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Molecular Mechanism of Polyprotein Processing of Hepatitis C Virus Nonstructural Protein

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Chapter 1

General Background

1.1 Characteristics of Hepatitis C Virus (HCV)

1.1.1 Significance for disease agents

In Japan, almost all infectious diseases have been brought under control by improvements in sanitary conditions, development of vaccines and so on. Viral hepatitis is one of the last infectious diseases for which control procedures have not yet been established. One reason for this has been delay in discovery of the viruses.

HCV was identified and molecularly cloned in the last few years (9, 31). HCV is the first virus to be discovered by molecular cloning without direct use of biological or biophysical methods. HCV was first recognized in 1974 as non-A, non-B (NANB) hepatitis resulting from blood transfusion (45). Therefore, it took almost 15 years to identify it by detecting a clone in a library of cDNA prepared from the nucleic acids extracted from plasma known to be infectious for chimpanzees.

There is a virus carrier state during HCV infection. HCV causes not only acute hapatitis by transient infection but also chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. In general, clinical symptoms and laboratory data on cases of acute HCV infection are milder than those on hepatitis B infection. But although the early stage of chronic HCV is generally mild, occasional elevation of serum transaminase is observed resulting in the patients finally developing liver cirrhosis.

From December 1990, screening for anti-HCV antibody in donor blood was carried out in blood banks across Japan using first generation enzyme linked immunosorbent assays

(ELISA) for anti HCV (9, 34, 57). In February 1992, this screening procedure was changed to a new one using the second generation anti-HCV assay. This is because the first generation assay, the anti-c100-3 antigen used in the first generation kit, was not so accurate. The sensitivity and specificity of HCV detection have been improved appreciably by this new ELISA. Anti-HCV positivity is age dependent in Japan; in subjects under 20 years, the prevalence is as low as 0.2 % whereas in those over 50 the prevalence is as high as 3.9 %. In Japan, it is estimated that 2.3 million have contracted the infection through contaminated blood. The actual number of anti-HCV positive persons may be even higher than 2.3 million.

Interferon remains the only accepted treatment for chronic HCV. Despite a large number of trials with a wide range of agents, no other effective treatment has yet been confirmed for patients. Methods for prevention and therapy of HCV must be developed. HCV specific drug design is necessary; for instance, a neutralizing antibody or inhibitory antibody for all HCV variants or specific inhibitors of HCV-derived enzymes such as RNA-dependent RNA polymerase, serine proteinase and helicase must be developed.

1.1.2 Classification and life cycle.

Classification and proposed genotypes of HCV are shown in Figure 1-1. The positive-stranded RNA virus family *Flaviviridae* consists of three genera (15). The largest genus, *Flavivirus*, contains many significant human

pathogens, for instance, yellow fever virus, Japanese encephalitis virus, dengue virus, and so on. The genus *Pestivirus* consists of three important serologically related animal pathogens, namely the bovine viral diarrhea virus (BVDV), hog cholera virus and border disease virus (BDV) of sheep. Virus of the final genus is HCV. At the molecular level, the flaviviruses have been the most extensively studied; however, rapid progress has been made in recent years toward elucidating the molecular details of viruses from the other two genera (24). Comparative analyses of the molecular features in members of these three virus groups have revealed both similarities and distinctions. Although little significant overall relatedness is apparent at the amino acid sequence level, the amino acid sequences of some of the proteins show small but significant sequence similarities with the corresponding proteins encoded by pesti and flaviviruses (6, 11, 16, 37). In particular, within the NS protein coding-region, there exist conserved sequence motifs in the analogous position within the ORF of members of all three virus groups that are predictive serine type proteinase and of a nucleoside triphosphatase (NTPase)/ RNA helicase (6, 16, 37). The motifs for both enzymatic activities lie within a single polypeptide coding region: the flavivirus NS3 protein, the pestivirus p125 (p80) protein, and HCV NS3 protein. The predicted Cpro-2 activity has been experimentally demonstrated for representatives of each of the three virus genera and shown to be required for viral polyprotein processing (18, 56).

Subtypes of HCV were determined by differences in nucleotide sequences; that is, low homologies of nucleotide sequences of different types. The first cDNA clone of the HCV genome was obtained from a cDNA library constructed from infected chimpanzee serum in USA (9). Soon afterwards, the HCV genome from Japanese infectious materials was isolated by an immunoscreening method. A marked difference was detected between the nucleotide sequence of original clone and the present Japanese clone. The homology between these two isolates in the NS5 region was 78.9% in nucleotide sequences, but 91.2% in amino acid sequences. Later many Japanese scientists cloned HCV genome from Japanese patients, and determined the sequence of the whole genome (31, 49).

Two other types of HCV have been isolated in Japan (41, 42). The homologies of the nucleotide sequences of these two isolates with each of the first two isolates were less than 70%, but that between the new isolate was a little higher (80%). However the heterogeneity of nucleotide sequences of HCV isolate belonging to the same type were less than 10%. No intermediate of these four types was detected. These four types named type I (American clone), type II (major clone in Japan), type III and type IV. The frequencies of these four types in Japan are 80% for type II, 10% for type III, and less than 5% each for types I and IV (42). The four types of HCV are easily distinguishable using different primer sequences specific for each type to amplify cDNA by the polymerase chain reaction (PCR). Recently the new types V and VI of HCV

were isolated from patients in Thailand (40). These data are summarized in Figure 1-1.

Schematic diagram of possible life cycle of HCV on the analogy of flavivirus is depicted in Figure 1-2. HCV is found only in low titers in the serum of infected humans and chimpanzees (37) and replicates poorly in cell culture, in hepatocytes cultured from chimpanzees with acute HCV infection (27) and in a human T-cell line (48). Consequently, traditional replication studies have not been performed. Therefore, for the most part of life cycle of the HCV is still obscure.

1.1.3 Organization and translation of the genome RNA.

HCV is a positive-stranded RNA virus of about 10 kilo bases (kb). The viral genome encodes a large polyprotein precursor of about 3000 amino acids. Typically, the noncoding region at the 5' and 3' termini of a viral genome contains regulatory elements important for viral gene expression and replication. Sequence analysis revealed the existence of a fairly long 5' untranslated region (UTR) that harbors three or four AUG sequences (19, 31, 49). This feature of the 5' UTR resembles that of picornaviruses rather than that of flaviviruses. Picornavirus RNAs are uncapped messengers and have unusually long 5' UTRs which contains many silent AUG sequences. It was proved that translation initiation on picornavirus RNAs occurs by binding of ribosomes to an internal sequence within the 5' UTR (28, 29, 43). An internal entry site for the ribosomes

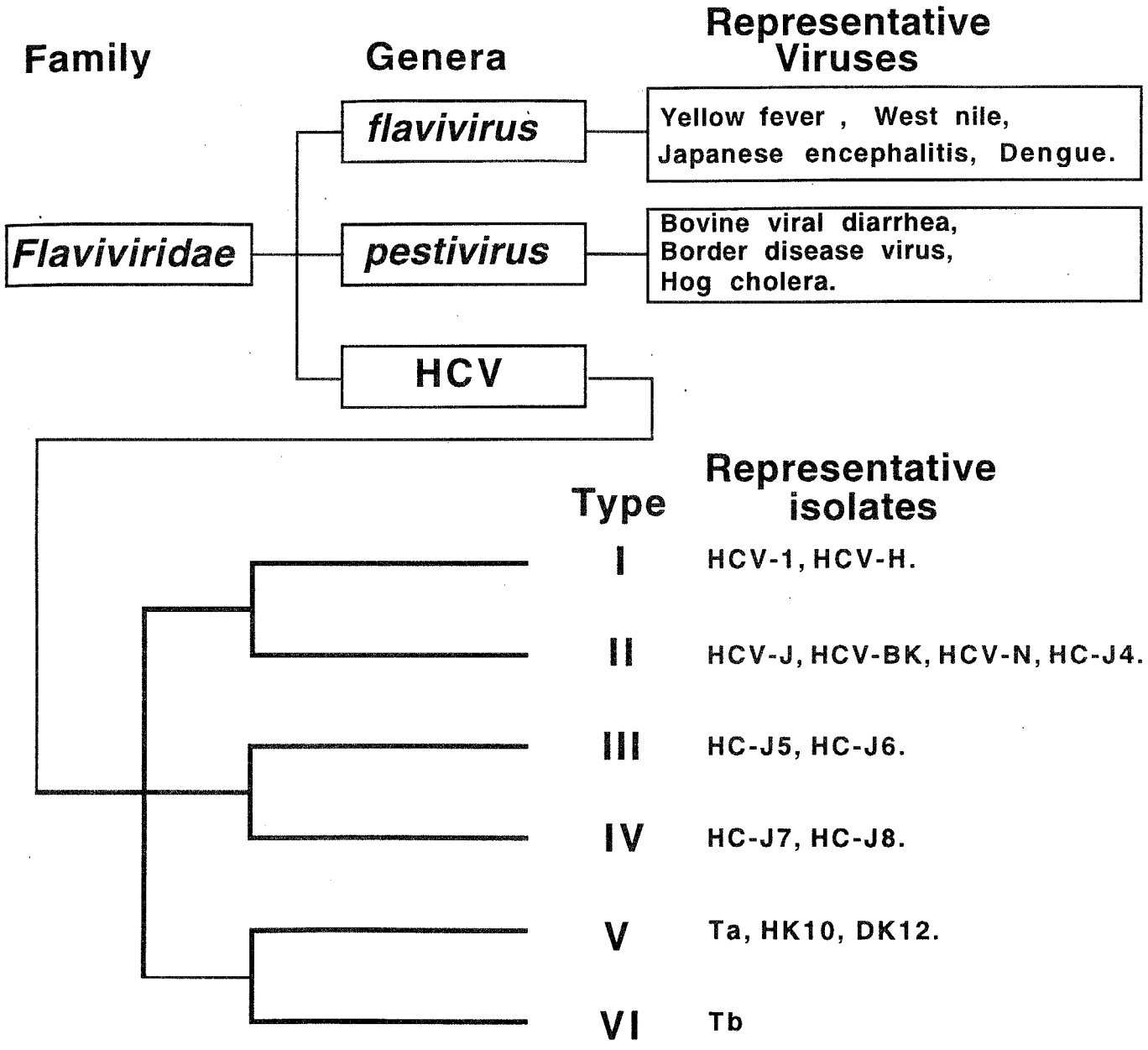


Fig. 1-1 Classification and genotypes of HCV.

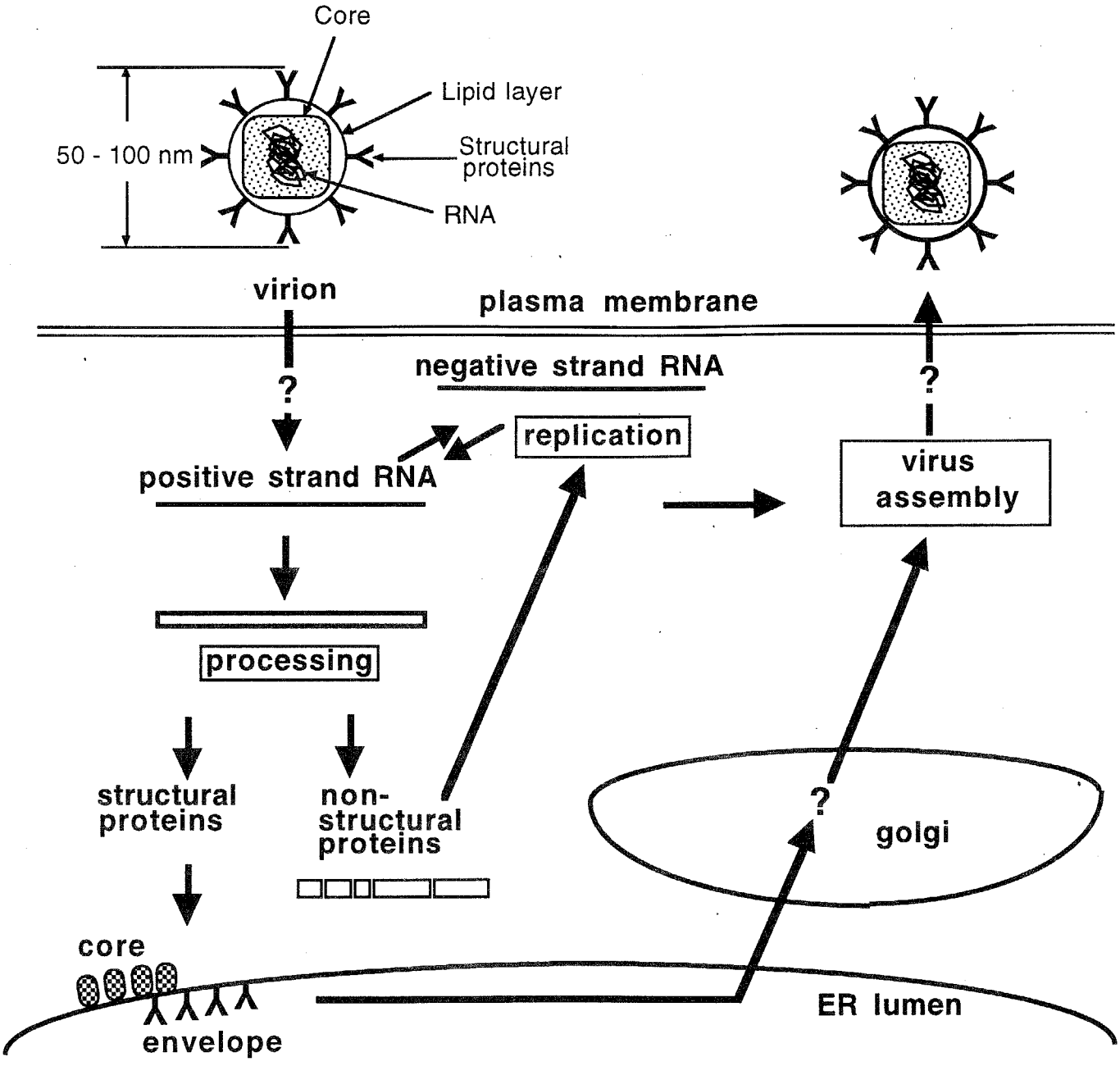


Fig. 1-2 Schematic diagram of possible life cycle of HCV.

has been called the IRES (internal ribosome entry site). Structural similarities of the 5' UTR of HCV RNA to those of picornavirus RNAs and cell-free protein synthesis system provide the possibility that IRES function resides in the 5' UTR of HCV RNA (55).

1.1.4 Protein processing of the viral polyprotein

The order of proteins encoded in the long ORF of HCV is 5'-C-E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3' as shown in figure 1-3. The 5' end of the HCV genome encodes structural proteins of the virion. The first gene product encodes a highly basic core (C) protein. Immediately downstream of C-protein are regions which encodes the envelope proteins (E1 and E2) of the virus. The locations of the possible cleavage sites between the structural proteins are based on determination of the N-terminal amino acid residues of E1 and E2 (20). E2 is presumed to be a typical type 1 membrane- associated glycoprotein with a membrane-anchoring portion in its C-terminal region. Hydrophobic amino acid sequences cluster in the C-terminal region of E2, from residue 715 to residue 809 of the HCV precursor polyprotein. The importance of this region for anchoring the E2 product to cellar membranes is suggested by the fact that deletion of this region results in the production of a soluble form of E2. Deletion analysis estimates place the possible C terminus of E2 around residue 740 (20). On the other hand, the possible N-terminal site of the adjacent NS2 is at residue 810 (38). Recently, the presence of a novel microsomal membrane-dependent cleavage site at amino

acid 746/747, about 60 amino acid residues upstream of the N-terminal end of the NS2 region of HCV precursor polyprotein was identified (39). Thus, the production of a protein of about 7 kDa (p7), from amino acid 747 to 809, was expected to be produced.

Two distinct proteinase activities are required for the production of NS3 (22). One of these proteinases is a novel zinc-dependent mettalloproteinase and is responsible for cleavage at the NS2/NS3 (abbreviated as 2/3) site. The other proteinase is a Cpro-2 which activity appears to be essential for the processing of NS4A, NS4B, NS5A, and NS5B (4, 12, 18, 21, 54).

1.2 Outline and scope of this study.

Virtual elimination of HCV-contaminated blood has greatly reduced the incidence of posttransfusion hepatitis; however, HCV remains responsible for a significant proportion of community-acquired hepatitis. In most cases, HCV is not cleared and establishes a chronic infection that can be associated with chronic hepatitis and more severe liver disease such as cirrhosis and hepatocellular carcinoma. For these reasons, there is considerable interest in developing additional HCV-specific antiviral agents that can complement currently available alpha interferon therapy, which effectively controls disease in only a minority of HCV-infected patients.

For developing a HCV-specific antiviral agents, better understanding of virus life cycle, especially in relation to functions of each viral protein is required.

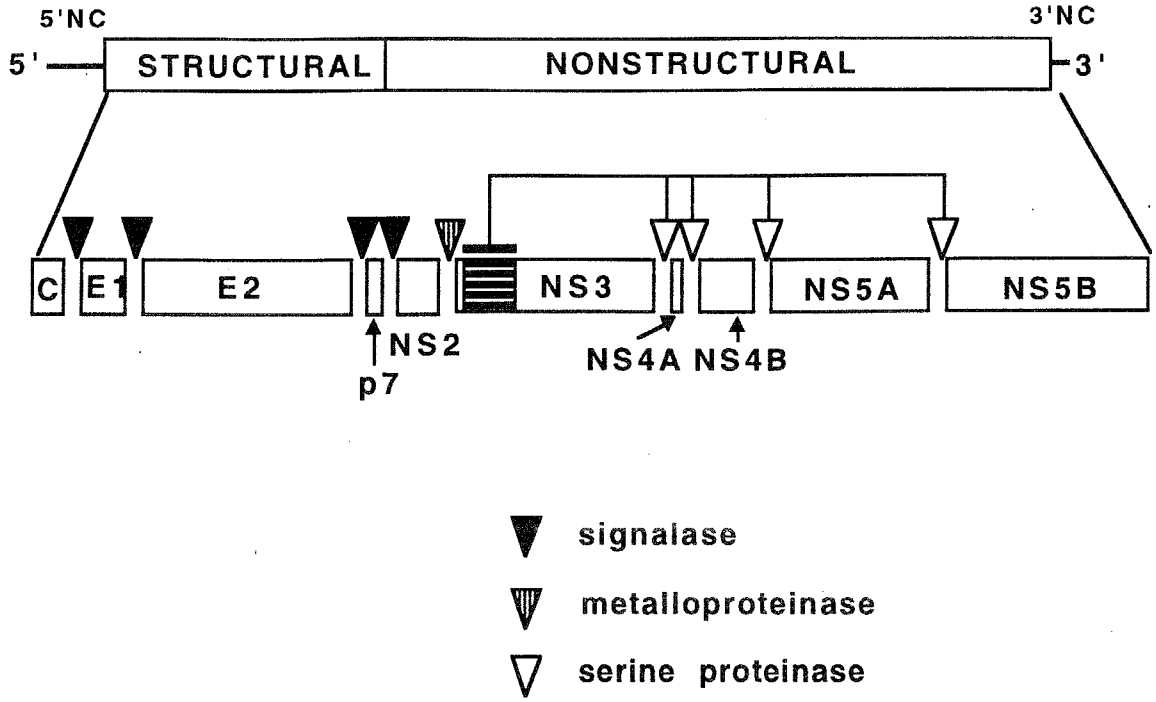


Fig. 1-3 Schematic of the processing events for the HCV polyprotein.

The top depicts the viral genome; the structural and nonstructural protein coding region and the 5' and 3' non coding (NC) regions are indicated. Boxes below the genome indicate mature proteins generated by the proteolitic processing cascade.

Virus encoded proteinase activity is the only function defined for HCV. Objects of this study are to promote a better understanding of this viral proteinase activity and to make an attempt to find out functions of virus encoded proteins. Because a cell culture system supporting efficient HCV replication is lacking, traditional replication studies could not be utilized. Therefore, cloned cDNA fragments encoding HCV polyprotein were expressed by using a transient expression system in cultured cells for the analyses in this study.

The outline of this study is shown in Figure 1-4. Composition of this thesis is as follows.

Chapter 2: Processing of the region downstream of NS3 appears to be dependent on the viral Cpro-2 located in the N-terminal part of NS3. However, the predicted location of the Cpro-2 active domain was based only on sequence analysis. In this chapter, Cpro-2 active domain was mapped by introducing deletion mutations into NS3. Furthermore, two different cleavage modes, intra-molecular (*trans*) and inter-molecular(*cis*) cleavages, were proposed to be employed in the Cpro-2 dependent polyprotein processing, depending on the location of cleavage sites.

Chapter 3: Pulse chase experiment was conducted to determine the order of nonstructural protein production. Furthermore, the effect of mutation at each cleavage site on the processing pathway was also analyzed.

Chapter 4: HCV encoded NS4A is a small peptide, composed of 53 amino acids, with its hydrophobic N-terminal half and hydrophilic C-terminal half. By analyzing the

processing of a mutated NSΔ4A polypeptide which encodes the entire HCV nonstructural protein except the region of NS4A, versatile functions of this peptide was revealed.

Chapter-5: NS5A is composed of 447 amino acids which has the potential to produce a 49 kDa protein. But actual molecular weight is 56 or 58 kDa. Thus, the nature of the modification of the NS5A products to clarify the difference between the actual molecular weight and calculated molecular weight was analyzed. In this chapter, NS5A was revealed to be phosphorylated at serine residues. Furthermore p56 was phosphorylated to produce p58 in a NS4A dependent manner.

Chapter-6: To deepen the understanding of NS5A phosphorylation, pulse-chase and mutation analyses were performed. Pulse-chase study showed that phosphorylation proceeded after the completion of NS5A processing. Mutation analysis identified the serine residues responsible for the NS4A-dependent phosphorylation and domain responsible for the NS4A-independent phosphorylation.

Chapter-7: Conclusions and problems remain to be solved of this study were summarized.

Molecular Mechanism of Polyprotein Processing of Hepatitis C Virus Nonstructural Protein

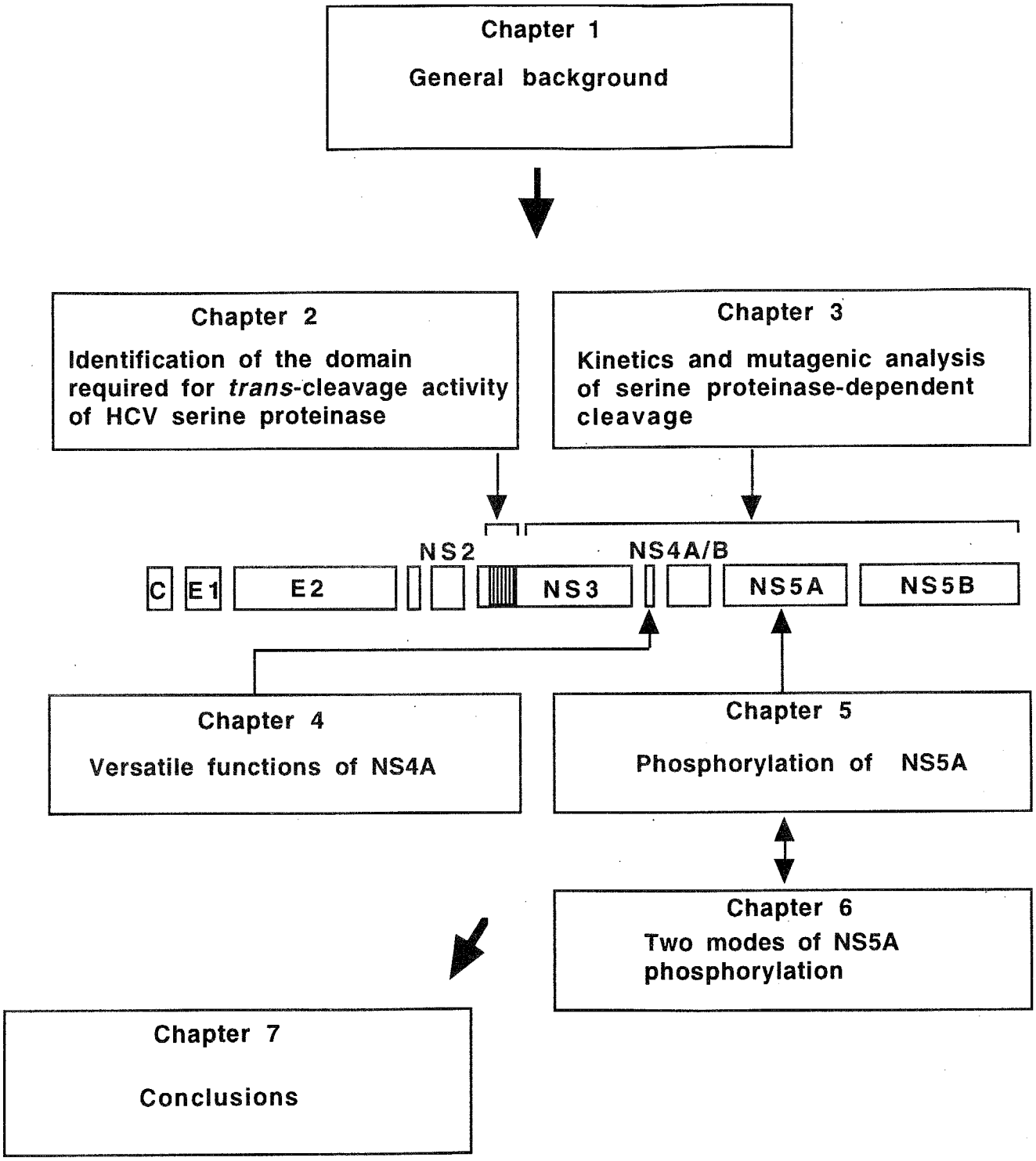


Fig.1-4 Outline of this dissertation.

Chapter 2

Identification of the domain required for *trans*-cleavage activity of H C V serine proteinase

2.1 Introduction.

The precursor polyprotein encoded in the HCV-ORF is cotranslationally and posttranslationally processed into viral proteins by viral proteinases and by host signal peptidase (4, 12, 18, 20, 21, 22, 54). Processing of the region downstream of NS3 in the polyprotein appears to be dependent on the serine proteinase activity (Cpro-2) located in the N-terminal part of NS3. However, the precise domain and structure of Cpro-2 is still obscure. In this chapter, Cpro-2 active domain by introducing deletion mutations into NS3 was mapped. And the mechanism of Cpro-2-dependent polyprotein processing using a transient protein production system in cultured cells was analyzed.

2.2 MATERIALS AND METHODS

2.2.1 Construction of plasmids

The series of HCV polyprotein regions used in this study is shown in Fig. 2-1. Plasmids pCMV/N729-3010, pCMV/N729-3010M1, pCMV/N729-1908, pCMV/H952A and pCMV/S1165A were reported previously (21, 22). The *Pst*I-*Hin*dIII fragment of the pCMV/N729-3010, which encodes a polypeptide corresponding to region from 2052 amino acid residue to 2951 amino acid residue of the HCV precursor polyprotein (N2052-2951), was inserted into the *Pst*I-*Hin*dIII site of pKS(+)/CMV (21) and the 8-mer *Nco*I linker (5'-GCCATGGC-3') (TAKARA Shuzo) was inserted into the *Pst*I site of this plasmid after blunt-ending the cohesive ends of the restriction site with T4 DNA polymerase. The resultant clone was named pCMV/N2052-2951. Plasmids

encoding part of the HCV polypeptide fused with the *E. coli* dhfr gene were made in the following ways. Two plasmids, pdhfrFX1 and pdhfrFX2, were prepared by inserting two types of synthetic linkers, FX1 and FX2, respectively, into the blunt-ended *Hin*dIII-*Bcl*I site of pTZSV2dhfr1sH. FX1 was made by annealing two synthetic oligonucleotides, 5'-AGCTTTCATCGAAGGTCGAGGTAT-3' and 5'-ATACCTCGACCTTCGATGAA-3'. FX2 was made by annealing 5'-AGCTTGCAATCGAAGGTCGAGGTAT-3' and 5'-ATACCTCGACCTTCGATTGCA-3'. The *Rsr*II-*Hin*dIII fragments of pHCN722-1325, pHCN722-1233 and pHCN722-1174 (21) were inserted into the *Rsr*II-*Hin*dIII site of pCMV/N729-3010 to obtain pCMV/N729-1325, pCMV/N729-1233 and pCMV/N729-1174, respectively. To fuse the dhfr gene to the 3'-terminal ends of the HCV-ORFs in pCMV/N729-1325 and pCMV/N729-1233 in frame, the *Hin*dIII-*Kpn*I fragment of pdhfrFX2 was inserted into the *Hin*dIII-*Kpn*I sites of pCMV/N729-1325 and pCMV/N729-1233. The resultant constructs were named pCMV/N729-1325D and pCMV/N729-1233d, respectively. For construction of pCMV/N729-1174D, the *Hin*dIII-*Kpn*I fragment of pdhfrFX1 was inserted into *Hin*dIII-*Kpn*I site of pCMV/N729-1174. To yield a series of 5' terminal deletion mutants of pCMV/N729-1233D, the *Pst*I-*Bln*I fragment of pCMV/N729-1233d was replaced by the *Pst*I-*Bln*I fragments of the products (N-P1, N-P2, N-P3 and N-P4) synthesized by polymerase chain reaction (PCR), in which the oligonucleotide N (5'-CACGGTGATCGATCCCGGAG-3') and oligonucleotide P1, P2, P3 and P4 (5'-TGCACTGCAGCCATGCTTACTCCACTGCGG-3', 5'-TGCACTGCAGCCATGGCGCCTATCACGGCCTAT-3', 5'-

TGCACTGCAGCCATGGGTCGGGACAAGAACCAG-3' and 5'-TGCACTGCAGCCATGAATGGCGTGTGTTGGACC-3') were used as a negative strand primer and positive strand primers, respectively. The resultant constructs were named pCMV/N953-1233D, pCMV/N1027-1234D, pCMV/N1049-1233D, and pCMV/N1075-1233D, respectively. To obtain pCMV/N1049-1215 in which the dhfr gene was not fused with the HCV-ORF, the stop codon linker (5'-TAGCTAGCTAGCTA-3') was inserted into the blunt-ended *Hind*III site of pCMV/N1049-1215D.

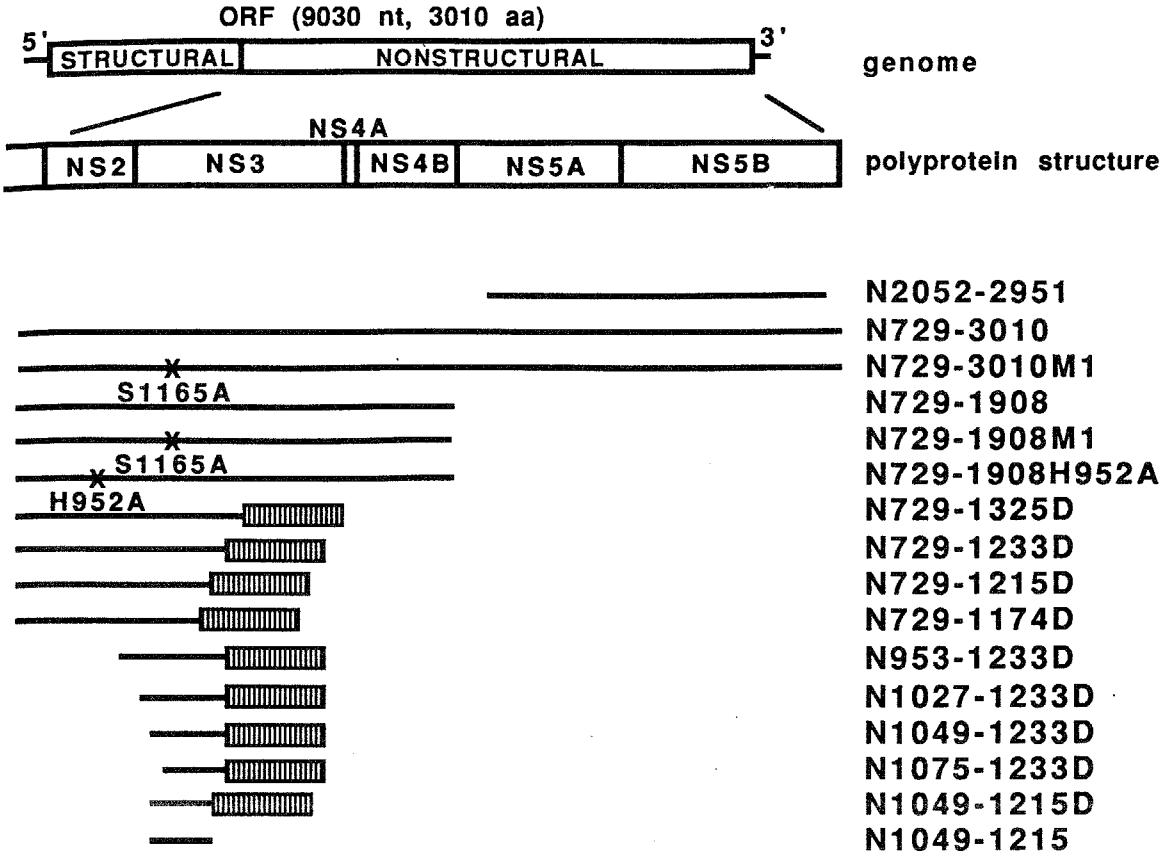


Fig. 2-1 Schematic representation of HCV protein regions encoded in the expression constructs.

General features of the HCV-IIj genome encoding the 5' and 3' noncoding regions, and the structural and nonstructural proteins encoding regions of the ORF are shown. The polyprotein structure of the nonstructural region from p21(NS2) to p66(NS5B) is shown enlarged below the genomic structure. Positions, in which mutations were introduced, are depicted by X with the name of the mutation. The HCV polypeptide regions expressed in COS-1 cells are shown with the name at right. Numbers in the name of each polypeptide indicates amino acid positions from the N- to C-teminal residues of the HCV precursor polyprotein regions. Hatched boxes indicate *E.coli* DHFR fused at C-terminal ends of HCV polypeptides in frame.

2.2.2 Transient expression assay in COS-1 cells

COS-1 cells were transfected by the calcium phosphate coprecipitation methods (8). Samples of the expression plasmids (10 μg) were transfected into $2x10^5$ cells in 35-mm dishes. Cells were harvested 48 h after transfection and resuspended in 2x sample buffer. Samples were fractionated on SDS-10 % polyacrylamide-gel electrophoresis (PAGE), and the proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Millipore; Immobilon). After incubation for 1 h in 150 mM sodium phosphate (pH 7.2) with 3% bovine serum albumin (BSA) for blocking, the membranes were incubated in the same buffer containing region specific antibody for 2 h and then washed extensively with phosphate buffered saline (PBS) containing 0.05% Tween 20. The antibodies bound to the membranes were detected with biotylated goat anti-rabbit or anti-mouse immunoglobulin G antibody and Vectastain ABC kit (Vector Laboratory).

2.2.3 Nucleotide sequence accession number.

The sequence of the cDNA of pCMV/N729-3010 has been deposited in the DDBJ/EMBL/Gene Bank DNA data bases under accession number D16435.

2.3 RESULTS

2.3.1 Production of HCV polyprotein in COS-1 cells

Productions of NS4A, p56(NS5A), p58(NS5A) and NS5B in the COS-1 cells transfected with pCMV/729-3010 were detected as described previously (22). Unprocessed NS

precursor polyprotein was produced in the COS-1 cells transfected with pCMV/N729-3010M1, encoding N729-3010M1 polypeptide in which Cpro-2 activity was inactivated by substitution of Ser-1165 to alanine residue (21, 22). Therefore, to analyze the mechanism of Cpro-2-dependent HCV polyprotein processing, the N729-1908, in which Cpro-1 (HCV proteinase 1) and Cpro-2 activities were intact but NS5A and the NS5B regions were not included (21) and the N729-3010M1 were coproduced in COS-1 cells as an enzyme and a substrate, respectively. The N729-1908M1 and N729-1908H952A polypeptides which are Cpro-2 and Cpro-1 defective mutants (21), respectively, were also used as a source of defective enzymes. When polypeptide for the substrate, N729-3010M1 and enzyme construct, N729-1908 or N729-1908H952A, were co-produced in the COS-1 cells, the production of p56 from the substrate polypeptide was detected while p58 was not (Fig. 2-2 A, lanes 7 and 9, upper panel). However, p56 was not produced from the substrate polypeptide co-produced with N729-1908M1, even though the enzyme polypeptide, p100 was detected (Fig. 2-2 A, lane 8, upper and lower panels). Production of NS5B was also observed in COS-1 cells co-producing the same enzyme/substrate combination (data not shown). These data indicated that p56(NS5A) was produced from the N729-3010M1 protein in a Cpro-2 *trans* cleavage activity-dependent manner. Since N729-1908H952A, which lacks the Cpro-1 activity (21), has the ability to produce p56(NS5A) from the substrate protein in a *trans* manner, the *trans* Cpro-2 activity appeared to be independent of the Cpro-1 activity

and the cleavage between NS2 and NS3. The *trans*-cleavage activity of Cpro-2 was also observed in an experiment in which the N2052-2951 polypeptide was used as a substrate and the size of the processed product detected by α-NS5A was 49 kDa (tentatively named p49) (Fig. 2-2 B, lane 2). The N-terminal residues of HCV-IIJ p56(NS5A) and NS5B, produced in insect cells using a baculovirus vector, were determined to be Ser-1972 and Ser-2420 in the precursor polyprotein by amino acid sequencing (23). Therefore, the substrate polypeptide contained only the NS5A/NS5B (abbreviated as 5A/5B) cleavage site and the 49 kDa product seems to be a polypeptide corresponding to the region from residue 2052 to residue 2419 of HCV precursor polyprotein.

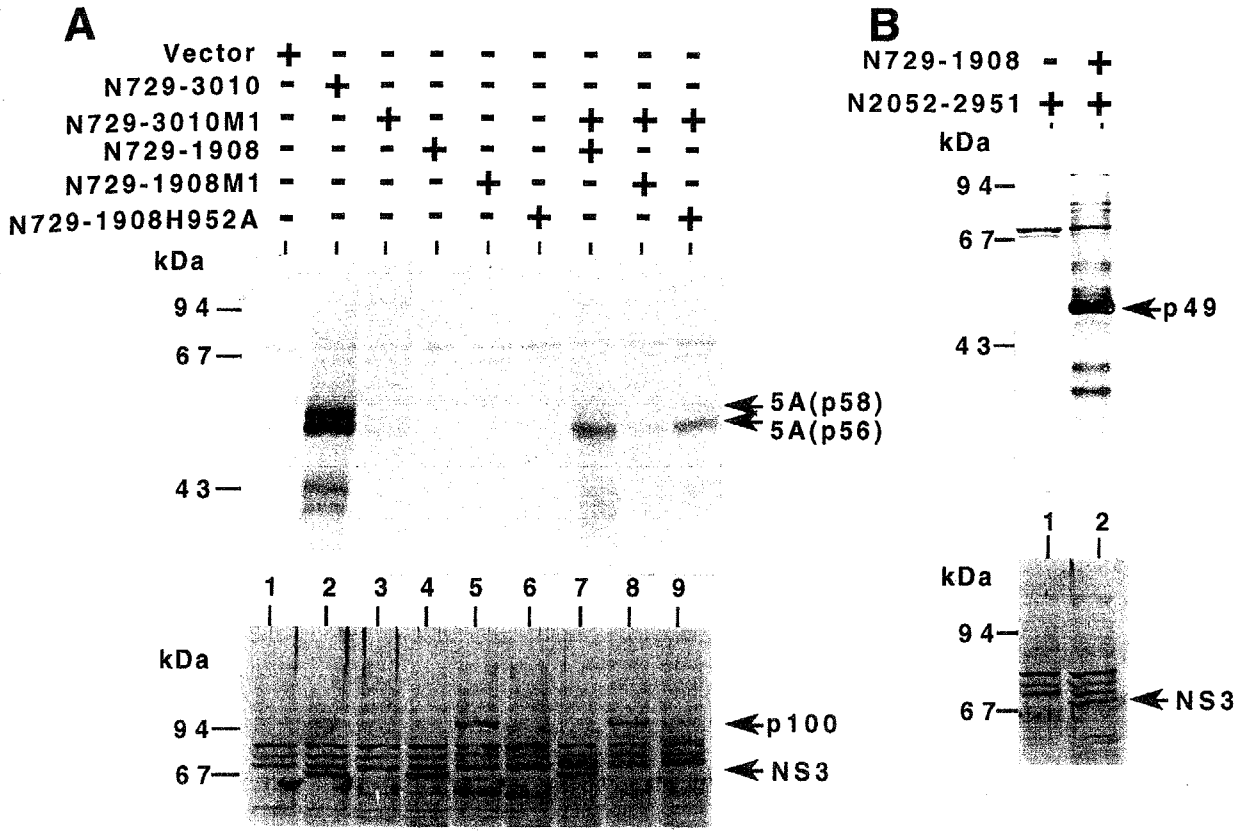


Fig. 2-2 Expression of HCV polyproteins in COS-1 cells for *trans*-cleavage assay.

Lysates of COS-1 cells transfected with the pCMV plasmids indicated above of the figure were fractionated on SDS-10% PAGE, followed by immunoblot analysis using polyvinylidene difluoride membranes (Millipore; Immobilon). Antibodies used in this experiment were α-NS5A (upper panel) and α-NS3 (lower panel).

2.3.2 Deletion mapping of the HCV serine proteinase.

The *trans* cleavage activity of Cpro-2 was further analyzed to determine the essential region for this activity. A series of deletion mutations in the N729-1908 polypeptide served as enzymes. The N2052-2951 polypeptide was used as a substrate and *trans*-cleavage activity of each mutants was determined by detection of p49 production. The *E. coli* DHFR protein was fused with the C-terminal end of each deletion mutant polypeptide to facilitate detection of the enzyme protein with α-DHFR (22) as shown in Figure 2.3 A and B lower panel. When N729-1325D, N729-1233D and N729-1215D were used as enzyme proteins, the p49 was detected by α-NS5A (Fig. 2-3 A, lanes 3, 5 and 7). However, this processed product was not detected in lysate in which N729-1174D was used as enzyme protein (Fig. 2-3 A, lane 9). These data indicated that the C-terminal part of the protein, downstream of residue 1216 of the HCV precursor polyprotein was not essential for the *trans*-cleavage activity of Cpro-2.

The NS3 is considered to have at least two distinct functions: a Cpro-2 activity and an RNA helicase activity (30). However, results suggested in this study that the possible RNA helicase region of NS3 was not necessary for Cpro-2 *trans* cleavage activity.

When N953-1233D, N1027-1233D and N1049-1233D, which were N-terminal deletion mutants of N729-1233D, were used as enzyme proteins, production of p49 from the substrate protein was detected (Fig. 2-3 B, lanes 3, 5 and 7, upper

panel). However, p49 was not processed from the substrate protein when N1075-1233D was used as an enzyme peptide (Fig. 2-3 B, lane 9, upper panel). From the results of these deletion analysis, Cpro-2 domain was supposed to be located from residue 1049 to residue 1215 of the HCV precursor polyprotein. When the region of this Cpro-2 domain (N1049-1215) was used as an enzyme in the *trans* cleavage assay, either with or without DHFR fusion, p49 was produced from the substrate, indicating that only this polypeptide region contained Cpro-2 *trans* cleavage activity (Fig. 2-3 C, lanes 3 and 5). The HCV Cpro-2 activity differed from the flaviviral serine proteinase in NS3 which was reported to require the presence of NS2B for the trans cleavage activity (7, 14). In addition, Cpro-2 location was determined to be within the N-terminal 167 amino acids of HCV NS3, from 1049 to 1215 amino acid of the HCV precursor polyprotein.

The region from residue 898 to residue 1233 of the HCV precursor polyprotein is shown to be necessary for the Cpro-1 activity (21). The data presented here indicated that Cpro-2 active domain was completely included in the region required for detection of Cpro-1 activity.

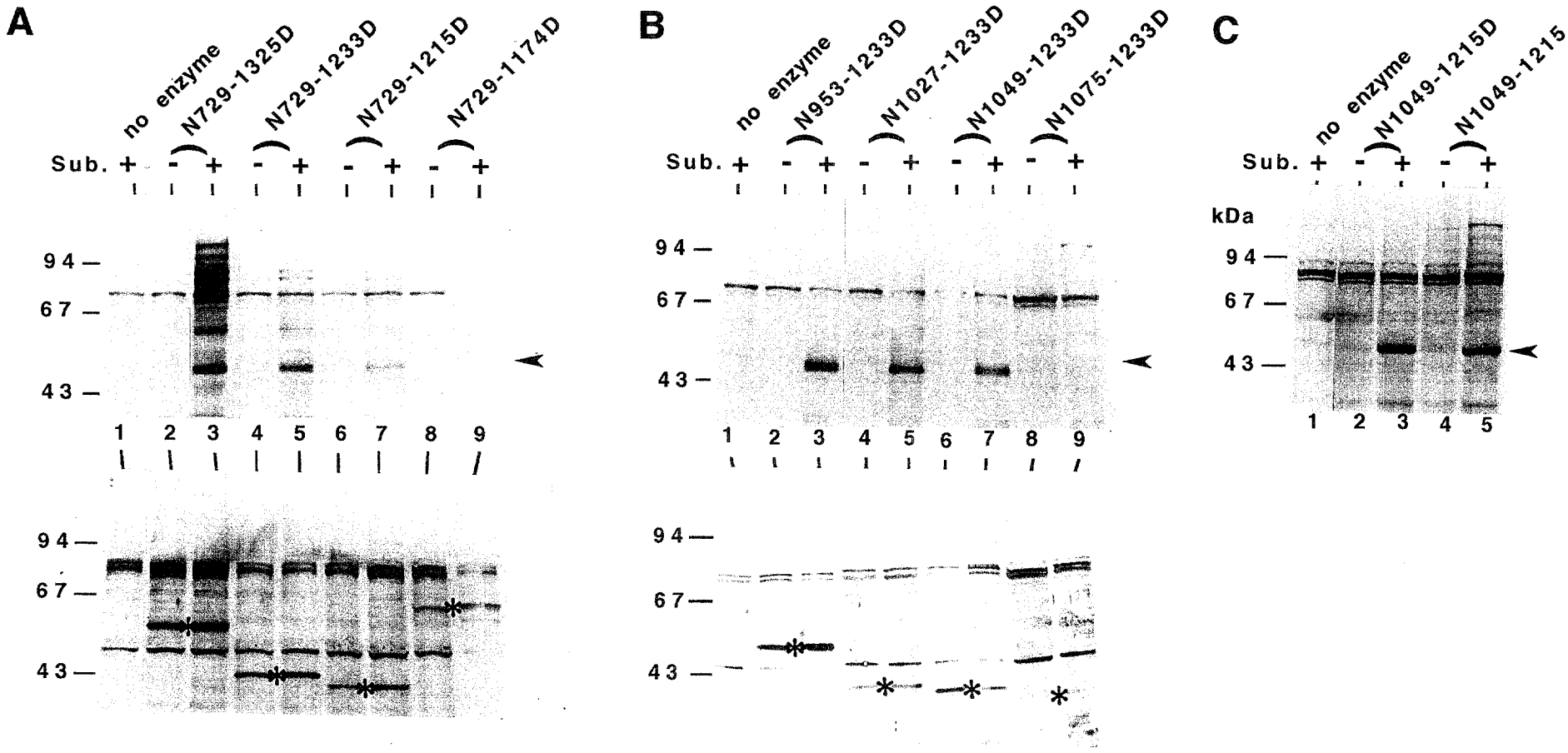


Fig. 2-3 Deletion mapping of the HCV Cpro-2.

Lysate of COS-1 cells transfected with plasmids encoding enzyme proteins in the absence (-) or presence (+) of pCMV/N2052-2951 were fractionated on SDS-10% PAGE and detected using α-NS5a (upper panel) and α-DHFR (lower panel) by the same method described in the figure 2-2 legend. Mapping of the HCV serine proteinase was performed by introducing deletion mutations into C-terminal regions (A) and N-terminal regions (B) around the predicted serine proteinase structure. Then, *trans*-cleavage activity of the mapped serine proteinase minimum domain was analyzed (C). The p49 and expressed enzyme proteins detected by α-DHFR are indicated by arrow heads (upper panel) and asterisks (lower panel), respectively.

2.3.3 Two cleavage modes of serine proteinase.

To study the mechanism of Cpro-2-dependent processing of the HCV precursor polyprotein, products cleaved from the substrate precursor protein, N729-3010M1, were analyzed in the *trans*-cleavage assay using N1049-1233D as an enzyme. In the cell lysate obtained from the COS-1 cells co-transfected with pCMV/N729-3010M1 and pCMV/N1049-1215D, a 74 kDa product was detected by either α-NS3 (21) or α-NS4A despite no detection of NS3 and NS4A, (Fig. 2-4 A, lane, 3, Fig. 2-4 B and 4 B', lanes 3). Apparently in this reaction, the NS4A/NS4B (abbreviated as 4A/4B) junction was cleaved but the NS3/NS4A (abbreviated as 3/4A) junction was not. It was reported that a continuous HCV NS3 region is required for Cpro-2-dependent 3/4A cleavage in an *in vitro* experiment (4). These facts suggested that the 3/4A junction is cleaved only by Cpro-2 auto-proteolytic activity (*cis* cleavage). In this study, while p56 and p66, which is located in the region downstream of NS4B/NS5A (abbreviated as 4B/5A), were produced in a *trans* cleavage reaction, production of p58 was not observed (Fig. 2-2A lanes 7 and 8, upper panel, Fig. 2-4C lane 3, Fig. 2-4D lane 3). Therefore, Cpro-2-dependent polyprotein processing seems to function in *cis*- and *trans*-cleavage modes.

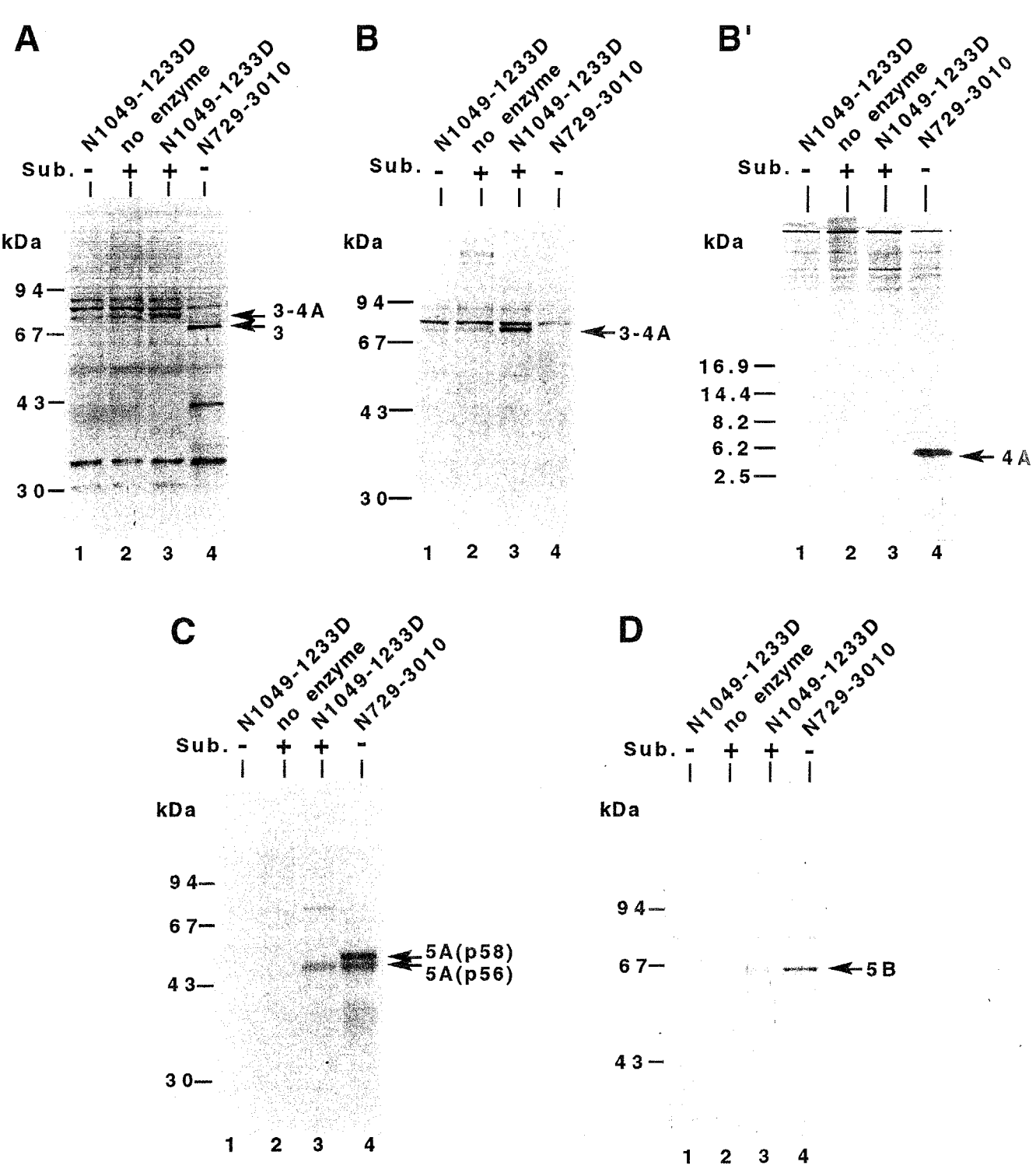


Fig. 2-4 Detection of processing products in Cpro-2-dependent *trans* cleavage manner.

The lysate of COS-1 cells transfected with pCMV/N1049-1233D (lane 1), pCMV/N729-3010M1 (lane 2), pCMV/N1049-1233D plus pCMV/N729-3010M1 (lane 3), or pCMV/N729-3010 (lane 4) were separated by SDS-10% PAGE (A, B, C, and D) or by tricine/SDS/16% PAGE (B'), and analyzed by immunoblotting using α-NS3 (A), α-NS4A (B, B'), α-NS5A (C), or α-NS5B (D).

2.4 Discussion

Amino acid sequences, Gly-Ser-Gly-Lys (1233 to 1236 amino acid) and Asp-Glu (1316 to 1317 amino acid) which are similar to the proposed NTP-binding sequence(Gly-X-Gly-Lys-(X)39-105-Asp-Glu) found in NS3 of flavivirus and in p80 of pestivirus, were located in the central region of NS3 of the HCV-IIJ. This region is considered to be involved in RNA-helicase activity (30, 16). A recent observation showed that a purified HCV polypeptide, corresponding to the region from residue 1193 to residue 1657 and produced in *E. coli*, has polynucleotide-stimulated nucleotide triphosphatase activity. Therefore, the NS3 is considered to have at least two distinct functions: a serine proteinase activity and an RNA helicase activity. However, results in this study suggested that the possible RNA helicase region in NS3 was not necessary for the Cpro-2 serine proteinase *trans* cleavage activity.

Comparing the amino acid sequences around the putative Cpro-2-dependent cleavage sites of the HCV polyprotein, a cysteine residue was found at the P1 position with the exception of the 3/4A junction where a threonine residue was positioned at the P1 site (18, 23). Further studies, for instance an analysis of the substrate specificity of the Cpro-2, may clarify the mechanisms of the two modes in Cpro-2-dependent processing of the HCV precursor polyprotein.

2.5 Summary of chapter 2.

A serine proteinase encoded in the HCV genome and named Cpro-2 is considered to be located in the N-terminal part of HCV NS3, one of the putative nonstructural proteins of HCV. Cpro-2 is suggested to be responsible for producing several kinds of nonstructural proteins by processing of the HCV precursor polyprotein. The active domain of Cpro-2 was identified and the mechanism of HCV polyprotein processing was clarified; various HCV mutants deleted around this Cpro-2 structure were co-produced with unprocessed HCV polypeptides containing Cpro-2-dependent cleavage sites in COS-1 cells. It was shown that Cpro-2 cleaved the HCV precursor polyprotein intramolecularly (*trans*) and that Cpro-2 domain which was necessary and sufficient for that cleavage mapped to within 167 amino acids, from Gly1049 to Ser1215 of the HCV precursor polyprotein.

Chapter 3

Kinetics and Mutagenic Analysis of Serine Proteinase-Dependent Cleavage

3.1 Introduction

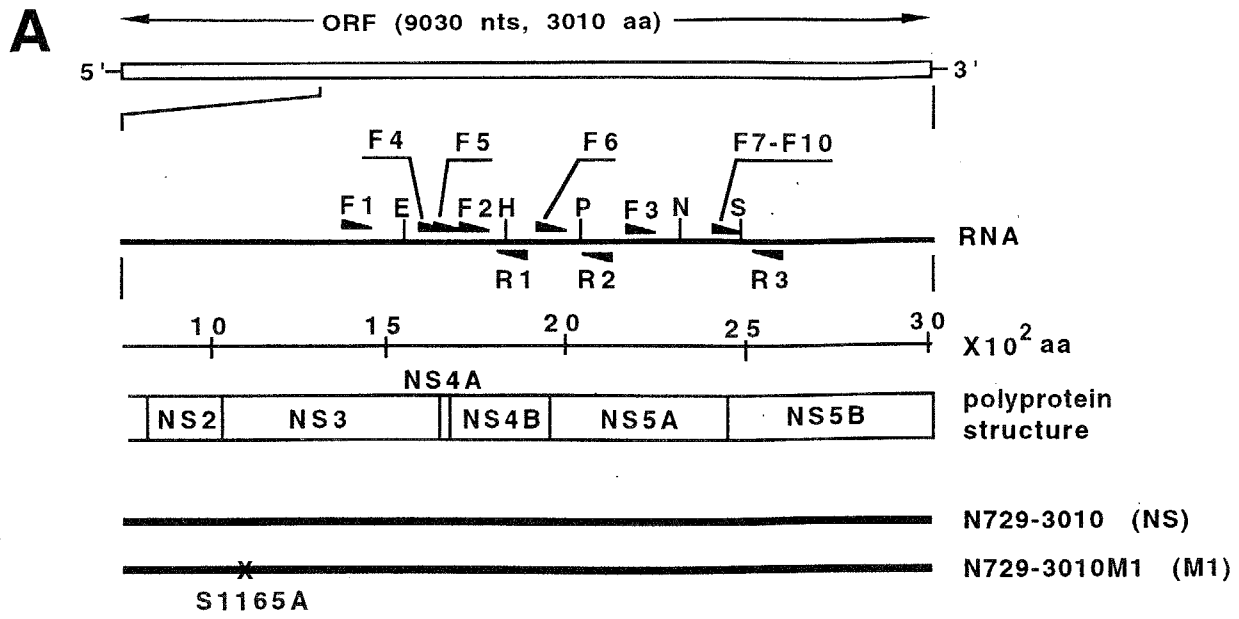
As indicated in the chapter 2, two different cleavage modes, *cis* and *trans* proteolytic cleavages, were proposed to be employed in the Cpro-2-dependent polyprotein processing. The 2/3 and 3/4A junctions were processed only in a *cis* cleavage manner. Furthermore, efficient cleavage at 4A/4B was shown to require the presence of the downstream NS5A region of the polyprotein (22). This result suggested that proteolytic cleavage at these processing sites might be strictly regulated. Partially or completely processed nonstructural proteins may associate with other nonstructural protein components (22) and thereby influence proteolytic processing at various steps in the pathway. To clarify this possibility, pulse chase experiment was conducted to determine the order of nonstructural protein production. Furthermore, the effect of amino acid mutation at each cleavage site on the processing pathway was also analyzed.

3.2 Materials and methods

3.2.1 Construction of plasmids.

Construction of plasmids pCMV/N729-3010 and pCMV/N729-3010M1 was described previously (22). Amino acid substitutions at the putative Cpro-2-dependent cleavage sites (18, 33), were introduced into the HCV polypeptides, NS and NSM1, encoded in pCMV/N729-3010 and pCMV/N729-3010M1, respectively, by using PCR. Sequences of the synthetic oligonucleotides (F1-F10 and R1-R3) used as primers in the PCR are indicated in Fig. 3-1B. Combinations

of primers were as follows: (F4-R1)-F1 for PCR product I; (F5-R1)-F1 for PCR product II; (F6-R1)-F1 for PCR product III; (F7-R2)-F2 for PCR product IV; (F8-R3)-F3 for PCR product V; (F9-R3)-F3 for PCR product VI; (F10-R3)-F3 for PCR product VII. The first PCR used the primers indicated in the parenthesis, then the resultant PCR product was used as a primer together with the third primer indicated at right side of the primer combinations, in the second PCR. To introduce the amino acid substitutions into the putative 3/4A and 4A/4B sites, the *Eco*T22I-*Hpa*I fragment of pCMV/N729-3010 were replaced by the *Eco*T22I-*Hpa*I fragments of the PCR products I and II, respectively. The respective resultant clones named pCMV/NS3/4NN and pCMV/NS4A/BNN produce polyproteins with aspargines substituted at positions p1 and p1' of 3/4A and 4A/4B sites, respectively. Plasmid pCMV/NS4/5NN was made by replacing the *Hpa*I-*Pst*I fragment of the pCMV/N729-3010 with the *Hpa*I-*Pst*I fragment of the PCR product III; this plasmid produces a polyprotein mutated at the p1 and p1' positions of 4B/5A site to aspargines. Mutations at the 5A/5B site were made by replacing the *Nhe*I-*Stu*I fragment of pCMV/N729-3010 with the *Nhe*I-*Stu*I fragments of the PCR products IV, V, VI, and VII, and were named pCMV/NS5A/BP1A, pCMV/NS5A/BP1N, pCMV/NS5A/BP1'N, and pCMV/NS5A/BNN, respectively. A plasmid pCMV/NS5A/BP1A produces a polyprotein mutated to alanine at P1 position of 5A/B site. Plasmids pCMV/NS5A/BP1N, pCMV/NS5A/BP1'N and pCMV/NS5A/BNN produce polyproteins mutated at P1, P1', and P1 plus P1' positions of 5A/B site to aspargines, respectively.



B

F1 : 5'-ACATGTGTCACCCAGACGGGT-3'
F2 : 5'-CTCGTGGCTTTTAAGG-3'
F3 : 5'-GTGGCAGTGCTCACTTCCAT-3'
F4 : 5'-CTGGAGGTCGTCAATAACACCTGGGTGCTG-3'
F5 : 5'-GAAATGGAAGAGAACAACTCACACCTCCCT-3'
F6 : 5'-TGCTCCACGCCAAACAACGGCTCGTGGCTA-3'
F7 : 5'-GACATCGTCTGCGCCTCAATGTCCTACACA-3'
F8 : 5'-GACATCGTCTGCAACTCAATGTCCTACACA-3'
F9 : 5'-GACATCGTCTGCTGCAACATGTCCTACACA-3'
F10 : 5'-GACATCGTCTGCAACAACATGTCCTACACA-3'
R1 : 5'-ACACACGATCCCGACGAC-3'
R2 : 5'-TGTGGTGTACGCGTTGAT-3'
R3 : 5'-GTCTTCCAGCAAGTCCTC-3'

Fig. 3-1 Schematic representation of HCV polyprotein structure, and oligonucleotides used to introduce mutations.

The genomic and polyprotein structures of the NS region from p21(NS2) to p66(NS5B) are shown enlarged below the HCV ORF with the scale for the amino acid position. The locations of *Eco*T22I (E), *Hpa*I (H), *Pst*I (P), *Nhe*I (N), and *Stu*I (S) restriction sites are shown on viral RNA. The positions of the primers used to introduce mutations into the original plasmids, pCMV/N729-3010, are indicated on the coding region of viral RNA with the half arrow heads, and sequences of those primers are shown in B. Underlined nucleotides indicate base changes introduced into primers. The position of the Ser to Ala mutation in N729-3010M1 is depicted by X.

3.2.2 Production of HCV polyproteins in COS-1 cells.

DNA transfections were performed as described in the chapter 2. Lysates of COS-1 cells transfected with the series of pCMV derived plasmids were fractionated by SDS-8% PAGE, followed by immunoblot analysis as described in the chapter 2. Antibodies used in this experiment were α-NS3, α-NS4A, α-NS4B, α-NS5A, and α-NS5B (21, 22, 36).

3.2.3 Pulse chase analysis.

After incubation for one day in a 35 mm dish, COS-1 cells transfected with a series of pCMV derived plasmids were used for pulse chase analysis. Cells were incubated in 0.5 ml methionine free Eagles' minimum essential medium (MEM, Flow Lab.) with 5% dialyzed-FCS for 1 hour, then the cells were labeled for 15-min in the medium supplemented with 400 μCi/ml[^{35}S]-methionine (ICN). After the pulse labeling, cells were either lysed immediately in 100 μl Laemmli's sample buffer without dye or lysed after following a chase for various times in Dulbecco's modified Eagle medium containing methionine and cysteine (75 μg/ml each).

3.2.4 Immunoprecipitation.

Cell lysates metabolically labeled with [^{35}S]-methinonine were boiled and diluted 10-fold with buffer (50 mM Tris-hydrochloride [pH 7.5]-150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF(phenyl methyl sulfonyl fluoride)). One ml of the diluted lysates was pre adsorbed with 50 μl

protein-G sepharose suspension (Pharmacia) and incubated with α-NS3, α-NS4A, α-NS4B, α-NS5A, and α-NS5B for 1 hour. The immunocomplex was recovered by the addition of 30 μl protein-G sepharose and was washed three times with one buffer (50 mM Tris-hydrochloride [pH 7.4], 500 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 5% sucrose) and once with another buffer (10 mM Tris-hydrochloride [pH 7.4], 50 mM NaCl, 1 mM EDTA). The immunoprecipitates were analyzed by 8% SDS-PAGE or tricine-sodium dodecyl sulfate-16% polyacrylamide gel electrophoresis (tricine/SDS/16% PAGE) (47). The gels were dried and were exposed to imaging plate (FUJI PHOTO FILM CO., LTD). The relative radioactivities of the immunoprecipitated [^{35}S]-methionine labeled products were calculated by a Bio Image Analyzer (BAS2000, FUJI PHOTO FILM CO., LTD) (2).

3.3 Results

3.3.1 Pulse chase analysis.

To study the kinetics of Cpro-2-dependent HCV polyprotein processing, COS-1 cells transfected with pCMV/N729-3010, which encodes the entire HCV putative nonstructural proteins region (22), were used in pulse chase analysis. The transfectant was pulse-labeled for 15 min with [^{35}S]-methionine, and chased for various times after addition of excess amounts of cold methionine. The cell lysates at chase time points of 0, 20, 60, 180, and 360 minutes were immunoprecipitated using α-NS3, α-NS4A, α-NS4B, α-NS5A and α-NS5B (Fig. 3-2). Production of NS3 in the lysate was detected by α-NS3 at 0 minutes of the chase

period and no precursor protein reactive with α-NS3 was detected. The relative abundance of NS3 was almost constant during 360-minutes chase, indicating that the processing of NS3 from the precursor polyprotein was almost completed within the 15 minutes pulse period and that NS3 was stable in the cell (Fig. 3-2A). Although the production of NS5B was detected by α-NS5B at the start of the chase, a small amount of precursor polypeptide of NS5B (p150 and p125) was detected, suggesting that processing to NS5B was not completed by the start of chase (Fig. 3-2E, lane 3). However, after 20 minutes of chase, only an increased amount of NS5B was detected, suggesting that processing of NS5B was almost completed by this time. On the contrary, productions of NS4A, NS4B, and NS5A, which were detected by α-NS4A, α-NS4B, and α-NS5A, respectively, were low at the start of the chase time, while 89 kDa protein (named p89) was immunoprecipitated by these three antibodies (Fig. 3-2, B, C, and D, lane 3). Since the size of this polypeptide, 89 kDa, was almost identical to the sum of the molecular masses of NS4A, NS4B, and NS5A, this polypeptide was likely to be a processing intermediate composed of these three viral proteins. The amount of p89 gradually decreased throughout the 180-minutes chase time, and disappeared at 360-minutes (Fig. 3-2B, C, and D, lanes 3-7). Quantitation of radioactivities of p89, p31, and NS4B immunoprecipitated by α-NS4B at each chase period (Fig. 3-2C) is shown in Figure 3-3A, and radioactivities of p89, p58, and p56 immunoprecipitated by α-NS5A at each chase period (Fig. 3-2D) are also shown in Figure 3-3B. During the chase,

production of both p58 and p56 increased from time 0 to 180 minutes and reached almost plateau levels. The decrease in the amount of p89 was accompanied by an increase in the amount of NS5A during the chase time from 20 to 180 minutes, and the level of p89 at 180 minutes became almost undetectable. Similarly, the decrease in the amount of p89 was accompanied by an increase in the level of NS4B (Fig. 3-3B). Because p31 is composed of NS4B and NS4A (22), the decrease of the p31 level is related in the increase of NS4A and NS4B levels (Fig. 3-2B' and C). An 85 kDa protein which was expected to be produced when the cleavage between 4A/4B proceeds prior to the cleavage between 4B/5A, was not detected in the chase period. Instead, the 31 kDa product of NS4A-NS4B was detected by both α-NS4A and α-NS4B (Fig. 3-2B, B', and C) early in the chase. For the first 60 minutes of the chase time, p31 was detectable and was not present at 180 minutes. The further processed products, NS4A and NS4B, accumulated at the late stage of the chase period. These observations suggested that p89 is processed into NS4A, NS4B, and NS5A through successive processing steps. It appeared that p89 was processed into NS5A and p31; p31, in turn, was cleaved into proteins NS4A and NS4B. Relative production of p58 and p56 were unchanged during the chase period from 60 to 360 minutes and the level of p56 was about two fold higher than that of p58 (Fig. 3-3B).

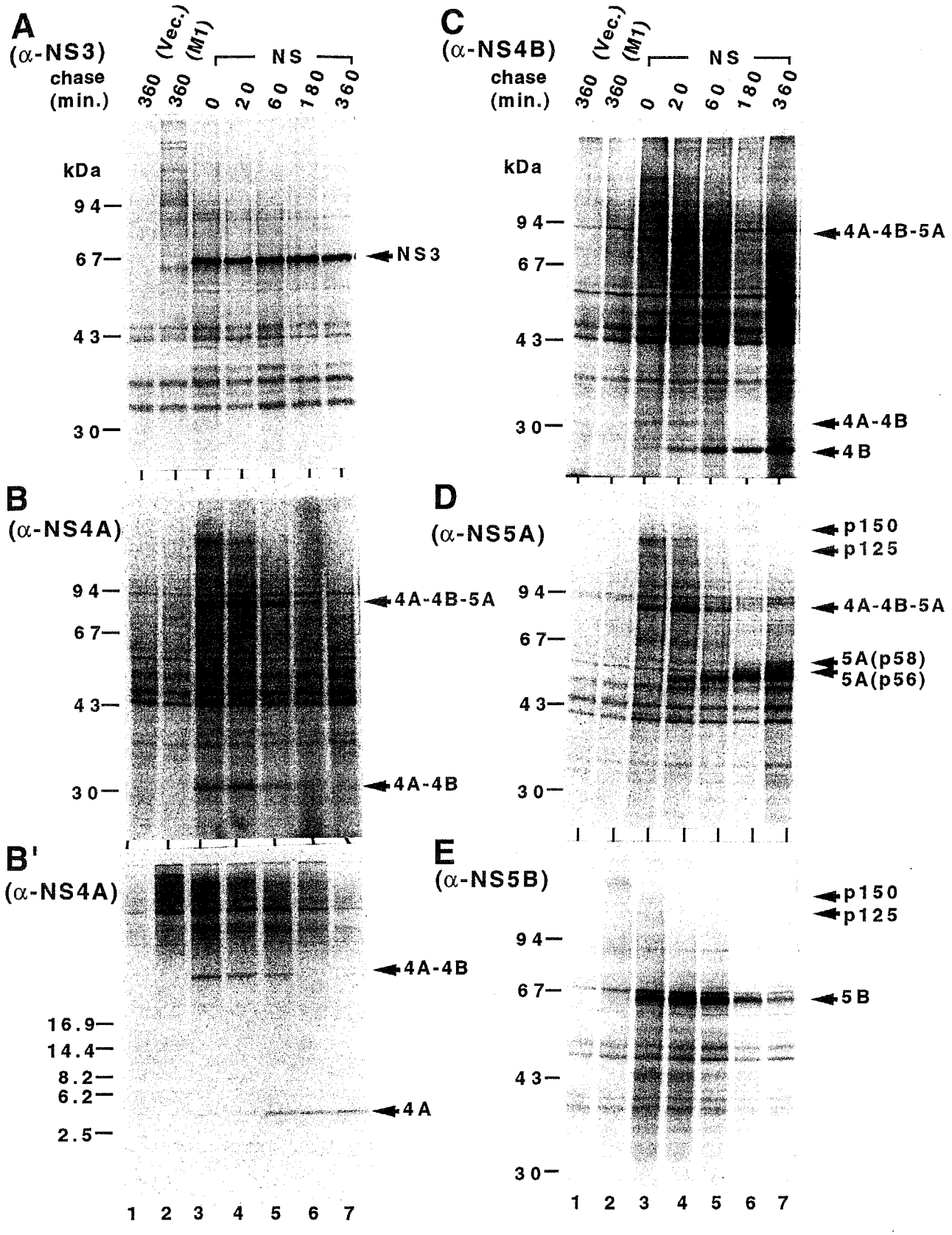

A
(α-NS3)
chase (min.)
360 (Vec.)
360 (M1)
NS
0
20
60
180
360
kDa
94
67
43
30
NS3
B
(α-NS4A)
4A-4B-5A
4A-4B
B'
(α-NS4A)
4A-4B
16.9
14.4
8.2
6.2
2.5
4A
1 2 3 4 5 6 7
C
(α-NS4B)
4A-4B-5A
4A-4B
4B
D
(α-NS5A)
p150
p125
4A-4B-5A
5A(p58)
5A(p56)
E
(α-NS5B)
p150
p125
5B

Fig. 3-2 Pulse chase analysis.

Cells were pulse labeled for 15 min, then chased as indicated. The lysate at each time point was immunoprecipitated with: α-NS3, panel **A**; α-NS4A, panel **B** and **B'**; α-NS4B, panel **C**; α-NS5A, panel **D**; and α-NS5B, panel **E**. Immunoprecipitation was performed with lysates transfected with: pKS(+)/CMV, an original plasmid without HCV insert, lane 1; pCMV/N729-3010M1, a Cpro-2 defective mutant, lane 2; and pCMV/N729-3010, lanes 3-7; Gel conditions were 8% SDS-PAGE for panels **A**, **B**, **C**, **D**, and **E**; and tricine/SDS/16% PAGE for panel **B'**. Molecular mass markers (in kDa) are shown on the left. The positions of processing products, NS3, NS4A, NS4B, NS5A, and NS5B, and precursor polyproteins, 4A-4B and p89(4A-4B-5A), are shown on the right with arrows.

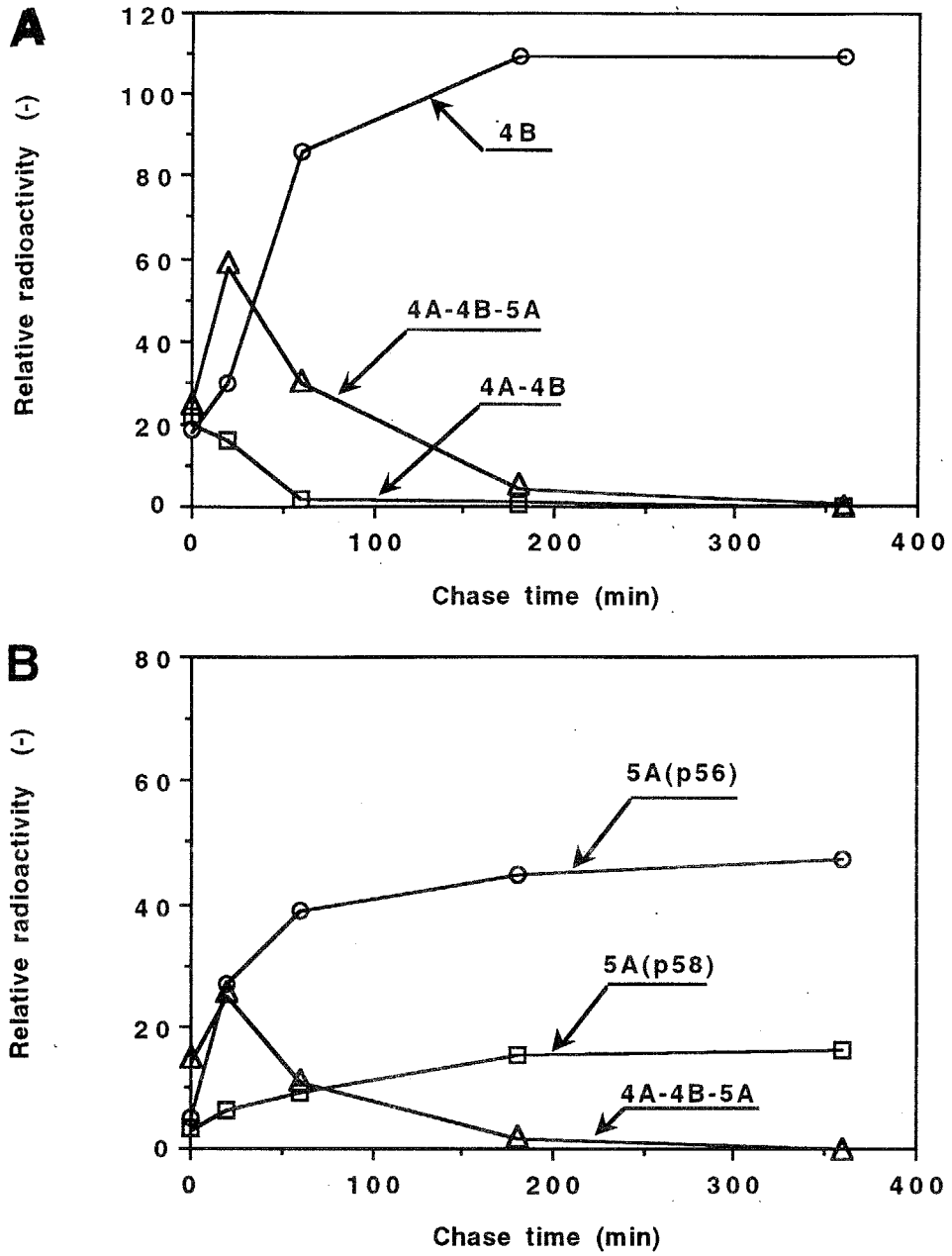


Fig. 3-3 Time courses of NS4B and NS5A(p58/p56) productions.

The relative radioactivities of the processing precursor polyprotein and final products were plotted. Panel A shows time course of relative radioactivity obtained from data of Fig. 3-2, panel C lanes 3-7. Open circle represents NS4B; open triangles, NS4A-4B-5A(p89) and open squares, NS4A-4B(p31). Panel B shows time course of relative radioactivity obtained from Fig. 3-2, panel D, lanes 3-7. Open circle represents 5A(p56); open squares, 5A(p58) and open triangle, NS4A-4B-5A(p89).

3.3.2 Mutation analysis.

To investigate the effect of mutations at each cleavage site on processing of the other sites, amino acid substitutions were introduced into each of the Cpro-2 dependent cleavage sites in the HCV polyprotein encoded by pCMV/N729-3010. Cleavage at the 5A/5B site occurred rapidly in the transient expression system using COS-1 cells as mentioned above, and in *E. coli* the amino acid substitutions in the P1 position (Cys) of this site to Ala or Asn are not cleaved by Cpro-2 (33). Therefore, the same amino acid substitutions were introduced in the cleavage site of the construct that could be analyzed in a eukaryotic transient expression system (Fig. 3-4A). When polyproteins, NS5A/BP1A and NS5A/BP1'N, encoded in pCMV/NS5A/BP1A and pCMV/NS5A/BP1'N, respectively, were produced in COS-1 cells, production of NS5B was unexpectedly observed. The cleavage seemed to be as efficient as that of wild type polyprotein, because there was no detectable amount of precursor polypeptide. This suggests that the substitutions with Ala and Asn at positions P1 and P1', respectively, do not dramatically impair the cleavage at the 5A/5B site (Fig. 3-4A, lanes 4 and 6). This observation was a contrast to that was seen in an *E. coli* expression system. However, when NS5A/BP1N and NS5A/BNN whose polyproteins have Asn substitution at the P1 and the P1 plus P1' positions, respectively, were expressed in COS-1 cells, very little of the NS5B protein and a significant amount of a possible 125 kDa processing intermediate accumulated (Fig. 3-4A, lanes 5 and 7). This

result indicated that the cleavage at this site was diminished in these mutated polypeptides. More of the 125 kDa protein was found in the pCMV/NS5A/BNN transfectant than in the pCMV/NS5ABP1N transfectant, which indicated that substitutions of Asn at both P1 and P1' of the putative cleavage site 5A/5B is likely to be more deleterious for Cpro-2 cleavage than that of single amino acid substitution at position P1. Effect of mutation at positions P1 and P1' of other sites for Cpro-2 cleavage was further analyzed. Amino acids substitutions to aspargine at the both P1 and P1' positions of the putative 3/4A, 4A/4B, 4B/5A cleavage sites of the nonstructural precursor polyprotein were independently introduced and cleavage at each site as well as the effects on the other cleavage sites was analyzed. The level of production of NS3, NS4A, and NS4B from NS5A/BNN was almost same as that from N729-3010. Thus, this mutant polypeptide probably does not impair other cleavages at 3/4A, 4A/4B, and 4B/5A sites (Fig. 3-4B, C, and E, lane 7). Since NS3/4NN encoded in pCMV/NS3/4NN produced NS3 and NS4A (Fig. 3-4B and E, lane 4), cleavage of the 3/4A site was not abolished in this mutant polypeptide. However, relative production of p58 to p56 from NS3/4NN was reduced (Fig. 3-4F, lane 4). NS4A/BNN encoded in pCMV/NS4A/BNN showed cleavages at 3/4A, 4B/5A, and 5A/5B sites which were as efficient as that of the wild type polyprotein, while the cleavage efficiency at 4A/4B was apparently reduced (Fig. 3-4B-G, lane 5). The accumulation of an uncleaved product of p31 was observed in the lysate of the transformant (Fig. 3-4C, D, and E, lane

5). The production of p58 from the mutant polypeptide NS4A/BNN was also reduced as seen in NS3/4NN (Fig. 3-4F, lane 5). Production of NS3, NS4A, and NS5B from NS4/5NN encoded in pCMV/NS4/5NN, suggested that cleavage at 3/4A, 4A/4B, and 5A/5B occurred efficiently in this polyprotein (Fig. 3-4B, E, and G, lane 6). In contrast, NS4/5NN generated an 85 kDa polyprotein (4B-5A) that was detected by α-NS5A (Fig. 3-4F, lane 6). This may imply a reduced efficiency of 4B/5A cleavage. These results suggested that the decrease of cleavage efficiencies at the 4A/4B, 4B/5A, and 5A/5B sites do not impair cleavages of the other sites except that the substitutions of amino acid at 3/4A and 4A/4B sites reduce the production of p58.

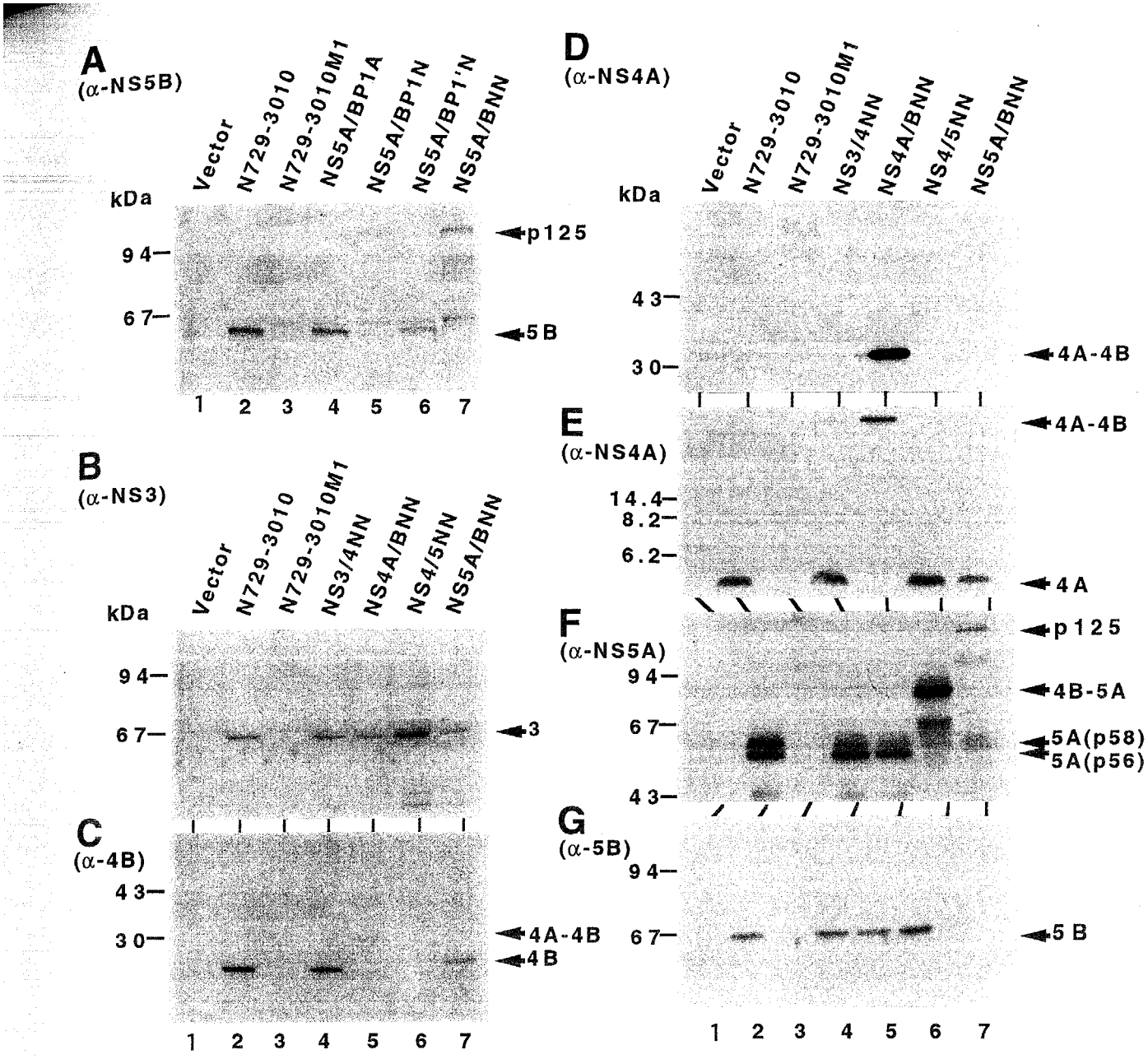


Fig. 3-4 Mutation analysis of the HCV precursor polyprotein processing.

Lysate of COS-1 cells transfected with the series of pCMV derived plasmids were fractionated on 8% SDS-PAGE (panels A, B, D, F, and G), 12% SDS-PAGE (panel C), or tricine/SDS/16% PAGE (panel E). Vector indicates the lysate of COS-1 cells transfected with pKS(+)/CMV. Antibodies used in this experiment were : α-NS5B, panel A and G; α-NS3, panel B; α-NS4B, panel C; α-NS4A, panel D and E; and α-NS5A, panel F. Molecular mass markers (in kDa) are shown on the left.

3.3.3 Possible pathway of HCV nonstructural protein processing.

A summary of possible HCV nonstructural polyprotein processing is shown in Figure 3-5. By the cleavages at 2/3 and 3/4A, NS3 was produced with the precursor protein p150 (NS4A-4B-5A-5B). Then, processing proceeded according to the order as follows. NS5B and precursor protein p89 (NS4A-4B-5A) were processed out by the cleavage at 5A/5B. Two forms of NS5A, p58 and p56, were produced from p89. Finally p31 (NS4A-4B) precursor protein splited into NS4A and NS4B.

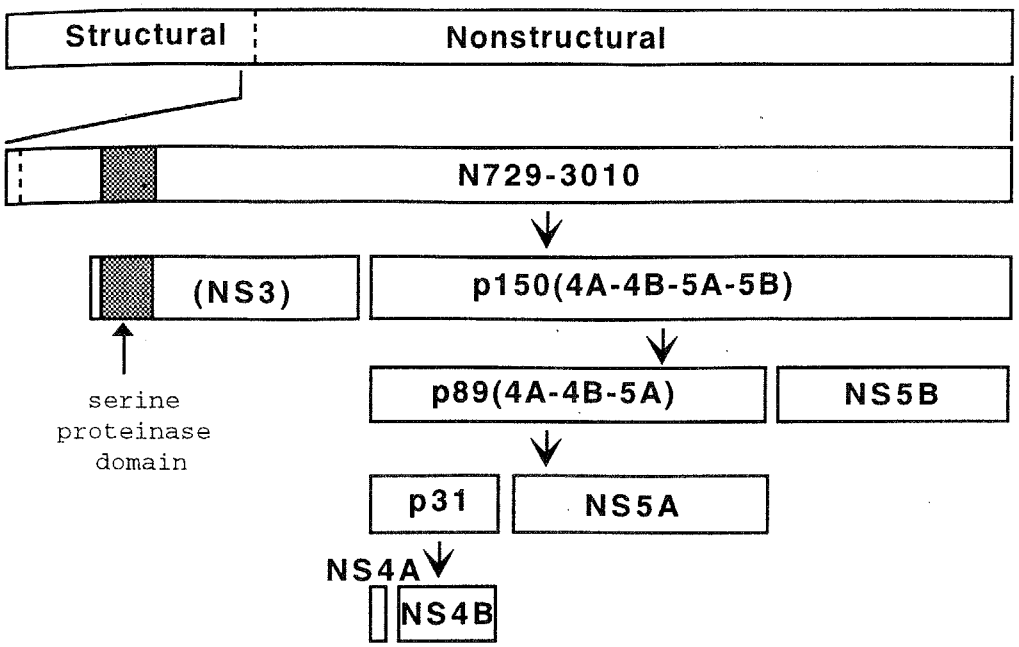


Fig. 3-5 Schematic illustration of the possible cleavage pathway of the polyprotein N729-3010.

Putative structural and nonstructural regions of HCV polyproteins are illustrated on the top. Dashed lines represent putative border between structural and nonstructural proteins. Polyproteins derived from pCMV/N729-3010 are indicated by a box in the second row from top with the name of primary product. The minimum domain required for Cpro-2 is indicated by shading.

3.4 Discussion.

From the analysis of genetic organization of HCV proteins, evidence is accumulating, which shows that HCV belongs to the family Flaviviridae in a genus apart from the flavivirus and pestivirus genera (37). In HCV at least 4 viral proteins are produced from the region downstream of NS3 (18, 21, 36, 54) whereas three and four proteins are produced from the corresponding region in flavivirus and pestivirus, respectively. The molecular sizes of the nonstructural proteins encoded by these viruses are different from each other. The NS5 and NS3 proteins share partial amino acid sequence similarity with other members of the Flaviviridae; in spite of this, the other nonstructural proteins are dissimilar. The processed structural proteins of HCV showed a similar genetic organization to that of the pestiviruses, suggesting a closer relationship to the genus pestivirus than to flavivirus (30, 49). However, this study disclosed that proteolytic processing of the HCV nonstructural polyprotein had a significantly different kinetic pattern from that of the pestiviruses, which supports the previously proposed classification of HCV. Bovine virus diarrhea virus (BVDV), a pestivirus, encodes 4 proteins downstream of a putative proteinase, the NS3 product. These are p10-p32-p58-p75 in this order from the C-terminus of NS3 (56), and p75 is likely to be RNA polymerase. Processing of p75 occurs in the late stage of nonstructural polyprotein processing (1) while the corresponding protein in HCV, NS5B, is produced during the early stage of proteolytic processing. HCV NS3

which has Cpro-2 activity, is produced by auto-catalytic cleavages. The N-terminus is cleaved by Cpro-1 whose active domain is presented between the C-terminus of NS2 and the N-terminus of NS3 regions (21). The C-terminus of NS3 was cleaved by the NS3 product in a *cis*-acting manner (chapter 2). In pulse-chase experiments with the HCV nonstructural polyprotein, maximum production of NS3 and NS5B was observed as early as the beginning of the chase period (Fig. 3-2). An 89 kDa protein, a processing intermediate composed of NS4A-4B-5A, was still detected at an early stage of the chase period. Assuming that the level of p89 was maximal at 20 minutes of chase time and that no further processing to this product occurred, then a half-life of p89 can be calculated to be about 100 minutes (Fig. 3-3A). Processing of p31 to NS4A and NS4B, seems to proceed faster than that of p89, suggesting that cleavage of p89 to p31 and NS5A was the rate-limiting step in processing of the HCV polyprotein. The region of NS5A promotes cleavage of 4A/4B (22). This may imply that NS5A acts efficiently to enhance the cleavage of this site in a trans-acting manner.

Because no polyprotein composed of NS4B-5A was detectable, it appears that the cleavage between 4B/5A occurs prior to the processing of 4A/4B. Thus, the 89 kDa protein was initially cleaved to form p31 composed of NS4A-4B, and NS5A, and then p31 was subsequently cleaved to form NS4A and NS4B. Cleavages at 2/3 and 3/4A, which are presumed to be cleaved by Cpro-1 and Cpro-2, respectively (20, 24), seemed to take place early in proteolytic processing pathway to produce NS3 and a putative precursor

polyprotein, p150. Further processing seemed to produce NS5B and the p89 precursor polyprotein.

Mutation analysis of the cleavages by Cpro-2 described in this work showed that mutations at both P1' and P1 positions in each Cpro-2 cleavage site reduced the level of its cleavage efficiency, but did not affect cleavage of other sites. The only exception was the reduced production of p58 from the polyproteins with mutations at the 3/4A or 4A/4B site. Because the mutation at the 3/4A site did not affect its cleavage efficiency, the reduction of p58 production was not simply due to reduction of any of the processed products. It was likely to be caused by some structural change of mutated precursor polyprotein. It was also possible that a slightly different structure of the NS3 or NS4A products produced from this mutated polyprotein may affect the production of p58. Alternately p58 might be a modified protein of p56 and such a modification might be suppressed in the products derived from mutated polyproteins at 3/4A and 4A/4B.

The difference in susceptibility to Cpro-2 of the mutated 5A/5B cleavage sites in COS-1 cell and in *E. coli* remains to be solved. Cleavage of this site by Cpro-2 expressed in *E. coli* was assayed using a chimeric protein composed of the maltose binding protein, 30 amino acids HCV sequence containing the 4A/4B junction and DHFR in this order from the N-terminus (33). Thus it may possible that conformation of the cleavage site in the chimeric substrate might differ from that used in this work. Alternately an additional viral protein(s) produced in this expression

system might change the biological character of Cpro-2 or the substrate.

In vitro translation/processing analysis of the HCV nonstructural polyprotein shows that NS4A helps to anchor NS3 on the surface of microsomal membranes (22). Furthermore, this complex is likely to mediate association with NS4B, NS5A and NS5B to form a complex structure (22) which may be involved in virus replication. Based on these observations, Cpro-2 dependent processing may be located on membrane. During the cleavage process, the association of various protein precursors with their products in assorted stage of processing may increase the efficiency of cleavage by Cpro-2 and may in effect regulate viral replication.

3.5 Summary of chapter 3.

HCV encoded Cpro-2 is suggested to be responsible for the processing of the region downstream of NS3 in the precursor polyprotein of HCV IIJ. At least 4 or 5 putative nonstructural proteins are located within the precursor polyprotein downstream of NS3; their order from amino terminus is NS4A, NS4B, NS5A and NS5B. To clarify the mechanism of Cpro-2-dependent processing of this region, pulse chase and mutation analyses were performed by using a transient protein production system in cultured cells. Pulse chase study revealed the sequential production of these nonstructural proteins. Productions of NS3 and NS5B were rapid and nearly completed within the 15 minutes pulse labeling period. An 89 kDa processing intermediate protein was observed during the early part of the chase. NS4A,

NS4B, and NS5A probably matured mainly through this 89 kDa protein intermediate. The 89 kDa intermediate seemed to be cleaved first into a 31 kDa protein and NS5A. The 31 kDa polypeptide was further processed into NS4A and NS4B. From estimation of the half-lives of the 89 kDa and 31 kDa intermediates, the rate limiting step of Cpro-2-dependent HCV polyprotein processing appeared to be the processing of the 89 kDa intermediate protein. Mutation analysis of the putative cleavage sites revealed that, with the exception of p58(NS5A) production, cleavage at each site was essentially independent from cleavage occurring at the other site.

Chapter 4

Versatile Functions of NS4A

4.1 Introduction.

In vitro transcription/translation analysis of the HCV nonstructural polyprotein showed that NS4A helps to anchor NS3 to the surface of the microsomal membrane (22). Furthermore, this complex associates with NS4B, NS5A, and NS5B to form a complex structure which may be involved in virus replication. These observations imply that NS4A plays an important role in this complex formation. Recently, it was reported that NS4A is necessary for cleavage at the 4B/5A site, and enhances cleavage at NS3-dependent cleavage sites (5, 13).

For this report, processing of a mutated NSΔ4A polypeptide which encodes the entire putative HCV nonstructural polyprotein except the region of NS4A was analyzed. Versatile functions of NS4A were revealed. NS4A acts not only in the cleavage at the 4B/5A site, but also in the stabilization and localization of NS3. Stoichiometrical assay and deletion analysis were also carried out to reveal the functions of NS4A.

4.2 Materials and methods.

4.2.1 Construction of plasmids.

The construction of plasmids pCMV/N729-3010 and pCMV/N1049-1215 has been described previously (21) and in the chapter-2. Combinations of positive and a negative stranded primers used for PCR (mentioned below) are indicated in parentheses after the PCR product. To obtain plasmid pCMV/NSΔ4A which encodes the entire HCV nonstructural polyprotein except NS4A, the *Bam*HI-*Pst*I

fragment of pCMV/N729-3010 was inserted into the *Bam*HI-*Pst*I site of pUC18 and used as a template for making PCR product-1 (F1, M13-RV primer) and PCR product-2 (M13-universal primer, R1). Both PCR products were ligated after phosphorylation and digested with *Bam*HI and *Pst*I, and then inserted into the *Bam*HI-*Pst*I site of pUC18. The resultant plasmid was pUC18/Δ4A which carries the *Bam*HI-*Pst*I fragment of pCMV/729-3010 except for the region encoding NS4A. pCMV/NSΔ4A was obtained by replacing the *Bam*HI-*Pst*I fragment of pCMV/N729-3010 with the *Bam*HI-*Pst*I fragment of pUC18/Δ4A. PCR product-3 (F2, R2) was digested with *Pst*I-*Eco*RI and inserted into the *Pst*I-*Eco*RI site of pKS(+)/CMV (21) to obtain pCMV/N1658-1711 which encodes NS4A. To obtain plasmids that produce proteins with 30 amino acids around the 4B/5A or 5A/5B cleavage sites fused in-frame to their C-termini by an *E. coli* DHFR, PCR product-4 (F3, R3) and product-5 (F4, R4) were digested with the *Pst*I and *Hin*dIII and were replaced with *Pst*I-*Hin*dIII fragment of pCMV/N729-1233D (chapter 2). The resultant plasmids were pCMV/N1953-1982D and pCMV/N2400-2429D, respectively. Deletion mutants of NS4A were constructed by replacing the *Pst*I-*Eco*RI fragment of PCR product-6 (F5, R2), product-7 (F6, R2), product-8 (F7, R2), product-9 (F2, R5), product-10 (F2, R6), and product-11 (F2, R7) with the *Pst*I-*Eco*RI fragment of pCMV/N1658-1711. The resultant plasmids were pCMV/N1668-1711, pCMV/N1678-1711, pCMV/N1688-1711, pCMV/N1658-1697, pCMV/N1658-1687, and pCMV/N1658-1677, respectively.

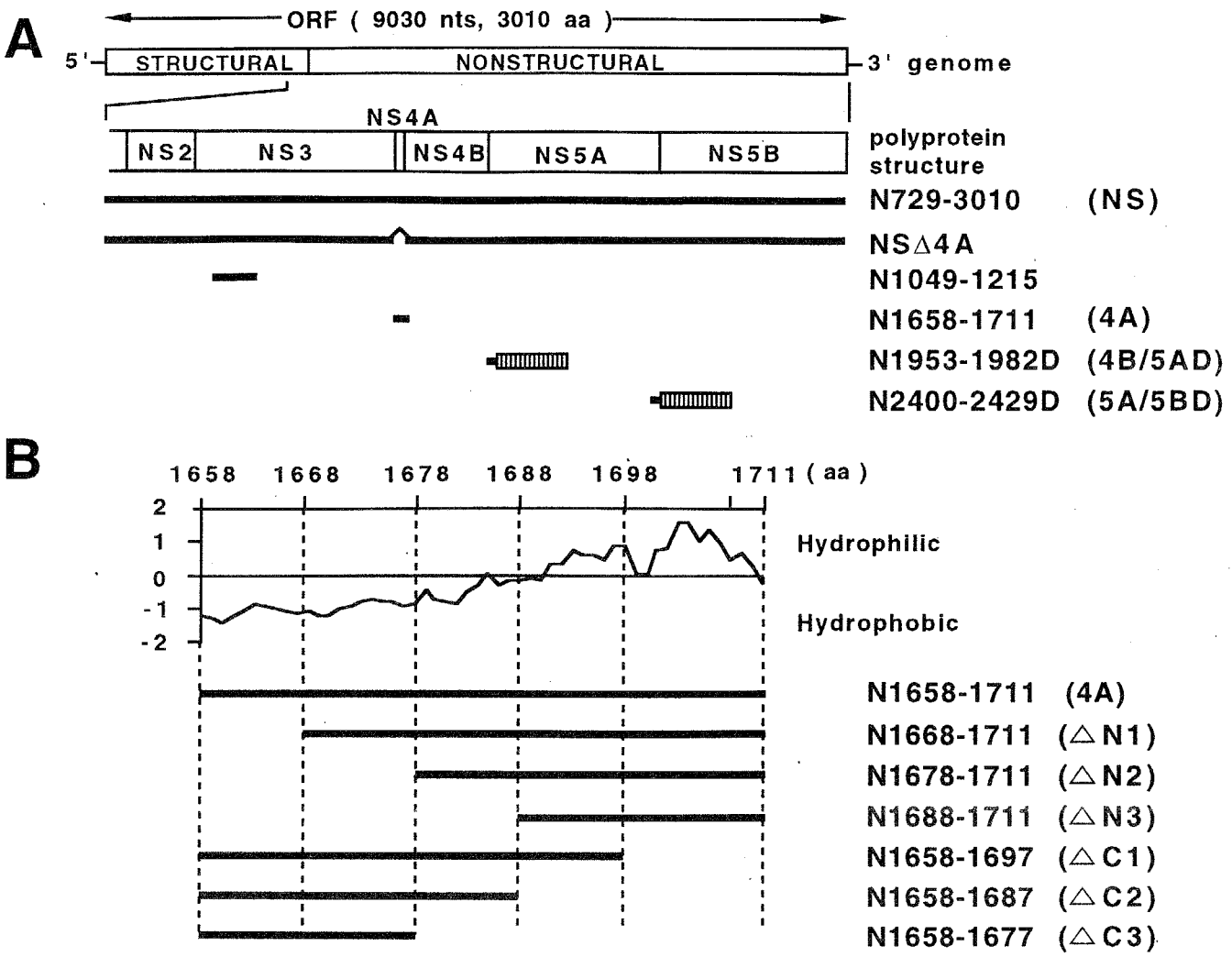


Fig. 4-1 Schematic representations of HCV polyprotein fragments produced by the expression constructs.

(A) The designation of HCV polypeptide regions synthesized in COS-1 cells is shown at the right. Numbers indicate amino acid positions from the N- to the C-terminus of the HCV precursor polyprotein. Abbreviations for the HCV polypeptides are indicated in parenthese. Hatched boxes indicate *E. coli* DHFR fused in frame at the C-terminal end of the HCV polypeptide (abbreviated as D). (B) HCV NS4A hydrophobicity profile as estimated by DNASIS program is shown in top. The program uses a moving-segment approach that continuously determines the avarage hydropathy within 7 amino acid as it advances through the sequence. The consecutive scores are plotted from the N- to C- terminus. At the same time, a midpoint line is printed that corresponds to the average of the hydropathy of the amino acid compositions found in most of the sequenced proteins (46). A series of peptides with deletions from the N- and C-termini of NS4A is shown below the hydrophobicity profile.

F1 :	5'-GCCTCACACCTCCCTTACATC-3'	(1712-1718)
F2 :	5'-GTCTGCAGATGAGCACCTGGGTGCTG-3'	(1658-1662)
F3 :	5'-TCGACTGCAGCCATGATCACTCAGCTGCTGAAG-3'	(1953-1958)
F4 :	5'-TCGACTGCAGCCATGCTCAGCGACGGGTCTTGG-3'	(2400-2405)
F5 :	5'-GACTGCAGATGGCAGCTCTGGCCGCA-3'	(1668-1672)
F6 :	5'-GCCTGCAGATGGGTAGTGTGGTCATT-3'	(1678-1682)
F7 :	5'-ATCTGCAGATGTTGTCCGGGAGGCCG-3'	(1688-1692)
R1 :	5'-AGTGACGACCTCCAGGTCGGC-3'	(1657-1651)
R2 :	5'-TCGAATTCTTAGCACGCTTCCATTTC-3'	(1711-1707)
R3 :	5'-CCGAAGCTTGTCCCAAACATCCCTTAG-3'	(1982-1977)
R4 :	5'-CCGAAGCTTCAAGGCACCTGTCCATGT-3'	(2429-2424)
R5 :	5'-ATGAATTCTTAGTCGGGAACAACAGC-3'	(1697-1693)
R6 :	5'-CAGAATTCTTAAATGATCCTACCCAC-3'	(1687-1683)
R7 :	5'-GCGAATTCTTAGGTTGTCAGGCAATA-3'	(1677-1673)

Fig. 4-2 Sequence of the primers.

Sequence of primers used for PCR in the construction of various plasmids are indicated. Designations of the primers used in the text are indicated at the left. Underlined nucleotides indicate sequences complementary to the genome of HCV IIj; the corresponding amino acid encoded in this complementary region are indicated at the right in parenthese.

4.2.2 Production of HCV polyproteins in COS-1 cells.

DNA transfections were performed as described in the chapter 2. Lysates of COS-1 cells transfected with the series of pCMV derived plasmids were fractionated by SDS-PAGE, and subjected to immunoblot analysis as described in the chapter-2. The antibodies used in this experiment were α-NS3, α-NS4A, α-NS4B, α-NS5A, α-NS5B, and α-DHFR (20, 21, 36).

4.2.3 Pulse chase analysis.

Pulse chase analysis was performed as described in the chapter 3.

4.2.4 Immunoprecipitation.

Immunoprecipitation was performed as described in the chapter 3.

4.2.5 Preparation of cytosol and membrane fractions.

pCMV-derived plasmids were transfected into COS-1 cells as described above. After incubation for one day in a 35 mm dish, the cells were incubated in 1 ml methionine free MEM with 5% FCS supplemented with 200 μCi/ml[^{35}S]-methionine for 4 hours. Cells were collected by a rubber scraper and homogenized in 200 μl MSB-medium (20 mM Tris-hydrochloride [pH 8], 25 mM $NaCl_2$, 0.5 mM $CaCl_2$, 1 mM PMSF, 1 mM dithiothreitol (DTT)). The homogenate was overlaid on 60 % (lower)-40 % (upper) sucrose solutions in MSB-medium and

centrifuged (200,000 x g, 60 minutes). After centrifugation, the portion on the 40 % sucrose layer was defined as the cytosol fraction, and the portion on the 60 % sucrose layer was defined as the membrane fraction. Both fractions were diluted 10-fold with extraction buffer and immunoprecipitated as described above.

4.3 Results.

4.3.1 Processing of proteins encoded in pCMV/NSΔ4A.

To understand the function of NS4A in HCV nonstructural polyprotein processing, pCMV/NSΔ4A, which encodes the entire putative HCV nonstructural polyprotein except the NS4A region, was expressed in COS-1 cells, and the protein products produced from this mutated polypeptide were analyzed by region-specific antisera. By comparing the products produced from NSΔ4A with those of the nonstructural region encoded in pCMV/N729-3010, which produces all HCV nonstructural proteins, the function of NS4A in HCV nonstructural polyprotein processing was analyzed. The production of NS3, NS4A, NS4B, NS5A, and NS5B from N729-3010 (abbreviated as NS in Fig. 4-3) was detected by α-NS3, α-NS4A, α-NS4B, α-NS5A, and α-NS5B, respectively (Fig. 4-3A, B, C, D, and E lanes 2). On the other hand, protein production from the NSΔ4A polypeptide was different from that of NS (Fig. 4-3A, B, C, and D lanes 3). Production of NS3 was quite low in NSΔ4A. No NS4B or NS5A could be detected by α-NS4B or α-NS5A, respectively, while an 85 kDa polyprotein (p85) was detected by α-NS5A. Since the size of this polypeptide, 85 kDa, was almost identical

to the sum of the molecular masses of NS4B and NS5A, this polypeptide was likely to be a processing intermediate composed of these two viral proteins. Failure to detect p85 by α-NS4B was possibly due to the low efficiency of blotting of the high molecular weight protein and the low titer of this antibody in this experiment. However, p85 was detected by α-NS4B antibody in the pulse chase experiment as described later. The production of NS5B as detected by α-NS5B indicates that cleavage at the 5A/5B site normally occurs in this mutant polypeptide. By co-production with NS4A, the production of NS3, NS4B, and NS5A from NSΔ4A was restored (Fig. 4-3A, C, and D lanes 3). The restoration of NS3 in the presence of NS4A seemed not to be due to efficient cleavage at the artificial NS3-NS4B site. Cleavage at this site occurred efficiently in the absence of NS4A, because the intermediate product, p85(NS4B-5A), accumulates in the absence of NS4A (Fig. 4-3D, lane 3). Instead, the restoration of NS3 by co-production with NS4A seemed to be caused by the stabilization of NS3. The production of p58(NS5A) relative to p56(NS5A) by co-production of NSΔ4A and NS4A was reduced compared with that of NS (Fig. 4-3D, lanes 2 and 4).

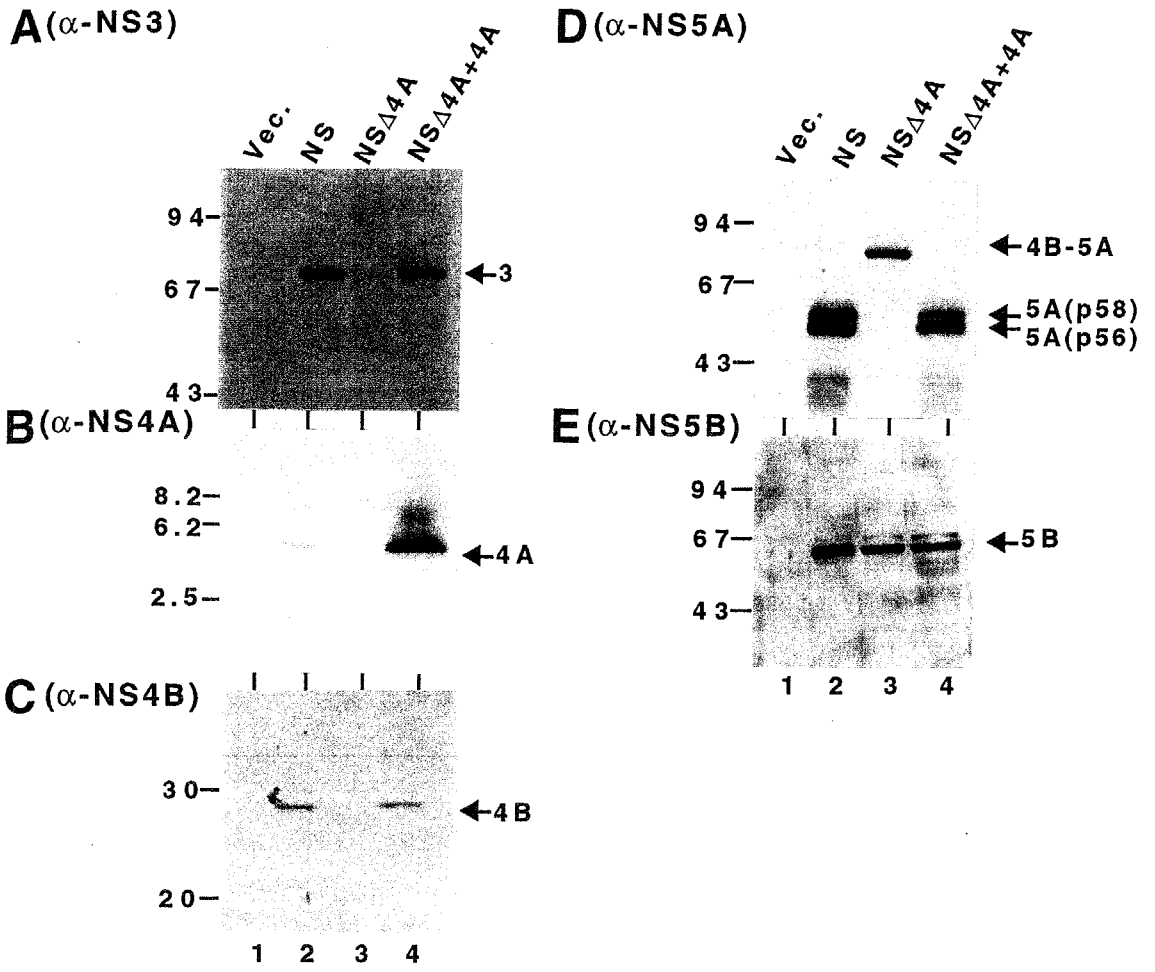


Fig. 4-3 Detection of processed products of NSΔ4A and the effect of NS4A on NSΔ4A processing.

Lysate of COS-1 cells transfected with: pKS(+)/CMV, an original vector plasmid without HCV insert, lane 1; pCMV/N729-3010, lane 2; pCMV/NSΔ4A, lane 3; pCMV/NSΔ4A plus pCMV/N1658-1711, lane 4, were separared by SDS-8% PAGE (A, D, and E), SDS-10% PAGE (C), or by tricine/SDS/16% PAGE (B), and analyzed by immunoblotting using α-NS3 (A), α-NS4A (B), α-NS4B (C), α-NS5A (D), or α-NS5B (E). The positions of processing products, NS3, NS4A, NS4B, NS5A(p58/p56), and NS5B, and precursor polyprotein NS4B-5A(p85), are indicated at the right with arrows.

4.3.2 NS3 stabilization by NS4A.

Since cleavage at NS2/3 was not affected by viral nonstructural proteins encoded in the region downstream of NS3 (22), and the viral proteins p85(NS4B-5A) and NS5B, encoded in the region downstream of NS3, were produced from NSΔ4A, the greatly reduced production of NS3 from this mutated polypeptide was thought to be due to the instability of NS3 in cells producing the mutated polypeptide. To clarify this possibility, pulse chase analysis was conducted using a transient expression system in COS-1 cells. Plasmid pCMV/NSΔ4A with or without pCMV1658-1711, which encodes NS4A, were used for transfection. Transfectants were pulse-labeled for 15 minutes with [^{35}S]-methionine, and chased for various times after the addition of excess amounts of unlabelled methionine. The cell lysates at chase time points 0, 20, 60, 180, 360, and 1440 (1 day) minutes were analyzed for the production of viral proteins by immunoprecipitation using α-NS3, α-NS4A, α-NS4B, α-NS5A, and α-NS5B.

In the chapter 3, it was shown that the processing of NS3 from the precursor polyprotein of N729-3010 was almost completed within the 15 minutes pulse period and that NS3 was stably present for more than one day of the chase period. Contrary to this observation, the presence of NS3 in NSΔ4A producing cell lysates was low at the start of the chase time, while a protein of approximately 150 kDa, immunoprecipitated by α-NS3 antibody and likely to be the processing intermediate of NS3-4B-5A, was detected in significant amount until 60 minutes of chase time (Fig. 4-

4A, lanes 2-5). The amount of NS3 reached a maximum at a chase time of 180 minutes, then started to decline. After one day (1440 minutes) of chase, no detectable NS3 was present, suggesting that NS3 does not exist stably in the cell without NS4A. On the other hand, when NS4A was co-produced with NSΔ4A, the NS3 level increased until 180 minutes of chase time, and the relative amount of NS3 remained almost constant thereafter, indicating that NS3 was stable in the cell (Fig. 4-4A, lanes 11-13). Co-produced NS4A was also stably present in the cell (Fig. 4-4B, lanes 8-13). A precursor polyprotein with a molecular weight of 85 kDa was detected by both α-NS4B and α-NS5A in lysates transfected with pCMV/NSΔ4A, but NS4B and NS5A were not detected throughout the chase period (Fig. 4-4C and D, lanes 2-7). On the other hand, NS4B and NS5A were produced when NS4A was co-produced with NSΔ4A (Fig. 4-4C and 4D lanes 8-13). This result was consistent with the above observation. The production of NS5B from NSΔ4A was seen at the start of the chase, and the level of the protein was not affected by co-production of NS4A (Fig. 4-4E, lanes 2-13).

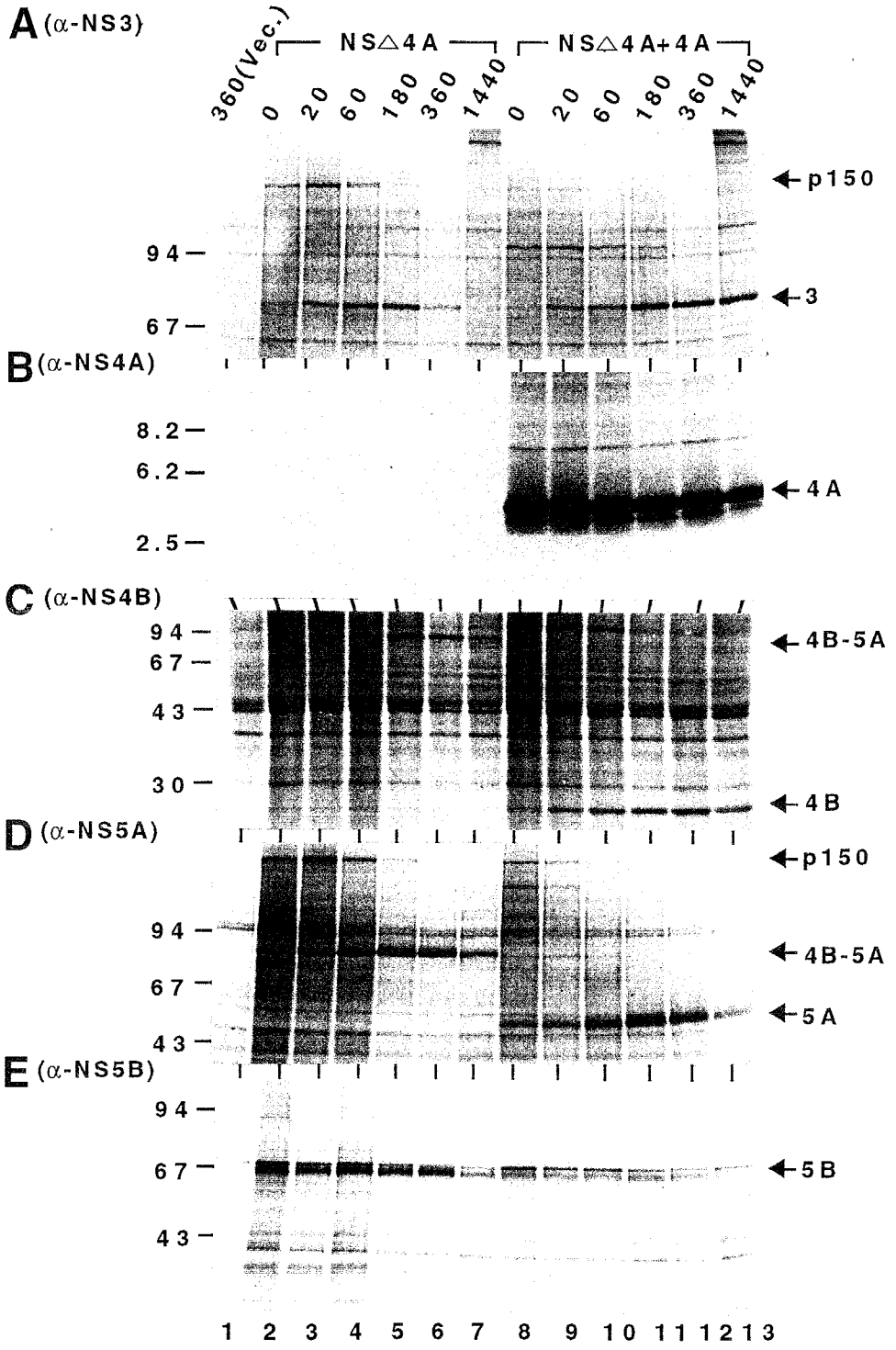

A (α-NS3)
360(Vec.)
NSΔ4A
NSΔ4A+4A
0 20 60 180 360 1440 0 20 60 180 360 1440
p150
3
94
67
B (α-NS4A)
8.2
6.2
2.5
4A
C (α-NS4B)
94
67
43
30
4B-5A
4B
D (α-NS5A)
p150
94
67
43
4B-5A
5A
E (α-NS5B)
94
67
43
5B
1 2 3 4 5 6 7 8 9 10 11 12 13

Fig. 4-4. Pulse chase analysis.

Cells were transfected with pCMV/NSΔ4A (5 μg) plus pKS(+)/CMV (5 μg) or pCMV/NSΔ4A (5 μg) plus pCMV/1658-1711 (5 μg) and were pulse labeled for 15 min and then chased as indicated. The lysate at each time point was immunoprecipitated with: α-NS3, panel **A**; α-NS4A, panel **B**; α-NS4B, panel **C**; α-NS5A, panel **D**; or α-NS5B, panel **E**. Immunoprecipitation was performed with lysates transfected with: pKS(+)/CMV, lane 1; pCMV/NSΔ4A, lanes 2-7; pCMV/NSΔ4A plus pCMV/N1658-1711, lanes 8-13. Gel conditions were SDS-8% PAGE for panels **A**, **D**, and **E**; SDS-10% PAGE for **C**; and tricine/SDS/16% PAGE for panel **B**. Molecular mass markers (in kDa) are shown on the left. The positions of processing products, NS3, NS4A, NS4B, NS5A, and NS5B, and precursor polyprotein p85(NS4B-NS5A), are shown at the right with arrows.

4.3.3 Subcellular localization of HCV proteins.

It is reported that NS4A is closely associated with NS3 and is responsible for the membrane association of NS3(22). Therefore, the prompt degradation of NS3 in the absence of NS4A was likely due to the loss of association of NS3 with the membrane. Association with the membrane may protect against proteolytic attack. To clarify this possibility, the role of NS4A in the localization of NS3 was examined.

NS3, produced from N729-3010, was found in both the cytosol and membrane fractions (Fig. 4-5A, lanes 2 and 6). However, the amount of NS3 present in the membrane fraction was larger than that in the cytosol fraction. On the other hand, a larger amount of NS3 produced from NSΔ4A was detected in the cytosol fraction (Fig. 4-5A, lanes 3 and 7). A drastic change in the subcellular localization of NS3 was found when NS4A was co-produced with NSΔ4A. The majority of NS3 was found in the membrane fraction (Fig. 4-5A, lanes 4 and 8). This result indicates that NS4A affects the localization of NS3, possibly by facilitating the membrane association of NS4A.

Besides NS3, the majority of NS4A, NS4B, NS5A, NS5B, and related precursor polyproteins were detected in the membrane fraction both with and without NS4A (Fig. 4-5B, C, D, and E).

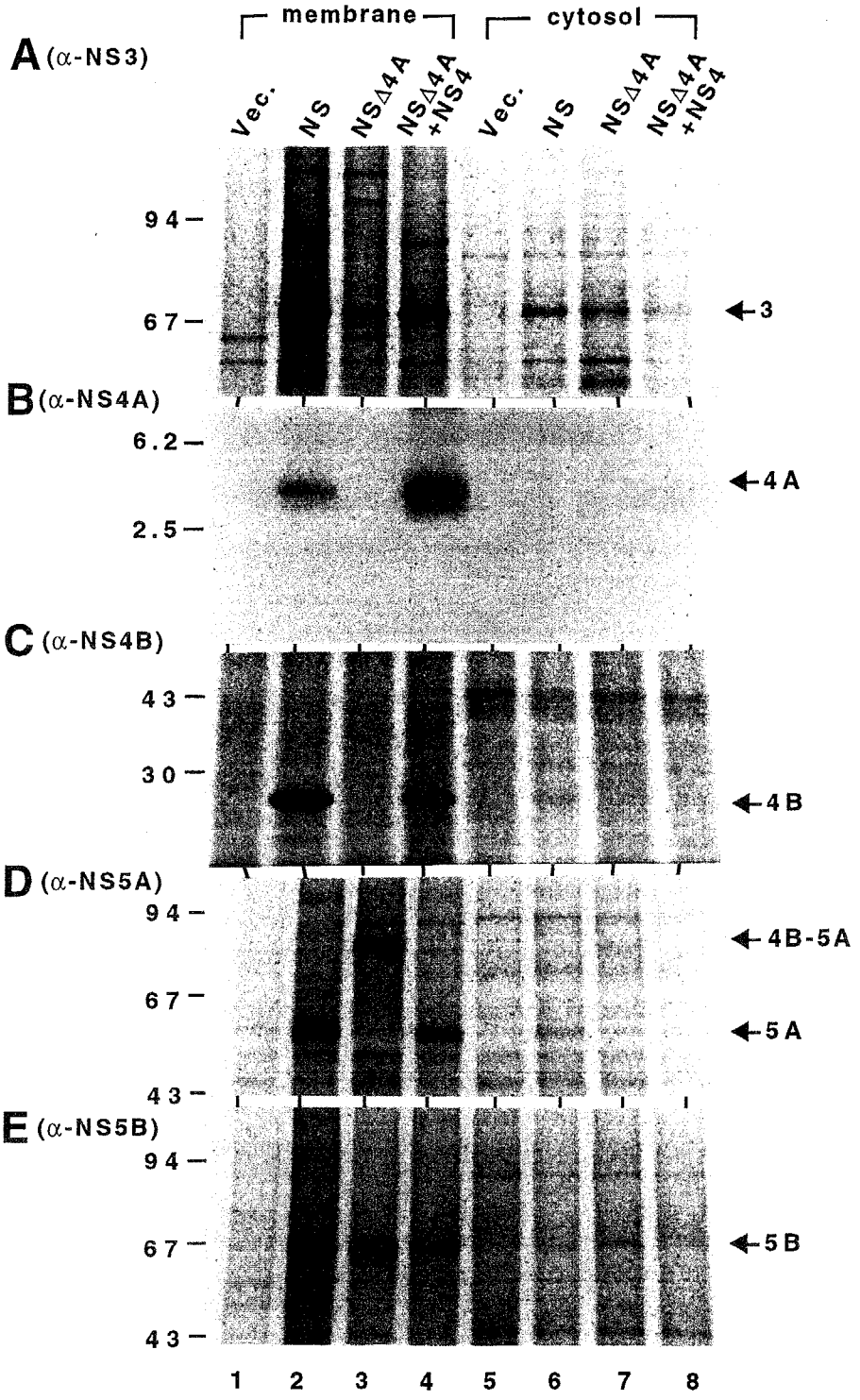


Fig. 4-5 Localization of processing products of NSΔ4A and the effect of NS4A on their sub-cellar localization.

Lysates of COS-1 cells transfected with: pKS(+)/CMV, lanes 1 and 5; pCMV/N729-3010 lanes 2 and 6; pCMV/NSΔ4A lanes 3 and 7; or pCMV/NSΔ4A plus pCMV/N1658-1711, lanes 4 and 8, were separated into membrane and cytosol fractions, and fractionated on SDS-PAGE, and analyzed by immunoblottting.

4.3.4 Role of NS4A in HCV nonstructural protein processing.

To investigate the role of NS4A on HCV nonstructural protein processing, a Cpro-2 dependent *trans* cleavage assay was performed. The function of NS4A in the processing of HCV nonstructural proteins was assayed by cleavage of DHFR fusion peptides containing 30 amino acid residues around the Cpro-2 cleavage site using a protein, N1049-1215, which has a minimum domain for Cpro-2 activity (chapter 2). The 30 amino acids used for this assay consisted of 20 amino acids located upstream of each cleavage site and 10 amino acids located downstream of each cleavage site. *E. coli* DHFR was fused in frame at the C-terminal end of each 30 amino acids segment to facilitate the detection of the C-terminal cleavage product by α-DHFR (21). Cleavage was detected in the substrate proteins of 4B/5AD and 5A/5BD. Both enzyme and NS4A proteins were required for the cleavage at 4B/5A (Fig. 4-6, lanes 1-3). The fact that NS4A was necessary for cleavage at the 4B/5A site implied that 30 amino acids around the 4B/5A site were sufficient for cleavage by N1049-1215 plus NS4A. On the other hand, approximately half of the 5A/5BD was processed by enzyme protein only, while co-production of NS4A led to an increase in cleavage efficiency (Fig. 4-6, lanes 4-6).

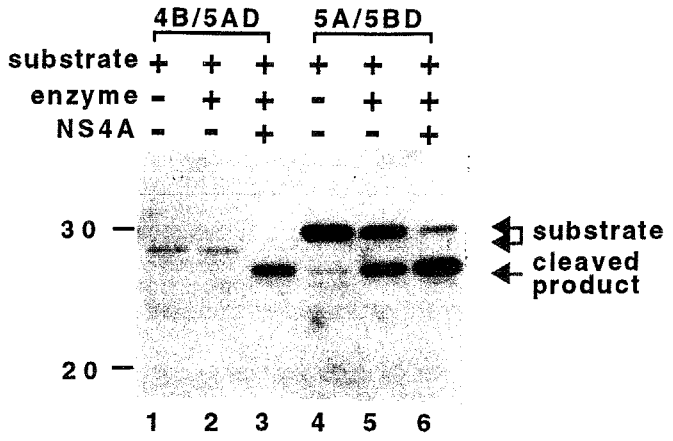


Fig. 4-6 Role of NS4A in HCV NS protein processing.

Lysates of COS-1 cells transfected with the plasmids indicated at the top of the figure were fractionated on SDS-10% PAGE, and analyzes by immunoblotting using α-DHFR. N1049-1215, encoding a minimum domain of Cpro-2 by pCMV/N1049-1215, was used as the enzyme protein. Substrate proteins were 4B/5AD, lanes 1-3 or 5A/5BD, lanes 4-6. The positions of substrate protein and cleaved products are indicated of the right with arrows.

4.3.5 Stoichiometrical assay of NS4A function.

To investigate the stoichiometrical relationship between NS4A and the enzyme-substrate protein of NSΔ4A, cleavage efficiency at the 4B/5A site using different amounts of pCMV/NSΔ4A and pCMV/N1658-1711 plasmids for transfection was examined. The efficiencies of 4B/5A cleavage were evaluated by detecting cleaved NS5A products by α-NS5A. As shown in Figure 4-7, the production of NS5A was reduced according to the decrease in the amount of NS4A encoding plasmid. However, production of p56(NS5A) was detected by α-NS5A even when the amount of NS4A encoding plasmid was far less than that of the NSΔ4A encoding plasmid. A proportional relationship between the production of protein and the amount the plasmid was found if the amount of plasmid used was less than 5 μg/well in this assay system (data not shown). Therefore, it was obvious that presence of the stoichiometrically least amount of NS4A relative to NS3 was sufficient to cleave at the 4B/5A site of NSΔ4A. For instance, even if the ratio of pCMV/NSΔ4A to pCMV/N1658-1711 was 99 μg to 1 μg (molar ratio of NS polyprotein to NS4A 38:1), more than half of the substrate protein NSΔ4A was cleaved at the 4B/5A site to produce p56(NS5A) (Fig. 4-7 lane 8).

The production of p58(NS5A) was strongly influenced by the amount of NS4A encoding plasmid. When excess amounts of NS4A to NSΔ4A were expressed in the cell, the relative production of p58(NS5A) to p56(NS5A) was almost the same as in lysates of NS producing cells (Fig. 4-7 lanes 2,3). On the other hand, when the least amount of NS4A to NSΔ4A was

expressed in the cell, the relative production of p58(NS5A) was reduced (Fig. 4-7 lanes 5-8). When 0.2 or 0.1 μg of NS4A encoded plasmid was used for transfection, no p58(NS5A) production was detected, while the uncleaved p85 product was detected (Fig. 4-7, lanes 7 and 8). These results suggest that NS4A also controls the production of p58(NS5A). Stoichiometrically equal or excess amounts of NS4A to the enzyme-substrate protein (NSΔ4A) were required for the production of p58(NS5A).

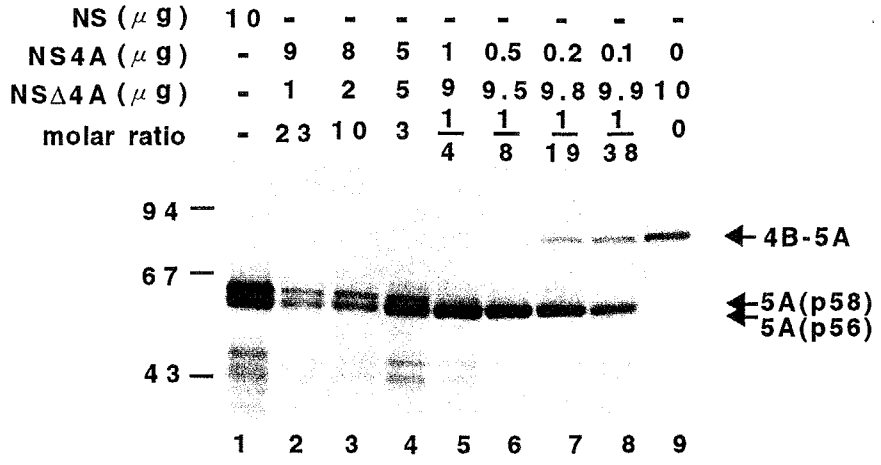


Fig. 4-7 Stoichiometrical assay of NS4A function in 4B/5A cleavage.

Lysates of COS-1 cells transfected with 10 μg of a plasmid encoding NS (lane 1), or with plasmids encoding NSΔ4A and NS4A in different relative proportions of amounts (lanes 2-9), were fractionated on SDS-8% PAGE. The amount of plasmids used and molar ratios of pCMV/N1658-1711 to pCMV/NSΔ4A are indicated at the top. The positions of the processing products are indicated at the right with arrows.

4.3.6 Deletion mapping of NS4A.

As shown in figure 4-1B, NS4A consists of two distinct parts, an N-terminal hydrophobic half and a C-terminal hydrophilic half. The role of these two regions in NS4A functioning is not known. An assay of NS4A-dependent cleavage at the 4B/5A site was used to determine the essential region of the NS4A protein in this function. A series of deletion mutants (Fig. 4-1B) derived from N1658-1711, NS4A, was used for the assay. The activity of each deletion mutant was determined by detecting NS5A production from NSΔ4A. When ΔN1, ΔN2, ΔC1, and ΔC2 were co-produced with NSΔ4A, different levels of p56(NS5A) were detected by α-NS5A (Fig. 4-8, lanes 3, 4, 6, and 7). The production of NS5A was greatly reduced when ΔN1 or ΔC2 was used as the source of NS4A. However, there was no NS5A production when ΔN3 or ΔC3 was co-produced. These data indicate that the C-terminal part of NS4A downstream of amino acid 1688 and the N-terminal part upstream of amino acid 1677 are not essential for NS4A activity, although a significant effect of these regions on cleavage at the 4B/5A site was observed. The minimum domain for NS4A activity appears to be from amino acid 1679 to 1687 of the HCV precursor polyprotein, although whether or not the expected minimum domain of N1679-1687 itself is functional remains to be determined.

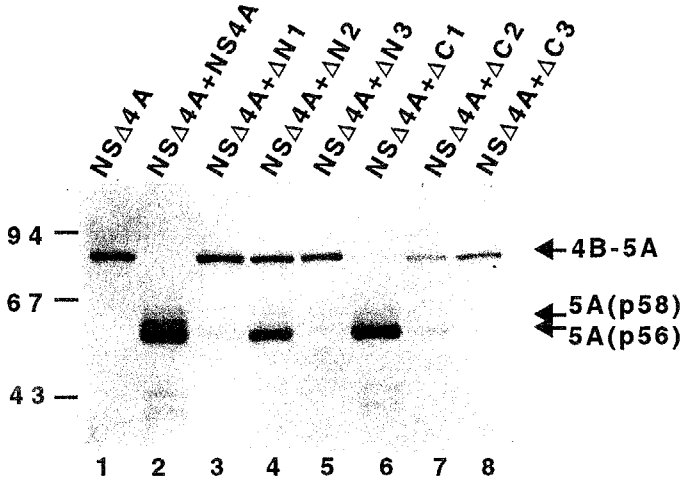


Fig. 4-8 Effect of deleted NS4A products on cleavage at the 4B/5A site.

Lysates of COS-1 cells transfected with plasmids encoding NSΔ4A (5 μg), plus: pKS(+)/CMV (5 μg), lane 1; NS4A or its deletion mutant encoded plasmids (5 μg each), lanes 2-8, were fractionated on SDS-8% PAGE, followed by immunoblot analysis using α-NS5A.

4.4 Discussion.

The transient expression system in COS-1 cells and pulse chase analysis showed the production of NS3 and NS5B from NSΔNS4A, indicating that cleavage at the 2/3 and 5A/5B sites proceeds without the presence of NS4A. However the rate of NS3 production was delayed in this construct compared with that in N729-3010 (chapter 3). During 180 minutes of chase, a putative precursor protein was detected by α-NS3, suggesting that processing to NS3 was not complete by the end of the 180 minute chase. Since NS4A was excluded from the mutated polypeptide NSΔ4A, NS4B was followed directly to NS3. Therefore, the amino acid sequence around the C-terminus end of NS3 was changed from DLEVVT/STW to DLEVVT/ASH (/ indicates the cleavage site). It was possible that the delayed processing of NS3 in NSΔ4A was caused by the artificial amino acid sequence between NS3 and NS4B. On the other hand, the production of NS5B in lysates transfected with pCMV/NSΔ4A was detected at the end of the pulse period and no precursor protein reactive with α-NS5B was detected. Therefore, efficient cleavage proceeds at the 5A/5B site of the NSΔ4A polyprotein in the absence of NS4A.

The amount of NS3 produced from NSΔ4A in the absence of NS4A reached a maximum after 180 minutes of chase, and then decreased gradually. Instead, the level of NS3 remained almost unchanged from the level after 180 minutes of chase in the presence of NS4A. Furthermore, a lower amount of NS3 production was observed in cells producing only the NSΔ4A polyprotein than in cells co-producing NSΔ4A

polyprotein and NS4A (Fig. 4-3A, lanes 3 and 4). Since the 85 kDa NS4B-5A precursor protein was clearly seen in this experiment (Fig. 4-3D, lane 3), processing of the C-terminus of NS3 must occur properly. These observations suggested that NS4A plays an important function in the stabilization of NS3.

An analysis of the subcellular localization of the nonstructural proteins revealed that without the co-existence of NS4A, more than 50 % of the NS3 produced from NSΔ4A was localized in the cytosol fraction, while under coproduction with NS4A, most of the NS3 was found in the membrane fraction. It was shown that a close association between NS4A and NS3 was important for the membrane anchoring of NS3 (22). In view of the previous data and of the evidence presented here, one aspect of NS4A functioning may be to anchor NS3 to the membrane of the endoplasmic reticulum.

Since NS5B was produced efficiently from NSΔ4A, it is believed that NS4A is not essential for cleavage at the 5A/5B as reported by other groups (5, 13). However, it is noteworthy that the cleavage at the 5A/5B site of the 5A/5BD protein was enhanced when NS4A was present (Fig.4-6 lanes 5,6). In this assay system the minimum Cpro-2 domain was supplied as *trans* to the substrate which contained only 30 amino acids residues surrounding the cleavage site; thus the efficiency of cleavage might be lowered. The molecular mechanism for the enhancement of 5A/5B cleavage by Cpro-2 with NS4A was not clear. However, stabilization of Cpro-2 and membrane association of Cpro-2 by NS4A, which might

facilitate close association to substrates, might be considered to enhance the cleavage function.

The fact that the presence of NS4A is indispensable for processing at the 4B/5A site was confirmed in this study as it has been by others (5, 13). The function of NS4A to stabilize and anchor NS3 to the membrane seems insufficient for this cleavage because the presence of NS3 associated with the membrane fraction could be detected in the absence of NS4A by the time that cleavage at the 4B/5A site was completed (Fig. 4-4A and 4D). Thus it is likely that an additional function of NS4A, which cooperates with the function of NS3, may involve cleavage at the 4B/5A site. For this function, 10 amino acid residues harboring the central region of NS4A (amino acid 1678 to 1687) were shown to be essential, although a great reduction in activity by this limited domain was evident (Fig. 4-8). The minimum domain of Cpro-2, amino acid 1049 to 1215, was sufficient to cleave at the 4B/5A site of a substrate containing only 30 amino acid residues surrounding the 4B/5A cleavage site in the presence of NS4A (Fig. 4-6). This result was in contrast to the results of Bartenschlager and coworkers (5), in which the N-terminal truncation of NS3 abolished NS4A dependent 4B/5A cleavage. The minimum domain of Cpro-2 (N1049-1215) used in the present study contains a further deletion of 15 amino acids from the N-terminus of their construct. The reason for the conflicting results is not known, however, it may be caused by using HCV clones with different sequences or by differences in the assay systems.

Proteins with different molecular weights, p56(NS5A) and p58(NS5A), were produced from NS5A. The biochemical natures of these proteins remain to be clarified. The production of p58(NS5A) was drastically reduced from mutated HCV-NS polyproteins in which amino acid at the P1 and P1' positions in the 3/4A or 4A/4B sites are substituted with asparagine leading to impaired cleavage at the each sites (chapter 3, 52). These results imply that only the authentic product of NS4A allows the production of p58(NS5A). This was in agreement with the observation that all of the truncated forms of NS4A fail to produce p58(NS5A).

Results from the stoichiometrical assay also revealed that the production of p58(NS5A) was strongly controlled by the level of NS4A. For the production of p58(NS5A), a stoichiometrically equal or greater amount of NS4A to NSΔ4A was required, although NS4A was likely to have the catalytic capability of cleavage at 4B/5A site. The mechanism for the control of the production of NS5A by NS4A remains to be clarified. The essential function of NS4A in the production p58(NS5A) differed from that in the cleavage at the NS4B/5A site.

In this chapter versatile functions of NS4A of HCV was described. The function of NS4A seemed to differ from that of the NS2B product of flaviviruses which is required for NS3 dependent proteolytic cleavage of the flavivirus precursor polyprotein (3, 7, 14, 44). The complicated function of NS4A seemed to be involved in the strict regulation of not only the proteolytic processing of the

precursor polyprotein, but also in the function of the processed protein, a function that may be crucial for viral proliferation.

4.5 Summary of chapter 4.

A transient protein expression system in COS-1 cells was used to study the role of HCV encoded NS4A protein on HCV nonstructural polyprotein processing. By analyzing the protein expression and processing of a deletion mutant polypeptide, NSΔ4A, which encodes the entire putative HCV nonstructural polyprotein except the region encoding NS4A, the versatile functions of NS4A were revealed. Most of the NS3 processed from NSΔ4A was localized in the cytosol fraction and was degraded promptly. Coproduction of NS4A stabilizes NS3 and assists in its localization in the membrane. NS4A was found to be indispensable for cleavage at the 4B/5A site but not essential for cleavage at the 5A/5B site in NSΔ4A. The functioning of NS4A as a cofactor for cleavage at the 4B/5A site was also observed when 30 amino acids around this site was used as a substrate and a minimum domain of viral Cpro-2 of 167 amino acids, from Gly-1049 to Ser-1215, was used as an enzyme protein, suggesting that possible domains for the interaction of NS4A were in those regions of the enzyme protein (NS3) and/or substrate protein. Two proteins, p58(NS5A) and p56(NS5A), were produced from the region of NS5A. For the production of p58(NS5A), stoichiometrically equal or excess molar amounts of NS4A to NSΔ4A were required. Deletion

analysis of NS4A revealed a minimum functional domain of NS4A of 10 amino acids, from Leu-1688 to Asp-1697.

Chapter 5

Phosphorylation of NS5A

5.1 Introduction.

During analysis of virus proteins processed from the nonstructural region, it was found that two proteins, a 56 kDa protein and a 58 kDa protein, were produced from NS5A as described in chapters 2, 3, and 4. The presence of a protein produced of NS4A has been shown to be important for production of the 58 kDa protein (13, 53). Since the NS4A was required for cleavage between NS4B and NS5A by a Cpro-2, it was supposed that additional cleavage upstream or downstream of NS5A occur. Both mutation analysis and deletion analysis of these regions revealed that this was not the case.

By analyzing possible modifications of these proteins, it was found that both proteins were phosphorylated and that p58 was a NS4A-dependent phosphorylated form of p56.

5.2 Materials and Methods.

5.2.1 Construction of plasmid .

The constructions of plasmid pCMV/N729-3010, which produces all of the processed HCV nonstructural proteins of HCV-IIJ, and of plasmid pCMV/NS3/4NN, which also produces most of the processed viral proteins but has mutations at positions p1 and p1' of the 3/4A cleavage site and thus produces only one type of protein from the NS5A region, were described in the chapter 3. Plasmid pCMV/N1973-2419, which produces NS5A protein, was constructed as follows. PCR was carried out using pCMV/N729-3010 as the template and the synthetic oligonucleotides 5'-CCGAAGCTTCTCGAGATGTCCGGCTCGTGGCTA-3' (the underlined

sequence represents 1973 to 1977 on the HCV ORF and the other region is the tagged sequence with a restriction enzyme site) and 5'-AAGGGATCCTTAGCAGCAGACGATGTC-3' (2415 to 2419) as the primers. The fragment obtained was digested with *Bam*HI and the ends were filled in with Klenow enzyme. The DNA fragment was further digested with *Hin*dIII and ligated into the *Hin*dIII-*Eco*RV site of pCMVS3. A cloning vector, pCMVS3, was derived from pKS(+)/CMV (22) by modifying it so that a *Pst*I fragment of 27 base pairs located downstream from the CMV promoter was deleted after the unique *Hin*dIII site was eliminated. The *Pst*I site was then converted to *Hin*dIII. Plasmid pCMV/N1658-1711, which produces NS4A protein was constructed as follows. A PCR fragment was obtained using pCMV/N729-3010 as the template and synthetic DNA fragments of 5'-GTCTGCAGATGAGCACCTGGGTGCTG-3' (1658 to 1662) and 5'-TCGAATTCTTAGCACGCTTCCATTTC-3' (1707 to 1711) as the primers. The fragment obtained was digested with *Pst*I and *Eco*RI, and then ligated into the *Pst*I-*Eco*RI site of pKS(+)/CMV.

The regions and names of the peptides produced from these plasmids on the HCV polyprotein are shown in Fig. 5-1 together with the genomic organization of HCV.

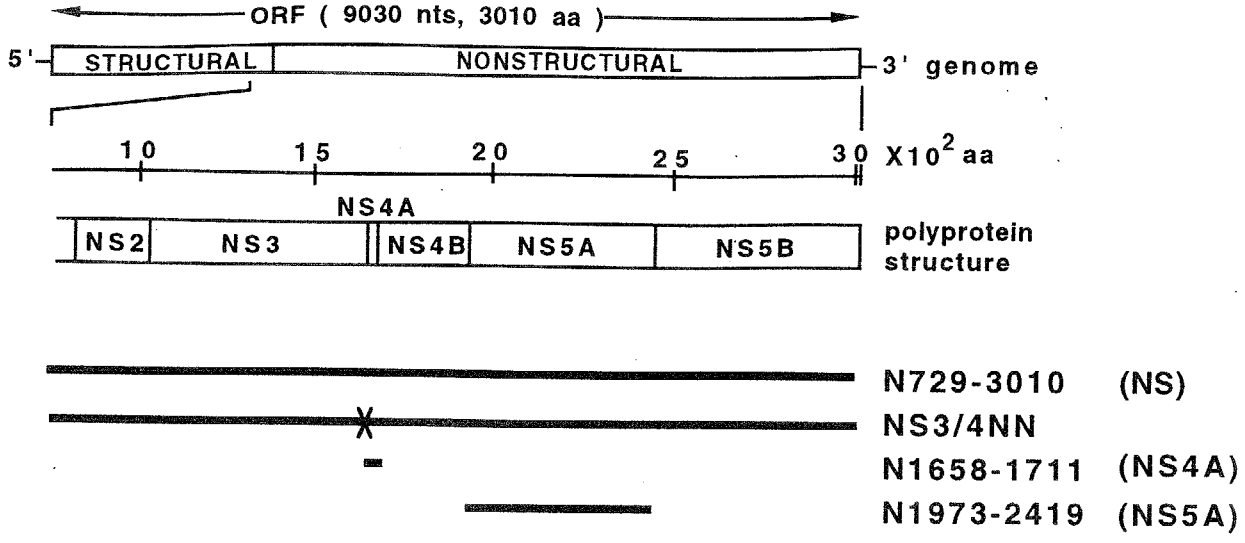


Fig. 5-1 Schematic map of the expression plasmids.

The HCV genomic ORF is represented as a box. The nonstructural region of the HCV precursor polyprotein structure is shown as a box with the names of the processing products. The cDNA fragments for the expression plasmids are represented by bars with their names of produced primary proteins. Mutations at p1 and p1' of NS3/NS4A sites were described by X.

5.2.2 Transient expression in COS-1 cells.

DNA transfection was performed as described in the chapter 2. Lysate of COS-1 cells transfected with a plasmid was fractionated by SDS-PAGE, and this was followed by immunoblot analysis. The antibodies used were α-NS4A and α-NS5A.

5.2.3 Analysis of phospho-amino acids.

Cells transfected with plasmids which produce HCV proteins were metabolically labeled with ^{32}P-ortho-phosphate. The cell lysate was immunoprecipitated with α-NS5A, and the immunoprecipitate was fractionated with SDS-PAGE and blotted on a nylon membrane filter. The bands corresponding to p56 and p58 were recovered and treated with 6N HCl for 2 hours. Phospho-amono-acids were electrophoretically separated and detected by autoradiography.

5.2.4 Dephosphorylation.

Cells transfected with plasmids were lysed and immunoprecipitated with α-NS5A. Samples bound to Protein G sepharose were suspended in 10 mM Tris-HCl(pH8)-2 mM $MgCl_2$-1 mM PMSF-1 mM DTT, and after adding 0.3 units of *E. coli* alkaline phosphatase, they were incubated at 56 °C. The reaction was terminated at various time points by the addition of Laemmli sample buffer and samples were subjected to SDS-PAGE, followed by immunoblot analysis or autoradiography.

5.3 Results.

5.3.1 Production of NS5A products in COS-1 cells.

Viral nonstructural proteins are produced by proteolytic processing of HCV precursor polyprotein. Viral proteinase, Cpro-2, is responsible for this cleavage (12, 17, 18, 54). Potential cleavage sites on the viral precursor polyprotein have been identified based on the results of an analysis of the N-terminal amino acid sequences of these products, and a protein with 447 amino acid residues (from amino acid 1973 to 2419 on HCV ORF) is expected from the NS5A region (17, 18, 23). Using a plasmid-based transient expression system for HCV nonstructural proteins in COS-1 cells, however, it was observed that two proteins, p56 and p58, were produced from the NS5A region (chapters 2, 3, and 4). Because of a lack of information concerning the C-terminus of the NS5A product, it was thought that products of different sizes might be derived based on differences in their C-termini. However, this was not the case. When the plasmid which produces the C-terminally deleted form of NS5A was transfected to COS-1 cells, two NS5A specific proteins were produced (data not shown). The possibility of the presence of an alternative cleavage site upstream of the authentic N-terminus of NS5A was unlikely because the production of a single discrete band of the juxtaposed product (NS4B) upstream of the NS5A region was always observed.

These findings suggested that production of two proteins from the NS5A region was not caused by different cleavages during proteolytic processing, but that it was probably

caused by some modification of the primary translated peptide backbone from NS5A. This speculation was supported by examination of the difference between the actual molecular weights (56 and 58 kDa) of the NS5A products and the calculated molecular weight (49 kDa). Thus, nature of the modification of the NS5A products was analyzed.

5.3.2 Phosphorylation of NS5A.

The plasmids which were used in this study produce proteins from the region coding the entire nonstructural region of the viral genome. When plasmid pCMV/N729-3010 was transfected to COS-1 cells, viral proteins NS2, NS3, NS4A, NS4B, p56/p58(NS5A), and NS5B were produced, in that order, from the amino-terminus of the viral precursor polyprotein. Production of p56 and p58 from NS5A was detected with α-NS5A (Fig. 5-2B, lane 1). COS-1 cells transfected with pCMV/NS3/4NN, which covers the same region of HCV ORF as pCMV/N729-3010 but has mutations at the 3/4A cleavage site, produced all viral nonstructural proteins except p58 (Fig. 5-2B, lane 2).

To analyze the possible modification of these proteins by phosphorylation, COS-1 cells transfected with these plasmids were metabolically labeled by culturing in medium containing ^{32}P-ortho-phosphate. The lysate was immunoprecipitated with α-NS5A, and the precipitate was analyzed for phosphorylation by SDS-PAGE followed by autoradiography (Fig. 5-2A, lanes 1 and 2). Two proteins, p56 and p58, of the same size observed by western blot using α-NS5A, were detected in their phosphorylated form.

Since the C-terminal amino acid sequence of the NS5A product has the characteristics of a certain Ras-related protein farnesylated at the site, the possibility of modification with fatty acids by palmytoylation or farnesylation was analyzed. However, it was unable to detect any such modifications (data not shown).

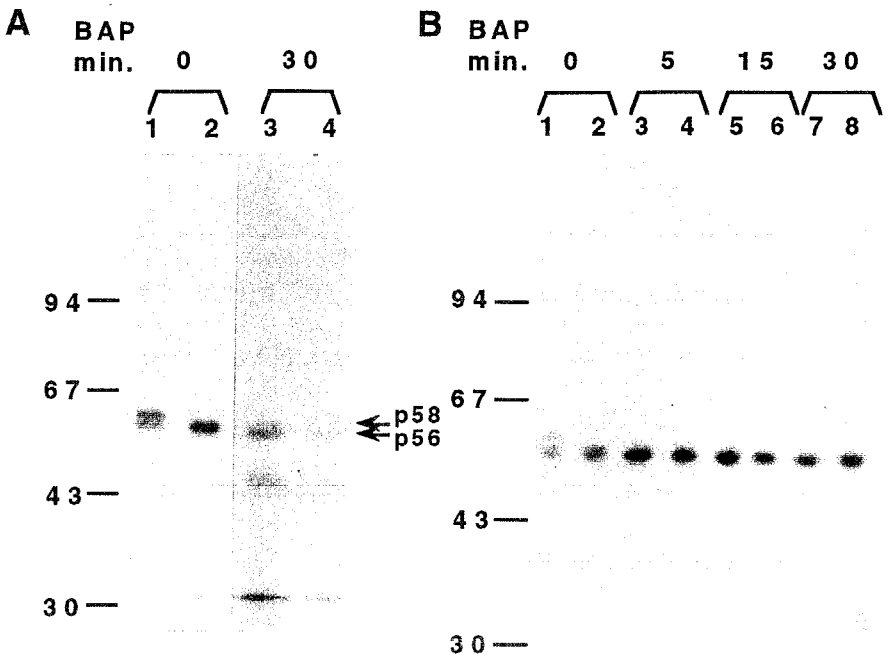


Fig. 5-2 Detection of HCV NS5A proteins expressed in COS-1 cells.

Autoradiograms of immunoprecipitated NS5A metabolically labelled with ^{32}P-ortho-phosphate (**A**). pCMV/N729-3010(**A**, lanes 1 and 3), and pCMV/NS3/4NN (**A**, lanes 2 and 4) were transfected into COS-1 cells. The proteins produced and processed in the cells were immunoprecipitated, treated with *E. coli* alkaline phosphatase for 0 minutes (lanes 1 and 2), or for 30 minutes (lanes 3 and 4), separated by SDS-PAGE on 7.5% gel and subjected to autoradiography..The 45 kDa band in lane 3 is likely to be *E. coli* alkaline phosphatase, which is believed to be intermediately bound to ^{32}P. Immunoblot detection of *E. coli* alkaline phosphatase treated NS5A(**B**). COS-1 cells were transfected with pCMV/N729-3010 (lanes 1, 3, 5 and 7) and with pCMV/NS3/4NN (lanes 2, 4, 6 and 8). Immunoprecipitated samples were treated with *E. coli* alkaline phosphatase for the times indicated above the lanes, and separated by SDS-PAGE.

5.3.3 Two degrees of NS5A phosphorylation.

To determine whether the difference in molecular weight of the two proteins encoded in NS5A is attributable to different degree of phosphorylation, the immunoprecipitates with α-NS5A were treated with *E. coli* alkaline phosphatase for 30 minutes at 56 °C. The radioactivity of p56 and p58 was lower after treatment, and the p58 band was no longer detectable after treatment (Fig. 5-2A, lanes 3 and 4). To confirm that the loss of radioactivity was not due to degradation of p56 and p58 by a contaminating proteinase during phosphatase treatment, the phosphatase-treated sample was fractionated by SDS-PAGE and analyzed by Western blot using α-NS5A. The 58 and 56 kDa bands were clearly detected in extracts obtained from COS-1 cells transfected with pCMV/N729-3010 and pCMV/NS3/4NN (Fig. 5-2B, lanes 1 and 2). After 5 minutes of digestion with alkaline phosphatase, only the p56 band was detected. The p56 band was still detectable after prolonged treatment (Fig. 5-2B lanes 3 to 8, up to 150 minutes data not shown). Since no degraded products of NS5A appeared during incubation, disappearance of p58 was not caused by proteolytic cleavage during incubation. This finding indicated that p56 and p58 were indistinguishable in size after dephosphorylation, that p58 was a additionally phosphorylated form of p56, and that the mobility of p56 was unaffected by dephosphorylation. These findings strongly suggested that p56 and p58 had identical peptide backbone which was produced from NS5A. The fact that p58

was the additionally phosphorylated form of p56 was further supported by the experiment described later.

To analyze phospho-amino-acid in p56 and p58, the bands corresponding to p56 and p58 were excised from the gel after SDS-PAGE, and the proteins extracted from the gel were treated with 6N HCl for 2 hours. The hydrolysate was then analyzed by electrophoresis followed by autoradiography (Fig. 5-3). Only serine residues were phosphorylated in both proteins. Since no phosphotyrosine was detected when the phospho-amino acids in proteins of p56 and p58 were analyzed after treatment by alkaline digestion (data not shown), it was concluded that phosphoserine was the unique phosphorylated amino acids in these proteins.

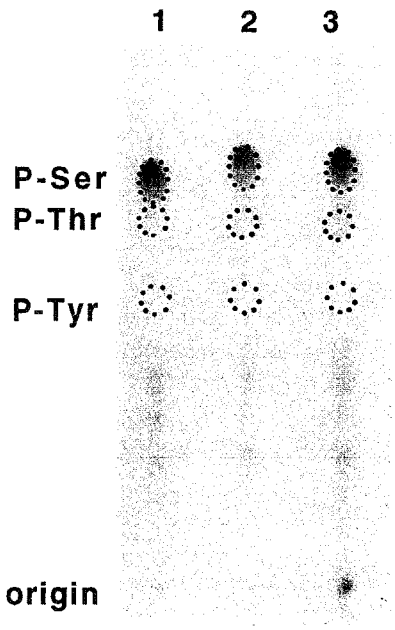


Fig.5-3 Autoradiogram of ^{32}P-labelled phospho-amino acids separated by thin layer electrophoresis.

p58 (lane 1), p56 (lane 2) expressed from pCMV/N729-3010, and p56 (lane 3) from pCMV/NS3/4NN were analyzed. Dotted circles represent the position of standard phospho-amino acids.

5.3.4 Role of NS4A on NS5A phosphorylation.

When a plasmid which produces an HCV nonstructural polyprotein, except the region for NS4A, was transfected into COS-1 cells, production of most processed viral proteins was observed as described in the chapter 4. The only exception was production of a p85(NS4B-5A) intermediate. When NS4A was co-produced in this system, cleavage at 4B/5A was completed and production of NS5A was detectable, however, when the level of production of NS4A was lower than that of other nonstructural proteins, p56 was only the major product of NS5A. Production of p58 became detectable when a stoichiometrical amount of NS4A to that of other nonstructural proteins was produced. The reason for the lack of p58 in pCMV/NS3/4NN transfected COS-1 cells was unknown. Production of processed NS4A product has been shown to occur normally from this construct. However, aberrant cleavage at the mutated site may produce non-functional NS4A product or the level of NS4A production may be insufficient to convert p56 to p58.

When NS5A was cotransfected with NS4A into COS-1 cells, production of both p58 and p56 was observed (Fig. 5-4, lane 2). Thus, NS4A was directly involved in the production of the additionally phosphorylated form of NS5A (NS4A dependent phosphorylation), although whether NS4A itself possesses kinase activity remains to be clarified.

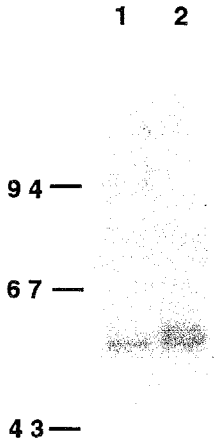


Fig.5-4 Immunoblot analysis of individually expressed NS5A with or without coexpression of NS4A.

pCMV/N1973-2419 (lane 1), pCMV/N1973-2419 plus pCMV/N11658-1711 (lane 2) were expressed in COS-1 cells. Samples were separated by SDS-PAGE and subjected to immunoblot detection.

5. 4. Discussion.

The function of the NS5A of HCV in viral replication remains to be determined, however, the function of NS5A is probably modulated by phosphorylation. Viruses of the Flaviviridae family, such as bovine virus diarrhea virus (BVDV), have a similar region corresponding to HCV NS5A, but the function of the product is unknown.

The HCV NS5A product was localized in the peripheral membrane fraction of cells transfected with plasmids which express viral nonstructural proteins (data not shown). It was demonstrated that the mutual interaction of most of the nonstructural viral proteins, and that this complex was associated with the microsomal membrane (22). Thus the NS4A-dependent kinase which phosphorylates p56 is probably associated with membranes. In this regard, cAMP-dependent protein kinases, casein kinases, protein kinases C and calmodulin kinases, which are known to be associated with microsomal membranes, are likely candidates, although an unidentified kinase may be involved in the phosphorylation of the NS5A product. Possible sites for proline-directed protein kinase, and for casein kinase II were detected in a search for consensus phosphorylation sites by amino acid sequence homology. Information on the site of phosphorylation in p58 may be important in further clarifying the mechanism of phosphorylation of NS5A products.

5.5 Summary of chapter 5.

The production of viral nonstructural proteins with an *in vivo* transient expression system using COS-1 cells was analyzed. Two proteins, a 56 kDa protein and a 58 kDa protein, were produced from NS5A, which has the potential to produce a 49 kDa protein. It was shown that these proteins were phosphorylated at the serine residues. The presence of the two proteins was reflected by different degrees of phosphorylation. Moreover, the production of p58 was shown to depend on the presence of NS4A.

Chapter 6

Two modes of NS5A phosphorylation

6.1 Introduction.

A 56 kDa (p56) and a 58 kDa (p58) were detected by NS5A specific antibody (α-NS5A) in the lysate of COS-1 cells transfected with the plasmid that encodes the entire nonstructural protein region (chapter2 to 5). In the chapter 5, it was indicated that p56 and p58, which were produced from the NS5A region, were phosphorylated at serine residues. Moreover, p58 was a NS4A-dependent phosphorylated form of p56.

In an attempt to obtain a better understanding of NS5A phosphorylation, pulse-chase and mutation analyses were performed. The results suggested that phosphorylation on NS5A took place after completion of the proteolytic cleavages at the N- and C- termini of NS5A. To identify the region important for NS4A-dependent phosphorylation in NS5A a series of C-terminal deletions in the region encoding the HCV nonstructural polyprotein were constructed by using pCMV/NS, a plasmid that produces the whole HCV nonstructural polyprotein. For identification of the possible serine residues in those regions, which the deletion analysis showed were important for the NS4A-dependent phosphorylation, serines were mutated to the neutral amino acid, alanine, and NS4A-dependent phosphorylation was assayed. To identify the region that was responsible for NS4A-independent phosphorylation, the same series of deletion mutants were introduced into a plasmid that produces only NS5A protein, and phosphorylation of each deleted NS5A protein was analyzed.

The subcellular localization of the NS5A products was also examined.

6.2 Materials and methods.

6.2.1 Construction of plasmids.

The HCV polypeptide regions synthesized in COS-1 cells for this study are shown in Fig. 6-1. Construction of plasmids pCMV/N729-3010, pCMV/N1658-1711, pCMV/N729-2052D, and pCMV/NS4A/BNN were reported previously (21, 22, 51, 52). To introduce deletion mutations into the HCV nonstructural polyprotein, a series of plasmids was constructed by using PCR. Sequences of the synthetic oligonucleotides used as primers in the PCR are indicated in Table.6-1. Combinations of positive (F) and negative (R) stranded primers used for PCR are indicated in the parentheses to the right of the PCR products designated PP1 to PP8: PP1 (F and R1); PP2 (F and R2); PP3 (F and R3); PP4 (F and R4); PP5 (F and R5); PP6 (F and R6); PP7 (F and R7); PP8 (F and R8). PP1 to PP8 were digested with *Mlu*I and *Hin*dIII, and inserted into the *Mlu*I and *Hin*dIII site of pCMV/N729-2472D (22) to obtain pCMV/N729-2431D, pCMV/N729-2417D, pCMV/N729-2350D, pCMV/N729-2300D, pCMV/N729-2250D, pCMV/N729-2200D, pCMV/N729-2150D, and pCMV/N729-2100D, respectively. The *Pst*I fragment of pCMV/N1973-2419 (chapter 5) was replaced with the *Pst*I fragments of those eight plasmids, respectively, to obtain pCMV/N1973-2431D, pCMV/N1973-2417D, pCMV/N1973-2350D, pCMV/N1973-2300D, pCMV/N1973-2250D, pCMV/N1973-2200D, pCMV/N1973-2150D, and pCMV/N1973-2100D.

Introduction of amino acid substitutions into N729-2431D was conducted as follows. The first PCR used the primers indicated in the parentheses shown below. The

resultant PPs were used as primers together with a third primer indicated to the right of the parenthesis of PCR products from the second PCR. PCR products and combinations of primers used were as follows: PP9 (F and R9)R1; PP10 (F and R10)R1; PP11 (F and R11)R1; PP12 (F and R12)R1; PP13 (F and R13)R1; PP14 (F and R14)R1; PP15 (F and R15)R1; PP16 (F and R16)R1; PP17 (F and R17)R1; PP18 (F and R18)R1; PP19 (F and R19)R1; PP20 (F and R20)R1. The resultant twelve PPs were digested with *Mlu*I and *Hin*dIII, and inserted into the *Mlu*I-*Hin*dIII site of pCMV/N729-2426D to obtain pCMV/S2194A, pCMV/S2197A, pCMV/S2200A, pCMV/S2201A, pCMV/S2202A, pCMV/S2204A, pCMV/S2207A, pCMV/S2210A, pCMV/S2221A, pCMV/SS2200/1AA, pCMV/SS2201/2AA, and pCMV/SSS2200/1/2AAA.

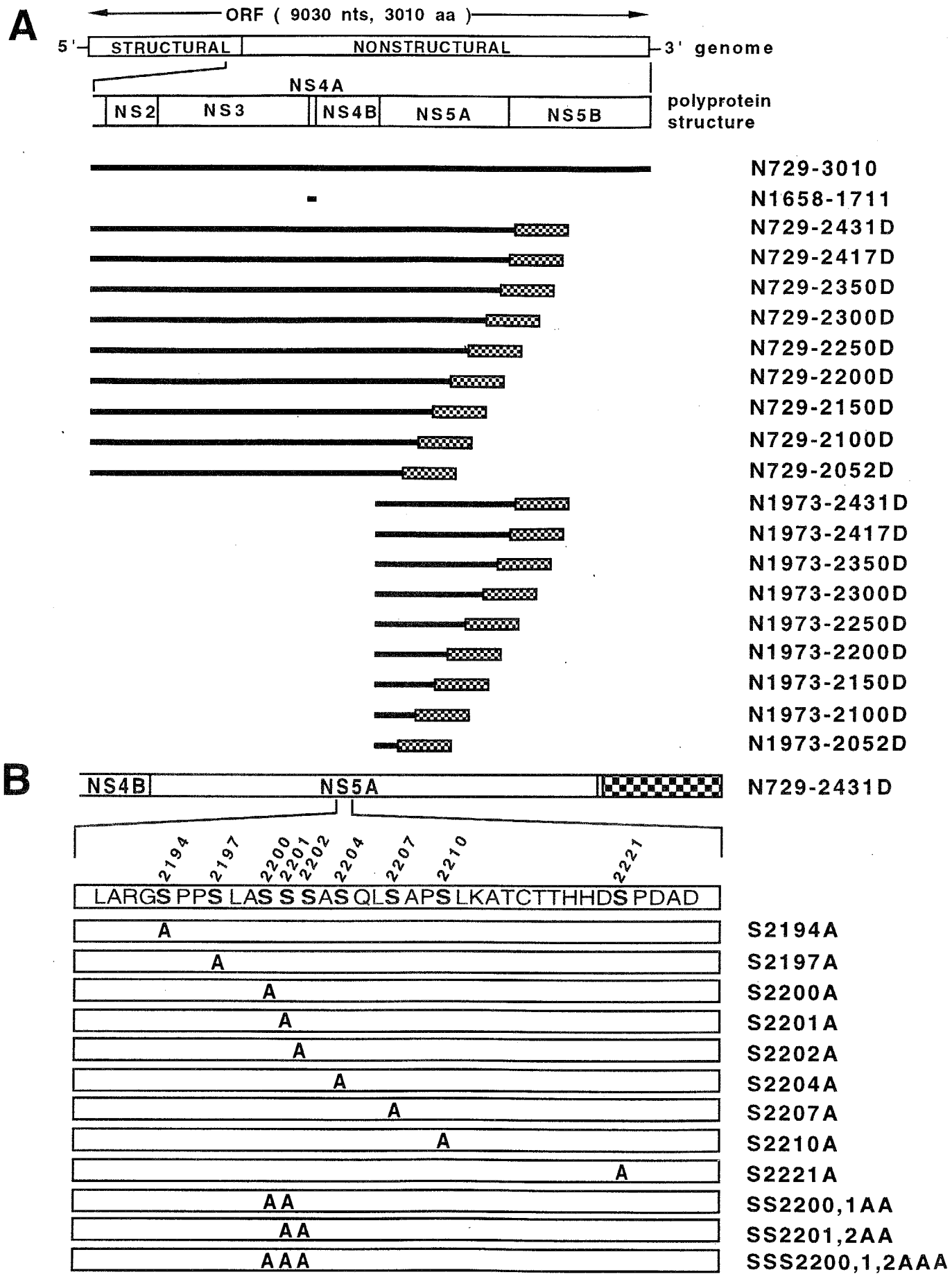

A
ORF (9030 nts, 3010 aa)
5'
STRUCTURAL
NONSTRUCTURAL
3' genome
NS4A
NS2
NS3
NS4B
NS5A
NS5B
polyprotein structure
N729-3010
N1658-1711
N729-2431D
N729-2417D
N729-2350D
N729-2300D
N729-2250D
N729-2200D
N729-2150D
N729-2100D
N729-2052D
N1973-2431D
N1973-2417D
N1973-2350D
N1973-2300D
N1973-2250D
N1973-2200D
N1973-2150D
N1973-2100D
N1973-2052D
B
NS4B
NS5A
N729-2431D
2194
2197
2200
2201
2202
2204
2207
2210
2221
LARGS PPS LAS S SAS QLS APS LKATCTTHHDS PDAD
A
S2194A
A
S2197A
A
S2200A
A
S2201A
A
S2202A
A
S2204A
A
S2207A
A
S2210A
A
S2221A
AA
SS2200,1AA
AA
SS2201,2AA
AAA
SSS2200,1,2AAA

Fig. 6-1. Schematic representations of HCV polyprotein fragments produced by the expression plasmids.

(**A**) The genomic and polyprotein structure of the nonstructural region from NS2 to NS5B are shown enlarged below the HCV ORF. The regions of polypeptides in the HCV precursor polyprotein are shown by thick bars. The designation of HCV polypeptide regions synthesized in COS-1 cells is shown at the right. Numbers indicate amino acid positions from the N- to the C- terminus of the HCV precursor polyprotein. Hatched boxes indicate *E. coli* DHFR fused in frame at the C-terminal end of the HCV polypeptide (abbreviated as D in the peptide designations). (**B**) The polyprotein structure around NS5A in N729-2431D. Below the polyprotein structure is the enlarged amino acid sequence, in single-letter code, showing amino acid substitution mutations. The serine residues in the HCV polyprotein that were mutagenized in this study are indicated by bold letters with the number of the amino acid position. Designations at the right indicate the positions in which serine residues were substituted to a neutral amino acid (alanine).

TABLE 6-1 Oligonucleotide primers for the PCR used in the construction of the pCMV plasmids.

Designation	Oligonucleotide[a]	aa[b]
F :	5'-TCAATGTCCTACACATGG-3'	(1973-1981)
R1 :	5'-CCGAAGCTTCGTGATCAAGGCACCTGT-3'	(2431-2426)
R2 :	5'-CCGAAGCTTGACGATGTCGTCGCCGGC-3'	(2417-2412)
R3 :	5'-CCGAAGCTTCTTAGTAGCCAGCTCCGC-3'	(2350-2345)
R4 :	5'-CCGAAGCTTGGACTCTAACAGTGGAGG-3'	(2300-2295)
R5 :	5'-CCGAAGCTTCACCTTATTCTCTGACTC-3'	(2250-2245)
R6 :	5'-CCGAAGCTTGCTGGCCAAGGAGGGGGG-3'	(2200-2195)
R7 :	5'-CCGAAGCTTCCCGACCTGGAATGTGAC-3'	(2150-2145)
R8 :	5'-CCGAAGCTTGTGGAAGTCCCCCACCCG-3'	(2100-2095)
R9 :	5'-CAAGGAGGGGGGAGCCCCCCTGGCCGG-3'	(S2194A)
R10 :	5'-AGAGCTGGCCAAGGCGGGGGGGAGACCC-3'	(S2197A)
R11 :	5'-GCTAGCTGAAGAGGCGGCCAAGGAGGG-3'	(S2200A)
R12 :	5'-TTGGCTAGCTGAAGCGCTGGCCAAGGA-3'	(S2201A)
R13 :	5'-CAATTGGCTAGCTGCAGAGCTGGCCAA-3'	(S2202A)
R14 :	5'-CGCAGACAATTGGGCAGCTGAAGAGCT-3'	(S2204A)
R15 :	5'-CAAGGAAGGCGCAGCCAATTGGCTAGC-3'	(S2207A)
R16 :	5'-TGTTGCCTTCAAGGCAGGCGCAGACAA-3'	(S2210A)
R17 :	5'-GTCAGCGTCCGGGGCGTCATGGTGGGT-3'	(S2221A)
R18 :	5'-TTGGCTAGCTGAAGCGGCGGCCAAGGAGGG-3'	(SS2200,1AA)
R19 :	5'-CAATTGGCTAGCTGCAGCGCTGGCCAAGGA-3'	(SS2201,2AA)
R20 :	5'-CAATTGGCTAGCTGCAGCGGCGGCCAAGGAGGG-3'	(SSS2200,1,2AAA)

[a] Underlined nucleotides indicate sequences complementary to the genome of HCV. Doubleunderlined nucleotides indicate sequences mutation was introduced.

[b] The corresponding amino acid numbers in the HCV genome (**F, R1-R8**). The amino acid positions of the Ser to Ala mutation (**R9-R20**).

6.2.2 Production of HCV polyprotein in COS-1 cells.

DNA transfections were performed as described in the chapter 2. The antibodies used in this experiment were α-NS4A, α-NS5A, and α-DHFR (20, 22, 36).

6.2.3 Pulse-chase analysis.

COS-1 cells seeded at a density of 2 X 10^5 cells per 3.5-cm plate were transfected with the pCMV plasmid for 24 h and used for pulse chase analysis. Cells were incubated in 0.5 ml phosphate free Eagles' minimum essential medium (MEM, Gibco BRL.) with 5% dialyzed-FCS for 1 h, and cultured in the medium supplemented with 400 μCi/ml ^{32}P-ortho-phosphate (10 mCi/ml, Amersham) for 15 min. After labeling, the cells were either lysed immediately in 100 μl Laemmli's sample buffer without dye or lysed following a chase for various times in Dulbecco's modified MEM (DMEM) containing 5% FCS. The conditions of pulse-chase labeling experiments using ^{35}S-methionine were described in the chapter 3.

6.2.4 Metabolic labeling with ^{32}P-ortho-phosphate.

COS-1 cells seeded at a density of 2 X 10^5 cells per 3.5-cm plate were transfected with the pCMV plasmid for 24 h and used for metabolic labeling. Cells were incubated in 1 ml phosphate free MEM (Gibco BRL.) with 5% dialyzed-FCS supplemented with 100 μCi/ml ^{32}P-ortho-phosphate for 3 h. After being labeled, the cells were further incubated for 3 h in DMEM containing 5% FCS.

6.2.5 Immunoprecipitation.

Immunoprecipitation of metabolically labeled with ^{32}P-ortho-phosphate was performed as described in the chapter 3.

6.2.6 Subcellular localization of NS5A.

As described above, pCMV-derived plasmids were transfected into COS-1 cells for 24 h, and switched to phosphate-free MEM with 5% dialyzed-FCS and 100 μCi/ml ^{32}P-ortho-phosphate for 4 h. Cells were harvested after trypsin treatment and washed with PBS containing 1 mM PMSF. The cells were lysed by adding 200 μl of buffer-A (10 mM Tris hydrochloride [pH 7.5], 10 mM KCl, 2 mM $MgCl_2$, 1 mM PMSF, 1 mM dithiothreitol). The homogenate was overlaid on sucrose solutions composed of 40% (upper layer) and 60% (lower layer) sucrose in buffer-A and centrifuged at 200,000 x g for 60 min to separate the cytosol, membrane, and nuclear fractions. All fractions were diluted 10-fold with extraction buffer and immunoprecipitated as described above.

6.3 Results.

6.3.1 Pulse chase analysis of NS5A phosphorylation.

COS-1 cells transfected with pCMV/N729-3010, which encodes the entire putative nonstructural protein region, were used for pulse-chase analysis. The transfectant was labeled for 15 min with ^{35}S-methionine or ^{32}P-ortho-phosphate, and chased for various times after addition of

DMEM containing 5% FCS. The cell lysates were immunoprecipitated using α-NS5A at chase time points of 0, 20, 60, 180, and 360 min (Fig. 6-2). Sequential production of the HCV nonstructural proteins was shown in the chapter 3. In this processing pathway, production of NS3 and NS5B was rapid and these proteins reached maximum after the end of the labeling period. An 89 kDa processing intermediate protein (NS4A-4B-5A) was observed during the early period of the chase, from 0 to 180 min. At the beginning of the chase period p58/p56 (NS5A) was not labeled, but the levels of p56 and p58 gradually increased, followed by a decrease in the p89 level. By re-examining the kinetics of p58/p56 production, previous results with ^{35}S-label were confirmed (Fig. 6-2A), and extended those results to include labeling with ^{32}P-ortho-phosphate. Incorporation of ^{32}P-ortho-phosphate, in contrast, was observed in p58/p56 at the beginning of the chase period, and was not detected throughout the chase period in the p89-precursor (Fig. 6-2B, lane 2 to 6). The level of ^{32}P-labeled p58/p56 became maximum after 20 min of chase, suggesting that phosphorylation was almost completed by this time. The relative abundance of detected p58 to p56 was nearly identical, indicating that p58 and p56 were phosphorylated at the same time (Fig. 6-2, lanes 2-6). This result suggested that phosphorylation of NS5A began after proteolytic processing of NS5A had finished.

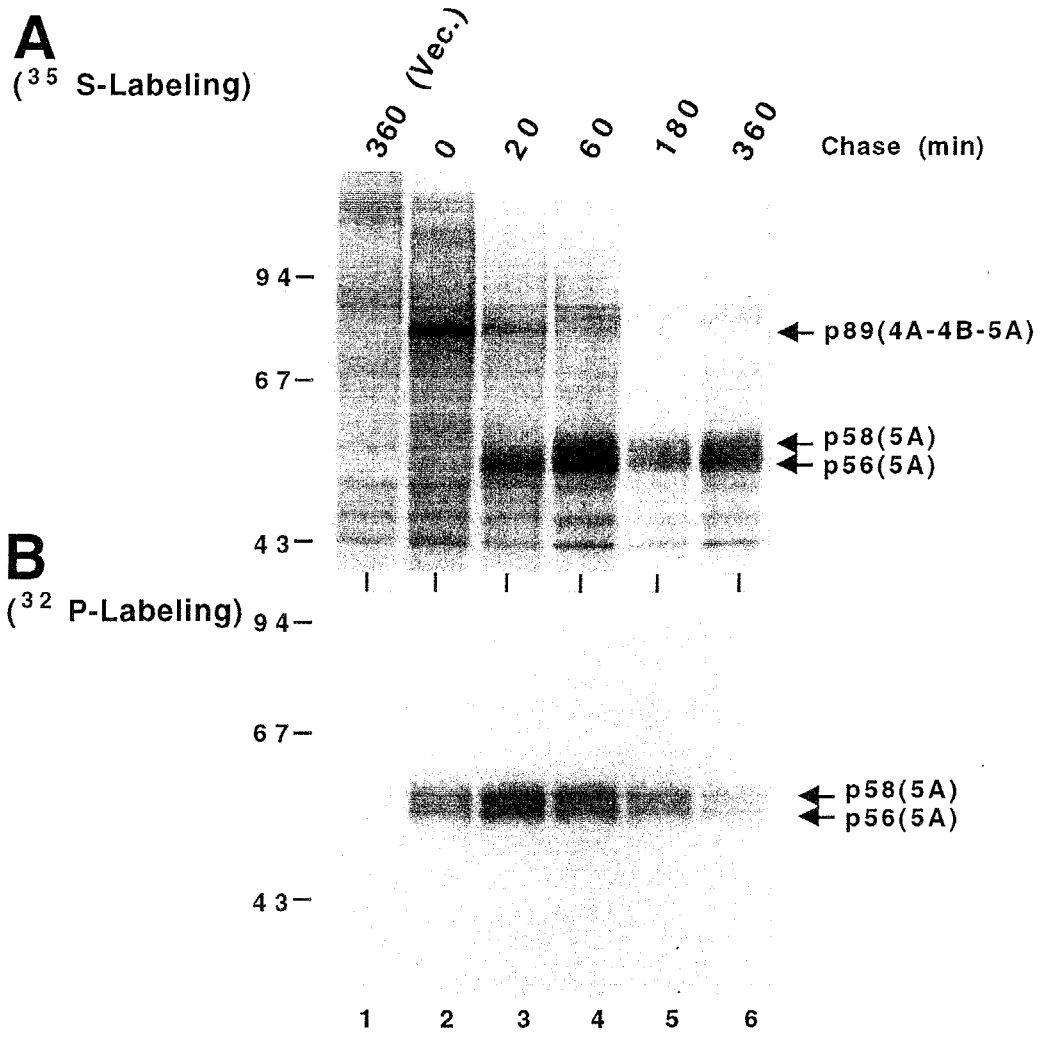


Fig. 6-2 Pulse chase analysis of phosphorylation of NS5A in the lysate transfected with pCMV/N729-3010.

Cells were pulse labeled for 15 min in the medium supplemented with ^{32}P-ortho-phosphate and then chased as indicated. The lysate at each time point was immunoprecipitated with α-NS5A. Immunoprecipitation was performed with lysates transfected with: pKS(+)/CMV, an original plasmid without HCV insert, lane 1 and pCMV/N729-3010, lanes 2-6. Gel condition was 8% SDS-PAGE. The gel was dried and was exposed to imaging plate. Molecular mass markers (in kDa) are shown on the left. The positions of p56 and p58 are shown on the right with arrows.

6.3.2 Identification of the region responsible for the NS4A-dependent phosphorylation.

Two NS5A products, p58 and p56, were phosphoproteins and that p58 was the NS4A-dependent phosphorylated form of p56 (chapter 5). To identify the region responsible for this NS4A-dependent phosphorylation, phosphorylation of products that were serially deleted from the C-terminus of NS5A was analyzed. In this study, the DHFR gene of *E. coli* was fused to the 3'-terminal ends of each of the deletion constructs that were derived from pCMV/N729-3010. The DHFR protein was used as the epitope tag for the detection of products. The results of these experiments are shown in Fig. 6-3. A comparison of N729-3010 and N729-2341D indicated, as expected, that the region downstream of the NS5A/5B cleavage site did not influence NS4A-dependent phosphorylation of NS5A (Fig. 6-3, lanes 1 and 2): N729-2431D includes the entire NS5A protein plus 10 amino acid residues from the N-terminus of NS5B, and yielded p58/p56 as efficiently as did N729-3010. When N729-2417D, which lacked two amino acid residues upstream of the NS5A/5B cleavage site, was produced in the cells, cleavage at the NS5A/5B site was impaired and two forms of NA5A fused with DHFR were detected by both α-NS5A and α-DHFR (Fig. 6-3A and B, lanes 3). This result indicated that the DHFR protein which was fused in frame at the C-terminal end of NS5A, did not interfere with the NS4A-dependent phosphorylation of NS5A. N729-2350D, N729-2300D, and N729-2250D also produced two distinct forms derived from NS5A plus DHFR, which were detectable by α-NS5A and α-DHFR (Fig. 6-3A and B, lanes 4,

5, and 6). However, from these three deletion mutant polypeptides the relative production of a slower migrating NS4A-dependent phosphorylated form to a faster migrating form was less than that of N729-3010 or N729-2431D. Products of N729-2200D and the further C-terminally deleted forms were not detected by the α-NS5A used in this work, but could be detected with α-DHFR (Fig. 6-3A and B, lanes 7 to 10). N729-2200D produced two forms, but production of the NS4A-dependent phosphorylated form was very low. Moreover, the difference in migration of the two forms produced in this polypeptide, N729-2200D, was slight, suggestig lesser extent of NS4A-dependent phosphorylation to the faster migrating protein. In contrast, N729-2150D, N729-2100D, and N729-2052D generated only single forms (Fig. 6-3B, lanes 8 to 10). These results indicated that deletion mutant polypeptides N729-2431D, N729-2417D, N729-2350D, N729-2300D and N729-2250D were NS4A-dependent phosphorylated although the efficiency of NS4A-dependent phosphorylation in the last three polypeptides was low, and the extent of NS4A-dependent phosphorylation in N729-2200D was reduced. Further deletion of the C-terminus region of NS5A abolished the NS4A-dependent phosphorylation, suggesting that the region responsible for this NS4A-dependent phosphorylation is located around residue 2,200 in the HCV precursor polyprotein.

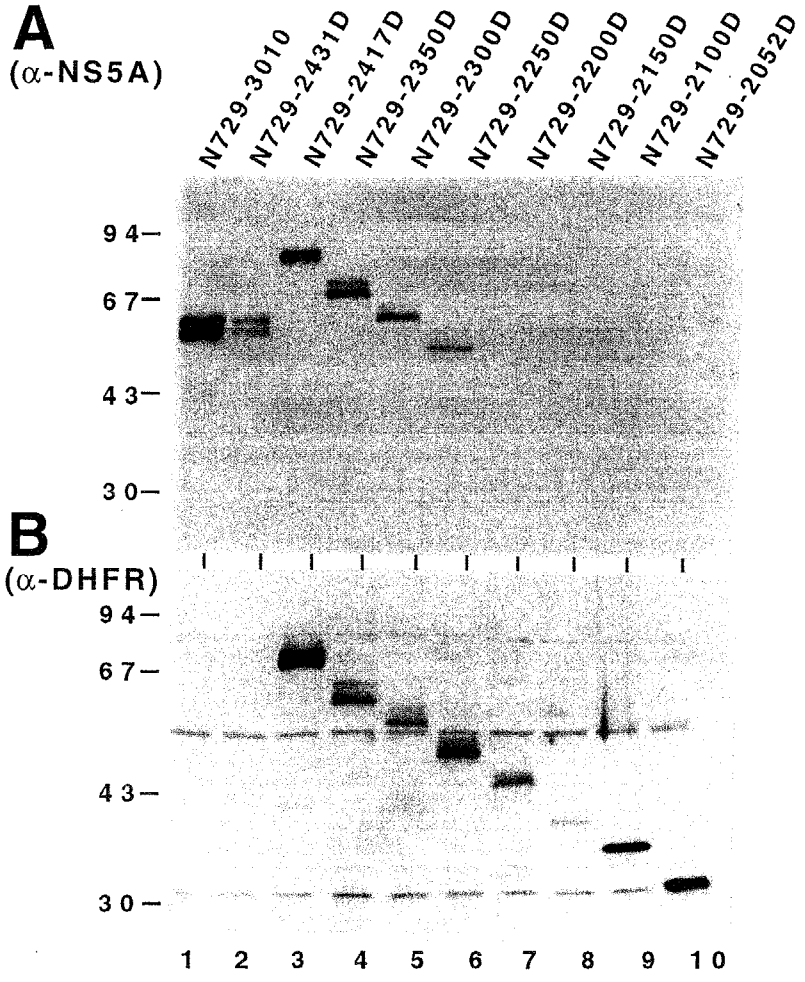


Fig. 6-3 Identification of the region responsible for the NS4A-dependent phosphorylation.

Lysate of COS-1 cells transfected with plasmids synthesizing fragments of polyprotein indicated above of the figure were fractionated on SDS-10% PAGE, followed by immunoblot analysis. Antibodies used in this experiment were α-NS5A (**A**), and α-DHFR (**B**). Molecular mass markers (in kDa) are shown on the left.

6.3.3 Identification of the serine residues responsible for the NS4A-dependent phosphorylation.

In order to determine the location of the serine residues responsible for the NS4A-dependent phosphorylation of NS5A, serine residues located around amino acid 2,200 were altered to alanine residues. In an attempt to determine whether one specific amino acid is the target for the NS4A-dependent phosphorylation or whether a specific cluster of serines is required for this reaction, mutated products shown in Fig. 6-1B for NS4A-dependent phosphorylation were examined. These included single mutations at amino acid 2,194 (S2194A), 2,197 (S2197A), 2,200 (S2200A), 2,201 (S2201A), 2,202 (S2202A), 2,204 (S2204A), 2,207 (S2207A), 2,210 (S2210A), and 2,221 (S2221A), double mutations at 2,200 plus 2,201 (SS2200/1AA), and 2,201 plus 2,202 (SS2201/2AA), and triple mutations at 2,200, 2,201, and 2,202 (SSS2200/1/2AAA).

Cells transfected with a series of mutant plasmids were analyzed by western blot using α-NS5A for detection of NS4A-dependent phosphorylation (Fig. 6-4A). The cells were simultaneously labeled with ^{32}P-ortho-phosphate, followed by immunoprecipitation with α-NS5A. Figure 4B shows production of phosphorylated NS5A in cells transfected with each mutant. It should be noted that the relative intensities of p58 and p56 in Fig. 6-4A and B were almost identical, indicating that the level of phosphorylation represented the amount of NS5A protein. The relative production of p58 to p56 in single mutants S2194A, S2200A, S2202A, S2207A, S2210A, and S2221A was almost identical to that of wild

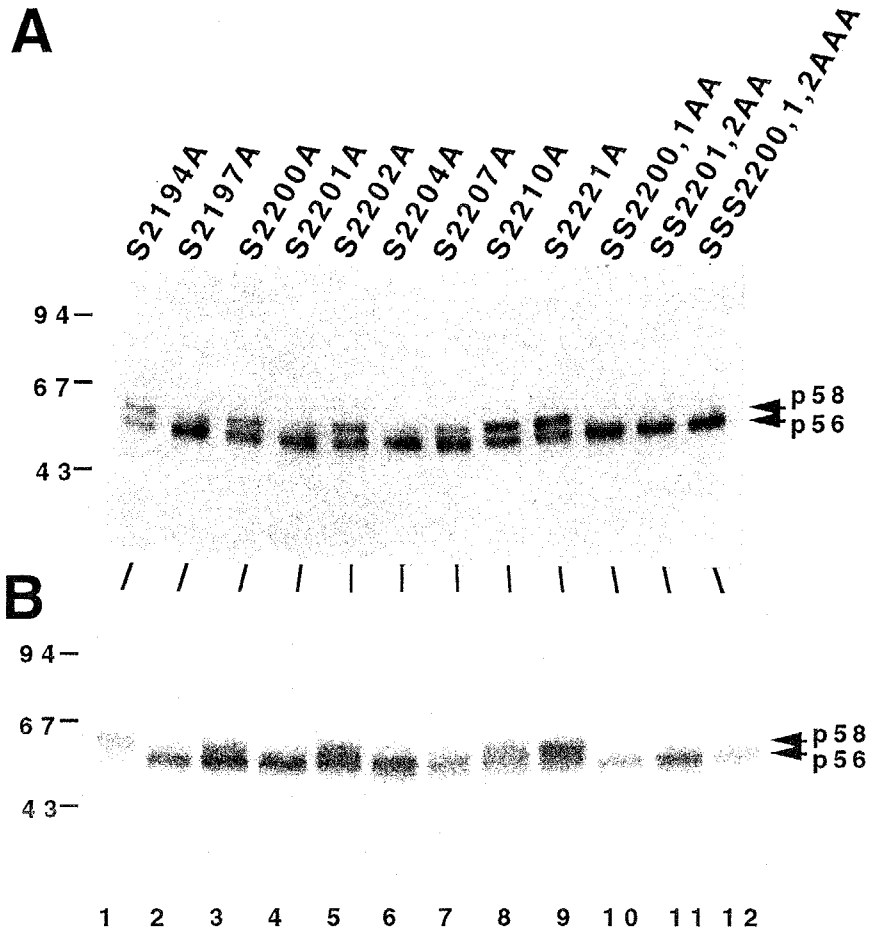


Fig. 6-4 Identification of serine residues responsible for the NS4A-dependent phosphorylation.

(**A**): COS-1 cells were transfected with plasmids synthesizing fragments of polyprotein (Fig. 6-1**B**) indicated above of the figure were fractionated on SDS-8% PAGE, followed by immunoblot analysis with α-NS5A. (**B**): Cells, transfected with plasmids, were labeled with ^{32}P-ortho-phosphate. The lysates were immunoprecipitated with α-NS5A, followed by fractionation on SDS-8% PAGE. Molecular mass markers (in kDa) are shown on the left. The positions of p58 and p56 are shown at the right with arrows.

type (Fig. 6-3A, lane 2, versus Fig. 6-4A or B, lanes 1, 3, 5, and 7 through 9). On the other hand, the production of the NS4A-dependent phosphorylated form of NS5A (p58) in S2197A, S2201A, S2204A, SS2200/1AA, SS2201/2AA, and SSS2200/1/2AAA was drastically reduced (Fig. 6-4A or B, lanes 2, 4, 6, 10, 11, and 12). These results suggested that serine residues at positions 2,197, 2,201, and 2,204 were important for the NS4A-dependent phosphorylation of NS5A.

6.3.4 NS4A-independent phosphorylation of NS5A.

It was shown that NS5A was phosphorylated at serine residue(s) even in the absence of NS4A product and gave a phosphoprotein of 56 kDa (chapter 5). To characterize NS4A independent phosphorylation of p56, plasmids expressing a series of the C-terminally deleted protein of NS5A fused with DHFR were transfected in COS-1 cells and the cell lysates were analyzed by immunoblot with α-DHFR. In contrast with the products of a series of C-terminally truncated NS5A protein produced from plasmids expressing nonstructural polypeptides (Fig. 6-3A and B, lanes 2 through 7), only a single form of NS5A product reactive with α-DHFR was produced from each deletion mutant (Fig. 6-5A, lanes 1 through 9). When the products were metabolically labeled with ^{32}P-ortho-phosphate and were immunoprecipitated with α-DHFR, a distinct 78 kDa NS5A product fused with DHFR was produced from N1973-2431D and N1973-2417D (Fig. 6-5B, lanes 1 and 2). Faster migrating bands of 50 kDa and 43 kDa were also detected. Although the nature of those faster migrating bands was not characterized, they seemed to be partially degraded forms of the fused proteins. The phosphorylated products reactive with α-DHFR were produced from N1973-2350D, N1973-2300D, and N1973-2250D, but the incorporation of radioactivity into these products was less than 1/10 that of N1973-2431D or N1973-2417D (Fig. 6-5B, lanes 1 to 5). Production of ^{32}P-labeled-phospho-proteins reactive with α-DHFR were not detected in N1973-2200D, N1973-2150D, N1973-2100D, and N1973-2052D (Fig. 6-5B, lanes 6 to 9). However, production

of these proteins was almost equally as detectable to that of N1973-2431D or N1973-2417D by western blot with α-DHFR (Fig. 6-5A). These results suggested the possibility that at least two regions were responsible for the NS4A-independent phosphorylation. One was in the C-terminal region between amino acid 2,351 to 2,419 of NS5A, which is the major region for NS4A-independent phosphorylation. Since no phosphorylated product was detected in the pCMV/N1973-2200D transfected cell lysate (Fig. 6-5B, lane 6), serine residues located in the region between amino acid 2,200 to 2,250 may be another region for the NS4A-independent phosphorylation.

When NS4A was coproduced (Fig. 6-5C, D, and E) with deletion mutant polypeptides, the slower migrating forms of NS5A products fused to DHFR (NS4A-dependent phosphorylated forms) were produced from the mutant polypeptides of N1973-2350D, N1973-2300D, and N1973-2250D (Fig. 6-5C and D, lanes 1, 2, and 3). These three mutant polypeptides lack the C-terminal region of NS5A, therefore, NS4A-dependent phosphorylation is likely to proceed independently from the major NS4A-independent phosphorylation at the C-terminal region of NS5A. Coproduction of NS4A with N1973-2150D, N1973-2100D, and N1973-2052D did not influence the production of the α-DHFR reactive proteins and did not yield phosphoprotein. This result supported previous evidence that the NS4A-dependent phosphorylation site is located in the region around amino acid 2200.

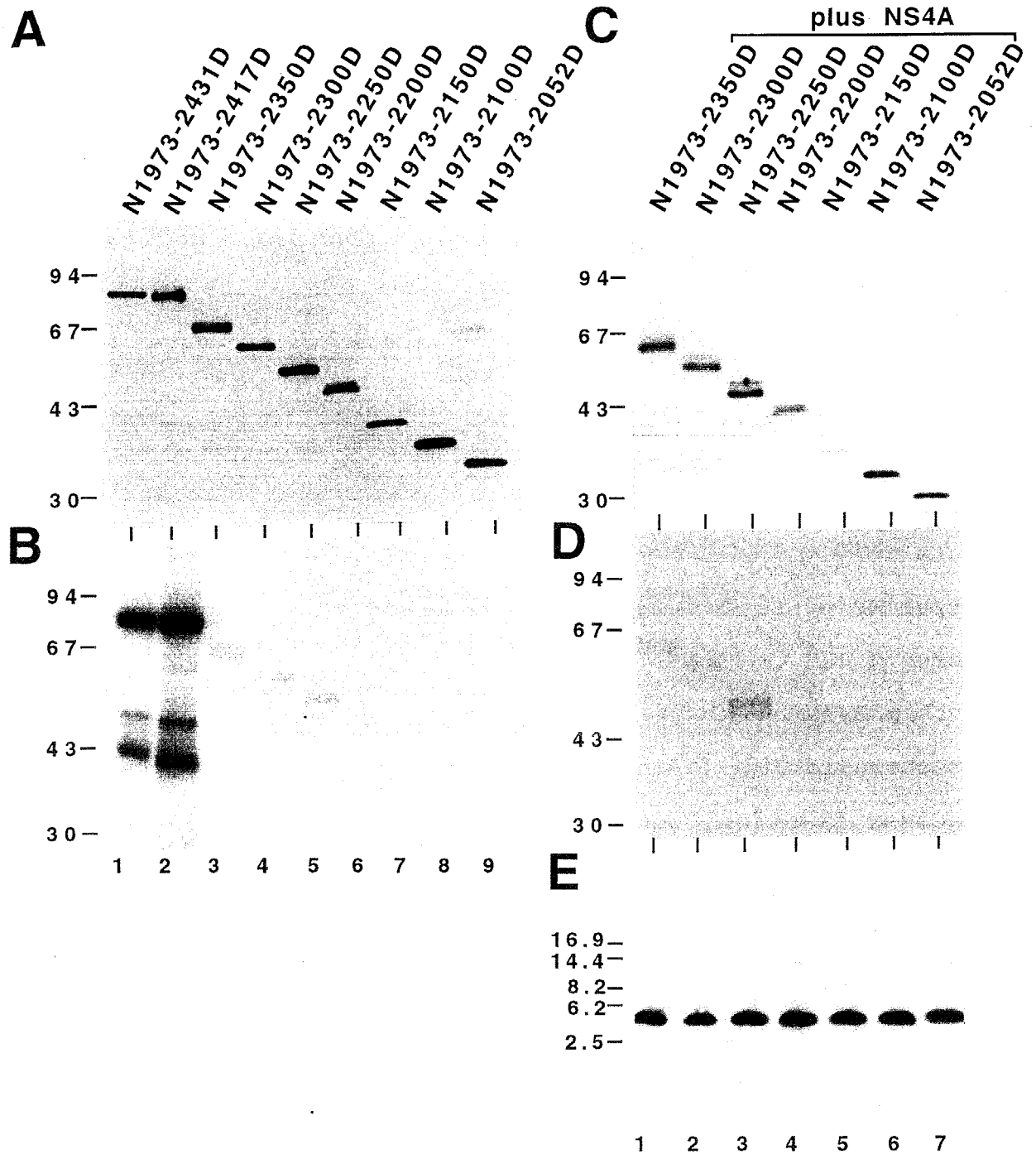


Fig. 6-5 NS4A-independent phosphorylation of NS5A.

COS-1 cells were transfected with plasmids synthesizing fragments of polyprotein indicated above of the figure. (**A**, **C**, and **E**): Cell lysate were fractionated on SDS-8% PAGE (**A** and **C**) or tricine/SDS/16% PAGE (**E**), followed by immunoblot analysis with: α-DHFR (**A** and **C**); α-NS4A (**E**). (**B** and **D**): Cells transfected with the plasmids, were labeled with ^{32}P-ortho-phosphate. Cell lysate were immunoprecipitated with α-DHFR, followed by fractionation on SDS 8% PAGE. Molecular mass markers (in kDa) are shown on the left.

6.3.5 Subcellular localization of NS5A.

To determine whether there are any differences in the subcellular locations of p58 and p56 in transiently expressing cells transfected with pCMV/N729-3010 or pCMV/NS4A/BNN, ^{32}P-ortho-phosphate labeled cell lysates were fractionated into cytosol, membrane, and nuclear fractions. COS-1 cells transfected with pCMV/NS4A/BNN, which contains the same region of the HCV ORF as pCMV/N729-3010 but has mutations at the NS4A/4B cleavage site, produces all the viral NS proteins except the NS4A-dependent phosphorylated form of NS5A (chapter 3). Whereas, COS cells transfected with pCMV/N729-3010 produce all the nonstructural proteins. As shown in Fig. 6-6, the majority of NS5A was found in the nuclear fraction, while a smaller amount was observed in the membrane fraction in both lysates (Fig. 6-6, lane 7, 8, 11, and 12). Indirect-immunofluorescence experiments of COS-1 cells transfected with pCMV/N729-3010 or pCMV/NS4A/BNN revealed NS5A products displayed on the nuclear membrane and distributed in the nuclear periplasmic membrane. No immunofluorescence was observed inside of the nuclei (data not shown).

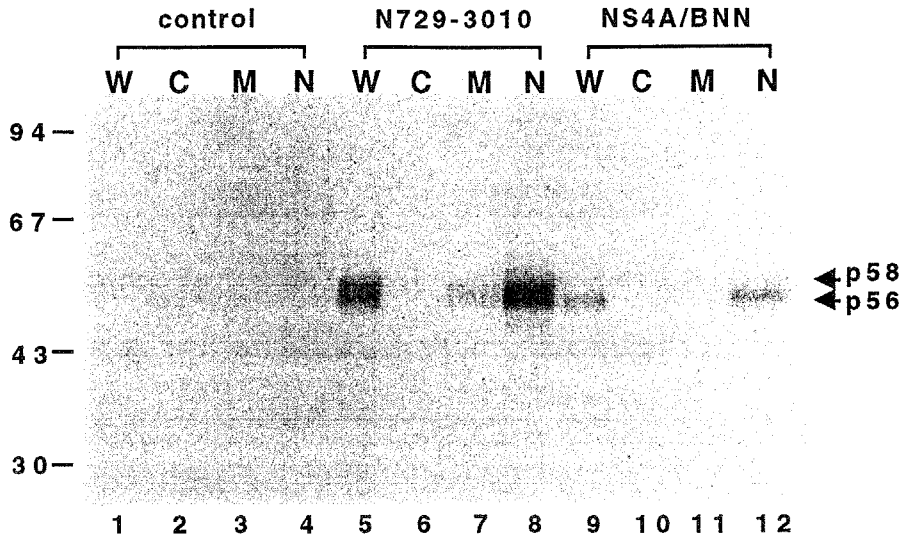


Fig. 6-6 Subcellular localization of NS5A.

Lysates of COS-1 cells transfected with: pKS(+)/CMV (lanes 1-4); pCMV/N729-3010 (lanes 5-8); or pCMV/NS4A/BNN (lanes 9-12), were separated into: whole cell (**W**) (lanes 1, 5, and 9); cytosol (**C**) (lanes 2, 6, and 10); membrane (**M**) (lanes 3, 7, and 11); and nuclear (**N**) (lanes 4, 8, and 12) fractions as indicated in the text. The lysate of each fraction was immunoprecipitated with α-NS5A. Gel condition was SDS-8% PAGE. Molecular mass markers (in kDa) are shown on the left. The positions of p58 and p56 are shown at the right with arrows.

6.4 Discussion.

It was shown that two phosphoproteins, p56 and p58, were produced from NS5A. The presence of the two proteins was reflected by different degrees of phosphorylation at serine residues. Moreover, the production of p58, the NS4A-dependent phosphorylated form of p56, depends on the presence of NS4A (chapter 5). In this work, serine residues at amino acid 2,197, 2,201, and 2,204 in the central region of NS5A were shown to be important for the NS4A-dependent phosphorylation. As shown in Fig. 6-7, amino acid sequences in the central part of NS5A, around amino acid 2,200, which was important for the NS4A-dependent phosphorylation, are highly conserved among HCV isolates. In particular, ten serine residues including these three serine residues, indicated with bold letters in the figure, are all conserved among these strains. Mutation of the serine residue at amino acid 2,197, 2,201, and 2,204 decreased the production of the NS4A-dependent phosphorylated form of NS5A, but mutation of the other serine residues did not show any effect (Fig. 4). However, the mutations at amino acid 2,197, 2,201, and 2,204 did not impair NS4A-dependent phosphorylation completely. This observation raises several possibilities. All these serine residues may be important for NS4A-dependent phosphorylation, and mutation at one of these sites may affect the efficiency of phosphorylation of the other two sites. Inefficient alternative phosphorylation might occur at other serine residues when mutations are introduced into one of the three serine residues. These three serine residues may not be

phosphorylation sites, but may affect the efficiency of phosphorylation at other serine residue(s). To clarify these possibilities, direct analysis of NS4A-dependent phosphorylated serine residue(s) in p58 is required.

Deletion analysis of NS5A also revealed the regions responsible for NS4A-independent phosphorylation. At least two regions, from amino acid 2,200 to amino acid 2,250 and from amino acid 2,350 to the C-terminus of NS5A, were important as NS4A-independent phosphorylation domains. The degree of the NS4A-independent phosphorylation in the C-terminal region of NS5A was more than 10 times higher than that of the region from amino acid 2,200 to 2,250. Different degrees of NS4A-independent phosphorylation in the two regions suggested the presence of multiple forms of phosphorylated p56 with indistinguishable mobilities on SDS-PAGE. Alternatively, the NS4A-independent phosphorylation in the region from amino acid 2,200 to amino acid 2,250 might be greatly influenced by deletion of the C-terminal NS5A region. When NS4A and N1973-2250D were coproduced in COS-1 cells, two distinct ^{32}P-labeled products were observed (Fig. 6-5D, lane 3), therefore, serine residues responsible for the NS4A-dependent phosphorylation and NS4A-independent phosphorylation in this region, from amino acid 2,200 to amino acid 2,250, were supposed to be different. The amino acid sequences of the C-terminal region, from 2351 to 2419 were compared among the HCV isolates (Fig. 6-7). Amino acids from 2381 to 2400 are well conserved among these 7 strains. Invariant serine residues in this region are shown with bold letters.

In the ^{35}S-methionine labeling experiment that was used to study the kinetics of processing of the nonstructural viral polyprotein, a trace amount of NS5A products was detected at the end of the pulse-labeling period, 0 min of chase period (Fig. 6-2A). And the level of NS5A products became maximum at 60 min after pulse-labeling. In contrast to this observation, ^{32}P-labeled NS5A products were detected at the beginning of the chase time and became maximum at 20 min of the chase period. This result suggested that phosphorylation proceeded after the proteolytic cleavages of both the N- and C- termini of NS5A were completed.

Protein phosphorylation is an important post-translational modification that has been shown to modulate a variety of macromolecular events such as transcription and translation (25). Although the function of NS5A in viral replication remains to be clarified, NS5A contains a nuclear localization-like signal sequence (PPRKKRTVV) that is present in the region proximal to the C-terminus from amino acid 2,326 to 2,334. Because of this the subcellular localization of the phosphorylated NS5A products was examined. Both p58 and p56 were mainly detected in the nuclear periplasmic membrane fraction. There was no significant difference in the subcellular localization of p56 and p58.

Clarification of the role of NS5A in relation to viral replication may be important for understanding regulation of viral replication. Regulation by phosphorylation also may be involved in the proliferation of HCV infected cells. NS4A-independent phosphorylation of NS5A was observed

without coproduction of any other viral proteins, suggesting that some cellular kinase mediates this phosphorylation, although whether or not NS5A itself has a kinase activity remains to be clarified. Whether the same kinase or a different kinase(s) is involved in NS4A-independent and NS4A-dependent phosphorylation of NS5A is not known. It should be noted that in the putative phosphorylation sites of the NS5A region there are no amino acid consensus sequences like those that surround the phosphorylation sites of known serine kinases, suggesting the possible involvement of an unidentified kinase(s).

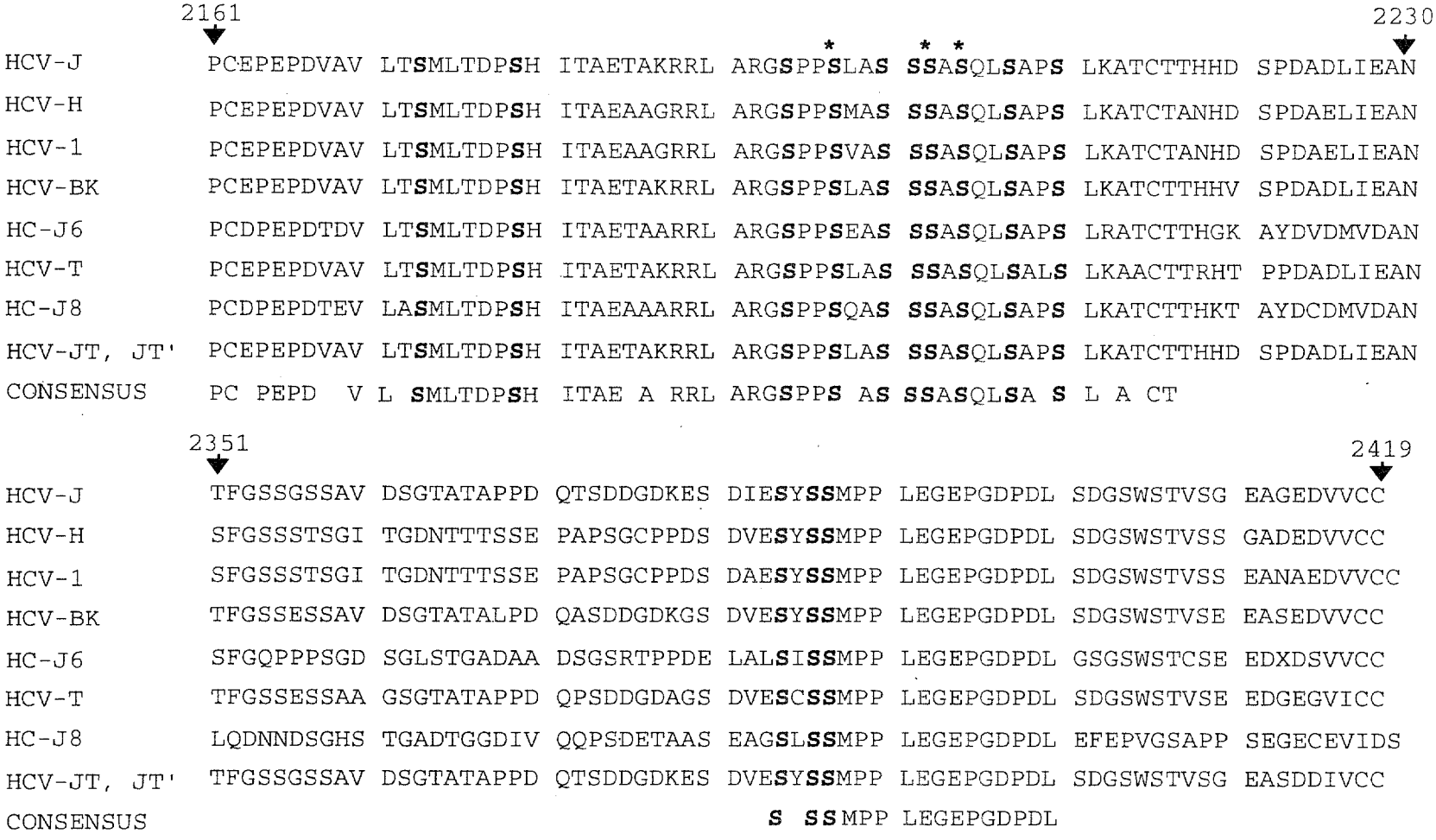


Fig. 6-7 Aligment of amino acid sequences of possible phosphorylation sites in HCV NS5.

The 70 amino acid residues surrounding the putative hyper-phosphorylation sites are aligned for the HCV-J strain as well as other published HCV sequences. Coserved serine residues in those HCV isolates are indicated by bold-letters. Possible NS4A-dependent phosphorylated serine residues are indicated by asterisks. Possible NS4A-independent phosphorylation domain, from 2351 aa to 2419 aa, is also indicated.

6.5 Summary of chapter 6.

Two proteins, a 56 kDa protein (p56) and a 58 kDa protein (p58), were produced from the HCV nonstructural region 5A (NS5A). Both proteins were phosphorylated at serine residues and that p58 was a NS4A-dependent phosphorylated form of p56. Furthermore, NS4A-dependent phosphorylation depended on the production of an intact form of the HCV NS4A protein. To clarify the nature of NS5A phosphorylation, pulse-chase analysis was performed by using a transient protein production system in cultured cells. The study indicated that phosphorylation of NS5A occurred after proteolytic production of NS5A was complete. In an attempt to identify the location of the NS4A-dependent phosphorylation sites in p58, proteins with sequential deletions from the C-terminal region of NS5A and with mutations of possible phosphorylated serine residues to a neutral amino acid, alanine, were constructed. The deleted or mutated proteins were then tested for NS4A-dependent phosphorylation in the presence of NS4A product. It was indicated that serine residues 2,197, 2,201, and/or 2,204 are important for NS4A-dependent phosphorylation. NS4A-independent phosphorylation sites were identified in the region from amino acid 2,200 to 2,250 and in the C-terminal region of NS5A product. Subcellular localization study showed that most of the NS5A products were localized in the nuclear periplasmic membrane fraction.

Chapter 7

Conclusions

In this thesis, the author presented analyses of HCV nonstructural polyprotein processing. HCV nonstructural proteins interacted essentially forming complex. This complex of associated nonstructural proteins may contain the RNA-dependent RNA polymerase enzymes (replicase), and roots that hold the genome on the surface of the endoplasmic reticulum during replication.

In chapter 2, identification of minimum serine proteinase domain was described. The essential domain for serine proteinase activity was located within the N-terminal 167 amino acids of HCV NS3, from amino acid 1049 to 1215 of the HCV precursor polyprotein. Serine proteinase dependent polyprotein processing was shown to function in *cis*- and *trans*- cleavage modes depending on the location of cleavage sites in the HCV precursor protein. The N-termini of NS4B, NS5A and NS5B were cleaved in a *trans*-cleavage manner, while that of NS4A was cleaved in a *cis*-cleavage manner.

As shown in this study, the N-terminal domain of NS3 contains proteolytic activity, while C-terminal region of NS3 contains conserved motifs in nucleotide-binding and helicase proteins. These two activities were located separately in the NS3.

In chapter 3, a possible processing pathway of HCV nonstructural protein was revealed. Production of NS3, which encodes serine proteinase domain, was first appeared. Then, the processing proceeded according to the order as follows. NS5B was produced by the cleavage at 5A/5B site. In turn, two NS5A products, p58 and p56, were processed

from the precursor polyprotein p89 (NS4A-4B-5A). Finally, p31 (NS4A-4B) split into NS4A and NS4B. Mutation analysis of cleavage sites revealed that cleavage at each site was essentially independent from cleavage occurring at the other site. Only exception was the processing of p58(NS5A). For the production of p58, efficient cleavages at 3/4A and 4A/4B sites were required.

Significance of the revealed sequential processing pathway of the HCV nonstructural protein in the formation of the functional replicase was not clear. Many RNA viruses produce their proteins as precursors which are subsequently cleaved into mature protein products. Rather than just being precursors, these intermediate proteins may perform functions in viral replication which are different from those of the individual proteins. These issues need to be reexamined in a system that supports HCV RNA replication.

In chapter 4, versatile functions of NS4A were shown. NS4A stabilizes NS3 and assisted in its localization in the membrane. Functioning of NS4A as a co-factor for cleavage at the 4B/5A site was observed when 30 amino acids around this site were used as a substrate and a serine proteinase domain identified in the chapter 2 was used as a enzyme protein. NS4A regulated the NS5A production. For the production of p58, equal or excess molar amounts of NS4A to enzyme-substrate protein were required. Deletion analysis of NS4A also revealed a minimum functional domain of NS4A of 10 amino acids.

The role of cytoplasmic membrane localization of the replicase complex in the initiation of RNA replication was

shown in many RNA viruses. HCV nonstructural protein complex is also predicted to be formed on the cytoplasmic membrane (22). Since all of the HCV nonstructural proteins were thought to be associated with NS3, NS4A mediated NS3 localization on the membrane was a essential step for the replicase complex formation.

In chapter 5, phosphorylation events on NS5A were analyzed. As was revealed in chapter 2 through 4, NS5A produced two forms of products, p56 and p58. Both proteins were phosphorylated at serine residues. And p58 was a additionally phosphorylated product of p56 depended on the presence of NS4A. The role of NS5A phosphorylation in HCV-RNA replication, if any, is remain to be clarified.

In chapter 6, phosphorylation of NS5A was analyzed further. Important serine residues for the NS4A-dependent phosphorylation were identified by deletion and mutation analyses. Alternation of serine residue(s) at 2197, 2201, and/or 2204 in HCV-IIJ to alanine residue(s) suppress the NS4A-dependent phosphorylation of NS5A. Two domains important for the NS4A-independent phosphorylation of NS5A were also identified as C-terminus region of NS5A and 2200-2250 amino acids in HCV-IIJ.

The amino acid sequence from residue 2631-2740 in NS5B, [Asp-$(X)_4$-Asp-$(X)_{56}$-Ser-Gly-$(X)_3$-Thr-$(X)_3$-Asn-$(X)_{25}$-Gly-Asp-Asp-X-Val] well matches the conserved motifs of RNA-dependent RNA polymerase. However, the function of NS5A in replicase complex is not known. One possibility is that NS5A is a regulatory cofactor of NS5B, and the phosphorylation of NS5A regulates replicase activity. In

any case, a biochemical approaches which allow a direct examination of active viral replicase complexes will ultimately be required.

REFERENCES

(1) **Akkina, R. K.** 1991. Pestivirus bovine viral diarrhea virus polypeptides: identification of new precursor proteins and alternative cleavage pathways. Virus Res. **19:**67-81.

(2) **Amemiya, Y., and J. Miyahara.** 1988. Imaging plate illuminates many field. Nature (London) **336:** 89-90.

(3) **Arias, C. F., F. Preugshat, and J. H. Strauss.** 1993. Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain. Virology **193:**888-899.

(4) **Bartenschlager, R., L. Ahlborn-Laake, J. Mous, and H. Jacobsen.** 1993. Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. J. Virol. **67:**3835-3844.

(5) **Bartenschlager, R., L. Ahlborn-Laake, J. Mous, and H. Jacobsen.** 1994. Kinetic and structural analyses of hepatitis C virus polyprotein processing. J. Virol. **68:**5045-5055.

(6) **Bazan, J. F., and R. J. Fletterick.** 1989. Detection of a trypsin-like serine proteinase domain in flaviviruses and pestiviruses. Virology **171:**637-639.

(7) **Chambers, T. J., A. Grakoui, and C. M. Rice.** 1991. Processing of the yellow fever virus nonstructural polyprotein: a catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites. J. Virol. **65:**6042-6050.

(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) **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 blood-borne non-A, non-B viral hepatitis genome. Science **244:**359-362.

(10) **Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, S. R. Medina, P. J. Barr, and a. l. et.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. U S A **88:**2451-2455.

(11) **Collett, M. S., D. K. Anderson, and E. Retzel.** 1988. Comparison of the pestivirus bovine viral diarrhea virus with members of Flaviviridae. J. Gen. Virol. **69:**2637-2643.

(12) **Eckart, M. R., M. Selby, F. Masiarz, C. Lee, K. Berger, K. Crawford, C. Kuo, M. Houghton, and Q.-L. Choo**. 1993. The hepatitis C virus encodes a serine protease involved in processing of the putative nonstructural proteins from the viral polyprotein precursor. Biochem. Biophys. Res. Commun. **192:**399-406.

(13) **Failla, C., L. Tomei, and R. D. Francesco.** 1994. Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. J. Virol. **68:**3753-3760.

(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) **Francki, R. I., C. M. Fauquet, D. L. Kundson, and F. Broun.** 1991. Classification and nomenclature of viruses. Arch. Virol. **52:**223-233.

(16) **Gorbalenya, A. E., E. V. Koonin, A. P. Donchenko, and V. M. Blinov.** 1989. N-terminal domains of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. Nucleic Acids Res. **17:**4713-4729.

(17) **Grakoui, A., D. Wychowski, C. Lin, S. M. Feinstone, and C. M. Rice,** 1993. Expression and identification of hepatitis C virus polyprotein cleavage products. J. Virol. 67:1385-1395.

(18) **Grakoui, A., D. W. Mccourt, C. Wychowski, S. M. Feinstone, and C. M. Rice.** 1993. Characterization of the hepatitis C virus-encoded serine proteinase: Determination of proteinase-dependent polyprotein cleavage sites. J. Virol. **67:**2832-2843.

(19) **Han, J. H., V. Shyamala, K. H. Richman, M. J. Brauer, B. Irvine, M. S. Urdea, P. Tekamp-Olson, G. Kuo, Q. L. Choo, and M. Houghton.** 1991. Characterization of the terminal regions of hepatitis C viral RNA: identification of conserved sequences in the 5' untranslated region and poly (A) tails at 3' end. Proc. Natl.. Acad. Sci. USA 88:5547-5551.

(20) **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. U S A **88:**5547-5551.

(21) **Hijikata, M., H. Mizushima, T. Akagi, S. Mori, N. Kakiuchi, N. Kato, T. Tanaka, K. Kimura, and K. Shimotohno.** 1993. Two distinct proteinase activities required for the processing of a putative nonstructural

precursor protein of hepatitis C virus. J. Virol. **67:**4665-4675.

(22) **Hijikata, M., H. Mizushima, Y. Tanji, Y. Komoda, Y. Hirowatari, T. Akagi, N. Kato, K. Kimura, and K. Shimotohno.** 1993. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proc. Natl. Acad. Sci. USA **90:**10773-10777.

(23) **Hirowatari, Y., M. Hijikata, Y. Tanji, & K. Shimotohno**, 1994. Expression and processing of putative nonstructural protein of hepatitis C virus in insect cells using baculovirus vector. Virus Res. in press.

(24) **Houghton, M., A. Weiner, J. Han, G. Kuo, and Q. L. Choo.** 1991. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology **14:**381-388.

(25) **Hunter, T., and M. Karin.** 1992 The regulation of transcription by phosphorylation. Cell **70:**375-387.

(26) **Inchauspe, G., S. Zebedee, D. -H. Lee, M. Sugitani, M. Nasoff, and A. M. Prince.** 1991. Genomic structure of the human prototype strain H of hepatitis C virus: comparison with the American and Japanese isolates. Proc. Natl.. Acad. Sci. USA **88:**10292-10296.

(27) **Jacob, J. R., J. W. Eichberg, G. R. Dreesman, and R. E. Lanford.** 1990. Expression of infectious viral particles by primary chimpanzee hepatocytes isolated during the acute phase of non-A, non-B hepatitis. J. Infect. Dis. **161:**1121-1127

(28) **Jang, S. K., H. G. Krausslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5' non translated region of encephalomyocarditis virus RNA directs internal entry of

ribosomes during in vitro translation. J. Virol. **62:**2636-2643.

(29) **Jang, S. K., M. V. Davies, R. J. Kaufman, and E. Wimmer.** 1989. Initiation of protein synthesis by internal entry of ribosomes into the 5' non translated region of encephalomyocarditis virus RNA in vivo. J. Virol. **63:**1651-1660.

(30) **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.

(31) **Kato, N., O. Yokosuka, M. Omata, K. Hosoda, and M. Ohto.** 1990. Detection of hepatitis C virus ribonucleic acid in the serum by amplification with polymerase chain reaction. J. Clin. Invest. **86:**1764-1767.

(32) **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. U S A **87:**9524-9528.

(33) **Komoda, Y., M. Hijikata, Y. Tanji, Y. Hirowatari, H. Mizusima, K. Kimura, and K. Shimotohno.** 1994. Processing of hepatitis C viral polyprotein in *Escherichia coli*. Gene **145:**221-226

(34) **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.

(35) **Lin, C., S. M. Amberg, T. J. Chambers, and C. M. Rice.** 1993. Cleavage at a novel site in the NS4A region by the yellow fever virus NS2B-3 proteinase is a prerequisite for processing at the downstream 4A/4B signalase site. J. Virol. **67:**2327-2335.

(36) **Manabe, S., I. Fuke, O. Tanishita, C. Kaji, Y. Gomi, S. Yoshida, C. Mori, A. Takamizawa, I. Yosida, and H. Okayama.** 1994. Production of nonstructural proteins of hepatitis C virus requires a putative viral protease encoded by NS3. Virol. **198:**636-644.

(37) **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 supergroups. Proc. Natl. Acad. Sci. U S A **87:**2057-2061.

(38) **Mizushima, H., M. Hijikata, Y. Tanji, K. Kimura, and K. Shimotohno.** 1994. Analysis of N-terminal processing of hepatitis C virus nonstructural protein 2. J. Virol. **68:**2731-2734.

(39) **Mizushima, H., M. Hijikata, S. Asabe, M. Hirota, K. Kimura, and K. Shimotohno.** 1994. Two hepatitis C virus glycoprotein E2 products with different C termini. J. Virol. **68:**6215-6222.

(40) **Mori, S., N. Kato, and A. Yagyu.** 1992. A new type of hepatitis C virus in Thailand. Biochem. Biophys. Res. Commun. **183:**334-342

(41) **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.

(42) **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.

(43) **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature (London). **334:**320-325.

(44) **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.

(45) **Prince, A. M., G. F. Grady, and C. Hazzi.** 1974. Long-incubation post transfusion hepatitis without serological evidence of exposure to hepatitis B virus. Lancet. **1:**241-249.

(46) **Russell, F. D., K. Jack.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105-132.

(47) **Schagger, H., and J. G. von**. 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.

(48) **Schimizu, Y. K., A. Iwamoto, M. Hijikata, R. H. Purcell, and H. Yosikura.** 1992. Evidence for *in vitro* replication of hepatitis C virus genome in a human T-cell line. Pro. Natl.. Acad. Sci. **89:**1105-1113

(49) **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.

(50) **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.

(51) **Tanji, Y., M. Hijikata, Y. Hirowatari, and K. Shimotohno**. 1994. Identification of the domain required for *trans*-cleavage activity of hepatitis C viral serine proteinase. Gene **145:**215-219.

(52) **Tanji, Y., M. Hijikata, Y. Hirowatari, and K. Shimotohno.** 1994. Hepatitis C virus polyprotein processing: kinetics and mutagenic analysis of serine proteinase-dependent cleavage. J. Virol. **68:**8418-8422.

(53) **Tanji, Y., M. Hijikata, S. Satoh, T. Kaneko, & K. Shimotohno**, Hepatitis C virus encoded nonstructural protein NS4A has versatile functions in viral protein processing. J. Virol. in press.

(54) **Tomei, L., C. Failla, E. Santolini, R. De Francesco, and N. La Monica**. 1993. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. J. Virol. **67:**4017-4026.

(55) **Tsukiyama-Kohara, K., N. Iizuka. M. Kohara, and A. Nomoto.** 1992. Internal ribosome entry site within hepatitis C virus RNA. J. Virol. 1992. **66:**1476-1483.

(56) **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.

(57) **Yano, M., H, Yatsuhasi, O. Inoue, and M. Koga.** 1991. Epideminology of hepatitis C virus in Japan: Role in chronic liver disease and hepatocellular carcinoma. J. Gastroenterol Hepatol. **6:**31-35

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List of author's published papers

(1) **Tanji, Y., Gennity, J., Pollitt, S., & M. Inouye,** 1991 Effect of OmpA signal peptide mutations on OmpA secretion, synthesis, and assembly. J. Bacteriol., **173:** 1997-2005.

(2) **Hirowatari, Y., M. Hijikata, Y. Tanji, H. Nyunoya, H. Mizushima, K. Kimura, T. Tanaka, Kato, N, & K. Shimotohno,** 1993. Two proteinase activities in HCV polypeptide expressed in insect cells using baculovirus vector. Arch. Virol. **133**:349-356.

(3) **Hijikata, M, H. Mizushima, Y. Tanji, Y. Komoda, Y. Hirowatari, T. Akagi, N. Kato, K. Kimura, & K. Shimotohno,** 1993. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Pro. Natl. Acad. Sci. USA **90**:10773-10777.

(4) **Mizushima, M., M. Hijikata, Y. Tanji, K. Kimura, & K. Shimotohno,** 1994. Analysis of N-terminal processing of hepatitis C virus nonstructural protein 2. J. Virol. **68**:2731-2734.

(5) **Tanji, Y., M. Hijikata, Y. Hirowatari, & K. Shimotohno,** 1994. Identification of the domain required for *trans*-cleavage activity of hepatitis C viral serine proteinase. Gene **145**:215-219.

(6) **Komoda, Y., M. Hijikata, Y. Tanji, Y. Hirowatari, H. Mizushima, K. Kimura, & K. Shimotohno,** 1994. Processing of hepatitis C viral polyprotein in *Escherichia coli*. Gene **145**:221-226.

(7) **Tanji, Y., M. Hijikata, Y. Hirowatari, & K. Shimotohno,** 1994. Hepatitis C virus polyprotein processing: kinetics and mutagenic analysis of serine proteinase-dependent cleavage. J. Virol. **68**:8418-8422.

(8) **Kaneko, T., Y. Tanji, S. Satoh, M. Hijikata, S. Asabe, K. Kimura, & K. Shimotohno,** 1994. Production of two phosphoprotein from the NS5A region of the hepatitis C viral genome. Biochem. Biophys. Res. Commun. **205**:320-326.

(9) **Hijikata, M., H, Mizushima, Y. Tanji, Y. Komoda, Y. Hirowatari, T. Akagi, N. Kato, T. Tanaka, K. Kimura, & K. Shimotohno,** 1994. Processing mechanisms of nonstructural proteins of hepatitis C virus. Viral Hepatitis and Liver Disease. p140-143.

(10) **Hirowatari,Y., M. Hijikata, Y. Tanji, & K. Shimotohno,** 1995. Expression and processing of putative nonstructural protein of hepatits C virus in insect cells using baculovirus vector. Virus Res. **35**:43-61.

(11) **Tanji, Y., M. Hijikata, S. Satoh, T. Kaneko, & K. Shimotohno,** 1995. Hepatitis C virus encoded nonstructural protein NS4A has versatile functions in viral protein processing. J. Virol. **69**: 1575-1581.

(12) **Tanji, Y., T. Kaneko, S. Satoh, & K. Shimotohno,** 1995. Phosphorylation of hepatitis C virus encoded nonstructural protein NS5A. J. Virol. **69**:in press.

(13) **Satoh, S., Y. Tanji, M. Hijikata, K. Kimura, & K. Shimotohno,** 1995. The N-terminal region of hepatitis C virus nonstructural protein 3 (NS3) is essential for stable complex formation with NS4A. J. Virol. in press.

(14) **Tanji, Y., & K. Shimotohno,** 1995. Mechanism of Hepatitis C virus protein processing. Nihon Rinshou. in press.