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Replication of hepatitis C virus RNA occurs in a membrane-bound replication complex containing nonstructural viral proteins and RNA

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Biochemical studies revealed that nonstructural proteins of hepatitis C virus (HCV) interacted with each other and were associated with intracellular membranes. The goals of this study were to determine whether nonstructural viral proteins are colocalized at specific intracellular sites where HCV RNA is replicated and to identify the virus components of the HCV replication complex (RC). Immunofluorescence and subcellular fractionation studies were performed to determine the intracellular colocalization of nonstructural HCV proteins and the replicating RNA in a human hepatoma cell line, Huh7, in which a subgenomic HCV RNA was replicated persistently. The replicating HCV RNA was labelled with 5-bromouridine 5′-triphosphate (BrUTP). Results show that each of the nonstructural HCV proteins was colocalized predominantly with the newly synthesized HCV RNA labelled with BrUTP and an endoplasmic reticulum (ER) protein, calnexin. Consistent with these findings, subcellular fractionation and Western blot analyses revealed that the nonstructural HCV proteins were colocalized with HCV RNA mainly in the membrane fractions. Conversely, the viral nonstructural proteins and RNA remained in the soluble fractions upon treatment with detergent, confirming the membrane association of the HCV RC. HCV RNA in the membrane-bound RC was resistant to RNase treatment, whereas it became sensitive to RNases once the membranes were disrupted by treatment with detergent, suggesting that the HCV RC is assembled within membrane structures. Collectively, these findings demonstrate that HCV RNA replication occurs in the perinuclear ER membrane-bound HCV RC, containing nonstructural viral proteins and RNA.

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped RNA virus containing a single, positive-stranded RNA approximately 9.6 kb in length (Choo *et al.*, 1989; Reed & Rice, 2000; Rice, 1996). The genomic RNA encodes a large viral polyprotein of about 3000 amino acids, which is proteolytically processed by

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cellular signal peptidases and viral proteases into structural (C, E1, E2 and possibly p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) viral proteins (Bartenschlager *et al.*, 1993, 1994, 1995; Choo *et al.*, 1989; De Francesco & Steinkuhler, 2000; Grakoui *et al.*, 1993; Hijikata *et al.*, 1991; Lin *et al.*, 1994; Reed & Rice, 2000; Rice, 1996; Selby *et al.*, 1994). Recently, subgenomic HCV RNAs containing the nonstructural genes NS2 or NS3 to NS5B were replicated in a human hepatoma cell line, Huh7, suggesting that the nonstructural proteins NS3, NS4A, NS4B, NS5A and NS5B are sufficient for HCV RNA replication in the cell (Blight *et al.*, 2000; Lohmann *et al.*, 1999a). However, the underlying molecular mechanism of HCV RNA replication and the roles of viral and cellular proteins in HCV RNA replication are poorly understood.

The replication of most RNA viruses is a complex process, occurring through protein–RNA and protein–protein interactions that require multiple proteins. RNA replication of positive-stranded RNA viruses occurs in a membrane-bound virus replication complex (RC) consisting of viral and cellular proteins and viral RNA (Egger *et al.*, 2000, 2002; Froshauer *et al.*, 1988; Restrepo-Hartwig & Ahlquist, 1996, 1999; Schwartz *et al.*, 2002; Suhy *et al.*, 2000; Westaway *et al.*, 1997, 1999). Brome mosaic virus (BMV) protein 1a induced the formation of perinuclear endoplasmic reticulum (ER) membrane-bound spherules, where BMV RNA was replicated in the presence of protein 2a (Schwartz *et al.*, 2002). For flaviviruses, such as dengue virus and Kunjin virus, viral RNAs are replicated in vesicle packets (VPs), membrane structures induced in the perinuclear region during virus infections (Mackenzie *et al.*, 1996; Westaway *et al.*, 1997). Virus infection-induced VPs contain viral RNA and nonstructural proteins required for viral RNA replication (Mackenzie *et al.*, 1996; Westaway *et al.*, 1997). Although little is known about the replication of the HCV RNA genome, increasing evidence suggests that HCV RNA is replicated in a membrane-bound protein complex formed by nonstructural HCV proteins and even cellular protein(s) (Egger *et al.*, 2002; Hwang *et al.*, 1997; Ishido *et al.*, 1998; Lin *et al.*, 1997; Mottola *et al.*, 2002; Tu *et al.*, 1999). This theory was based on the fact that most nonstructural HCV proteins were found to interact with each other and to colocalize to the ER membranes (Egger *et al.*, 2002; Hwang *et al.*, 1997; Ishido *et al.*, 1998; Lin *et al.*, 1997; Mottola *et al.*, 2002; Tu *et al.*, 1999). For instance, NS5B was colocalized and coprecipitated with the nonstructural proteins NS3 and NS4A, and NS4A interacts with NS4B and NS5A (Ishido *et al.*, 1998; Lin *et al.*, 1997). In addition, both NS5B and NS5A interact with the N and C termini of a cellular protein, human vehicle-associated membrane protein (hVAP-33). The hVAP-33 protein is associated predominantly with cellular membranes, suggesting membrane association of the HCV RNA RC (Tu *et al.*, 1999). Furthermore, NS5A and NS5B were found to interact directly with the ER membranes via the N-terminal amphipathic α-helix and the C-terminal hydrophobic domains, respectively (Brass *et al.*, 2002; Schmidt-Mende *et al.*, 2001). Moreover, the nonstructural HCV proteins were found to colocalize to the ER membranes in Huh7 cells harbouring a replicating subgenomic HCV RNA (Gosert *et al.*, 2003; Mottola *et al.*, 2002; Shi *et al.*, 2003).

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The goals of this study were to determine whether the nonstructural proteins are colocalized at specific intracellular sites where HCV RNA replication occurs and to identify virus components of the HCV RC. A functional virus RC in Huh7 cells maintaining and replicating a subgenomic HCV RNA was characterized by double-label immunofluorescence and subcellular fractionation. Our findings demonstrate that nonstructural HCV proteins and the replicating viral RNA were colocalized to the perinuclear ER membranes. Subcellular fractionation analysis demonstrated further that the virus RC was associated with the ER membranes and that HCV RNA packaged in the RC was resistant to RNase treatment. *In vitro* RNA-dependent RNA polymerase (RdRp) assays revealed that the membrane-bound RC was active in RNA synthesis catalysed by the RdRp. Taken together, these findings demonstrate that HCV RNA is replicated in a membrane-bound RC that contains viral nonstructural proteins and RNA.

METHODS

Cell culture. Huh7 cell lines harbouring a replicating HCV replicon RNA were maintained in DMEM (Invitrogen) containing 10 % FBS and 1 mg G418 sulfate ml^{-1}. Huh7 cells were grown in DMEM with 10 % FBS.

Antibodies. A monoclonal anti-BrdU antibody was purchased from Sigma. Monoclonal antibodies (mAbs) against NS3, NS4A, NS4B and NS5A were purchased from Biodesign. An NS5B-specific polyclonal antibody (pAb) was raised against purified recombinant NS5B of the HCV replicon (genotype 1b) in rabbits and was purified by affinity chromatography (unpublished data). An NS4B pAb was kindly provided by R. Bartenschlager (University of Heidelberg, Germany). Rabbit pAbs against NS3 and NS5A were purchased from ViroStat and ViroGen, respectively. A pAb specific to calnexin was a gift from A. Sinai (University of Kentucky, USA). Secondary fluorescent antibodies were purchased from Molecular Probes, including Alexa Fluor 488 donkey anti-rabbit IgG (for primary pAbs reacting with NS3, NS4B, NS5A, NS5B and calnexin) and Alexa Fluor 594 donkey anti-mouse IgG (for primary anti-BrdU NS3, NS4A, NS4B and NS5A mAbs).

***In vitro* RdRp assay.** To determine the RdRp activity of the membrane-bound HCV RC, 50 ng of negative-sense 3′UTR RNA template was incubated with 10 μl of membrane-bound HCV RC in 25 μl containing 20 mM HEPES (pH 8.0), 1.5 mM $MnCl_2$, 100 mM ammonium acetate, 1 mM DTT, 40 U RNasin (Promega), 500 μM ATP, CTP and GTP, 10 μM UTP, 10 μCi [α^{32}-P]UTP (ICN) and 20 μg actinomycin D ml^{-1} (Luo *et al.*, 2000). RNA products were purified by phenol/chloroform extraction and collected by ethanol precipitation. RNA products were analysed by electrophoresis on a 6 % polyacrylamide/7 M urea denaturing gel.

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Dual immunofluorescence and confocal microscopy. Localization of nonstructural HCV proteins and newly synthesized viral RNA was determined by double-label immunofluorescence and confocal microscopy analyses. Huh7 cells harbouring an HCV replicon RNA were grown on coverslips in a 24-well plate. The newly synthesized HCV RNA in Huh7 cells was labelled with 5-bromouridine 5′-triphosphate (BrUTP) (Sigma). At 30 min prior to BrUTP transfection, 10 μg actinomycin D ml^{-1} was added to cell culture medium to block RNA transcription by cellular DNA-dependent RNA polymerases. BrUTP was then transfected into Huh7 cells using FuGENE lipofection, following the manufacturer's instructions (Roche) (Haukenes & Kalland, 1998). Briefly, FuGENE was diluted to 10 % with serum-free MEM (Invitrogen). After 5 min of incubation at room temperature, BrUTP was added to the diluted FuGENE solution at a final concentration of 3 mM with an additional 15 min of incubation. Subsequently, 20 μl BrUTP/FuGENE mixture was added to each well (24-well plate) containing 500 μl medium. After 1 h of incubation at 37 °C, BrUTP-transfected cells were washed with 1× PBS, fixed with 3 % formaldehyde and permeabilized with 0.1 % Triton X-100. Cells were then incubated with 1× PBS containing 1 % BSA, 1–5 % normal donkey serum and each of the primary antibodies. All primary antibodies specific to NS3, NS4A, NS4B, NS5A, NS5B and calnexin were diluted to 1:500. BrUTP-labelled HCV RNA was detected using an anti-BrdU mAb diluted to 1:50. After washing three times, Huh7 cells were incubated with the secondary antibodies Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 594 donkey anti-mouse IgG, both diluted to 1:1000. After immunostaining, coverslips were washed with 1× PBS and mounted with moviol antifade solution. Confocal microscopy was performed using the Leica TCS laser scanning microscope system (Leica).

Subcellular fractionation and membrane flotation analyses. HCV replicon-containing Huh7 cells were washed twice with 1× PBS, detached by scraping and collected by centrifugation. The cell pellet was resuspended in a hypotonic buffer (20 mM HEPES, pH7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF and 2 μg leupeptin ml^{-1}) and kept on ice for 20 min. Cells were homogenized by passing 20 times through a 27-gauge needle. Unlysed cells and cell nuclei were removed by centrifugation at 1000 ***g*** for 5 min at 4 °C. The supernatant was mixed with sucrose solution in isotonic buffer (35 mM HEPES, pH 7.4, 146 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF and 2 μg leupeptin ml^{-1}) and subjected to density sucrose gradient (10–50 %) flotation centrifugation. The sucrose gradient was centrifuged at 150 000 ***g*** in a Beckman SW55Ti rotor for 16–20 h at 4 °C. A total of 10 fractions was collected (top to bottom) from the sucrose gradient. Half of each fraction was used for RNA extraction with Trizol reagent (Invitrogen) and the other half for protein extraction using the chloroform/methanol method (Wessel & Flugge, 1984).

The crude RC was prepared exactly in the same way as described recently (Hardy *et al.*, 2003). Briefly, the replicon-containing Huh7 cells were washed with 1× PBS and collected by scraping. Cells were then

centrifuged at 1000 ***g*** for 10 min. Cell pellets were resuspended in a hypotonic buffer (10 mM Tris/HCl, pH 7.8, and 10 mM NaCl), incubated on ice for 15 min and homogenized with 50 strokes in a Dounce homogenizer. Undisrupted cell debris and nuclei were removed by centrifugation at 900 ***g*** for 5 min. The membrane-bound crude RC in the supernatant was collected by centrifugation at 15 000 ***g*** for 20 min and resuspended in hypotonic buffer, described above, with 15 % glycerol. The isolated crude RC is referred to as P15 hereafter.

Western blot analysis. HCV nonstructural proteins were analysed by electrophoresis on a 10 % SDS-PAGE gel and then transferred to a nitrocellulose membrane. Immunoblot analysis was performed by blocking overnight with a Superblock dry blend (TBS) blocking buffer (Pierce) and then incubated with primary antibodies. mAbs were used to detect the NS3, NS4A and NS5A proteins and pAbs were used to detect the NS4B and NS5B proteins together with an ER-resident protein, calnexin. Proteins were visualized by staining with horseradish peroxidase-conjugated goat anti-mouse (for primary mAbs) or goat anti-rabbit (for pAbs) antibody (Pierce) and chemiluminescent substrate (Roche).

RNase protection assay (RPA). The procedure for RPA experiments was the same as that described using the RPA kit from Ambion (Luo *et al.*, 2003). Positive-stranded HCV RNA in each fraction was determined using a negative-sense 3′UTR RNA probe. The negative-sense 3′UTR RNA probe was transcribed by T7 RNA polymerase from pUC19/T7(–)3′UTR DNA linearized with *Hin*dIII and labelled with [α^{32}-P]UTP. HCV RNA extracted from each fraction was hybridized with 10^5 c.p.m. [α^{32}-P]UTP-labelled negative-sense 3′UTR RNA probe. After digestion with RNase A/T1, RNA products were analysed on a 6 % polyacrylamide/7.7 M urea gel and visualized by autoradiography (Luo *et al.*, 2003).

RESULTS

The molecular basis underlying HCV replication is poorly understood due largely to the lack of efficient cell culture systems for HCV propagation and manipulation. The development of a subgenomic HCV replicon replication system, however, provides a foundation towards understanding the molecular mechanisms of HCV RNA replication (Blight *et al.*, 2000; Lohmann *et al.*, 1999a). In an effort to characterize the HCV RC, we have constructed human hepatoma cell lines (Huh7) in which a subgenomic HCV RNA was replicated constitutively (Luo *et al.*, 2003). Huh7 cells maintaining an efficiently replicating HCV RNA were therein used in this study for identification and isolation of the HCV RC (Luo *et al.*, 2003).

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Colocalization of nonstructural HCV proteins and replicating HCV RNA

Dual-label immunofluorescence experiments were performed to determine the colocalization of nonstructural HCV proteins in Huh7 cells harbouring a replicating subgenomic HCV RNA. Two different nonstructural proteins in various combinations were immunostained with a mAb for one protein and a pAb for the other. Localization of nonstructural proteins was determined by immunostaining with secondary Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 594 donkey anti-mouse IgG antibodies and visualization under a confocal microscope. Findings from these experiments demonstrate that all nonstructural HCV proteins were colocalized predominantly to the perinuclear region together with an ER-resident protein, calnexin (data not shown). Our results confirmed the findings reported recently that nonstructural HCV proteins in Huh7 cells carrying a replicating HCV RNA were colocalized to the perinuclear ER membranes (Gosert *et al.*, 2003; Mottola *et al.*, 2002; Shi *et al.*, 2003).

To determine the localization of replicating HCV RNA in the cell, BrUTP was used to label the newly synthesized HCV RNA. BrUTP is an efficient nucleotide substrate that can substitute for UTP, as demonstrated by an *in vitro* RdRp assay using purified recombinant NS5B (data not shown). Incorporation of BrUTP into HCV RNAs could be visualized by indirect immunofluorescence analysis using a BrdU-specific mAb (Gosert *et al.*, 2003; Shi *et al.*, 2003). Intracellular localization of the newly synthesized HCV RNA with BrUTP incorporation and nonstructural HCV proteins was determined by dual immunofluorescence and confocal microscopy analysis. Representative immunofluorescence images derived from a number of experiments are presented in Fig. 1. Three images of a single focal plane of Huh7 cells are shown with nonstructural HCV proteins in green (Fig. 1, left panels), BrUTP-labelled replicating HCV RNA in red (Fig. 1, middle panels) and superimposed images of both (Fig. 1, right panels). Each of the nonstructural HCV proteins was localized predominantly to the perinuclear rim during HCV RNA replication, similar to the staining of the ER-resident protein, calnexin (Fig. 1; data not shown). In particular, nonstructural proteins were stained strongly on the perinuclear ER membranes as dense spots. The shape and size of the spots varied between nonstructural proteins and from cell to cell. Some of the spots were fused to form irregularly shaped inclusions (Fig. 1, left panels, green). Likewise, nascent BrUTP-labelled HCV RNA was localized on perinuclear ER membranes with the strongest staining in dense spots (Fig. 1, middle panels, red). When the paired images were superimposed, there was strong colocalization between each of the nonstructural HCV proteins and the replicating HCV RNA labelled with BrUTP. Strikingly, HCV proteins and RNA showed the strongest colocalization in the dense spots on the perinuclear ER membranes (Fig. 1, right panels). These findings suggest that HCV RNA was replicated primarily in the perinuclear ER membrane regions where nonstructural HCV proteins and RNA were colocalized.

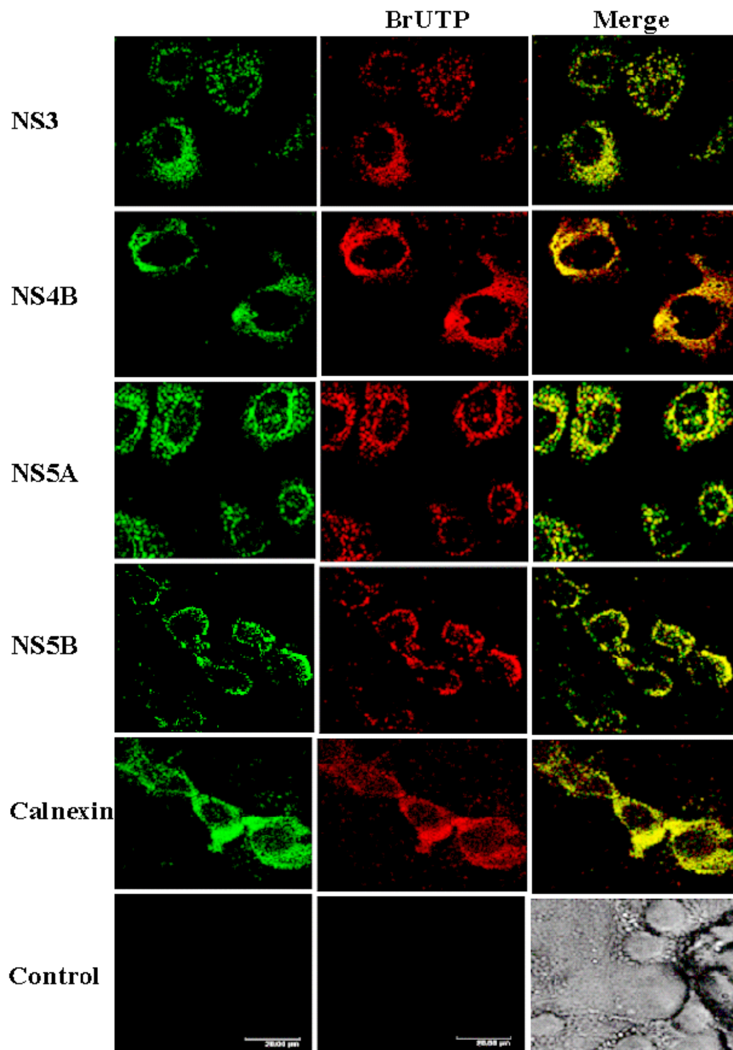

Fig. 1. Colocalization of newly synthesized HCV RNA with nonstructural proteins. The newly synthesized HCV RNA in the HCV replicon-harbouring Huh7 cells was labelled with BrUTP. Cells were fixed, permeabilized and double-labelled with an anti-BrdU mAb and with pAbs reacting with nonstructural HCV proteins or an ER-resident protein, calnexin. Nonstructural HCV proteins were immunostained with Alexa Fluor 488 donkey anti-rabbit IgG, while BrUTP-labelled HCV RNA was stained with Alexa Fluor 594 donkey anti-mouse IgG (diluted to 1:1000). Images were taken under a Leica TCS laser scanning microscope. Images of the nonstructural HCV proteins are shown in the left panels, BrUTP-labelled HCV RNA in the middle panels and superimposed images in the right panels.

Membrane association of the HCV RC

Previous studies have shown that the nonstructural HCV proteins were associated predominantly with the intracellular membranes when expressed from DNA vectors or in Huh7 cells harbouring a replicating HCV RNA (Egger *et al.*, 2002; Hwang *et al.*, 1997; Mottola *et al.*, 2002; Shi *et al.*, 2003). To determine further the colocalization and membrane association of the nonstructural HCV proteins and RNA, we performed membrane flotation and Western blot analyses of the cell extracts prepared from Huh7 cells in which a subgenomic HCV RNA was replicated persistently. Membrane-associated proteins were expected to float to an equilibrium density within the sucrose gradient. Subsequently, each fraction was examined simultaneously for the presence of the nonstructural HCV proteins, an ER-resident protein, calnexin, and HCV RNA. Strikingly, the NS3, NS4A, NS4B, NS5A and NS5B proteins were detected predominantly in the same membrane fraction on the top (fraction 2) of the sucrose gradient (Fig. 2A). The ER marker, calnexin, was also detected in the membrane factions on the top of the sucrose gradient (Fig. 2A). The subgenomic HCV replicon RNA in each fraction was extracted with Trizol and then determined by an RPA using an [α^{32}-P]UTP-labelled negative-sense 3′UTR RNA probe. The results are shown in Fig. 2(B). The subgenomic HCV RNA was detected primarily in the same membrane fraction as

the nonstructural viral proteins. The negative-stranded RNA was also detected in the same membrane fraction (data not shown). These findings suggest that the virus RC was associated with the ER membranes and contained the nonstructural viral proteins and RNA.

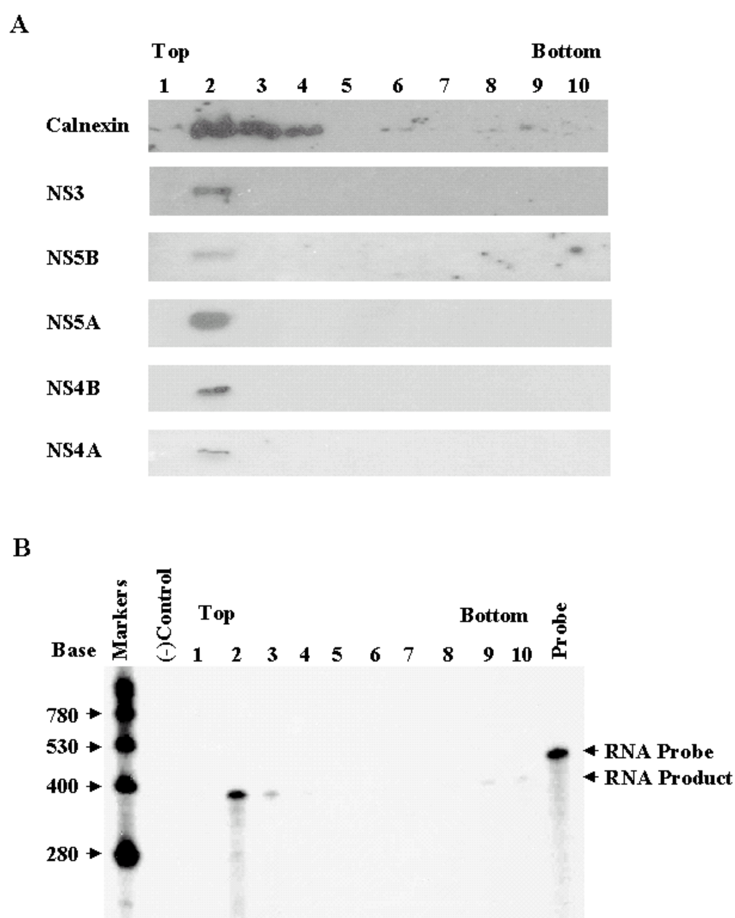

Fig. 2. Subcellular fractionation of the membrane-bound HCV RC by membrane flotation analysis. Huh7 cells carrying a replicating HCV RNA were harvested and lysed. Subcellular fractionation of the Huh7 cell lysates was done by 10–50 % sucrose gradient flotation centrifugation. Fractions were collected from the top to the bottom of the sucrose gradient. RNA and proteins in each fraction were extracted using Trizol and chloroform/methanol, respectively. HCV nonstructural proteins and an ER marker protein were detected by Western blot analysis (A) and the positive-strand HCV RNA was determined by RPA (B), as described in Methods. Fractions are numbered as indicated on the top. Nonstructural HCV proteins and an ER-resident protein are indicated on the left (A). The sizes of the RNA markers are indicated on the left and HCV RNA is highlighted by an arrow on the right (B).

To analyse further the membrane association of the virus RC, cell lysates of the HCV replicon-containing Huh7 cells were treated with Nonidet-P40 prior to fractionation. The membrane association of the RC would be disrupted by treatment with a detergent such as Nonidet-P40. The nonstructural HCV proteins and RNA present in each fraction were detected by Western blot analysis and RPA, respectively (Fig. 3). When the cell extract was treated with a detergent prior to subcellular fractionation, all nonstructural HCV proteins remained predominantly in the cytosolic fractions at the bottom of the sucrose gradient (Fig. 3A). This is contrary to a recent report that the HCV RC was resistant to detergent treatment (Shi *et al.*, 2003). Also, the HCV RNA was present mainly in the cytosolic fractions, similar to the nonstructural proteins (Fig. 3B). Clearly, treatment of the cell extract with Nonidet-P40 resulted in the disassociation of the RC with the ER membrane. Collectively, these findings demonstrate that the virus RC was associated with the ER or with membrane structures that originated from the ER.

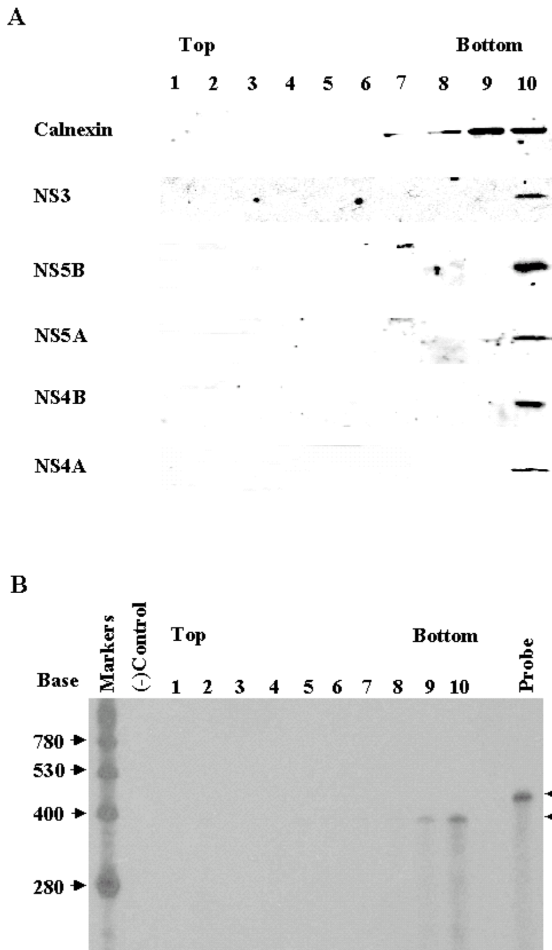

Fig. 3. Effect of detergent on fractionation of the HCV RC. Cell lysates were treated with 1 % Nonidet-P40 prior to membrane flotation centrifugation; otherwise, details are as described for Fig. 2.

Resistance of the membrane-bound HCV RC to RNase treatment

The results described above show that HCV RNA colocalized with the nonstructural proteins on perinuclear ER membranes. Findings from studies of other virus RCs revealed that viral RNAs were replicated in enclosed membrane structures that originated from the ER (Mackenzie *et al.*, 1996; Schwartz *et al.*, 2002; Westaway *et al.*, 1997). The 1a protein of BMV actually rendered the viral RNA resistant to nuclease (Schwartz *et al.*, 2002). To determine whether the HCV RC remained in or outside of the perinuclear ER membranes, the membrane-bound HCV RC was treated with RNases either with or without detergent treatment. The membrane-bound RC was isolated by sucrose density gradient centrifugation. The membrane fraction (Fig. 2, fraction 2) was then treated or not with detergent prior to RNase A/T1 digestion. As shown in Fig. 4, HCV RNA in the RC was strongly resistant to RNases A/T1 digestion (Fig. 4, lane 2). Upon quantification, 56 % of the HCV replicon RNAs were resistant to RNase treatment. It is not known how the remaining 44 % of RNA remained sensitive to RNase A/T1. It is possible that the replicon RNA sensitive to RNases might be associated with polyribosomes rather than assembled in the RC. Conversely, HCV RNA became sensitive to RNase A/T1 once the membranes were disrupted with detergent (Fig. 4, lane 4). Nearly all replicon RNAs were degraded by RNase A/T1 following membrane disruption by a detergent. Clearly, the membranes protected HCV RNA from RNase digestion, suggesting that the HCV RC was assembled within either the ER lumen or membrane structures that originated from the ER.

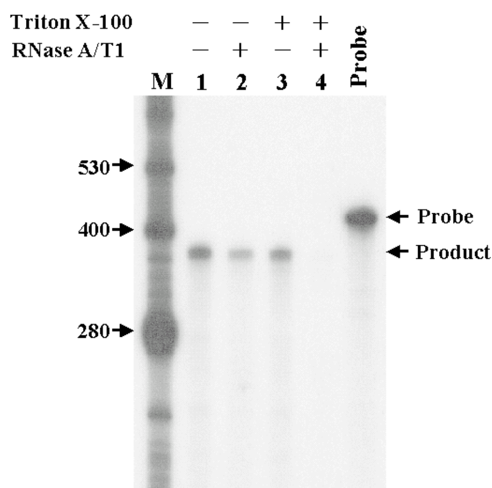

Fig. 4. Resistance of the membrane-bound HCV RC to RNase treatment. The membrane-bound HCV RC was isolated by sucrose gradient flotation centrifugation. Membrane fraction 2 (Fig. 2) was treated with or without 1 % Triton X-100. The sensitivity of the HCV RNA contained in the RC in the presence or absence of Triton X-100 was determined by RNase A/T1 digestion. After 30 min of incubation with RNase A/T1, RNA was extracted with Trizol. The positive-stranded HCV RNA was quantified subsequently by RPA using an [α^{32}-P]UTP-labelled negative-sense 3′UTR RNA probe, as described previously. RNA products were analysed on a 6 % polyacrylamide/7.7 M urea gel. The sizes of the RNA markers are indicated on the left and arrows on the right highlight the negative-sense 3′UTR RNA probe and the protected RNA product.

RdRp activity of the membrane-bound RC

To determine further whether the membrane-bound HCV RC was active in RNA synthesis, an *in vitro* RdRp assay was performed using a procedure described previously (Luo *et al.*, 2003). Upon subcellular fractionation, the membrane-bound RC was collected by centrifugation. The negative-sense HCV 3′UTR RNA probe (377 nt in length) was used as a template for *in vitro* RNA synthesis by the fractionated RC. As a control, the crude HCV replicase complex was prepared from HCV replicon-carrying Huh7 cells (Hardy *et al.*, 2003; Lai *et al.*, 2003). The results are shown in Fig. 5. The RdRp activity of the crude replicase complex was detected when exogenous RNA was used as a template (Fig. 5, P15). Interestingly, replicase activity was only detected in the membrane fractions (Fig. 5, combined fractions 1 and 2), confirming that the membrane-bound HCV RC containing the nonstructural HCV proteins and RNA was enzymatically active in RNA synthesis.

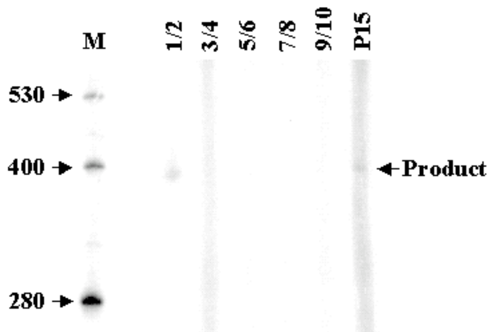

Fig. 5. RdRp activity of the membrane-bound HCV RC isolated by subcellular fractionation, as described in Methods. RdRp activity present in each fraction was determined by its ability to synthesize RNA *in vitro*. The negative-sense 3′UTR RNA template was incubated with the membrane-bound HCV RC in the presence of [α^{32}-P]UTP and 10 μg actinomycin D ml^{-1}. RNA products were analysed on a 6 % polyacrylamide/7.7 M urea gel. Fractions are numbered as indicated on the top. Fractions 1 and 2 (1/2), 3 and 4 (3/4), 5 and 6 (5/6), 7 and 8 (7/8) and 9 and 10 (9/10) were combined. P15 is the crude RC, as described in Methods. The sizes of RNA markers are indicated on the left and the RNA product is highlighted on the right (arrow).

DISCUSSION

The molecular mechanism of HCV RNA replication has been studied mainly using *in vitro*-purified recombinant HCV RdRp (Behrens *et al.*, 1996; Lohmann *et al.*, 1997, 1999b; Luo *et al.*, 2000; Oh *et al.*, 1999). Although purified recombinant HCV RdRp catalysed RNA synthesis *in vitro*, it lacked template specificity, suggesting that other viral/cellular factors are required for the formation of the authentic HCV RC *in vivo*. Analyses of the underlying molecular basis and/or composition of the HCV RC have been hampered by the lack of an efficient cell culture system for HCV propagation. This difficulty has been circumvented recently by the successful development of a subgenomic HCV RNA replication system in Huh7 cells (Lohmann *et al.*, 1999a). The subgenomic HCV replicon RNAs were replicated efficiently in the cells as a result of adaptive mutations that occurred in the nonstructural proteins (Blight *et al.*, 2000; Lohmann *et al.*, 2001). Replication of HCV RNA in the cells induced the intracellular membrane alterations that resemble the structural changes observed in HCV-infected human and chimpanzee liver tissues (Gosert *et al.*, 2003; Mottola *et al.*, 2002). Despite the report that a subgenomic HCV RNA that replicated efficiently in cell culture was attenuated in a chimpanzee model (Bukh *et al.*, 2002), accumulating evidence points to the cell-based HCV replicon replication system as a good model system to study HCV RNA replication (Blight *et al.*, 2000; Guo *et al.*, 2001; Ikeda *et al.*, 2002; Lohmann *et al.*, 1999a; Luo *et al.*, 2003).

It has been speculated that HCV RNA might be replicated on perinuclear ER membranes, based on the localization of nonstructural proteins expressed from DNA vectors and by analogy to other positive-stranded RNA viruses (Hwang *et al.*, 1997; Reed & Rice, 2000; Rice, 1996; Schwartz *et al.*, 2002). By examining Huh7 cells harbouring a replicating subgenomic HCV RNA, a number of studies revealed recently that HCV nonstructural proteins were colocalized and associated with the ER membranes, confirming previous findings derived from biochemical studies (Gosert *et al.*, 2003; Mottola *et al.*, 2002; Shi *et al.*, 2003). We also found that the nonstructural proteins NS3, NS4A, NS4B, NS5A and NS5B were all colocalized predominantly to the perinuclear ER membranes in the HCV replicon-harbouring Huh7 cells (Fig. 1; data not shown). In addition, we found that each of the nonstructural HCV proteins was colocalized with the replicating HCV RNA in the perinuclear region (Fig. 1). Interestingly, nonstructural proteins and the replicating HCV RNA were colocalized in the perinuclear rim as dense spots, suggesting that the strongly immunostaining spots are the sites of HCV RNA replication (Fig. 1). The dense spots may represent some types of membranous structures/alterations induced by nonstructural HCV proteins (Egger *et al.*, 2002) or HCV RNA replication (Gosert *et al.*, 2003). Several types of ER membrane alteration were induced by virus infection and/or viral proteins in other positive-stranded RNA viruses (Mackenzie *et al.*, 1996; Schwartz *et al.*, 2002; Suhy *et al.*, 2000; Westaway *et al.*, 1997). Poliovirus infection causes invagination of the ER membrane to form double-membraned vesicles (Suhy *et al.*, 2000). Dengue virus infection induces VPs from the ER membrane; this was also observed in

Kunjin virus-infected Vero cells (Mackenzie *et al.*, 1996; Westaway *et al.*, 1997). Nonstructural viral proteins and double-stranded RNAs were colocalized in the VP, suggesting that the VP was the site of viral RNA replication for dengue and Kunjin viruses (Mackenzie *et al.*, 1996; Westaway *et al.*, 1997). For BMV, protein 1a alone was able to induce expansion of the ER lumen and result in invagination of the ER membrane to form membrane-bound spherules (Schwartz *et al.*, 2002). Convoluted membranes and paracrystalline structures were observed in Huh7 cells carrying a replicating HCV replicon (Gosert *et al.*, 2003; Mottola *et al.*, 2002). NS4B was found to induce a membranous web, an ER membrane alteration containing membranous vesicles (Egger *et al.*, 2002). Whether the dense spots on the perinuclear membrane of the Huh7 cell harbouring a replicating HCV RNA are clusters of specific membrane alteration (e.g. a membranous web) induced by HCV nonstructural proteins remains to be verified.

Membrane association of RNA replication is an important feature common to all known positive-stranded RNA viruses (Egger *et al.*, 2000, 2002; Froshauer *et al.*, 1988; Gosert *et al.*, 2003; Mackenzie *et al.*, 1996; Mottola *et al.*, 2002; Schwartz *et al.*, 2002; Shi *et al.*, 2003; Suhy *et al.*, 2000; Westaway *et al.*, 1997). Findings from this study confirm further that the HCV RC was associated with the ER or with membranes derived from the ER. Membrane flotation analysis of the HCV RC demonstrated that HCV nonstructural proteins were cofractionated with the HCV RNA and the ER-resident protein, calnexin (Fig. 2). When membranes were disrupted with a detergent, nonstructural proteins and RNA all remained in the soluble fractions at the bottom of the sucrose density gradient (Fig. 3), confirming the membrane association of the HCV RC. Moreover, HCV RNA contained in the RC was resistant to RNases, while disruption of the membranes with detergent rendered the viral RNA sensitive to RNases (Fig. 4). These findings suggest that the HCV RC was assembled in the membrane-associated structures that protected HCV RNA from RNase digestion. The question arises as to why RNA replication always occurs on intracellular membranes (e.g. membranous vesicles/spherules induced by viral proteins). First of all, many of the nonstructural proteins required for RNA replication were targeted to the ER membranes by hydrophobic membrane-anchoring domains (Brass *et al.*, 2002; Hugle *et al.*, 2001; Schmidt-Mende *et al.*, 2001). For instance, HCV NS5A and NS5B proteins interact with ER membranes via the N-terminal amphipathic α-helix and the C-terminal hydrophobic transmembrane domains, respectively (Brass *et al.*, 2002; Schmidt-Mende *et al.*, 2001). In addition, the membranous structures such as vesicles and spherules induced by viral proteins separate the virus RC from the hostile environment of the cytosol. Otherwise, the viral RNA exposed to the cytosol may be vulnerable to degradation by various nucleases. It was demonstrated unequivocally that the BMV protein 1a was able to transfer viral RNA to a membrane-associated and nuclease-resistant state (Schwartz *et al.*, 2002). Findings from our studies demonstrate that membrane structures protected the HCV RNA assembled in the RC from degradation by RNases (Fig. 4). Furthermore, RNA replication requires membrane-bound cellular proteins. A recent study using a yeast two-hybrid system revealed that both HCV NS5A and NS5B proteins interact with hVAP (Tu *et al.*,

1999). There may be other undetermined factors accounting for the membrane association of viral RNA replication.

The results derived from this study also indicate that all nonstructural proteins (NS3, NS4A, NS4B, NS5A and NS5B) expressed from the HCV replicon RNA were assembled in the HCV RC. Each of these proteins was colocalized with the replicating HCV RNA to perinuclear ER membranes and cofractionated with HCV RNA in the membrane-bound complex (Figs 1 and 2). However, we do not know whether all of these nonstructural proteins are required for HCV RNA replication. Deletion analysis of the subgenomic HCV replicon indicated that NS4B and NS5A are essential for HCV RNA replication (unpublished data). However, the exact roles of NS4B and NS5A in HCV RNA replication are not known (Tu *et al.*, 1999). Although NS3 has multiple enzymatic activities, its role in HCV RNA replication is not clear either (Banerjee & Dasgupta, 2001; De Francesco & Steinkuhler, 2000). NS5B was found to interact with the cellular protein hVAP, suggesting a role for cellular protein(s) in HCV RNA replication (Tu *et al.*, 1999). There are probably cellular proteins other than hVAP that are required for HCV RNA replication. The potential cellular components of the HCV RC may also include those interacting with the 5′- and 3′UTR of the HCV RNA genome, including polypyrimidine tract-binding protein, hnRNP-C, and GAPDH (Gontarek *et al.*, 1999; Ito & Lai, 1997; Luo, 1999; Petrik *et al.*, 1999; Tsuchihara *et al.*, 1997). However, the exact cellular proteins of the HCV RC and their roles in HCV RNA replication remain to be determined.

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