al.*, 1991). In the viral particle, HCV genomic RNA is complexed with the highly basic capsid protein. On its surface, the viral particle bears two envelope glycoproteins E1 and E2 that are anchored in the lipid bilayer. Both these proteins have been shown to accumulate in the endoplasmic reticulum (ER), where the particles are probably assembled (Op De Beeck *et al.*, 2001). A small integral membrane protein, p7, has been reported to function as an ion channel (Griffin *et al.*, 2003; Pavlovic *et al.*, 2003). Among the NS proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B, which coordinate the intracellular processes of the virus life cycle, only proteins NS3 through to 5B are sufficient to support the HCV RNA replication (Lohmann *et al.*, 1999). In addition to the polyprotein, a new HCV protein with an unknown function has also been reported. The so-called F-protein (frameshift) or ARFP (alternative reading frame protein) is generated by ribosomal frameshifting into an alternative reading frame within the

capsid-coding sequence (Roussel *et al.*, 2003; Varaklioti *et al.*, 2002; Walewski *et al.*, 2001; Xu *et al.*, 2001).

Despite intensive research efforts, the HCV life cycle and host–virus interactions have been difficult to investigate due to the lack of efficient cell culture and small animal models. Nevertheless, significant progress has been made by using heterologous expression systems. Infectivity of some cDNA-derived HCV RNAs has been demonstrated in chimpanzees upon intrahepatic inoculation (Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997, 1999). Also, the development of a functional cell-based replication system allowing efficient replication of HCV subgenomic RNAs (replicons) has provided an important tool for studying the HCV RNA replication or for evaluating potential antiviral compounds (Blight *et al.*, 2000; Lohmann *et al.*, 1999). Several surrogate systems have also been developed to palliate the difficulties in studying interactions between several candidate HCV receptors and the HCV glycoproteins. Thus, retrovirus-based pseudoparticles (pp) or HCVpp has provided the first insight into HCV entry (Bartosch *et al.*, 2003; Hsu *et al.*, 2003). But the major breakthrough arose recently with the propagation of virus in a human liver hepatoma cell line, Huh-7 (Wakita *et al.*, 2005), by transfecting these cells with an RNA transcribed from a full-length cDNA cloned initially from a patient with a fulminant hepatitis and infected with a genotype 2a isolate (Kato *et al.*, 2001). Unfortunately, it was reported by this group that the efficacy of the infection was low. Subsequently, different papers reported a robust production of infectious virus obtained with a homologous chimeric FL-J6/JFH-1 (Lindenbach *et al.*, 2005) or obtained into Huh-7.5.1 cells (Zhong *et al.*, 2005), derived from a cell line (Huh-7.5) having a defect in the RIG-I pathway (Sumpter *et al.*, 2005).

In this study, a highly efficient *in vitro* infection system based on Huh-7 cell line was obtained. The transcribed genomic JFH-1 RNA was used to produce infectious virus. The viral titre was initially low; however, successive infections of naïve Huh-7 cells led to a robust production of virus. The sequencing of the viral genome revealed only a few mutations located in the E2 glycoprotein. Furthermore and based on the characterization of a 1a–2a chimeric virus, we showed by site-directed mutagenesis that 2 aa present in the C-terminal part of the capsid-coding sequence were important for the production of high titres. Consequently, a robust HCV particle production was obtained independently of the Huh-7.5.1 cells or JFH-1 recombinant viral genome.

METHODS

Cell culture. Cell monolayers of human hepatoma cell line Huh-7 (Nakabayashi *et al.*, 1982) were grown in Dulbecco's modified essential medium (DMEM; Invitrogen) supplemented with 100 nmol non-essential amino acids l^{-1} and 10 % fetal bovine serum (FBS).

Antibodies. Rat monoclonal antibody (mAb) 3/11 (Flint *et al.*, 1999), kindly provided by J. McKeating (Institute of Biomedical Research, Birmingham University, UK) was produced *in vitro* by using a MiniPerm apparatus (Heraeus) as recommended by the manufacturer. Anti-C (ACAP27) mouse mAb was kindly provided by J. F. Delagneau (Bio-Rad, Marne-La-Coquette 92430, France). Anti-E2 mouse mAb AP33 was kindly provided by A. Patel (MRC Virology Unit, Institute of Virology, Glasgow, UK). Goat anti-β-actin polyclonal antibodies were from Santa Cruz. Alexa 488-conjugated and Alexa 555-conjugated goat anti-mouse secondary antibodies were from Molecular Probes.

Plasmid construction. The plasmids pJFH-1 containing the full-length cDNA of JFH-1 isolate, belonging to subtype 2a (GenBank accession no. AB047639), pJFH-1/GND and pJFH-1/ΔE1-E2 were described previously (Wakita *et al.*, 2005). Individual or combined viral mutations N534K (N6), F172C and P173S (FP→CS) were introduced into the pJFH-1 plasmid by sequential PCR steps as described using the high fidelity deep vent DNA polymerase (New England Biolabs), then assembled by a second PCR amplification (Goffard *et al.*, 2005), followed by restriction digestions and ligation. The resulting plasmids were named pJFH-1/N6 (N534K) and pJFH-1/CS. The plasmid pJFH-1/CS-N6 was obtained by inserting the fragment *Bsi*W1–*Not*I obtained from pJFH-1/N6 into the plasmid pJFH-1/CS. All constructs were verified by DNA sequencing.

***In vitro* transcription.** To generate genomic HCV RNA, the plasmid pJFH-1 and derivatives were linearized at the 3′ end of the HCV cDNA with the restriction enzyme *Xba*I (New England Biolabs). Following treatment with Mung Bean Nuclease, the linearized DNAs were then precipitated overnight and resuspended in RNase-free water to a concentration of 1 μg μl^{-1}. *In vitro* transcripts were generated using Megascript (Ambion) according to the manufacturer's protocol. The *in vitro* reaction was set up and incubated at 37 °C for 4 h. To degrade the DNA template, DNase I was added and incubated for another 20 min at 37 °C. The *in vitro* transcripts were then precipitated by the addition of LiCl and the precipitates were recovered by centrifugation. The concentration of each transcript was determined by measurement of the absorbance at 260 nm. *In vitro* transcribed RNA was delivered to cells by electroporation as described previously (Kato *et al.*, 2003a). Viral stocks were obtained by harvesting cell culture supernatants at 1 week post-transfection. Secondary viral stocks were obtained by additional infections of naïve Huh-7 cells.

Successive infections and titration of HCV RNA by RT-PCR. Huh-7 cells were seeded at 7×10^5 cells in T25 flask and inoculated with 2 ml supernatant medium from cells transfected with the infectious JFH-1 RNA. At 24 h, the cells were supplemented with 4 ml complete DMEM. At day 3 post-infection, infected cells were trypsinized and then replated at 2×10^6 cells in a T75 flask. Indirect immunofluorescence was used to estimate the levels of infectivity of the amplified virus. In addition, for quantification of HCV RNA, the RNA was extracted from the supernatant of infected cells and titrated by quantitative real-time RT-PCR assay (RT-qPCR) (Castelain *et al.*, 2004).

HCV RNA genome sequencing. Five microlitre aliquots of the RNA solutions were subjected to reverse transcription with random hexamer and moloney murine leukemia virus reverse transcriptase (Superscript II; Invitrogen) at 42 °C for 1 h. PCR primers of 20-mer designated on the sequence of JFH-1 were used to amplify five fragments of HCV cDNA (nt 129–626, 467–2367, 2285–4665, 4594–7003 and 6950–9634) to cover most of the HCV genome. One microlitre of the cDNA was subjected to PCR with TaKaRa LA *Taq* polymerase (Takara Biochemicals), and PCR conditions consisted of 30 cycles each with a denaturing cycle at 95 °C for 30 s, an annealing cycle at 60 °C for 30 s and an extension cycle at 72 °C for 2 min. The sequence of each amplified DNA was determined.

Titration of HCV cell culture (cc). Huh-7 cells were seeded at 8×10^4 cells per well in a 24-well plate. The next day, cell supernatants of transfected or infected Huh-7 cells were serially diluted in DMEM and used to infect naïve Huh-7 cells. The inoculum was incubated for 2 h at 37 °C, washed with DMEM and then overlaid with complete DMEM. The viral titre was then determined at 3 days post-infection by indirect immunofluorescence staining of the capsid protein and expressed as focus-forming unit per millilitre (f.f.u. ml^{-1}) as described previously (Zhong *et al.*, 2005).

Western blot analysis. Cells were lysed in a buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5 % (v/v) Igepal and a mixture of protease inhibitors (Complete; Roche). Protein content of pre-cleared cell lysates was determined by the BCA method as recommended by the manufacturer (Sigma), using BSA as a standard. Total proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (Hybond-ECL; Amersham) by using a Trans-Blot apparatus (Bio-rad) and revealed with a specific mAb followed by goat anti-mouse or anti-rat IgG conjugated to peroxidase (Jackson Immunoresearch) and donkey anti-goat conjugated to peroxidase. The immune complexes were visualized by enhanced chemiluminescence detection (ECL; Amersham) as recommended by the manufacturer.

Indirect immunofluorescence microscopy. Infected Huh-7 cells grown on coverslips were fixed in 4 % paraformaldehyde. Immunostaining was performed as described previously (Rouille *et al.*, 2006) using anti-C ACAP27 mouse mAbs and anti-E2 3/11 rat mAb, followed by species-specific-conjugated secondary antibodies. Image acquisition was carried out using an Axiophot 2 microscope (Zeiss) equipped with a camera. For confocal microscopy, double-label staining was performed with anti-E2 mouse mAb AP33 (IgG1) and anti-C mouse mAb ACAP27 (IgG2a) followed by Alexa 488-conjugated goat anti-mouse IgG2a and Alexa 555-conjugated goat anti-mouse IgG1. Fluorescent signals were collected with a Leica SP2 confocal microscope equipped with a PL APO × 100/1.4 immersion objective.

RESULTS

Production of infectious virus in Huh-7 cell line by a transcribed genomic JFH-1 RNA

In an attempt to generate higher infection titres for HCV, Huh-7 cells were electroporated with an *in vitro* transcribed genomic JFH-1 RNA (Wakita *et al.*, 2005). Transfected cells were then passaged every 5–7 days in order to maintain subconfluent cultures during the experiment. Immunofluorescence staining for capsid and E2 proteins revealed that the percentage of positive cells increased from 30 % at day 2 to 80 % at day 12, after two passages (Fig. 1a). These results suggest that the virus spread within the cell culture allowing the untransfected cells to be infected. Western blot analyses of transfected cells showed that the capsid and E2 proteins were still detected after 33 days (Fig. 1b) and even after 90 days (data not shown). Similar results were obtained for NS3 (data not shown). Virus released in the supernatant of transfected cells was then used to inoculate naïve Huh-7 cells. Immunofluorescence staining revealed that a low percentage of cells was positive (Fig. 1c), but after several passages, all cells were infected (data not shown). Controls for transfection and infection were also performed with JFH-1/ΔE1-E2 and JFH-1/GND. No infection was observed with these constructs as described previously (data not shown) (Wakita *et al.*, 2005). Titration of HCV RNA by RT-qPCR assay revealed that after the first passage of transfected cells the level of detection of HCV RNA was low (Table 1). However, the extracellular HCV RNA increased slowly in the supernatant of transfected cells reaching a maximal level of 2×10^6 genome equivalent (GE) ml^{-1} after four passages (P_4). In the same time, the infectious viral titre was only approximately 10^3 f.f.u. ml^{-1}. These results indicate that our Huh-7 cells can replicate JFH-1 RNA and can be infected with HCV particles. However, in transfected cells maintained in culture, the production of infectious viral particles remained low.

Increase in HCVcc infectivity after several rounds of infection

As a first approach, successive infections of naïve Huh-7 cells were performed to obtain higher titres of infectious virus. The scheme of infection is presented in Fig. 2. To follow the release of infectious virus particles in the supernatant, the viral RNA was extracted from supernatant and titrated by RT-qPCR. The successive infections, performed on Huh-7 cells with JFH-1 (noted I_1–I_6), led to a progressive release of viral genomes in the supernatant of inoculated cells, which reached a maximal level of 2.9×10^8 GE ml^{-1} after six successive infections (Table 1). Substantially more viral RNA was released into supernatant fluids of infected cells in I_5 or I_6 than in transfected cells. Interestingly, infectious titres ranging between 10^5 and 10^6 f.f.u. ml^{-1} were obtained at I_5 and I_6, indicating that a robust infection could be obtained with Huh-7 cell line after successive infections.

Identification of mutations in the JFH-1 virus produced in I_6

Since a highly infectious JFH-1 virus was obtained after several passages, we wanted to define whether some mutations were selected during the successive infections of Huh-7 cells. To identify potential mutations in JFH-1 isolate, total cellular RNAs were prepared from infected cells in I_6 and the full HCV RNA genome was sequenced by RT-PCR. This approach should allow us to determine the major modifications selected during the successive infections. Surprisingly, the sequencing revealed the presence of only three mutations that were located in the E2-coding sequence. Two of them were silent mutations found at positions 1843 and 1912 (G→A and U→C). The third mutation, at position 1942 (U→A), led to a change in amino acid from Asn 534 to a Lys (N534K), which is a potential site of *N*-glycosylation (N6 site) (Fig. 3a). Thus, to verify that the core glycosylation was modified in E2 of JFH-1 produced in I_6, the E2 glycoprotein resulting of the first transfection was compared with that resulting of the last infection (I_6). As confirmed by immunoblotting, a slight shift in the E2 migration was observed (Fig. 3b, lanes I_6 and wt). Furthermore, no differences in the migration

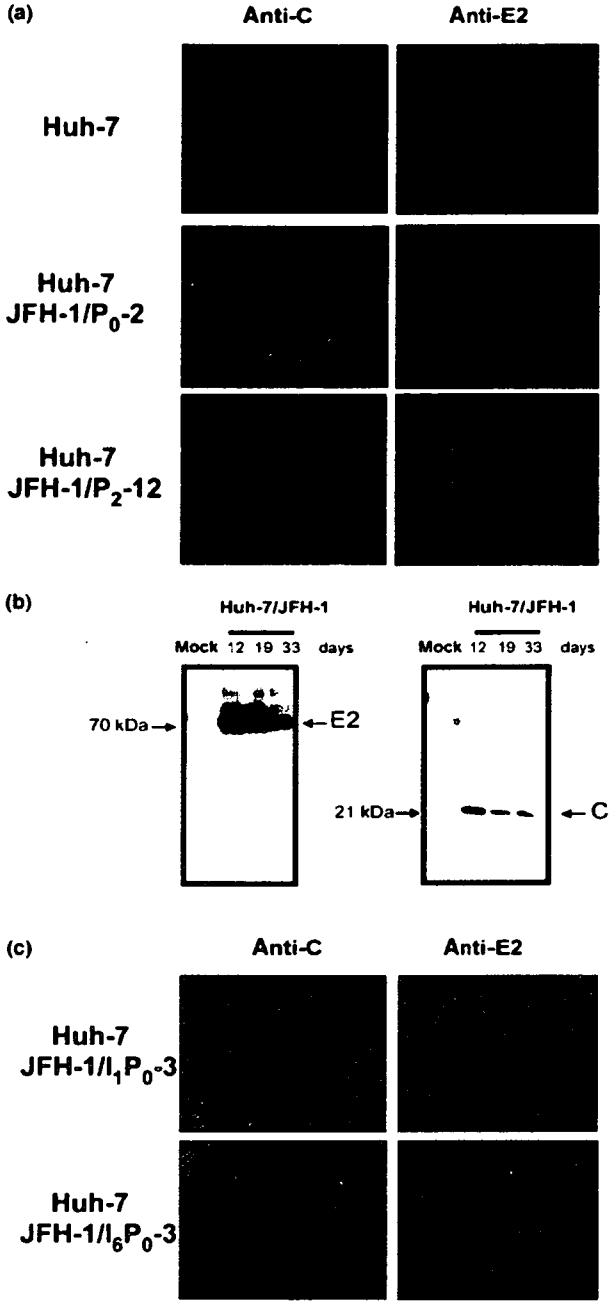

Fig. 1. Detection of HCV structural proteins in transfected Huh-7 cells. (a) Huh-7 cells were electroporated with the RNA transcript of JFH-1. Transfected cells grown on coverslips were fixed and processed for double-label immunofluorescence for capsid protein (green) and E2 (red) after 2 days (noted JFH-1/P_0-2). Transfected cells were passaged in order to maintain subconfluent cultures. After two passages transfected cells grown on coverslips were fixed and processed as described (noted JFH-1/P_2-12). Non-transfected Huh-7 cells were also used as control. (b) At indicated times, cell extracts were prepared and total proteins were separated by SDS-PAGE and revealed by Western blotting with mAbs ACAP27 (anti-C) and 3/11 (anti-E2). (c) Huh-7 cells were infected with supernatant of transfected or infected cells and processed 3 days later as described above (noted JFH-1/I_1P_0-3 and JFH-1/I_6P_0-3, respectively).

profiles of E2 were observed after deglycosylation by PNGase F treatment, indicating that the difference observed in the molecular mass of E2 is due to the glycosylation. Due to the higher infectious titre obtained with this final virus, we can speculate that the lack of a glycan at position N6 of E2 might favour a better interaction with an HCV receptor. Alternatively, we cannot exclude that this mutation improves the assembly and/or release of infectious particles.

N534K mutation facilitates the infection of JFH-1 in Huh-7 cells

We focused our subsequent analyses on the role of the N534K mutation in order to determine whether this mutation may favour the infection of Huh-7 cells with JFH-1 virus. The mutation N534K was therefore introduced into the parental JFH-1 sequence. The *in vitro* transcribed recombinant JFH-1 was electroporated into the human hepatoma cells, and the ability of this virus to propagate in naïve cells was examined. As shown in Table

Table 1. Titration of RNA and infectious viruses during the successive infections

Evolution of RNA and infectivity titres titrated by RT-qPCR [estimated in log/genome equivalent per ml (GE ml^{-1})] or determined by immunofluorescence (log/f.f.u. ml^{-1}) at P_1 of transfected or infected Huh-7 cells with JFH-1 and mutated JFH-1. Mean ± standard deviations have been calculated from three determinations. I, Infection.

Viruses		RNA titres log (GE ml^{-1})	Infectivity titres log (f.f.u. ml^{-1})
JFH-1	/	5.8±0.3	2.7±0.2
	/I_1	5.9±0.2	2.9±0.2
	/I_2	6.0±0.2	3.3±0.3
	/I_3	6.5±0.3	3.7±0.2
	/I_4	7.0±0.3	4.0±0.2
	/I_5	8.1±0.3	5.2±0.2
	/I_6	8.2±0.3	5.2±0.2
JFH-1/N6	/	5.3±0.2	2.4±0.3
	/I_1	5.7±0.3	2.3±0.2
	/I_2	7.0±0.3	3.9±0.2
JFH-1/CS	/	7.0±0.3	3.9±0.2
	/I_1	8.5±0.4	5.3±0.4
	/I_2	9.0±0.4	5.8±0.2
JFH-1/CS-N6	/	8.0±0.4	5.1±0.3
	/I_1	8.4±0.4	5.7±0.3

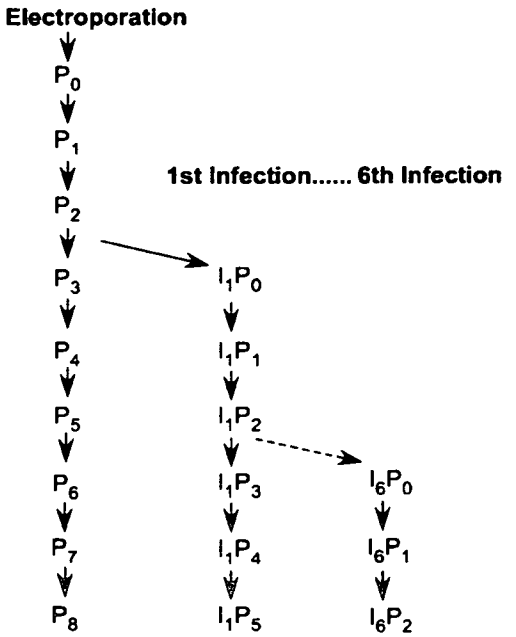

Fig. 2. Schematic representation of successive infections. The supernatant of transfected cells or infected cells prepared at 9–14 days was used for a new infection of naïve Huh-7 cells. Passaged transfected cells were noted P1, P2, etc., the first infection was noted I_1 with the corresponding passages, the second infection I_2 and so forth.

1, infectious virus in the supernatant of transfected cells was initially low. Indeed, the RNA and viral titres of the JFH-1/N6 (N534K) were initially comparable to the original JFH-1 virus. However, after only two successive amplifications in naïve cells, the JFH-1/N6 virus spread faster than the wild-type JFH-1 virus leading to a better production of infectious particles (Table 1). Together, these data indicate that the N534K mutation facilitates the amplification of JFH-1 virus in Huh-7 cells.

Release of infectious particles is improved by mutations in the capsid-coding sequence

Recently, a chimeric virus containing the genotype 1a capsid-coding sequence in the context of the full-length 2a sequence [JFH-1/C(+)6-1a2a] was constructed in order to analyse the expression of F protein (D. Delgrange, T. Wakita & C. Wychowski, unpublished data). Interestingly, higher levels of infectious particles were detected in the supernatant of cells transfected with the virus JFH-1/C(+)6-1a2a (1.5×10^8 GE ml^{-1}, 10^5 f.f.u. ml^{-1}), suggesting that some residues present in the genotype 1a capsid protein might improve the infectivity of JFH-1. Taking advantage of this result and of previous published data with FL-J6/JFH-1 chimeric construct (Lindenbach *et al.*, 2005), the capsid-coding sequences of genotype 1a, 2a (FL-J6) and 2a (JFH-1) were aligned to identify residues that might potentially improve the JFH-1 infectivity. A sequence alignment was performed as presented in Fig. 4 and differences in the amino acid sequence of JFH-1 capsid were identified at positions 20, 48, 81, 145, 151, 152, 172 and 173. We were particularly interested by the differences

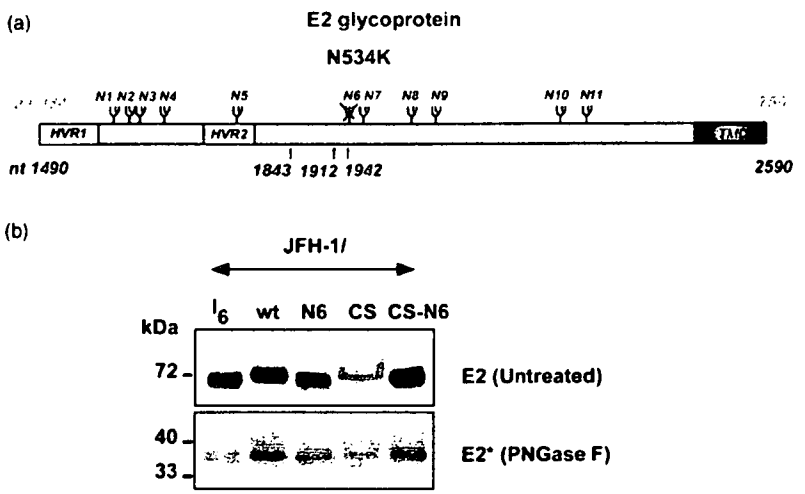

Fig. 3. Characterization of the N534K mutation. (a) A schematic diagram of the primary sequence of E2 glycoprotein is shown. E2 glycoprotein is located between aa 384 and 750 of JFH-1 polyprotein or between nt 1490 and 2590 of JFH-1 sequence. *N*-Glycosylated sites are indicated by branched structures and noted N1–N11. N534K is a modification of Asn→Lys residue of the glycosylation site N6 of E2. Numbers 1843, 1912 and 1942 indicate the 3 nt changes detected in the E2-coding sequence of JFH-1. (b) Analysis of glycans associated with HCV glycoprotein E2. Lysates of HCV-transfected cells [JFH-1 (wt), JFH-1/N6, JFH-1/CS and JFH-1/CS-N6] or HCV-infected cells (JFH-1/I_6) were prepared and total proteins were immunoprecipitated with anti-E2 mAb AP33. The immunoprecipitates were then treated or not treated with PNGase F. Proteins were separated by SDS-PAGE and then revealed by Western blotting with the anti-E2 mAb 3/11. E2* represents the unglycosylated protein.

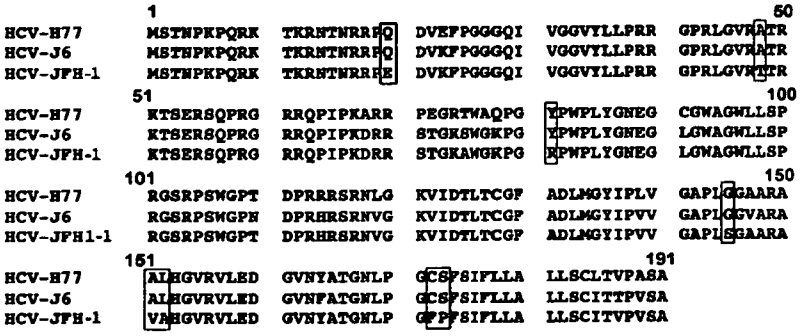

Fig. 4. Alignment of amino acids of capsid proteins of genotype 1a and 2a. The sequences of amino acids corresponding to the genotype 1a (HCV-H77) and genotype 2a (HCV-J6 and HCV-JFH-1) were aligned. The boxes indicate modifications that were detected in JFH-1 but not in strains J6 and H-77.

in amino acids at positions 172 and 173 because they correspond to drastic mutations in the context of JFH-1. Furthermore, another study relating to the genotype 2a capsid protein has shown that some modifications in the C-terminal 31 aa of core protein were important for its processing and/or its morphogenesis (Kato *et al.*, 2003b). Consequently, the mutations F172C and P173S (FP→CS) were introduced by site-directed mutagenesis in JFH-1 capsid-coding sequence to determine whether the release of infectious viral particles could be improved (Fig. 5a). HCV RNA was quantified and the secretion of particles was analysed by successive passages of the transfected cells or by successive infections on naïve Huh-7 cells as initially described in this study (Table 1). High viral titres of JFH-1/CS were obtained faster and were higher than those obtained with JFH-1. These data suggest that the replacement of FP to CS residues confers an advantage for the virus and these modifications might improve the morphogenesis and/or the release of viral particles.

A previous study has shown that the capsid protein and E2 glycoprotein do not colocalize in JFH-1 infected cells (Rouille *et al.*, 2006). We next wanted to determine whether the JFH-1/CS mutant might affect the subcellular localization of the capsid protein, leading to some colocalization with the envelope proteins. As shown in Fig. 5(b), no differences were observed between the two viruses in the intracellular distribution of the capsid protein and E2 glycoprotein. For both clones, E2 colocalized with ER markers, whereas capsid protein was associated with lipid droplets (data not shown). In addition, no differences were observed in the capsid protein when analysed by Western blotting (Fig. 5c). Consequently, no detectable differences in the capsid processing and intracellular localization were observed that could explain the enhanced production of viral particles.

N534K, F172C and P173S mutations improve the infection of JFH-1 in Huh-7 cells

In order to produce a higher infectious JFH-1 virus in cell culture, we introduced three mutations N534K, F172C and P173S into JFH-1 (JFH-1/CS-N6). Moreover, to visualize more efficiently the infectivity of this mutant on cell culture, Huh-7 cells were transfected with the *in vitro* transcribed JFH-1, JFH-1/N6, JFH-1/CS and JFH-1/CS-N6 RNAs (Fig. 6a) and the supernatants obtained at 3 days post-transfection were used in the infection of naïve Huh-7

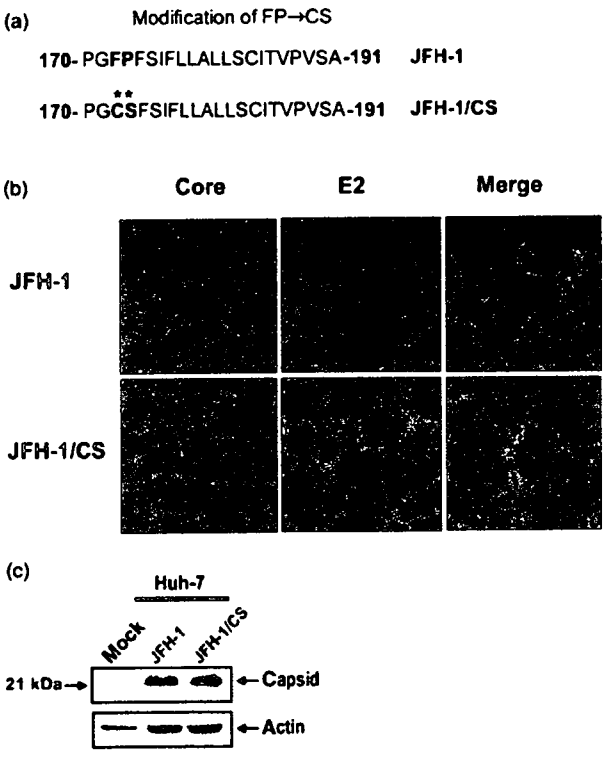

Fig. 5. Some characteristics of JFH-1/CS virus. (a) Modifications introduced in the capsid protein of JFH-1. The numbers at each side indicate the location of the first and last amino acid in the sequence. (b) Intracellular localization of HCV capsid and E2 proteins analysed by confocal immunofluorescence microscopy on cells infected either by JFH-1 or JFH-1/CS. (c) Total proteins of lysates of Huh-7 cells infected with JFH-1 or JFH-1/CS were separated by SDS-PAGE and revealed by Western blotting with the anti-C mAb ACAP27. Then, the membrane was stripped and processed for the detection of actin used as control.

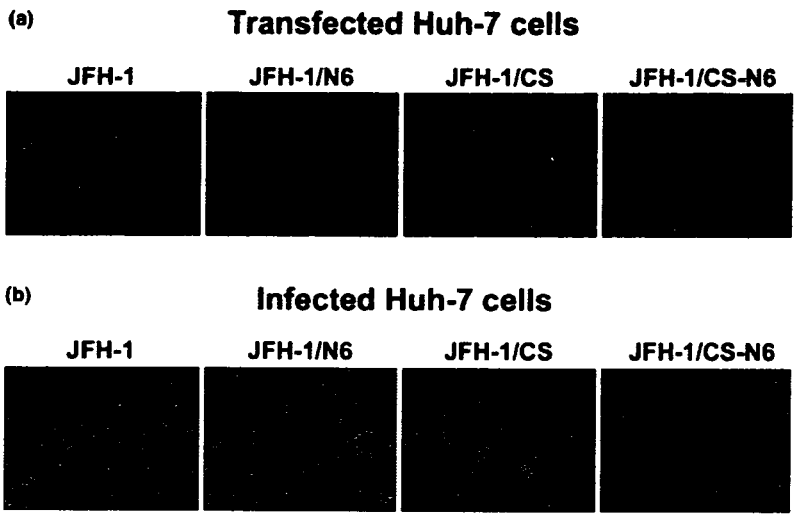

Fig. 6. Comparison of infectivity of each mutant by immunofluorescence. (a) Huh-7 cells were electroporated with RNA transcripts of JFH-1, JFH-1/N6, JFH-1/CS and JFH-1/CS-N6. Transfected cells were grown on coverslips. After 3 days, the cells were fixed and processed for double-label immunofluorescence for capsid protein (green) and nuclei (blue, Hoescht). (b) Naïve Huh-7 cells seeded on coverslips were infected with the supernatant obtained at 3 days post-transfection, and then fixed and processed at 3 days post-infection for double-label immunofluorescence for capsid protein (green) and nuclei (blue, Hoescht).

cells (Fig. 6b). The profile of E2 produced by each virus was also analysed before and after treatment with PNGase F and was consistent with predicted results (Fig. 3b). Our results suggest that the JFH-1/CS-N6 virus expands more rapidly and reaches higher titres than the JFH-1, JFH-1/N6 and JFH-1/CS viruses (Fig. 6b and Table 1). As for JFH-1/CS, no differences were observed in the intracellular distribution of the capsid protein and E2 glycoprotein of JFH-1/CS-N6 (data not shown). Furthermore, we also observed that after a single round of amplification the JFH-1/CS-N6 virus displayed an accelerated cytopathic effect, which was faster than what we observed for the JFH-1/CS virus (data not shown). These data suggested that the combined mutations located in the capsid- and E2-coding sequences resulted in an enhanced virus infectivity.

DISCUSSION

Although a low efficiency of infection has been detected by transfection of Huh-7 cells with the RNA generated from a genomic JFH-1 viral clone, this discovery has been a major breakthrough in HCV research (Wakita *et al.*, 2005). Different groups have developed robust cell culture systems for the propagation of HCV, and the data led to the conclusions that the Huh-7 cell culture is important for the propagation of the virus and that each Huh-7 cell line can display different permissiveness to the virus. In this study, we established a strong production of infectious HCV particles by using successive infections of naïve Huh-7 cells or by introducing specific mutations into the JFH-1 genome.

Contrary to what was observed in other groups (Lindenbach *et al.*, 2005; Zhong *et al.*, 2005), the robust production of HCVcc in our Huh-7 cell line was only obtained after several successive infections. Initially, the JFH-1 virus released into the culture medium after the first transfection was low, which was consistent with a previous report (Wakita *et al.*, 2005). It is worth noting that several passages of transfected cells did not change the viral titre. However, after repeated infections of naïve Huh-7 cells, analyses of HCV RNA in the medium of infected cells revealed an increase in the particle release, which was correlated with a higher titre of infectious virus. The direct sequencing of HCV RNA genome was used to determine the major modifications appearing in JFH-1. Surprisingly, the only coding mutation identified was an Asn to Lys mutation located at aa 534 (N534K) in E2. This modification, which is characterized as preventing the addition of an *N*-glycan at the E2 glycosylation site 6, may favour the interaction between HCV E2 glycoprotein and a cellular receptor. Indeed, the introduction of this mutation in JFH-1 leads to a higher infectious titre after only two successive infections. This suggests that the particles produced by JFH-1/N6 are more effective than those produced by JFH-1 for the reinfection of Huh-7 cells. This is also true when the mutation N534K is combined with the modifications FP→CS at positions 172 and 173 in JFH-1 (Fig. 6b). Consequently these mutations might lead to a better viral expansion on Huh-7 cells. There is some evidence that CD81 is essential for the entry of HCVpp (Hsu *et al.*, 2003; Lavillette *et al.*, 2005) or HCVcc (Lindenbach *et al.*, 2005; Zhong *et al.*, 2005) into hepatoma cells via an interaction with the HCV E2 glycoprotein (Pileri *et al.*, 1998). Residues critical for the CD81 binding have been identified in the HCV glycoprotein E2. These residues are located at positions 420, 437, 438, 441, 442, 529, 530 and 535 of E2 glycoprotein (Drummer *et al.*, 2006; Owsianka *et al.*, 2006). Moreover, replacement of Thr at the E2 glycosylation site 6 results in moderately increased CD81 binding (Owsianka *et al.*, 2006). This is consistent with the hypothesis that the loss of the *N*-linked E2 glycosylation site 6 favours a better exposure of E2 to the E2-binding site of CD81 and then the JFH-1 reinfection. In a similar way, Zhong *et al.*, (2006)

have identified a mutation located at aa 451 of HCV E2 glycoprotein, which displays an accelerated spreading of the virus. This G451A mutation is located between HVR1 and HVR2 of E2 in a region that has been reported to modulate the accessibility of the CD81-binding site (Roccasecca *et al.*, 2003).

In the present study, the substitution of amino acids FP→CS at positions 172 and 173 of the capsid protein leads to an increased viral production. The amino acids Cys and Ser appear to be important determinants for the spreading of the virus and they are probably involved in the morphogenesis and/or the release of the viral particles. The C172 and S173 residues are highly conserved among the HCV isolates. In a former *in vitro* study, we showed that several amino acids located at the C terminus of the capsid protein were important for the mature p21 protein, and FP→CS mutations showed a higher level of the immature capsid protein (p23) (Kato *et al.*, 2003b). The hydrophobic sequence at the C terminus of the capsid protein was described as the signal sequence necessary for the translocation of E1 glycoprotein into the ER lumen (Hijikata *et al.*, 1991). Recently, this signal sequence was also described as a substrate for signal peptide peptidase (SPP) (McLauchlan *et al.*, 2002). During the assembly of the virus, two consecutive membrane-dependent cleavages are responsible for the production of p23 and p21 forms of the capsid protein (Liu *et al.*, 1997). Detected in particles isolated from the blood of infected patients (Yasui *et al.*, 1998), the p21 mature capsid protein is produced by cleavage of the p23 immature capsid protein by a cellular protease identified as SPP (Hussy *et al.*, 1996; Lemberg & Martoglio, 2002; McLauchlan *et al.*, 2002). It may be hypothesized that the production of p23 and then p21 is important for the morphogenesis of the virus and the production of infectious viral particles. In this hypothesis, the immature capsid protein p23 would be necessary for an initial step of the particle formation, for example its accumulation and its oligomerization at the ER membrane where a progressive maturation would be introduced by SPP cleavage. The completion of the maturation of the viral particle could then occur after the budding process. However, immature capsid protein (p23) has not been detected in an *in vivo* study using JFH-1/CS probably due to the low production or a completion of the cleavage during the preparation of cell extracts. Additional experiments have to be conducted to understand the function of these amino acids in the morphogenesis of the viral particle.

In conclusion, the data presented in this study show that few modifications are sufficient for a more efficient production of HCVcc in Huh-7 cells. These mutations are located in the structural proteins and likely affect the recognition of a cellular receptor and/or the morphogenesis of the viral particle. Extensive modifications introduced in the C terminus of the capsid protein and analyses of the resulting viruses will help the understanding of the role of individual amino acids in particle assembly.

ACKNOWLEDGEMENTS

We thank Véronique Descamps, Sophana Ung, Tomoko Imamura and Sayoko Ishizeki for their technical assistances. We are grateful to J. F. Delagneau, A. Patel and J. McKeating for providing us with reagents. This work was supported by grants from the 'Agence Nationale de Recherche sur le Sida et les Hépatites virales' (ANRS) (C. W., Y. R., G. D.). T. W. was supported partially by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science and from the Ministry of Health, Labor, and Welfare of Japan; and by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation. D. D. was supported by fellowships from the ANRS. J. D. is an international scholar of the Howard Hughes Medical Institute.

REFERENCES

Bartosch, B., Dubuisson, J. & Cosset, F. L. (2003). Infectious hepatitis C virus pseudo-particles containing functional E1–E2 envelope protein complexes. *J Exp Med* **197**, 633–642.

Blight, K. J., Kolykhalov, A. A. & Rice, C. M. (2000). Efficient initiation of HCV RNA replication in cell culture. *Science* **290**, 1972–1974.

Castelain, S., Descamps, V., Thibault, V., Francois, C., Bonte, D., Morel, V., Izopet, J., Capron, D., Zawadzki, P. & other authors (2004). TaqMan amplification system with an internal positive control for HCV RNA quantitation. *J Clin Virol* **31**, 227–234.

Drummer, H. E., Boo, I., Maerz, A. L. & Poumbourios, P. (2006). A conserved Gly436-Trp-Leu-Ala-Gly-Leu-Phe-Tyr motif in hepatitis C virus glycoprotein E2 is a determinant of CD81 binding and viral entry. *J Virol* **80**, 7844–7853.

Flint, M., Maidens, C., Loomis-Price, L. D., Shotton, C., Dubuisson, J., Monk, P., Higginbottom, A., Levy, S. & McKeating, J. A. (1999). Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. *J Virol* **73**, 6235–6244.

Goffard, A., Callens, N., Bartosch, B., Wychowski, C., Cosset, F. L., Montpellier, C. & Dubuisson, J. (2005). Role of N-linked glycans in the functions of hepatitis C virus envelope glycoproteins. *J Virol* **79**, 8400–8409.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M. & Rice, C. M. (1993). Expression and identification of hepatitis C virus polyprotein cleavage products. *J Virol* **67**, 1385–1395.

Griffin, S. D., Beales, L. P., Clarke, D. S., Worsfold, O., Evans, S. D., Jaeger, J., Harris, M. P. & Rowlands, D. J. (2003). The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett* **535**, 34–38.

Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M. & Shimotohno, K. (1991). Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. *Proc Natl Acad Sci U S A* **88**, 5547–5551.

Houghton, M. (1996). Hepatitis C viruses. In *Fields Virology*, 3rd edn, pp. 1035–1058. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott-Raven.

Hsu, M., Zhang, J., Flint, M., Logvinoff, C., Cheng-Mayer, C., Rice, C. M. & McKeating, J. A. (2003). Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc Natl Acad Sci U S A* **100**, 7271–7276.

Hussy, P., Langen, H., Mous, J. & Jacobsen, H. (1996). Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. *Virology* **224**, 93–104.

Kato, T., Furusaka, A., Miyamoto, M., Date, T., Yasui, K., Hiramoto, J., Nagayama, K., Tanaka, T. & Wakita, T. (2001). Sequence analysis of

hepatitis C virus isolated from a fulminant hepatitis patient. *J Med Virol* **64**, 334–339.

Kato, T., Date, T., Miyamoto, M., Furusaka, A., Tokushige, K., Mizokami, M. & Wakita, T. (2003a). Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* **125**, 1808–1817.

Kato, T., Miyamoto, M., Furusaka, A., Date, T., Yasui, K., Kato, J., Matsushima, S., Komatsu, T. & Wakita, T. (2003b). Processing of hepatitis C virus core protein is regulated by its C-terminal sequence. *J Med Virol* **69**, 357–366.

Kolykhalov, A. A., Agapov, E. V., Blight, K. J., Mihalik, K., Feinstone, S. M. & Rice, C. M. (1997). Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* **277**, 570–574.

Lavillette, D., Tarr, A. W., Voisset, C., Donot, P., Bartosch, B., Bain, C., Patel, A. H., Dubuisson, J., Ball, J. K. & other authors (2005). Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. *Hepatology* **41**, 265–274.

Lemberg, M. K. & Martoglio, B. (2002). Requirements for signal peptide peptidase-catalyzed intramembrane proteolysis. *Mol Cell* **10**, 735–744.

Lindenbach, B. D., Evans, M. J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., Hynes, R. O., Burton, D. R. & other authors (2005). Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623–626.

Liu, Q., Tackney, C., Bhat, R. A., Prince, A. M. & Zhang, P. (1997). Regulated processing of hepatitis C virus core protein is linked to subcellular localization. *J Virol* **71**, 657–662.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110–113.

Major, M. E., Rehermann, B. & Feinstone, S. M. (2001). Hepatitis C viruses. In *Fields Virology*, 4th edn, pp. 1127–1161. Edited by D. M. Knipe, P. M. Howley, R. M. Chanock, T. P. Monath, B. Roizman & S. E. Straus. Philadelphia, PA: Lippincott-Raven.

McLauchlan, J., Lemberg, M. K., Hope, G. & Martoglio, B. (2002). Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J* **21**, 3980–3988.

Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T. & Sato, J. (1982). Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. *Cancer Res* **42**, 3858–3863.

Op De Beeck, A., Cocquerel, L. & Dubuisson, J. (2001). Biogenesis of hepatitis C virus envelope glycoproteins. *J Gen Virol* **82**, 2589–2595.

Owsianka, A. M., Timms, J. M., Tarr, A. W., Brown, R. J., Hickling, T. P., Szwejk, A., Bienkowska-Szewczyk, K., Thomson, B. J., Patel, A. H. & other authors (2006). Identification of conserved residues in the E2 envelope glycoprotein of the hepatitis C virus that are critical for CD81 binding. *J Virol* **80**, 8695–8704.

Pavlovic, D., Neville, D. C., Argaud, O., Blumberg, B., Dwek, R. A., Fischer, W. B. & Zitzmann, N. (2003). The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. *Proc Natl Acad Sci U S A* **100**, 6104–6108.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., Houghton, M., Rosa, D. & other authors (1998). Binding of hepatitis C virus to CD81. *Science* **282**, 938–941.

Poynard, T., Yuen, M. F., Ratziu, V. & Lai, C. L. (2003). Viral hepatitis C. *Lancet* **362**, 2095–2100.

Roccasecca, R., Ansuini, H., Vitelli, A., Meola, A., Scarselli, E., Acali, S., Pezzanera, M., Ercole, B. B., McKeating, J. & other authors (2003). Binding of the hepatitis C virus E2 glycoprotein to CD81 is strain specific and is modulated by a complex interplay between hypervariable regions 1 and 2. *J Virol* **77**, 1856–1867.

Rouille, Y., Helle, F., Delgrange, D., Roingeard, P., Voisset, C., Blanchard, E., Belouzard, S., McKeating, J., Patel, A. H. & other authors (2006). Subcellular localization of hepatitis C virus structural proteins in a cell culture system that efficiently replicates the virus. *J Virol* **80**, 2832–2841.

Roussel, J., Pillez, A., Montpellier, C., Duverlie, G., Cahour, A., Dubuisson, J. & Wychowski, C. (2003). Characterization of the expression of the hepatitis C virus F protein. *J Gen Virol* **84**, 1751–1759.

Sumpter, R., Jr, Loo, Y. M., Foy, E., Li, K., Yoneyama, M., Fujita, T., Lemon, S. M. & Gale, M., Jr (2005). Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol* **79**, 2689–2699.

Thomas, D. L. (2000). Hepatitis C epidemiology. *Curr Top Microbiol Immunol* **242**, 25–41.

Tsukiyama-Kohara, K., Iizuka, N., Kohara, M. & Nomoto, A. (1992). Internal ribosome entry site within hepatitis C virus RNA. *J Virol* **66**, 1476–1483.

Varaklioti, A., Vassilaki, N., Georgopoulou, U. & Mavromara, P. (2002). Alternate translation occurs within the core coding region of the hepatitis C viral genome. *J Biol Chem* **277**, 17713–17721.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G. & other authors (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **11**, 791–796.

Walewski, J. L., Keller, T. R., Stump, D. D. & Branch, A. D. (2001). Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. *RNA* **7**, 710–721.

Xu, Z., Choi, J., Yen, T. S., Lu, W., Strohecker, A., Govindarajan, S., Chien, D., Selby, M. J. & Ou, J. (2001). Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. *EMBO J* **20**, 3840–3848.

Yanagi, M., Purcell, R. H., Emerson, S. U. & Bukh, J. (1997). Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc Natl Acad Sci U S A* **94**, 8738–8743.

Yanagi, M., Purcell, R. H., Emerson, S. U. & Bukh, J. (1999). Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. *Virology* **262**, 250–263.

Yasui, K., Wakita, T., Tsukiyama-Kohara, K., Funahashi, S. I., Ichikawa, M., Kajita, T., Moradpour, D., Wands, J. R. & Kohara, M. (1998). The native form and maturation process of hepatitis C virus core protein. *J Virol* **72**, 6048–6055.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & other authors (2005). Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A* **102**, 9294–9299.

Zhong, J., Gastaminza, P., Chung, J., Stamataki, Z., Isogawa, M., Cheng, G., McKeating, J. & Chisari, F. V. (2006). Persistent hepatitis C virus infection in vitro: co-evolution of virus and host. *J Virol* **80**, 11082–11093.

JOURNAL OF VIROLOGY, Aug. 2007, p. 8814–8820
0022-538X/07/$08.00+0 doi:10.1128/JVI.02824-06

Vol. 81, No. 16

Monitoring the Antiviral Effect of Alpha Interferon on Individual Cells[▽]

Chon Saeng Kim,[1] Jong Ha Jung,[1] Takaji Wakita,[2] Seung Kew Yoon,[3] and Sung Key Jang[1]*

PBC, Department of Life Science, Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea[1]; Department of Virology II, National Institute of Infectious Diseases, Shinjuku, Tokyo 162-8640, Japan[2]; and Department of Internal Medicine, College of Medicine, Catholic University of Korea, Seoul, Republic of Korea[3]

Received 21 December 2006/Accepted 21 May 2007

An infectious hepatitis C virus (HCV) cDNA clone (JFH1) was generated recently. However, quantitative analysis of HCV infection and observation of infected cells have proved to be difficult because the yield of HCV in cell cultures is fairly low. We generated infectious HCV clones containing the convenient reporters green fluorescent protein (GFP) and *Renilla* luciferase in the NS5a-coding sequence. The new viruses responded to antiviral agents in a dose-dependent manner. Responses of individual cells containing HCV to alpha interferon (IFN-α) were monitored using GFP-tagged HCV and time-lapse confocal microscopy. Marked variations in the response to IFN-α were observed among HCV-containing cells.

It is estimated that 170 million individuals worldwide and about 1% of the population in developed countries are chronically infected with hepatitis C virus (HCV) (17). Most acute HCV infections become chronic, and some progress to liver cirrhosis or hepatocellular carcinoma (1, 7). A protective vaccine does not yet exist, and therapeutic options are limited. Alpha interferon (IFN-α) in combination with ribavirin is the only recommended therapy (5).

HCV contains a single-stranded, positive-sense RNA genome of approximately 9.6 kb, which encodes a polyprotein that is flanked by nontranslated regions at its 5′ and 3′ ends (2). The polyprotein precursor is co- and posttranslationally processed by cellular and viral proteases to yield the mature structural and nonstructural proteins, which are arranged in the sequence NH_2-C-E1-E2-P7-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH.

The availability of a cell culture system is a prerequisite to study the proliferation cycle of a virus and to devise strategies for prophylactic and therapeutic interventions (3). The most recent advance is the development of a virus production system that is based on transfection of the human hepatoma cell line Huh 7 with genomic HCV RNA (JFH1) isolated from a patient with fulminant hepatitis (11, 16, 18). This cell culture-based model allows the study of all stages of the HCV life cycle. Recently, two studies demonstrated the mechanism of HCV entry into tissue culture cells using this infection system (9, 15). To facilitate studies of HCV infection, those authors generated luciferase reporter viruses using heterologous controlling elements such as the internal ribosome entry site of encephalomyocarditis virus and the 2A protease of foot-and-mouse disease virus.

In the present study, we generated new infectious HCV clones containing reporters without addition of heterologous sequences to facilitate expression of viral gene products. We incorporated the green fluorescent protein (GFP) and *Renilla* luciferase (Rluc) reporters after amino acid 2394 (amino acid 418 of NS5a) of the JFH construct (Fig. 1A). Insertion of heterologous sequences at this insertion site has previously been shown to allow replication of subgenomic replicons (13).

First, we compared the parental JFH1 genome with its derivatives containing GFP or Rluc reporter genes (JFH 5a-GFP and JFH 5a-Rluc) in terms of viral protein expression in transfected cells (Fig. 1B). RNAs transcribed in vitro were introduced into the Huh 7.5.1 cell line (18) by electroporation as described by Wakita et al. (16). Three days after transfection, cell lysates were prepared and the levels of the NS5a protein and core protein were assessed by Western blot analysis using anti-NS5a and anticore antibodies (gifts from Ralf Bartenschlager, University of Heidelberg) (Fig. 1B). NS5a-GFP and NS5a-Rluc fusion proteins with the predicted molecular masses were well expressed, as shown in Fig. 1B (lanes 3 and 4, respectively). Similar levels of core protein were expressed in the cells transfected with JFH and JFH 5a-GFP RNAs (Fig. 1B, lanes 1 and 3). Interestingly, greater amounts of core protein were observed in cells transfected with JFH 5a-Rluc RNA than in cells transfected with JFH RNA (compare lane 4 with lane 1 in Fig. 1B). More cells transfected with JFH and JFH 5a-GFP RNAs died compared with cells transfected with JFH 5a-Rluc RNA, for unknown reasons (data not shown). This cytopathic effect of JFH and JFH 5a-GFP RNAs may be attributed to the difference in the amount of viral protein. However, neither NS5a nor core protein was detected in cells transfected with JFH Pol^- RNA, which was used as a negative control because it contains a mutation at the catalytic site of the RNA polymerase NS5b (Fig. 1B, lane 2).

Replication of HCV was also monitored using the reporter Rluc integrated into NS5a in the JFH 5a-Rluc construct (Fig. 1C). At 3 days after transfection, luciferase activity in the cells transfected with JFH 5a-Rluc RNA increased by about 1,000-fold compared with the activity in the cells transfected with the JFH, JFH Pol^-, or JFH 5a-GFP RNA (Fig. 1C).

We then examined the subcellular localization of core and NS5a-GFP proteins in cells transfected with JFH, JFH 5a-

* Corresponding author. Mailing address: PBC, Department of Life Science, Pohang University of Science and Technology, San31, Hyoja-Dong, Pohang 790-784, Republic of Korea. Phone: 82-54-279-2298. Fax: 82-54-279-8009. E-mail: sungkey@postech.ac.kr.
▽ Published ahead of print on 30 May 2007.

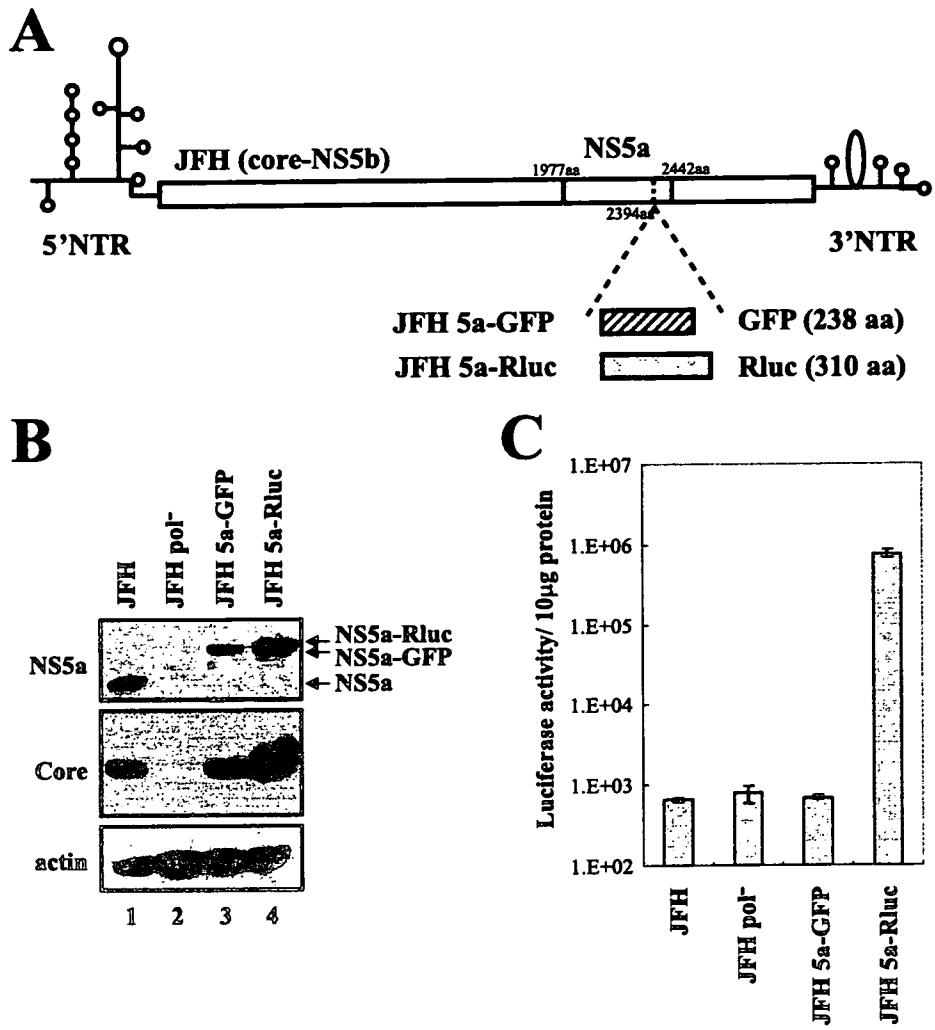

FIG. 1. Generation of infectious HCV containing GFP or Rluc fused with NS5a. (A) Schematic diagrams of JFH and its derivatives containing the GFP or Rluc gene within the NS5a gene. NTR, nontranslated region; aa, amino acids. (B) Western blot analysis of Huh 7.5.1 cells transfected with JFH, JFH Pol$^-$, JFH 5a-GFP, or JFH 5a-Rluc RNA. Protein levels were analyzed by Western blotting with anti-NS5a, anticore, or antiactin antibodies. (C) The expression of NS5a-Rluc was monitored by measuring luciferase activity in the cells transfected with JFH, JFH Pol$^-$, JFH 5a-GFP, or JFH 5a-Rluc RNA. The luciferase activities in the cells were normalized by the protein concentrations determined by the Bradford assay. Experiments were performed three times. Means and standard deviations are shown.

GFP, or JFH 5a-Rluc RNAs (Fig. 2A). Three days after transfection, cells were fixed and fluorescence microscopy was performed as described previously (8). Core protein was visible in a ring-like pattern around lipid droplets in the JFH RNA-transfected cells (Fig. 2A, panel a; see Fig. S1A at http://www.postech.ac.kr/dept/life/mv1/figure_s1.pdf), as previously reported (14). The same patterns of localization of core protein were observed in the cells transfected with JFH 5a-GFP and JFH 5a-Rluc RNAs (compare panels d and g with panel a in Fig. 2A). No signal was detected by the antibody against core protein in cells transfected with JFH Pol$^-$ RNA (data not shown). Huh 7.5.1 cells transfected with JFH 5a-GFP RNA displayed bright punctate patterns in the cytoplasm (Fig. 2A, panel e). This 5a-GFP fluorescence was perfectly colocalized with the signal of NS5a displayed by an anti-NS5 monoclonal antibody (AUSTRAL Biologicals) (Fig. 2B). Similar cytoplasmic punctate patterns of NS5a protein were observed in the cells transfected with JFH and JFH 5a-Rluc RNAs (compare panels a and b in Fig. S1B at http://www.postech.ac.kr/dept/life/mv1/figure_s1.pdf with panel a in Fig. 2B). GFP fluorescence was not detected in cells transfected with other constructs (Fig. 2A, panels b and h). A partial colocalization of core and NS5a-GFP, shown as yellow signals, was observed when the core and NS5a-GFP images were merged (Fig. 2A, panel f). The pattern of colocalization of core and NS5a proteins was similar to that of core and NS3 proteins reported by Rouille et al. (14).

Next, we assessed the ability of the reporter constructs to release infectious virion particles and whether it was possible to quantify their infectivity using fluorescence microscopy and a luciferase assay. We transfected the JFH, JFH Pol$^-$, JFH 5a-GFP, and JFH 5a-Rluc RNAs into Huh 7.5.1 cells and then harvested the culture supernatants at 8 days after transfection.