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Two Distinct Proteinase Activities Required for the Processing of a Putative Nonstructural Precursor Protein of Hepatitis C Virus

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Gene products of hepatitis C virus (HCV), a possible major causative agent of posttransfusion non-A, non-B hepatitis, are considered to be produced from a precursor polyprotein via proteolytic processing mediated by either host cell or viral proteinases. The presence of HCV serine proteinase has been proposed from analyses of amino acid sequence homology. To examine the processing mechanism of the HCV precursor polyprotein, the amino-terminal region of the putative nonstructural protein region of the HCV genome, containing the serine proteinase motif, was expressed and analyzed by using an in vitro transcription/translation system and a transient expression system in cultured cells. Two distinct proteinase activities which function in the production of a 70-kDa protein (p70) from the precursor polyprotein were detected. One of these proteinase activities, which cleaved the carboxyl (C)-terminal side of p70, required the presence of the serine proteinase motif, which is located in the amino (N)-terminal region of p70. That suggested that the predicted HCV serine proteinase was functional. The other activity, which was responsible for the cleavage of the N-terminal side of p70, required the expression of the region upstream and downstream of that cleavage site, including the p70 serine proteinase domain. From the results of pulse-chase analysis, using proteinase inhibitors coupled with a point mutation analysis, the latter activity was proposed to be a novel zinc-dependent metalloproteinase.

Infection with the hepatitis C virus (HCV) is reported to be closely related to posttransfusion non-A, non-B hepatitis (10, 27). The HCV genome has recently been cloned and sequenced (11, 23, 32, 33, 38, 39). The positive-strand RNA genome of this virus is composed of about 9,400 nucleotides and includes a single large open reading frame (ORF) encoding a polypeptide of 3010, 3011, or 3033 amino acid (aa) residues (11, 23, 32, 33, 38, 39). Analyses of amino acid sequence homology have revealed that HCV is related to the flaviviruses and the pestiviruses (10, 22, 23, 29, 38), suggesting that the genomic organization of HCV is homologous to those of these two related viruses. Therefore, functional HCV gene products seem to be produced by both cotranslational and posttranslational processing of the large polyprotein precursor, as in the cases of these related viruses (for a review, see reference 35). Using an in vitro processing system, we have shown that three processed products of the putative viral structural proteins, p22, gp35, and gp70, are initially cotranslationally produced from the N-terminal region of the HCV precursor polyprotein, possibly by a host cell signal peptidase (18). However, the processing mechanism of the C-terminal region of the precursor polyprotein has not been clarified. This latter region, which constitutes about 75% of the HCV ORF 3′ terminus, is suggested to encode the nonstructural (NS) proteins that are essential for replication of the HCV genome; it contains conserved amino acid sequence motifs of a chymotrypsin-like serine proteinase, RNA helicase, and RNA-dependent RNA polymerase. The sequence motifs are also found in sequences of flavivirus and pestivirus NS proteins (22, 23, 29). Recently, putative viral serine proteinases were shown to be functional in the proteolytic processing of viral precursor polyproteins by in vitro and in vivo experiments. This is the case in the NS proteins of yellow fever virus and dengue virus type 2 and type 4 (DEN2 and DEN4), all members of the *Flavivirus* genus, and it is also true for bovine viral diarrhea virus, a pestivirus (5, 14, 34, 43); however, the predicted RNA helicase and RNA polymerase activities were not detected. These data strongly suggest that the structure homologous to serine proteinase found in the HCV ORF has enzymatic activity and is responsible for processing the primary HCV gene products.

To determine the molecular mechanism of HCV viral protein production and gene organization, cloned cDNA fragments corresponding to the middle of the genome, in which the putative serine proteinase motif is located, were expressed by using an in vitro transcription/translation system and in cultured cells by using a transient expression system. Two distinct proteinase activities functioning in the production of a 70-kDa polypeptide (p70) were detected. p70 was presumably an HCV homolog of the flaviviral NS3 and the 80-kDa protein (p80) encoded by the bovine viral diarrhea virus genome, which has a serine proteinase domain (2). One of the proteinase activities, which cleaved the C-terminal end of p70, required the alignment of predicted serine proteinase structure in the N-terminal portion of p70. The other, which cleaved the p70 N terminus, is proposed to be zinc dependent and is required the polypeptide region upstream and downstream of the cleavage site, which includes the complete serine proteinase motif.

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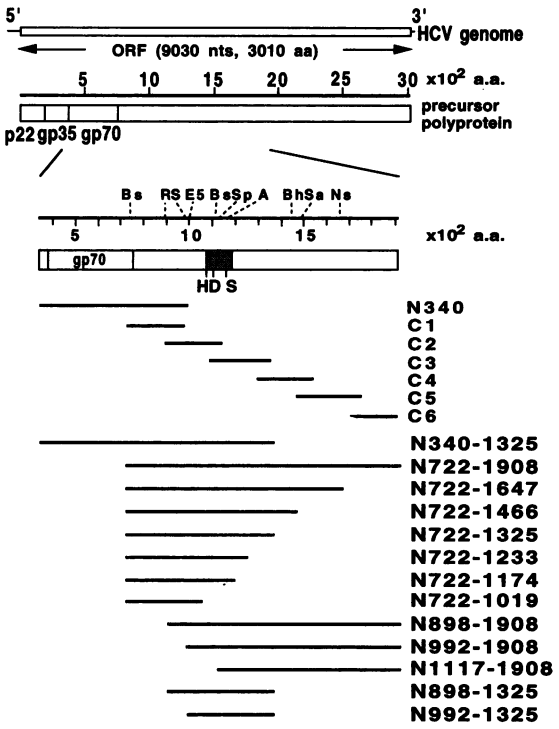

FIG. 1. Constructions of cDNA fragments used in in vitro transcription/translation experiments and regions encoded by these constructs in the HCV ORF. HCV polyprotein precursors are boxed. The p22, gp35, and gp70 are putative initial processing products of the structural protein region of HCV ORF (18). H, D, and S indicate His at aa 1083, Asp at aa 1107, and Ser at aa 1165 of the HCV precursor protein. These residues are predicted to form the catalytic triad of the chymotrypsin-like serine proteinase, and a highly homologous sequence motif has been found in the HCV precursor polyprotein indicated by the shaded box. The cDNA fragments of N340, C1, C2, C3, C4, C5, and C6 were from pN340 (18), clones 6, 7, 8, and 9 (23), and clones 6 and 18 (39), respectively. The locations of *Bss*HII (Bs), *Rsr*II (R), *Sac*II (S), *Avr*II (A), *Eco*52I (E5), *Sph*I (Sp), *Bam*HI (Bh), *Sac*I (Sa), and *Nsi*I (Ns) restriction sites are shown on the scale for the amino acid position.

MATERIALS AND METHODS

Construction of an in vitro expression plasmid. A cDNA fragment covering the HCV ORF region from aa positions 722 to 1908 was constructed with six overlapping cDNAs, C1, C2, C3, C4, C5, and C6 (Fig. 1). These cDNA fragments were from clones 6, 7, 8, and 9 (23) and clones 6 and 18 (39), respectively. A translation initiation codon in the 5'-terminal end of the C1 fragment was constructed by inserting the 12-mer *Nco*I linker (5'-CAGCCATGGCTG-3') (Takara Shuzo) into the *Eco*RI site of pC1, which is a recombinant plasmid carrying the C1 fragment in the *Hin*cII site of the multicloning region of pTZ18U vector (U.S. Biochemicals), after filling in the cohesive ends of the restriction sites with T4 DNA polymerase. The 5'-terminal end of the ORF of the insert in the resultant clone, named pC1N, encoded four extra amino acids (methionine, alanine, glutamic acid, and phenylalanine). The *Eco*RI-*Sph*I fragment of the C2 fragment and the *Sph*I-*Bam*HI fragment of the C3 fragment were subcloned into the *Eco*RI-*Bam*HI site of the pTZ18U vector simultaneously to obtain the pC2-3 clone. When no adequate restriction enzyme site was present in the overlapping region, we used the polymerase chain reaction method for the ligation of the two overlapping fragments (21). The primers used for the ligation reactions were as follows: 5'-TTTTG TACGGGCTCAGGGGCT-3', 5'-TCCATGTCAGAGAAG ACGAC-3', 5'-GCGGTGGCAGTAGAGCCCGTC-3', and 5'-ACCCCTGCTCTCCAAAACTA-3' for ligation of C1 and C2 to give the C1-2 fragment; 5'-GTCGTCTTCTCTGACAT GGA-3', 5'-TGGTAGTCGAGTCAGTTGAGT-3', 5'-ACTC AACTGACTCGACTACCA-3', and 5'-TCAGCGGGCGTG AGCTCATA-3' for ligation of C2-3 and C4 to give the C2-4 fragment; 5'-ACATGTGTCACCCAGACAGT-3', 5'-TCAGCGGGCGTGAGCTCATA-3', 5'-ATGACGCAGGCT GTGCTTGG-3', and 5'-CTGCATTCCTTGCTCGATGT-3' for ligation of C4 and C5 to obtain the C4-5 fragment; 5'-ATGACGCAGGCTGTGCTTGG-3', 5'-CTGCATTCCTT GCTCGATGT-3', 5'-GTTCGATGAGATGGAAGAGT-3', and 5'-CATCCACTGCACAGCCCCCTC-3' for ligation of C5 and C6 to obtain the C5-6 fragment. The *Sph*I-*Hin*dIII fragment of pC1N was replaced by the *Sph*I-*Eco*52I fragment of C1-2 and the *Eco*52I-*Hin*dIII fragment of the pC2 insert to yield pC1-2N. pC1-3N was obtained by replacing the *Bss*HII-*Hin*dIII fragment of pC1-2N with the *Bss*HII-*Hin*dIII fragment of pC2-3. The *Bss*HII-*Bam*HI fragment of pC1-3N was replaced by the *Bss*HII-*Bam*HI fragment of pC2-4 to yield pC1-4N. The *Bam*HI-*Sac*I fragment of pC4-5 and the *Sac*I-*Sma*I fragment of pC5-6 were inserted between *Bam*HI and the blunt-ended *Pst*I sites of pC1-4N to yield pC1-6N. The insert cDNA of pC1-6N encodes the amino acid sequence corresponding to the region from aa 722 to aa 1908 of the HCV precursor protein, which is thus termed pHCN722-1908. The pC1-3N fragment was inserted into the *Rsr*II-*Hin*dIII site of pN340 (18) to yield pHCN340-1325, the insert of which encodes the region from the putative second HCV envelope protein (gp70) region to the serine proteinase motif in the HCV precursor polyprotein.

Construction of the deletion mutants and point mutants. Four 5'-terminal deletion mutants, pHCN729-1908, pHCN898-1908, pHCN992-1908, and pHCN1117-1908, were constructed from pHCN722-1908 by deleting the *Eco*RI-*Bss*HII fragment, the *Eco*RI-*Rsr*II fragment, the *Eco*RI-*Sac*II fragment, and the *Eco*R-*Bss*HII fragment, respectively, and by ligating with 12-mer (CAGCCATGGCTG [for pHCN729-1908, pHCN898-1908, and pHCN1117-1908]) and 8-mer (GC CATGGC [for pHCN992-1908]) *Nco*I linkers after filling in the reaction as described above. The 3'-terminal deletion mutants of pHCN722-1908 were constructed as follows. pHCN722-1647 was constructed by using the *Sac*I-*Nsi*I fragment of pC5-6 instead of the *Sac*I-*Sma*I fragment during the construction of pC1-6N. pHCN722-1466 and pHCN722-1325 were pC1-4N and pC1-3N, respectively, intermediates during construction of pHCN722-1908. pHCN722-1233, pHCN722-1174, and pHCN722-1019 were constructed by deleting the 3'-terminal portion of pHCN722-1325 with exonuclease III after cutting the *Xba*I and *Pst*I sites of the plasmid (17). To obtain pHCN898-1325 and pHCN992-1325, the *Avr*II-*Hin*dIII fragments of pHCN898-1908 and pHCN992-1908 were replaced by the *Avr*II-*Hin*dIII fragment of pHCN722-1325.

The single-amino-acid mutations were introduced into pHCN722-1908 by site-directed mutagenesis methods (21, 26). The mutation primers used, the name of the resultant clones, and the amino acid substitutions were as follows: 5'-CACCAGCGTAGACGGTCCAA-3' was used for production of pH1083A (His-1083 to Ala-1083); 5'-GACGAGGT TCTGGTCTACAT-3' was used for production of pD1107N (Asp-1107 to Asn-1107); and 5'-GTCCACCCGCGGAGCC CTTC-3' was used for production of pS1165A (Ser-1165 to

Ala-1165). The series of mutants pH932A, pH952A, pH1083A, pH1136A, pH1175A, pH1227A, and pH1229A, in which a single histidine residue at a numbered amino acid position was changed to an alanine residue, were produced by using the primers 5'-ATTTGGACATAGGCGCCTCC-3', 5'-TGGAGTAAGAGCGTCATATAC-3', 5'-CACCAGCGT AGACGGTCCA-3', 5'-GACATCGGCAGCCCTCGTGA-3', 5'-ATGCCTACAACGGCCCCCGA-3', 5'-AGCGTGTAAA GCTGCCACTTG-3, and 5'-AGTGGGAGCGGCTAAATG TG-3', respectively. The series of mutants pE972Q, pE980Q, pE1009Q, pE1058Q, pE1199Q, and pE1202Q, in which a single glutamic acid residue at a numbered amino acid position was changed to a glutamine residue, were produced by using the primers 5'-AAGACGACGGGCTG TACTGC-3', 5'-GTTTAGTCTGCATGTCAGAG-3', 5'-GA AGTATCTGCTTCCCCCTT-3', 5'-ACCTGAACCTGCCC ATCGAC-3', 5'-TCCATAGACTGAACGGGTAT-3', and 5'-ATGGTAGTTTGCATAGACTC-3', respectively. The series of mutants pC922A, pC993A, pC1123A, pC1125A, pC1171A, and pC1185A, in which a single cysteine residue at a numbered amino acid position was changed to an alanine residue, were produced by using the primers 5'-ATCAA CATGGCTGCACGGATG-3', 5'-ATGATGTCCCCAGCCG CCGC-3', 5'-CCGCAGGTGGCCGGTGTCAT-3', 5'-GAGC TGCCGGCGGTGGCCGGT-3', 5'-AAGGGGCAAGCAGT GGTCCA-3', and 5'-GGTGGCAGCAGCAGCCCGGAAG AT-3', respectively. The substituted nucleotide sequences of all of the clones obtained were verified by sequencing.

Construction of in vivo expression plasmids. For expression of HCV proteins in vivo, a eukaryotic cell expression plasmid bearing the enhancer and promoter of the human cytomegalovirus immediate early gene was constructed as follows. The 8-mer *Bam*HI-linker was inserted into the *Hin*dIII site of pCMVcat (15) after *Hin*dIII digestion and a filling-in reaction. The *Xba*I-*Bam*HI fragment, containing the human cytomegalovirus enhancer-promoter, was inserted into the *Xba*I-*Bam*HI sites of pBluescript II KS(+) plasmid (Stratagene), and then the *Hin*cII-*Xho*I fragment of the multicloning site of the plasmid was replaced by a *Bam*HI-*Sal*I fragment containing the simian virus 40 polyadenylation signal taken from pSVL (Pharmacia) after filling in of the *Bam*HI site. The resulting expression plasmid was named pKS(+)/CMV. The *Eco*RI-*Hin*dIII fragments of pHCN729-1908, pS1165A, and pH952A were inserted into the *Eco*RI-*Hin*dIII site of pKS(+)/CMV to construct pCMV/HCN729-1908, pCMV/S1165A, and pCMV/H952A, respectively.

Antibody. A polyclonal antibody to the HCV p70 product was raised in rabbits by immunization with the HCV viral protein 07, which was expressed in a fused form with β-galactosidase in *Escherichia coli* (31) and was purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretic elution.

In vitro transcription/translation and immunoprecipitation. In vitro transcription/translation was performed with T7 RNA polymerase, a rabbit reticulocyte lysate, a canine pancreatic microsomal membrane fraction (Promega), and [^{35}S]methionine (Amersham), as described previously (18). All in vitro expression plasmids were linearized by cleavage of the unique *Hin*dIII site, located at the 3' ends of the inserts, before being used in in vitro transcription. After in vitro translation at 30°C for 90 min, part of the translation product was treated with proteinase K (200 μg/ml) to determine the location of the product in the lysate containing microsomal membranes, as described previously (18). In some cases, the translation product was suspended in 1 ml of 50 mM Tris (pH 7.2)–150 mM NaCl–1% Triton X-100–1% sodium deoxycholate–0.1% SDS (RIPA buffer), and the suspension was incubated with anti-p70 antibodies for 1 h. The immune complex was precipitated with Pansorbin (Calbiochem). The immunoprecipitates was washed three times with RIPA buffer and suspended in 2× sample buffer (28). The in vitro translation products were analyzed directly or after immunoprecipitation by SDS-PAGE (19, 28, 36) and then by fluorography.

Proteinase inhibitor study. The effects of various proteinase inhibitors were studied by their addition to the in vitro translation mixture during the chase period of pulse-chase analysis. The in vitro transcripts from pHCN722-1325 were translated in vitro during a pulse period of 30 min at 30°C in the presence of [^{35}S]methionine. The chase period was started by the addition of cycloheximide (1 mg/ml) and cold methionine (1 mM) for termination of the translation reaction in the presence or absence of a proteinase inhibitor. The proteinase inhibitors used were aprotinin (10 μg/ml), [(4-amidinophenyl)-methane sulfonyl fluoride] (APMSF) (0.2 mg/ml), E64 (1 mg/ml), bestatin (50 μg/ml), pepstatin (0.7 μg/ml), EDTA (2 mM), antipain (0.2 mg/ml), chymostatin (0.2 mg/ml), and phosphoramidon (0.2 mg/ml). All of these proteinase inhibitors were purchased from Boehringer Mannheim. The proteinase (Cpro-1) activity was measured as an increase in the amount of p21 radioactivity during the chase period by using an imaging plate and BAS2000 (Fuji Photo Film Co.) (1), because the production of p21 is dependent on Cpro-1 activity and because it was one of the most stable of the in vitro translation products of the HCV ORF (see Results).

Transient expression assay and immunoblotting. COS-1 cells and Huh-7 cells were transfected by the calcium phosphate coprecipitation method (7). Samples of the expression plasmids (10 μg) were transfected into 4×10^5 cells in 60-mm dishes. Cells were harvested 48 h after transfection and resuspended in 2× sample buffer (28). Samples were fractionated on SDS-11% PAGE, and the proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Millipore; Immobilon). After incubation for 1 h at 25°C in 150 mM sodium phosphate (pH 7.2) with 3% bovine serum albumin for blocking, the membranes were incubated in the same buffer containing anti-p70 antibodies for 2 h at 25°C and then washed extensively with phosphate-buffered saline containing 0.05% Tween 20. The antibodies bound to the membranes were detected with biotinylated goat anti-rabbit immunoglobulin G antibody and a Vectastain ABC kit (Vector Laboratory) under the conditions specified by the supplier.

Nucleotide sequence accession number. The sequence of the cDNA of pHCN722-1908 has been deposited in the DDBJ/EMBL/GenBank DNA data bases under accession number D11397.

RESULTS

Detection of viral protein processing in in vitro transcription/translation products. To detect HCV polyprotein processing and processed viral products, the polypeptide of HCV precursor protein from residue 340 to residue 1325 (N340-1325) (Fig. 1), was expressed in an in vitro transcription/translation system with pHCN340-1325 as the template for the transcription. This region of the precursor protein includes the amino acid sequences of the putative HCV envelope protein, gp70 (18), and the predicted serine proteinase. Previously, two products, gp70 and p19, were

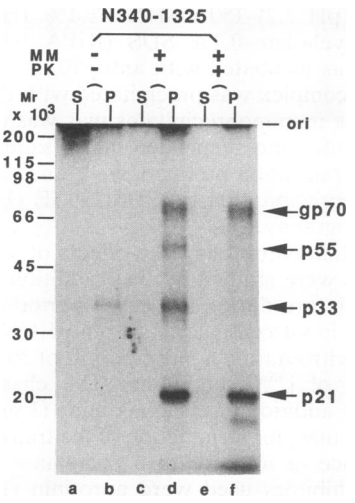

FIG. 2. Detection of proteinase activity cleaves in vitro translation product. RNA transcribed from pN340-1325 in vitro was translated in the absence (lanes a and b) or presence (lanes c, d, e, and f) of microsomal membranes (MM). After translation in the presence of microsomal membranes, the translate was incubated with proteinase K (PK) (lane f). The supernatant (S) (lanes a, c, and e) and particulate (P) (lanes b, d, and f) fractions of reaction mixtures obtained by centrifugation were analyzed. The amount of supernatant analyzed was equivalent to one-fifth of the particulate fraction. The position of the origin on the SDS-polyacrylamide gel is indicated by ori. Molecular size markers are shown on the left.

produced in a microsomal membrane-dependent manner from the region spanning residues 340 to 980 (pN340) (18). In addition to gp70, three other polypeptides with molecular masses of 55, 33, and 21 kDa made by N340-1325 in the presence of microsomal membranes (Fig. 2, lane d) were detected, and we named these products p55, p33, and p21, respectively. Product p55 was revealed to be a precursor of p33 and p21 by deletion mutant analysis (see Fig. 4A, lane h) and by pulse-chase analysis (see Fig. 6A). Since gp70 is already known to be produced from the N-terminal half of this polypeptide (18), p21 and p33 were likely to be produced from its C-terminal half. Production of p33 occurred even in the absence of microsomal membranes (Fig. 2, lanes a and b). This result suggested the possibility that the translated polypeptide bears the proteinase activity which excises p33 from the precursor polypeptide, because no well-known site-specific proteinase activity was present in the reticulocyte lysate. It is supposed that p33 is located in the C-terminal region of the translated polypeptide. The production of p21 is known to require microsomal membranes. Therefore, if p33 is located on the N-terminal side of p21, it would also be expected to require microsomal membranes for its production. The production of p33 in the reaction in the absence of microsomal membranes suggested that these three processed products were likely to be arranged as follows: NH_2-gp70-p21-p33-COOH. The order of the arrangement of these products was further confirmed by deletion analysis as described below. Translation products produced in the reaction in the absence of microsomal membranes seemed to be stacked at the origin of the polyacrylamide gel, as previously reported (18) (Fig. 2, lane b). Unprocessed and unglycosylated polypeptides, including the gp70 region, especially its C-terminal hydrophobic region, were difficult to separate by the gel electrophoresis systems used in our experiment (data not shown). The weak p33 signal in lane b of Fig. 2 may have been caused by the coexistence of an aberrant form of unprocessed gp70 region. Such a difference was not observed in the cleavage reaction products of N722-1325, with most of the gp70 region deleted, as shown below (see Fig. 4A, lanes g and h).

The production of p21 required the presence of microsomal membranes (Fig. 2, lane d). Cleavage of the region next to the gp70 C terminus yielded p19 (18), which was a C-terminal truncated form of p21. The supernatant and particulate fractions of the translation reaction mixture contained p33; however, p21, like gp70, was present in only the particulate fraction. Resistance to proteinase K added after the translation reaction (Fig. 2, lane f) suggested that p21 associated with microsomal membranes. As reported previously, p19 was sensitive to proteinase K treatment under the same experimental conditions (18). Several other C-terminal deletion mutants of p21 showed different sensitivities to proteinase K digestion under the same experimental conditions (data not shown). Proteins located in the lumen of microsomal membranes are known to be fully resistant to proteinase K treatment. The results obtained in the present study, together with the above-mentioned unpublished data, imply that p21 is not located in the lumen of the microsomal membranes. More probably, the intact form of p21 may be buried in the lipid bilayer of microsomal membranes, in which it would be protected from proteinase K digestion.

Gene mapping of the N-terminal portion of the NS protein region of the HCV ORF. To assess the molecular nature of HCV proteinase, residues 722 to 1908 of the HCV precursor polyprotein and its N-terminal and C-terminal deletion mutants were expressed in an in vitro transcription/translation system. The template plasmids used for in vitro transcription were pHCN722-1908, pHCN722-1647, pHCN722-1466, pHCN722-1325, pHCN722-1233, pHCN722-1174, pHCN 722-1019, pHCN898-1908, pHCN992-1908, and pHCN1117-1908 (Fig. 1). Two major products with molecular masses of 70 and 21 kDa and seven minor products with molecular masses of 125, 100, 91, 44, 29, 26, and 24 kDa were detected in the cleavage reaction products of N722-1908 in the presence of microsomal membranes and were named p70, p21, p125, p100, p91, p44, p29, p26, and p24, respectively (Fig. 3A, lane c). The products detected from this cleavage reaction in the absence of the microsomal membranes were the same as those detected in the presence of the microsomal membranes, except that p26 increased and p21 disappeared (Fig. 3A, lane a). These observations indicated that the main proteolytic processing events in this reaction occurred in a microsomal membrane-independent manner. From the calculated molecular weight of the expressed polypeptide, p125 seemed to be an intermediate that was processed in the N-terminal small portion of the precursor only in the presence of microsomal membranes. A product slightly larger than p125, possibly the unprocessed precursor, was found in the cleavage reaction products in the absence of microsomal membranes (Fig. 3A, lane a). All of the other minor polypeptides except p26 (shown in lanes a and c of Fig. 3A) are likely to represent intermediates, artificially processed products, or degradation products (25), because they decreased during prolonged incubation. This contrasted with p21, p26, and p70, which accumulated (data not shown) and were likely to be the processed products, although p26 was detected as a faint, smeared band. The precursor N722-1647, which lacked the C-terminal 261 aa residues of the N722-1908 polypeptide, still yielded p21 and p70 after cleavage, although p26 was not detected (Fig. 3B, lane c; Fig. 4A, lane

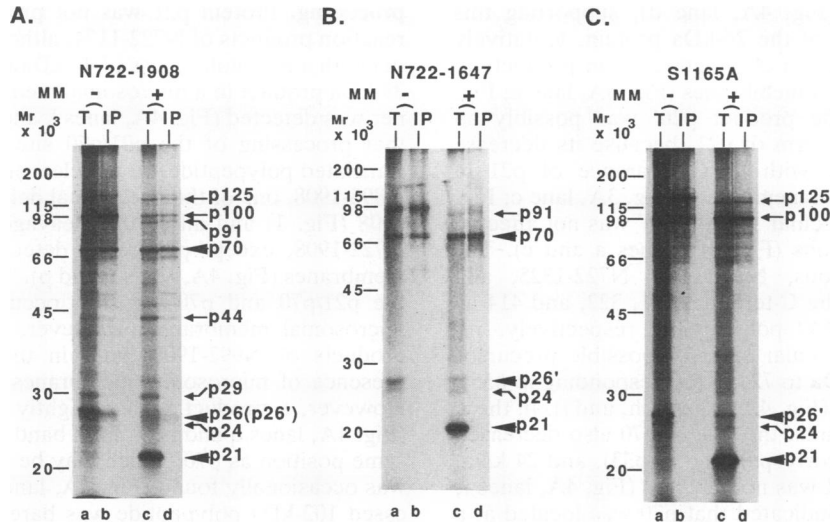

FIG. 3. Detection of processed products of HCV polypeptides expressed in vitro by immunoprecipitation analysis. RNAs transcribed from pN722-1908 (A), pN722-1647 (B) and pS1165A (C) in vitro were translated in the absence (lanes a and b) or presence (lanes c and d) of microsomal membranes (MM). Translated products (T) (lanes a and c) and products immunoprecipitated (IP) (lanes b and d) with antibody prepared against the polypeptide from residue 1308 to residue 1533 of HCV precursor (anti-p70 antibody) (see text) were analyzed by electrophoresis on an SDS-11% polyacrylamide gel. The major processing products, p70, p26, and p21, are indicated by arrowheads. Proteins precipitated with the antibody are identified at the right. The possible processing intermediates or artificially processed products are indicated by arrows (see Results). The sizes of protein molecular mass markers are indicated at the left.

d). This result suggests that p26 originated from the C-terminal region of the N722-1908 polypeptide. The cleavage reaction products of N722-1647 lacked p100, and p91 was found as the largest precursor protein (Fig. 3B, lane c, and Fig. 4A, lane d). These data suggested that p100 is an intermediate form of p70 and p26 and that p91 is an intermediate composed of p70 and p21. The sizes of p21 and p70 were unchanged in the C-terminal deletion mutant, N722-

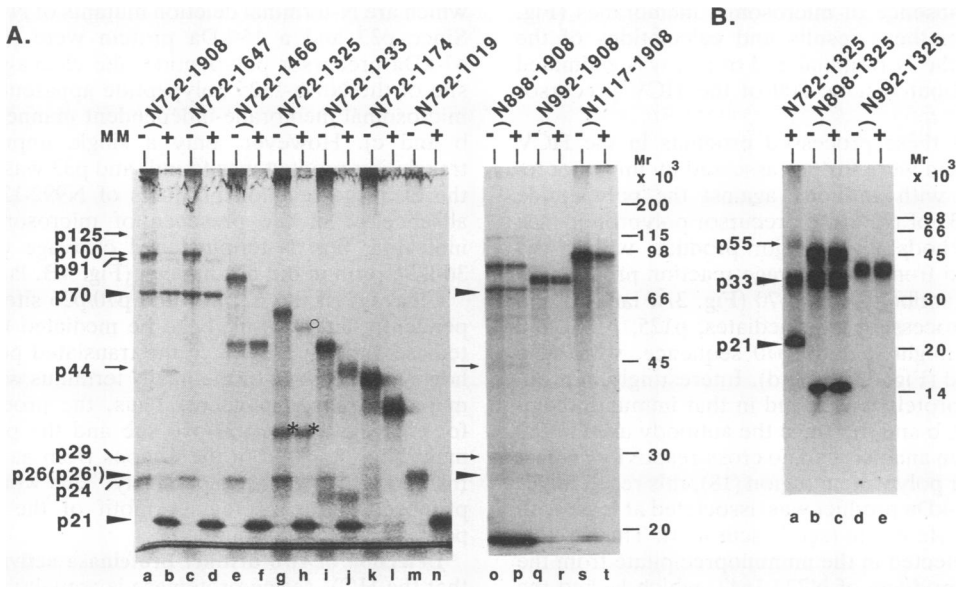

FIG. 4. Gene mapping of the N-terminal region of the putative NS protein-encoding region of the HCV genome. (A) Series of C-terminal (lanes c, d, e, f, g, h, i, j, k, l, m, and n) and N-terminal (lanes o, p, q, r, s, and t) deletion mutant constructs of pN722-1908 (lanes a and b) were used as templates for the in vitro transcription, and the resultant RNAs were translated in the absence (lanes a, c, e, g, i, k, and m) or presence (lanes b, d, f, h, j, l, and n) of microsomal membranes (MM). (B) RNAs transcribed in vitro with the N-terminal deletion mutant constructs of pN722-1325 (lane a), pN898-1325 (lanes b and c), and pN992-1325 (lanes d and e) as templates were translated in the absence (lanes b and d) or presence (lanes a, c, and e) of microsomal membranes. Translation products were analyzed by electrophoresis on an SDS 11%-polyacrylamide gel (A) (28) or a tricine-SDS-16% polyacrylamide gel (B) (19, 36). The products detected in the cleavage reaction of N722-1908 in the presence of microsomal membranes are marked by arrows or arrowheads, with their names on the left. The p55 and p33 shown in Fig. 2 are indicated by an open circle and asterisks, respectively.

1647 (Fig. 3B, lane c; Fig. 4A, lane d), supporting this possibility. The product of the 26-kDa protein, tentatively named p26′, was detected in cleavage reaction products in the absence of microsomal membranes (Fig. 3A, lane a; Fig. 3B, lanes a and c). The product p26′ was possibly an N-terminal unprocessed form of p21, because its decrease occurred simultaneously with the appearance of p21 in reactions with microsomal membranes (Fig. 3A, lane c; Fig. 3B, lane c), and the molecular size of p26′ was not affected by the C-terminal deletions (Fig. 3B, lanes a and c). The other C-terminal deletions, N722-1466, N722-1325, and N722-1233, which lack the C-terminal 181, 322, and 414 aa residues of the N722-1647 polypeptide, respectively, resulted in decreased molecular sizes of possible precursor polypeptides, from 91 kDa to 72, 55 (corresponding to p55), and 45 kDa, as expected (Fig. 4A, lanes f, h, and j). In these C-terminal deletion mutants, the size of p70 also decreased from 70 kDa to 50, 33 (corresponding to p33), and 24 kDa, but the production of p21 was not affected (Fig. 4A, lanes f, h, and j). These results indicated that p70 was located at a region downstream of p21 in the HCV precursor polyprotein and that p33 is a C-terminal truncated form of p70, as suggested above. Each molecular size lost in the precursor polypeptides and derivatives of p70 by the C-terminal deletions of the N722-1647 polypeptide was in good agreement with that calculated from the deleted amino acid sequence of each deletion mutant. Therefore, the cleavage site between p70 and p26 (p70/p26) seemed to be located at around aa residue 1650 in the HCV precursor polyprotein. The cleavage site of p21/p70 was estimated to be at about residue 1020 of the HCV precursor polyprotein, because a 21-kDa polypeptide was produced in the cleavage reaction products of N722-1019 in the presence of microsomal membranes (Fig. 4A, lane n). The product with a size almost similar to that of p26′ was observed in the translation products of N722-1019 in the absence of microsomal membranes (Fig. 4A, lane m). From these results and calculations of the molecular weight, the N-terminal end of p21 was estimated to be located at about residue 800 of the HCV precursor polyprotein.

The locations of these processed products in the HCV precursor polyprotein were further assessed by immunoprecipitation analysis with antibody against the polypeptide from aa 1308 to 1533 of the HCV precursor polyprotein (see Materials and Methods). The major product, which was immunoprecipitated from the cleavage reaction products of N722-1908 by this antibody, was p70 (Fig. 3A, lanes b and d). The possible processing intermediates, p125, p100, and p91, all of which might include p70 sequence, were also immunoprecipitated (Fig. 3A, lane d). Interestingly, a product of the 26-kDa protein was found in that immunoprecipitate (Fig. 3A, lanes b and d). Since the antibody used in the immunoprecipitation analysis had no cross-reactivity against any other precursor polyprotein region (18), this result might indicate that the 26-kDa product was associated at least with p70 in the reticulocyte lysate (see Discussion). This 26-kDa product was not detected in the immunoprecipitate from the cleavage reaction products of N722-1647, which lacked the C-terminal 261 aa residues of the N722-1908 polypeptide, indicating that p26 was mapped in the deleted C-terminal region of the N722-1908 polypeptide (Fig. 3B, lanes b and d). From these results, we concluded that the major processed products from N722-1908 are p21, p70, and p26 and that these products were arranged in the order NH_2-p21-p70-p26-COOH, as summarized in Fig. 8.

Detection of the region required for HCV polyprotein processing. Protein p21 was not produced in the cleavage reaction products of N722-1174, although a translation product with a molecular mass of 44 kDa that was processed to a 39-kDa product in a microsomal membrane-dependent manner was detected (Fig. 4A, lanes k and l). This result implied that processing of the p21/p70 site did not occur in this translated polypeptide. In the cleavage reaction products of N898-1908, one of the N-terminal deletion mutants of N722-1908 (Fig. 1) and all of the cleavage reaction products of N722-1908, except p21, were detected in the absence of membranes (Fig. 4A, lanes o and p). Thus, cleavages at both the p21/p70 and p70/p26 sites occurred independently of microsomal membranes. However, the cleavage reaction products of N992-1908, both in the absence and in the presence of microsomal membranes, did not contain p70. However, a product with a slightly larger size was found (Fig. 4A, lanes q and r). A faint band migrating at nearly the same position as p70, which may be a degradation product, was occasionally found (Fig. 4A, lane q). Since the unprocessed 102-kDa polypeptide was barely detected, and since p26 was found in the N992-1908 cleavage reaction products, cleavage of the p70/p26 site apparently occurred. Therefore, it was concluded that cleavage of the p21/p70 site did not occur in the N992-1908 polypeptide and that consequently a product composed of the C-terminal portion of p21 plus p70 migrated slightly slower, to a position of 70 kDa. Furthermore, only a single unprocessed polypeptide of the 90-kDa protein was found in the N1117-1908 cleavage reaction products, in which half of the serine proteinase motif was deleted (Fig. 4A, lanes s and t), which suggested that the serine proteinase motif present in the HCV ORF was functional and was required at least for p70/p26 site cleavage.

The loss of the p21/p70 site cleavage in this N-terminal deletion mutant was further supported by the results from translations of the N898-1325 and N992-1325 polypeptides, which are N-terminal deletion mutants of N722-1325 (Fig. 1). Since p33 and a 15-kDa protein were produced from a 47-kDa precursor polypeptide, the cleavage at the p21/p70 site of the N898-1325 polypeptide apparently occurred in a microsomal membrane-independent manner (Fig. 4B, lanes b and c). However, only a single unprocessed 36-kDa translation product was found, and p33 was not produced in the cleavage reaction products of N992-1325 either in the absence or in the presence of microsomal membranes, indicating that N-terminal end cleavage of p33 from the 36-kDa protein did not happen (Fig. 4B, lanes d and e).

Cleavage of the p21/p70 and p70/p26 sites occurred independently and was likely to be mediated by the viral proteinase activity present in the translated polypeptide itself; however, cleavage of the p21 N terminus was only microsomal-membrane dependent. Thus, the processing activities for cleavage of the p21/p70 site and the p70/p26 site were most likely located in the regions from aa 898 to 1233 and from aa 992 to 1908, respectively. This would be within the proposed serine proteinase motif of the HCV precursor polyprotein (see Discussion).

Detection of two distinct proteinase activities. To confirm that the HCV serine proteinase is actually functional and is required for processing of viral proteins, the processing potentials of three point mutants derived from N722-1908 were analyzed (Fig. 5). The point mutants used were pH1083A, pD1107N, and pS1165A, in which His-1083, Asp-1107, and Ser-1165, which were presumed to form the catalytic triad of the serine proteinase on the basis of amino acid sequence homology with other chymotrypsin-like serine proteinases (22), were independently replaced by Ala-1083,

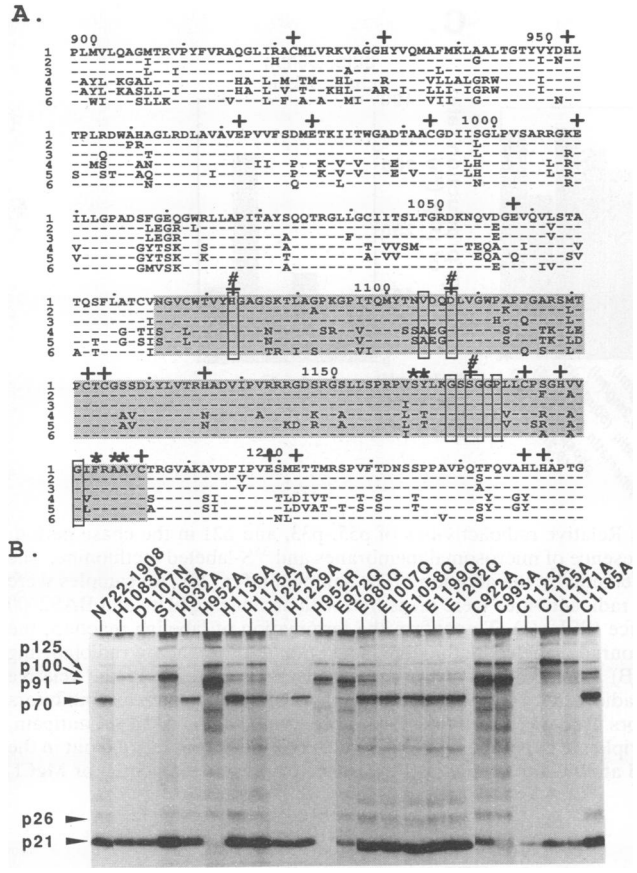

FIG. 5. Analysis for proteinases Cpro-1 and Cpro-2 with mutant constructs. (A) The amino acid sequence (line 1) of the region from residue 898 to residue 1233 of the HCV precursor polyprotein, in which Cpro-1 activity was detected, is aligned with those of the corresponding regions of other reported HCV clones, HCV-BK (line 2) (38), HCV-TW (line 3) (8), HC-J6 (line 4) (33), HC-J8 (line 5) (32), and HCV-US (line 6) (11). Standard one-letter amino acid abbreviations are used. Hyphens indicate amino acid residues identical to those derived from the N722-1908 sequence at the same positions. Predicted domains homologous with that of chymotrypsin-like serine proteinase are shaded (22). Conserved residues in the serine proteinase motifs of HCV, flaviviruses, and pestiviruses are boxed. #, the residues predicted to form a catalytic triad, histidine 1083, aspartic acid 1107, and serine 1165; *, residues predicted to contribute to substrate binding; +, histidine, glutamic acid, and cystein residues that are relatively conserved in these clones and were independently substituted. (B) RNAs from each of the 22 templates, which encoded a single-amino-acid mutant polypeptide of N722-1908, were translated in the presence of microsomal membranes. The cleavage reaction products were analyzed by electrophoresis on an SDS-11% polyacrylamide gel. Intermediates and products are indicated by arrows and arrowheads, respectively, on the left.

Asn-1107, and Ala-1165, respectively. The processing patterns of the cleavage reaction products of these mutants were identical. Products p91, p70, and p26 were not found in these cleavage reaction products, but the accumulation of p100 and p21 was observed (Fig. 5B). These results suggested that the proteinase activity required for cleavage of the p70/p26 site was lost in these mutants. Six other independent point mutations at amino acid residues in the putative serine proteinase domain carried in mutants H1136A, H1175A, C1123A, C1125A, C1171A, and C1185A did not essentially affect the cleavage at the p70/p26 site. Some reduction in cleavage activity was found in three of the four mutants of cysteine residues as described below. To confirm that the p70/p26 site cleavage did not occur in the mutant S1165A, processing of the mutant polypeptide was further examined by immunoprecipitation analysis with the anti-p70 antibody (see Materials and Methods). The major products immunoprecipitated from the S1165A cleavage reaction by this antibody were p125 and p100 (Fig. 3C, lane d). However, p26 was not detected in that immunoprecipitate in either the absence or the presence of microsomal membranes (Fig. 3C, lanes b and d). These data confirm that the serine proteinase identified from the HCV ORF was actually functional and responsible for processing of the C-terminal end of p70. Cleavage of the p21/p70 site, however, does not require the serine proteinase motif but the presence of this region as described above. Therefore, these proteinases, which cleave the p21/p70 site and the p70/p26 site, were tentatively named HCV proteinase 1 (Cpro-1) and HCV proteinase 2 (Cpro-2), respectively.

Characterization of Cpro-1 activity. The amino acid sequence of the Cpro-1 region from aa 898 to 1233 did not show homology to any known proteinase structures except the serine proteinase motif. Therefore, Cpro-1 was characterized by testing the effects of known proteinase inhibitors on its activity during pulse-chase analysis of in vitro translations (Fig. 6). During the chase period, the amount of radioactivity of p21 in the N722-1325 pulse-labelled translation products increased, while those of p55 and p33 gradually decreased (Fig. 6A). Because the translation was terminated by the addition of cycloheximide and unlabelled methionine to the reaction mixture, the accumulation of p21 during the chase period implied that p55 had been processed. An essentially identical result was seen during pulse-chase analysis in the absence of microsomal membranes, in which an increasing amount of radioactive p26′ instead of that of p21 during the chase period was detected (data not shown). Product p21 should be stable in this reaction mixture, because this product was resistant to exogenous proteinase K treatment, as mentioned above (Fig. 2, lane f). However, p33, the C terminally truncated form of p70, might be unstable and thus degraded in the reticulocyte lysate during the chase period. Because the pattern of p26′ increase was not linear with time, in contrast to that of p21 (data not shown), p26′ was also likely to be slightly degraded during the chase period. Therefore, Cpro-1 activity was measured by counting the increase in the amount of relative radioactivity in p21 during the chase period. As shown in Fig. 6B, when the Cpro-1 activity in the absence of any proteinase inhibitor is considered to be 100%, it was found that the production of p21 was suppressed to 22% of the control level only in the presence of EDTA (2 mM). Other proteinase inhibitors had no significant effect on p21 production. The inhibition of Cpro-1 activity by EDTA was decreased by the addition of $MgCl_2$ or $ZnCl_2$ (2 mM) (Fig. 6C). The Cpro-1 activity was increased to three times the control level by the addition of $ZnCl_2$ but not of $MgCl_2$ (Fig. 6C). Other metal ions, Ni^{2+} and Co^{2+}, also had no significant effects on Cpro-1 activity (data not shown). Furthermore, the amount of radioactive p33, which decreased rather gradually without the addition of $ZnCl_2$ as shown in Fig. 6A, was also observed to be increased by the addition of $ZnCl_2$ (data not shown). These results indicated that production of p21 during pulse-chase analysis was due to a site-specific cleavage reaction rather than to degradation of the precursor polypeptide and suggested that Cpro-1 is a zinc-dependent metalloproteinase.

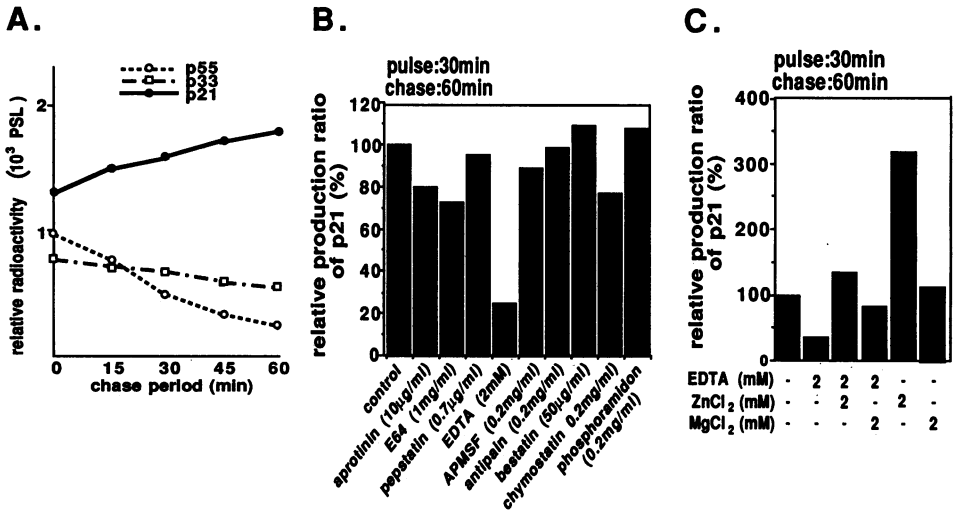

FIG. 6. Effects of various proteinase inhibitors on Cpro-1 activity. (A) Relative radioactivities of p55, p33, and p21 in the chase period. RNA transcribed from pN722-1325 in vitro was translated in vitro in the presence of microsomal membranes and ^{35}S-labeled methionine. The chase reaction was terminated after incubation at 30°C for 15, 30, 45, and 60 min by the addition of 2× sample buffer (28). All samples were separated by electrophoresis on an SDS-12% polyacrylamide gel. Relative radioactivities were measured with an imaging plate and BAS2000 (Fuji Photo Film Co.) and are represented as photostimulated luminescence (PSL) (1). To confirm the termination of labeling reaction, the translation reaction was carried out in the presence of cycloheximide and nonradiolabeled methionine in addition to the standard radiolabeling condition. Open circles, p55; open squares, p33; closed circles, p21. (B) Cpro-1 activity was measured as an increase in the relative radioactivity of p21 during the 60-min chase. The increase in the relative radioactivity of p21 in the control without any proteinase inhibitors was arbitrarily designated as 100% Cpro-1 activity. The proteinase inhibitors used were aprotinin, E64, pepstatin, EDTA, APMSF, antipain, bestatin, chymostatin, and phosphoramidon. Data are average values for triplicate experiments. (C) The chase reaction was carried out in the absence (−) or presence (+) of EDTA. Reaction mixtures were incubated at 30°C for 60 min in the absence or presence of $ZnCl_2$ or $MgCl_2$ as indicated.

Mutation analysis of Cpro-1 activity. There was no amino acid sequence motif in the Cpro-1 region that was homologous with those found in the other known metalloproteinases. Therefore, candidate residues coordinating with zinc ion in the Cpro-1 region were determined through site-directed mutagenesis. Almost all metalloproteinases and metalloendopeptidases share common zinc-binding amino acids; histidine and glutamic acid residues are well known as coordinators of zinc in these enzymes (30, 41; for a review, see reference 12). The cysteine residue is suggested to be essential for the catalytic properties of several metal enzymes and metal-binding proteins (41; for a review, see reference 12). Site-specific mutations at zinc-binding ligands of a metalloprotease lead to complete abolition of the protease activity and concomitant accumulation of an inactive precursor polypeptide (6). Therefore, a series of pN722-1908 point mutants were constructed, in which the histidine, glutamic acid, and cysteine residues in the Cpro-1 region, normally conserved among all reported HCV strains, were independently replaced by alanine, glutamine, and alanine residues, respectively (Fig. 5A). The substituted amino acids were His-932, -952, -1136, -1175, -1227, and -1229; Glu-972, -980, -1009, -1058, -1199, and -1202; and Cys-922, -993, -1123, -1125, -1171, and -1185 in the HCV precursor proteins; the translation products of these point mutants were named H932A, H952A, H1136A, H1175A, H1227A, H1229A, E972Q, E980Q, E1009Q, E1058Q, E1199Q, E1202Q, C922A, C993A, C1123A, C1125A, C1171A, and C1185A, respectively. Then, the catalytic properties of Cpro-1 and Cpro-2 of these mutants were compared with those of the wild-type construct (Fig. 5B). Since the Cpro-1 and Cpro-2 activities were both required for the production of p70, the presence of p70 in the cleavage reaction products of the mutant RNAs indicated the retention of both proteinase activities in a single mutant protein. The absence of p21 and p70 and the accumulation of the 91-kDa processing intermediate may have implied the loss of Cpro-1 activity. Of these single point mutants, H952A and C993A were found not to produce p21 and p70 but did accumulate p26 and 91-kDa products (Fig. 5B), which suggested that they had lost Cpro-1 activity but retained Cpro-2 activity. The same result was obtained by in vitro translation of the point mutant H952R, in which His-952 was replaced by arginine (Fig. 5B). All mutants in which glutamic acid residues were substituted retained both proteinase activities, although the Cpro-1 activity of E972Q was reduced (Fig. 5B). All mutants of cysteine residues, except C993A, produced p21 and p70, although in various amounts. With C1123A and C1171A, p70 was detectable only after prolonged incubation (data not shown). The main cleavage reaction product of C1123A, C1125A, and C1171A was the 125-kDa precursor polypeptide, and no significant accumulation of intermediates was observed (Fig. 5B). This indicated that the Cpro-1 and Cpro-2 activities were probably both reduced in these mutants and that cysteines 1123, 1125, and 1171, which are located in the serine proteinase domain of the HCV precursor polyprotein (Fig. 5A), are important but not essential for both Cpro-1 and Cpro-2 activity. From these results, the candidates for coordination with zinc ion in the Cpro-1 region are proposed to be His-952 and Cys-993 of the HCV precursor polyprotein, although further biochemical and structural analyses of Cpro-1 are required to precisely determine the metal coordinators.

Expression of HCV proteins in cultured cells. To confirm that similar processing occurs in eukaryotic cells, expression of the genes for polypeptides N729-1908, S1165A, and

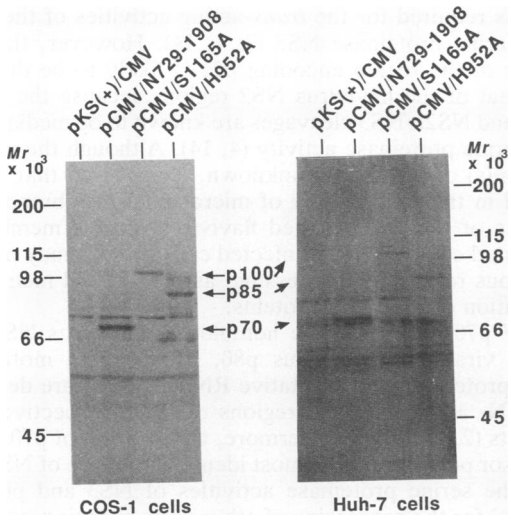

FIG. 7. Expression of HCV polypeptides in COS-1 and Huh-7 cells. Expression plasmids pCMV/N729-1908, pCMV/S1165A, and pCMV/H952A were transfected into COS-1 or Huh-7 cells, and the proteins expressed by these cell lines were analyzed by immunoblotting with anti-p70 antibody after separation by electrophoresis on an SDS-11% polyacrylamide gel as described in Materials and Methods.

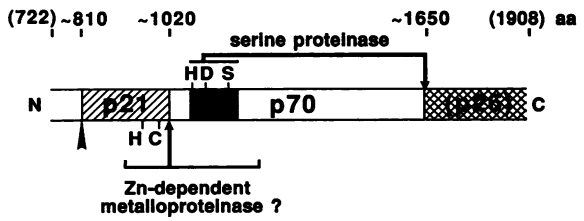

FIG. 8. Summary of processing and production of HCV p21 and p70 products. The box indicates a part of the HCV precursor polyprotein. The N- and C-terminal sides of the precursor are shown by N and C on the left and right of the box. The p21 and p26 regions of the HCV precursor polyprotein are indicated by hatched and latticed boxes, respectively. Cleavages that have been shown or proposed to occur in a microsome-dependent manner and by proteinases encoded in the HCV genome are indicated by an arrowhead and arrows, respectively. The approximate locations of the cleavage sites are indicated at the top. The putative Cpro-2 serine proteinase domain is shaded, and the predicted catalytic triad, His-1083, Asp-1107, and Ser-1165, of the serine proteinase is indicated by H, D, and S, respectively, above the box. The region required for detection of Cpro-1 activity is bracketed. The proposed zinc (Zn)-coordinating residues, His-952 and Cys-993, of Cpro-1 are indicated by H and C, respectively, below the box.

H952A in COS-1 cells and Huh-7 cells was examined with the expression vector pKS(+)/CMV (see Materials and Methods). The expression products were analyzed by immunoblotting with anti-p70 antibody. As shown in Fig. 7, p70, p100, and an 85-kDa intermediate (p85) were detected in both COS-1 and Huh-7 cells transfected with pCMV/N729-1908, pCMV/S1165A, and pCMV/H952A, respectively, and scarcely any p91 was found. The appearance of p85 might have meant that further processing of p21 occurred in vivo. Therefore, it was concluded that the processing of the p70 precursor in the HCV ORF characterized in an in vitro protein expression system reflected protein processing in vivo.

DISCUSSION

Two proteinases required for HCV precursor polyprotein processing. Two distinct proteinases, Cpro-1 and Cpro-2, functioned in the production of p70 from the putative nonstructural region of HCV precursor polyprotein. Those activities were demonstrated by cleavage of cloned partial HCV precursor polyprotein in an in vitro transcription/translation system and by transient expression of cloned genes in cultured cells. The results suggested that both proteinase activities were located in the HCV polypeptide. This suggestion may be supported by the recent observation that p70 was produced from HCV polypeptide expressed in *E. coli* via proteolytic processing identical to that found in the present paper (24). However, further biochemical and structural characterizations of purified enzymes will be required for the final identification. It is possible that an unknown proteinase(s) commonly present in the rabbit reticulocyte lysate, eukaryotic cultured cells, and *E. coli* can specifically recognize regions of the HCV precursor polyprotein. The multiple proteinases encoded by the genomes of some other viruses, such as poliovirus (40) and plant potyvirus (42), have been reported and characterized. Cpro-1 and Cpro-2 were responsible for the cleavages of the N- and C-terminal ends of p70, respectively. Both cleavage reactions seemed to occur mainly intramolecularly (in a *cis*-dependent manner) because p70 production could not be detected in either in vitro translation reaction mixtures or in cell lysates which coexpressed S1165A and H952A (data not shown). Although no inhibitor assay for Cpro-2 could be performed, point mutation analysis indicated that Cpro-2 is a serine proteinase, as predicted by a sequence alignment and modeling study (22). The results of inhibitor assays and point mutation analyses suggested that Cpro-1 can be classified as a zinc-dependent metalloproteinase.

The catalytic activities of these two proteinases are separable, because some mutants, such as S1165A and H952A, retained only one of these activities (Fig. 5B). However, the regions required for detection of these activities in the HCV precursor polyprotein overlapped. The Cpro-2 serine proteinase is likely to be located in the region from residue 1075 to 1185 of the HCV precursor polyprotein (22), corresponding to the N-terminal region of p70. At least the region from residue 898 to 1233 of the HCV precursor polyprotein was revealed to be essential for the detection of Cpro-1 activity by analyses of a series of deletion mutants. The results suggested that the candidates for the coordinators of zinc ion in the Cpro-1 region are His-952 and Cys-993, both of which are located in the C-terminal region of p21 (Fig. 8). However, further experimentation with the authentic product, containing the Cpro-1 region, is required to confirm this suggestion, since the possibility that each point mutation causes a change in the tertiary structure of p21 and/or another unknown type(s) of amino acid residue(s) which contributes to zinc binding cannot be completely ruled out. In addition, the C-terminal region just downstream of the serine residue in the serine proteinase domain was essential for the cleavage mediated by Cpro-1 activity, because the C-terminal deletion mutant, N722-1174, was not cleaved at the p21/p70 site (Fig. 4A, lane 1). In that mutant, only the small region corresponding to the C-terminal substrate-binding pocket of the serine proteinase structure was deleted (Fig. 5A). Larger amounts of unprocessed products accumulated in the cleavage reactions of N722-1477, N722-1325, and

N722-1233 than in that of N722-1908 (Fig. 4A), indicating that even a deletion in the p70 C-terminal region reduced Cpro-1 activity. Furthermore, the mutations of cysteine residues at aa 1123, 1125, and 1171 to alanine residues reduced both the Cpro-1 and the Cpro-2 activities (Fig. 5B), suggesting the requirement of tertiary structure for full activity. There seem to be at least two possible explanations for why overlapping regions of the HCV precursor polyprotein might be required for the detection of two distinct proteinase activities. One is that the entire tertiary structure of the p21-p70 precursor protein or at least the overlapping serine proteinase domain is required for its recognition as a substrate by the active domain of Cpro-1. Cpro-1 might be located in the C-terminal half of p21 or in the serine proteinase structure. The other possibility is that the sequence of the C-terminal half of p21 plus the active domain of Cpro-2 serine proteinase functions as a metalloproteinase with Cpro-1 activity. In the former case, Cpro-1 would be the smallest known metalloproteinase that specifically recognizes the tertiary structure of a large polypeptide as a substrate. In the latter case, the structure of Cpro-1 may be partly constructed with the serine proteinase structure, and its activity should be inactivated after autocatalytic *cis* cleavage, implying that two proteinase domains of the HCV precursor polyprotein overlap and form the enzyme in enzyme structure. Although there has been no direct report of a proteinase like that described in the first case, a domain-overlapping structure in the structural core protein of Sindbis virus has been recently reported (9, 16). The core protein was revealed to contain a C terminally truncated form of a chymotrypsin-like serine proteinase structure, the proteinase activity of which is responsible for releasing the core protein from the precursor protein by autocatalytic *cis* cleavage (9, 16). This indicated that this viral core protein has two overlapping functional domains, one for structural protein and another for enzyme. The serine proteinase activity in the precursor of the core protein is suicidal, because the cleavage site of the proteinase is located within the substrate-binding region of the proteinase itself (9). If the Cpro-1 and Cpro-2 domains overlap, then the Cpro-1 activity may have a similar suicidal function that releases Cpro-2 from the Cpro-1 structure and the HCV precursor polyprotein.

Putative NS proteins of HCV. The region spanning residue 722 to residue 1908 of the HCV precursor polyprotein produced three major processed products, p21, p70, and p26 (Fig. 8). This region may also have produced smaller processed polypeptides that were not detectable by the method used. The functional properties of p21 and p70 as proteinases were suggested, but the function of the wild-type form of p26 is still unknown. An immunoprecipitation experiment, shown in Fig. 3, suggested that p26 was likely to be associated with p70 at least in the in vitro translation mixture. Recent in vitro transcription/translation experiments with the entire NS protein region from the HCV genome suggest that p26 is a C-terminal truncated form of the 31-kDa (p31) product which is dependent on Cpro-2 activity (20). In vitro, p31 is also associated with p70, although the significance of this molecular association is still unknown (20). Moreover, p31 is likely to be further processed to smaller products when expressed in vivo, suggesting the presence of a cellular proteolytic site in p31 (20).

The location and size of p21 in the viral precursor polyprotein are likely to be similar to those of flavivirus NS2a or NS2b (37). The protein NS2a is suggested to be a proteinase that cleaves the N-terminal end of its molecule (13), and NS2b is required for the *trans*-acting activities of the flavivirus serine proteinase NS3 (3, 4, 14). However, the processing of the region encoding p21 is likely to be different from that of the flavivirus NS2 region, because the NS2a/NS2b and NS2b/NS3 cleavages are known to be mediated by NS3 serine proteinase activity (4, 14). Although the precise function(s) of p21 is still unknown, it appeared that it was located in the lipid bilayer of microsomal membranes. NS proteins of the HCV-related flavivirus form a membrane-associated complex in the infected cell (35). p21 may play an analogous role in the processing, assembly, and membrane association of HCV NS proteins.

HCV p70 seems to be a homolog of flavivirus NS3 and bovine viral diarrheal virus p80, because the motifs for serine proteinase and a putative RNA helicase are detected in the N- and C-terminal regions of these respective viral products (2, 22, 23). Furthermore, the location of p70 in the precursor polyprotein is almost identical to those of NS3 and p80. The serine proteinase activities of NS3 and p80 are essential for the processing of other parts of their respective NS proteins (3, 4, 14). The data presented here suggested that the Cpro-2 serine proteinase activity was required for the processing of HCV NS proteins. Recently, it was shown that processing of the C-terminal portion of the HCV precursor polyprotein is abolished by a point mutation of serine at aa 1165 to alanine in the domain of Cpro-2 proteinase (20).

ACKNOWLEDGMENTS

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REFERENCES

1. **Amemiya, Y., and J. Miyahara.** 1988. Imaging plate illuminates many fields. Nature (London) **336**:89–90.
2. **Bazan, J. F., and R. J. Fletterick.** 1989. Detection of a trypsin-like serine protease domain in flaviviruses and pestiviruses. Virology **171**:637–639.
3. **Cahour, A., B. Falgout, and C.-J. Lai.** 1992. Cleavage of the dengue virus polyprotein at the NS3/NS4A and NS4B/NS5 junctions is mediated by viral protease NS2B-NS3, whereas NS4A/NS4B may be processed by a cellular protease. J. Virol. **66**:1535–1542.
4. **Chambers, T. J., A. Grakoui, and C. M. Rice.** 1991. Processing of the yellow fever virus non-structural polyprotein: a catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites. J. Virol. **65**:6042–6050.
5. **Chambers, T. J., R. C. Weir, A. Grakoui, D. W. McCourt, J. F. Bazan, R. J. Fletterick, and C. M. Rice.** 1990. Evidence that the N-terminal domain of nonstructural protein NS3 from yellow fever virus is a serine protease responsible for site-specific cleavages in the viral polyprotein. Proc. Natl. Acad. Sci. USA **87**:8898–8902.
6. **Chang, P.-C., and Y.-H. W. Lee.** 1992. Extracellular autoprocessing of a metalloprotease from Streptomyces cacaoi. J. Biol. Chem. **267**:3952–3958.
7. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**:2745–2752.
8. **Chen, P.-J., M.-H. Lin, K.-F. Tai, P.-C. Liu, C.-J. Lin, and D.-S. Chen.** 1992. The Taiwanese hepatitis C virus genome: sequence determination and mapping the 5′ termini of viral genomic and antigenomic RNA. Virology **188**:102–113.
9. **Choi, H.-K., L. Tong, W. Minor, P. Dumas, U. Boege, M. G. Rossmann, and G. Wengler.** 1991. Structure of Sindbis virus core protein reveals a chymotrypsin-like serine proteinase and the organization of the virion. Nature (London) **354**:37–43.

10. **Choo, Q.-L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a bloodborne non-A, non-B viral hepatitis genome. Science **244**:359–362.
11. **Choo, Q.-L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, A. Medina-Selby, P. J. Barr, A. J. Weiner, D. W. Bradley, G. Kuo, and M. Houghton.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA **88**:2451–2455.
12. **Coleman, J. E.** 1992. Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. Annu. Rev. Biochem. **61**:897–946.
13. **Falgout, B., R. Chanock, and C.-J. Lai.** 1989. Proper processing of dengue virus nonstructural glycoprotein NS1 requires the N-terminal hydrophobic signal sequence and the downstream nonstructural protein NS2a. J. Virol. **63**:1852–1860.
14. **Falgout, B., M. Pethel, Y.-M. Zhang, and C.-J. Lai.** 1991. Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. J. Virol. **65**:2467–2475.
15. **Foecking, M. K., and H. Hofstetter.** 1986. Powerful and versatile enhancer-promoter unit for mammalian expression vectors. Gene **45**:101–105.
16. **Hahn, C. S., and J. H. Strauss.** 1990. Site-directed mutagenesis of the proposed catalytic amino acids of the Sindbis virus capsid protein autoprotease. J. Virol. **64**:3069–3073.
17. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted break points for DNA sequencing. Gene **28**: 351–359.
18. **Hijikata, M., N. Kato, Y. Ootsuyama, M. Nakagawa, and K. Shimotohno.** 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by *in vitro* processing analysis. Proc. Natl. Acad. Sci. USA **88**:5547–5551.
19. **Hijikata, M., N. Kato, T. Sato, Y. Kagami, and K. Shimotohno.** 1990. Molecular cloning and characterization of a cDNA for a novel phorbol-12-myristate-13-acetate-responsive gene that is highly expressed in an adult T-cell leukemia cell line. J. Virol. **64**:4632–4639.
20. **Hijikata, M., H. Mizushima, Y. Komoda, Y. Tanji, Y. Hirowatari, T. Akagi, T. Sato, S. Mori, N. Kato, T. Tanaka, K. Kimura, and K. Shimotohno.** Unpublished data.
21. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77**:51–59.
22. **Kato, N., M. Hijikata, M. Nakagawa, Y. Ootsuyama, K. Muraiso, S. Ohkoshi, and K. Shimotohno.** 1991. Molecular structure of the Japanese hepatitis C viral genome. FEBS Lett. **280**:325–328.
23. **Kato, N., M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, T. Sugimura, and K. Shimotohno.** 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B, hepatitis. Proc. Natl. Acad. Sci. USA **87**:9524–9528.
24. **Komoda, Y., M. Hijikata, H. Mizushima, Y. Tanji, Y. Hirowatari, N. Kato, T. Tanaka, and K. Shimotohno.** Unpublished data.
25. **Krausslich, H.-G., and E. Wimmer.** 1988. Viral proteinases. Annu. Rev. Biochem. **57**:701–754.
26. **Kunkel, T. A., J. D. Roberts, and R. A. Zabour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
27. **Kuo, G., Q.-L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, G. E. Tegtmeier, F. Bonino, M. Colombo, W.-S. Lee, C. Kuo, K. Berger, J. R. Shuster, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science **244**:362–364.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
29. **Miller, R. H., and R. H. Purcell.** 1990. Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus subgroups. Proc. Natl. Acad. Sci. USA **87**:2057–2061.
30. **Monzingo, A. F., and B. W. Matthews.** 1984. Binding of N-carboxymethyl dipeptide inhibitors to thermolysin determined by X-ray crystallography: a novel class of transition-state analogues for zinc peptidase. Biochemistry **23**:5724–5729.
31. **Mori, S., S. Ohkoshi, M. Hijikata, N. Kato, and K. Shimotohno.** 1992. Serodiagnostic assay of hepatitis C virus infection using viral proteins expressed in *Escherichia coli*. Jpn. J. Cancer Res. **83**:264–268.
32. **Okamoto, H., K. Kurai, S. Okada, K. Yamamoto, H. Lizuka, T. Tanaka, S. Fukuda, F. Tsuda, and S. Mishiro.** 1992. Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology **188**:331–341.
33. **Okamoto, H., S. Okada, Y. Sugiyama, K. Kurai, H. Iizuka, A. Machida, Y. Miyakawa, and M. Mayumi.** 1991. Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. J. Gen. Virol. **72**:2697–2704.
34. **Preugschat, F., C.-W. Yao, and J. H. Strauss.** 1990. In vitro processing of dengue virus type 2 nonstructural proteins NS2A, NS2B, and NS3. J. Virol. **64**:4364–4374.
35. **Rice, C. M., E. G. Strauss, and J. H. Strauss.** 1986. Structure of the flavivirus genome, p. 279–326. *In* S. Schlesinger and M. J. Schlesinger (ed.), The *Togaviridae* and *Flaviviridae*. Plenum, New York.
36. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166**:368–379.
37. **Speight, G., G. Coia, M. D. Parker, and E. G. Westaway.** 1988. Gene mapping and positive identification of non-structural proteins NS2a, NS2b, NS3, NS4b, and NS5 of the flavivirus Kunjin and their cleavage sites. J. Gen. Virol. **69**:23–34.
38. **Takamizawa, A., C. Mori, I. Fuke, S. Manabe, S. Murakami, J. Fujita, E. Onishi, T. Andoh, I. Yoshida, and H. Okayama.** 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. J. Virol. **65**:1105–1113.
39. **Tanaka, T., N. Kato, M. Nakagawa, Y. Ootsuyama, M.-J. Cho, T. Nakazawa, M. Hijikata, Y. Ishimura, and K. Shimotohno.** 1992. Molecular cloning of hepatitis C virus genome from a single Japanese carrier: sequence variation within the same individual and among infected individuals. Virus Res. **23**:39–53.
40. **Toyoda, H., M. J. H. Nicklin, M. G. Murray, C. W. Anderson, J. J. Dunn, F. W. Studier, and E. Wimmer.** 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell **45**:761–770.
41. **Vallee, B. L., and D. S. Auld.** 1990. Active-site zinc ligands and activated H_2O of zinc enzymes. Proc. Natl. Acad. Sci. USA **87**:220–224.
42. **Verchot, J., E. V. Koonin, and J. C. Carrington.** 1991. The 35-kDa protein from the N-terminus of the potyviral polyprotein functions as a third virus-encoded proteinase. Virology **185**:527–535.
43. **Wiskerchen, M., and M. S. Collett.** 1991. Pestivirus gene expression: protein p80 of bovine viral diarrhea virus is a proteinase involved in polyprotein processing. Virology **184**:341–350.