JOURNAL OF VIROLOGY, July 1995, p. 3980–3986 Vol. 69, No. 7
0022-538X/95/$04.00+0

Phosphorylation of Hepatitis C Virus-Encoded Nonstructural Protein NS5A

YASUNORI TANJI, TAKASHI KANEKO, SHINYA SATOH, AND KUNITADA SHIMOTOHNO*

Virology Division, National Cancer Center Research Institute, Chuo-ku, Tokyo 104, Japan

Received 9 January 1995/Accepted 20 March 1995

Two proteins, a 56-kDa protein (p56) and a 58-kDa protein (p58), are produced from the hepatitis C virus (HCV) nonstructural region 5A (NS5A). Recently, we found that both proteins are phosphorylated at serine residues and that p58 is a hyperphosphorylated form of p56. Furthermore, hyper-phosphorylation depends on the production of an intact form of the HCV NS4A protein. To clarify the nature of NS5A phosphorylation, pulse-chase analysis was performed with a transient protein production system in cultured cells. The study indicated that basal and hyperphosphorylation of NS5A occurred after proteolytic production of NS5A was complete. In an attempt to identify the location of the hyperphosphorylation 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 hyperphosphorylation in the presence of the NS4A product. Here, we report that serine residues 2197, 2201, and/or 2204 are important for hyper-phosphorylation. Important sites for basal phosphorylation were identified in the region from residues 2200 to 2250 and in the C-terminal region of the NS5A product. A subcellular localization study showed that most of the NS5A products were localized in the nuclear periplasmic membrane fraction.

Hepatitis C virus (HCV) is a positive-stranded RNA virus of about 9.5 kb (3, 14, 20). The viral genome encodes a large polyprotein precursor of about 3,000 amino acids (aa). The processed gene products include a putative core (C), three putative envelopes (E1, E2 type A, and E2 type B), and six nonstructural (NS) proteins. The order of individual proteins in the polyprotein is NH_2-C-E1-E2 (type A/type B)-(p7)-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. Recently, a 7-kDa cleavage product was shown to be produced from the region between E2 and NS2 (15, 17).

Processing of the precursor polyprotein requires both host and viral proteinases. A signalase(s) associated with the membrane of the endoplasmic reticulum is responsible for the production of C, E1, E2 type A, E2 type B, and p7 (6–8, 17). Cleavage at the NS2/NS3 junction depends on the virus-encoded metalloproteinase Cpro-1 (8). The remaining cleavages at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B sites are conducted by a virus-encoded serine proteinase, Cpro-2 (1, 4, 6, 8, 25). Furthermore, the NS4A protein appears to function as a cofactor required for cleavage at the NS4B/NS5A site (5, 24).

A 56-kDa (p56) and a 58-kDa (p58) protein are detected by NS5A-specific antibody (α-NS5A) in a lysate of COS-1 cells transfected with a plasmid that encodes the entire nonstructural protein region (9, 13, 22–24). Recent findings indicate that p56 and p58, which are produced from the NS5A region, are phosphorylated at serine residues (13). Moreover, p58 is a hyperphosphorylated form of p56, which can be phosphorylated in an NS4A-dependent manner.

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 hyperphosphorylation 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 hyperphosphorylation, serines were mutated to the neutral amino acid alanine, and hyper-phosphorylation was assayed. To identify the region that was responsible for NS4A-independent phosphorylation (basal 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.

MATERIALS AND METHODS

Construction of HCV protein expression plasmids. The HCV polypeptide regions synthesized in COS-1 cells for this study are shown in Fig. 1. The construction of plasmids pCMV/N729-3010, pCMV/N1658-1711, pCMV/N729-2052D, and pCMV/NS4A/BNN was reported previously (8, 9, 22, 23). To introduce deletion mutations into the HCV nonstructural polyprotein, a series of plasmids were constructed by PCR. The sequences of the synthetic oligonucleotides used as primers in the PCR are indicated in Table 1. The combinations of positive-stranded (F) and negative-stranded (R) primers used for PCR for PCR products PP1 to PP8 were: 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; and PP8, F and R8. PP1 to PP8 were digested with *Mlu*I and *Hin*dIII and inserted into the *Mlu*I and *Hin*dIII sites of pCMV/N729-2472D (9) 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 (13) was replaced with the *Pst*I fragments from each of the eight plasmids to obtain pCMV/N1973-2431D, pCMV/N1973-2417D, pCMV/N1973-2350D, pCMV/N1973-2300D, pCMV/

* Corresponding author. Mailing address: Virology Division, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104, Japan. Phone: 81-3-3542-2511, ext. 4700. Fax: 81-3-3543-2181. Electronic mail address: kshimoto@gan.ncc.go.jp.

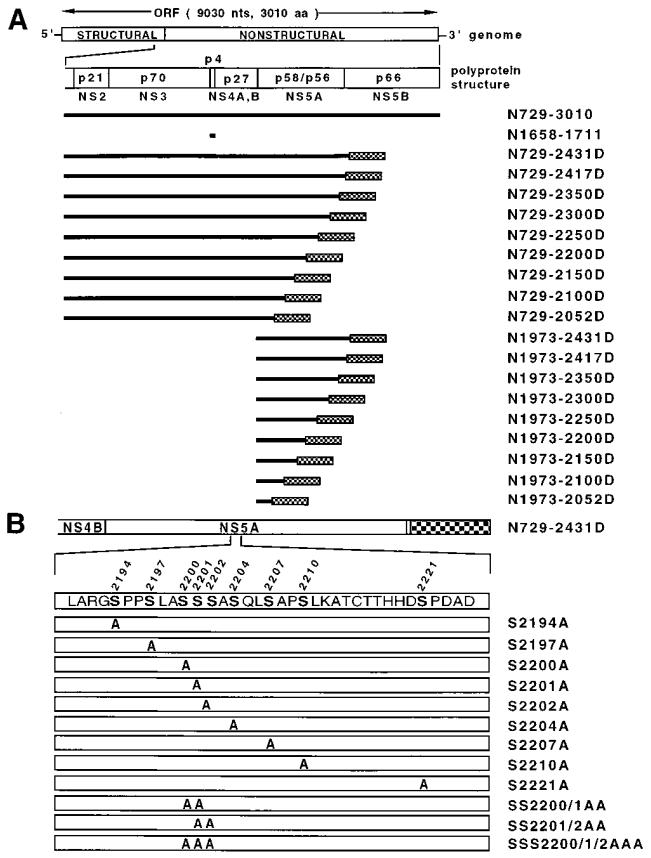

FIG. 1. Schematic representations of HCV polyprotein fragments produced by the expression plasmids. (A) The genomic and polyprotein structures of the NS region from p21 (NS2) to p66 (NS5B) are shown enlarged below the HCV open reading frame (ORF). The regions of polypeptides in the HCV precursor polyprotein 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 (14). Hatched boxes indicate *E. coli* DHFR fused in frame at the C-terminal end of the HCV polypeptide (abbreviated D in the peptide designations). nt, nucleotide. (B) Polyprotein structure around NS5A in N729-2431D. Below the polyprotein structure is the enlarged amino acid sequence, in the single-letter code, showing amino acid substitution mutations. The serine residues in the HCV polyprotein that were mutagenized in this study are indicated by boldface letters, with the amino acid position given. Designations at the right indicate the positions at which serine residues were changed to a neutral amino acid (alanine).

N1973-2250D, pCMV/N1973-2200D, pCMV/N1973-2150D, and pCMV/N1973-2100D, respectively.

Amino acid substitutions N729-2431D were introduced as follows. The first PCR used the primers indicated below. The resultant products were used as primers together with a third primer indicated in parentheses after the PCR products from the second PCR: 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); and PP20, F and R20 (R1). The resultant 12 PCR products were digested with *Mlu*I and *Hin*dIII and inserted into the *Mlu*I and *Hin*dIII sites 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, respectively.

Expression of HCV polyproteins in COS-1 cells. DNA transfections were performed as described previously (7). Lysates of COS-1 cells transfected with the series of pCMV-derived plasmids were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western immunoblot analysis as described previously (7). The antibodies used in this experiment were anti-NS4A antibody (α-NS4A), anti-NS5A antibody (α-NS5A), and antidihydrofolate reductase antibody (α-DHFR) (7, 9, 16). α-NS4A and α-NS5A were generous gifts from A. Takamizawa, Osaka University, Osaka, Japan.

TABLE 1. PCR oligonucleotide primers used to construct pCMV plasmids[a]

Primer	Sequence	Position
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'-AGAGCTGGCCAAGGCGGGGGGAGACCC-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] Sequences complementary to the HCV genome are underlined. Mutated nucleotides are double underlined. For F and R1 to R8, numbering corresponds to positions in the HCV genome. For R9 to R20, the position of the Ser-to-Ala mutation(s) is shown.

Pulse-chase labeling of NS5A products. COS-1 cells seeded at a density of 2×10^5 cells per 3.5-cm plate were transfected with a pCMV plasmid for 24 h and used for pulse-chase analysis. The cells were incubated in 0.5 ml of phosphate-free Eagle's minimal essential medium (MEM; Gibco BRL) with 5% dialyzed fetal calf serum (FCS) for 1 h and cultured in this medium supplemented with 400 μCi of $^{32}P_i$ (10 mCi/ml, Amersham) per ml for 15 min. After labeling, the cells were either lysed immediately in 100 μl of 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 with [^{35}S]methionine were described previously (23).

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

Immunoprecipitation of metabolically labeled cell extract with $^{32}P_i$. Cell lysates metabolically labeled with $^{32}P_i$ were boiled and diluted 10-fold with extraction buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride [PMSF]). One milliliter of the diluted lysates was pre-adsorbed with 50 μl of a protein G-Sepharose suspension (Pharmacia) and incubated with α-NS5A or α-DHFR for 1 h. The immunocomplex was recovered by addition of 30 μl of protein G-Sepharose and washed three times with 50 mM Tris-HCl (pH 7.4)–500 mM NaCl–5 mM EDTA–1% Nonidet P-40–5% sucrose and once with 10 mM Tris-HCl (pH 7.4)–50 mM NaCl–1 mM EDTA. The immunoprecipitates were analyzed by SDS–8% PAGE. The gels were dried and exposed to an imaging plate (Fuji Photo Film Co., Ltd.).

Subcellular localization of viral proteins. As described above, pCMV-derived plasmids were transfected into COS-1 cells for 24 h, after which the cells were switched to phosphate-free MEM with 5% dialyzed FCS and 100 μCi of $^{32}P_i$ per ml for 4 h. Cells were harvested after trypsin treatment and washed with phosphate-buffered saline (PBS) containing 1 mM PMSF. The cells were lysed by adding 200 μl of buffer A (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 2 mM $MgCl_2$, 1 mM PMSF, 1 mM dithiothreitol). Under these conditions of cell lysis, almost all of the nuclei were free of cytoplasmic fraction by microscopic observation. The lysate was overlaid on sucrose solutions composed of 40% (upper layer) and 60% (lower layer) sucrose in buffer A and centrifuged at 200,000 × *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.

Nucleotide sequence accession number. The sequence of the cDNA of pCMV/N729-3010 has been deposited in the DDBJ, EMBL, and GenBank DNA databases under accession number D16435.

RESULTS

Pulse-chase analysis for production of phosphorylated NS5A products. COS-1 cells transfected with pCMV/N729-3010, which encodes the entire putative nonstructural protein

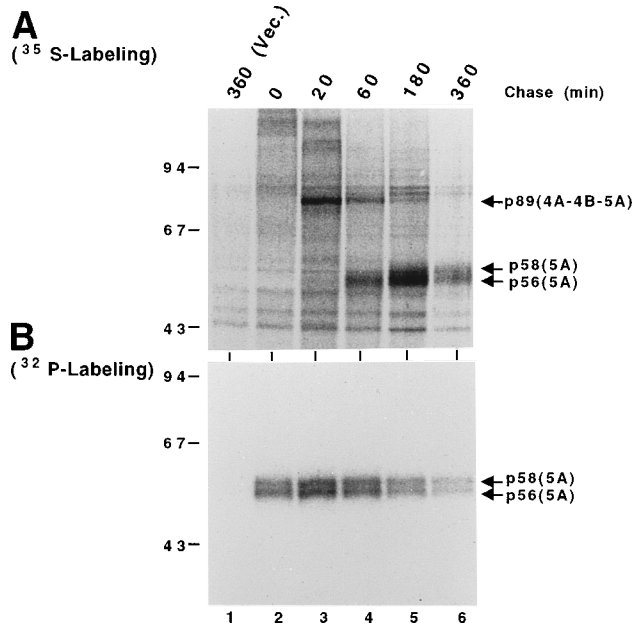

FIG. 2. Pulse-chase analysis of NS5A phosphorylation in a lysate transfected with pCMV/N729-3010. Cells were pulse labeled for 15 min in medium supplemented with [^{35}S]methionine (A) or $^{32}P_i$ (B) and then chased as indicated. The lysate at each time point was immunoprecipitated with α-NS5A. Immunoprecipitation was performed with lysates transfected with pKS(+)/CMV (8), an original plasmid without an HCV insert (Vec.) (lane 1), and pCMV/N729-3010 (lanes 2 to 6). The immunoprecipitates were separated by SDS–8% PAGE, and autoradiographed. The positions of molecular mass markers (in kilodaltons) are shown on the left. The positions of p89, p58, and p56 are shown on the right with arrows.

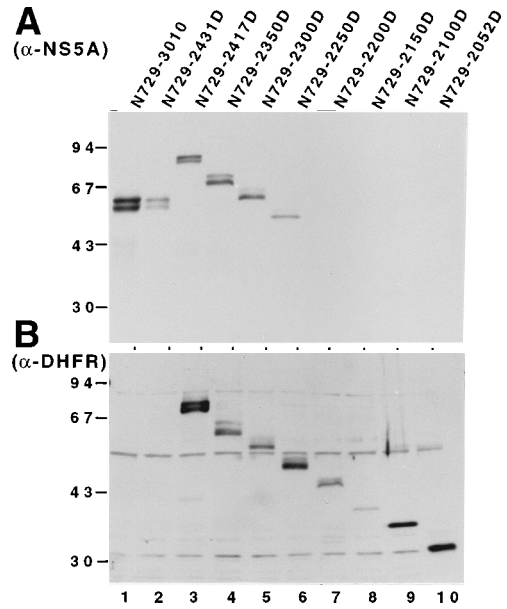

FIG. 3. Identification of the region responsible for hyperphosphorylation. Lysates of COS-1 cells transfected with polyprotein-producing plasmids, denoted above on each lane, were fractionated on SDS–10% PAGE followed by immunoblot analysis. The antibodies used in this experiment were α-NS5A (A) and α-DHFR (B). The positions of molecular mass markers (in kilodaltons) are shown on the left.

region (9), were used for pulse-chase analysis. The transfectant was labeled for 15 min with [^{35}S]methionine or $^{32}P_i$ and chased for various times after addition of DMEM containing 5% FCS. The cell lysates were immunoprecipitated with α-NS5A at chase time points of 0, 20, 60, 180, and 360 min (Fig. 2). We previously showed that the HCV nonstructural proteins are produced sequentially (23). In this processing pathway, production of NS3 and NS5B is rapid, and these proteins reach maximum levels after the end of the labeling period. An 89-kDa processing intermediate protein (NS4A-4B-5A) is observed during the early period of the chase, from 0 to 180 min. At the beginning of the chase period, p58/p56 (NS5A) is 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, we confirmed our previous results with ^{35}S labeling (Fig. 2A) and extended those results to include labeling with $^{32}P_i$. Incorporation of $^{32}P_i$, 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. 2B, lanes 2 to 6). The level of ^{32}P-labeled p58/p56 became maximal after 20 min of chase, suggesting that phosphorylation was almost complete by this time. The relative abundances of detected p58 and p56 were nearly identical, indicating that p58 and p56 were phosphorylated at the same time (Fig. 2, lanes 2 to 6). This result suggested that phosphorylation of NS5A began after proteolytic processing of NS5A had finished.

Identification of region in NS5A responsible for hyperphosphorylation. We showed previously that two NS5A products, p58 and p56, are phosphoproteins and that p58 is the hyperphosphorylated form of p56 (13). To identify the region responsible for this hyperphosphorylation, we analyzed the phosphorylation of products that were serially deleted from the C terminus of NS5A. In this study, the DHFR gene of *Escherichia 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. 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 the hyperphosphorylation of NS5A (Fig. 3, lanes 1 and 2). N729-2431D includes the entire NS5A protein plus 10 aa residues from the N terminus of NS5B and yielded p58/p56 as efficiently as did N729-3010. When N729-2417D, which lacked two 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 with both α-NS5A and α-DHFR (Fig. 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 hyperphosphorylation of NS5A. N729-2350D, N729-2300D, and N729-2250D also produced two distinct forms derived from NS5A plus DHFR, which were detectable by using α-NS5A and α-DHFR (Fig. 3A and B, lanes 4, 5, and 6). However, less of a slower-migrating hyper-phosphorylated form relative to a faster-migrating form was produced from these three deletion mutants than from N729-3010 or N729-2431D. Products of N729-2200D and the further C-terminally deleted forms were not detected with the α-NS5A used in this work but could be detected with α-DHFR (Fig. 3A and B, lanes 7 to 10). N729-2200D produced two forms, but production of the hyperphosphorylated form was very low. Moreover, the difference in migration of the two forms produced in this polypeptide, N729-2200D, was slight, suggesting lower hyperphosphorylation in the faster-migrating

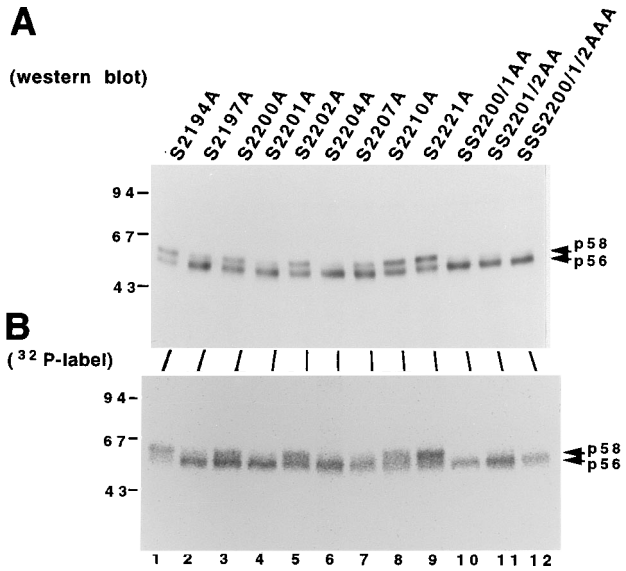

FIG. 4. Identification of serine residues responsible for hyperphosphorylation. (A) COS-1 cells were transfected with polyprotein-producing plasmids, as denoted above each lane, and lysates were fractionated on SDS–8% PAGE, followed by Western blot analysis with α-NS5A. (B) COS-1 cells transfected with the same plasmids were labeled with $^{32}P_i$. The lysates of transfected cells were immunoprecipitated with α-NS5A, followed by fractionation on SDS–8% PAGE and autoradiography. The positions of molecular mass markers (in kilodaltons) are shown on the left. The positions of p58 and p56 are shown at the right with arrows.

protein. In contrast, N729-2150D, N729-2100D, and N729-2052D generated only single forms (Fig. 3B, lanes 8 to 10). These results indicated that deletion mutant polypeptides N729-2431D, N729-2417D, N729-2350D, N729-2300D, and N729-2250D were hyperphosphorylated, although the efficiency of hyperphosphorylation in the last three polypeptides was low and the extent of hyperphosphorylation in N729-2200D was reduced. Further deletion of the C-terminal region of NS5A abolished the hyperphosphorylation, suggesting that the region important for this hyper-phosphorylation is located around aa 2200 in the HCV precursor polyprotein.

Locations of serine residues responsible for hyper-phosphorylation of NS5A protein. In order to determine the locations of the serine residues responsible for the hyperphosphorylation of NS5A, serine residues located around aa 2200 were altered to alanine residues. In an attempt to determine whether one specific amino acid is the target for the hyperphosphorylation or whether a specific cluster of serines is required for this reaction, we examined the mutated products shown in Fig. 1B for hyperphosphorylation. These included single mutations at aa 2194 (S2194A), 2197 (S2197A), 2200 (S2200A), 2201 (S2201A), 2202 (S2202A), 2204 (S2204A), 2207 (S2207A), 2210 (S2210A), and 2221 (S2221A), double mutations at aa 2200 plus 2201 (SS2200/1AA) and aa 2201 plus 2202 (SS2201/2AA), and triple mutations at aa 2200, 2201, and 2202 (SSS2200/1/2AAA).

Cells transfected with a series of mutant plasmids were analyzed by Western blot with α-NS5A for detection of hyperphosphorylation (Fig. 4A). The cells were simultaneously labeled with $^{32}P_i$ and then immunoprecipitated with α-NS5A. Figure 4B shows the production of phosphorylated NS5A in cells transfected with each mutant. It should be noted that the relative intensities of p58 and p56 in Fig. 4A and B were almost identical, indicating that the level of phosphorylation represented the amount of NS5A protein. The relative production of p58 and p56 in single mutants S2194A, S2200A, S2202A, S2207A, S2210A, and S2221A was almost identical to that in the wild type (Fig. 3A, lane 2, versus Fig. 4A and B, lanes 1, 3, 5, and 7 through 9). On the other hand, the production of the hyperphosphorylated form of NS5A (p58) in S2197A, S2201A, S2204A, SS2200/1AA, SS2201/2AA, and SSS2200/1/2AAA was drastically reduced (Fig. 4A and B, lanes 2, 4, 6, 10, 11, and 12). These results suggested that serine residues at positions 2197, 2201, and 2204 were important for the hyperphosphorylation of NS5A.

Basal phosphorylation domains in NS5A. We have shown previously that NS5A is phosphorylated at serine residue(s) even in the absence of NS4A product (basal phosphorylation) and gives a phosphoprotein of 56 kDa (13). To characterize NS4A-independent phosphorylation of p56, plasmids expressing a series of the C-terminally deleted protein of NS5A fused with DHFR were transfected into COS-1 cells, and the cell lysates were analyzed by immunoblot with α-DHFR. In contrast to the products of a series of C-terminally truncated NS5A proteins produced from plasmids expressing nonstructural polypeptides (Fig. 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. 5A, lanes 1 through 9). When the products were metabolically labeled with $^{32}P_i$ and immunoprecipitated with α-DHFR, a distinct 78-kDa NS5A product fused with DHFR was produced from N1973-2431D and N1973-2417D (Fig. 5B, lanes 1 and 2). Faster-migrating bands of 50 and 43 kDa were also detected. Although we did not characterize the nature of those faster-migrating bands, they seemed to be partially degraded forms of the fused proteins. 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. 5B, lanes 1 to 5). The production of ^{32}P-labeled phosphoproteins reactive with α-DHFR was not detected in N1973-2200D, N1973-2150D, N1973-2100D, and N1973-2052D (Fig. 5B, lanes 6 to 9). However, production of these proteins was almost equal to that of N1973-2431D or N1973-2417D by Western blot with α-DHFR (Fig. 5A). These results suggested that at least two regions were responsible for basal phosphorylation. One was in the C-terminal region between aa 2351 and 2419 of NS5A, which is the major region for basal phosphorylation. Since no phosphorylated product was detected in the pCMV/N1973-2200D-transfected cell lysate (Fig. 5B, lane 6), serine residues located in the region between aa 2200 and 2250 may be another site for NS4A-independent phosphorylation.

When NS4A was coproduced (Fig. 5C, D, and E) with deletion mutant polypeptides, the slower-migrating forms of NS5A products fused to DHFR (hyperphosphorylated forms) were produced from the mutant polypeptides of N1973-2350D, N1973-2300D, and N1973-2250D (Fig. 5C and D, lanes 1, 2, and 3). These three mutant polypeptides lack the C-terminal region of NS5A, and therefore, hyperphosphorylation is likely to proceed independently of the major basal 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 hyperphosphorylation site is located in the region around aa 2200.

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, $^{32}P_i$-labeled cell lysates

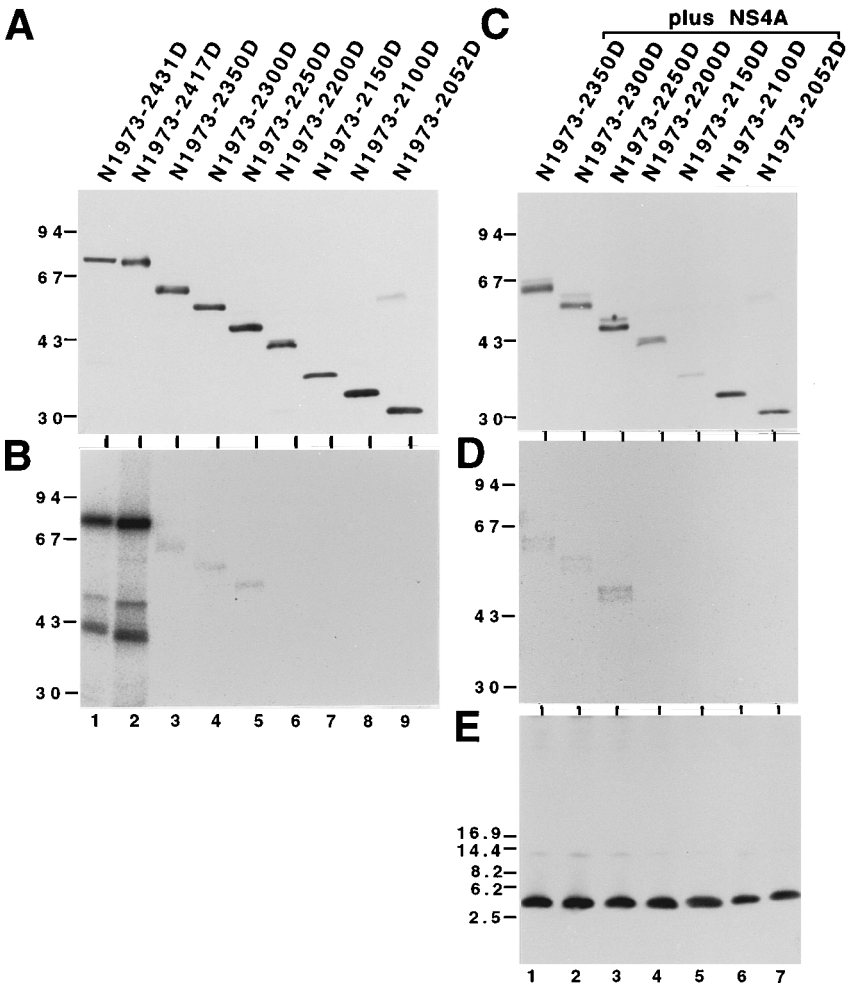

FIG. 5. Basal phosphorylation of NS5A. COS-1 cells were transfected with polyprotein-producing plasmids as denoted above each lane (A and B) or cotransfected with each of these plasmid plus a plasmid expressing NS4A (C, D, and E). Cell lysates were fractionated on SDS–10% PAGE (A and C) or tricine–SDS–16% PAGE (E), followed by Western blot analysis with α-DHFR (A and C) or α-NS4A (E). Transfected cells were metabolically labeled with $^{32}P_i$, and the cell lysates were immunoprecipitated with α-DHFR, followed by fractionation on SDS–8% PAGE and autoradiography (B and D). The positions of molecular mass markers (in kilodaltons) are shown on the left.

were fractionated into cytosol, membrane, and nuclear fractions. COS-1 cells transfected with pCMV/NS4A/BNN, which contains the same region of the HCV open reading frame as pCMV/N729-3010 but has mutations at the NS4A/4B cleavage site, produce all the viral NS proteins except the hyperphosphorylated form of NS5A (23), whereas COS cells transfected with pCMV/N729-3010 produce all the nonstructural proteins. As shown in Fig. 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, lanes 7, 8, 11, and 12). Indirect immunofluorescence experiments with COS-1 cells transfected with pCMV/N729-3010 or pCMV/NS4A/BNN revealed NS5A products on the nuclear membrane and distributed throughout the nuclear periplasmic membrane. No immunofluorescence was observed inside the nuclei (data not shown).

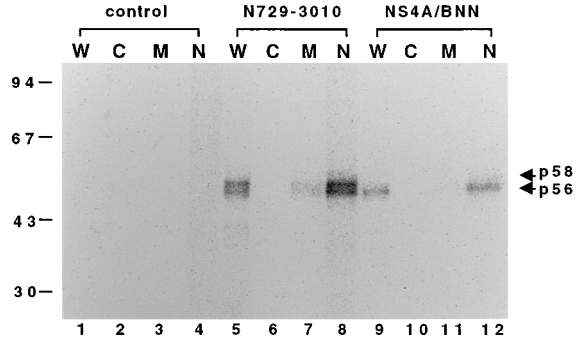

FIG. 6. Subcellular localization of NS5A. Lysates were made of COS-1 cells transfected with a control plasmid, pKS(+)/CMV (lanes 1 to 4), pCMV/N729-3010 (lanes 5 to 8), or pCMV/NS4A/BNN (lanes 9 to 12). The lysates were separated into whole-cell extract (W) (lanes 1, 5, and 9), cytosol (C) (lanes 2, 6, and 10), membrane (M) (lanes 3, 7, and 11), and nuclear (N) (lanes 4, 8, and 12) fractions. After immunoprecipitation with α-NS5A, proteins were fractionated on SDS–8% PAGE, followed by autoradiography.

DISCUSSION

We previously showed that two phosphoproteins, p56 and p58, are produced from NS5A. The presence of the two proteins is reflected by different degrees of phosphorylation at

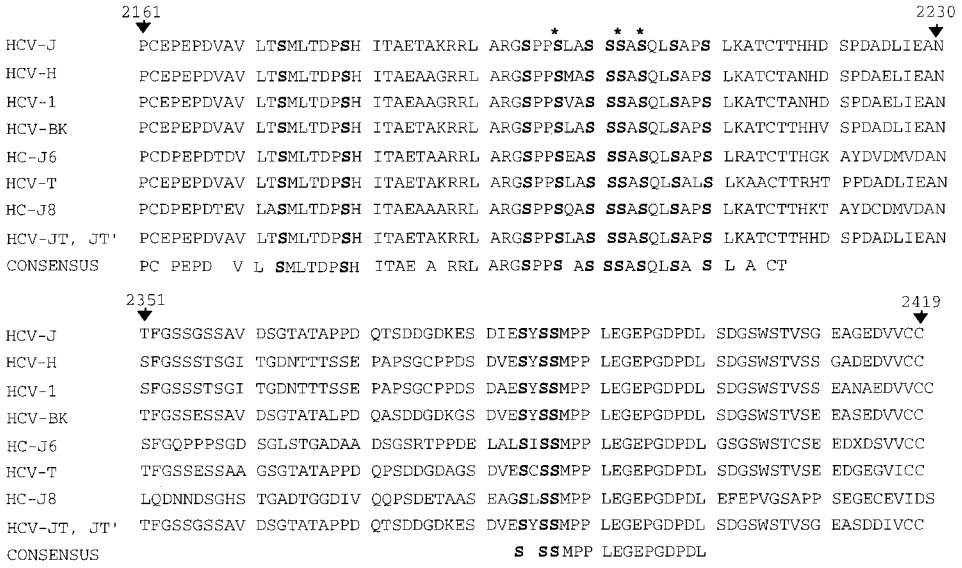

FIG. 7. Alignment of amino acid sequences of the regions important for basal and hyperphosphorylation of HCV NS5A. The 70 aa residues surrounding the possible hyper-phosphorylation sites were identified by mutation analysis and are aligned with the sequence of the HCV J strain as well as with other published HCV sequences: HCV J (14), HCV H (12), HCV-1 (3), HCV BK (20), HCV J6 (18), HCV T (2), HC J8 (18), and HCV JT and JT′ (21). Conserved serine residues in these HCV isolates are indicated by boldface letters. Serine residues important for hyperphosphorylation are indicated with asterisks.

serine residues. Moreover, the production of p58, the hyperphosphorylated form of p56, depends on the presence of NS4A (13). In this work, we found that serine residues at aa 2197, 2201, and 2204 in the central region of NS5A were important for hyperphosphorylation. As shown in Fig. 7, amino acid sequences in the central part of NS5A, around aa 2,200, which was important for hyperphosphorylation, are highly conserved among HCV isolates. In particular, 10 serine residues, including these 3 serine residues, indicated with boldface letters in the figure, are all conserved among these strains. Mutation of the serine residues at aa 2197, 2201, and 2204 decreased the production of the hyper-phosphorylated form of NS5A, but mutation of the other serine residues did not have any effect (Fig. 4). However, the mutations at aa 2197, 2201, and 2204 did not impair hyper-phosphorylation completely. This observation raises several possibilities. All of these serine residues may be important for hyperphosphorylation, 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 another serine residue(s). To clarify these possibilities, direct analysis of hyperphosphorylated serine residue(s) in p58 is under way.

Deletion analysis of NS5A also revealed the regions responsible for NS4A-independent phosphorylation (basal phosphorylation). At least two regions, from aa 2200 to aa 2250 and from aa 2350 to the C terminus of NS5A, were important as basal phosphorylation domains. The degree of basal phosphorylation in the C-terminal region of NS5A of N1973-2431D and N1973-2417D was more than 10 times higher than that of the region from aa 2200 to 2250 in N1973-2350D, N1973-2300D, and N1973-2250D. Different degrees of basal phosphorylation in the two regions suggested the presence of multiple forms of phosphorylated p56 with indistinguishable mobilities on SDS-PAGE. Alternatively, basal phosphorylation in the region from aa 2200 to aa 2250 might be greatly influenced by deletion of the C-terminal NS5A region. When NS4A and N1973-2250D were coproduced in COS-1 cells, we observed two distinct ^{32}P-labeled products (Fig. 5D, lane 3); therefore, we think that the serine residues responsible for hyperphosphorylation and basal phosphorylation in this region, from aa 2200 to aa 2250, are different. The amino acid sequences of the C-terminal region from aa 2351 to 2419 were compared among the HCV isolates (Fig. 7). The amino acids from 2381 to 2400 are well conserved among these seven strains. Invariant serine residues in this region are shown with boldface letters. Studies are under way to determine the locations of the phosphorylated serine residues in this region.

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. 2A), and the level of NS5A products became maximal 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 maximal 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 (11). 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 aa 2326 to 2334. Because of this, we examined the subcellular localization of the phosphorylated NS5A products. Both p58 and p56 were detected mainly 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 may also be in-

volved in the proliferation of HCV-infected cells. Basal phosphorylation of NS5A was observed without coproduction of any other viral proteins, suggesting that some cellular kinase mediates this phosphorylation, although whether NS5A itself has kinase activity remains to be clarified. Whether the same kinase or a different kinase(s) is involved in basal and hyperphosphorylation 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).

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare for a second-term Comprehensive 10-Year Strategy for Cancer Control and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. **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.
2. **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.
3. **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, A. J. Weiner, D. W. Bradley, G. Kuo, and M. Houghton.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:**2451–2455.
4. **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.
5. **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.
6. **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.
7. **Hijikata, M., N. Kato, Y. Ootsuyama, M. Nakagawa, and K. Shimotohno.** 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc. Natl. Acad. Sci. USA **88:**5547–5551.
8. **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.
9. **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.
10. **Hirowatari, Y., M. Hijikata, Y. Tanji, and K. Shimotohno.** 1995. Expression and processing of putative nonstructural proteins of hepatitis C virus in insect cells using baculovirus vector. Virus Res. **35:**43–61.
11. **Hunter, T., and M. Karin.** 1992. The regulation of transcription by phosphorylation. Cell **70:**375–387.
12. **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.
13. **Kaneko, T., Y. Tanji, S. Satoh, M. Hijikata, S. Shinichi, K. Kimura, and K. Shimotohno.** 1994. Production of two phosphoproteins from the NS5A region of the hepatitis C viral genome. Biochem. Biophys. Res. Commun. **205:**320–326.
14. **Kato, N., M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, T. Sugimura, and K. Shimotohno.** 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. USA **87:**9524–9528.
15. **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.
16. **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. Virology **198:**636–644.
17. **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.
18. **Okamoto, H., K. Kurai, S.-I. Okada, K. Yamamoto, H. Iizuka, 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.
19. **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.
20. **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.
21. **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.
22. **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.
23. **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.
24. **Tanji, Y., M. Hijikata, S. Satoh, T. Kaneko, and K. Shimotohno.** 1995. Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing. J. Virol. **69:**1575–1581.
25. **Tomei, L., C. Failla, E. Santolini, R. De Francisco, and N. La Monica.** 1993. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. J. Virol. **67:**4017–4026.