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Gene mapping of the putative structural region of the hepatitis C virus genome by *in vitro* processing analysis

(RNA virus/structural proteins/glycoproteins/signal peptidase)

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ABSTRACT **Processing of the putative structural proteins of hepatitis C virus was examined by using an *in vitro* expression system. An RNA transcript for cell-free translation was prepared from a cDNA construct that encompasses the region encoding the 980 amino-terminal residues of the viral polyprotein precursor. Processing of the *in vitro* translation product proceeded cotranslationally in the presence of microsomal membranes and generated four major membrane-associated products. Two of these four major products, named gp35 and gp70, were shown to be transported into microsomes and heavily glycosylated, suggesting that the processing events are partly mediated by the signal peptidase of the endoplasmic reticulum. The other two products, p19 and p21, were probably associated with the outer surface of the microsomal membrane. Analysis of processed proteins translated from a series of truncated forms of the cDNA construct as well as determination of amino-terminal amino acid sequences of gp35 and gp70 indicated that these four products are arranged from the amino-terminal end of the polyprotein precursor in the order: NH_2–p22–gp35–gp70–p19. Both gp35 and gp70 could be candidates of initially processed forms of envelope proteins of the hepatitis C virus.**

Hepatitis C virus (HCV) is considered to be a causative agent of post-transfusion non-A, non-B hepatitis (1, 2). The enveloped virions consist of unknown species of structural proteins encoded by positive-stranded RNA genomes. This virus is probably related to pestiviruses and to flaviviruses judging from the similarities of the deduced amino acid sequences of the putative viral proteins (refs. 3 and 4 and unpublished results). Our recent study showed that genomic RNA of HCV from Japanese patients with non-A, non-B hepatitis is more than 9413 nucleotides long and includes a single open reading frame (ORF) encoding a precursor polyprotein of 3010 amino acids (4). This precursor polyprotein has many basic amino acid residues clustered in its 120 amino-terminal residues deduced from the sequence of the ORF, as in the amino-terminal regions of nucleocapsid (C) proteins of flaviviruses (4, 5). It also has 15 potential aspargine-linked glycosylation (N-glycosylation) sites clustered in the region of amino acid residues 196–645 like those found in putative envelope (E) glycoproteins of pestiviruses (4, 6). These data suggest that the genetic organization of HCV is almost identical to those of pestiviruses or flaviviruses and that the 5′ portion of the genome encodes viral structural proteins.

The gene order in the genome of flaviviruses has been determined to be C–premembrane (preM)–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5 (5), where NS is nonstructural protein. The viral gene products of flaviviruses have been postulated to be released from a polyprotein precursor co- and post-translationally by both cellular and viral proteinases (5, 7–9). Recent *in vitro* studies on processing of precursor proteins of flaviviruses using fractionated microsomal membranes have indicated that the initial processing events to generate the viral structural proteins are mediated by a host signal peptidase located in the lumen of the endoplasmic reticulum (10–12).

In this work, to characterize the putative structural proteins encoded by the HCV genome, we studied the processing of a truncated polyprotein precursor of HCV using *in vitro* transcription/translation system in the presence of a microsomal membrane fraction.

MATERIALS AND METHODS

Construction of Expression Plasmids. An expression plasmid, pC980, containing an 81-base-pair 5′ noncoding region and a 2940-base-pair coding region of the HCV cDNA sequence,* which, therefore, encodes the 980 amino-terminal residues of HCV ORF, and its serial deletion mutants were constructed as follows (see Fig. 1). The *Eco*RI fragment of HCV-J clone 2 (4) was subcloned into the *Eco*RI site of the pTZ18U vector, which contains T7 phage promoter just upstream of the multicloning region, to construct expression plasmid pC163. The *Nar* I–*Hin*dIII fragment of pC163 was replaced by the *Nar* I–*Hin*dIII fragment of HCV-J clone 3 (4) to yield pC511. pC740 was obtained by replacing the *Mlu* I–*Hin*dIII fragment of pC511 by the *Mlu* I–*Hin*dIII fragment of clone 33, which overlapped the cDNA clone for HCV-J (4). pC980 was then constructed by replacing the *Bss*HII–*Hin*dIII fragment of pC740 by the *Bss*HII–*Hin*dIII fragment of HCV-J clone 6 (4). pN124 and pN340 were constructed from pC980 by deleting the *Nru* I–*Cla* I fragment and *Sma* I–*Bam*HI fragment, respectively, and ligating using *Sph* I linker (Takara Shuzo, Kyoto) after filling-in the cohesive ends of the restriction sites by using the Klenow fragment of DNA polymerase I. pN124 was constructed by deleting 187 base pairs of the 3′ terminus by *Sph* I digestion.

***In Vitro* Transcription.** All purified plasmids were linearized by cutting them at either the unique *Hin*dIII site of multicloning region downstream of the insert or at appropriate sites within the insert. They were then used as templates for *in vitro* transcription. RNA transcripts were synthesized *in vitro* using T7 RNA polymerase (United States Biochemical) as described (13). C281, C381, N340B, and N340N were obtained as transcripts after *in vitro* transcription by using pC511 truncated with *Bam*HI or *Hin*cII and pN340 truncated with *Bss*HII or *Not* I, respectively, as templates (Fig. 1).

***In Vitro* Processing Assay and Protein Analyses.** The transcripts were translated using a rabbit reticulocyte lysate

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Abbreviations: HCV, hepatitis C virus; NS, nonstructural protein; ORF, open reading frame; BVDV, bovine viral diarrhea virus.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. D90208 and D00757).

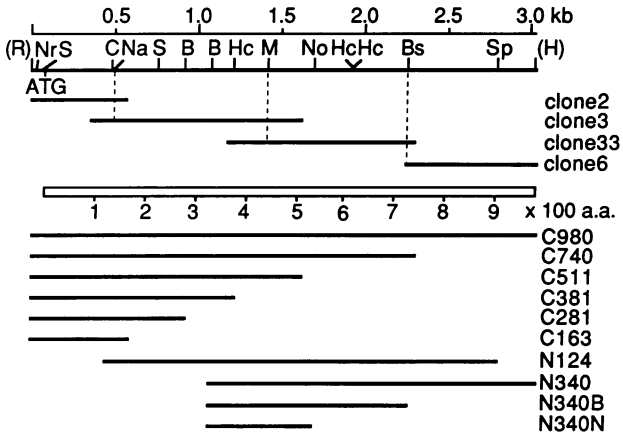

FIG. 1. Diagram of the strategy for construction of expression plasmids and a map of deletion constructs used in *in vitro* processing analysis. Positions of the initiator methionine and the ORF are indicated by "ATG" and a box, respectively. B, *Bam*HI; Bs, *Bss*HII; C, *Cla* I; H, *Hin*dIII; Hc, *Hin*cII; M, *Mlu* I; Na, *Nar* I; No, *Not* I; Nr, *Nru* I; R, *Eco*RI; and S, *Sma* I; aa, amino acid(s); kb, kilobase(s). Restriction sites originating from the vectors are in parentheses.

(Amersham) in the presence or absence of canine pancreatic microsomal membrane (Promega) with [^{35}S]methionine (Amersham) for labeling. Samples of translation mixtures were then treated with proteinase K (200 μg/ml) in the presence or absence of detergents (1% sodium deoxycholate/1% Triton X-100) at 0°C for 30 min. The mixtures were centrifuged at 10,000 × *g* for 20 min, and samples of the pellets and supernatants were analyzed by SDS/PAGE and fluorography (13, 14). Treatment with endoglycosidase H (Boehringer Mannheim) was performed as described by Ruiz-Linares *et al.* (12). Partial amino-terminal amino acid sequences of the translation products labeled with [^{35}S]methionine plus [^{3}H]-valine, [^{35}S]methionine plus [^{3}H]tyrosine, or [^{35}S]methionine plus [^{3}H]histidine (New England Nuclear) were determined manually essentially by the procedure of Preugschat *et al.* (8).

RESULTS

Processing of Truncated Polyprotein Precursor of HCV *in Vitro*. The results of an *in vitro* processing assay using pC980 are shown in Fig. 2*A*. The translation product (C980) from pC980 was found to be processed and to generate four major cleavage products, a (maximum 70 kDa), b (35 kDa), c (22 kDa), and d (19 kDa) (Fig. 2*A*, lane 5), only when microsomal membranes were present during translation *in vitro*. The unprocessed form synthesized in the absence of microsomes was not detected except as a possible stacked form at the origin of the gel (Fig. 2*A*, lane 3). The bands seen between bands b and c would be immature products, or products of degradation of C980, because similar bands are also observed in Fig. 2*A*, lane 3. Two smaller products (17 and 15 kDa) were occasionally detected. These seemed to originate from the amino-terminal region of this ORF, because both products were detectable among the translation products of C163, even in the absence of microsomal membranes, on long exposure (data not shown). These four major products, a, b, c, and d, were all found in the pelleted microsomal membrane fraction. Products a and b were resistant to the proteinase K treatment, whereas products c and d were degraded by this enzyme (Fig. 2*A*, lane 7). The resistance to proteinase treatment was abolished by treatment with detergents (Fig. 2*A*, lane 9), indicating that products a and b were translocated into the microsomes. The cleavage of the polyprotein precursors probably occurred cotranslationally because such processing was not observed after addition of cycloheximide

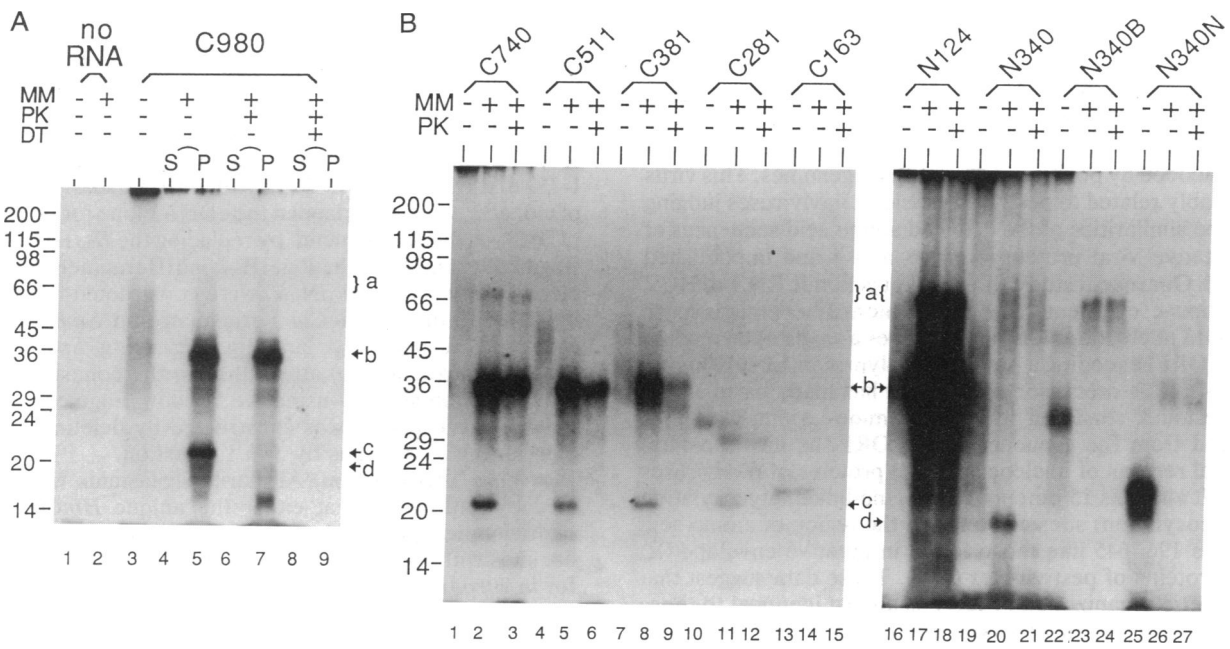

FIG. 2. Analysis of the *in vitro* translation products by SDS/PAGE. (*A*) The *in vitro* transcripts of pC980 were translated with a rabbit reticulocyte lysate in the presence (+, lanes 4–9) or absence (−, lane 3) of microsomal membranes (MM), followed by proteinase K (PK) treatment in the presence (+, lanes 8 and 9) or absence (−, lanes 6 and 7) of detergents (DT). S (lanes 4, 6, and 8) and P (lanes 5, 7, and 9) indicate supernatant and particle fractions, respectively, obtained by centrifugation after treatment with proteinase K. The products translated without addition of RNA in the presence (+, lane 2) or absence (−, lane 1) of microsomal membranes are shown as controls. The four major products detected are indicated as a, b, c, and d on the right. (*B*) *In vitro*-processed products of deletion mutants of C980. Transcripts synthesized *in vitro* were translated *in vitro* in the presence (lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, 15, 17, 18, 20, 21, 23, 24, 26, and 27) or absence (lanes 1, 4, 7, 10, 13, 16, 19, 22, and 25) of microsomal membranes (MM), followed by proteinase K (PK) treatment (lanes 3, 6, 9, 12, 15, 18, 21, 24, and 27). Only particle fractions of translation mixtures in the presence of microsomal membranes are shown. (*A* and *B*) Positions of molecular mass markers are indicated at the left in kDa.

to reaction mixture in which the precursor protein was still present (data not shown).

Gene Mapping of the Processed Products on the HCV Genome. To determine the order of the four processed products encoded by the HCV ORF, we examined the *in vitro* processing of the precursor protein using various deletion mutants of C980 (Fig. 2*B*). The *in vitro* translation products C740, C511, C381, C281, and C163 had serial carboxyl-terminal deletions of C980 and included 740, 511, 381, 281, and 163 residues, respectively, of the amino-terminal sequences. In the products of pN124 and pN340, respectively, 123 and 339 residues of the amino-terminal sequence of the product of pC980-ORF were replaced with the amino acid sequence Met-Pro. pN124-ORF lacks the sequence for the 59 carboxyl-terminal residues of pC980. N340B and N340N lacked 252 and 438 residues, respectively, of the carboxyl-terminal sequence of N340. C740, C511, and C163 included 18 extra residues (Met-Glu-Phe-Glu-Leu-Gly-Thr-Arg-Gly-Ser-Ser-Arg-Val-Asp-Leu-Gln-Ala-Cys, Arg-Asn-Ser-Ser-Ser-Val-Pro-Gly-Asp-Pro-Leu-Glu-Ser-Thr-Cys-Arg-His-Ala, and Trp-Asn-Ser-Ser-Ser-Val-Pro-Gly-Asp-Pro-Leu-Glu-Ser-Thr-Cys-Arg-His-Ala, respectively) at their carboxyl termini. C980 and N340 included 4 extra residues (Pro-Ala-Gly-Met) at their carboxyl termini. All these extra peptides originated from the multicloning sites of pTZ18U. Each transcript was translated *in vitro* in the presence or absence of microsomal membranes and the reaction products were tested for sensitivity to proteinase K (Fig. 2*B*). Since proteinase-resistant product a (70 kDa) could be detected only in the processed forms of C740, N124, N340, and N340B (Fig. 2*B*, lanes 3, 18, 21, and 24), the location of this protein in the HCV ORF should be roughly from residue 340 to residue 740, which includes a possible signal sequence for this protein. The proteinase-resistant product of the processed form of N340N (34–40 kDa) was found to be smaller than that of N340B, also supporting this estimation (Fig. 2*B*, lane 27). The other proteinase-resistant product b (35 kDa) was not detected in the processed forms of C281, C163, N340, N340B, and N340N (Fig. 2*B*, lanes 12, 15, 21, 24, and 27), although a smaller proteinase-resistant product (≈30 kDa) was found in processed forms of C281 (Fig. 2*B*, lane 12). This indicates that product b with the signal sequence is located in the region from about residue 163 to residue 381 in the HCV ORF. Product c (22 kDa) should originate from the amino-terminal end of HCV ORF, because the band for this protein was missing in the processed reaction products from the amino-terminal deletion mutants pN124, pN340, pN340B, and pN340N (Fig. 2*B*, lanes 17, 20, 23, and 26, respectively). The fact that band of the proteinase-sensitive translation product of C163 migrated slightly slower than product c may be due to the presence of the extra peptide at its carboxyl terminus (Fig. 2*B*, lane 14). Similar abnormal migration of the product in SDS/PAGE was observed in our previous study (15). Product d (19 kDa) was detected only in the processed product of C980 and of N340, which has the same carboxyl-terminal sequence as C980 (Fig. 2 *A*, lane 5, and *B*, lane 20), indicating that this protein is encoded by the carboxyl-terminal region of the C980-ORF. From these results, we concluded that the order of the processed proteins in the HCV ORF was NH_2–c(22 kDa)–b(35 kDa)–a(70 kDa)–d(19 kDa)–COOH.

Endoglycosidase H Treatment of Proteinase-Resistant Products. We found 15 potential N-glycosylation sites in the region covering products a and b of the HCV ORF (4), suggesting that both products were N-glycosylated. Therefore, both products translated *in vitro* were treated with endoglycosidase H to remove possible N-linked high-mannose-type glycans and then analyzed by SDS/PAGE. As shown in Fig. 3, the sizes of proteinase-resistant products a and b decreased after treatment with endoglycosidase H, indicating that both products are glycoproteins. The molecular masses of the deglycosylated forms of gp35 and gp70

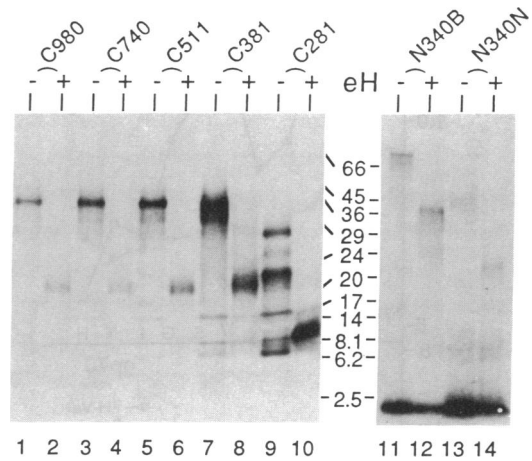

FIG. 3. Digestion of proteinase-resistant translation products with endoglycosidase H. Proteinase K-resistant translation products (−, lanes 1, 3, 5, 7, 9, 11, and 13) of deletion mutants of C980 were digested with endoglycosidase H (eH) to remove N-linked high-mannose-type glycans (+, lanes 2, 4, 6, 8, 10, 12, and 14) and were analyzed by SDS/PAGE. Molecular masses are indicated in kDa.

were estimated to be about 21 kDa and 38 kDa (Fig. 3, lanes 2, 4, 6, 8, and 12). The products of C281 and N340N were reduced in size about 10 kDa and 22 kDa, respectively, by deglycosylation as expected from deletion analysis (Fig. 3, lanes 10 and 14). Hence, we tentatively named products b and a, as gp35 and gp70, respectively.

Determination of Amino-Terminal Amino Acid Sequences of gp35 and gp70. To determine the exact cleavage sites of the HCV polyprotein precursor, we examined the amino-terminal amino acid sequences of gp35 and gp70 by using the proteinase-resistant *in vitro* translation products of C281 and N340N, respectively. The amino acid sequences determined from the peaks of ^3H-labeled amino acids in Edman degradation cycles were compared with the deduced amino acid sequence of the C980-ORF. Sequencing of the *in vitro* processed products from C281 and N340N identified the amino-terminal residues of gp35 and gp70 as Tyr-192 and His-384, respectively, of the sequence deduced from the C980-ORF, as shown in Fig. 4. We found signal sequence homologs at residues 175–191 and residues 370–383 in the deduced sequence of HCV ORF, just preceding the amino-terminal ends of gp35 and gp70, respectively (Fig. 5, double underlines) (16). We also found two segments that may act as membrane-spanning segments in the carboxyl-terminal regions of gp35 and gp70 (Fig. 5, underlines), suggesting that both products are type I membrane-anchored proteins (17).

DISCUSSION

In this work, using an *in vitro* processing system, we detected four major products (p22, gp35, gp70, and p19) cotranslationally generated from the 980 amino-terminal residues encoded by the HCV ORF. Cleavage of the polyprotein precursor was dependent on the presence of microsomal membranes during *in vitro* translation. gp70 and gp35 were shown to be transported into the microsomal membrane and to be putative membrane-anchored proteins that are heavily glycosylated, and p22 and p19 were probably associated with the outer surface of the microsomal membrane. These four products were shown to be arranged at the amino-terminal end of the polyprotein encoded by the HCV ORF in the order NH_2–p22–gp35–gp70–p19–COOH. We found two hydrophobic segments (residues 174–191 and residues 371–383) that may act as signal sequences (16), preceding the amino termini of gp35 and gp70. These findings suggest that the amino

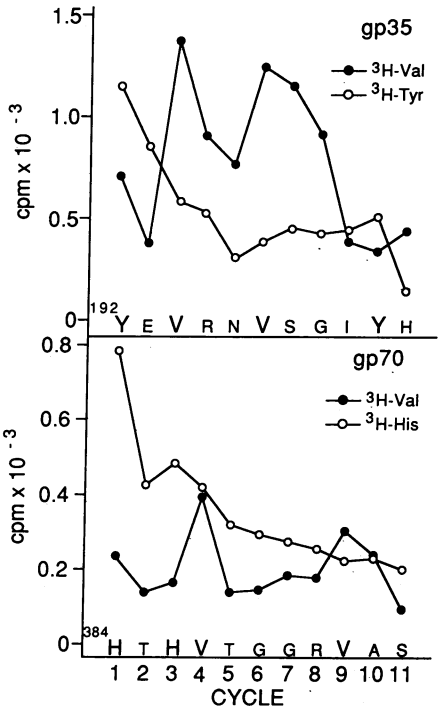

FIG. 4. Partial amino-terminal sequence analyses of proteinase-resistant translation products derived from C281 and N340N. Proteinase-resistant translation products that were labeled with individual tritiated amino acids were deglycosylated with endoglycosidase H and fractionated by SDS/PAGE followed by electroblotting onto an Immobilon membrane (Millipore). Radiolabeled products on the membrane were cut out and subjected to manual sequential Edman degradation. The release of radioactive amino acids at each cycle of degradation is plotted. In the partial amino acid sequences, residues (large characters) that aligned with the corresponding amino acid sequences of C980-ORF are shown above the abscissa; residues (small characters) that were the deduced amino acids from the nucleotide sequence of these regions are also shown.

terminal 980 residues encoded by the HCV ORF are processed to the four major viral proteins and that the cleavage of p22, gp35, and gp70 is mediated by the signal peptidase(s) of the endoplasmic reticulum lumen in the host cell, as in the processing of the structural proteins of flaviviruses (10–12). However, cleavage between gp70 and p19 does not seem to be due to a signal peptidase, because p19 was not transported into the microsomal membrane. The location of the region encoding p19 in the HCV ORF is similar to that of the region encoding NS2A in the flavivirus ORF. Cleavage of the N terminus of NS2A of Dengue virus type 4 was shown to be observed only when NS2A was intact and suggested to be a cis-acting proteinase that cleaves itself from the preceding NS1 or provides sequences for recognition by some unknown cellular proteinase that cleaves the NS1–NS2A junction (18). p19 was generated only when the entire carboxyl-terminal portion of C980 was present, suggesting that p19 is generated by the same mechanism as that producing NS2A protein of Dengue virus type 4.

The genetic organization of HCV was suggested to be related to those of flaviviruses or pestiviruses from the partial homologies of the amino acid sequences among these viruses (3, 4). The proteins encoded in the genomes of flaviviruses have been shown to be arranged on the polyprotein precursor in the order NH_2–C–preM–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5–COOH (5), and the genomic organization of bovine viral diarrhea virus (BVDV), a member of the pestiviruses, was suggested to be NH_2–p20–gp62–gp53–NS2–NS3–NS4–NS5–COOH (6, 19). The basic protein located at the amino-terminal of the polyprotein encoded by the HCV ORF was suggested to be C protein, as in flaviviruses (4). Product p22, therefore, is considered to be an initially processed form of C protein of HCV. In flaviviruses, C protein that is initially produced in a membrane-associated form, like HCV p22 generated *in vitro*, is converted to mature C protein by cleavage of its carboxyl-terminal hydrophobic segment by some unknown proteinase, prior to or during virus assembly (10). So HCV p22 may also be subjected to further processing to generate mature C protein in the virus particles *in vivo*.

We found two putative membrane glycoproteins encoded by the HCV genome and tentatively named these proteins gp35 and gp70 on the basis of their maximum molecular weights. However, the exact sizes of the mature forms of these proteins in HCV-infected cell are unknown because both glycoproteins seem to be transported to the cell surface through the endoplasmic reticulum–Golgi pathway as host plasma membrane proteins (20) and, therefore, these proteins may undergo further proteolytic cleavage(s) and/or modifications of N-linked glycans (21, 22). The location of the

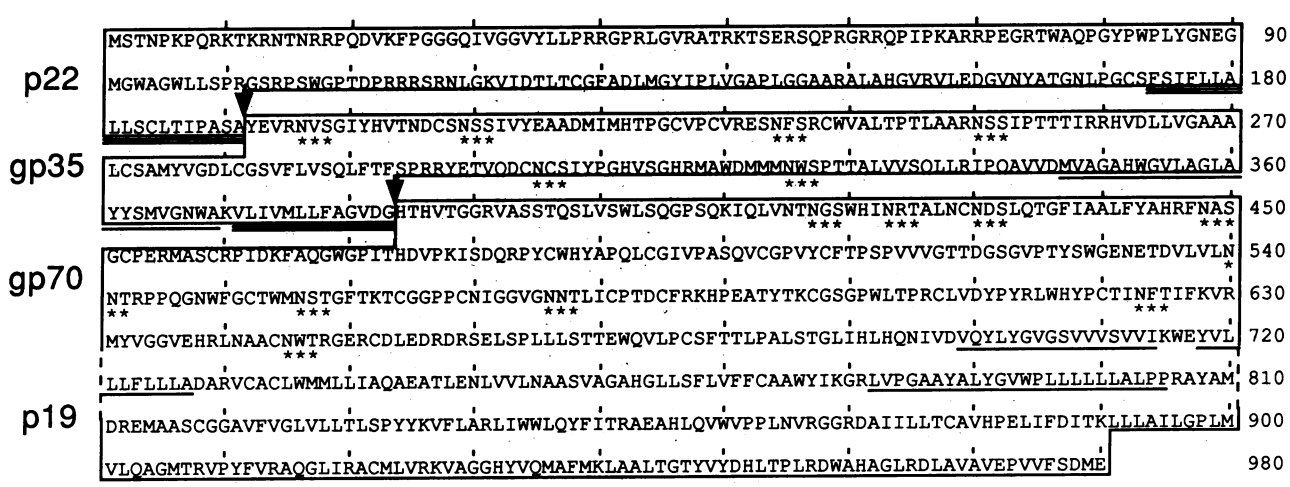

FIG. 5. Organization of proteins encoded by the amino-terminal portion of the HCV ORF. The amino acid sequence deduced from the C980-ORF is shown. Standard one-letter amino acid abbreviations are used. The regions corresponding to p22, gp35, gp70, and p19 are boxed. The putative junctional region between gp70 and p19 is shown by broken lines. The amino-terminal ends of gp35 and gp70 are indicated by arrows. Putative membrane-spanning segments (underlines), signal sequences (double underlines), and potential N-glycosylation sites (asterisks) are indicated.

region encoding gp70 in the HCV ORF probably corresponds to that of the region encoding NS1 in the flavivirus ORF, judging from the similarity in the distances between the amino-terminal residues of these products and conserved Gly-Asp-Asp motifs, which are found in the NS5 regions of flavivirus ORFs and the corresponding region of the HCV ORF (ref. 4 and data not shown). The NS1 protein of flaviviruses is considered to be a nonstructural membrane glycoprotein and to be able to elicit a protective immune response of the host against lethal virus infection (23). However, the genetic organization of the glycoprotein-encoding region of HCV polyprotein precursor is different from those of flaviviruses. Only two products of HCV were found to be translocated into the microsomal membrane initially in our experiment, whereas three proteins, preM, E, and NS1, of flaviviruses are translocated (10–12, 18). The region from residue 190 to residue 730 of the HCV ORF, which fully covers the region encoding gp35 and gp70, was suggested to correspond to that encoding HCV envelope protein(s), judging from the evidence that the hydropathy profile of the encoded sequence and the number of potential N-glycosylation sites in this region, which was quite similar to that of putative envelope glycoproteins (gp25, possibly a processed form of gp62, and gp53) of BVDV rather than that of any part of flaviviral glycoproteins (4, 27). In this study, we showed that both gp35 and gp70 are actually heavily glycosylated, like BVDV glycoproteins, and that the genetic organization of these products (NH_2–p22–gp35–gp70–p19) in the HCV ORF is similar to that of BVDV putative structural genes (NH_2–p20–gp62–gp53), which is followed by the equivalent nonstructural gene NS2 (6, 23). gp53 of BVDV and gp55 of hog cholera virus (another member of the pestiviruses) have been shown to be targets of virus-neutralizing antibodies (24, 25). Therefore, the mature form of gp70 may play an important role in infection by HCV.

Recently, we found two hypervariable regions (HVR1 and HVR2) in the glycoprotein-encoding regions of Japanese HCV isolates (28). Variability of the region around HVR1 in isolates in the United States has also been reported (26). Residues 391–400 of HVR1 and residues 474–480 of HVR2 were both found to be located in the amino-terminal portion of gp70 in this study. Although the biological implication of these variabilities in gp70 is unknown, HVR2 was suggested to be possible candidate for the favorable antigenic region (28). For development of effective therapeutic agents against HCV infection, further analyses of the molecular mechanisms of virus assembly are necessary, including identification of the nature of the viral structural proteins.

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