JOURNAL OF VIROLOGY, Dec. 1995, p. 7461–7471 Vol. 69, No. 12
0022-538X/95/$04.00+0

The NS2 Protein of Hepatitis C Virus Is a Transmembrane Polypeptide

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Received 6 July 1995/Accepted 5 September 1995

The NS2 protein of hepatitis C virus (HCV) is released from its polyprotein precursor by two proteolytic cleavages. The N terminus of this protein is separated from the E2/p7 polypeptide by a cleavage thought to be mediated by signal peptidase, whereas the NS2-3 junction located at the C terminus is processed by a viral protease. To characterize the biogenesis of NS2 encoded by the BK strain of HCV, we have defined the minimal region of the polyprotein required for efficient cleavage at the NS2-3 site and analyzed the interaction of the mature polypeptide with the membrane of the endoplasmic reticulum (ER). We have observed that although cleavage can occur in vitro in the absence of microsomal membranes, synthesis of the polyprotein precursor in the presence of membranes greatly increases processing at this site. Furthermore, we show that the membrane dependency for efficient in vitro processing varies among different HCV strains and that host proteins located on the ER membrane, and in particular the signal recognition particle receptor, are required to sustain efficient proteolysis. By means of sedimentation analysis, protease protection assay, and site-directed mutagenesis, we also demonstrate that the NS2 protein derived from processing at the NS2-3 site is a transmembrane polypeptide, with the C terminus translocated in the lumen of the ER and the N terminus located in the cytosol.

The molecular cloning of the cDNA of hepatitis C virus (HCV), the most common cause of non-A non-B hepatitis (4, 29), has opened new avenues to the study of viral replication of this important human pathogen. The viral genome consists of a positive-sense RNA of about 9.5 kb (5, 27, 44), and the mature enveloped virion has a diameter of approximately 60 nm (26). Sequence analysis of the cDNA isolated from different sources has allowed the identification of at least five major genotypes and accompanying subtypes and has indicated that the virus distantly resembles human flaviviruses and animal pestiviruses (3, 34). Thus, HCV has been classified as a separate genus in the *Flaviviridae* family (13).

The genetic organization of the HCV genome has been characterized by means of transient expression of HCV cDNA in transfected cells (1, 17, 42, 45) and by cell-free translation studies of in vitro-synthesized transcripts (20). The putative structural proteins core, E1, and E2 are found in the N-terminal quarter of the 3,010-residue polyprotein that represents the primary translation product of the viral RNA. Core, a basic protein with RNA binding activity (41), is thought to be the nucleocapsid component of the virion, whereas E1 and E2 are glycoproteins that are probably associated with the viral envelope. The E2 glycoprotein exists in two different forms: as a fully processed polypeptide or fused to the adjacent downstream p7 protein, which may be an additional structural component of the viral particle (30, 35). The nonstructural polypeptides NS2, NS3, NS4a, NS4b, NS5a, and NS5b are localized within the remainder of the polyprotein and are likely to be involved in processing of the viral polypeptide and possibly in the replication of viral RNA (24). The N terminus of all processed HCV polypeptides has been determined by direct sequencing of proteins expressed in vitro and in transfected cells (15, 16, 23, 31, 36, 39).

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Previous studies have shown that the putative structural polypeptides of HCV are released from the polyprotein precursor by host protease, most likely the endoplasmic reticulum (ER) signal peptidase, which is responsible for the removal of a hydrophobic domain at the C terminus of the core polypeptide (20, 41) and catalyzes the primary cleavages at the E1-E2, E2-p7, and p7-NS2 sites (20, 30, 36). However, although a hydrophobic domain located upstream of the N terminus of NS2 could function as signal or signal-anchor sequences (35), processing at the p7-NS2 junction and the overall membrane topology of NS2 are not clear, particularly in view of the posttranslational nature of the cleavage at the N terminus of this viral polypeptide (9). The HCV-encoded serine protease, localized in the N-terminal region of the NS3 protein, mediates in conjunction with the NS4a polypeptide processing of the putative nonstructural proteins by cleaving at the following sites: NS3-NS4a, NS4a-NS4b, NS4b-NS5a, and NS5a-NS5b (1, 2, 10, 12, 15, 31, 45). Cleavage at the N terminus of the NS3 protein is mediated by yet another virus-encoded protease, which includes the NS2 region and the serine protease domain of the NS3 protein (16, 21). However, the molecular mechanism of the proteolytic processing at the NS2-3 site is poorly understood.

To further characterize the biogenesis of the NS2 protein of HCV and to define the membrane topology of this polypeptide, we have examined the processing and membrane interaction of this viral protein in in vitro translation studies utilizing the cDNA of the BK type of HCV. We have observed that efficient cleavage at the NS2-3 site is dependent on the synthesis of the protein precursor in the presence of functional microsomal membranes. Furthermore, there is an apparent difference in cleavage efficiency between the HCV-BK and -J precursors in the absence of membranes. Also, we show that processing at the NS2-3 site resulted in the integration of the NS2 protein into the ER membrane as a bona fide transmembrane polypeptide.

MATERIALS AND METHODS

Construction of recombinant plasmids. Clones expressing HCV E2-NS2-NS3 sequences are derived from cDNA fragments of the HCV-BK strain (44) from nucleotides (nt) 2496 to 5303 (amino acid [aa] residues 722 to 1657) unless otherwise specified. Cloning of the cDNA fragments in the desired expression vectors was achieved by PCR amplification of the area of interest, using synthetic oligonucleotides with the appropriate restriction sites, or by standard DNA protocols.

Plasmid pCITE(722-1325) was obtained by cloning into the *Msc*I-*Xba*I sites of the expression vector pCITE (Novagen) (11) nt 2496 to 4307 to which the *Msc*I and *Xba*I sites were engineered by PCR. The plasmid contains the HCV cDNA fragment downstream of a T7 promoter and of the 5′ untranslated region of encephalomyocarditis virus (25). pN(722-1325) was provided by K. Shimotohono and has already been described (21). It contains nt 2496 to 4307 of the HCV-J strain downstream of a T7 promoter.

pCD(810-1615) contains HCV sequences from an *Nco*I site at position 2758 to a *Bam*HI site engineered at position 5177. The cDNA fragment was cloned in the expression vector pCDNA3 (Invitrogen) downstream of a T7 promoter and of the 5′ untranslated region of the β-globin gene of *Xenopus laevis* (28).

Plasmids containing specific mutations were obtained by oligonucleotide-directed mutagenesis, and the mutations were confirmed by sequence analysis. Mutations are denoted by the parental amino acid residue and its position in the HCV-BK strain, followed by the substituted residue. pCD(810-1615; C993A) was created by inserting the mutation in the PCR primers that were used to generate the mutant DNA fragment according to the procedure of Higuchi et al. (19). The mutant DNA fragment was cloned into the parent plasmid by using flanking restriction sites. The triplet coding for cysteine 993, TGT, was replaced by GCT, which codes for alanine. pGEM(810-1615; L1026ΔQ) was created by changing the HCV nucleotide sequence starting at position 3402 from CGACTCCTC GCG into CGACTGCAG according to the procedure of Higuchi et al. (19). The amplified DNA fragment derived from pCITE(810-1615) was inserted into plasmid pGEMBP1 (6).

Site-directed mutagenesis of aa residues 983 and 988 was carried out by inserting the mutations in the PCR primers that were used to generate the mutant DNA clone pGEM(810-1026G) according to the procedure of Hemsley et al. (18). Plasmid pGEM(810-1026) (see below) was used as the template for PCR amplification of the target DNA. The triplets coding for isoleucine 983, ATC, and alanine 988, GCA, were replaced by AAC, which codes for asparagine. To construct plasmid pGEM(810-1615G), pGEM(810-1026G) was cleaved with *Sac*II and *Eco*RI and a *Sac*II-*Eco*RI DNA fragment derived from pCD(810-1615) containing nt 3307 to 5177 (aa residue 992 to 1615) was inserted into the plasmid.

pCITE(849-1615) was obtained by cloning into the pCITE vector digested with *Msc*I and *Xba*I an *Ssp*I-*Xba*I cDNA fragment derived from plasmid pCITE(623-1615) (7). The construct contains nt 2872 to 5177 downstream of a T7 promoter.

pCITE(849-1325) was obtained by subcloning an *Ssp*I-*Pst*I fragment derived from plasmid pN(722-1325) into the pCITE vector digested with *Msc*I and *Pst*I. The construct contains nt 2872 to 4307 of the HCV-J strain.

pCITE(923-1615) was constructed by cloning into the pCITE vector digested with *Bst*XI and *Pst*I an *Sph*I-*Pst*I fragment derived from plasmid pCITE(849-1615) after treatment of both DNAs with T4 DNA polymerase. The plasmid contains nt 3098 to 5177 downstream of a T7 promoter.

pCITE(992-1615) was constructed by cloning a *Sac*II-*Pst*I fragment derived from plasmid pCITE(849-1615) into the pCITE vector digested with *Bst*XI and *Pst*I. Prior to ligation, both DNAs were treated with T4 DNA polymerase. The plasmid contains nt 3301 to 5177.

pCITE(960-1615) was derived from PCR amplification of nt 3208 to 5177 with sequence-specific primers using pCITE(849-1615) as the template. The amplified DNA was digested with *Pvu*II, whose site had been included within the 5′ end primer, and *Bst*XI and inserted into the pCITE vector that had been digested with *Msc*I and *Bst*XI.

pGEM(810-1026) was obtained by PCR amplification of nt 2760 to 3410 (aa residues 810 to 1026) with sequence-specific primers. The amplified DNA fragment derived from pCITE(810-1615) was digested with *Xba*I and inserted into plasmid pGEMBP1.

pT7(1027-1657) contains nt 3410 to 5303 (7).

In vitro transcription and translation. All plasmids used as templates for in vitro RNA synthesis were linearized with the appropriate restriction enzyme and transcribed in vitro with either T7 or SP6 RNA polymerase as previously described (45). Transcripts encoding aa residues 849 to 1083, 849 to 1115, 849 to 1137, 849 to 1237, 849 to 1355, 849 to 1401, and 849 to 1462 were obtained by runoff transcription of pCITE(849-1615) digested with *Bst*XI (nt 3578), *Sma*I (nt 3675), *Aat*II (nt 3744), *Sca*I (nt 4041), *Bst*EII (nt 4394), *Sac*I (nt 4536), and *Sal*I (nt 4716), respectively (44).

In vitro translation reactions (25 μl of mixture) were performed for 60 min at 30°C with nuclease-treated reticulocyte lysate (Promega). Unless stated otherwise, canine rough microsomes (RM) were used at a concentration of 1 eq/25 μl. After translation, samples were either diluted in sample buffer and directly analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or treated as described below. Incorporation of radioactivity into specific bands was determined by direct analysis of the dried gel with a PhosphorImager (Molecular Dynamics). The processing efficiency was estimated on the basis of the incorporation of radioactivity in translation products according to the following formula:

$$\% \text{ of total cpm} = \frac{\mathrm{NS}(a/b)}{\mathrm{NS}(a/b) + P} \quad (1)$$

where NS is counts per minute of the processed protein, a is the number of methionine residues present in the precursor, b is the number of methionine residues present in the processed protein, and P is the counts per minute of the unprocessed precursor.

Preparation of microsomes and of SRP receptor fragment. Canine pancreatic RM were prepared as described elsewhere (37) and washed with 0.5 M potassium acetate (KOAc) and 10 mM EDTA. KOAc-EDTA-washed membranes were subjected to limited proteolysis after resuspension in buffer A (0.25 M sucrose, 50 mM triethanolamine [pH 7.5], 5 mM $CaCl_2$, 5 mM $MgCl_2$, 2 mM dithiothreitol) to a concentration of 1 eq/μl. Trypsin (0.5 mg/ml in buffer A) was added to a final concentration of 5 μg/ml, and the microsome suspension was incubated for 60 min on ice. Proteolysis was terminated by the addition of ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*, *N*′, *N*′-tetraacetic acid (EGTA) and phenylmethylsulfonyl fluoride (PMSF) to final concentrations of 20 and 5 mM, respectively. KOAc-EDTA-washed membranes at a concentration of 1 eq/μl in buffer A were alkylated for 30 min at 22°C in the presence of 2.5 mM *N*-ethylmaleimide (NEM). Alkylation reactions were quenched by the addition of dithiothreitol to a final concentration of 20 mM. Trypsinized microsomes (TRM) and alkylated microsomes (NRM) were collected by centrifugation through a cushion of 0.5 M sucrose–50 mM triethanolamine, pH 7.5, for 20 min at 65,000 rpm in a TL 100 rotor (Beckman Instruments Inc.) and resuspended in 0.25 M sucrose–50 mM triethanolamine, pH 7.5, to a concentration of 1 eq/μl. Under these conditions, the cytoplasmic domain of the signal recognition particle (SRP) receptor is either removed (TRM) or inactivated (NRM) and the microsome fraction is translocation incompetent. Control membranes were mock treated and processed similarly (37).

The 52-kDa cytoplasmic fragment of the SRP receptor (37) was prepared as follows: 20 ml of KOAc-EDTA-washed membrane suspension, at a concentration of 1 eq/μl in buffer A, was supplemented with 10 U of aprotinin (Boehringer Mannheim) per ml and digested with elastase (Boehringer Mannheim), at a final concentration of 1 μg/ml, for 60 min on ice. After addition of PMSF and KOAc to concentrations of 1 mM and 0.5 M, respectively, microsomes were collected by centrifugation at 4°C for 35 min at 40,000 rpm in a 50.2 Ti rotor (Beckman Instruments Inc.). The proteolytic digestion was repeated, and the supernatants from the two treatments were combined and dialyzed versus buffer B (50 mM Tris-HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4], 150 mM KOAc, 2 mM dithiothreitol). The dialyzed fraction was centrifuged for 1 h at 45,000 rpm in a 50.2 Ti rotor to remove the precipitated material and applied to a 1-ml column of CM Sephadex equilibrated in buffer B. The column was washed with 10 column volumes of buffer B and 10 column volumes of buffer B containing 0.25 M KOAc. The 52-kDa SRP receptor fragment was eluted with buffer B containing 0.425 M KOAc and concentrated by ultrafiltration with Centricon 30 (Amicon Corp., Danvers, N.H.).

Sedimentation of membrane-associated proteins on sucrose step gradients and urea extraction. Ten microliters of the translation reaction mixtures was supplemented with an equal volume of TKM buffer (20 mM Tris-Cl [pH 7.5], 100 mM KOAc, 2 mM $MgCl_2$); after 10 min of incubation on ice, samples were overlaid on an 80-μl cushion of 0.4 M sucrose and centrifuged at 60,000 rpm for 8 min in a Beckman TLA-100 rotor at 4°C. Alternatively, 10 μl of the translation reaction mixtures was supplemented with 90 μl of 5 M urea–11.1 mM Tris-Cl (pH 7.5)–55.5 mM KOAc–1.1 mM $MgCl_2$; after 10 min at room temperature samples were overlaid on 100 μl of 0.25 M sucrose containing 4.5 M urea and centrifuged at 70,000 rpm in a Beckman TLA-100 rotor for 15 min at 20°C. The supernatants and cushion fractions were precipitated with 2 volumes of saturated ammonium sulfate, washed with 5% trichloroacetic acid, and resuspended in sample buffer. Pellet fractions were directly resuspended in sample buffer and analyzed by SDS-PAGE.

Protease digestion. Five microliters of the translation mixtures was diluted with 5 μl of solution I (6 mM tetracaine), II (6 mM tetracaine, 0.4 mg of proteinase K [PK] per ml), or III (6 mM tetracaine, 0.4 mg of PK per ml, 2% Triton-X) and incubated for 60 min on ice. Proteolysis was terminated by adding 1 μl of 100 mM PMSF and incubating the samples for 10 min on ice. Samples were then diluted with sample buffer and analyzed by SDS-PAGE. For protease protection assay of in vivo-synthesized protein, HeLa cells were infected and transfected and labeled with [^{35}S]Promix (Amersham, Little Chalfont, United Kingdom) as described below. After metabolic labeling, cells (approximately 2×10^6) were resuspended in 400 μl of H buffer (20 mM Tris-Cl [pH 7.5], 250 mM sucrose, 50 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA) and homogenized with a 0.5-ml glass Teflon homogenizer. The homogenate was centrifuged for 4 min at 1,000 × *g* in a Haereus bench top centrifuge. Aliquots (100 μl) of the postnuclear supernatant were supplemented with an equal volume of either solution I, II, or III and incubated for 1 h on ice. Proteolysis was terminated by adding PMSF and SDS to final concentrations of 10 mM and 2%, respectively, and by incubating the samples for 5 min at 95°C. Samples were diluted with 1.5 ml of 20 mM Tris-Cl (pH 7.5)–150 mM NaCl–0.5 mM EDTA–0.5

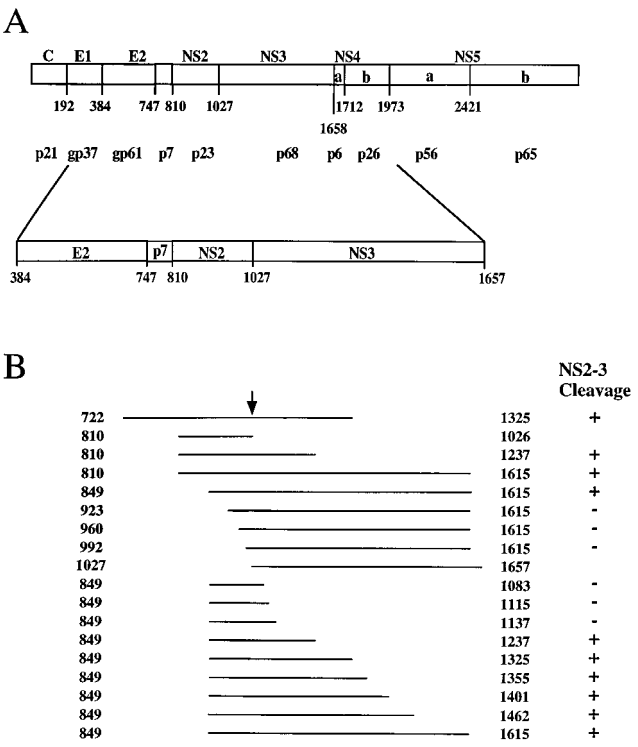


FIG. 1. Schematic representation of the HCV genome and of the transcripts used in this study. (A) Diagram of the HCV polyprotein, showing the cleavage products. The amino acid residues representing the N termini and the molecular sizes of the processed viral proteins are indicated (16, 20, 21). The lower diagram indicates the NS2 coding sequence and its surrounding region. (B) Transcripts expressing the HCV polyprotein. Lines describe the HCV protein expressed in a variety of constructs and are drawn to scale and oriented with respect to the lower diagram in panel A. Numbers refer to the first and last amino acids of the HCV polyprotein expressed in each transcript; the arrow indicates NS2-3 cleavage site. Processing at the NS2-3 junction is indicated at the right.

mM EGTA–1.25% Triton X-100 and immunoprecipitated as described previously (45).

Cell transfections. HeLa cell monolayers were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells were infected with vaccinia virus vTF7-3 and subsequently transfected by calcium phosphate DNA precipitation as previously described (45). Immunoprecipitations of transfected cell lysates were performed with HCV-specific rabbit polyclonal antisera as previously described (45).

Endoglycosidase H treatment. Eighty microliters of transfected cell lysate was immunoprecipitated with NS2-specific rabbit polyclonal antiserum (45), and the pelleted samples were resuspended in 20 μl of buffer C (10 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% SDS, and 0.5 mM PMSF) and denatured at 95°C for 5 min. The samples were then diluted with 80 μl of buffer D (50 mM sodium acetate [pH 5.2], 1.25% Triton X-100 and 0.5 mM PMSF). Deglycosylation was carried out by adding 2 mU of endoglycosidase H (Boehringer Mannheim), and the mixture was incubated at 37°C for 4 h. The protein samples were then precipitated for 1 h on ice with 10% trichloroacetic acid and processed for SDS-PAGE analysis.

RESULTS

Effect of deletions and point mutations on NS2-3 cleavage. To determine the HCV sequence requirements for cleavage at the NS2-3 site, a series of 5′ and 3′ deletions were introduced in the cDNA encoding the HCV polyprotein and used for cell-free translation studies. Figure 1 shows a diagram of all transcripts used in this study. To define the importance of membranes for cleavage efficiency, translations were performed in the absence or the presence of canine pancreatic microsomal membranes. The results of in vitro processing assays using transcripts spanning the NS2 and most of the NS3 polypeptides are shown in Fig. 2. The transcript encoding aa 810 to 1615 yielded an 88-kDa unprocessed precursor as well as two proteins of 65 and 23 kDa corresponding to the cleaved NS3 and NS2 proteins, respectively (Fig. 2A, lane 2). Processing was clearly evident when the translation reaction was carried out in the presence of microsomal membranes, whereas the cleaved products were almost undetectable when translation was performed in the absence of membranes (Fig. 2A, lane 1). The N-terminal boundary of the NS2 portion required for efficient processing appears to be located between aa 849 and 923, since polyproteins starting at or downstream of residue 923 did not yield cleaved NS2 and NS3 polypeptides (Fig. 2A, lanes 3 to 10). Similarly, the C-terminal boundary of the NS3 portion required for processing appears to be located between aa 1137 and 1237 because proteins terminating beyond aa 1237 were properly cleaved (Fig. 2B, lanes 7 to 16), whereas precursors terminating at or upstream of aa 1137 were not processed at the NS2-3 site (Fig. 2B, lanes 1 to 6). Interestingly, efficient processing at the NS2-3 site could not be reestablished by extending the truncated NS2-3 precursor that comprised aa 849 to 1083 with aa 2885 to 3010 derived from the NS5b region (data not shown), suggesting that the NS3 sequences located downstream of the NS2-3 junction play an active role in the reaction and are therefore specifically required for proper cleavage.

The results described above could be interpreted as indicative of a signal peptidase-mediated cleavage of the NS2-3 precursor that requires microsomal membranes and thus is distinct from viral proteolytic processing at the NS2-3 site. To ascertain that the cleavage observed in the cell-free translation of the NS2-3 polyprotein was indeed due to the viral protease, we introduced by site-directed mutagenesis a cysteine-to-alanine substitution at residue 993. This mutation inactivates the HCV NS2-3 protease encoded by the H and J strains of HCV (16, 21). Furthermore, to confirm the substrate specificity of the BK NS2-3 protease, mutant L1026ΔQ, in which the amino acid sequence LLAP spanning residues 1025 to 1028 and including the cleavage site (16, 21) was mutated into LQP with a net deletion of residue 1026 and an alanine-to-glutamine substitution at residue 1027, was constructed. Mutations were introduced into a plasmid encoding aa 810 to 1615, and the translation products of the in vitro-transcribed mutant RNAs were compared with those of the parental transcript. As shown in Fig. 2C, both mutations resulted in the lack of processing at the NS2-3 junction and did not yield processed NS2 and NS3 polypeptides. Thus, these data are in agreement with the interpretation of a viral protease-mediated cleavage at the NS2-3 site and also confirm the observation that amino acid changes in the NS2-3 junction have a significant effect on the processing efficiency (23).

Membrane dependence for in vitro NS2-3 cleavage varies among different HCV cDNAs. The results described above indicate that efficient in vitro processing of the NS2-3 site requires a specific segment of the HCV polyprotein, in agreement with recent published data (1, 16, 21). However, it appeared that in vitro cleavage of the NS2-3 site of the HCV-BK strain was heavily dependent on the presence of microsomal membranes in the translation reaction, more than what has been described by other groups that have utilized a cDNA clone of HCV strains other than HCV-BK. Specifically, Hijikata et al., using the cDNA clones of the HCV-J strain, have clearly shown that the processing of the NS2-3 site in vitro could occur efficiently in the absence of the microsomal membranes (21).

To ascertain whether this different requirement for microsomal membranes is due to a real diversity between HCV

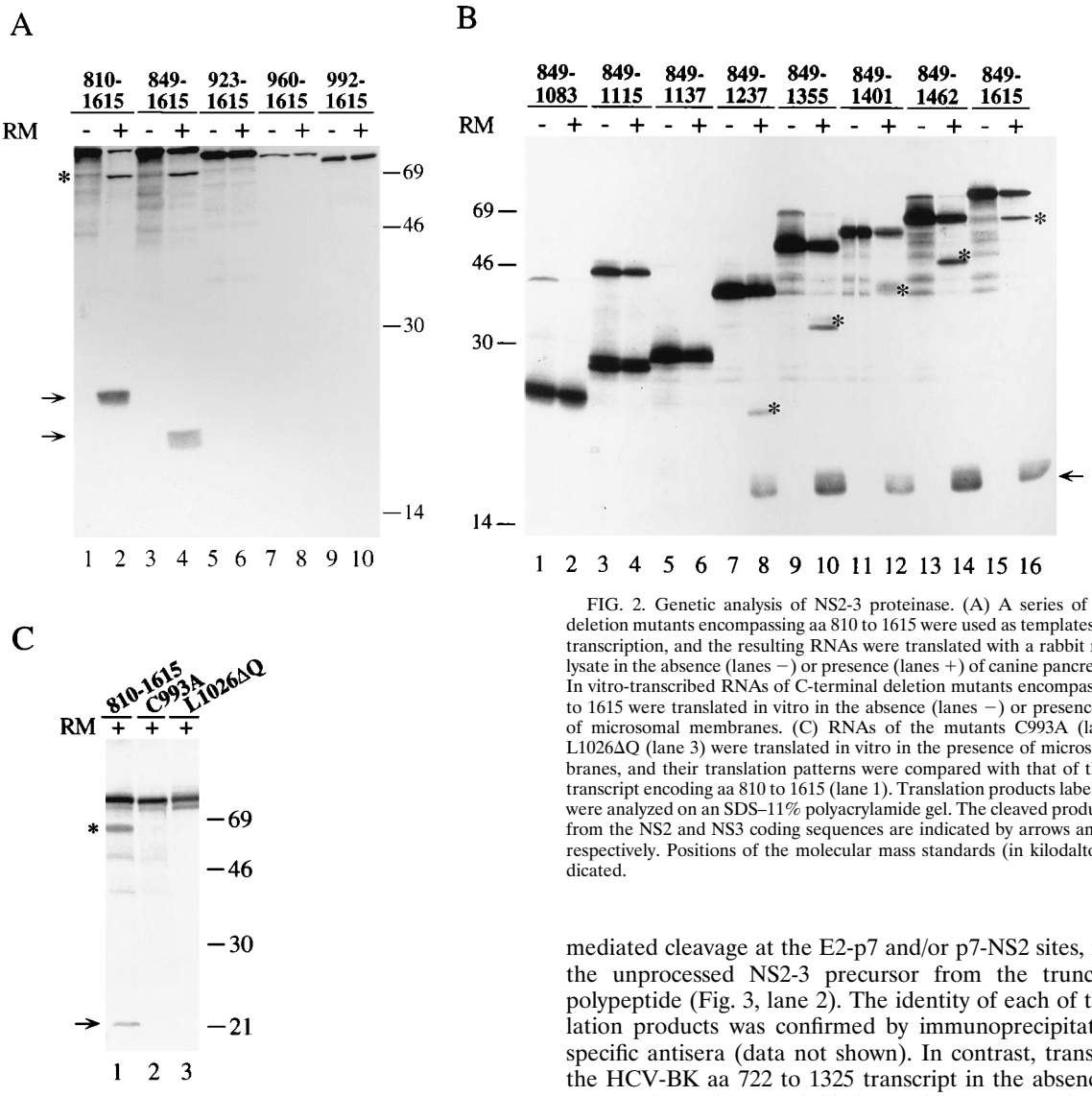


FIG. 2. Genetic analysis of NS2-3 proteinase. (A) A series of N-terminal deletion mutants encompassing aa 810 to 1615 were used as templates for in vitro transcription, and the resulting RNAs were translated with a rabbit reticulocyte lysate in the absence (lanes −) or presence (lanes +) of canine pancreas RM. (B) In vitro-transcribed RNAs of C-terminal deletion mutants encompassing aa 849 to 1615 were translated in vitro in the absence (lanes −) or presence (lanes +) of microsomal membranes. (C) RNAs of the mutants C993A (lane 2) and L1026ΔQ (lane 3) were translated in vitro in the presence of microsomal membranes, and their translation patterns were compared with that of the parental transcript encoding aa 810 to 1615 (lane 1). Translation products labeled with ^{35}S were analyzed on an SDS–11% polyacrylamide gel. The cleaved products derived from the NS2 and NS3 coding sequences are indicated by arrows and asterisks, respectively. Positions of the molecular mass standards (in kilodaltons) are indicated.

strains rather than to distinct experimental procedures, we compared the effects of microsomal membranes on the processing efficiency of the NS2-3 protein encoded by the HCV-J and HCV-BK strains. The transcripts utilized in this comparison comprise part of the E2 glycoprotein as well as most of the NS2-3 region. Thus, to generate a mature NS2 protein, the polyprotein precursor encoded by these RNAs must be processed at the E2-NS2 junction, presumably by signal peptidase, and at the NS2-3 site by the viral protease (21). Translation of the HCV-J aa 722 to 1325 RNA in the absence of microsomal membranes yielded a major precursor band of approximately 66 kDa as well as two smaller proteins of 32 and 26 kDa corresponding to the NS3 and E2-NS2 proteins, respectively (Fig. 3, lane 1). The 26-kDa band decreased in intensity when translation was carried out in the presence of microsomal membranes with the concomitant appearance of the 23-kDa protein, indicating that the NS2 product had been released from the E2 protein (21, 36). Furthermore, an additional 56-kDa protein running as a doublet was also observed. We interpret this polypeptide to be derived by the signal peptidase-mediated cleavage at the E2-p7 and/or p7-NS2 sites, releasing the unprocessed NS2-3 precursor from the truncated E2 polypeptide (Fig. 3, lane 2). The identity of each of the translation products was confirmed by immunoprecipitation with specific antisera (data not shown). In contrast, translation of the HCV-BK aa 722 to 1325 transcript in the absence of microsomal membranes yielded a major precursor band of 66 kDa, but little or no processed NS3 and E2-NS2 could be detected (Fig. 3, lane 3). However, when translation was carried out in the presence of microsomal membranes, processing of the NS2-3 site as well as at the E2-NS2 junction was evident. Notably, the 56-kDa unprocessed precursor migrated as a single band, suggesting that cleavage at the N terminus of NS2 also varies between different HCV strains (Fig. 3, lane 4). The differential requirement of microsomal membranes was also observed when shorter transcripts of the two strains were analyzed. Translation of the HCV-J transcript encoding aa 849 to 1325 yielded a 53-kDa unprocessed precursor as well as two proteins of 32 and 19 kDa corresponding to the cleaved NS3 and to the truncated NS2 (NS2′) proteins, respectively. The processed NS2 and NS3 polypeptides were more visible when translation was carried out in the presence of microsomal membranes in the reaction mixture, suggesting that processing of the HCV-J strain was also affected, at least in part, by the presence of membranes (Fig. 3, lanes 5 and 6). Additionally, translation of the HCV-BK transcript encoding aa 849 to 1355 in the absence of membranes yielded only a 53-kDa precursor, and the NS2 and NS3 proteins could be detected only when translation was carried out in the presence of microsomal

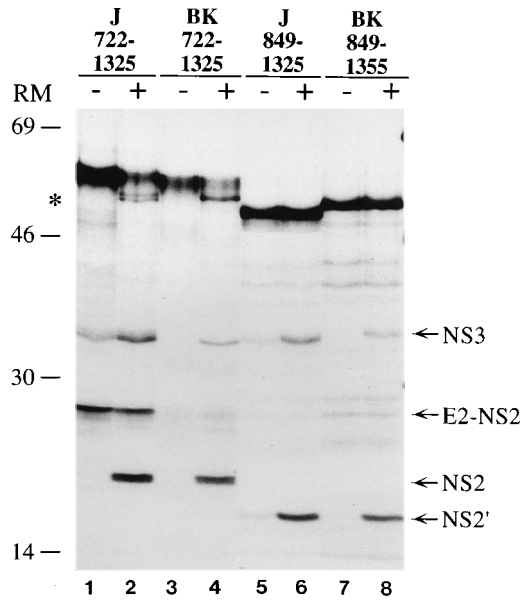


FIG. 3. Comparison of in vitro processing efficiencies at the NS2-3 site between the HCV-BK and HCV-J polyproteins. Transcripts encoding the indicated amino acids of the HCV-J strain (J) and the HCV-BK strain (BK) were translated in vitro with a rabbit reticulocyte lysate in the absence (lanes −) or presence (lanes +) of RM. Translation products labeled with ^{35}S were analyzed on an SDS–11% polyacrylamide gel. The positions of the processed NS3, E2-NS2, NS2, and NS2′ polypeptides are indicated. The asterisk indicates the partially processed NS2-3 precursor. Positions of the molecular mass standards (in kilodaltons) are indicated.

membranes (Fig. 3, lanes 7 and 8). These results demonstrate that, independent of the length of the transcript, the HCV-J polyprotein precursor is less dependent on microsomal membranes than is that of HCV-BK for efficient in vitro cleavage at the NS2-3 site. This finding confirms the observation that different strains of HCV have, at least in vitro, different biological requirements for processing of the NS2-3 junction (1, 8, 16, 21).

Role of membranes in the NS2-3 cleavage. To investigate the role of microsomal membranes in the in vitro cleavage at the NS2-3 site, translation reactions were carried out with the HCV-BK transcript encoding aa 849 to 1615 in the presence of increasing amounts of microsomal membranes. As shown in Fig. 4A, translation in the absence of membranes produced an 84-kDa unprocessed precursor and little or no 65- and 19-kDa protein product, corresponding to the cleaved NS3 and NS2 polypeptides, could be detected. However, an increase in processed NS2 and NS3 proteins could be detected as a function of the microsomal membrane concentration in the translation reaction. Although we cannot rule out the possibility that the presence of microsomal membranes in the translation reaction had a stabilizing effect on the processed polypeptides, it is apparent that membranes contributed to an increase in the processing efficiency at the NS2-3 site, as indicated by a decreased amount of the unprocessed precursor concomitant with the increase in the amounts of cleaved NS3 and NS2 (Fig. 4). The yield of processed NS2 and NS3 reached a plateau at around 2 membrane equivalents per 25 μl of translation mixture.

To determine whether the increase in efficiency of cleavage at the NS2-3 site observed in the presence of microsomal membranes was merely due to the hydrophobic interaction between the precursor protein and the membranes or whether a specific host factor contributed to the processing at the NS2-3 junction, we examined the effects on the cleavage at the NS2-3 site of trypsin treatment of the microsomal membranes. This treatment was chosen on the basis of its capacity to inactivate protein components involved in protein translocation (37). Also, we compared the effect of membrane treatment on the processing efficiency of the NS2-3 protein encoded by both the HCV-J and HCV-BK strains.

As shown in Fig. 5, translation of the HCV-BK transcript encoding aa 722 to 1325 in the presence of TRM yielded only the 66-kDa precursor (lane 11) whereas translation in the presence of mock-treated membranes produced the 66-kDa precursor, the 56-kDa partially processed polypeptide, and the NS2 and NS3 proteins of 23 and 32 kDa, respectively (lane 9). Similarly, translation of the HCV-BK transcript encoding aa 849 to 1355 was also affected by the trypsin treatment of the membranes, since no processed products could be detected (Fig. 5, lane 17). Thus, trypsin treatment of microsomal membranes completely inhibited processing at all sites of the HCV-BK precursor. As expected, membrane inactivation resulted in the loss of processing at the E2-p7 and p7-NS2 sites of HCV-J precursor comprising aa 722 to 1325, whereas cleavage at the NS2-3 site showed a similar level of processing efficiency compared with translation reactions carried out in the absence of microsomal membranes (Fig. 5, lanes 1 and 5), in agreement with the observation that cleavage at NS2-3 junction of the HCV-J strain is less membrane dependent than that in HCV-BK.

A similar inactivation of efficient cleavage of the HCV-BK NS2-3 precursor was obtained when membranes (NRM) were treated with the sulfhydryl-directed alkylating agent NEM or when transcripts encoding aa 810 to 1615 were utilized in the translation reactions (data not shown). This result indicated that both treatments impaired the ability of microsomal membranes to contribute to the increased processing efficiency at the NS2-3 site independently of the transcript length utilized in the experiment, suggesting that the stimulating activity required a proteinaceous component located on the cytosolic side of the ER membrane and containing at least one free sulfhydryl group. Furthermore, addition of the cytoplasmic fragment of the α subunit of the SRP receptor to the translation reactions restored, at least in part, the processing-stimulating activity of both TRM and NRM (Fig. 5, lanes 6, 12, and 18, and data not shown). Conversely, addition of the SRP receptor α subunit to reactions performed in the absence of membranes or in the presence of mock-treated membranes (Fig. 5, lanes 2, 4, 8, 10, 14, and 16) did not change the protein processing pattern, indicating that the SRP receptor was necessary but not sufficient to stimulate processing at the NS2-3 site. These results indicate that the nascent NS2-3 protein is targeted to the ER membrane by the SRP-SRP receptor targeting machinery and that this targeting event results in an increased processing efficiency at the NS2-3 site.

Membrane interaction of the NS2 protein. The data reported above established that the cleavage of the NS2-3 site is enhanced by the interaction between the newly synthesized NS2-3 protein and the ER membrane, possibly by determining a proper folding and/or correct localization on the membrane of the polyprotein precursor that ultimately results in the increased processing efficiency at the NS2-3 site. To define whether the interaction between the nascent HCV protein and the ER membrane resulted in a stable association between the NS2 polypeptide and the ER membrane, the sedimentation profiles of the in vitro translation products of HCV-BK transcripts encoding aa 810 to 1615 (NS2-3), 810 to 1026 (NS2), and 1027 to 1657 (NS3) were analyzed by ultracentrifugation

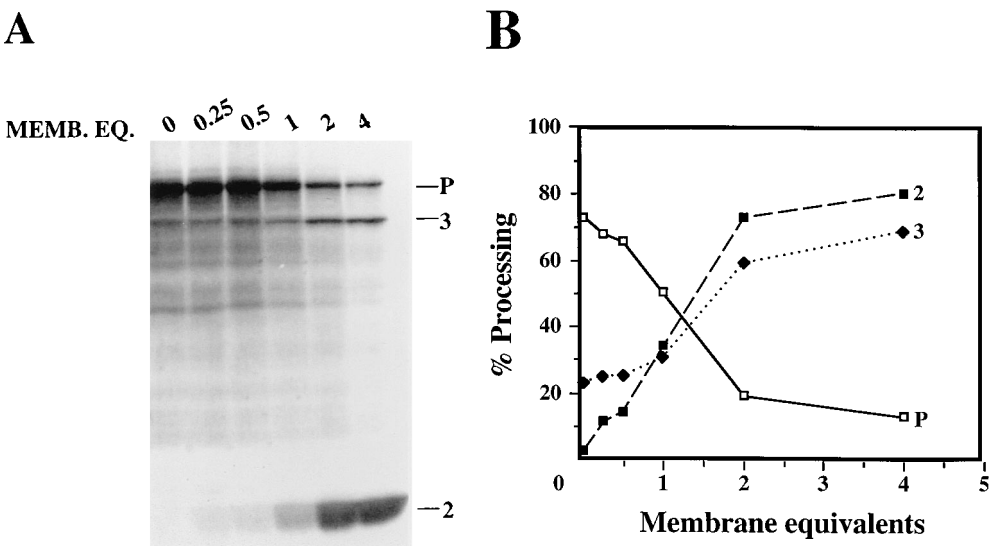


FIG. 4. Effect of microsomal membrane concentration on NS2-3 processing efficiency. Transcript encoding HCV-BK aa 849 to 1615 was translated in vitro with a rabbit reticulocyte lysate in the presence of different amounts of microsomal membrane (membrane equivalents). (A) Translation products labeled with ^{35}S were analyzed on an SDS–11% polyacrylamide gel. Unprocessed precursor protein (P) and cleaved NS2 and NS3 proteins are indicated. (B) Percentage of processing was calculated as described in Materials and Methods and plotted as a function of membrane equivalents present in the translation reaction.

on sucrose step gradients. The effect of cleavage at the NS2-3 site on the sedimentation of the HCV proteins was determined by comparing the sedimentation of the NS2 and NS3 polypeptides derived from processing at the 2-3 site with that of the same proteins translated from two separate transcripts. The translations were performed in the absence or in the presence of microsomal membranes. Furthermore, the influence of membrane components on the sedimentation profile of the HCV proteins was assessed by translating their corresponding RNAs in the presence of microsomal membranes that had been treated with trypsin or NEM.

NS2-3 precursor protein synthesized in the absence of microsomal membranes or in the presence of pretreated membranes was found mostly in the supernatant, whereas it was recovered in equal amounts in the pellet and supernatant when the translation was carried out in the presence of untreated microsomal membranes (Fig. 6A, lanes 1 to 8). Independent of the presence of biologically active or pretreated membranes, the majority of the NS3 protein derived from processing of the NS2-3 precursor or from the translation of the RNA encoding aa 1027 to 1657 was recovered in the supernatant, although a portion of this protein was present also in the pellet (Fig. 6A, lanes 3, 4, and 9 to 16). In contrast, the NS2 polypeptide derived from processing of the NS2-3 precursor was mostly found in the pellet fraction (Fig. 6A, lanes 3 and 4) whereas the same 23-kDa protein when expressed as the polypeptide

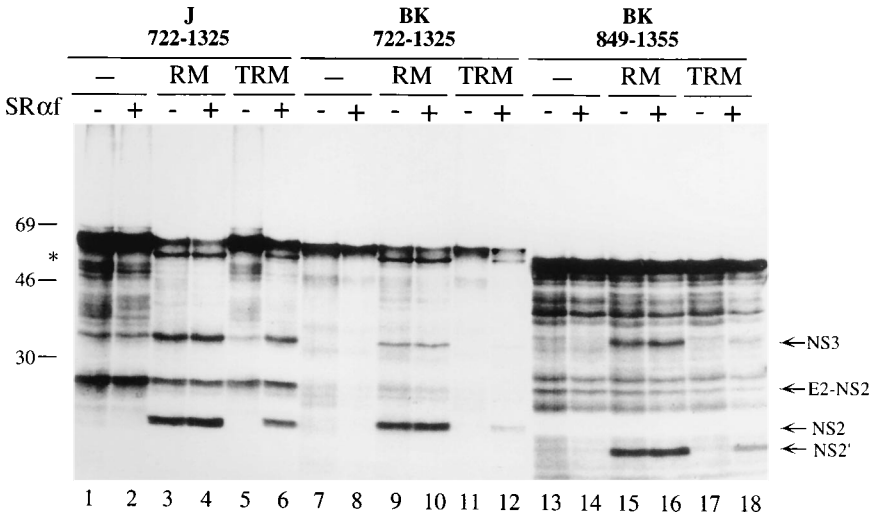


FIG. 5. The microsomal membrane-dependent enhancement of NS2-3 processing efficiency requires an active SRP receptor. Transcripts encoding the indicated amino acids of the HCV-J strain (J) and the HCV-BK strain (BK) were translated in vitro with a rabbit reticulocyte lysate either in the absence of microsomal membranes (lanes 1, 2, 7, 8, 13, and 14) or in the presence of mock-treated membranes (lanes RM) or trypsinized membranes (lanes TRM). Where indicated (+) the 52-kDa cytoplasmic fragment of the α subunit of the SRP receptor (SRαf) was present at a concentration of 3 μg/ml. The positions of the processed NS3, E2-NS2, NS2, and NS2′ polypeptides are indicated. The asterisk indicates the partially processed NS2-3 precursor. Positions of molecular mass standards (in kilodaltons) are indicated.

encompassing aa 810 to 1026 in the presence of active membranes was equally distributed between the pellet and supernatant fractions (Fig. 6A, lanes 11 and 12). However, the 23-kDa polypeptide sedimented mostly in the supernatant when synthesized in the absence or presence of treated membranes (Fig. 6A, lanes 9, 10, and 13 to 16). These data indicated that, unlike the NS2-3 precursor and the NS3 protein, which did not show a strong association with the ER membrane, the NS2 polypeptide was intimately associated with the ER membrane. Interestingly, the association of NS2 with membranes was more pronounced for the protein derived from processing of the aa 810 to 1615 precursor than for the aa 810 to 1026 polypeptide.

To analyze whether the association with the ER membrane of the NS2 peptide was indicative of its integration in the bilayer, we performed sedimentation analysis of the in vitro-translated protein after urea extraction, as this treatment empirically distinguishes peripheral membrane proteins from transmembrane integral proteins. Translation reactions were carried out with untreated membranes or membranes inactivated with trypsin or NEM. The aa 810 to 1615 precursor sedimented mostly in the supernatant independently of the presence of biologically active or pretreated membranes (Fig. 6B, lanes 1 to 8). Similarly, the NS3 protein synthesized from a transcript encoding either aa 810 to 1615 or 1027 to 1657 was found in the supernatant independently of the presence of and the type of membranes used in the translation reaction (Fig. 6B, lanes 1, 2, and 9 to 16). In contrast, the NS2 protein derived from processing of the aa 810 to 1615 precursor sedimented almost entirely in the pellet (Fig. 6B, lanes 1 and 2), whereas the 23-kDa polypeptide derived from the translation of the transcript encoding aa 810 to 1026 was mostly found in the supernatant independently of the presence of membranes (Fig. 6B, lanes 9 to 16). These results suggest that the NS2 protein derived from processing of the aa 810 to 1615 precursor is integrated in the membrane in a manner indistinguishable from that of a conventional integral membrane protein and that the integration event is preceded by SRP receptor-mediated targeting of the nascent polypeptide to the ER membrane and processing of the precursor protein.

Orientation of the NS2 protein with respect to the ER membrane. To characterize the topology of the NS2 protein in the membrane, we investigated the sensitivity to PK digestion of the NS2 and NS3 polypeptides synthesized in the presence or in the absence of microsomal membranes. As shown in Fig. 7A, PK digestion of the aa 810 to 1615 protein synthesized in the presence of membranes yielded a 17-kDa undigested peptide (lane 5). This peptide derives from NS2 since it could be immunoprecipitated with anti-NS2 antibodies (data not shown). No protected bands could be observed when translation was carried out in the absence of microsomal membranes (Fig. 7A, lane 2). The resistance to PK treatment was abolished by treatment with detergents (Fig. 7A, lane 6), suggesting that the NS2 protein has a 17-kDa portion that was protected from PK digestion by virtue of its interaction with the microsomal membrane. In view of the observation that the association of NS2 with membranes was dependent on the cleavage at the NS2-3 site, we determined whether any correlation existed between processing at the NS2-3 site and translocation across the membranes, as judged by sensitivity to PK digestion. Translation of a series of NS2-3 proteins that started from aa 849 and contained C-terminal deletions (Fig. 1) followed by PK digestion showed that only those polypeptides that terminated at or beyond aa 1237, and therefore were processed, gave rise to the 17-kDa protected band. In contrast, those precursors that were

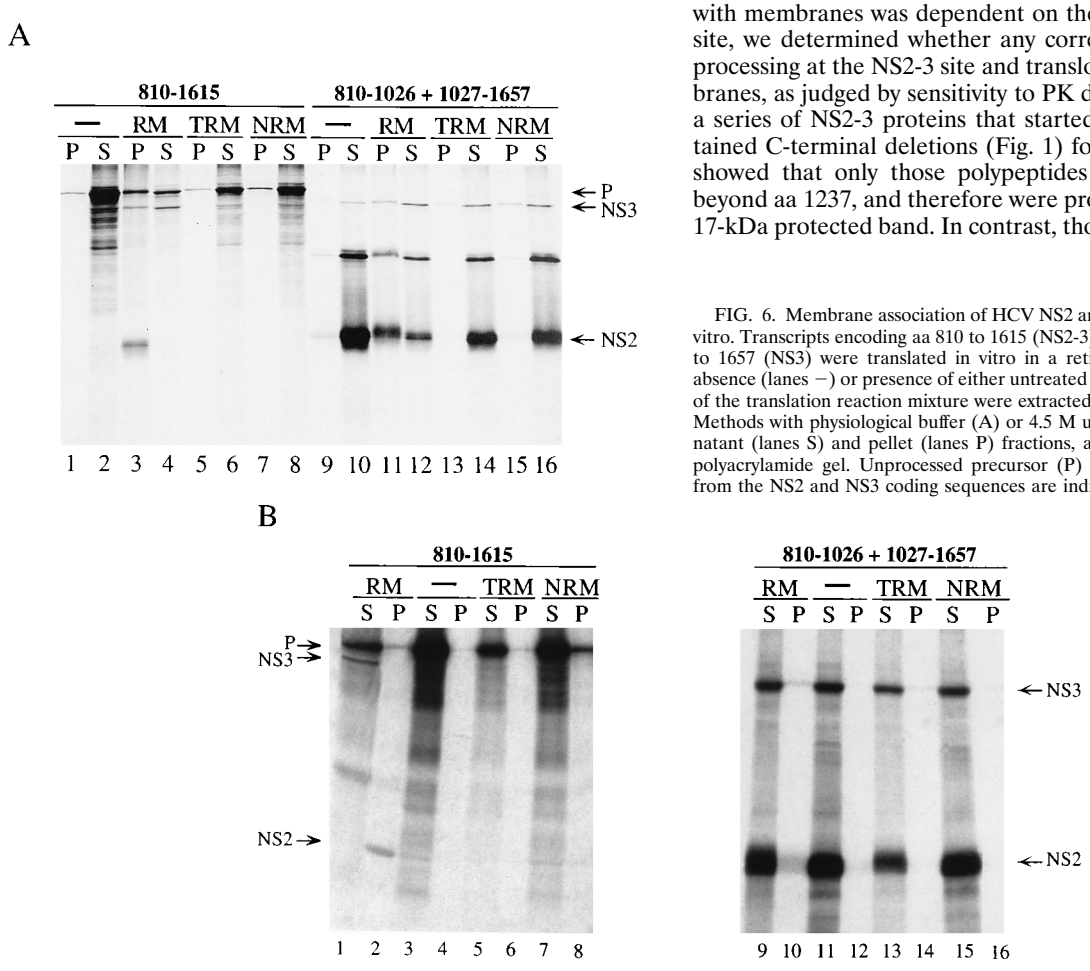


FIG. 6. Membrane association of HCV NS2 and NS3 proteins synthesized in vitro. Transcripts encoding aa 810 to 1615 (NS2-3), 810 to 1026 (NS2), and 1027 to 1657 (NS3) were translated in vitro in a reticulocyte lysate system in the absence (lanes −) or presence of either untreated RM, TRM, or NRM. Aliquots of the translation reaction mixture were extracted as described in Materials and Methods with physiological buffer (A) or 4.5 M urea (B), separated into supernatant (lanes S) and pellet (lanes P) fractions, and analyzed on an SDS–11% polyacrylamide gel. Unprocessed precursor (P) and the polypeptides derived from the NS2 and NS3 coding sequences are indicated.

not cleaved did not yield any PK-resistant band (data not shown). This result was confirmed by PK treatment of NS2 and NS3 proteins synthesized from separate transcripts. In fact, when HCV-BK aa 810 to 1026 and 1027 to 1657 transcripts were cotranslated in the presence of microsomal membranes, no protein resistant to PK digestion could be observed (Fig. 7A, lanes 8 and 11). Thus, these data indicate that in spite of their ability to partially bind to microsomes, neither the uncleaved precursors nor the NS2 protein derived from the aa 810 to 1026 transcript could be efficiently inserted in the membrane and thus protected from PK digestion.

To determine what domain of NS2 was inserted in the membrane and to ascertain that the NS2 protein of the HCV-J strain behaved in a fashion similar to that of the HCV-BK protein, the PK protection patterns of the HCV-J translation products spanning aa 722 to 1325 and 849 to 1325 were compared with those of the HCV-BK polypeptides spanning aa 722 to 1325 and 849 to 1355 (Fig. 7B). Translation of all transcripts in the presence of microsomal membranes yielded a PK-resistant fragment of 17 kDa (Fig. 7B, lanes 5, 11, 17, and 23). The 17-kDa fragment comigrates with the protected fragment obtained with the precursor spanning aa 810 to 1615 (data not shown). Taken together, these data indicate that the NS2 translocation across the ER membrane is a common characteristic of both the HCV-BK and HCV-J strains and that it can be observed independently of the transcript length as long as processing at the NS2-3 junction is preserved. Furthermore, the observation that transcripts containing progressive N-terminal deletions of NS2 yield a protected fragment of the same size suggests that the N-terminal region of NS2 is exposed, at least in part, to the cytosol.

To determine whether the integration of NS2 in the ER membranes was also observed in vivo, a plasmid encoding aa 810 to 1615 was transiently expressed in HeLa cells by the T7-vaccinia virus system (14) and the PK protection assay was performed on the transfected cell lysate. As shown in Fig. 8, the 17-kDa protein band resistant to PK digestion was immunoprecipitated from the lysate (lane 2) and resistance to the PK digestion was abolished if detergents were included during proteolysis (lane 3). A 12-kDa polypeptide resistant to PK digestion was also detected in transfected cells, although as a minor species (Fig. 8, lane 2). The exact nature of this protein species is not clear and is currently being investigated. A similar PK protection pattern was observed upon expression in transfected cells of a full-length cDNA copy of the viral genome (data not shown). Thus, these data indicate that in transfected cells the NS2 protein is inserted in the ER membrane with an orientation similar to that observed in vitro.

To investigate if the NS2 peptide was indeed a true transmembrane protein, we tested whether glycosylation signals introduced at the C-terminal region of the NS2 protein were recognized by the oligosaccharyl-transferase located in the ER lumen. To that end, we introduced by site-directed mutagenesis two Asn residues at positions 983 and 988 of NS2, thus obtaining the Asn-X-Thr consensus sequence required for N-linked glycosylation. Plasmid pGEM(810-1615G) bearing the two Asn substitutions was transfected, and the NS2 profile was compared with that of the wild-type clone expressing the same polypeptide. Figure 8 shows that besides the 23-kDa band corresponding to mature NS2, a protein of 28 kDa was immunoprecipitated with anti-NS2 antiserum from cells transfected with pGEM(810-1615G) and not with pCD(810-1615) (Fig. 8, compare lanes 6 and 8). The size of this polypeptide is consistent with that of the mature NS2 bearing two N-linked oligosaccharide chains. Furthermore, this protein was sensitive to endoglycosidase H treatment (Fig. 8, lanes 7 and 9), indicating that high-mannose sugars had been attached to the polypeptide backbone. Thus, these results confirm the cell-free translation data and indicate that the NS2 protein spans the ER membrane at least once, with the C-terminal region located in the lumen and the N-terminal domain exposed in the cytosol.

DISCUSSION

We have examined by cell-free protein synthesis and transfection studies the biogenesis and membrane localization of the NS2 protein of HCV with particular emphasis on the C-terminal processing event. The results obtained indicate that the NS2 polypeptide is a transmembrane protein and that cleavage at the NS2-3 junction involves a cellular component(s) as a cofactor(s) and has important implications for the localization of the viral polypeptide.

The data reported here indicate that in analogy to what has been described for the HCV-J and -H strains (16, 21), processing of the NS2-3 junction of the BK strain is mediated by the viral autoprotease that spans most of the NS2 polypeptide and the entire NS3 serine protease domain. Deletion analysis of the NS2-3 region shows that efficient cleavage at the NS2-3 site of HCV-BK requires the expression of aa 849 to 1237. Also, substitution of Cys-993 of the BK polyprotein abolishes the NS2-3 cleavage similarly to what has been described for the HCV-J and -H strains (16, 21) (Fig. 2). Thus, although the biochemical requirements for efficient in vitro cleavage at this site appear to differ from one strain to another (see below), it is reasonable to conclude that cleavage at the NS2-3 site of these three HCV strains is mediated by the same autoproteolytic activity. Furthermore, although the role of the NS3 domain in the processing reaction is not clear, our data demonstrate that NS3 sequences cannot be substituted by other segments of the HCV polyprotein.

The HCV-J and -BK strains display distinct degrees of membrane dependency for efficient in vitro cleavage at the NS2-3 site. Direct comparison of the in vitro synthesis of the NS2-3 polyproteins of these two strains clearly shows that, independently of the transcript length, the BK strain requires the presence of membranes during translation for efficient processing at the NS2-3 junction (Fig. 3). However, translation in the presence of membrane and expression of the NS2-3 autoprotease in transfected cells (data not shown) indicate that the overall processing efficiencies of these two strains are not dissimilar. The contribution of the microsomal membranes to BK NS2-3 processing is not limited to a stabilizing effect on the reaction products but rather causes an enhancement of cleavage (Fig. 4). Also, our data indicate that the membrane-stimulating activity of the NS2-3 protease requires targeting of the nascent precursor to the membrane via the SRP-SRP receptor machinery (Fig. 5). Thus, although the exact role of membranes in the cleavage reaction remains to be defined, it is conceivable that they affect the processing at the NS2-3 junction by assisting the proper folding of the nascent precursor. Thus, it is likely that the differences in amino acid sequence of the HCV-BK and -J strains (27, 44) influence the capacity of the nascent protein precursor to properly fold in the absence of membranes, rather than compromising its intrinsic protease activity.

It is not clear what the biological significance of the distinct in vitro requirements for cleavage at the NS2-3 site of the BK and J strains is. Interestingly, the processing at the p7-NS2 junction appears also to differ in efficiency between the HCV-J and -BK strains (Fig. 3). Furthermore, the kinetics of association between the E1 and E2 polypeptides derived from the BK strain has been shown to differ from that of the H strain (9),

suggesting that observed differences among HCV strains are not limited to processing at the NS2-3 junction but are a more general phenomenon. It is tempting to speculate that the above-described differences influence the replication capacity of the HCV strains and affect their pathogenic properties. Alternatively, the observed differences in enzymatic activity and processing kinetics may be the results of the changes in the protein sequence introduced during cloning of the cDNAs of these viruses and therefore may not be representative of their actual biological properties.

The results presented in this study reveal that the NS2 protein is a transmembrane polypeptide with the C-terminal region translocated in the ER lumen and the N-terminal region exposed, at least in part, in the cytoplasm. Several lines of evidence support this conclusion. Resistance to urea extraction of the processed NS2 protein is consistent with the sedimentation profile of an integral membrane polypeptide (Fig. 6). Also, the NS2 protein is protected by the membranes from digestion by exogenously added PK both in cell-free translation studies and in transfected cells (Fig. 7 and 8). Furthermore, the C terminus of the NS2 protein is available for N-linked glycosylation in transfected cells once the appropriate signals are introduced in the protein backbone (Fig. 8).

Interestingly, although NS2 targeting to the ER membrane appears to involve SRP and its receptor, much like all known secretory and transmembrane polypeptides (40), the mode of interaction with the targeting machinery as well as the mechanism of insertion across the ER bilayer present some peculiar features. In fact, analysis of the amino acid sequence of the NS2 polypeptide revealed no long stretches of hydrophobic residues that could be identified as a signal sequence(s) and/or transmembrane domain(s). However, the inefficient targeting to the membrane of the aa 810 to 1026 NS2 precursor suggests that the functional signal sequence responsible for proper targeting is located across or downstream from the cleavage site, at the C terminus of the translocated polypeptide. This observation is in line with the data presented by Hijikata et al. (20, 21), in which the authors failed to observe an NS2 protein species resistant to PK digestion when they expressed trun-

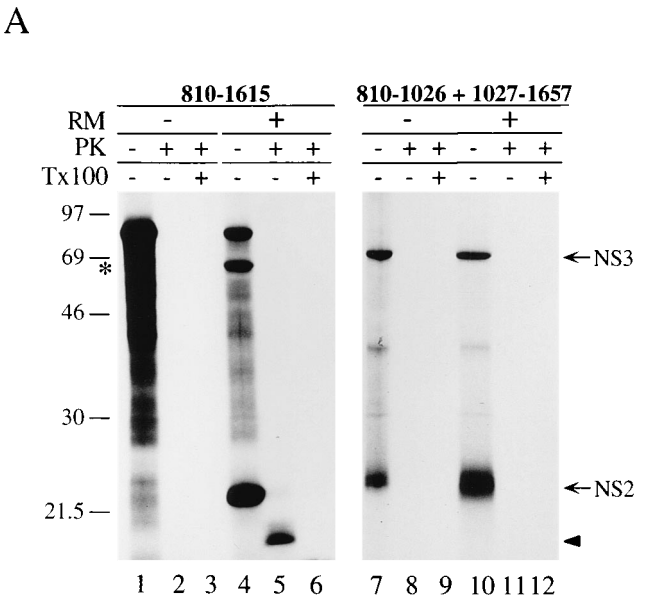
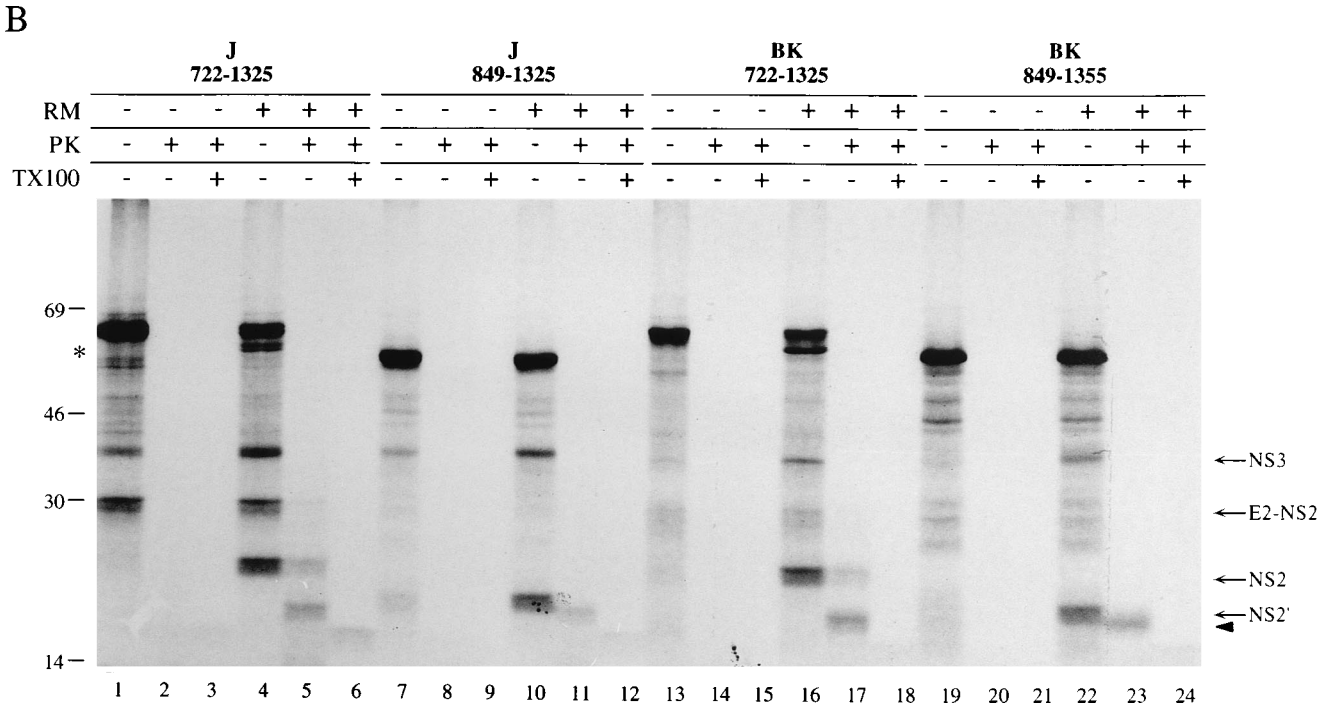


FIG. 7. PK protection assay of HCV proteins synthesized in vitro. (A) Transcripts encoding aa 810 to 1615 and aa 810 to 1026 and 1027 to 1657 were translated in vitro in the absence or presence of RM. (B) Transcripts encoding the indicated amino acids of the HCV-J strain (J) and the HCV-BK strain (BK) were translated in vitro with a rabbit reticulocyte lysate either in the absence or in the presence of microsomal membranes. Aliquots of the translation mixtures were treated with PK or with PK and Triton X-100 (Tx100) as described in Materials and Methods and subsequently were analyzed on an SDS–11% polyacrylamide gel. The positions of the processed NS3, E2-NS2, NS2, and NS2′ polypeptides are indicated. Asterisks indicate the partially processed NS2-3 precursor. Arrowheads indicate the protected NS2 protein. Positions of molecular mass standards (in kilodaltons) are indicated.



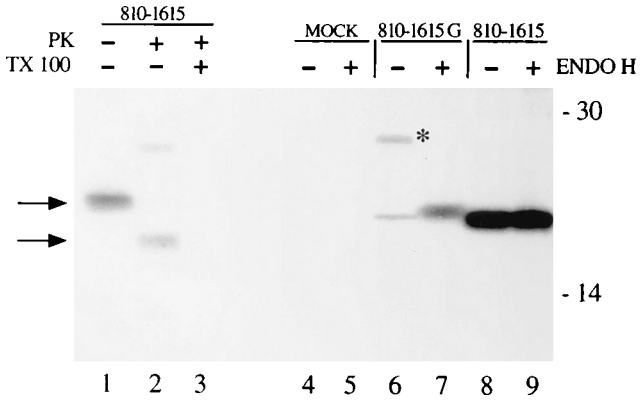


FIG. 8. NS2 is translocated across the ER membrane in transfected cells. Recombinant plasmids pCD(810-1615) and pGEM(810-1615G) were transfected into vaccinia virus vT7F-3-infected HeLa cells as previously described (45). Lysates were immunoprecipitated with anti-NS2 antiserum (45). Prior to immunoprecipitation, lysates from cells transfected with pCD(810-1615) were subjected to a PK protection assay as described in Material and Methods (lanes 1 to 3). The immunoprecipitates were digested with endoglycosidase H (lanes 5, 7, and 9) and analyzed on an SDS–12.5% polyacrylamide gel. Mock-transfected cell lysate was also treated. + and −, digested and mock digested, respectively. Arrows indicate unglycosylated and protected NS2 protein. The asterisk indicates glycosylated NS2 polypeptide. Positions of molecular mass standards (in kilodaltons) are shown.

cated forms of the NS2 polypeptide that did not require processing at the C terminus for correct maturation.

Although our results do not address whether the NS2 protein spans the bilayer more than once, they indicate that at least one membrane-spanning domain is located in the N-terminal third of the protein, possibly between residues 50 and 80 (Fig. 7). However, the localization of the extreme N terminus of NS2 as proposed by Mizushima et al. (36) cannot be substantiated by our experiments. Furthermore, our data provide evidence that cleavage at the NS2-3 site is a prerequisite for NS2 translocation. In fact, the perfect correlation between the ability of the various deletion mutants to be processed at the NS2-3 site and their translocation competence strongly suggests that only the NS2 protein derived from the processed NS2-3 precursor can be efficiently translocated into the ER membrane. This conclusion is strengthened by the observation that the NS3 polypeptide is neither protected nor associated with the membrane (Fig. 6 and 7) and that the C993A and L1026ΔQ mutants, which are no longer processed at the NS2-3 site, have also lost their translocation competence into the membrane bilayer (data not shown). The inference that processing at its C terminus precedes NS2 insertion in the membrane has important implications for the mechanism by which this protein is translocated. In fact, it confirms previous observations that targeting to and translocation across the ER membrane are distinct events (37, 38) and indicates that at variance with the majority of mammalian secretory and membrane proteins, the NS2 polypeptide is translocated entirely posttranslationally. This conclusion raises the intriguing possibility that proper topogenesis of NS2 involves folding of the polyprotein precursor into an active autoprotease and subsequent unfolding of the cleaved NS2 polypeptide to allow translocation across the ER membrane.

The functional role of the NS2-3 protease as well as that of the NS2 protein in the life cycle of HCV remains to be elucidated. The NS3 serine protease of the J, H, and BK strains appears to be largely insensitive to cleavage at the NS2-3 site, since inactivation of the NS2-3 protease by site-directed mutagenesis does not perturb the processing capacity of the serine protease (16, 21; data not shown). Thus, it appears that cleavage at the NS2-3 site has a regulatory role in the biogenesis of the HCV proteins limited to the processing and membrane topology of the NS2 polypeptide and does not affect the functionality of the serine protease. However, recent data have indicated that the presence of uncleaved NS2 sequences may severely interfere with the NS3-mediated proteolysis at downstream sites in the polyprotein of a United Kingdom HCV isolate (8). These conflicting observations need further investigation but could be the result of dissimilar experimental approaches utilized to investigate this problem or, alternatively, may reflect differential dependence of various HCV strains on NS2-3 cleavage for NS3 activity.

The finding that NS2 is a transmembrane peptide is in agreement with the observation that in cells expressing the entire polyprotein of HCV the NS2 polypeptide is associated with the E1 and E2 glycoproteins, as judged by coimmunoprecipitation with monospecific antibodies (32, 43; data not shown). These data prompt the provocative speculation that NS2 is a structural rather than a nonstructural viral component (43). Protease protection experiments suggest that both HCV glycoproteins do not contain a cytoplasmic tail suitable for a specific interaction with the viral nucleocapsid (20, 33, 35). It is thus conceivable that NS2 functions during the assembly process as a structural bridge between the envelope glycoproteins and the nucleocapsid. However, the NS2 polypeptide has been also immunoprecipitated with NS5A and NS5B, which are the putative components of the replication complex (22). Obviously, the definitive classification of NS2 as a structural component awaits a precise biochemical characterization of the HCV virion.

ACKNOWLEDGMENTS

We thank K. Shimotohno for providing the HCV-J subclone pN(722-1325) and R. De Francesco for plasmids pCITE(623-1615) and pT7(1027-1657). We also thank S. Bonatti, J. Jiricny, and C. Steinkühler for critical review; M. Emili for graphics; and P. Neuner for oligonucleotide synthesis.

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