The molecular mechanism of hepatitis C virus (HCV) RNA replication is still unknown. Recently, a cell culture system in which the HCV subgenomic replicon is efficiently replicated and maintained for a long period in Huh-7 cells has been established. Taking advantage of this replicon system, we detected the activity to synthesize the subgenomic RNA in the digitonin-permeabilized replicon cells. To elucidate how and where this viral RNA replicates in the cells, we monitored the activity for HCV RNA synthesis in the permeabilized replicon cells under several conditions. We obtained results suggesting that HCV replication complexes functioning to synthesize the replicon RNA are protected from access of nuclease and proteinase by possible cellular lipid membranes. We also found that a large part of the replicon RNA, including newly synthesized RNA, was present in such a membranous structure but a large part of each NS protein was not. A small part of each NS protein that is resistant to the proteinase action was shown to contribute sufficiently to the synthesis of HCV subgenomic RNA in the permeabilized replicon cells. These results suggested that a major subcellular site of HCV genome replication is probably compartmentalized by lipid membranes and that only a part of each NS protein forms the active replication complex in the replicon cells.

Infection of hepatitis C virus (HCV) is estimated to occur in about 3% of the world’s population. HCV infection frequently causes chronic hepatitis, which often leads to the development of liver cirrhosis and hepatocellular carcinoma after a long period (1–3). Current combination therapy with interferon-α and ribavirin, a nucleotide analogue, is effective in many patients with chronic hepatitis C (4, 5). There still are, however, a large number of patients who do not respond to these treatments. Therefore, extensive studies have been performed to develop highly effective anti-HCV drugs. Such a drug, however, has not been produced yet, possibly because of the lack of detailed information about the life cycle of this virus.

HCV is a member of the Flaviviridae family and contains a single-strand RNA genome of positive polarity (6). The RNA genome is ~9.6 kb in length and consists of a 5′-untranslated region of 341 nucleotides, a large open reading frame encoding a single precursor polyprotein of ~3500 amino acids, and a 3′-untranslated region of variable length (6–8). The polyprotein is processed by the host and viral proteinases to generate at least 10 functional viral proteins: core (C), envelope (E) 1, E2, p7, non-structural protein (NS) 2, NS3, NS4A, NS4B, NS5A, and NS5B (from the amino- to the carboxyl-terminal) (9–12). C, E1, and E2 are believed to form viral particles as structural proteins. p7 was recently reported to form an ion channel-like structure (13, 14). NS molecules have been considered to function in the replication of HCV subgenomic RNA (15). Using recombinant proteins produced in either bacterial or insect cells, the proteinase and helicase activities of NS3 and RNA-dependent RNA polymerase activity of NS5B have been biochemically characterized (16–20). However, it was not clear whether these recombinant proteins function in HCV genomic replication as it has been revealed that HCV genomic sequences are highly variable among all isolates and furthermore, it is unclear whether the genes for these viral enzymes were derived from infectious HCV genomes.

Recently, HCV subgenomic RNA that replicates efficiently and is maintained for a long period in the human hepatoma cell line Huh-7 was developed and called the HCV subgenomic replicon (15). Functional replicons originating from different HCV isolates have been reported (15, 21–23). The HCV subgenomic RNA was constructed by replacing the structural and part of the non-structural protein-encoding regions (C-NS2) of the HCV genome with the neomycin phosphotransferase gene (neo) and an internal ribosome entry site of encephalomyocarditis virus (21). This implies that HCV proteins encoded in this subgenomic RNA (NS3-NS5B) are functional and sufficient for this RNA replication. In this model system, it has been suggested that mutations of particular amino acids in the NS region enhanced the efficiency of the replication (24–27). It was also demonstrated that the existence of the cis-acting elements in either the 5′- or 3′-untranslated regions were required for efficient replication (27–29). Recent observations indicated that the replication of the replicon RNA could be reproduced in vitro using particular cellular fractions from replicon cells (30–32). It remains, however, to elucidate how and where the HCV RNA is synthesized in the cells. So, we intended to clarify these points using digitonin-treated replicon cells of which plasma membranes were permeabilized. This permeabilized cell system is often used to monitor several cellular events,
such as a nuclear protein transport as well as replication of positive-strand RNA viruses.

Cell biological and biochemical analyses have demonstrated that all HCV NS proteins are directly or indirectly associated with inner cellular membranes and colocalize on the rough endoplasmic reticulum (ER) membranes (33–36). So, we expected the active HCV replication complexes to be retained on the inner cellular membranes in permeabilized replicon cells. In this paper, we report that the functional replication complexes are retained in permeabilized replicon cells and its activity is enhanced by using this system. We also obtained data suggesting that a part of each NS protein in the cell, which is probably located in a membranous compartment, forms the active replication complex and contributes to the synthesis of HCV subgenomic RNA.

EXPERIMENTAL PROCEDURES

Cell Cultures—The human hepatoma cell line HuH-7 was grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml nonessential amino acids (Invitrogen), and 100 μg/ml penicillin and streptomycin sulfate (Invitrogen). MH-14 cells were cultured in the same medium with 500 μg/ml G418 (GENETICIN, Invitrogen). MH-14 cells were cultured in the same medium with 500 μg/ml penicillin and streptomycin sulfate (Invitrogen).

Preparation of RNA and Northern Blot Analysis—RNA was extracted from cells and a reaction mixture with Sepasol RNA I and II as described previously (37). For the preparation of the 32P-labeled probe, the EcoRI fragment of pNNRZ2 was labeled with a Ready-to-Go DNA labeling beads kit (Promega, Madison, WI) into the TA site of pGEM-T-Easy vector (Promega, Madison, WI), and the cDNA fragment of the subgenomic replicon from pNNRZ2 and used as a template for in vitro transcription with MEGA script SP6 or T7 kit (Ambion, Austin, TX), respectively.

In Vitro Transcription—pGEM-NN was linearized by PvuI or SpeI for plus or minus strand subgenomic HCV RNA synthesis, respectively, and used as a template for in vitro RNA synthesis with MEGA script SP6 or T7 kit (Ambion, Austin, TX), respectively.

Preparation of RNA and Northern Blot Analysis—RNA was extracted from cells and a reaction mixture with Sepasol RNA I and II super reagent (Nacalai Tesque, Kyoto, Japan), respectively, according to the manufacturer’s protocol. Northern blot analysis was performed as described previously (37). For the preparation of the 32P-labeled probe, the EcoRI fragment of pNNRZ2 was labeled with a Ready-to-Go DNA labeling beads kit (Ambersham Biosciences) in the presence of 50 μCi of [α-32P]UTP (Ambersham Biosciences).

Western Blotting—The preparation of the cell lysate, SDS-PAGE, and immunoblotting were performed as previously described (10). The antibodies used in the immunoblotting were those against HCV NS3, NS4A (anti-NS4A), NS4B (NS4B-52), NS5A (anti-NS5A), NS5B (NS5B-14) (21), dihydrofolate reductase (DHFR) (34), BiP/Grp78 (StressGen, Victoria, BC, Canada), and Calnexin-NT (StressGen). Anti-NS3 antibody was a gift from Dr. M. Kohara (Tokyo Metropolitan Institute of Medical Science).

 indirect Immunofluorescence—indirect immunofluorescence analysis was essentially performed as described previously (38), with minor modifications. 1.0 × 10⁵ cells were seeded on poly-L-lysine (Sigma)-coated coverslips. Three days post-seeding, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄) for 30 min at room temperature. The cells were treated with pre-chilled 100% methanol for 10 min at −20 °C after the fixation. In the case of permeabilized cells, permeabilization with digitonin followed by washing with buffer B (see below) was performed before or after fixation with 4% paraformaldehyde and then the cells were permeabilized completely with washed 100% methanol after fixation. The antibodies against NS5A (anti-NS5A), NS5B (NS5B-12), and protein-disulfide isomerase (StressGen) were used as primary antibodies. NS5B-12 was a gift from Dr. M. Kohara (Tokyo Metropolitan Institute of Medical Science).

Cell Permeabilization and Synthesis of HCV Subgenomic RNA—Cells of about 80% confluency in 12- or 6-well plates were precultured in complete Dulbecco’s modified Eagle’s medium containing 5 μg/ml actinomycin D (Nacalai Tesque) for 2 h, then washed with cold buffer B: 20 mM HEPES-KOH (pH 7.7 at 27 °C), 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, and 2 mM dithiothreitol. The cells were permeabilized by incubation in buffer B containing 50 μg/ml digitonin for 5 min at 27 °C and the reaction was stopped by washing twice with cold buffer B. The permeabilized cells were then, incubated for 4 h at 27 °C in the labeling reaction mixture: 2 mM manganese(II) chloride, 1 mg/ml acetylated bovine serum albumin (Nacalai Tesque), 5 mM phosphocreatine (Sigma), 20 units/ml creatine phosphokinase (Sigma), 50 μg/ml actinomycin D, 500 μM ATP, CTP, and GTP (Roche Diagnostics), and 10 μCi of [α-32P]UTP (Ambersham Biosciences) in buffer B (pH 7.7), for 4 h at 27 °C unless otherwise specified. The reaction was terminated by the addition of Sepasol RNA I or II super reagent.

For the experiments with micrococcal nuclease and/or Nonidet P-40, the permeabilized cells were pre-washed twice with buffer D: 20 mM HEPES-KOH (pH 7.7 at 27 °C), 110 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, and 1 mM CaCl₂. Then the permeabilized cells were incubated in buffer D containing 0.1 unit/ml micrococcal nuclease (United States Biochemical Corp.), with or without 0.5% Nonidet P-40 for 15 min at 37 °C or 0.5% Nonidet P-40 for 15 min on ice.

Slot Blot Hybridization—RNA products synthesized in the permeabilized cells in the presence of 200 μCi of [α-32P]UTP were fractionated by denaturing agarose gel. The 8-κb RNA bands were eluted from the gel by using RNAse (BioOne, Carlsbad, CA). To increase the hybridization signal, the 32P-labeled 8-κb RNA eluted from the gel was subjected to alkaline hydrolysis to generate fragments of ~250 nucleotides in length and used in hybridization. Newly synthesized replicon RNA in intact replicon cells was metabolically labeled by adding 1200 μCi of [32P]orthophosphate to the culture medium (see below) and handled in the same manner. For detection of plus and minus strand replicon RNA, minus and plus strand replicon RNA were prepared as riboprobes by in vitro transcription, respectively, as described above. Then 2 μg of each riboprobe was applied to a nylon membrane filter (Hybond-N, Ammon). Slot blot hybridization was performed as described previously (39), except that UTRAHYb (Ambion) was used for hybridization buffer.

Metabolic Labeling—For metabolic labeling of the replicon RNA, after a preculture in phosphate-free Dulbecco’s modified Eagle’s medium (Invitrogen) containing 2% dialyzed fetal bovine serum, 200 μg/ml G418, and 5 μg/ml actinomycin D for 2 h, 4 × 10⁵ cells were cultured for 12 h in the same medium with 100 μCi of [32P]orthophosphate (Amersham Biosciences), as described previously (21).

Protease K Treatment—After permeabilization of the replicon cells, the cells were scraped into 400 μl of cold buffer B and transferred to a siliconized tube. The cells were treated with various concentrations of protease K at 37 °C for 5 min. The reaction was terminated by the addition of 1 μl of methylmethanesulfonyl fluoride, and followed by trichloroacetic acid precipitation. The trichloroacetic acid precipitates were solubilized in 1× sample buffer containing 1 μl of methylmethanesulfonyl fluoride and used in Western blotting analysis.

RESULTS

Selection of Replicon Cell Clones in Which Replicon RNA Efficiently Replicated—We reported the establishment of a cell clone, NN 50-1, in which HCV subgenomic replicon RNA originating from the HCV genome isolated from the cultured human T cell line MT-2C infected with HCV in vitro efficiently replicated (21). Furthermore, we obtained a cell clone, MH-14, in which the HCV subgenomic RNA level is nearly 5-fold higher than that in NN 50-1 (data not shown). Nucleotide sequence analysis revealed that the replicon RNA in MH-14 cells bore two point mutations in the NS4B and NS5A encoding regions. One was a thymine to cytosine transition at nucleotide position 5985 (the number corresponds to the nucleotide number of the HCV genotype 1b genome) in the NS4B-encoding region without an amino acid substitution. Another was a cytosine to adenine transition at nucleotide 6953 in the NS5A-encoding region without an amino acid position 2204. The substitution at amino acid 2204 is essential for the maintenance of the active replication complex and contributes to the synthesis of HCV subgenomic RNA.
experiments to analyze the mechanism of HCV genomic replication.

Replicon RNA and NS Proteins Were Retained in Digitonin-permeabilized Replicon Cells—Previous papers suggested that all HCV NS proteins are directly or indirectly associated with the inner cellular membranes, especially rough ER membranes, and form replication complexes on the membranes (10, 40). This suggested to us that functional HCV replication complexes could be retained in the replicon cells whose plasma membranes had been permeabilized with digitonin. To test this possibility, first, the fate of NS proteins in the replicon cells was investigated after digitonin treatment by Western blotting. In this experiment, ectopically expressed mouse DHFR was used as a cytoplasmic protein marker. After digitonin treatment, DHFR was not detected in the permeabilized cells (Fig. 1A, lanes 2 and 3), indicating that cytoplasmic soluble proteins were washed efficiently out of the cells under these conditions. On the other hand, HCV NS proteins (NS3-NS5B) were detected just like BiP/Grp78, an ER marker, in the permeabilized replicon cells as in the intact cells that were not treated with digitonin, as expected (Fig. 1A, lanes 2 and 3). Moreover, when we analyzed the RNA by Northern blotting, the retention of replicon RNA in the permeabilized replicon cells was observed (Fig. 1B, lanes 2 and 3). Treatment of the permeabilized replicon cells with the high salt buffer containing 2 M KCl did not greatly influence the amount of replicon RNA and NS proteins retained in the cells (data not shown). To investigate whether newly synthesized replicon RNA in the intact replicon cells was retained after permeabilization with digitonin, we performed metabolic labeling of the cells with [32P]orthophosphate. As shown in Fig. 1C, newly synthesized replicon RNA was detected after permeabilization (Fig. 1C, lane 3), although the amount was slightly decreased compared with that in the intact cells (Fig. 1C, lane 2). The localization of NS5A and NS5B in the permeabilized replicon cells was also analyzed by indirect immunofluorescence. These proteins were seen to accumulate around the perinuclear region and to be mostly colocalized with protein-disulfide isomerase, an ER marker, in the permeabilized replicon cells (Fig. 1D, panels i–p), just as in the intact replicon cells (Fig. 1D, panels a–h). This indicated that treatment with digitonin did not markedly affect subcellular localization of these proteins. From all of these results, it seemed likely that the replication complexes including replicon RNA were retained in the permeabilized replicon cells just like in the intact cells.

The Replication Complexes in the Permeabilized Replicon Cells Functioned to Synthesize the HCV Subgenomic RNA—To see whether the replication complexes in the permeabilized replicon cells were active in HCV RNA synthesis, permeabilized or intact cells were incubated in reaction mixtures including [α-32P]UTP for 4 h. Actinomycin D, which showed no inhibitory effect on the RNA-dependent RNA polymerase activity of HCV NS5B (15), was also added to the reaction mixture to inhibit the cellular activities of DNA-dependent DNA and RNA synthesis. After the reaction, total RNA of the cells and the reaction supernatants were extracted and analyzed by denaturing agarose gel electrophoresis followed by autoradiography. A radiolabeled product ~8 kb in length, which was equivalent to the subgenomic replicon RNA in size, was found in RNA from the permeabilized replicon cells but not from HuH-7 cells (Fig. 2A, lanes 1–4). When overexposed, the 8-kb band became detectable in RNA sample from the intact replicon cells, although the signal was much lower than that from the permeabilized replicon cells (data not shown). This 8-kb product, however, was not detected in all the reaction supernatants (Fig. 2A, lanes 5–8). The radiolabeled 8-kb product was degraded by treatment with RNase A (data not shown), suggesting that it was HCV subgenomic RNA synthesized by the replication complexes in the permeabilized replicon cells. To examine whether the 8-kb RNA synthesized in the permeabilized cells was actually HCV subgenomic RNA, we performed slot blot hybridization analysis using each plus and minus strand HCV...
RNA synthesized in vitro as probes on the nylon membrane filter. After denaturing agarose gel electrophoresis, the radiolabeled 8-kb RNA derived from the permeabilized replicon cells was eluted from the gel and hybridized with the probes on the filter. Then the radioactivity hybridized with either the plus or minus strand-specific probe on the filter was detected by autoradiography (Fig. 2B, 8 kb RNA), whereas radiolabeled RNA prepared from permeabilized Huh-7 cells in the same way did not show any hybridization signals on the filter (data not shown). By the same procedure, we also confirmed that the metabolically radiolabeled 8-kb RNA in Fig. 1C was actually replicon RNA (data not shown). The ratio of plus to minus strand RNA synthesized in the permeabilized replicon cells was estimated to be 2.7 ± 0.4 (average ± S.D.) by three independent experiments. These results indicated that the radiolabeled 8-kb RNA was actually HCV subgenomic RNA synthesized in the permeabilized replicon cells and contained both plus and minus strands of the HCV RNA, implying that the functional HCV replication complexes were present in the permeabilized replicon cells.

**Replicon RNA in the Permeabilized Replicon Cells Was Resistant to Nuclease**—The production of radiolabeled HCV subgenomic RNA in the permeabilized cells seemed to continue until 4 h and reached a maximum until ~5 to 6 h (data not shown). Then the amount of radiolabeled HCV RNA was stably maintained even after 8 h. One possible explanation for this stability was the removal of RNase from the cell by permeabilization. Therefore, we examined the sensitivity of the newly synthesized HCV RNA in the permeabilized replicon cells to exogenously added nuclease. After the reaction for RNA synthesis, the permeabilized replicon cells were treated with micrococcal nuclease. As shown in Fig. 3A, the radiolabeled HCV RNA remained almost intact even after nuclease treatment (lanes 1 and 2), although 28 S rRNA was efficiently degraded under the same condition (lanes 2 and 3). This lower sensitivity of the replicon RNA to nuclease suggested that one of the reasons for the stability of the RNA in the permeabilized cells was its resistance to RNase activity. On the other hand, the radiolabeled HCV RNA was sensitive to nuclease in the presence of a nonionic detergent, Nonidet P-40 (Fig. 3A, lane 3), although a small portion of the HCV RNA was likely to remain resistant to nuclease. When the permeabilized replicon cells were treated with only Nonidet P-40, the radiolabeled HCV RNA in the cells was still detectable but apparently diminished. However, the radiolabeled molecules became detectable in the reaction supernatant, possibly because of leakage from the cells because of Nonidet P-40 (Fig. 3A, lanes 4 and 8). From these results, HCV subgenomic RNA newly synthesized in the permeabilized cells seemed resistant to nuclease in a cellular lipid membrane-dependent manner. Moreover, we also observed that the replicon RNA synthesis was equally carried out when the permeabilized cells were pretreated with nuclease before the reaction of the RNA synthesis (data not shown). This suggested that the HCV RNA used as a template in the viral RNA synthesis was also resistant to nuclease and present in the same environment as the newly synthesized RNA products mentioned above. Furthermore, we investigated the replicon RNA that was newly synthesized in the intact replicon cells. After metabolic labeling of the replicon cells with [32P]orthophosphate, the cells were permeabilized with digitonin and treated with micrococcal nuclease as above. As shown in Fig. 3B, about 30% of radiolabeled replicon RNA in the intact replicon cells was found to be lost after permeabilization (lanes 1 and 2), probably implying that a part of the newly synthesized RNA flowed out with the cytoplasm from the permeabilized cells (see discussion). A large part of newly synthesized replicon RNA retained in the permeabilized cells showed resistance to the nuclease action in the absence of Nonidet P-40 (Fig. 3B, lane 3) but was tuned to be sensitive to nuclease in the presence of Nonidet P-40 (Fig. 3B, lane 4). This suggested that the fate of the newly synthesized replicon RNA in the permeabilized cells was similar to that retained in intact cells after permeabilization. Then we analyzed the nuclease sensitivity of total replicon RNA in replicon cells by Northern blotting. After permeabilization, replicon cells were treated with nuclease in the presence or absence of Nonidet P-40 as described above. As shown in Fig. 3C, we found that most of the replicon RNA was intact even after treatment (Fig. 3C, lanes 2 and 3). As in the case of the newly synthesized replicon RNA, the replicon RNA pre-existing in the replicon cells was sensitive to nuclease in the presence of Nonidet P-40 (Fig. 3C, lane 4).
results, it was suggested that replicon RNA existed in a subcellular compartment that was formed with cellular lipid membranes.

A Large Amount of Each NS Protein Was Not Required for Nuclease Resistance of Replicon RNA—To investigate the contribution of HCV NS proteins to the resistance of replicon RNA against nuclease, the sensitivity of the replicon RNA to nuclease was examined in permeabilized replicon cells after treatment with proteinase K at various concentrations. The condition of the replicon RNA and NS proteins in the cells after treatment was analyzed by Northern and Western blotting, respectively. An endogenous Calnexin, a type I transmembrane protein, was metabolically labeled with [32P]orthophosphate. After incubation, the cells were permeabilized and treated with (lanes 3, 4, 7, and 8) or without micrococcal nuclease (lanes 2, 5, 6, and 9). The reactions were performed in the presence (Nonidet P-40 +, lanes 3, 4, 7, and 8) or absence of 0.5% Nonidet P-40 (Nonidet P-40 −, lanes 1, 2, 5, and 6). Total RNA extracted from the cells (Cell, lanes 1−4) and the reaction supernatant (Sup, lanes 5−8) was similarly analyzed as described in the legend to Fig. 2. B, resistance of the newly synthesized replicon RNA in the intact replicon cells to nuclease action. The intact replicon cells were metabolically labeled with [32P]orthophosphate. After incubation, the cells were permeabilized and treated with (lanes 3, 4, 7, and 8) or without micrococcal nuclease (lanes 2, 5, 6, and 9), in the presence (lanes 4, 5, 8, and 9) or absence of Nonidet P-40 (lanes 2, 3, 6, and 7), as described above. C, resistance of the replicon RNA in replicon cells against nuclease action. After permeabilization of the replicon cells with digitonin, the cells were treated with (lanes 3, 4, 7, and 8) or without micrococcal nuclease (lanes 2, 5, 6, and 9), in the presence (lanes 4, 5, 8, and 9) or absence of (lanes 2, 3, 6, and 7) Nonidet P-40 as above. Total RNA from the cell fraction or the reaction supernatant after treatment was analyzed by Northern blotting using a HCV genome-specific probe to detect the replicon RNA (indicated by the arrowhead with replicon, upper panel). 28 S rRNA (28S) was detected by staining with ethidium bromide (A−C, lower panels). In A, the amount of 28 S rRNA seemed to be low probably because of degradation during incubation in the reaction mixture for 4 h.

**Fig. 3.** A large part of the replicon RNA was located in the nuclease-resistant environment in the replicon cells. A, resistance of the newly synthesized HCV RNA in the permeabilized replicon cells to nuclease action. After the RNA synthesis reaction in the presence of [32P]UTP, the permeabilized replicon cells were treated with (nuclease +, lanes 2, 3, 6, and 7) or without micrococcal nuclease (nuclease −, lanes 1, 4, 5, and 8). The reactions were performed in the presence (Nonidet P-40 +, lanes 3, 4, 7, and 8) or absence of 0.5% Nonidet P-40 (Nonidet P-40 −, lanes 1, 2, 5, and 6). Total RNA extracted from the cells (Cell, lanes 1−4) and the reaction supernatant (Sup, lanes 5−8) was similarly analyzed as described in the legend to Fig. 2. B, resistance of the newly synthesized replicon RNA in the intact replicon cells to nuclease action. The intact replicon cells were metabolically labeled with [32P]orthophosphate. After incubation, the cells were permeabilized and treated with (lanes 3, 4, 7, and 8) or without micrococcal nuclease (lanes 2, 5, 6, and 9), in the presence (lanes 4, 5, 8, and 9) or absence of Nonidet P-40 (lanes 2, 3, 6, and 7), as described above. C, resistance of the replicon RNA in replicon cells against nuclease action. After permeabilization of the replicon cells with digitonin, the cells were treated with (lanes 3, 4, 7, and 8) or without micrococcal nuclease (lanes 2, 5, 6, and 9), in the presence (lanes 4, 5, 8, and 9) or absence of (lanes 2, 3, 6, and 7) Nonidet P-40 as above. Total RNA from the cell fraction or the reaction supernatant after treatment was analyzed by Northern blotting using a HCV genome-specific probe to detect the replicon RNA (indicated by the arrowhead with replicon, upper panel). 28 S rRNA (28S) was detected by staining with ethidium bromide (A−C, lower panels). In A, the amount of 28 S rRNA seemed to be low probably because of degradation during incubation in the reaction mixture for 4 h.

The Active Replication Complex Was in a Similar Environment to the Replicon RNA—As shown above, the majority of each NS protein in digitonin-permeabilized replicon cells were sensitive to proteinase treatment. Furthermore, all NS proteins (NS3-NS5B) in replicon cells were detectable by indirect immunofluorescent analysis after permeabilization only with digitonin following fixation with 4% paraformaldehyde, although the proteins such as protein-disulfide isomerase and BiP/Grp78 that are present in the ER lumen were not detected under this condition (data not shown). These results indicated that the precise subcellular localization of replicon RNA was different from that of the majority of each NS protein.

The Active Replication Complex Was in a Similar Environment to the Replicon RNA—As shown above, the majority of each NS protein in digitonin-permeabilized replicon cells were sensitive to proteinase treatment. Furthermore, all NS proteins (NS3-NS5B) in replicon cells were detectable by indirect immunofluorescent analysis after permeabilization only with digitonin following fixation with 4% paraformaldehyde, although the proteins such as protein-disulfide isomerase and BiP/Grp78 that are present in the ER lumen were not detected under this condition (data not shown). These results indicated that the precise subcellular localization of replicon RNA was different from that of the majority of each NS protein.
HCV Replication Complex in Membranous Compartments

**Fig. 4.** A large part of each NS protein was not required for the nucleic resistance of the replicon RNA. The digitonin-permeabilized replicon cells were treated with proteinase K at various concentrations (0 µg/ml for lanes 2 and 3, 0.01 µg/ml for lanes 4 and 8, 0.1 µg/ml for lanes 5 and 9, 1 µg/ml for lanes 6 and 10, 10 µg/ml for lanes 7 and 11), followed by treatment with (lanes 3 and 8–11) or without (lanes 2 and 4–7) micrococcal nuclease. The samples from intact replicon cells without any treatments were similarly analyzed as shown in lane 1. After these treatments, total RNA and protein in the cells were analyzed by Northern and Western blotting, respectively. Antibodies used in the Western blotting were anti-NS4B (NS4B), anti-NS5A (NS5A), anti-NS5B (NS5B), anti-Calnexin (Calnexin-NT), and anti-KDEL (BiP/Grp78) antibodies. Each protein with original size is indicated by an arrowhead. An asterisk denotes the position of the Calnexin NH2-terminal segment that is located in the lumen of the ER. BiP/Grp78, which located on the luminal side of the ER membrane, was used as a negative control for proteinase digestion.

**Fig. 5.** A, the activity for the synthesis of HCV RNA in the permeabilized replicon cells was intact even after degradation of a large part of each NS protein by proteinase K treatment. After treatment of the permeabilized replicon cells with proteinase K as described in the legend to Fig. 4, the RNA synthesis reaction was performed. The concentrations of proteinase K used in this experiment were 0 (lane 1), 0.01 (lane 2), 0.1 (lane 3), 1 (lane 4), or 10 µg/ml (lane 5). Total protein of the cells prior to the RNA synthesis reaction was analyzed by Western blotting using anti-NS4B (NS4B), anti-NS5A (NS5A), anti-NS5B (NS5B), anti-Calnexin (Calnexin-NT), and anti-KDEL (BiP/Grp78) antibodies. Newly synthesized replicon RNA that was labeled with [32P]UTP was indicated by the arrowhead with replicon, upper panel. B, a small part of each NS protein was located in the proteinase-resistant environment in the replicon cells. After permeabilization of the replicon cells with digitonin, fractions of the cells were treated with 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4), 50 (lane 5), and 100 µg/ml (lane 6) proteinase K. After the reaction, total protein was analyzed by Western blotting using anti-NS3 (NS3), anti-NS4A (NS4A), anti-NS4B (NS4B), anti-NS5A (NS5A), anti-NS5B (NS5B), anti-Calnexin (Calnexin-NT), and anti-KDEL (BiP/Grp78) antibodies.

**DISCUSSION**

By monitoring the synthesis of HCV RNA in permeabilized HCV replicon cells, we obtained results suggesting that the active HCV replication complexes function to synthesize the replicon RNA in subcellular compartments, which are probably formed by cellular lipid membranes. Recently, electron microscopic analysis showed that the expression of HCV proteins in Huh-7 cells induced the formation of a distinct membrane structure, designated a "membranous web," and all HCV proteins were found in the structure (40). Moreover, a similar web-like structure in livers of HCV-infected chimpanzees and HCV subgenomic replicon cells was reported (41, 42). Therefore, it seems likely that this membrane structure is a candidate for the site where the HCV RNA genome is mainly located and replicated. Until now the genomes of many positive-strand RNA viruses, such as brome mosaic virus, murine hepatitis virus, and Kunjin virus have been reported to replicate on inner cellular membranes in association with vesicles or other membrane structures (43–45). These membrane structures seemed to be constructed by viral proteins. For example, it was reported that brome mosaic virus la, the multifunctional RNA replication protein, selectively recruits brome mosaic virus 2a polymerase and viral RNA and forms membrane-bound spheres (43, 46). A subcellular site for the genome replication of these viruses including HCV has been suggested by the localization of brome uridine-incorporated genomic RNA, which is a...
product of the replication reaction, in the cells (43, 47–49). It has not been biochemically analyzed, however, whether viral RNA is actually synthesized in or around such a membrane structure. In this paper, we showed that a quite similar activity for the synthesis of HCV subgenomic RNA was present in the permeabilized replicon cells even after proteinase treatment, which digested almost all the NS proteins including NS5B, to that in the cells not treated with proteinase. Furthermore, we found that a small part of each NS protein is actually present in the proteinase-resistant environment in the replicon cells. This suggested, therefore, that a small part of each NS protein forms the replication complex and functions in the membranous compartment.

A fairly large amount of each HCV protein accumulated in the perinuclear fraction in the replicon cells, which is exposed to the cytoplasmic environment. In this protein complex, no association of the HCV subgenomic RNA was observed and there was no activity to synthesize the viral RNA. Thus, the significance of the HCV protein complex regarding multiplication of the virus genome remains to be clarified. The replicon cells originating from HuH-7 may produce large numbers of HCV proteins in the perinuclear fraction as a consequence of overproduction and these proteins may play less important roles in the replication of the HCV genome than the active replication complex that we have noted in this paper. Conversely, the protein complex may play important roles in the regulation of not only the multiplication of the HCV genome but also further processes during the maturation of the virus.

In this regard, it is important to analyze the presence as well as the function of the HCV protein complexes in cells where HCV proliferates with different degrees of multiplication. To date, several cellular proteins that interact with particular NS proteins have been reported. For example, double strand RNA-dependent protein kinase, soluble NSF attachment protein receptors-like protein, and karyopherin β3 are reported to interact with NS5A (50–52), although the physiological importance of these interactions has been obscure. This larger part of each NS protein might, therefore, participate in several cellular events and/or modulate the replication of the HCV genome through interactions with these cellular proteins.

The ratio of minus to strand RNA synthesized in the permeabilized replicon cells was estimated to be 2.7 ± 0.4 by slot blot hybridization analysis as shown in Fig. 2. On the other hand, that in the intact replicon cells was estimated to be 11.9 ± 2.0 as reported previously (data not shown and Ref. 15), when intact replicon cells were metabolically labeled with [32P]orthophosphate and the newly synthesized and radiolabeled replicon RNA in the cells was analyzed by slot blot hybridization in a similar manner. The discrepancy in the ratio between the permeabilized and intact cells may be explained by the release of the replicon RNA synthesized in the permeabilized cells from the membranous compartment and degraded in the reaction mixture, although the possibility that the regulation of the minus to strand ratio of newly synthesized replicon RNA may be lost in the permeabilized replicon cannot be completely ruled out. Approximately 50% of the replicon RNA newly synthesized in the intact cells was actually lost by nuclease treatment following permeabilization (Fig. 3B, lanes 1 and 3), suggesting that some parts of replicon RNA newly synthesized in intact replicon cells is present in the cytoplasm. Slight degradation of the replicon RNA was also seen in permeabilized cells in the presence of Nonidet P-40 (Fig. 3). We also observed that in vitro synthesized replicon RNA added exogenously to the permeabilized cells was unstable in the reaction mixture (data not shown), as was observed in a recent report in which the replication activity of the HCV replicon was detected using the cell lysate fraction of replicon cells (30). These observations seem to support the former possibility indirectly. As the radiolabeled nucleotide substrate was incorporated in the newly synthesized replicon RNA in the permeabilized replicon cells, a channel-like structure should be present in the membranous compartment including the replication complex. In the case of spherules of brome mosaic virus, the channel-like structure connecting to the cytoplasm with the inside of the spherule was actually detected by the electron microscopic technique (43). This supported the idea that the replicon RNA that was present in the cytoplasmous compartment should be present in the membranous compartment including the replication complex. A similar phenomenon was already reported for reovirus in that relatively large reoviral mRNA was exported from the viral core particles to the cytoplasm by the channel formed by a viral protein (53). The pore size of the putative channel of HCV, however, seemed to be limited, because nuclease and proteinase did not pass through the channel. The replicon RNA may be specifically recognized by some viral proteins and exported through the channel post- or co-translationally. These mechanisms, however, have remained to be elucidated.

During the preparation of this article, Shi et al. (54) reported that almost all of the NS5A and part of the NS5B proteins were present in the membrane fraction that was resistant to treatment with 1% Nonidet P-40. Because the replicational activity in that fraction from the cell lysate was not demonstrated, we do not know whether that kind of membrane fraction from the cell lysate would include the replication activity observed here. Furthermore, a part of replicon RNA was reported to localize in the non-ionic detergent-insoluble membrane fractions (54), whereas we showed that the RNase resistance of both total and newly synthesized replicon RNA was reduced in the presence of Nonidet P-40. This discrepancy may be explained by the different methods used for the detection of the replicon RNA, reverse transcriptase-PCR and Northern blotting in that paper and ours, respectively. We also showed data indicating that a negligible amount of replicon RNA was released from the permeabilized cells into the reaction mixture by the detergent as shown in Fig. 3. It may also be relevant to this discrepancy that we and others have observed instability of the free replicon RNA in the reaction mixture (50). We also observed the existence of nuclease-resistant replicon RNA, even in the presence of nonionic detergent (Fig. 3). Such RNA may represent the replicon RNA in the non-ionic detergent-insoluble fractions.

Here we showed the stable nature of the active HCV RNA replication complex in the replicon cells by permeabilization of the cells. Our data suggests that only a small part of each NS protein contributes to RNA synthesis in the replicon cells. This implied that careful investigation would be required for identification of the precise subcellular sites of replication in the replicon cells. Further investigation to reveal how and where this complex is formed in the cells and what is essential for its activity is required for understanding the mechanism of viral replication and the life cycle of HCV.

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