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Biosynthesis and Biochemical Properties of the Hepatitis C Virus Core Protein

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The biosynthesis and biochemical properties of the putative nucleocapsid protein of hepatitis C virus (HCV) were investigated. RNA transcripts for cell-free translation were prepared from truncated forms of the cDNA construct encoding the structural proteins of HCV. Processing of the translation products was dependent on microsomal membranes and signal recognition particle, suggesting that release of the 21-kDa core protein from the polyprotein precursor is mediated solely by the signal peptidase of the endoplasmic reticulum (ER) and is achieved by the removal of a putative signal sequence of approximately 18 residues located at its C terminus. The core protein was found to bind membranes in vitro and in transfected cells, as shown by centrifugation analysis of in vitro translation products and transfected-cell lysates. Immunofluorescence of transfected cells showed that the core protein colocalized with the E2 glycoprotein as well as with a cellular ER membrane marker. The nucleocapsid protein expressed by in vitro translation in rabbit reticulocyte lysates cosedimented with the large ribosomal subunit in sucrose gradients. The ribosome binding domain was mapped to the N-terminal region of the core protein. Moreover, the same region was shown to bind RNA in vitro, suggesting that cosedimentation of core protein with ribosomes may be mediated by the RNA binding of the nucleocapsid protein of HCV. These studies indicate that the HCV core protein is a cytoplasmic protein associated with the ER membranes and possesses RNA binding activity.

Hepatitis C virus (HCV), the most common cause of non-A non-B hepatitis (7, 24), is thought to be an enveloped virus with a diameter of 30 to 60 nm (1). The viral genome consists of a positive-strand RNA of about 9.5 kb (8, 23, 49), which distantly resembles human flaviviruses and animal pestiviruses in its organization (32). As in the better-characterized flaviviruses and pestiviruses, the genome of HCV includes a single open reading frame that encodes a large polyprotein that is cleaved by host- and virus-encoded proteases to yield mature viral polypeptides (22). The putative structural proteins core (C), E1, and E2 are localized in the N-terminal quarter of the polyprotein, and the nonstructural proteins NS2, NS3, NS4a, NS4b, NS5a, and NS5b are found within the remainder of the polyprotein (18, 51). The genetic order of the HCV proteins on the viral polyprotein has been determined, and the NS3 protein has been shown to be a serine protease implicated in the release of all putative nonstructural proteins from the polyprotein precursor with the exception of NS2 (2, 17, 51). N-terminal sequencing of the processed nonstructural proteins has indicated that the viral protease cleavage specificity is characterized by residues with small side chains, Ser or Ala at the P1′ position and Cys or Thr at the P1 position (17, 36).

In vitro translation studies of the 5′ region of the HCV genome encoding the structural proteins have shown that these proteins are present in the order NH_2-C-E1-E2. Furthermore, these studies indicated that the structural proteins of HCV are released from the polyprotein precursor by cleavages that are presumably catalyzed in the endoplasmic reticulum (ER) lumen by host signal peptidase (21, 51). Amino acid sequencing of the cleaved products identified the N termini of E1 at amino acid (aa) residue 192 and of E2 at aa residue 384. Both of these residues are preceded by two hydrophobic segments (residues 174 to 191 and 371 to 383) that may act as signal sequences to direct the integration of the viral glycoproteins into the membrane of the ER (21). We have arbitrarily designated these segments domains H1 and H2, respectively.

The core protein is probably the nucleocapsid component of the virion, because it is highly basic and corresponds to the C protein found in flaviviruses. This protein appears to be well conserved among several HCV strains isolated in different regions worldwide (6). Interestingly, processing of C of the flavivirus West Nile virus requires cleavages mediated by both host proteases and by the viral serine protease. Host signal peptidase releases C from the viral polyprotein as a core precursor (called anchored core) characterized by a hydrophobic segment which is subsequently removed by the NS2b-NS3 protease complex (29, 34). In contrast, the NH_2 terminus of the nucleocapsid protein of the pestiviruses is released from the polyprotein precursor by a virus-encoded protease, whereas the C terminus is processed by cellular signal peptidases (38, 47). For HCV, a conserved C-S sequence is found upstream of the H1 domain located at the C terminus of the core protein (21). Thus, the biogenesis of the HCV core protein could involve, at least in principle, proteolytic cleavages mediated by both a cellular signal peptidase which would recognize the signal sequence and generate the NH_2 terminus of the E1 glycoprotein and the viral protease which could recognize the C-S signal and release the hydrophobic tail from the C terminus of the core protein.

To understand the biogenesis and biochemical characteristics of the HCV core protein, we examined the processing and membrane interactions of the core protein in both in vitro translation studies and transfected cells. We observed that the release of the mature core protein from the polyprotein precursor does not require the viral NS3 protease but that it is dependent on the cellular signal peptidase. Two cleavages are probably required for the removal of the H1 domain, releasing a polypeptide that is associated with but not integrated into the membranes of the ER. Both of these cleavages are probably mediated by the signal peptidase. We have also found that the

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core protein localizes in the cytoplasm of transfected cells and that it can bind RNA in vitro. The core protein also binds the 60S ribosomal subunit in vitro, and the binding domain is localized in the N-terminal region of the viral polypeptide.

MATERIALS AND METHODS

Construction of recombinant plasmids. Clones expressing HCV core sequences are derived from HCV cDNA fragments from nucleotides 1 to 934 (49). Cloning of the HCV fragments in the desired expression vectors was achieved by PCR amplification of the area of interest, using synthetic oligonucleotides containing the appropriate restriction sites, or by in-frame fusion of cDNA fragments by standard DNA protocols.

Plasmid pT7(CΔ-1) contains HCV sequences from an *Nde*I site engineered at nucleotide 333 to an *Xho*I site introduced at nucleotide 934. The cDNA fragment that encodes aa residues 1 to 201 lacks the ATG codon and untranslated region of HCV and was inserted downstream of a T7 promoter in the plasmid vector pT7-7 (48). pT7(CΔ-3) is derived from pT7(CΔ-1) and contains the HCV sequence from nucleotides 333 to 851 (aa residues 1 to 173) downstream of a T7 promoter in plasmid vector pT7-7.

pSK(CΔ-2) was derived by reverse transcription PCR amplification of HCV RNA with sequence-specific primers. The amplified DNA fragment extends from nucleotides 10 to 905 and contains one *Eco*RI site and one *Spe*I site engineered at the 5′ and 3′ ends, respectively. The cDNA fragment was cloned downstream of a T7 promoter in the pBluescript vector SKII; it encodes aa residues 1 to 191.

Plasmid pGEM(HCV1b) contains HCV sequences from an *Xba*I site engineered at nucleotide 313 to another *Xba*I site constructed at nucleotide 2915. The 2.6-kb fragment was cloned into the *Xba*I sites of plasmid pGEMBP1 (9), and it encodes aa residues 1 to 861, followed by an additional leucine residue and a stop codon.

The same 2.6-kb fragment was also cloned into plasmid pHMTIIa (40) and was utilized for the selection of stable cell lines expressing the HCV cDNA (see below). Plasmid pHMT(HCV1b) was obtained by blunt-end ligation of the HCV cDNA fragment into the expression vector which had been cleaved with *Nco*I and blunted with mung bean nuclease.

Plasmid pCD(38-9.4) was described previously and it encodes the HCV sequence from nucleotides 1 to 9416 downstream of a T7 promoter (51).

Constructs for expression of TrpE fusion proteins with selected regions of the C coding sequence were made with pATH plasmids (46). Recombinant plasmids were transformed into *Escherichia coli* DH5α.

Preparation of fusion proteins and Northwestern blot (protein-RNA immunoblot) assays. TrpE fusion proteins were induced in *E. coli* DH5α cells harboring recombinant plasmids. The TrpE-core fusion proteins encoding aa 1 to 201, 70 to 160, 122 to 201, and 122 to 201 accumulated in the inclusion bodies of *E. coli* and were prepared as insoluble fractions as previously described (51). Fusion protein preparations were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroeluted, and used for Northwestern blot assays as previously described (27).

In vitro transcription and translation. The NH_2, M, COOH, and 1-73 cDNA fragments used as templates for in vitro RNA synthesis were obtained from PCR amplification of plasmid pT7(CΔ-1) with sequence-specific primers. Each sense primer was designed to have a bacteriophage T7 promoter (AATAC GACTCACTATAAGG) at the 5′ end and an HCV sequence of 15 nucleotides at the 3′ end, in frame with an ATG codon. Antisense primers contain two UAA codons in frame with a sequence complementary to nucleotides 534 to 551 or 679 to 699 of the HCV cDNA. The amplified NH_2, M, COOH, and 1-73 cDNA fragments encode aa residues 1 to 122, 68 to 173, 122 to 173, and 1 to 73, respectively. Each PCR product or linearized plasmid was transcribed by T7 RNA polymerase in a 50-μl reaction mixture volume as previously described (51).

In vitro translation reactions (10 to 50 μl) were performed for 45 to 90 min at 25 to 30°C with either a nuclease-treated reticulocyte lysate or a wheat germ translation system (Promega). Rough microsomes (RM), RM washed with a high salt solution and EDTA (RMek), and signal recognition particles (SRP) were prepared as described previously (31). Where indicated, RM and RMek were present at a concentration of 1 equivalent per 25-μl reaction mixture (31). SRP were used at a final concentration of 30 nM. After translation, samples were either diluted in sample buffer and directly analyzed by SDS-PAGE or treated as described below.

Sedimentation of membrane-associated proteins on sucrose step gradients. Aliquots (10 μl) of the translation reaction mixtures were supplemented with 1 volume of TKM buffer (20 mM Tris-Cl [pH 7.5], 100 mM KCl, 2 mM $MgCl_2$) supplemented with 1 mM dithiothreitol (DTT) (buffer A) or with buffer triethanolamine acetic acid containing 40 mM EDTA (pH 7.5) (buffer B), 1 M KCl (buffer C), or 9 M urea (buffer D). After a 10-min incubation on ice, samples were overlaid onto an 80-μl cushion containing either 0.5 (buffers A, B, and C) or 0.25 (buffer D) M sucrose and centrifuged for 8 min at 60,000 rpm (buffers A, B, and C) or for 10 min at 70,000 rpm (buffer D) in a Beckman TLA-100 rotor at 4°C. The supernatant and cushion fractions were harvested and proteins were precipitated by the addition of 2 volumes of saturated ammonium sulfate as previously described (31). Pellet fractions were directly resuspended in sample buffer and processed for SDS-PAGE analysis.

Sedimentation of ribosome-associated proteins on continous sucrose gradients. In vitro translation reaction mixtures were chilled on ice and diluted to 50 μl with TKM buffer. The samples were further diluted with 1 volume of TKM buffer containing either 40 mM EDTA (pH 7.5) or 1.25 mg of RNase A per ml or 2 M NH_4Cl and incubated for 1 h on ice. Samples were overlaid onto a 5-ml 10 to 30% sucrose gradient in TKM buffer and centrifuged for 1 h at 50,000 rpm in a Beckman SW 50.1 rotor at 4°C. Gradients were collected from the bottom and separated into 0.5-ml fractions and a pellet fraction. Proteins in each fraction were precipitated with ammonium sulfate and prepared for SDS-PAGE analysis. The pellet fraction was directly resuspended in SDS-PAGE sample buffer.

Subcellular fractionation and flotation of membrane-associated proteins on discontinous sucrose gradients. CV1 cell monolayers (approximately 10^8 cells) were incubated for 12 h with 5 μM $CdCl_2$, harvested, and resuspended in 4 ml of hypotonic buffer (H buffer) [20 mM triethanolamine acetic acid (TEA-AA) (pH 7.5), 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. Cells were incubated for 5 min on ice and homogenized with 20 strokes of a tight-fitting glass-Teflon homogenizer. The cell homogenate was adjusted to isotonic conditions by the addition of 800 μl of compensating buffer (20 mM TEA-AA [pH 7.5], 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1.5 M sucrose, 0.6 M KCl) and homogenized with 20 more strokes. The homogenate was centrifuged for 4 min at 3,700 rpm at 4°C in a Haereus Megafuge. The postnuclear supernatant (4 ml) was loaded

onto a 4-ml cushion (20 mM TEA-AA [pH 7.5], 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 0.25 M sucrose, 80 mM KCl) and centrifuged for 2 h at 45,000 rpm in a Beckman Ti90 rotor at 4°C. The microsomal pellet was resuspended in 400 μl of buffer H and 100 μl of compensating buffer, gently homogenized with a Teflon pestle, and stored at −80°C.

Samples containing the microsomal pellet fraction (80 μg of protein) were adjusted to 50 μl with TKM buffer and extracted for 10 min on ice by the addition of 1 volume of TKM buffer containing either 40 mM EDTA or 1 M KCl or 9 M urea. Portions (0.9 ml) of 2.3 M sucrose in TKM buffer were then added, and the samples were layered under a discontinous sucrose gradient consisting of 1 ml of 1.9 M sucrose, 1 ml of 1.6 M sucrose, 1 ml of 1.3 M sucrose, 0.8 ml of 1.1 M sucrose, and 0.2 ml of TKM buffer. The gradients were centrifuged for 14 h at 50,000 rpm in a Beckman SW 50.1 rotor at 4°C, and the fractions (0.33 ml) were collected from the bottom. After dilution with 0.3 ml of a solution of 12.5 μg of bovine serum albumin per ml, proteins in each fraction were precipitated for 1 h on ice with 10% trichloroacetic acid and prepared for SDS-PAGE analysis. The pellet fraction was directly resuspended in SDS-PAGE sample buffer.

Cell transfections. Cells were transfected by calcium phosphate DNA precipitation. Stable transformants were obtained by cotransfecting CV1 cells with plasmid pHMT(HCV1b) and pSV2neo in a molar ratio of 10:1. G418-resistant clones were screened for viral antigen expression by indirect immunofluorescence.

Indirect immunofluorescence. Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 20 min at room temperature and made permeable with 0.1% Triton X-100 in phosphate-buffered saline. Cells were labeled with the appropriate antibodies and with secondary antibodies and photographed with fluorescein and rhodamine filters. Coverslips were mounted in Moviol containing 1 mg of *para*-phenylenediamine per ml and photographed by epifluorescence on a Leica Diaplan photomicroscope with a 100× planar objective.

RESULTS

In vitro processing of HCV core. To examine in detail the biogenesis of the putative nucleocapsid protein of HCV, cell-free protein synthesis experiments were performed with mRNAs encoding truncated forms of the core protein. These RNAs were transcribed in vitro from plasmid templates containing fragments of the HCV cDNA downstream of a T7 promoter; plasmid pT7(CΔ-1) encodes aa residues 1 to 201, pSK(CΔ-2) encodes aa residues 1 to 191, and pT7(CΔ-3) encodes aa residues 1 to 173. The translation products of the mRNAs were compared with those of plasmid pGEM(HCV-1b) which expresses the entire structural region of the HCV genome (aa 1 to 861) (Fig. 1A).

Translation of the RNA derived from pGEM(HCV-1b) in the absence of microsomal membranes yielded a major precursor band of approximately 90 kDa as well as several minor bands, most likely due to internal initiation of translation (Fig. 1B, lane 1). Translation of the same RNA in the presence of microsomal membranes yielded three major protein bands of 67, 37 to 35, and 21 kDa representing the E2, E1, and C proteins, respectively (Fig. 1B, lane 2), in agreement with the observation that processing of the HCV structural proteins is dependent upon the presence of ER membranes (21). In vitro translation of pT7(CΔ-1) and pSK(CΔ-2) transcripts in the absence of membrane yielded bands of 24 and 23 kDa, respectively (Fig. 1B, lanes 3 and 5). When translated in the presence of microsomal membranes, both RNAs yielded a processed core protein with an apparent molecular mass of 21 kDa, indistinguishable from the protein derived from the translation of pGEM(HCV-1b) (compare lanes 2, 4, and 6 of Fig. 1B). The CΔ-3 protein also has a molecular mass of 21 kDa, but its size is not affected by the presence of microsomal membranes in the translation reaction mixture (Fig. 1B, lanes 7 and 8), suggesting that the protein expressed by plasmid pT7(CΔ-3) is not subject to further processing and may represent the mature form of HCV nucleocapsid. These results indicate that mature core protein terminates around residue 174 and demonstrate that the H1 domain, which was postulated to act as a signal sequence to specify the integration in the rough ER membrane of the E1 protein (21), is required for efficient, membrane-dependent processing of the core protein. Furthermore, these data suggest that the generation of the mature form of core protein occurs as a consequence of an endoproteolytic cleavage resulting in the removal of the H1 domain itself.

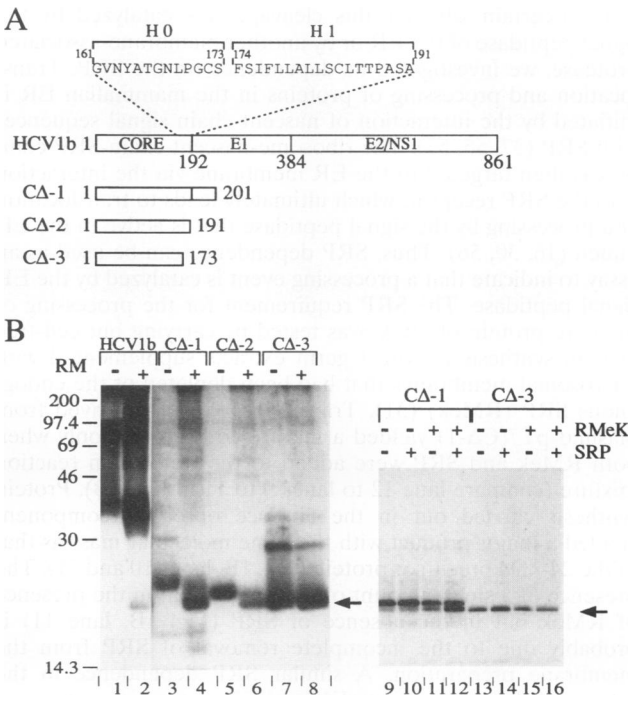

FIG. 1. In vitro translation of mRNAs coding for HCV core protein. (A) Schematic representation of the RNA transcripts (open boxes) used in this experiment. The viral proteins and amino acid residues encoded by each transcript are shown. The positions of the N termini of E1 and E2 at residues 192 and 384 (21) are also indicated (vertical lines). The amino acid sequences of the H0 and H1 domains are shown at the top of the figure (21). The name of each RNA is shown on the left. (B) Transcripts HCV1b, CΔ-1, CΔ-2, and CΔ-3 were translated in vitro with a rabbit reticulocyte lysate in the presence (+) or absence (−) of canine pancreas rough microsomes (RM). Transcripts CΔ-1 and CΔ-3 were translated in wheat germ extracts that were supplemented with microsomal membranes (RMek) (lanes 11, 12, 15, and 16) and SRP (lanes 10, 12, 14, and 16). Translation products labeled with [^{35}S]methionine were analyzed on an SDS–12.5% polyacrylamide gel. Lanes 1 to 8 are a composite of different exposures of the same gel. Lanes 9 to 12 were run on an SDS–12.5% polyacrylamide gel containing 4 M urea. The positions of the processed core protein (arrows) and of the molecular mass standards (in kilodaltons) are indicated at the sides of the gels.

To ascertain whether this cleavage was catalyzed by the signal peptidase of the ER or by another membrane-associated protease, we investigated its dependence on the SRP. Translocation and processing of proteins in the mammalian ER is initiated by the interaction of nascent chain signal sequences with SRP (37, 55, 57). The ribosome-nascent chain-SRP complex is then targeted to the ER membrane via the interaction with the SRP receptor, which ultimately leads to translocation and processing by the signal peptidase that is active in the ER lumen (16, 30, 56). Thus, SRP dependence can be used as an assay to indicate that a processing event is catalyzed by the ER signal peptidase. The SRP requirement for the processing of the core protein of HCV was tested by carrying out cell-free protein synthesis in wheat germ extracts supplemented with microsomal membranes that had been depleted of the endogenous SRP (RMek) (31). Translation of RNA derived from plasmid pT7(CΔ-1) yielded a mature core protein only when both RMek and SRP were added to the translation reaction mixture (compare lane 12 to lanes 9 to 11 in Fig. 1B). Protein synthesis carried out in the absence of either component yielded a major product with the same molecular mass as that of the 24-kDa precursor protein (Fig. 1B, lanes 10 and 11). The presence of a small amount of mature protein in the presence of RMek but in the absence of SRP (Fig. 1B, lane 11) is probably due to the incomplete removal of SRP from the membrane preparation. A similar SRP dependence in the release of the mature core, E1, and E2 proteins was observed in the translation of pGEM(HCV1b) transcript (data not shown). As expected, the size of the translation product derived from the pT7(CΔ-3) transcript was not affected by the presence of membranes or SRP in the reaction mixture (Fig. 1B, lanes 13 to 16). These results demonstrate that the generation of the HCV structural proteins involves a bona fide cotranslational translocation event and strongly argue for ER signal peptidase being the enzyme responsible for the two endoproteolytic cleavages that generate the C terminus of the core protein and the N terminus of E1.

To determine whether the core protein synthesized in vitro was similar in size to that observed in cells, plasmid pT7(CΔ-3) was expressed in HeLa cells using the vaccinia virus T7 transient expression system (12). For comparison, plasmid pCD(38-9.4) which encodes the entire polyprotein was also transfected (51). The cells were harvested 6 h posttransfection, and the lysates were analyzed by Western blotting using human antisera with antibodies against the core protein. An immunoreactive band of approximately 21 kDa was detected in lysates from cells transfected with plasmid pT7(CΔ-3) and pCD(38-9.4) and was not detected in mock-transfected cells. This band comigrates with the core protein synthesized by in vitro translation of pT7(CΔ-3) transcript, suggesting that, within the limitations of gel electrophoresis analysis, the in vitro translation system faithfully mimics the co- and posttranslational modifications of the core protein synthesized in transfected cells (data not shown).

Membrane interactions of the core protein. The data reported above established that the biogenesis of the core protein is dependent on the interaction of the nascent polypeptide with the ER membrane. To characterize the association of the newly synthesized HCV nucleocapsid protein with the ER membrane and to determine the role of the H1 domain in this interaction, the sedimentation profiles of the in vitro translation products of pT7(CΔ-1), pT7(CΔ-3), and pGEM(HCV1b) transcripts were analyzed by ultracentrifugation on sucrose step gradients. The precursor form of the core protein encoded by pT7(CΔ-1) and pT7(CΔ-3) when synthesized in the absence of microsomal membranes was found in the supernatant fraction (Fig. 2A, lanes 5 and 6 and lanes 9 and 10), whereas the 90-kDa precursor protein encoded by pGEM(HCV1b) was recovered in equal amounts in the supernatant (S) and pellet (P) fractions (Fig. 2A, lanes 1 and 2). The sedimentation profile of the HCV structural proteins was significantly different when the three transcripts were translated in the presence of microsomal membranes. The mature CΔ-1 species was found almost completely in the pellet, whereas the p24 precursor was still predominantly recovered in the supernatant (Fig. 2A, lanes 7 and 8). Similarly, the processed core, E1, and E2 proteins encoded by pGEM(HCV1b) were found in the pellet, whereas the 90-kDa precursor was still equally distributed between S and P fractions (Fig. 2A, lanes 3 and 4). In contrast, the CΔ-3 protein was recovered mainly in the S fraction, although a significant portion was found in the pellet (Fig. 2A, lanes 11 and 12). These results indicate that similar to the glycoproteins E1 and E2, the processed core protein associates with ER membranes.

To further investigate the nature of the core-membrane interaction, the effects of EDTA, KCl, and urea extractions on the sedimentation pattern of the HCV structural proteins were analyzed. It is known that EDTA treatment results in a significant removal of membrane-bound ribosomes (39) and also causes ribosome disassembly and subsequent release of the ribosome-associated nascent chain (9). Extractions with KCl and with urea are conventionally used to differentiate peripheral membrane proteins from transmembrane integral proteins.

Although EDTA treatment caused a shift in the S fraction of the 90-kDa precursor protein encoded by pGEM(HCV1b) (Fig. 2B, lanes 1 and 2), it did not have a major effect on the sedimentation patterns of both precursor and mature core proteins, indicating that membrane association of the core protein is not mediated by ribosomes (Fig. 2B, lanes 5 to 12). In contrast, extraction with KCl had a significant effect on the membrane association of the core protein. The mature form of CΔ-1 generated in the presence of membranes was partially shifted into the S fraction (Fig. 2C, lanes 7 and 8), whereas the sedimentation of CΔ-3 was not affected by the salt treatment and the protein was recovered mostly in the supernatant (Fig. 2C, lanes 11 and 12). However, the mature form of the core protein generated from the translation of pGEM(HCV1b) transcript in the presence of membranes was only minimally affected by KCl extraction and was still recovered in the P fraction (Fig. 2C, lanes 3 and 4). Similarly, the E1 and E2 glycoproteins were quantitatively recovered in the pellet.

When the translation reaction mixtures were extracted with urea and analyzed by sedimentation, both the precursor and mature forms of the core protein generated by the translation of any of the three transcripts were found entirely in the supernatant fraction independently of the presence of microsomal membranes during translation (Fig. 2D). Under these extraction conditions, the 37- and 35-kDa doublet and the 67-kDa species encoded by plasmid pGEM(HCV1b) that correspond to the glycoproteins E1 and E2 were significatively enriched in the P fraction (Fig. 2D, lanes 3 and 4). This result is consistent with the observation that E1 and E2 are integral membrane glycoproteins (21) and thus cannot be extracted from the membranes unless detergents are used.

These extraction-sedimentation experiments suggest that the in vitro-synthesized core protein associates with ER membranes and that this association is strongly enhanced by an SRP-dependent targeting event mediated by the H1 domain. Salt extraction does not release soluble translocated proteins from the ER membranes. Thus, the core protein is probably associated with the cytoplasmic side of the ER membrane. This

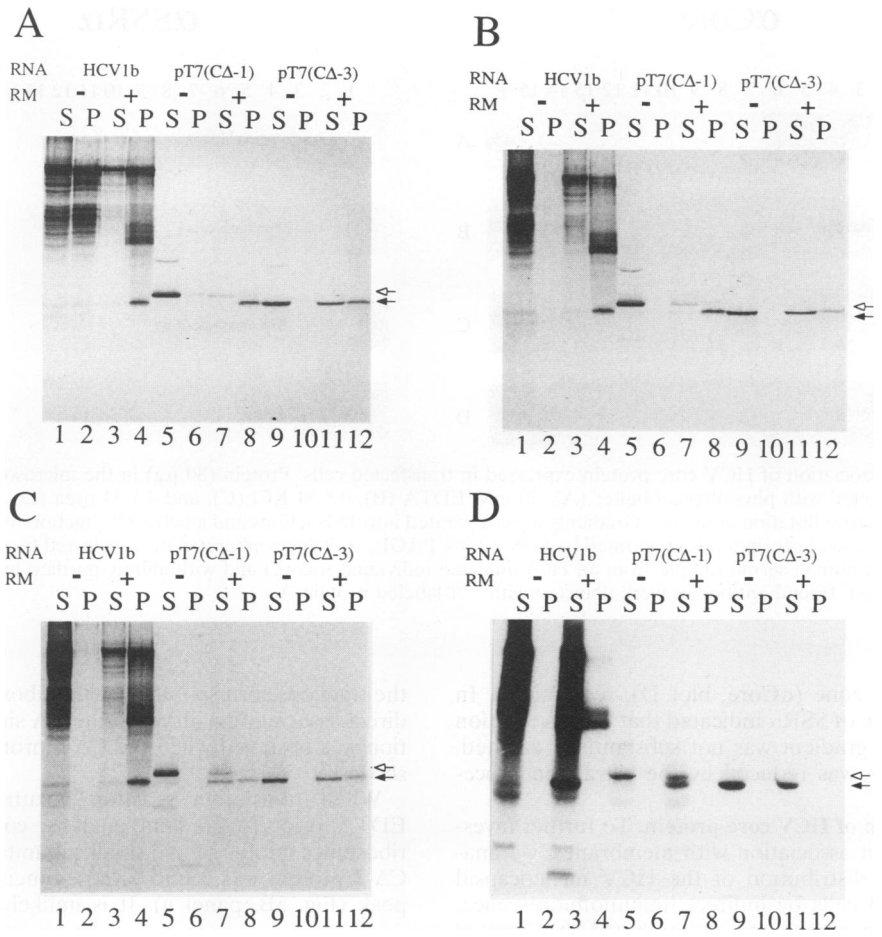

FIG. 2. Membrane association of HCV core protein synthesized in vitro. RNAs transcribed in vitro from plasmids pGEM(HCV1b), pT7(CΔ-1), and pT7(CΔ-3) were translated in a reticulocyte lysate system in the absence (−) or presence (+) of canine pancreas rough microsomes (RM) for 1 h at 30°C. Aliquots of the translation reaction mixture were extracted as described in Materials and Methods with physiological buffer (A), 20 mM EDTA (B), 0.5 M KCl (C), and 4.5 M urea (D), separated into supernatant (S) and pellet (P) fractions and analyzed by SDS–12.5% PAGE and autoradiography. The black arrows indicate the mature form of core protein, and the white arrows indicate the unprocessed core precursor.

topology is confirmed by the sensitivity to proteinase K digestion of core protein generated by translation of any of the three transcripts in the presence of microsomal vesicles (21) (data not shown).

To investigate whether the core association with membranes was also observed in vivo, the subcellular distribution of core protein in transfected cells was examined. To this end, we used a CV1 cell clone (HCV1-b4) permanently transformed with plasmid pHMT(HCV1b), a eukaryotic expression vector containing the same HCV sequences present in plasmid pGEM(HCV1b). Upon conventional subcellular fractionation, the core protein was detected by immunostaining mostly in the nuclear and microsomal pellets (data not shown). This result indicates that similar to the in vitro-synthesized protein, the core protein expressed in transfected cells does not behave as a soluble monomeric polypeptide does, and the core protein may be associated with membranes.

To confirm that the sedimentation behavior of in vivo-synthesized core protein is indeed due to its association with membranes, the microsomal fraction derived from HCV1b-4 cells was analyzed by equilibrium ultracentrifugation on sucrose flotation gradients and the distribution of core protein was assessed by immunoblotting. As shown in Fig. 3, although a small but significant portion of the core protein was found in the pellet, the majority of the protein floated up in the gradient in fractions with densities between 1.19 and 1.25 g/ml, which is compatible with the predicted density of the rough ER of cultured cells (αCore, blot A). Flotation of the rough ER membranes in these fractions was confirmed by the detection of the α subunit of signal sequence receptor (SSRα) (αSSRα, blot A), an integral membrane protein of the ER (54). This result indicates that similar to the in vitro-synthesized protein, the core protein expressed in transfected cells may be associated with membranes.

The type of interaction between the core protein and the rough ER membrane was probed by treatment with EDTA (Fig. 3, blots B), KCl (blots C), and urea (blots D). EDTA treatment did not substantially affect the distribution of either the core protein or SSRα in the gradient, although both proteins were shifted into the lighter zone of the gradient (compare Fig. 3, blots B to blots A). This shift probably reflects a decrease in the density of ER membranes occurring as a consequence of EDTA-mediated ribosome stripping. In contrast, KCl and urea treatments had a dramatic effect on the distribution of the core protein, which was almost completely recovered in the pellet fraction (αCore, blot C) or in the pellet

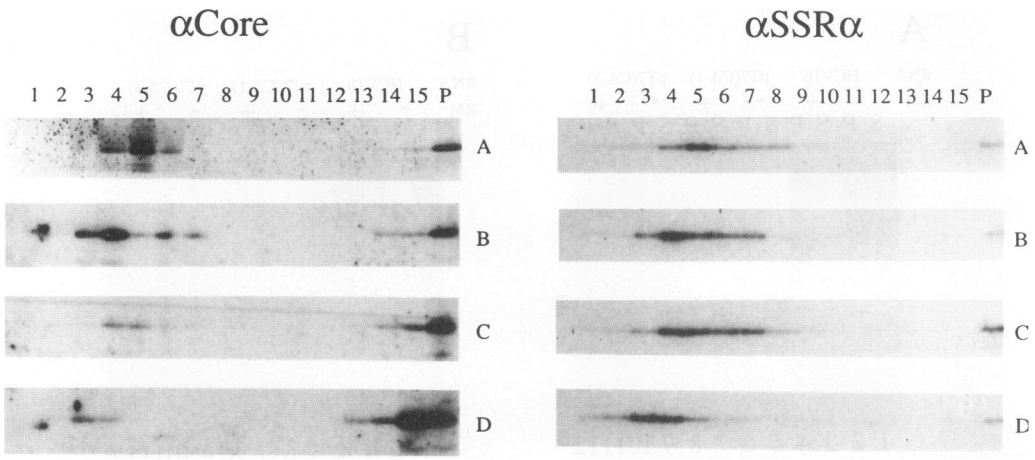

FIG. 3. Membrane association of HCV core protein expressed in transfected cells. Protein (80 μg) in the microsomal fraction derived from HCV1b-4 cells was extracted with physiological buffer (A), 20 mM EDTA (B), 0.5 M KCl (C), and 4.5 M urea (D) and analyzed by isopycnic ultracentrifugation on sucrose flotation gradients. Gradients were separated into 15 fractions and a pellet (P) fraction. Protein in each fraction was collected by trichloroacetic acid precipitation, separated by SDS–12.5% PAGE, and electrophoretically transferred to a nitrocellulose membrane. Blots were probed with a human serum sample from an HCV-infected individual (αcore) and with affinity-purified immunoglobulin G directed against the SSRα (αSSRα). Bound antibodies were detected with ^{125}I-labeled protein A.

fraction and the load zone (αCore, blot D), respectively. In both cases, the position of SSRα indicated that the distribution of membranes in the gradient was not substantially affected, although their density was reduced by the extraction procedure.

Cellular localization of HCV core protein. To further investigate the core protein association with membranes, we analyzed the subcellular distribution of the HCV nucleocapsid protein in transfected cells by indirect immunofluorescence. Staining with a human monoclonal antibody (MAb) directed against the core protein (5) was specifically observed in cells transfected with pHMT(HCV1b) and not in cells transfected with pHMT(HCV2b) (data not shown). The signal was localized exclusively in the cytoplasm and had a characteristic reticular distribution, which overlapped the signal observed with anti-E2 and anti-SSRα antisera (Fig. 4, compare A to B and C to D, respectively). This labeling pattern strongly reinforces the idea that the protein is associated with the ER membranes. However, in a high percentage of transfected cells, the MAb against the core protein also revealed perinuclear structures (Fig. 4A and C) and peripheral small round structures (data not shown) that were not labeled by the anti-E2 and anti-SSRα antisera. A similar overall labeling pattern has been observed in COS and HUH-7 cells transfected with a variety of plasmids encoding the core protein (19, 42), in infected lymphoblastoid cells (41, 43), and in HeLa cells transfected with plasmid pCD(38-9.4) (data not shown).

Ribosome binding activity of HCV core protein. When we analyzed the association of in vitro-synthesized CΔ-1 and CΔ-3 proteins with membranes, we noticed that if centrifugation conditions that caused pelleting of intact ribosomes were employed, core protein was almost entirely found in the pellet, even when membranes were not included in the reaction mixture (data not shown). This unexpected sedimentation pattern prompted us to investigate the potential association of the core protein with ribosomes. For this purpose, translation reaction mixtures programmed with pT7(CΔ-3) transcript were analyzed on sucrose velocity gradients after treatment with several agents. As shown in Fig. 5A, under physiological salt conditions, the CΔ-3 protein was almost entirely recovered in the fractions corresponding to the ribosome peak, suggesting a direct association with ribosomes. A similar ribosome association was observed when the CΔ-1 protein was used (data not shown).

When translation reaction mixtures were treated with EDTA prior to gradient analysis, complete dissociation of ribosomes into large and small subunits was observed, and the CΔ-3 protein was found to cosediment with the 60S subunit peak (Fig. 5B, panel a). It is unlikely that the core protein

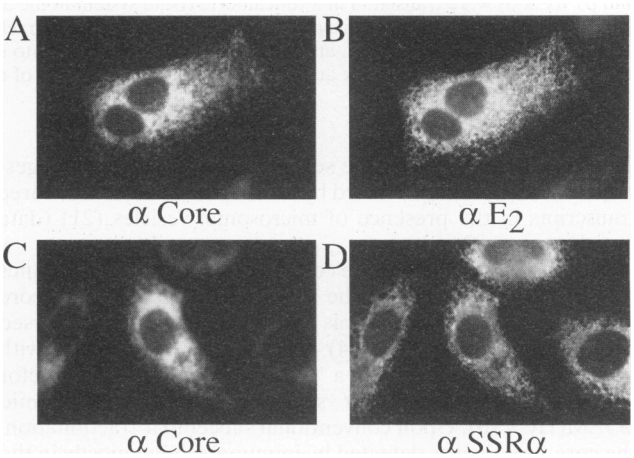

FIG. 4. Indirect immunofluorescence analysis of cells expressing HCV structural proteins. HCV1b-4 cells were treated for 12 h with 5 μM $CdCl_2$, fixed, and made permeable. Double staining was performed with a human MAb directed against the core protein (α Core) and affinity-purified rabbit anti-SSRα IgG (αSSRα) (C and D) or with the α Core MAb together with a rabbit anti-E2 antiserum (αE_2) (A and B). Secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin G and tetramethyl rhodamine isocyanate-conjugated goat anti-rabbit immunoglobulin G. Panels A and C were photographed with a fluorescein filter. Panels B and D were photographed with a rhodamine filter and show the same cells as in panels A and C, respectively.

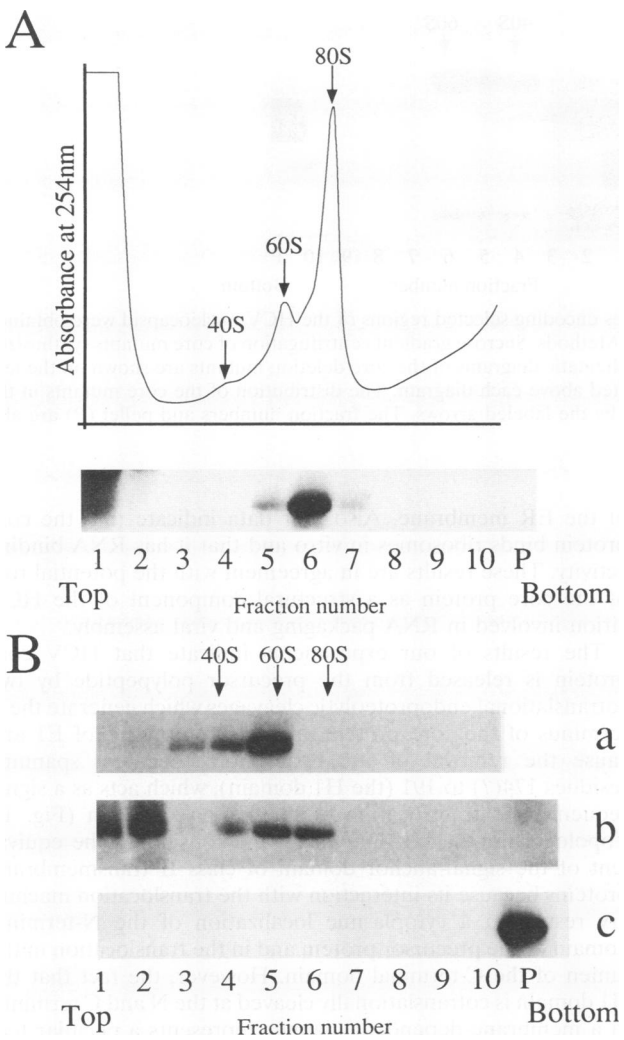

FIG. 5. Ribosome binding of HCV core synthesized in vitro. (A) RNA transcribed in vitro from plasmid pT7(CΔ-3) was translated in a reticulocyte lysate system for 1 h at 30°C. Aliquots of the translation reaction mixture were diluted with TKM buffer, overlaid onto a 10 to 30% sucrose gradient, and centrifuged for 1 h at 4°C. Gradient fractions were collected from the bottom, and proteins in each fraction were precipitated and analyzed by SDS-PAGE. The distribution of the optical density of each fraction and the distribution of the CΔ-3 protein in the gradient fractions are shown. (B) The effects of several agents on the core association with ribosomes were tested by incubating translation reaction mixture aliquots with 20 mM EDTA (panel a), 1 M NH_4Cl (panel b), and 25 μg of RNase A per ml (panel c) prior to centrifugation. The ribosome peaks are indicated by the labeled arrows. The fraction numbers and pellet (P) are also indicated.

association with ribosomes results from lack of translational termination, because EDTA treatment is known to terminate translation and to cause discharge of the nascent chain (9). This conclusion is confirmed by the observation that puromycin treatment did not affect cosedimentation of the core protein with ribosomes but effectively dissociated a control protein encoded by an mRNA lacking a termination codon (data not shown).

Treatment with NH_4Cl prior to gradient analysis did not affect ribosome sedimentation but altered the sedimentation profile of the core protein, which was found in the ribosome peak as well as on top of the gradient (Fig. 5B, panel b), suggesting that an increase in ionic strength induced a partial dissociation of the core protein from ribosomes and that the dissociated protein is in a soluble, possibly monomeric form. Additionally, when ribosomes were disassembled by extensive digestion with RNase A, the core protein was quantitatively recovered in the pellet (Fig. 5B, panel c).

To identify which domain of the core protein was responsible for the observed binding to the 60S ribosomal subunit, we constructed four deletion mutants encompassing aa 1 to 122 (mutant A), 68 to 173 (mutant B), 122 to 173 (mutant C), and 1 to 73 (mutant D) and tested their ribosome binding properties. As shown in Fig. 6, mutant A, containing the two-thirds N-terminal domain of the core protein, is mostly found in the 60S subunit peak, indicating that this mutant maintains almost all of the ribosome binding activity. Mutant D, which contains the highly basic N-terminal domain of the core protein, was only partially associated with the 60S subunit peak, the remainder of the protein being found in the top gradient fractions. Mutants B and C, which contain the two-thirds and one-third C-terminal domains of the protein, respectively, did not cosediment with ribosomes. Mutant B was predominantly found in the pellet fraction and partly in the top gradient fractions, whereas mutant C was almost entirely recovered in the top gradient fractions. These data indicate that the highly charged N-terminal region of the core protein is sufficient for ribosome binding. However, the capacity to associate with ribosome seems to be enhanced by the presence of the central domain of the protein.

To investigate whether a similar ability to bind ribosomes was detectable also for the core protein synthesized in transfected cells, we analyzed the postnuclear supernatants derived from HCV1b-4 cells and HeLa cells transfected with pCD(38-9.4) and infected with vaccinia virus T7 (12) on identical sucrose sedimentation gradients. In both cases, the core protein was quantitatively recovered in the pellet fraction and little if any was found in the ribosome peak fractions (data not shown), confirming our observation that in vivo, in a steady-state situation, the core protein is stably associated with membranes.

RNA binding of HCV core protein. Since the core protein of HCV is a highly basic polypeptide, as predicted from its amino acid composition, and is postulated to be a structural component of the virion (50), it might be associated with HCV genomic RNA in the virion or with other cellular RNA in the infected cells. Furthermore, the core protein association with ribosomes observed in vitro may also be determined, at least in part, by the RNA binding activity of the HCV nucleocapsid protein. To determine whether the HCV core might contain an RNA binding activity, we constructed a series of plasmids encoding TrpE-HCV fusion proteins expressing the core polypeptide. To localize a potential RNA binding domain of the HCV core protein, the nucleocapsid protein was divided into four separate domains, analogous to those employed for ribosome binding studies, and expressed as TrpE fusion proteins. The fusion proteins were assayed for RNA binding by a Northwestern procedure by incubation with in vitro-transcribed ^{32}P-labeled RNA (27) (Fig. 7). The TrpE-core fusion protein (aa 1 to 201), but not the TrpE protein alone, bound the labeled RNA, indicating that the TrpE sequences did not contribute to the interaction of the fusion protein with the labeled transcript. However, the TrpE-core protein bound with equal efficiency to a transcript representing the entire genome of HCV as well as to the genomic hepatitis B virus (HBV) ^{32}P-labeled RNA, which was used as a heterologous transcript. No preferential binding of the TrpE-core fusion protein to the

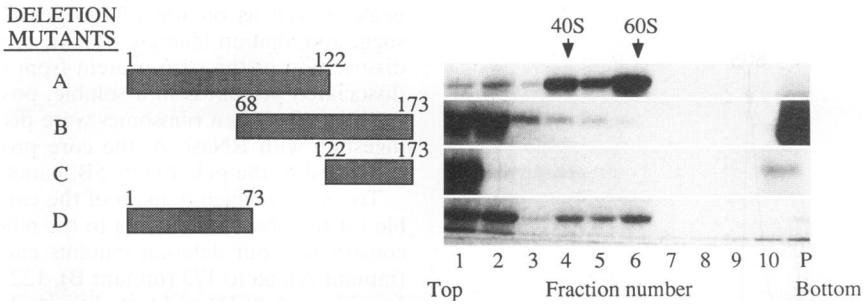

FIG. 6. Ribosome binding of HCV core deletion mutants. RNA templates encoding selected regions of the HCV nucleocapsid were obtained by PCR amplification and in vitro transcription as described in Materials and Methods. Sucrose gradient centrifugation of core mutants synthesized by in vitro translation was carried out as described in the legend to Fig. 5. Schematic diagrams of the core deletion mutants are shown on the left. The amino acid residues of HCV core contained in each protein are indicated above each diagram. The distribution of the core mutants in the gradient fractions is shown on the right. The ribosome peaks are indicated by the labeled arrows. The fraction numbers and pellet (P) are also indicated.

labeled HCV transcript could be shown, even when other heterologous transcripts were used in the binding assay or when unlabeled transcripts were used in competition experiments (data not shown). RNA binding was also observed with TrpE fusion proteins containing the amino-terminal half (aa 1 to 122) as well as the N terminus (aa 1 to 75) of the core protein. In contrast, the middle fusion domain (aa 70 to 160) and the C-terminal region of the core protein (aa 122 to 201) did not bind RNA at all. Thus, these experiments indicate that the RNA binding domain of the core protein is localized in the highly basic N-terminal region of the polypeptide (aa 1 to 75). However, the TrpE-core fusion protein does not display, at least under these conditions, RNA binding that is specific for the HCV RNA.

DISCUSSION

The biogenesis of the HCV proteins is a complex event mediated by cellular and viral proteases that specifically cleave the nascent polypeptide into discrete subunits. In this study, we examined the biosynthesis of the putative nucleocapsid protein of HCV and its expression in eukaryotic cells. The data reported here indicate that the core protein is released from the polyprotein precursor by cellular signal peptidase and that the released polypeptide associates with the cytoplasmic side

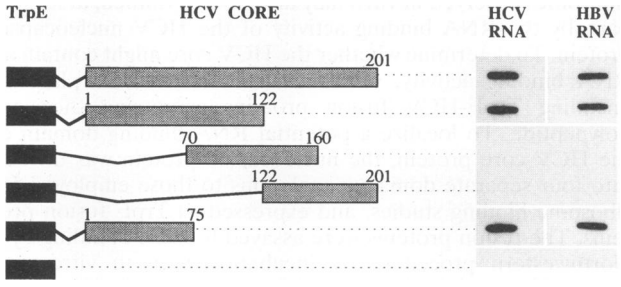

FIG. 7. RNA binding activity of recombinant HCV core. The various TrpE-core fusion proteins were separated by SDS-PAGE and electroeluted, and 0.5 μg of each protein was blotted onto a nitrocellulose membrane. The proteins were then incubated with ^{32}P-labeled HCV or HBV genomic RNA as previously described (27), and autoradiographed. Schematic diagrams of the fusion proteins are shown at the left. The amino acid residues of the HCV core protein contained in each protein are indicated above each diagram. The TrpE sequences derived from plasmid pATH are shown (black boxes).

of the ER membrane. Also, our data indicate that the core protein binds ribosomes in vitro and that it has RNA binding activity. These results are in agreement with the potential role of the core protein as a structural component of the HCV virion involved in RNA packaging and viral assembly.

The results of our experiments indicate that HCV core protein is released from the precursor polypeptide by two cotranslational endoproteolytic cleavages which generate the C terminus of the core protein and the N terminus of E1 and cause the removal of the hydrophobic segment spanning residues 174(?) to 191 (the H1 domain), which acts as a signal sequence for translocation of the E1 glycoprotein (Fig. 1). Topologically, the H1 domain can be considered the equivalent of the signal-anchor domain of class II transmembrane proteins because its interaction with the translocation machinery results in a cytoplasmic localization of the N-terminal domain of the precursor protein and in the translocation in the lumen of the C-terminal domain. However, the fact that the H1 domain is cotranslationally cleaved at the N and C terminus in a membrane-dependent manner represents a peculiar feature of the HCV biogenesis and poses several questions about the mechanisms of translocation and cleavage of the H1 domain.

It is currently believed that protein translocation across the ER membrane occurs through an aqueous channel or pore that does not create a directional bias in polypeptide movement and that vectorial translocation is driven and sustained by posttranslational events that prevent retrograde movement (10, 15, 19, 33, 35, 44). Also, the active site of signal peptidase is thought to be localized on the lumenal side of the ER membrane, and all the signal peptidase cleavage sites described so far are located at the C termini of the hydrophobic domains which act as signal sequences. On the basis of the SRP dependence, we interpret the cleavage at the N terminus of the H1 domain as mediated by signal peptidase. A possible explanation of how signal peptidase can cleave at this site is that the stretch of partially hydrophobic residues 161 to 173 (H0 domain) immediately preceding the H1 domain (Fig. 1A) (21) plays a role in the biogenesis of the core protein. The cleavage at the N terminus of the H1 domain could be interpreted as a cleavage at the C terminus of the H0 domain. If this is indeed the case, one could imagine a scenario in which translocation of the HCV nascent chain is initiated by the SRP-dependent interaction of the H0-H1 domains with the translocation machinery. The H0-H1 domains would then be translocated, possibly in a loop configuration, until signal peptidase can

cleave H1 on both sides. Subsequently, translocation of the N-terminal core protein would be aborted because of folding in the cytoplasm or interaction with cytoplasmic components, whereas translocation of E1 would proceed, driven by folding in the lumen and/or interaction with lumenal components. A similar case of transient translocation of a partially hydrophobic sequence accompanied by removal of the following hydrophobic domain by signal peptidase has been observed in the biogenesis of the structural glycoproteins of Sindbis and Semliki Forest viruses (26, 28). Abortive translocation after signal peptidase cleavage was also observed for the precore protein of HBV (13), albeit in this case the cleavage was canonically found at the C terminus of the putative signal sequence.

Our data indicate that both in vivo and in vitro, core protein released from the polyprotein precursor is not a soluble protein but that it stably associates with the cytoplasmic side of the ER membrane (Fig. 2 and 3). The observation that the mature core protein encoded by the CΔ-1 mutant associates with membranes much more efficiently than the same protein encoded by the CΔ-3 transcript suggests that the H1 domain not only acts as a signal sequence for the E1 glycoprotein but also facilitates docking of the nascent core protein to the cytoplasmic side of the ER membrane. Nonetheless, the observation that the mature core protein encoded by the CΔ-3 mutant also associates with membranes indicates that the H1 domain is not absolutely necessary for this interaction. It is conceivable that in the case of the CΔ-3 mutant the interaction with the membrane is initiated, albeit inefficiently, by the H0 domain, which could also play a relevant role in maintaining this interaction.

The sensitivity of membrane association to aqueous perturbants, such as KCl and urea, empirically defines the core protein as a peripheral membrane protein (Fig. 2 and 3). Our experiments indicate that independently of the polyprotein precursor, mature core protein is efficiently extracted from the membranes with urea. However, the sensitivity of the core protein to KCl extraction depends on the polyprotein precursor: the in vitro-synthesized protein derived from pT7(CΔ-1) and pT7(CΔ-3) is partially released from the membranes by salt treatment, whereas the core protein generated in vitro from the HCV1b precursor is almost completely resistant to KCl extraction. It is tempting to speculate that the different sensitivity to KCl extraction may be indicative of an interaction between the core protein and the E1 and E2 glycoproteins, although alternative possibilities cannot be excluded. Also, our data indicate that in contrast with the protein synthesized in vitro, the core protein produced in transfected cells expressing the HCV1b coding region is readily released from the membrane by KCl treatment. This discrepancy between in vivo and in vitro results may be a consequence of the different experimental procedure. Alternatively, it may reflect the fact that the in vitro translation experiment describes the interaction with the membrane of the newly synthesized protein, whereas the in vivo experiment delineates the steady-state condition of the protein produced over a prolonged period of time. This possibility is supported by our immunofluorescence analysis which shows that in transfected cells the core protein does not colocalize entirely with the E2 protein and with the SSRα marker (Fig. 4). This observation suggests that within the time frame of the experiment, the core protein may have moved from the ER to an undefined subcellular compartment.

Does core association with membranes have any role in the life cycle of HCV? Ultrastructural studies indicate that flavivirus and pestivirus morphogenesis occurs in association with intracellular membranes. HCV is expected to follow a similar pathway of assembly. In this scenario, the core protein association with membranes may play a role in facilitating assembly and/or budding of the virus.

In view of the amino acid sequence of the core protein that is characterized by 16.8% basic amino acid residues clustered in the N-terminal region of this protein (50), it is not surprising that the TrpE-core protein is indeed capable of binding RNA (Fig. 7). Interestingly, the RNA binding activity is localized in the region with aa residues 1 to 75; this region is the most conserved domain of the putative nucleocapsid protein and it contains a large number of arginine residues (50). Basic amino acids play a major role in the RNA binding activities of several proteins, for example, the RNA binding domain of Sindbis capsid protein (14), the arginine-rich motif of proteins such as the human immunodeficiency virus Tat transactivator (58), the nucleocapsid of HBV (20), and the hepatitis delta antigen (25). Although the RNA targets of these proteins vary considerably, the RNA binding activities of these polypeptides depend upon the interaction of basic amino acid side chains with the sugar-phosphate backbone of RNA.

The true biological significance of the RNA-core interaction must be assessed with soluble core protein. It should be possible to determine whether the lack of specificity observed in the RNA binding assay described in this study is due to the expression of the core protein as a fusion polypeptide that could preclude the core protein from folding into the correct structure, thereby interfering with the specific interaction between the viral protein and genomic RNA, or whether the specific interaction between the core protein and the HCV genomic RNA may be dependent on other viral or cellular components that are required for correct recognition. For example, the cooperative action of the HBV core protein and P gene product is required for the correct packaging of the HBV pregenome into viral particles (3). Nonetheless, the observed HCV core-RNA interaction, albeit not specific, is in agreement with the putative structural role of this nucleocapsid protein.

Our data also show that the HCV core protein binds ribosomes in vitro, as indicated by centrifugation analysis of translation reaction mixtures (Fig. 5). The interaction of viral proteins and ribosomal subunits has been extensively studied in alphaviruses (52, 59). It has been shown that the core protein binds to ribosomes both in vitro and in vivo cosedimenting with 60S ribosomal subunits (60, 61). Also, the ribosome binding site in the alphavirus core protein has been identified (62). These studies indicate that core interaction with ribosomes is an important step in assembly and uncoating of the virion. Singh and Helenius (45) have suggested that the alphavirus C protein binding sites on ribosomes contain exposed rRNA. They have reported that isolated 28S rRNA causes complete dissociation of the C protein from the viral RNA in vitro and it is possible that the C protein binds preferentially to the 28S RNA during uncoating. The observed in vitro interaction of the HCV core protein with the 60S ribosomal subunits is reminiscent of the similar behavior detected with the alphavirus nucleocapsid polypeptide. Furthermore, the binding of the core protein to RNA detected by Northwestern assay suggests that the RNA binding capacity of the HCV nucleocapsid may contribute to the in vitro association of the core protein with the ribosomes. This possibility is also supported by the deletion analysis of the core protein that shows that both the RNA and ribosome binding properties of the HCV nucleocapsid protein reside in the N-terminal region of this polypeptide (Fig. 6 and 7).

The functional significance of this interaction is still unclear. The core protein expressed in cells does not bind ribosomes, suggesting that the observed in vitro association of the core

protein with the 60S subunits does not mimic the steady-state situation. However, this association could reflect a transient interaction observed only in the early stages of infection and therefore represent the uncoating mechanism of the viral particle which would result in the release of the genomic RNA in close proximity to the cellular translation apparatus. It is conceivable therefore that the nucleocapsid protein expressed in transfected cells has a greater affinity for the ER membranes where the processing events that lead to its biogenesis and assembly take place.

Recent data suggested that the HCV core protein may have a regulatory role because it has been implicated in the regulation of HBV gene expression in cells cotransfected with the HCV nucleocapsid. The researchers suggested that the N-terminal 122 aa residues of the HCV core protein may bind HBV RNA and somehow inhibit the assembly of the HBV core particles (42). The nucleocapsid protein of Semliki Forest virus has also been shown to be multifunctional, since besides binding viral RNA, it regulates viral and host protein synthesis (4, 11, 53). Thus, it is tempting to speculate that the core nucleic acid binding property and the ribosome interaction could influence the expression of HBV genes or, more generally, may play a regulatory role in the gene expression of the infected cells.

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