The Lipid Droplet Binding Domain of Hepatitis C Virus Core Protein Is a Major Determinant for Efficient Virus Assembly*

Anna Shavinskaya1, Steeve Boulant3,1, François Penin6, John McLauchlan2, and Ralf Bartenschlager1,2

From the 1Department of Molecular Virology, University of Heidelberg, 69120 Heidelberg, Germany, 2Medical Research Council Virus Unit, Institute of Virology, Church Street, Glasgow G11 5JR, Scotland, United Kingdom, and 3Institut de Biologie et Chimie des Protéines, UMR 5086, CNRS, Université de Lyon, IFR 128, BioSciences Lyon-Gerland, F-69397 Lyon, France

Hepatitis C virus core protein forms the viral capsid and is targeted to lipid droplets (LDs) by its domain 2 (D2). By using a comparative analysis of two hepatitis C virus genomes (JFH1 and Jc1) differing in their level of virus production in cultured human hepatoma cells, we demonstrate that the core of the genotype 2a isolate J6 that is present in Jc1 mediates efficient assembly and release of infectious virions. Mapping studies identified a single amino acid residue in D2 as a major determinant for enhanced assembly and release of infectious Jc1 particles. Confocal microscopy analyses demonstrate that core protein in JFH1-replicating cells co-localizes perfectly with LDs and induces their accumulation in the perinuclear area, whereas no such accumulation of LDs and only a partial co-localization of core and LDs were found with the Jc1 genome. By using a fluorescence recovery after photobleaching assay, we found that green fluorescent protein-tagged D2 variants are mobile on LDs and that J6- and JFH1-D2 differ in their mobility. Taken together, our results demonstrate that the binding strength of the D2 domain of core for LDs is crucial for determining the efficiency of virus assembly.

HCV3 infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. About 170 million individuals worldwide are currently infected with HCV (1). A protective vaccine does not exist to date, and the therapeutic options are still limited (2). HCV has been classified in the Hepacivirinae genus within the family Flaviviridae (3). The viral genome is a 9.6-kb plus-strand RNA, which encodes a polyprotein of about 3,000 amino acids in a single open reading frame flanked at the 5′ and 3′ ends by nontranslated regions (NTRs). The NTRs are required for RNA translation and replication (reviewed in Refs. 4 and 5). An internal ribosome entry site resides in the 5′ NTR, which allows expression of the polyprotein. It is co- and post-translationally processed by cellular and viral proteases to yield the mature structural and nonstructural (NS) proteins. The structural proteins include the core protein, which forms the viral capsid, and the envelope glycoproteins E1 and E2. They are separated from the NS proteins by p7, which is assumed to be a viroporin. The NS proteins include the NS2-3 autoprotease, the NS3 serine protease, a nucleotide triphosphatase/RNA helicase located in the C-terminal two-thirds of NS3, the NS4A serine-protease cofactor, NS4B, which induces membrane alterations, the RNA binding protein NS5A, and the NS5B RNA-dependent RNA polymerase (reviewed in Refs. 4–6).

The HCV core protein is a highly basic, RNA-binding protein. It is 191 aa in length and consists of three distinct domains: an N-terminal hydrophilic domain 1 (D1) formed by the first 117 aa; a hydrophobic D2 directly C-terminal of D1 and reaching to about aa 169; and a highly hydrophobic domain 3 (D3) spanning the C-terminal 20 aa, which serves as the signal peptide of the C-terminal E1 protein (7, 8). This immature form of core protein (p23) generated by the primary cleavage at the C terminus by signal peptidase (SP) undergoes additional C-terminal processing leading to the removal of most or all of the D3 by the intramembrane-cleaving protease signal peptide peptidase (SPP) (9). The resulting mature p21 core, whose C terminus has not yet been mapped precisely, thus includes D1 and D2. Mature core is a dimeric, α-helical protein exhibiting features that are consistent with those of a membrane protein (7).

D1 contains numerous positively charged aa residues and is mainly involved in RNA binding and oligomerization of the core protein (7). In addition, D1 is involved in interaction with numerous cellular factors and thus may contribute to alterations of host cell functions upon HCV infection (reviewed in Refs. 8 and 10). The N terminus of D1 contains immunodominant antigenic sites and a helix-loop-helix motif between aa residues 17 and 37 (11). Folding of D1 appears to depend on the presence of D2 (7), which consists of two amphipathic α-helices connected by a hydrophobic loop (HL) (12). D2 is known to mediate core association with lipid droplets (LDs) and endoplasmic reticulum (ER) membranes (12–15). The interaction between HCV core and LDs in the viral replication cycle has not
yet been elucidated in detail, but recent evidence suggests that LDs are involved in the production of infectious virus particles (16, 17).

In this study, we investigate the role of HCV core protein in virus particle production. Using the novel HCV infection system and a combination of reverse genetics and biochemical studies, we demonstrate that D2 of core protein is a major determinant for high titer virus production. We characterized the essential role of D2 for efficient assembly and release of infectious virions and provide novel insights into the role of core and its interaction with LDs for virus morphogenesis.

EXPERIMENTAL PROCEDURES

Sequence Analyses—Sequence analyses were performed using the European HCV data base (euHCVdb) website facilities at the Institute de Biologie et Chimie des Protéines (18). Multiple sequence alignments and amino acid conservation were carried out with the Clustal W program using default parameters (19). The repertoire of residues at each amino acid position and their frequencies observed in natural sequence variants were computed by using the tool “Extract” in the euHCVdb.

Plasmids—Plasmids pFK-JFH1, pFK-JFH1ΔE1E2, pFK-Jc1, and pFK-Jc1Δp7 have been described recently (20–22). Plasmids expressing GFP-D2 and pSFV/CE1E2 have been described in Ref. 14. Individual mutants were generated by standard PCR-based cloning techniques, and DNA fragments were verified by nucleotide sequence analysis. Detailed cloning information is available upon request.

Cell Culture—Huh7-derived cell clones Huh7.5 and Huh7-Lunet that are both highly permissive for HCV RNA replication (24, 25) were used for transfection and infection assays. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 2 mM L-glutamine, nonessential amino acids, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal calf serum (DMEM complete). Because of high permissiveness for HCV RNA replication upon transfection and infection experiments, most experiments were performed with Huh7-Lunet cells. Huh7.5 cells were primarily used in case of infection experiments because of their high level CD81 expression (26).

In Vitro Transcription and RNA Transfection—For in vitro transcription, SFV and HCV constructs were linearized with SpeI and MluI, respectively, whereas pSFV-JFH1CE1E2 was linearized with SapI. In vitro transcription reaction mixtures with SFV plasmids contained 1 unit of 5× SP-6 buffer (Promega), 3.125 mM of each nucleotide, 1 unit of RNasin (Promega, Mannheim, Germany) per μl, 1 mM cap analogue m7G(5′)ppp(5′)G (Roche Applied Science) per μl, 0.1 μg of plasmid DNA/μl, and 1.8 units of SP-6 RNA polymerase (Promega) per μl. After 2 h at 37 °C, an additional 1 unit of SP-6 RNA polymerase/μl was added, and the reaction mixture was incubated for another 2 h. Transcription was terminated by a 30-min incubation at 37 °C with 1.2 units of RNase-free DNase (Promega) per μg of plasmid DNA. In vitro transcription and electroproportion of HCV RNAs has been described elsewhere (27). Transfection efficiency of authentic genomes was controlled by measurement of intracellular core amounts 4 h post-transfection by using core ELISA. For SFV constructs, transfection efficiency was determined by core-specific indirect immunofluorescence analysis 16 h post-transfection.

Transfection of Enhanced GFP Plasmids—Enhanced GFP DNA constructs were transfected into Huh7-Lunet cells by using Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. Cells were incubated overnight at 37 °C and analyzed 24 h after transfection.

Intra- and Extracellular Infectivity Assay—Cell-associated infectivity was determined according to a published protocol (28). Briefly, cells were extensively washed with PBS, scraped off the plate, and centrifuged for 5 min at 400 × g. Cell pellets were resuspended in 1 ml of DMEM complete and subjected to three cycles of freezing and thawing using liquid nitrogen and a thermo block set to 37 °C. Samples were then centrifuged at 10,000 × g for 10 min at 4 °C to remove cell debris. Infectivity titers of these lysates and cell culture supernatants were determined by using limited dilution assay on Huh7.5 cells as described recently (27, 29). Tissue culture 50% infective dose (TCID50) was calculated according to the method of Spearman (30).

Quantitative Detection of HCV Core Protein by ELISA—HCV core protein was quantified using the Trak-C Core ELISA (Ortho Clinical Diagnostics, Neckargemünd, Germany) according to the instructions of the manufacturer. For quantification of intracellular core protein amounts, cells were lysed directly on the plate with 1 ml of ice-cold lysis buffer (0.1% Triton X-100, 25 mM glycyglycine, 15 mM MgSO4, 4 mM EGTA, and 1 mM dithiothreitol, pH 7.8). Lysates were cleared at 20,000 × g for 10 min, and the amount of core was measured at a dilution of 1:100 in PBS.

Indirect Immunofluorescence—Transfected cells were seeded on glass coverslips in 24-well plates at a density of ~2 × 104 per well. At time points specified under “Results,” cells were fixed by 20 min of treatment with methanol at ~ −20 °C or 4% paraformaldehyde. After washing with PBS and blocking with PBS containing 5% normal goat serum, cells were incubated with primary antibody diluted in PBS/goat serum for 1 h at room temperature. Cells were washed extensively with PBS and then incubated with conjugated secondary antibody (either anti-rabbit or anti-sheep IgG) for 1 h at room temperature in darkness. DNA was stained with 4′,6′-diamidino-2-phenylindole (Molecular Probes, Karlsruhe, Germany) for 1 min at room temperature. After washing with PBS, cells were mounted on slides using SlowFade® Gold antifade reagent (Invitrogen).

For the three-dimensional reconstructions in Fig. 5B, cells were imaged with an Ultraview ERS spinning disk (PerkinElmer Life Sciences) on a Nikon TE2000-E inverted confocal microscope equipped with a Plan-Apochromat VC 100 l (NA 1.4). Channels were recorded sequentially onto an EM-CCD camera using 488 nm excitation, 527/55 nm emission and 568 nm excitation, 615/70 nm emission for BODIPY™ and Alexa 568 labels, respectively. Optical slices were acquired at 0.15-μm Z spacing generating a stack of ~30–40 slices per cell. These

---

4 S. Boulant, A. Shavinskaya, F. Penin, P. Targett-Adams, R. Bartenschlager, and J. McLaughlan, submitted for publication.
Role of HCV Core Domain 2 in Virus Assembly

stacks were deconvolved based on a theoretical point spread function using Huygens Essential software version 3.0 (Scientific Volume Imaging BV). Three-dimensional projections of deconvoluted data were generated as Iso-Data Surface renderings using the same software.

Antibodies—Antibodies used to detect HCV core (rabbit antisera R308 and C-830) and human adipose differentiation-related protein have been described previously (14, 31, 32).

Drug Treatment of Cells—Huh7-Lunet cells were propagated in DMEM Complete and treated with MG132 as described previously (9). For treatment with (Z-LL)_2 ketone, Huh7-Lunet cells were propagated in DMEM Complete and treated 3 h post-electroporation with complete DMEM containing 100 μM of the drug for 14 h (9, 33).

Preparation of Cell Extracts, PAGE, and Western Blot Analysis—To prepare extracts, electroporated cells were harvested by removing the growth medium and washing the cell monolayers with PBS. Cells were scraped into sample buffer (160 mM Tris, pH 6.7, 2% SDS, 700 mM β-mercaptoethanol, 10% glycerol, 0.004% bromphenol blue) and solubilized at a concentration of ~4 × 10^6 cell eq/ml sample buffer. Samples were sonicated (Heinemann Ultraschall and Labortechnik) and heated to 98 °C for 5 min to fully denature proteins and nucleic acids. Samples were loaded onto a 12.5% SDS-polyacrylamide gel, and after electrophoresis proteins were transferred to polyvinylidene fluoride membrane (PerkinElmer Life Sciences). Blots were blocked overnight at 4 °C in blocking solution (5% milk powder and 0.5% Tween 20 in PBS). Incubation with the primary antibody was performed in blocking solution for 1 h at room temperature. Blots were washed three times for 10 min in washing solution (0.5% Tween 20 in PBS), incubated for 1 h with the horseradish peroxidase-conjugated secondary antibody in blocking solution, and washed as described above. Antibody-protein complexes were detected using the ECL Plus Western blotting detection system (Amersham Biosciences).

FRAP—Huh7 cells, seeded onto 35-mm glass bottom microwell dishes (MatTek Cultureware), were transfected with plasmids encoding the GFP-tagged proteins by using Lipofectamine (Invitrogen). FRAP analysis and image recording were conducted with an LSM510 META inverted confocal microscope (Zeiss) at 37 °C in a humidified chamber with an atmosphere of 5% CO_2. Images were recorded with a Plan-Apo-microscope (Zeiss) at 37 °C in a humidified chamber with an atmosphere of 5% CO_2. Images were recorded with a Plan-Apo-microscope (Zeiss) at 37 °C in a humidified chamber with an atmosphere of 5% CO_2. Images were recorded with a Plan-Apo-microscope (Zeiss) at 37 °C in a humidified chamber with an atmosphere of 5% CO_2. Images were recorded with a Plan-Apo-microscope (Zeiss) at 37 °C in a humidified chamber with an atmosphere of 5% CO_2. Imaging was stored with a ZEN software (Zeiss) as Z-stacks. The cutoff of the TCID50 assay (20). Because of the chimeric nature of the Jc1 genome, we assumed that the higher titer and faster kinetics of virus production must be due to determinants residing in the J6 sequence. To identify these determinants, we initially focused on the core protein and constructed J6/JFH1 chimeras as follows: one analogous to Jc1 but encoding the core gene of the JFH1 isolate (JFH1core/Jc1), and another containing the J6 core coding region in the context of the JFH1 genome (Fig. 1A, J6core/JFH1). These chimeras were transfected into cells of the highly permissive cell clone Huh7.5, in parallel with Jc1, after abbreviated to J6) and the remainder of the genome of the JFH1 isolate (Fig. 1A), replicates to similar levels as JFH1wt but supports much higher production of infectious HCV particles (20). Because of the chimeric nature of the Jc1 genome, we assumed that the higher titer and faster kinetics of virus production must be due to determinants residing in the J6 sequence. To identify these determinants, we initially focused on the core protein and constructed J6/JFH1 chimeras as follows: one analogous to Jc1 but encoding the core gene of the JFH1 isolate (JFH1core/Jc1), and another containing the J6 core coding region in the context of the JFH1 genome (Fig. 1A, J6core/JFH1). These chimeras were transfected into cells of the highly permissive cell clone Huh7.5, in parallel with Jc1,
HCV first detectable 24 h after transfection were found in the supernatant of JFH1wt-transfected cells, and virus titers increased to \( \sim 10^4 \) by 72 h. By contrast, Jc1-transfected cells released high levels of virus with titers of \( > 10^6 \) by 24 h, and the amount of infectious HCV detected at later times remained at approximately similar titers. Cells transfected with the JFH1core/Jc1 chimera released up to 100-fold less infectious HCV as compared with the parental Jc1 by 48 and 72 h, arguing that J6 core supports more efficient virus production. In agreement with this assumption, insertion of J6 core into JFH1 enhanced virus titers at least 100-fold as compared with the parental JFH1 strain. Titers achieved with J6core/JFH1 were comparable with those obtained with the Jc1 chimera, although kinetics of virus release was slower. Replication levels as determined by the amounts of intracellular core protein by ELISA were similar for all tested genomes (data not shown). These results thus show that J6 core contains determinant(s), which enhance virus production.

Core Domain 2 Determines Efficiency of Virus Formation—To identify the determinant in J6 core responsible for efficient virus production, we constructed JFH1 genomes, in which individual core domains were replaced by those of J6 (Fig. 2A, chimeras J6D1/JFH1, J6D2/JFH1, and J6D3/JFH1). Levels of intracellular core protein accumulating in transfected cells were comparable between the core gene chimeras, JFH1wt and Jc1, arguing for equivalent levels of RNA replication (Fig. 2B). In contrast, infectivity release obtained with the J6D1/JFH1 chimera was delayed, and titers were about 100-fold lower as compared with JFH1wt (data not shown). The exchange of the JFH1 core domain 3 by the corresponding J6 domain had no effect on virus titer (J6D3/JFH1). In contrast, in the case of the JFH1 chimera with the J6-D2 replacement, infectious virions were detected already by 12 h p.t. (data not shown), and peak titers were only slightly lower than titers attained by the J6core/JFH1 chimera, which
Role of HCV Core Domain 2 in Virus Assembly

contains the entire J6 core gene. The differences in virus production observed with the core chimeras were comparable with the differences of the amounts of core protein released from cells that had been transfected with these chimeras (Fig. 2D). The results thus argue that primarily virus production rather than specific infectivity was affected by the exchange of core domains.

Having found that D2 of J6 increases virus production by JFH1, we next tested whether this domain is sufficient for the enhancement or whether the introduction of longer J6 core sequences enhances virus production even further. We therefore generated J6/JFH1 core chimeras each containing a combination of two core domains as depicted in Fig. 2A (right panel). In agreement with the first series of results, the exchange of core domains had no significant effect on RNA replication as deduced from the amounts of intracellular core protein accumulating in transfected cells (Fig. 2B). The combination of domains 1 and 3 of J6 reduced infectivity titers ~50-fold compared with JFH1wt (J6D1D3/JFH1; Fig. 2C). In contrast, chimeras containing the J6-D2 domain in combination with either D1 or D3 (J6D1D2/JFH1 and J6D2D3/JFH1) supported highly efficient virus particle production with faster kinetics of virus release as compared with the D2 chimera (compare the 24 h values in Fig. 2C). Both the D1D2 and the D2D3 chimeras yielded slightly higher titers than the J6D2/JFH1 genome at 24 h p.t., whereas peak titers of all three chimeras were almost comparable (Fig. 2C). Moreover, as deduced from the enhanced titers of the J6D1D2/JFH1 chimera, the negative effect of the J6-D1 domain on JFH1 virus production was not observed in the presence of J6-D2 arguing for cross-talk between the D2 and D1 domains. This is in keeping with the D2-dependent folding of D1 we reported previously (7). Infectivity correlated well with the amount of extracellular core protein released from transfected cells (Fig. 2D) showing that the primary effect of core domain 2 of J6 is to enhance virus assembly and release without detectably affecting specific infectivity of released viruses.

Mapping of Residues in D2 of J6 Responsible for Efficient Virus Production—To identify the residue(s) in D2 of J6 responsible for enhanced virus titers, we first aligned the aa sequences of J6 and JFH1 core and found four aa differences in D2 between the two genotype 2 (GT2) isolates (Fig. 3A). It should be noted that among the 71 GT2 sequences available in the data base, alanine at position 147 and valine at position 151 have been reported in only one other sequence and phenylalanine at position 164 in only 11% of GT2 sequences. These low frequencies were also observed at 72 h p.t. (data not shown). These results suggest that D2 of J6 core (containing V147) enhances the release of infectious HCV particles.

Next, we wanted to know whether J6 core and the A147V exchange enhance assembly of JFH1 particles. To this end, we compared total (intracellular plus extracellular) infectivity of JFH1wt and core gene chimeras obtained with Huh7-Lunet cells at 48 h p.t. As shown in Fig. 4B, total infectivity of JFH1wt and J6D1D3/JFH1 was lowest, demonstrating that assembly of infectious particles was inefficient. A significantly higher amount of infectious virus was found with the A147V mutant, the J6D2, and the J6 core chimeras, but these titers remained below the level of virus production achieved with Jc1. It should be noted that this reduced virus production was not observed at later time points, arguing that virus particle release of Jc1 follows more rapid kinetics (see the 24-h values in Fig. 3E). Taken together, these data show that D2 of J6 core contains determin-
FIGURE 3. Mapping of mutations in D2 responsible for efficient virus production. A, schematic representation shows the amino acid sequence of D2 aligned with the limits of helix I, the hydrophobic loop (HL), and helix II deduced from NMR analyses (12). Residues that are different between JFH1 and J6 sequences are shaded (Val-147 in magenta, Leu-151 in dark gray, and Phe-164 in violet). The shading includes the corresponding positions in aa repertoires of the D2 sequences deduced from the ClustalW multiple alignments of 71 sequences of GT2 (middle) and the 26 representative D2 sequences of confirmed HCV genotypes/subtypes (any GT, listed with accession numbers in Table 1 in Simmonds et al. (43)) (bottom). Amino acids are listed in decreasing order of observed frequencies, from top to bottom. Amino acids observed at a given position in less than 1% of the sequences were not included. The asterisks indicate the aa in JFH1 that were replaced by those occurring in J6. B, model of the amphipathic helix II of J6 core and expected location at the membrane interface. This model of helix II (amino acids 148–164) was deduced from the NMR analysis we reported recently (12). The structure is represented as ribbon in the side view (left) and as backbone in the axial helix view (right). Side chains are shown in stick representation; hydrogen atoms are not shown. The residues mutated in this study are colored as indicated in A. Other hydrophobic residues are colored gray. Hydrophilic residues are color-coded as follows: acidic residues (Glu, Asp) are red; basic residues (Lys, Arg) are blue; His is cyan; Asn is yellow; and Gly is light gray. This figure was generated with Rasmol 2.7 program (23). The phospholipid bilayer is schematically represented. The positioning of the helix between the polar heads and the hydrophobic tails of the phospholipids is tentative. C, replication of JFH1wt and given mutants in transfected Huh7.5 cells as determined by quantification of intracellular amounts of core protein using an ELISA. Values were normalized for transfection efficiency by using core protein amounts determined 4 h after transfection. Mean values of duplicate measurements and the standard errors are given. D, efficiency of core release into the culture supernatant 48 h p.t. The percent values of released core protein relative to total core protein amounts are shown. Mean values of duplicate measurements and the standard errors are given. E, infectious virus release obtained with JFH1 D2 mutants. Twenty-four, 48, and 72 h post-transfection, cell-free supernatants were harvested from transfected Huh7.5 cells and used for TCID₅₀ determinations. The result of a representative experiment of three independent experiments is shown. For further details see legend to Fig. 1.
Role of HCV Core Domain 2 in Virus Assembly

FIGURE 4. Detection of intracellular and total infectivity in Huh7-Lunet cells transfected with core gene mutants. A, Huh7-Lunet cells were electroporated with the genomes specified at the bottom of the panel and 48 h.p.t. cell-free culture fluid was harvested for quantification of infectivity by TCID50 assay. In parallel, virus-producing cells were lysed by repetitive cycles of freezing and thawing, and intracellular infectivity was determined by limiting dilution assay. The percentage of cell-associated and released infectivity expressed relative to the total amount of infectivity is shown. Data are derived from a single representative experiment of three independent repetitions. B, sum of intra- and extracellular infectivity of genomes specified at the bottom is expressed as total infectivity obtained per transfection. Mean values of at least two independent experiments and the means ± S.D. are shown.

nant(s) such as valine at position 147 that enhance the efficiency of both assembly and release of infectious virus particles.

Association of HCV Core with Lipid Droplets—Next, we analyzed association of the core variants in the context of infectious genomes with LDs. To this end, we investigated the co-localization of core protein variants of JFH1wt and J6/JFH1 chimeras with LDs in transfected Huh7-Lunet cells by using confocal microscopy. As shown in Fig. 5A, in the majority of cells expressing the JFH1wt genome, core protein co-localized perfectly with ADRP, a cellular marker for LDs (32) (for a summary of core and intracellular LD distribution observed with all mutants, see Table 1). A higher magnification of the image revealed that the core in JFH1wt-transfected cells, together with ADRP, is organized in ring-like structures, corresponding to the surfaces of LDs (Fig. 5A, crop panel). Furthermore, the intracellular localization of LDs in cells transfected with JFH1wt displayed accumulation of LDs in the perinuclear region, whereas in mock-transfected cells, LDs were more evenly distributed in the cytoplasm (compare Fig. 5A, panels vi and xxii). By contrast, in almost all cells transfected with the highly assembly and release-competent Jc1 genome, LDs did not cluster in the perinuclear region, but rather were evenly distributed throughout the cytoplasm, thus resembling more the staining pattern in naive cells (compare Fig. 5A, panels xviii and xxii). Moreover, only a partial co-localization of J6 core with LDs was found, with core showing a punctate staining pattern on LDs, in contrast to the ring-like staining pattern of JFH1 core protein (compare Fig. 5A, panels xx and vii, respectively). Furthermore, in cells expressing the Jc1 genome, only about 22% of LDs were co-localizing with the J6 core, whereas almost all LDs were covered with the JFH1 core (92%; Table 1). In the case of the J6core/JFH1 (data not shown) and J6D2/JFH1 transfected cells, an intermediate phenotype between Jc1 and JFH1wt with respect to core-LD association was observed. Neither the A147V mutation nor the replacement of the JFH1 D1 and D3 domains by those of J6 had a considerable effect on the staining pattern for JFH1wt core (Table 1). These patterns were independent from the chosen Huh7 clone and the detection method because the same results were found with Huh7 cells from other sources and by using the C7–50 core-specific monoclonal antibody (34) and Oil Red O for LD staining (data not shown).

The clearly different core-specific staining patterns observed with JFH1 and Jc1 argue that the level of association of the respective core proteins with LDs is an important determinant for efficient virus production. Alternatively, the extent of core association with LDs visualized by immunofluorescence might not be the cause but rather a consequence of efficient virus production. To clarify this point we analyzed a Jc1 mutant unable to support virus production because of a deletion of a part of the p7 gene (Fig. 1A) presumably assisting a late step of assembly (Jc1Δp7) (22). In Jc1-transfected cells, the core protein was barely detectable (Fig. 5A, panel xvii, and Fig. 6), which was primarily because of its homogeneous distribution rather than the expression level, which was comparable with JFH1wt (see Figs. 2B and 3C) and Jc1Δp7 (data not shown). More importantly, in the majority of cells expressing the Jc1Δp7 mutant, we observed an LD distribution and a core LD co-localization very similar to that observed in JFH1wt replicating cells (i.e. perinuclear accumulation of LDs, complete core ADRP co-localization, and about 90% of LDs decorated with core) (Fig. 5A, compare panels iii and iv with panels vii and viii).

To gain more accurate information about a relative location of different core variants on LDs, we performed deconvolution of confocal image stacks of cells stained with a core protein antibody and BODIPY for LDs. To represent the relative positions of LDs and core, three-dimensional views of deconvolved data were generated with an iso-surface rendering for cells expressing the indicated constructs (Fig. 5B). In agreement with the results shown in Fig. 5A, only local association between LDs and J6 core (Jc1) or J6-D2-chimeric core was found. The extent...
Role of HCV Core Domain 2 in Virus Assembly

Intracellular distribution of HCV core and LDs in cells transfected with JFH1wt and JFH1/J6 chimeras

| Construct   | Titer 48 h.p.t. | Cells punctate core* | Cells LDs covering | LDs associated with core/cell* |
|-------------|-----------------|----------------------|--------------------|-------------------------------|
| Jc1Δp7      | 0               | 22 ± 2               | 78 ± 2             | 93 ± 2                        |
| J6 D1D3/JFH1| 3 × 10^4        | 16 ± 7               | 84 ± 7             | 88 ± 6                        |
| JFH1wt      | 2 × 10^4        | 9 ± 3                | 91 ± 3             | 92 ± 3                        |
| A147V       | 2 × 10^4        | 22 ± 5               | 78 ± 5             | 80 ± 6                        |
| J6 D2/JFH1  | 4 × 10^4        | 35 ± 9               | 65 ± 9             | 79 ± 11                       |
| J6 core/JFH1| 10^5            | 75 ± 2               | 25 ± 2             | 44 ± 21                       |
| Jc1         | 10^6            | 98 ± 2               | 2 ± 2              | 22 ± 5                        |

* Shown is the percentage of cells with complete co-localization of core and LDs and perinuclear accumulation of LDs (48 h.p.t.).

From all these results we conclude that comparable with JFH1wt core protein, the J6 core protein itself is able to associate with LDs and to induce accumulation of core on LDs, which in turn may affect the intracellular distribution of LDs.

Association of HCV core with LDs in cells transfected with JFH1wt or core gene mutants.

Intracellular distribution of HCV core and LDs in cells transfected with JFH1wt and JFH1/J6 chimeras. A, Huh7-Lunet cells were electroporated with genomes specified at left and seeded onto glass coverslips. Forty-eight h.p.t. cells were fixed, probed with the core-specific (green) or the ADRP-specific antiserum (red; panels marked Core or LDs, respectively), and examined by confocal microscopy. The scale bar in panel i represents 10 μm. An enlargement of the overlay indicated by white squares in the Merge panels is shown in the right (Crop). In case of merged and cropped images, nuclei are labeled with 4′,6′-diamidino-2-phenylindole. The scale bar in panel iv represents 1 μm. B, three-dimensional reconstruction of HCV core on LDs. Huh7-Lunet cells were electroporated with genomes specified to the left of A and seeded onto glass coverslips. Forty-eight h.p.t. cells were fixed, probed with the core-specific antibody (green) or BODIPY for LD staining (red; panels marked Core or LDs, respectively), and examined by using an Ultra-View spinning disk (PerkinElmer Life Sciences) on a Nikon TE2000-E inverted confocal microscope. Z-stack analysis of core and BODIPY was performed by recording a series of ~30–40 images depending on cell thickness. Three-dimensional reconstructions of core and LDs are shown.

Co-localization of HCV Core with NS5A—Because core protein is directed to LDs whereas RNA replication most likely is mediated by a replicase complex associated with ER or ER-derived membranes, we recently suggested that at some stage during virus assembly the RNA genome is transferred from the replicase complex to the core protein (17). This requires interactions between core and the replicase complex, presumably with involvement of NS5A, which is an RNA-binding protein assumed to play a role in virus assembly (6, 35). We therefore analyzed whether the differences in virus titers achieved with the core chimeras were because of different efficiencies of interaction with the viral replication complex.

To this end, we compared the co-localization of core and NS5A in Huh7-Lunet cells transfected with various HCV genomes. As shown in Fig. 6, in all cases we observed co-localization between NS5A and core proteins. Moreover, the ring-like staining pattern of NS5A (best visible in the case of JFH1wt) indicates that this protein also associates with LDs. This distribution was confirmed by NS5A-ADRP co-staining (data not shown). Nevertheless, the similar co-localization pattern observed with all core gene chimeras and mutants indicates that differences in core-NS5A (replicase) interactions do not detectably account for the different levels of virus production.

Intracellular Localization of Core Protein Variants in Cells Transfected with SFV1/Core-E1-E2 Constructs—To compare the intracellular distribution of JFH1 and J6 core variants independent from HCV RNA replication, assembly, and release of viral particles, core variants were expressed as part of a core-E1-E2 polyprotein fragment by using a recombinant Semliki Forest virus (SFV) replicon. Twenty four hours after electropo-

TABLE 1

Intracellular distribution of HCV core and LDs in cells transfected with JFH1wt and JFH1/J6 chimeras

| Construct   | Titer 48 h.p.t. | Cells punctate core* | Cells LDs covering | LDs associated with core/cell* |
|-------------|-----------------|----------------------|--------------------|-------------------------------|
| Jc1Δp7      | 0               | 22 ± 2               | 78 ± 2             | 93 ± 2                        |
| J6 D1D3/JFH1| 3 × 10^4        | 16 ± 7               | 84 ± 7             | 88 ± 6                        |
| JFH1wt      | 2 × 10^4        | 9 ± 3                | 91 ± 3             | 92 ± 3                        |
| A147V       | 2 × 10^4        | 22 ± 5               | 78 ± 5             | 80 ± 6                        |
| J6 D2/JFH1  | 4 × 10^4        | 35 ± 9               | 65 ± 9             | 79 ± 11                       |
| J6 core/JFH1| 10^5            | 75 ± 2               | 25 ± 2             | 44 ± 21                       |
| Jc1         | 10^6            | 98 ± 2               | 2 ± 2              | 22 ± 5                        |

* Shown is the percentage of cells with punctate co-localization of core and LDs and the homogeneous cytoplasmic distribution of LDs (48 h.p.t.).

a Shown in the percentage of cells with complete co-localization of core and LDs and perinuclear accumulation of LDs (48 h.p.t.).

b Shown is the percentage of cells with complete co-localization of core and LDs and perinuclear accumulation of LDs (48 h.p.t.).

For all these results we conclude that comparable with JFH1wt core protein, the J6 core protein itself is able to associate with LDs and to induce accumulation of core on LDs, which in turn may affect the intracellular distribution of LDs.
Role of HCV Core Domain 2 in Virus Assembly

Intracellular localization, core protein stability, and proteolytic processing of core as specified in a SFV-based expression system. A, Huh7-Lunet cells were electroporated with the SFV/C-E1-E2 RNAs or only the SFV vector RNA (mock) and seeded onto glass coverslips. Sixteen h.p.t. cells were fixed, probed with a core-specific (green) or an ADRP-specific (red) antisera, and examined by confocal microscopy. Only merged images are shown. Scale bar, 10 μm. B, cells were cultured in the presence or absence of MG132 or its solvent Me2SO (DMSO) as indicated, and extracts were analyzed by Western blot analysis using the core-specific antiserum C830. β-Actin was used as a loading control. C, cells were cultured in the presence or absence of (Z-LL)2-ketone or its solvent Me2SO (DMSO), and extracts were analyzed by Western blot as specified in B.

Processing and Stability of Core Protein Variants—To examine whether the differences in virus production obtained with the core variants are because of variable protein stability, we determined core protein levels in the absence and presence of the proteasome inhibitor MG132. Cells were transfected with SFV vectors expressing core variants in the context of a core-E1-E2 polyprotein fragment and treated for 12 h with MG132 (Fig. 7B). Apart from the F130E mutant, recently shown to be degraded by the proteasome (12) and used here as a positive control, all other core variants were detected at comparably high levels and with an apparent molecular mass of 21 kDa. Addition of MG132 did not affect the abundance of any of the core proteins. The same result was found when cells were analyzed by indirect immunofluorescence analysis (data not shown). Confocal microscopy studies also revealed that treatment with MG132 did not affect core accumulation on LDs (data not shown).

In addition, with the exception of the F130E mutant, a minor core-reactive protein of about 16 kDa was detected with all core-expressing constructs. The nature of this product, most prominent in the case of SFV/J6core, is not clear, but it may represent a specific cleavage product of core or a core + 1 protein generated by ribosomal frameshift (36).

Because the maturation of HCV core protein requires two cleavage events, first by SP and then by SPP, we compared the processing pattern of the different core variants. To distinguish between the SP-cleaved immature form of core (p23) and SPP-cleaved mature p21, we used HCV core isolated from cells, which were treated with a specific SPP inhibitor ((Z-LL)2-ketone (33, 37)) as a size control. As expected, two core species representing SP- and SPP-cleaved core were detected in (Z-LL)2-ketone treated cells (Fig. 7C). In the absence of the inhibitor, the major product of all core variants was mature core with an apparent molecular mass of 21 kDa. From all these data we conclude that the stability and the processing of the core variants are comparable.

Mobility of Core D2 Variants on LDs—Recently, we demonstrated that D2 can direct GFP to the surface of LDs, and unlike cellular proteins, the domain is highly mobile on LD surfaces. Small changes in the D2 sequence influence the extent of mobility, most likely through alterations in the structure of the domain and/or interaction with the phospholipid layer of LDs. Moreover, mutations in D2 that strongly restrict mobility also abolish virus production. Therefore, we wished to determine whether the alanine to valine substitution at position 147, responsible for the higher efficiency of virus production by the J6 sequence, affected mobility. To this end, we constructed GFP-D2 fusion proteins for the JFH1 and J6-D2 domains as well as the individual point mutants that had been tested in infectivity assays (S145G, A147V, V151L, and Y164F). All of the fusion proteins were directed to ring-like structures corresponding to LDs in Huh7 cells (Fig. 8A, and data not shown). Next, we analyzed recovery of GFP fluorescence after photobleaching of selected intracellular regions containing GFP-D2 attached to LDs. Regions of interest were exposed to six bleaching itera-
tions (100% laser power, 488 nm laser line) to reduce fluorescence intensity. Before and after bleaching, images were recorded with 2% laser power at ~1-s intervals.

Comparison of the D2 domains of JFH1 and J6 revealed that they gave different patterns of behavior by FRAP; J6 was more readily bleached and showed a slower rate of recovery compared with JFH1. The pattern of bleaching and recovery was highly reproducible between experiments, and the slower rate of recovery was reflected in the calculated $t_{1/2}$ values, which measured the time taken for half of the fluorescence to recover (8 s for JFH1-D2 and 14 s for J6-D2). In addition, the D2 domain of JFH1 consistently gave a higher mobile fraction. FRAP analysis of the individual amino acid variants between the two strains revealed that the A147V conversion generated a D2 domain whose pattern of bleaching and fluorescence recovery was almost indistinguishable from that for JFH1 (Fig. 8B). By comparison, two of the variants (S145G and Y164F) gave fluorescence recovery curves, which were almost identical to that for JFH1. The third variant (V151L) had slightly slower recovery, but its overall pattern of fluorescence loss and recovery matched more closely JFH1 as compared with J6. The lower mobility of V151L could result from the greater hydrophobicity of leucine as compared with valine, which may increase the binding strength of D2 to LDs. For these variants, the same results were obtained when protein synthesis was blocked by the addition of cycloheximide 30 min prior to FRAP analysis until the end of measurements. Fluorescence recovery of all GFP-D2 proteins was comparable with that in nontreated cells supporting the notion that FRAP analysis measured D2 mobility in membranes rather than replacement of bleached protein with newly synthesized GFP-D2 (data not shown). Therefore, the amino acid variants, which are not involved in the higher infectivity of the J6 chimera, have no appreciable affect on protein mobility on LDs compared with JFH1. However, the mutation of alanine to valine at position 147, which increases virus particle production, has a mobility that matches the D2 domain from J6. Hence, there is close correspondence between the behavior of the A147V variant and J6 by FRAP analysis and in infectivity assays arguing that the nature of the interaction between core and LDs is critical for virus production.

**DISCUSSION**

In this study we have identified the lipid droplet binding domain of HCV core as a major determinant for efficient virus production. We demonstrate that insertion of J6 core into the JFH1wt genome is sufficient to increase virus titer by about 100-fold, and thus to a level attained with Jc1, the most efficient HCV genome. Although the single point mutation A147V in D2 was found to contribute mostly to the enhancement of JFH1 virus titer, it is interesting to note that this mutation has not been observed during several independent cell culture adaptation experiments in which JFH1 variants were selected for high level virus production (38–40). One possible explanation is that even though A147V enhances virus titer, the kinetics of virus release observed with JFH1-A147V are delayed by about 24 h as compared with Jc1 or the JFH1 chimera containing J6 core. Taking into consideration the important role of p7 for virus production (22), we generated an additional JFH1 chimera, containing both core and p7 from J6. This chimera supported high virus titers with the same kinetics as Jc1, suggesting that p7 has a major impact on the kinetics of virion production, whereas core protein primarily determines virus titer. 5

In the course of our mapping studies of core domains contributing to efficient virus production, we found that D1 of J6 decreases the titers about 100-fold (chimera J6D1/JFH1 in Fig. 2C) compared with JFH1wt, arguing for incompatibilities between D1 of J6 and D2 of JFH1. In line with this assumption, titers achieved with the J6D1D2/JFH1 chimera in which both D1 and D2 of J6 were inserted into JFH1wt were increased almost 10,000-fold as compared with J6D1/JFH1. It has been shown that folding of D1 depends on the presence of D2 (7). Therefore, we assume that JFH1 D2 does not support the optimal stabilization of J6 D1 folding, in contrast to autologous J6-D2. As deduced from the high titers of the J6D2/JFH1 chimera, JFH1 D1 folding is either efficiently stabilized in the presence of J6-D2 or a disadvantage of D1 folding is compensated by another titer-enhancing effect.

The insertion of J6 D3 into JFH1wt had no effect on virus titer. This domain is the signal sequence of E1 and is cleaved internally by SPP around aa position 170 resulting in mature core protein (9). Within D3, the J6 and JFH-1 core differs at three aa positions (172, 173, and 187). Because only a fully processed form (p21) was observed for J6 and JFH1 core proteins (Fig. 7C), we conclude that the poorly conserved sequences

---

5 A. Shavinskaya, E. Steinmann, and R. Bartenschlager, unpublished observations.
Role of HCV Core Domain 2 in Virus Assembly

Phe-Pro in JFH1 and Cys-Ser in J6 at positions 172–173 in the context of a core to E2 polyprotein or the complete polyprotein, support comparable levels of core processing and virus production, respectively.

By mutagenesis analysis we found that the single point mutation A147V within D2 increased JFH1 titer to a level similar to J6D2/JFH1 (Fig. 3E), whereas the other three aa substitutions had no considerable effect. Based on NMR studies of D2 (12), we established a structural model of helix II, according to which the A147V substitution resides at the very N-terminal end of the helix as the Ncap residue and is exposed to the hydrophobic core of the ER membrane or the LD (Fig. 3B). According to the scale of free energies established by White and Wimley (41) for transfer of amino acids from water to octanol, which mimics the hydrophobic core of the membrane, transfer of alanine is unfavorable by 0.50 kcal mol⁻¹, whereas transfer of valine is favorable by −0.46 kcal mol⁻¹. It is thus likely that the enhancing hydrophobicity of valine stabilizes both the N-terminal helix fold as well as the helix-membrane interaction. Consequently, the A147V mutation is expected to enhance and stabilize core-membrane interactions but might also play a role in the oligomerization of the core protein subunits during assembly or the stability of core protein itself. Western blot analysis and treatment with a proteasome inhibitor demonstrated similar stability of core protein variants (Fig. 7B). Nevertheless, it remains to be clarified whether there are some minor differences in half-life of the core proteins. Unfortunately we could not address this question by pulse-chase analysis of radiolabeled core, probably because of an insufficient number of methionine residues and poorly reactive antibodies.

Several reports have demonstrated that HCV core protein associates with LDs (13, 15). More recently, it was shown that core D2 is sufficient to target core to these organelles (12). From our results we conclude that J6-D2 as well as JFH1-D2, if expressed alone, associates with LDs. Because of its higher hydrophobicity, the valine at position 147 (present in J6 core and the A147V variant) likely increases the binding strength of the protein for LDs. This would lead to more stable binding of J6 core to LDs, which is reflected by lower mobility of J6-D2 on LDs (Fig. 8) and which correlates with higher virus titers (Fig. 3E). Mutants $145G and Y164F, which produce virus titers comparable with JFH1, have high mobility on LDs, whereas mutant V151L had slightly lower mobility even though virus titers were not affected by this mutation (Fig. 3E). The most likely explanation is that valine at position 147, which is the Ncap residue of helix II, has an additional influence on stabilization of helical folding. Such an effect would not be expected for leucine at position 151, which resides within helix II. Thus, the V151L variant is slightly less mobile because of increased interaction with the core of the LD membrane; in the case of the A147V mutation, there is not only an increase in hydrophobic interaction but also the position of this residue at the beginning of helix II is likely to have an additional effect on the stabilization of the helix structure.

One speculation emerging from this observation is that the lower mobility of J6 core may facilitate the interaction with the viral replicate delivering the RNA genome to the core protein. Alternatively, reduced core mobility may enhance some other assembly step such as envelopment, which may occur in close connection to very low density lipoprotein formation (42).

In the context of Jc1 full-length virus, J6 core only partially co-localizes with LDs and does not induce redistribution of LDs (Fig. 5A). These characteristics do not arise from an intrinsic property of J6 core as the protein coats LDs under conditions whereby virus particle assembly and release are not possible. In a recent study, we have demonstrated that loading of LDs by the JFH1 core is a progressive process during infection as the protein increases in abundance (16). At early times after infection, JFH1 core is located at a punctate site on LDs, and complete loading does not occur until between 48 and 72 h. Our conclusion from observations in this study is that rapid assembly and release of virus particles by J6 core prevents accumulation of the protein, and thus coating of LDs may be delayed until later time points or does not occur at all.

The staining pattern of NS5A supports the notion that this protein associates with ER membranes, core protein on LDs, and/or directly with LDs. The core-NS5A co-localization may indicate an interaction of the replicase complex with the core protein to deliver the RNA genome and to initiate nucleocapsid formation. In fact we have recently proposed a model in which HCV assembly occurs in close proximity to LDs and that LDs are an important element of the assembly reaction (17).

In conclusion, we show that D2 in HCV core is a critical determinant for efficient virus assembly and that small numbers of variations in this highly conserved domain can exert a significant effect on production of infectious HCV. We provide additional evidence that association of core with LDs is an essential step of HCV morphogenesis and that mobility of core on LDs seems to be a critical parameter for the efficiency of virus production.

Acknowledgments—We thank Ulrike Herian and Stephanie Kallis for excellent technical assistance. We are also grateful to Ulrike Engel and Christian Ackermann (Nikon Imaging Center at the University of Heidelberg) for providing access to confocal fluorescence microscopes and for support in generation of high resolution images, and to Bernhard Dobberstein and Katja Kapp for provision of SPP inhibitor.

REFERENCES
1. Poynard, T., Yuen, M. F., Ratziu, V., and Lai, C. L. (2003) Lancet 362, 2095–2100
2. McHutchison, J. G., and Fried, M. W. (2003) Clin. Liver Dis. 7, 149–161
3. Van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGeoch, D. J., Pringle, C. R., and Wickner, R. B. (eds) (2000) Virus Taxonomy: The VIIth Report of the International Committee on Taxonomy of pp. 872–878, Academic Press, San Diego
4. Bartenschlager, R., Frese, M., and Pietschmann, T. (2004) Adv. Virus Res. 63, 71–180
5. Moradpour, D., Penin, F., and Rice, C. M. (2007) Nat. Rev. Microbiol. 5, 453–463
6. Appel, N., Schaller, T., Penin, F., and Bartenschlager, R. (2006) J. Biol. Chem. 281, 9833–9836
7. Boulong, S., Vanbelle, C., Ebel, C., Penin, F., and Lavergne, J. P. (2005) J. Virol. 79, 11333–11365
8. McLauchlan, J. (2000) J. Viral Hepat. 7, 2–14
6 A. Shavinskaya and R. Bartenschlager, unpublished observations.
Role of HCV Core Domain 2 in Virus Assembly

9. McLauchlan, J., Lemberg, M. K., Hope, G., and Martoglio, B. (2002) EMBO J. 21, 3980–3988
10. Giannini, C., and Brechot, C. (2003) Cell Death. Differ. 10, S27–S38
11. Jolivet, M., Penin, F., Dalbon, P., Ladaviere, L., and Lacoux, X. (March 5, 1997) Patent application EP 1015481, WO 98/39360
12. Boulant, S., Montserret, R., Hope, R. G., Ratiniere, M., Targett-Adams, P., Lavergne, J. P., Penin, F., and McLauchlan, J. (2006) J. Biol. Chem. 281, 22236–22247
13. Barba, G., Harper, F., Harada, T., Kohara, M., Goulain, S., Matsuura, Y., Eder, G., Schaff, Z., Chapman, M. J., Miyamura, T., and Brechot, C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1200–1205
14. Hope, R. G., and McLauchlan, J. (2000) J. Gen. Virol. 81, 1913–1925
15. Moradpour, D., Englert, C., Wakita, T., and Wands, J. R. (1996) Virology 222, 51–63
16. Boulant, S., Targett-Adams, P., and McLauchlan, J. (2007) J. Gen. Virol. 88, 2204–2213
17. Miyanari, Y., Atsuzawa, K., Usuda, N., Watashi, K., Hishiki, T., Zayas, M., Bartenschlager, R., Wakita, T., Hijikata, M., and Shimotohno, K. (2007) Nat. Cell Biol. 9, 1089–1097
18. Combet, C., Garnier, N., Charavay, C., Grando, D., Crisan, D., Lopez, J., Dehne-Garcia, A., Geourjon, C., Bettler, E., Hulo, C., Le Mercier, P., Bartenschlager, R., Diepolder, H., Moradpour, D., Pawlotsky, J. M., Rice, C. M., Trepo, C., Penin, F., and Deleage, G. (2007) Nucleic Acids Res. 35, D363–D366
19. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
20. Pietschmann, T., Kaul, A., Koutsoudakis, G., Ravindranathan, A., Kallis, S., Steinmann, E., Abid, K., Negro, F., Dreux, M., Cosset, F. L., Bartenschlager, R. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 7408–7413
21. Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G., Mizokami, M., Bartenschlager, R., and Liang, T. J. (2005) Nat. Med. 11, 791–796
22. Steinmann, E., Penin, F., Kallis, S., Patel, A. H., Bartenschlager, R., and Pietschmann, T. (2007) Plos Pathog. 3, e103
23. Sayle, R. A., and Milner-White, E. J. (1995) Trends Biochem. Sci. 20, 374
24. Bligh, G. R., McKeating, J. A., and Rice, C. M. (2002) J. Virol. 76, 13001–13014
25. Friebe, P., Boudet, J., Simorre, J. P., and Bartenschlager, R. (2005) J. Virol. 79, 380–392
26. Koutsoudakis, G., Herrmann, E., Kallis, S., Bartenschlager, R., and Pietschmann, T. (2007) J. Virol. 81, 588–598
27. Koutsoudakis, G., Kaul, A., Steinmann, E., Kallis, S., Lohmann, V., Pietschmann, T., and Bartenschlager, R. (2006) J. Virol. 80, 5308–5320
28. Gastaminza, P., Kapadia, S. B., and Chisari, F. V. (2006) J. Virol. 80, 11074–11081
29. 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., McKeating, J. A., and Rice, C. M. (2005) Science 309, 623–626
30. Spearman, C. (1908) Br. J. Psychology 2, 227–242
31. Hussy, P., Langen, H., Mous, J., and Jacobsen, H. (1996) Virology 224, 93–104
32. Targett-Adams, P., Chambers, D., Gledhill, S., Hope, R. G., Coy, J. F., Girod, A., and McLauchlan, J. (2003) J. Biol. Chem. 278, 15998–16007
33. Weihofen, A., Lemberg, M. K., Ploegh, H. L., Bogoy, M., and Martoglio, B. (2000) J. Biol. Chem. 275, 30951–30956
34. Moradpour, D., Wakita, T., Tokushige, K., Carlson, R. L., Krawczynski, K., and Wands, J. R. (1996) J. Med. Virol. 48, 234–241
35. Schaller, T., Appel, N., Koutsoudakis, G., Kallis, S., Lohmann, V., Pietschmann, T., and Bartenschlager, R. (2007) J. Virol. 81, 4591–4603
36. Xu, Z., Choi, J., Yen, T. S., Lu, W., Sterkecker, A., Govindarajan, S., Chien, D., Selby, M. J., and Ou, J. (2001) EMBO J. 20, 3840–3848
37. Weihofen, A., Lemberg, M. K., Friedrich, E., Rueeger, H., Schmitt, A., Paganetti, P., Rovelli, G., and Martoglio, B. (2003) J. Biol. Chem. 278, 16528–16533
38. Kaul, A., Worel, I., Meuleman, P., Leroux-Roels, G., and Bartenschlager, R. (September 7, 2007) J. Virol. 10.1128/jvi.01362-07
39. Zhong, J., Gastaminza, P., Chung, J., Stamatatos, Z., Isogawa, M., Cheng, G., McKeating, J. A., and Chisari, F. V. (2006) J. Virol. 80, 11082–11109
40. Delgrange, D., Pilleux, A., Castelain, S., Coquerel, L., Rouille, Y., Dubuisson, J., Wakita, T., Duverlie, G., and Wychowski, C. (2007) J. Gen. Virol. 88, 2495–2503
41. White, S. H., and Wimley, W. C. (1998) Biochim. Biophys. Acta 1376, 339–352
42. Huang, H., Sun, F., Owen, D. M., Li, W., Chen, Y., Gale, M., Jr., and Ye, J. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 5848–5853
43. Simmonds, P., Bukh, J., Combet, C., Deleage, G., Enomoto, N., Feinstone, S., Halfon, P., Inchauspe, G., Kuiken, C., Maertens, G., Mizokami, M., Moradpour, D., Okamoto, H., Pawlotsky, J. M., Penin, F., Sablon, E., Shin, I., Stuyver, L., Theil, H. J., Viazov, S., Weiner, A. J., and Widell, A. (2005) Hepatology 42, 962–973