

Critical Point Mutations for Hepatitis C Virus NS3 Proteinase

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The hepatitis C virus NS3 proteinase plays an essential role in processing of HCV nonstructural precursor polyprotein. To detect its processing activity, we developed a simple *trans*-cleavage assay. Two recombinant plasmids expressing the NS3 proteinase region and a chimeric substrate polyprotein containing the NS5A/5B cleavage site between maltose binding protein and protein A were co-introduced into *Escherichia coli* cells. The proteinase processed the substrate at the single site during their polyprotein expression. Deletion analysis indicated that the functionally minimal domain of the NS3 proteinase was composed of 146 amino acids, 1059 to 1204. We isolated several cDNA clones encoding the functional domain of the NS3 proteinase from the sera of patients chronically infected with HCV and determined their proteinase activity by this *trans*-cleavage assay. Both active and inactive clones existed in the same patients. Comparative sequence analyses of these clones suggested that certain point mutations seemed to be related to the loss of proteolytic activity. This was confirmed by back mutation experiments. Among the critical mutations, Pro-1168 to Thr and Arg-1135 to Gly were intriguing. These amino acids, which are situated near the oxyanion hole, seem to be essential for maintaining the conformation of the active center of the NS3 proteinase. © 1998 Academic Press

INTRODUCTION

Hepatitis C virus (HCV) is the major etiological agent of posttransfusion non-A, non-B hepatitis worldwide (Choo *et al.*, 1989; Kuo *et al.*, 1989). HCV infection results in mild and acute liver disease, but chronic infections are common and may eventually develop into cirrhosis or hepatocellular carcinoma (Saito *et al.*, 1990). Although interferons are currently used for the treatment of chronic hepatitis, their efficacy is limited to a small portion of patients owing to insufficient suppression of HCV replication. Therefore, another reliable anti-HCV agent is necessary to control HCV hepatitis.

HCV has positive-strand RNA approximately 9400 nucleotides long which encodes a single polyprotein of about 3010 amino acids (aa) (Choo *et al.*, 1989, 1991; Kato *et al.*, 1990; Takamizawa *et al.*, 1991). Since its genomic organization is similar to those of flaviviruses and pestiviruses, HCV is classified as a member of the family Flaviviridae (Miller and Purcell, 1990; Takeuchi *et al.*, 1990). Following the 5'-untranslated region, the viral structural proteins are located at the amino (N)-terminal region of the polyprotein in the order of core, E1 and E2. After being translated as a precursor polyprotein, the structural proteins are processed by a host cell signal

peptidase(s), perhaps with no apparent involvement of virus-coded proteinase. The nonstructural (NS) proteins, which represent the essential machinery for viral replication, are located in the carboxyl (C)-terminal region in the order of NS2-NS3-NS4A-NS4B-NS5A-NS5B (Rice, 1996). In contrast to the cleavage of the structural proteins by the host enzyme, the NS proteins are processed by virus-coded proteinases. The HCV has two proteinases, NS2/3 proteinase and NS3 serine proteinase. The cleavage at NS2-NS3 is mediated by the former proteinase encoded within a region composed of the C-terminal portion of the NS2 gene and the N-terminal portion of the NS3 gene. Most likely this enzyme is a zinc-dependent metalloproteinase whose His-952 and Cys-993 were involved in catalysis (Grakoui *et al.*, 1993a; Hijikata *et al.*, 1993a). This putative metalloproteinase partially overlaps with the NS3 serine proteinase and presumably autocleaves the NS2/NS3 junction. The NS3 serine proteinase was shown to cleave the NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B junctions (Manabe *et al.*, 1994). The active site of this proteinase is composed of three highly conserved aa residues, His-1083, Asp-1107, and Ser-1165, which are well known as a catalytic triad of the serine proteinase family (Bazen and Fletterick, 1990; Bartenschlager *et al.*, 1993). It was confirmed that the Ser-1165 in the NS3 protein was essential for cleaving the downstream portion of the polyprotein by using *in vitro* transcription-translation systems and some mammalian cell culture systems (Chambers *et al.*, 1990; Gra-

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koui *et al.*, 1993b,c; Hijikata *et al.*, 1993b; Tomei *et al.*, 1993). Comparison of the sequences around each cleavage site revealed the unique substrate specificity of the proteinase: Cys or Thr at the P1 position, Ser or Ala at the P1' position, and Asp or Glu at the P6 position (Grakoui *et al.*, 1993b). Among other NS proteins, NS4A, a 54-residue amphipathic peptide, was shown to act as a cofactor for the NS3 proteinase interacting with the N-terminal portion of the enzyme (Bartenschlager *et al.*, 1995). Recently, the crystal structures of NS3 serine proteinase were reported from two groups. The tertiary structure of the proteinase was revealed to adopt a chymotrypsin-like folding, and its unique conformational aspects including a zinc-binding site and its complex formation with an NS4A peptide were elucidated (Kim *et al.*, 1996; Love *et al.*, 1996).

Since the viral serine proteinase is an attractive target for antiviral therapy, several assay systems of NS3 proteinase using *in vitro* transcription-translation systems and some mammalian cell culture systems have already been developed. Previously, we constructed an enzymatic assay system using one of our original HCV clones, D51, which displays functional activity of the purified enzyme *in vitro*, and studied the characteristics

of the proteinase (Mori *et al.*, 1996). We also reported the importance of the N-terminal part of NS3 for its proteinase activity (Mori *et al.*, 1997). In parallel with these previous studies, we newly cloned HCV proteinase genes from HCV-positive sera of chronic hepatitis patients into a plasmid of *Escherichia coli* and examined their ability to cleave a coexpressed substrate fusion protein containing the NS5A/5B cleavage site between maltose binding protein (MBP) and protein A. It was found that the isolated NS3 proteinase clones had variety of activities and some of them were inactive. In this study, we identified critical point mutations at Pro-1168 and Arg-1135, which lead to loss of proteinase activity, and discuss how these amino acids contribute to the processing activity from a structural point of view.

RESULTS

Construction of *trans*-cleavage assay system in *E. coli*

To evaluate the activity of isolated HCV NS3 proteinase clones, we developed an assay system which detects the proteinase activity by cotransformation of *E. coli* with expression plasmids containing a cloned NS3 pro-

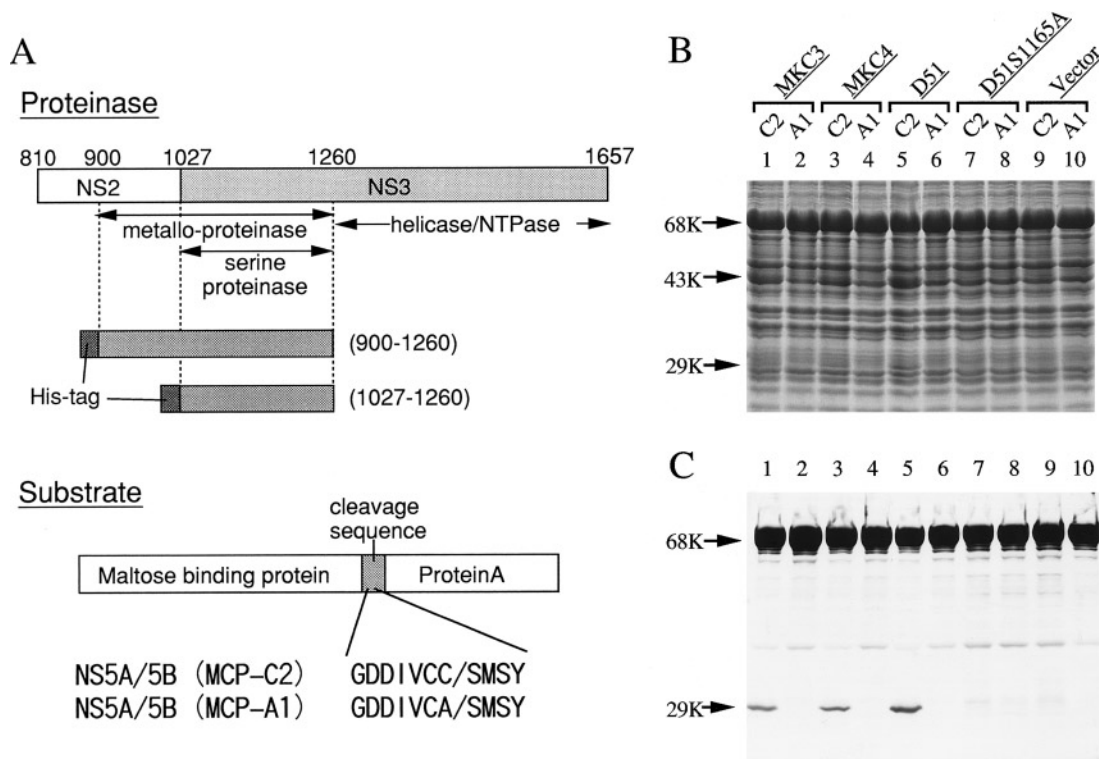


FIG. 1. Construction of *trans*-cleavage assay system in *E. coli*. (A) Schematic representation of the recombinant substrates and the HCV NS3 proteinases produced by the expression plasmids. (B) A substrate (MCP-C2, lanes 1, 3, 5, 7, and 9; MCP-A1, lanes 2, 4, 6, 8, and 10) and an enzyme clone (MKC3, lanes 1 and 2; MKC4, lanes 3 and 4; D51, lanes 5 and 6; D51S1165A, lanes 7 and 8; vector only, lanes 9 and 10) were coexpressed in double transformants of the substrate and the enzyme expression vectors by IPTG induction. The proteolytic activity was analyzed by SDS-PAGE followed by CBB staining. The substrate (68 kDa) and the products (43 and 29 kDa) are indicated by arrows. (C) Western blotting of the same sample as (B) using HRP-conjugated goat IgG anti-rabbit IgG. Only the substrate and the processed C-terminal product (68 and 29 kDa), which contained a protein A domain, were detected. The details are given under Materials and Methods.

teinase and a recombinant substrate. First, our proteinase-active original cDNA clones pMKC3, pMKC4, and pD51, which encode the residues 900–1260 of the viral polyprotein, were introduced into *E. coli* together with substrate expression plasmid pMCP-C2, which encodes a fusion protein containing the NS5A/5B cleavage sequence between MBP and protein A junctions (Fig. 1A). As shown in Fig. 1B, we found at the CBB staining level that the substrate (68 kDa) was cleaved into two polypeptides, MBP (43 kDa) and protein A (29 kDa). The processing activity was confirmed by Western blotting analysis using HRP-conjugated IgG (Fig. 1C). N-terminal sequence analysis of the protein A band produced by the proteinase revealed that the substrate was indeed processed at the NS5A/5B cleavage site (data not shown). When the Ser-1165 of the enzyme was replaced with Ala (Figs. 1B and 1C, lanes 7) or the P1 Cys residue of the recombinant substrate was replaced with Ala (Figs. 1B and 1C, lanes 2, 4, 6, 8, and 10), the cleavage was not observed. These results indicated that this assay is useful for screening for functional cDNA clones with proteinase activity.

Deletion analysis of the proteinase region

Using this *E. coli trans*-cleavage assay, we then determined the minimal region to maintain the proteinase

activity. A series of N- and C-terminal deletion mutants from the D51 clone were constructed and subjected to the *trans*-cleavage assay (Figs. 2A–2C). Although the cleavages by the proteinase region (900–1260) and (1059–1214) were not efficient for detection by CBB staining (Fig. 2B), they were clearly detected by Western blotting (Fig. 2C). The N-terminal deletion experiments (lanes 2, 5, 7–14) indicated that the N-terminal border essential for *trans* cleavage of the substrate at the 5A/5B site was Val-1059. On the other hand, the C-terminal deletion experiments (lanes 2–6, 15) indicated that the functional C-terminal border was Thr-1204. From these results, the minimal NS3 proteinase region was narrowed down to the region between aa residues 1059 and 1204. To detect the proteinase activity sensitively, we adopted Western blotting analysis in the following experiments.

cDNA cloning of NS3 proteinase region from a patient and determination of its activity

Using RT-PCR methods, we isolated cDNA fragments of the HCV genome coding NS3 proteinase region 1027–1260 from two patients' sera (N and U). From each serum, we obtained several clones and examined them individually for their proteinase activity (Fig. 3). Several clones did not cleave the substrate (Fig. 3, clones MKC2, N-A1,

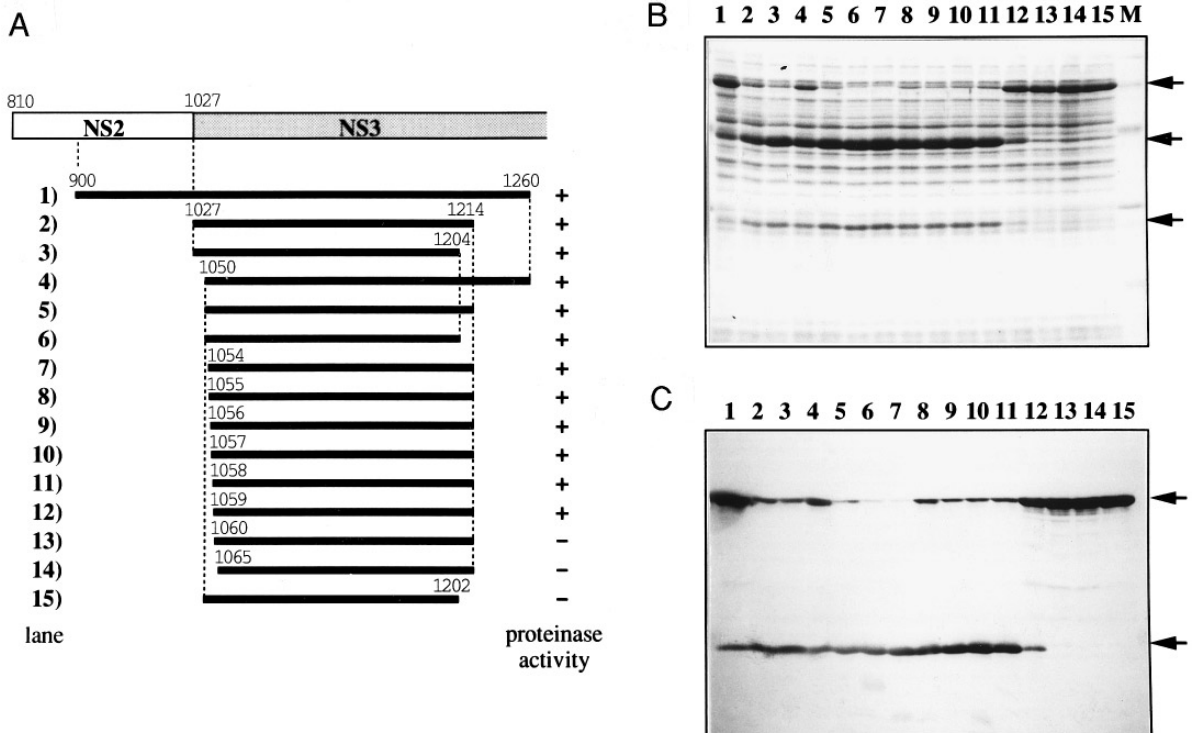


FIG. 2. Mapping of the minimal NS3 region required for cleavage at the NS5A/NS5B site. A series of N- and C-terminal deletion mutants from D51 proteinase clone were constructed and subjected to the *trans*-cleavage assay. (A) Schematic representation of the deletion mutants used in the assay. The numbers indicated on the left side correspond to the lane numbers in (B) and (C). (B) The processing activities of each deletion mutant were determined by SDS-PAGE followed by CBB staining. (C) The substrate and the processed C-terminal product, which contained a protein A domain, were detected by Western blotting using HRP-conjugated goat IgG anti-rabbit IgG.

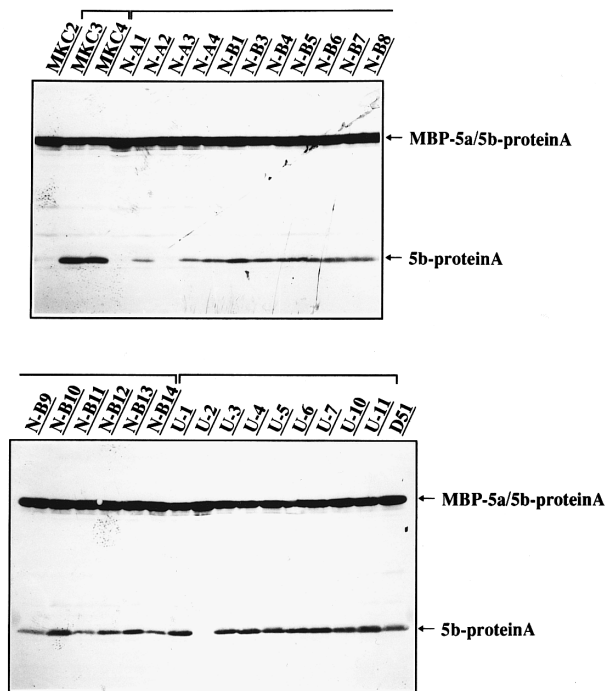


FIG. 3. Determination of processing activities of NS3 proteinase cDNA clones obtained from HCV-infected patients' sera. HCV NS3 proteinase cDNAs encoding the aa sequence from 1027 to 1260 of the HCV precursor polyprotein were cloned into the expression plasmid as described under Materials and Methods. Each clone was coexpressed with the substrate expression plasmid pMCP-C2 (Fig. 1A) in *E. coli* and its processing activity was determined. Both precursor and processed polyproteins containing a fused protein A domain were detected by Western blotting with HRP-conjugated IgG. Clones N-A1–A4 and N-B1–B14 were isolated from the serum of patient N. Also, clones U-1–11 were isolated from another sole serum source, patient U.

N-A3, and U-2). Since the expression of the NS3 enzyme was detected in each assay sample except for clone N-A1 (data not shown), the cDNA clones MKC2, N-A3, and U-2 were considered not functional.

To study the correlation between their aa sequences and their cleavage activities, we analyzed DNA sequences of each isolated clone. In the total nucleotide sequences, 1.18 and 0.27% of base substitutions from the consensus sequence were found in the clones derived from sera N and U, respectively (data not shown). These mutation rates account for 0.85% of the combined total sequences. However, 85% of the substitutions occurred at the third codon and aa changes were not frequent. Figure 4 shows aa sequences of each clone. The sequences of clone N-B5, N-B6, N-B9, N-B11, and N-B13 were the same as that of N-A2, and the sequence of N-B12 was the same as that of N-B3. Similarly, sequences of U-3, U-6, U-7, and U-11 were identical to that of the U-1 clone. From the comparative alignment, it was revealed that the sequences could be classified into two groups by their serum source, N or U at six positions (at 1115, Pro and Ser; at 1148, Asn and Thr; at 1151, Ser and Ala; at 1196, Val and Ile; at 1222, Ala and Thr; at 1239, Lys

and Arg, respectively). In addition to such diversity, each clone had a few extra minor point mutations. However, the majority of such minor mutations did not affect proteinase activity (Fig. 3). Among four inactive clones, clone N-A1 had a single base pair deletion which made a stop codon at aa 1079 (Fig. 5A). The appearance of a stop codon was consistent with the finding that the enzyme expression was not detected in clone N-A1 (data not shown). The aa sequence of its back mutant N-A1N, instead of the sequence of N-A1, is shown in Fig. 4. The other inactive clones, MKC2, N-A3, and U-2, had a replacement of Arg-1135 by Gly, Pro-1168 by Thr, and His-1083 by Leu, respectively. These point mutations seemed to cause the loss of activity of the NS3 enzyme.

Analysis of the inactive proteinase clones

The comparative alignment revealed that the aa sequence of clone U-2 was identical to that of clone U-1 except for His-1083. Therefore, it was concluded that the loss of the proteinase activity was due to the point mutation at His-1083 to Leu. Since His-1083 is one of the catalytic triad residues (His-1083, Asp-1107, Ser-1165), it was clear that the point mutation at this site caused the loss of proteinase activity (Hijikata *et al.*, 1993a).

To examine whether the aa substitutions in other inactive clones caused the loss of activity, we next constructed a back mutant for each inactive clone. A corresponding nucleotide was inserted to correct the nonsense mutation in clone N-A1 and a single base pair substitution was introduced in MKC2 and N-A3, respectively (Fig. 5A). When the back mutant clones N-A1N, MKC2N, and N-A3N were introduced in *E. coli* together with the substrate expressing plasmid, cleavages of the recombinant substrate were observed in all cases (Fig. 5B). These results confirmed that each predicted aa change caused the loss of proteinase activity. The immunoblot analysis using anti-NS3 polyclonal antibody showed that each enzyme, except for the clone N-A1, normally expressed in *E. coli* (Fig. 5B, bottom), eliminating the possibility that the failure of detection of the proteinase activity was due to the enzyme instability or low-level expression. To further analyze the contribution of positive charge at the side chain of position 1135 to the processing activity, we prepared additional mutants from clone D51, in which Arg-1135 was substituted by Lys (D51R1135K) or Gln (D51R1135Q) as well as Gly (D51R1135G). Interestingly, it was found that Gln as well as Lys could substitute for Arg. However, when the residue was changed to Gly, the D51 clone lost its activity, like the MKC2 clone (Fig. 5B). This finding indicates that any factor other than a positive charge at position 1135 may contribute to the NS3 proteinase activity. Furthermore, mobility shift of the enzyme was observed in the mutants at position 1135, suggesting the importance of this aa position for enzyme conformation (Fig. 5B, bottom).

	1050	1083	1100	1107	
D51	1027: APTTAYSQOTRGLLGCIIITSLTGRKKNVEGEVQVWSTATQSFLLATCVNGVQVSVYHGAGSKTLAGFKGPITQMYINVDOLGWPAPPGARSMPTCTCGSSDLVLRHADVI FVRRRG	★ #		#	
MKC2	1027:	A . T . F			L G
MKC3	1027:		V L Q L		
MKC4	1027:		V L Q L		
N-A1N	1027:	T		Q L	
N-A2	1027:	T		Q L	
N-A3	1027:	T		Q L	
N-A4	1027:	T		Q L I	
N-B1	1027:	T		Q L	
N-B3	1027:	T		Q L	
N-B4	1027:	T		Q L	
N-B7	1027:	T		Q L	
N-B8	1027:	T		Q L	
N-B10	1027:	T		Q L	
N-B14	1027:	T		Q L	
U-1	1027:	T		Q . S L	
U-2	1027:	T . L		Q . S L	
U-4	1027:	G		Q . S L	
U-5	1027:	T	I	Q . S L	
U-10	1027:	R		Q . S L	
	*****	*	*****	*****	*****

	1150	1165	1200	1250	
D51	1147: DSRGSLSPRPISYLGKSSGGPLLCPSCSHVVGIFRAAVCTRGVAKAVDFIPVESMETIMRSPVFTDNSTPPAVPQSFQVAHLHAPTIGSGKSTKVPAAVAAQGYKVLVLPNSVAA	#			(1260)
MKC2	1147: T			T	(1260)
MKC3	1147:	A	Y S	T	(1260)
MKC4	1147:	A	Y S	T	(1260)
N-A1N	1147: N	A	V	S A	(1260)
N-A2	1147: N	A	V	S A	(1260)
N-A3	1147: N	T	V	S A	(1260)
N-A4	1147: N	A	V	S A	(1260)
N-B1	1147: N	A	V	S A A	(1260)
N-B3	1147: N	A	V	S A L	(1260)
N-B4	1147: N	A	V	S A A	(1260)
N-B7	1147: N	A	V	S A G	(1260)
N-B8	1147: N	A	V . V	S A	(1260)
N-B10	1147: N	A	V	K . S A	(1260)
N-B14	1147: N	A	V	S . L A	(1260)
U-1	1147: T . A	A	S	T R	(1260)
U-2	1147: T . A	A	S	T R	(1260)
U-4	1147: T . A	A	S	T R	(1260)
U-5	1147: T . A	A . L	S	T R	(1260)
U-10	1147: T . A	A	S	T R	(1260)
	***	*****	*****	*****	*****

FIG. 4. Comparative alignment of the deduced amino acid sequences of the NS3 proteinase genes. MKC3, MKC4, and N-A1-A4 were initially obtained as 1196-bp DNA fragments encoding the aa sequence from 900 to 1260 of the HCV precursor polyprotein. The others were obtained as 716-bp DNA fragments encoding the aa sequence from 1027 to 1260 of the HCV precursor polyprotein. Although MKC3 and MKC4 are identical within the NS3 region, they are different within the NS2 region. The clones isolated from the same patient's serum are indicated by brackets. N-A1N is a proteinase-positive revertant of N-A1, which was mutated by a single base pair (G/C) insertion at the starred position (Fig. 5). Amino acid residues predicted to be null mutations are indicated in bold capitals. The residues predicted to form a catalytic triad (H1083, D1107, and S1165) are indicated by #.

DISCUSSION

We developed a *trans*-cleavage assay of HCV NS3 proteinase which detected its specific cleavage activity in *E. coli*. By using a coexpressed proteinaceous substrate, the cleavage activity was easily detected by SDS-PAGE and Western blotting. Using this *trans*-cleavage assay, we determined the minimal proteinase region in the N-terminal third of the NS3 gene by constructing a series of deletion mutants of the D51 clone. Previously, the proteinase domain was identified within region 1049–1215 (Tanji *et al.*, 1994) and we also used the region 1050–1214 as an active NS3 proteinase for its characterization (Mori *et al.*, 1996). In this study, the analyses of additional deletion mutants revealed that the minimal proteinase region was mapped between Val-1059 and Thr-1204 of HCV precursor protein, which was constructed with 146 aa residues (Fig. 2). The tertiary structure of the proteinase was revealed to adopt a chymotrypsin-like folding (Kim *et al.*, 1996; Love *et al.*, 1996). It

was reported that positions of secondary structure elements were well matched to those of chymotrypsin, although some β strands did not superimpose with the equivalent strands in other chymotrypsin-like proteinases. Both the terminal residues of the minimal region, Val-1059 and Thr-1204, are located at the N-terminal end of β strand A1 and the end of C-terminal α helix, respectively (Kim *et al.*, 1996; Love *et al.*, 1996). NS3(1059–1204) covers the minimal region containing all secondary structures which are conserved among chymotrypsin-like proteinase family members. Our results suggest that this region forms a core domain essential for processing activity.

In the course of studying the activity of HCV proteinase cDNA clones obtained from HCV-infected patients, we found both functionally active and inactive clones of NS3 proteinase by the *trans*-cleavage assay (Fig. 3). It is interesting that some inactive clones (N-A1, N-A3 from serum N and U-2 from serum U) existed together with

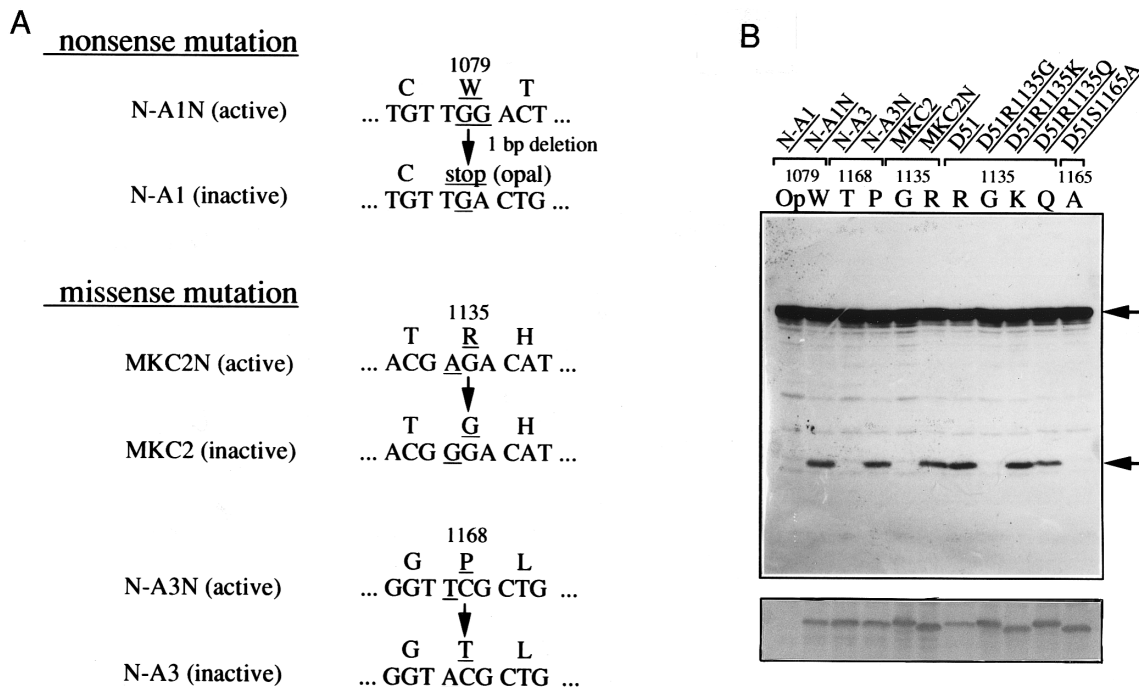


FIG. 5. Mutation analysis of proteinase-defective clones. (A) Predicted mutation sites of proteinase-defective clones. Underlines indicate replaced aa residues and nucleotides. (B) To confirm the predicted null mutation sites shown in (A), back mutation experiments were performed. The Opal mutation (indicated as Op in the figure) in N-A1 was repaired by single G/C base pair insertion. The aa sequence of the resultant clone designated as N-A1N is shown in Fig. 4. The Thr-1168 in N-A3 and the Gly-1135 in MKC2 were replaced by Pro (N-A3N) and Arg (MKC2N), respectively. Furthermore, the Arg-1135 in D51 (an active clone) was replaced by Gly (D51R1135G), Lys (D51R1135K), or Gln (D51R1135Q) residues. D51S1165A, in which the Ser-1165 was replaced by Ala residue, was used as inactive control. The proteinase activities of these clones were determined by *trans*-cleavage assay in *E. coli*. The bottom part of this figure shows the expression of NS3 proteinase analyzed by Western blotting using rabbit polyclonal anti-NS3 antibody.

active clones in the same patient's serum. The sequencing analysis of the cloned NS3 genes revealed that the base substitutions amounted to 0.85% in the total nucleotide sequences (data not shown). Among the substitutions, 85% of the cases occurred at the third codon, which did not change the aa sequences. Since the analysis of the sequence of HCV genome might inevitably involve the possibility of nucleotide change due to PCR error, we are not able to entirely exclude such possibility. However, these biased nucleotide mutations may reflect the characteristics of an error-prone RNA-dependent RNA polymerase and the lack of an associated repair mechanism in the viral replication, which may cause the quasispecies of the HCV genome within infected individuals (Bukh *et al.*, 1995). Among such quasispecies, some mutations may happen to change an aa residue(s) critical for proteinase activity. In this study, we identified four cases of such critical mutations in sera of patients chronically infected with HCV.

Among the four inactive clones, N-A1 was found to have a nonsense mutation at position 1079 (Fig. 5A). Since the translation of the HCV polyprotein may be terminated at the stop codon, N-A1 is considered to be a clone defective in HCV replication. In another inactive clone, U-2, a point mutation occurred at a histidine residue of the catalytic triad (His-1083) and directly made

the proteinase inactive. In the third case of N-A3, mutation at Pro-1168 was revealed to induce inactivation of the enzyme. The proline residue follows the "oxyanion-stabilizing loop" (Leu-1161 to Ser-1165), which constitutes an active center. It is possible that this proline residue is structurally critical and its substitution for threonine distorted the conformation of the main chain within the loop and caused loss of the enzyme activity. In the fourth inactive clone, MKC2, Arg-1135 was replaced by glycine, which was located on a loop between β strands A2 and B2 in the C-terminal domain and was positioned just behind the oxyanion hole of the active center (Kim *et al.*, 1996; Love *et al.*, 1996). The side chain at position 1135 may contribute to the processing activity by certain interactions with amino acids constituting the "oxyanion-stabilizing loop." Since a glutamine residue could substitute for Arg-1135 as shown in Fig. 5B, a positive charge at this position may not be very important for the interactions. The volume of the side chain might be critical in interactions such as hydrogen bonds, and the smallest side chain of glycine might lead to loss of such interaction causing the enzyme to be inactive in the clone MKC2.

The *trans*-cleavage assay described here could be used for analyses to measure the activity of NS3 proteinase clones and to identify quasispecies of the proteinase

sequences. The system would be also useful for revealing the amino acids critical in inactive HCV clones. Accumulation of such information should be helpful for understanding the relationship between the structure and the activity of the enzyme.

MATERIALS AND METHODS

Construction of substrate expression vectors

The expression plasmid pMCP1, which encodes *E. coli* MBP and *Staphylococcus aureus* protein A in tandem, is a derivative of pMAL-c2 (New England BioLabs, Inc., MA) and pRIT2 (Pharmacia Biotech, Inc., Uppsala, Sweden). Plasmid pMCP1 also contains multiple cloning sites (*Hind*III to *Xba*I site of pUC19) between the MBP gene and the protein A gene. Substrate expression plasmids, pMCP-C2 and pMCP-A1, which encode fusion proteins containing appropriate aa sequences around the NS3 proteinase cleavage site between the MBP and protein A junctions (Fig. 1A), were constructed by inserting phosphorylated linkers into the *Hind*III-*Xba*I site of pMCP1. The synthetic oligonucleotide pairs used in these constructions are as follows: for plasmid pMCP-C2, 5'-AGCT-TGGCGACGACATCGTCTGCTGCTCAATGTCCTACT-3' and 5'-CTAGAGTAGGACATTGAGCAGCAGACGATGTCGTCGCCA-3'; for plasmid pMCP-A1, 5'-AGCTTGGCGACGACATCGTCTGCGCCTCAATGTCCTACT-3' and 5'-CTAGAGTAGGACATTGAGGCGCAGACGATGTCGTCGCCA-3'.

Cloning of NS3 proteinase genes

The expression plasmids pWNH71 and pWB298 each contain a 13 N-terminal amino acid leader sequence derived from a nitrile hydratase gene, which are driven under the control of the *trc* promoter. This leader sequence possesses a consecutive stretch of histidine (His) residues which allows the fusion protein to be purified with ease in a single step by metal chelating affinity chromatography.

HCV RNA molecules were purified from 50 μ l of an HCV patient's serum (a gift from Dr. N. Hayashi) by using RNAzol B (Tel-Test, Inc., Texas) and subjected to cDNA synthesis, followed by the reverse transcription-polymerase chain reaction (RT-PCR). To amplify the NS3 region, the following oligonucleotides were used as nested PCR primers: forward primers, YH351 (5'-TTAA-GCTTCTCGGTCCGCTCATGGTRCTCCARGC-3') and YH357 (5'-TTAAGCTTGCGCCYATCACGGCCTAYTCCC-3'); backward primers, YH354 (5'-TAAGATCTARGCRGCAACRGA-IGGRAGGACG-3') and KY390 (5'-TAGGATCCTARGCRG-CAACRGAIGGRAGGACG-3') (Underlines denote the restriction sites used for subcloning, and nucleotides in bold type indicate the stop codon.) To amplify the cDNA fragment encoding aa residues 900 to 1260 in the HCV polyprotein, a primer set of YH351 and YH354 was used.

To amplify the cDNA fragment encoding aa residues 1027 to 1260 in the HCV polyprotein, a primer set of YH357 and KY390 was used.

The *Hind*III-*Bgl*II fragment and the *Hind*III-*Bam*HI fragment of the PCR products were ligated into the *Hind*III-*Bgl*II site of pWNH71 and the *Hind*III-*Bam*HI site of pWB298, respectively. To screen enzymatically active NS3 proteinase clones, as described later, the ligated plasmid DNA and the substrate expression plasmid pMCP-C2 were co-introduced into *E. coli* DH5 cells. Since the substrate and the enzyme expression vectors have different replication origins and different antibiotic-resistant markers, both plasmids can be maintained under selective pressure. Ampicillin and kanamycin double-resistant colonies were selected as the transformants harboring the two plasmids, and the plasmid DNAs were subjected to restriction endonuclease analysis.

Detection of NS3 proteinase activity in *E. coli* expression system

Recombinant *E. coli* strains harboring the two plasmids were precultured overnight in LB broth (Difco) containing ampicillin (50 μ g/liter) and kanamycin (30 μ g/liter) at 30°C with shaking. The cultured cells were inoculated at 1/50 dilution into the same broth and incubated further at 37°C. When the OD₆₅₀ reached 0.8, isopropyl- β -D-thiogalactopyranoside (IPTG) was added at the final concentration of 0.5 mM, and the culture was further incubated for 4 h. After the cultured cells were harvested and suspended in 0.85% NaCl, a portion of the suspension was mixed with Laemmli's sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was carried out in a 14% acrylamide slab gel and the gel was stained with Coomassie brilliant blue (CBB). In addition, Western blot analysis was carried out after SDS-PAGE. The processed products from the C-terminal region of the substrate protein, containing protein A-fused polypeptide, was detected with horseradish peroxidase (HRP)-conjugated goat IgG anti-rabbit IgG. To confirm the expression of the NS3 protein in *E. coli* cells, rabbit anti-NS3 IgG prepared from a rabbit immunized with purified recombinant protein (aa 900 to 1260; expressed in *E. coli*) was used as the first antibody, and HRP-conjugated goat IgG anti-rabbit IgG as the second.

Mutagenesis

Site-specific mutagenesis was accomplished by using a PCR-based method (Ho *et al.*, 1989). All constructs were confirmed by sequencing analysis. The PCR primers used were as follows: Vector primers; forward primer, 5'-TGTGAGCGGATAACAATTTCCGGATC-3', and backward primer, 5'-TCGGCCGCCGACTATCACCGCCC-3'; mutagenesis primers; for MKC2 (G1135→R): 5'-CTTTACCTG-

GTCACGAGACATGCTGATGTC-3' and 5'-GACATCAGCATGTCTCGTGACCAGGTAAG-3'; for N-A1 (amber → W1079; 1 G/C bp insertion): 5'-AACGGCGTGTGGAC-TGTCTACCATGG-3' and 5'-ACCATGGTAGACAGTCCAA-CACACGCCGTT-3'; for N-A3 (T1168 → P): 5'-GCTCTTC-GGGTGGTCCGCTGCTTTGCC-3' and 5'-GGCAAAGCAG-CGGACCACCCGAAGAGC-3'; for D51 (R1135 → G): 5'-CTTTACTTGGTCACGGGACATGCTGATGTC-3' and 5'-GACATCAGCATGTCCCGTGACCAAGTAAAG-3'; for D51 (R1135 → K): 5'-CTTTACTTGGTCACGAAACATGCTGATGTC-3' and 5'-GACATCAGCATGTTTCGTGACCAAGTAAAG-3'; for D51 (R1135 → Q): 5'-CTTTACCTGGTCACGC-AACATGCTGATGTC-3' and 5'-GACATCAGCATGTTGCG-TGACCAGGTAAG-3'; for D51 (S1165 → A): 5'-TGAAG-GGTTCTCGCGGTGGTCC-3' and 5'-GGACCACCGCAGG-AACCCTTCA-3', forward and backward primers, respectively.

Construction of deletion mutants

All deletion mutants were constructed by the PCR. The *HindIII*-*BglII* fragment and the *HindIII*-*BamHI* fragment of PCR products were ligated into the *HindIII*-*BglII* site of pWNH71. All constructs were confirmed by sequencing analysis.

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