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Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus

(RNA virus/serine proteinase)

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ABSTRACT **By using a plasmid-based transient protein expression system in cultured cells and an *in vitro* transcription/translation system, we analyzed the proteolytic processing of the putative nonstructural protein region of the precursor polyprotein from a Japanese type of hepatitis C virus. In addition to the previously reported viral proteins, p21 and p70, we identified products of 4 kDa (p4), 27 kDa (p27), 56 kDa (p56), 58 kDa (p58), and 66 kDa (p66). These products were produced in a viral serine proteinase (proteinase 2)-dependent manner from the region downstream of p70 in the precursor polyprotein and were arranged as NH_2-p70–p4–p27–p58(p56)–p66-COOH as determined with region-specific antibodies. We showed that p56 was an N-terminally truncated form of p58, which suggested that a small polypeptide of 2 kDa (p2) was produced from the N-terminal part of p58. Cleavage between p4 and p27 was inefficient *in vitro* and we saw the 31-kDa precursor polypeptide (p31) accumulate. Furthermore, efficient cleavage at this site *in vivo* required the presence of p58/p56. Immunoprecipitation analysis *in vitro* also suggested the mutual interaction of those nonstructural protein products. An especially close association of p4 with p70 may contribute to association of p70 with microsomal membranes.**

Hepatitis C virus (HCV) was first discovered as one of the causative agents of non-A, non-B hepatitis by using molecular cloning techniques (1, 2). Cloning and sequence analyses of cDNAs including the entire HCV genome showed that the positive-stranded RNA genome is 9400 nt long and contains a single large open reading frame (ORF). The HCV ORF encodes a polypeptide of 3010, 3011, or 3033 aa residues (3–9). This is a precursor polyprotein from which the following nine viral proteins are proteolytically processed: NH_2-core (C)–envelope 1 (E1)–envelope 2 (E2)–nonstructural protein 2 (NS2)–NS3–NS4A–NS4B–NS5A–NS5B-COOH (10–12). We used an *in vitro* processing system to show that three putative viral structural proteins, p22 (C), gp35 (E1), and gp70 (E2), are processed possibly by a signal peptidase of the host cell (13). We determined that production of p70 (NS3) requires two distinct proteinase activities (14). One of these proteinases, HCV proteinase 1 (Cpro-1), may be a metalloproteinase and is responsible for cleavage at the p21 (NS2)/p70 (NS3) site. The other proteinase is a serine proteinase located in the N-terminal portion of p70 (NS3), named Cpro-2, and cleaves at the C-terminal side of p70 (NS3). Cpro-2 serine proteinase activity appears to be essential for the processing of downstream NS4A, NS4B, NS5A, and NS5B (12, 14–17). However, the molecular mechanism of the proteolytic processing of these proteins is still unknown. With the intention of uncovering that mechanism, we expressed the cloned cDNA fragments encompassing the entire putative NS-encoding region of the HCV genome using a plasmid-based transient expression system in cultured cells and an *in vitro* transcription/translation system.‡

MATERIALS AND METHODS

Construction of an *in Vitro* Expression Plasmid. A cDNA fragment covering the HCV precursor polyprotein region from aa 729 to 3010 was constructed by using overlapping cDNAs reported previously (3, 8, 14) and subcloned into pTZ18U vector to obtain pHCN729-3010. The 3′-terminal deletion mutants pHCN729-2472 and pHCN729-2052 were obtained, respectively, by deleting the 3′-terminal *Bst*EII–*Hin*dIII and *Pst* I–*Hin*dIII fragments from the pHCN729-3010 insert, respectively. For construction of pHCN729-1658, the *Nsi* I–*Hin*dIII fragment of pHCN729-1908 (14) was replaced by a synthetic adaptor made of 5′-TGTCGGCCGACCTGGAGGTCGTCACTAGTA-3′ and 3′-ACGTACAGCCGGCTGGACCTCCAGCAGTGATCATTCGA-5′. The same fragment was replaced with the *Nsi* I–*Hin*dIII fragment of the PCR product from template pHCN729-1908 and primers 5′-TCATGGCATGCATGTCGGC-3′ and 5′-GAAGCTTCTGCATTCCTTGCTC-3′ to yield pHCN729-1723. To obtain a mutant construct, pHCN729-3010M1, without Cpro-2 serine proteinase activity, the *Rsr* II–*Bam*HI fragment of pHCN729-3010 was replaced by the *Rsr* II–*Bam*HI fragment of pS1165A (14).

Construction of *in Vivo* Expression Plasmids. Plasmids pCMV/N729-3010, pCMV/N729-3010M1, pCMV/N729-2472, and pCMV/N729-2052 were made by inserting the *Rsr* II–*Sal* I fragment of pHCN729-3010, pHCN729-3010M1, pHCN729-2472, and pHCN729-2052, respectively, into the *Rsr* II–*Hin*dIII site of pCMV/N729-1908 (14). The *Sal* I sites of the inserts and the *Hin*dIII sites of the vector were blunt-ended prior to ligation.

Construction of Deletion Mutants Fused with the Dihydrofolate Reductase (DHFR) Gene. To fuse the DHFR gene of *Escherichia coli* with a series of HCV ORF deletion constructs, primary constructs pFXdhfr0SH and pFXdhfr2SH were made by inserting adaptors made of 5′-AGCTTGATCGAAGGTCGAGGTAT-3′ and 3′-ACTAGCTTCCAGCTCCATA-5′ and 5′-AGCTTGCAATCGAAGGTCGAGGTAT-3′ and 3′-ACGTTAGCTTCCAGCTCCATA-5′, respectively, into the *Hin*dIII–*Bcl* I site of the pTZSV2dhfr vector (18), followed by inserting a *Sal* I–*Hin*dIII–*Sal* I linker

Abbreviations: HCV, hepatitis C virus; NS protein, nonstructural protein; Cpro, HCV proteinase; MM, microsomal membrane; DHFR, dihydrofolate reductase; α-p70, α-p27, etc., anti-p70, anti-p27, etc.; ORF, open reading frame.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. for the pHCN729-3010 insert, D16435).

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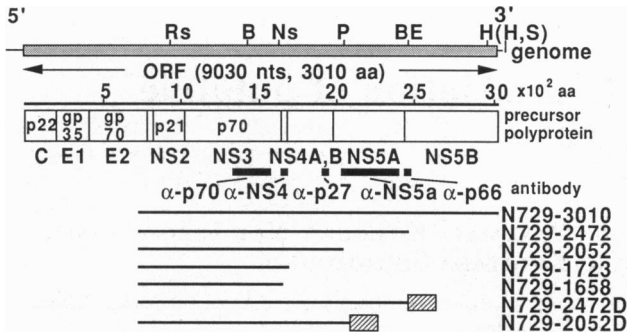

FIG. 1. Schematic diagrams of the map of deletion and fusion constructs. The HCV genomic ORF is represented as a shaded box. HCV precursor polyprotein structure is indicated by boxes with names of processing products (10–17). Hatched boxes indicate the *E. coli* DHFR gene ORF. Target regions for region-specific antibodies are shown by thick bars under the boxes. The cDNA fragments for HCV ORF are shown by bars. The locations of *Rsr* II (R), *Bam*HI (B), *Nsi* I (Ns), *Pst* I (P), *Bst*EII (BE), *Hin*dIII (H), and *Sal* I (S) sites are shown on the shaded box. Restriction sites in parentheses originated from the linker sequence.

(5′-CGTCGACAAGCTTGTCGACG-3′) into the blunt-ended *Bam*HI site of the vector. The *Bcl* I sites of the vector were blunt-ended prior to ligation. The *Hin*dIII fragments of pFXdhfr0SH and pFXdhfr2SH were inserted into the *Hin*dIII sites of pCMV/N729-2052 and pHCN729-2472 to obtain pCMV/N729-2052d and pHCN729-2472d, respectively.

***In Vitro* Transcription/Translation and Immunoprecipitation.** The *in vitro* transcription/translation experiment was performed with a rabbit reticulocyte lysate (Promega) and [^{35}S]methionine (Amersham) as described (13, 14). The translation reaction mixture was suspended in 1 ml of 50 mM Tris·HCl, pH 7.2/150 mM NaCl/0.1% Triton X-100/0.5% sodium deoxycholate [RIPA(-SDS) buffer] for immunoprecipitation analysis using region-specific antibodies as described (14). The *in vitro* translation products were analyzed directly or after immunoprecipitation by SDS/PAGE (14, 19, 20), followed by fluorography.

Transient Expression Assay in COS-1 Cells and Immunoblot Analysis. DNA transfection experiment in COS-1 cells and immunoblot analysis were performed as described (14).

Antibodies. Anti-p70 antibody (α-p70) for detection of HCV p70 was as described elsewhere (14). Polyclonal antibodies, anti-p27 (α-p27), and anti-p66 (α-p66), were raised in rabbits by immunization with synthetic oligopeptides, SRGNHVSPTHYVPESDAAAR and STTSRSASLRQKKVTC, respectively (Fig. 1). Mouse monoclonal antibodies, α-NS4 and α-NS5a, were generously supplied by A. Takamizawa (Osaka University). Anti-DHFR rabbit antibody (α-DHFR) was a generous gift from T. Osumi (Himeji Institute of Technology).

RESULTS

Expression of the HCV Type II Putative NS Protein Region in Cultured COS-1 Cells. The products that were transiently expressed and processed in COS-1 cells transfected with pCMV/N729-3010 were detected by immunoblot analysis with the region-specific antibodies (Fig. 1). p70 (NS3) was detected using α-p70 in this cell lysate, as reported (Fig. 2, lane b) (14). The production of the proteins of 4 kDa (p4) and 66 kDa (p66), which were likely to correspond to NS4A and NS5B, respectively, were specifically detected by α-NS4 and α-p66 (Fig. 2, lanes e and q). With α-NS5a, however, two products of 56 kDa (p56) and 58 kDa (p58) were detected in the lysate (Fig. 2, lane l). All these processed products were not detected in the lysate of COS-1 cells transfected with pCMV/HCN729-3010M1, in which Cpro-2 activity was destroyed by an amino acid substitution (Ser-1165 → Ala) (14) (Fig. 2, lanes c, f, m, and r). These data indicate that production of these products is dependent on the Cpro-2 serine proteinase activity. These results were mostly consistent with the results of others (10–12); the detection of two products by α-NS5a was the only exception.

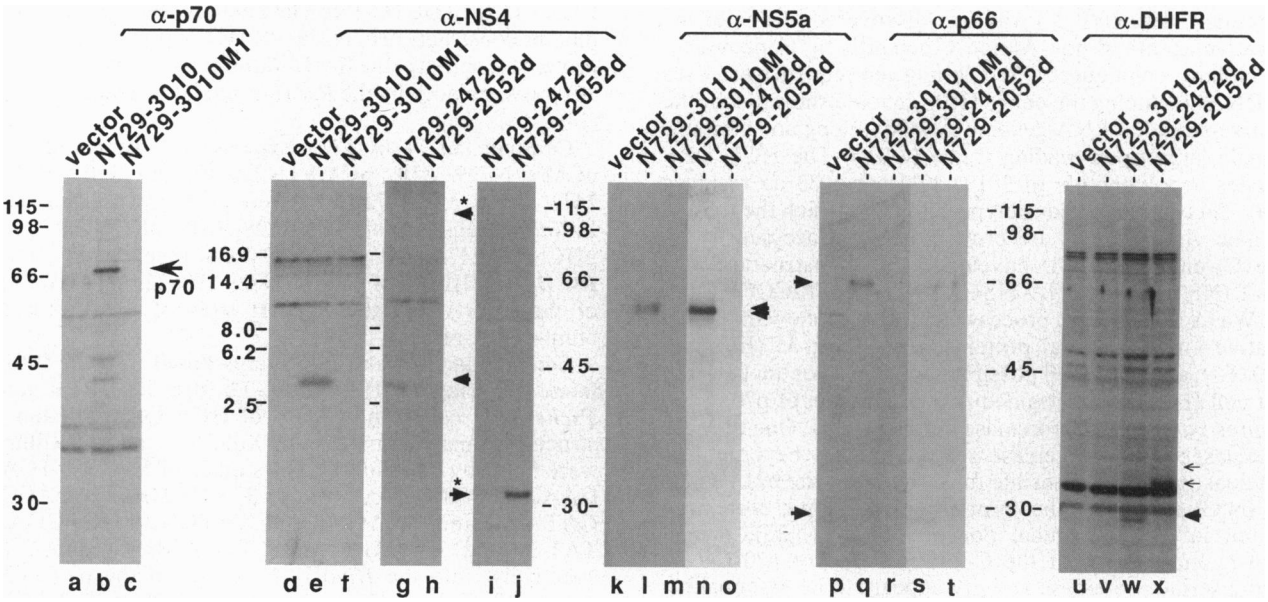

FIG. 2. Immunoblot detection of HCV proteins expressed and processed in COS-1 cells. Expression plasmids pCMV/N729-3010 (lanes b, e, l, q, and v), pCMV/N729-3010M1 (lanes c, f, m, and r), pCMV/N729-2472d (lanes g, i, n, s, and w), and pCMV/N729-2052d (lanes h, j, o, t, and x) were transfected into COS-1 cells. The proteins expressed and processed in the cells were analyzed on an immunoblot using the region-specific antibodies indicated above the lanes; separation was by electrophoresis on an 11% polyacrylamide/SDS gel (lanes a–c and i–x) or a tricine/SDS/16% polyacrylamide gel (lanes d–h) (19). The lysate of cells transfected with expression vector pKS+/CMV (14) was analyzed as a negative control (lanes a, d, k, p, and u). p70 is indicated by the large arrow. Products that are detected specifically by region-specific antibodies and anti-DHFR antibody are indicated by small arrows. Arrows with asterisks indicate an identical product separated in different electrophoresis systems.

Epitope Tag Scanning Analysis. To clarify the difference between p58 and p56, we performed an epitope tag scanning analysis. In this experiment, the DHFR gene of *E. coli* was fused in-frame to the 3′-terminal ends of 3′-terminal deletion constructs of pCMV/N729-3010 (Fig. 1), and DHFR protein was used as the epitope tag for the detection of the C-terminal processed product. We detected p4, p58, and p56 in the lysate of COS-1 cells transfected with pCMV/N729-2472d, in which the C-terminal 538 residues of the HCV precursor polyprotein, corresponding to most of p66, was replaced by DHFR (Fig. 2, lanes g and n). These results indicated that very little p66 is required for HCV polyprotein processing. Instead of p66, a 29-kDa product was detected in this lysate using either α-p66 and α-DHFR (Fig. 2, lanes s and w). In the lysate of COS-1 cells transfected with pCMV/N729-2052d, in which the C-terminal 958 residues of the precursor polyprotein was replaced by DHFR, production of only a small amount of p4 and accumulation of a 31.5-kDa product (p31), which was likely to be a precursor polypeptide of p4 and a product of p27 (see below) (11, 12), was found with α-NS4 (Fig. 2, lanes h and j). Two products of 32 kDa and 34 kDa were detected by α-DHFR in this lysate (Fig. 2, lane x, indicated by small arrows). These results suggest that the difference in size between p58 and p56 is due to a difference in the N-terminal ends of these products. Detection of a small amount of p4 and accumulation of p31 caused by deletion of a large part of p58/p56 suggests that the p58/p56 region is required for efficient cleavage at the p4/p27 site.

Expression of the Putative HCV NS Proteins *in Vitro*. The molecular mechanism of HCV polyprotein processing was examined using an *in vitro* transcription/translation system plus or minus the presence of the microsomal membranes (MMs) and with pHCN729-3010 as a template for the transcription. Furthermore, processed products in the reaction mixture were located by fractionation of the mixture by centrifugation at 7000 × *g* for 5 min. Processed products in both the supernatant and pelleted fractions were detected by immunoprecipitation using region-specific antibodies. In the translation reaction mixture without MMs, all products were present in the supernatant fraction (Fig. 3*A*). Proteins p70 and p31 and a 91-kDa product, which seemed to be a processing intermediate of p70 and p26′ (14), were found in this mixture (Fig. 3*A*, lanes b–f). Although p27 and p56 were not detected, p58 and p66 were detected after prolonged exposure (data not shown). These results indicated that production of p70, p31, p58, and p66 was not dependent on the presence of MMs. Only the unprocessed precursor polypeptide and the degradation products were present in the translation reaction mixture of the Cpro-2 mutant construct, N729-3010M1. These processing events, therefore, with exception of p21 production were likely to depend on Cpro-2 activity (Fig. 3*B*) (14).

Almost all of the antibodies used in this experiment precipitated p70 to some degree (Fig. 3*A*). In immunoblot analysis, p70 was detected only with α-p70 (Fig. 2) and p70 expressed *in vitro* without other NS proteins located downstream of p70 was immunoprecipitated only by α-p70 (Fig. 3*C*, lanes b and c; data not shown). This coprecipitation of p70 with other processing products was probably the result of a preexisting protein–protein association. Similarly, p31 was coprecipitated with p70 by α-p70 (Fig. 3*A*, lanes n and t), supporting this suggestion. Similar results were also observed in the translation reaction mixture in the presence of MMs (Fig. 3*A*). Production of p27 was apparent in the pelleted fraction of this mixture (Fig. 3*A*, lane v), although its precursor, p31, was also observed in the supernatant (Fig. 3*A*, lanes n–p). Therefore, cleavage between p4 and p27 seemed to require the presence of MMs but was less than optimal *in vitro*. Although p4 was not detected by the analysis because of a low content of methionine residues in the

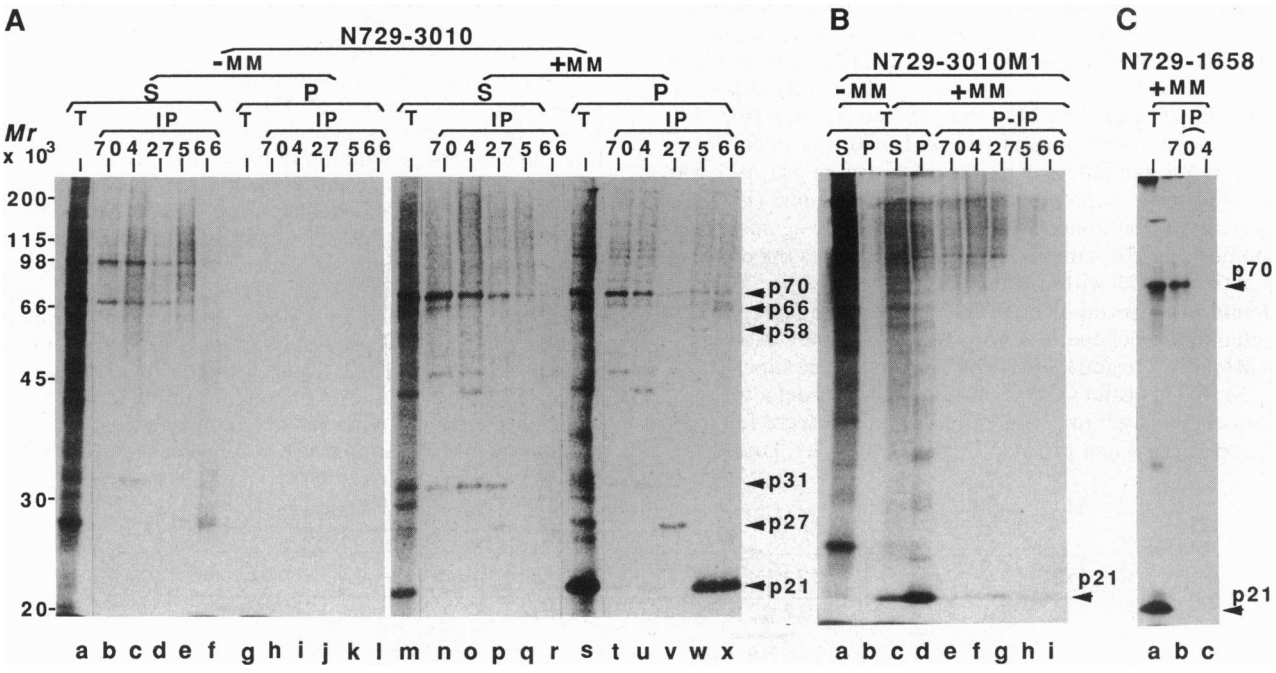

FIG. 3. Immunoprecipitation detection of HCV proteins expressed *in vitro*. RNAs transcribed from pHCN729-3010 (*A*), pHCN729-3010M1 (*B*), and pHCN729-1658 (*C*) *in vitro* were translated in the absence (*A*, lanes a–l, and *B*, lanes a and b) or presence (*A*, lanes m–x, *B*, lanes c–i, and *C*, lanes a–c) of MMs. After fractionation by centrifugation at 7000 × *g*, 5 min, translated products (T) in the supernatant (S) (*A*, lanes a and m, and *B*, lanes a and c) and pelleted fraction (p) (*A*, lanes g and s, and *B*, lanes b and d) and products immunoprecipitated from these fractions with region-specific antibodies (IP) were analyzed by SDS/PAGE on 11% gels and by fluorography. Antibodies used were α-p70 (shown as 70 in *A*, lanes b, h, n, and t, in *B*, lane e, and in *C*, lane b), α-NS4 (shown as 4, in *A*, lanes c, i, o, and u, in *B*, lane f, and in *C*, lane c), α-p27 (shown as 27 in *A*, lanes d, j, p, and v, and in *B*, lane g), α-NS5a (shown as 5 in A, lanes e, k, q, and w, and in *B*, lane h), and α-p66 (shown as 66 in *A*, lanes f, l, r, and x and in *B*, lane i). Processing products p70, p66, p58, p31, p27, and p21 are indicated by arrowheads. Molecular weight markers are shown at the left.

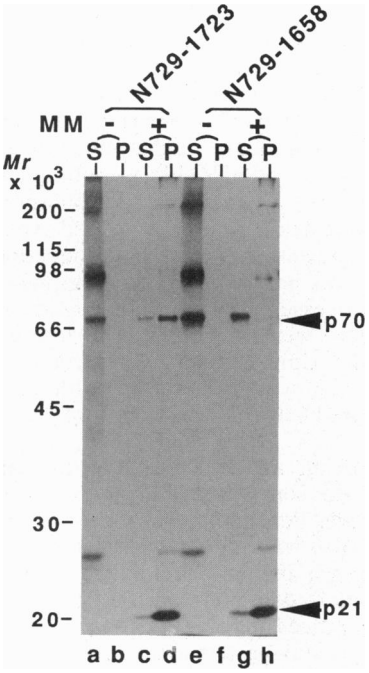

FIG. 4. RNAs transcribed from pHCN729-1723 and pHCN729-1658 *in vitro* were translated in the absence (−; lanes a, b, e, and f) or presence (+; lanes c, d, g, and h) of MMs. After fractionation by centrifugation at 7000 × *g*, 5 min, translated products of the supernatant (S) (lanes a, c, e, and g) and pelleted fraction (P) (lanes b, d, f, and h) were analyzed by SDS/PAGE on 11% polyacrylamide gels and by fluorography. Arrowheads indicate p70 and p21. Molecular weight markers are shown at the left.

corresponding region, this product seemed to be produced in the pelleted fraction. Because p70 in the pelleted fraction was effectively precipitated by α-NS4 (Fig. 3*A*, lanes o and u), despite detection of only a small amount of p31 in this fraction (Fig. 3*A*, lanes t–v). Since p70 was not effectively coprecipitated with p27 using α-p27 in the pelleted fraction (Fig. 3*A*, lane v), close association of p31 with p70 was likely to be caused by interaction through the p4 region in p31. p21 was also found to be coprecipitated with p58 and p66 (Fig. 3*A*, lanes w and x), although p21 was not reactive against these antibodies (Fig. 3*B*, lanes h and i). This indicates the physical association of p21 with p58 and p66.

Membrane Association of HCV Proteins. Although p21 was detected in the pelleted fraction, which is closely associated with MMs (14), residual MMs were present in the supernatant (Fig. 3*A*, lanes m and s). One protein, p66, was detected only in the pelleted fraction, whereas p70 and p58 were found in both supernatant and pelleted fractions (Fig. 3*A*, lanes n, q, t, and w). These results suggested that almost all HCV proteins are, more or less, associated with MMs.

Membrane association of p70 was even seen in the translation reaction mixture of pHCN729-1723, in which the HCV NS region downstream of p4 was deleted (Fig. 4, lane c and d). However, virtually all p70 remained in the supernatant of the pHCN729-1658 translation reaction mixture, in which most of the p4 region was further deleted from the pHCN729-1673 insert (Fig. 4, lanes g and h). These results strongly suggest that membrane association of p70 requires the presence of p4.

DISCUSSION

The processed HCV products we detected were generally consistent with the data generated from other HCV strains in systems using a vaccinia virus vector to express proteins; p21, p70, p4, p27, and p66 corresponded to HCV NS2, NS3, NS4A, NS4B, and NS5B, respectively (Fig. 5) (10–12). However, we detected two additional products, p58 and p56, that corresponded to HCV NS5A. Results of epitope tag scanning analysis indicated that the difference between p58 and p56 was an alteration in the N termini of these products. Only p31, the precursor polypeptide of p4 and p27, was processed from the region just upstream of p58 and p56; therefore, it is possible that p58 is a precursor of p56 and a small polypeptide of ≈2 kDa (tentatively named p2/NS5C). The smaller protein was probably produced from the N-terminal portion of p58 (Fig. 5). This means that p56 may be a processed product corresponding to NS5A as reported by other groups (10–12). Interestingly, p56 was not detected in processed products *in vitro*, whereas in lysates of COS-1 cells cotransfected with pCMV/N729-3010M1 and pCMV/N729-1908, p56 but not p58 was found. This implied that p56 was a trans cleavage product (unpublished results). Production of HCV NS5A in the same manner was recently reported (12). We examined trans cleavage activity of Cpro-2 by *in vitro* cotranslation of transcripts from pHCN729-3010M1 and pHCN729-1908, used as substrate and enzyme constructs, respectively. Production of p31, p58, and p66 was not observed in the reaction mixture (data not shown), suggesting that the efficient production of each viral product, including p58, occurs in a cis-acting manner *in vitro*. Therefore, we consider that the N-terminal sites of p58 and p56 were cleaved in cis-acting and trans-acting manners, respectively. Furthermore, the lack of Cpro-2 *in vitro* trans-cleavage activity suggested that at least processing of p31, p58, and p66 was directly mediated by Cpro-2 and not by an unknown cellular proteinase(s) possibly activated by Cpro-2 in trans.

Recent studies (11, 12, 17) suggested that cleavage of the HCV NS4A/NS4B site is dependent upon an HCV serine proteinase activity. Our results indicated that a complex processing mechanism promoted this cleavage. For efficient

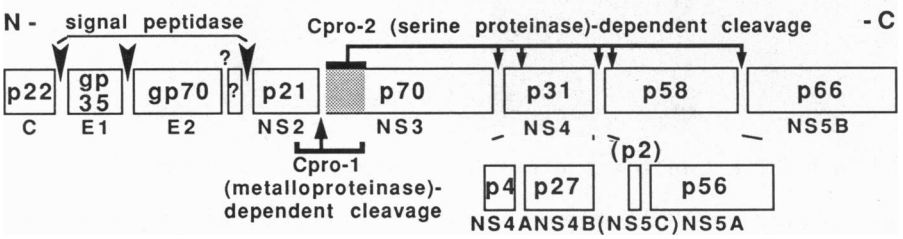

FIG. 5. Summary of proteolytic processing of the HCV precursor polyprotein. Detected or proposed Japanese type HCV proteins are shown by boxes with tentative names. The N- and C-terminal sides of the precursor are shown by N and C on the left and right, respectively. C, E1, and E2 indicate core, envelope 1, and envelope 2, respectively. A box with a question mark is a possible processing product (unpublished results and refs. 10 and 11) and (p2)/(NS5C) is a hypothetical product proposed in this report. The putative Cpro-2 serine proteinase domain is shaded. Cleavages that were proposed to be mediated by the host signal peptidase (13) and by two distinct proteinases, Cpro-1 and Cpro-2 (14), encoded in the HCV genome are indicated by arrowheads and arrows, respectively. The region required for detection of Cpro-1 activity is bracketed below the boxes.

cleavage of this site, the presence of MMs and p58/p56 polypeptides was required. Although the mechanism of participation of NS5A in the processing of NS4AB (p31) was not clear, the N-terminal portion of flaviviral NS5 is also reported to influence the cleavage of the flaviviral NS4A/NS4B site (21, 22). The function of p58 and/or p56 may be to interact with preexisting p31 and change the conformation of this hydrophobic product to a form that is highly accessible to Cpro-2 on endoplasmic reticulum membranes, as suggested in processing of NS4A and NS4B from dengue virus type 2 (23).

All the putative HCV NS proteins processed *in vitro*, except p31, were proposed to be associated with MMs. Half of p70 and half of p58 were also found as membrane-free forms (Fig. 3*A*). These results are partly consistent with subcellular localization of HCV-1 proteins based on expression in cultured cells and indirect immunofluorescence detection (10). Our results differed in that at least parts of HCV NS3 and NS5B were associated with membranes. Immunoprecipitation and deletion analyses indicated that p4 was closely associated with p70 (Fig. 3) and was responsible for the membrane association of p70 (Fig. 4). These results indicated that one aspect of the function of p4 may be to anchor p70 on the surface of the endoplasmic reticulum. Replication of the flaviviral RNA genome is considered to be closely associated with host cell membranes (24, 25). The NS proteins are thought to function in the replication of the viral genome. Immunoprecipitation analysis suggested that the putative HCV NS proteins interacted essentially forming a complex. This complex of associated NS proteins may contain the replicatory enzymatic machinery and roots that hold the genome on the surface of the endoplasmic reticulum during replication.

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