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Sequence Analysis of NS4 Region of HCV Isolated from Korean Patient

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Hepatitis C virus (HCV) has been considered as a major causative agent of post-transfusion related non-A, non-B hepatitis. In this study, the cDNA sequence of NS4 region of HCV (HCV-S) obtained from a Korean patient's plasma was determined. Comparative nucleotide sequence analysis between HCV-S and other foreign isolates showed 77.4~78.5% homology to type I, 90.4~92.6% homology to type II, 67.2% homology to type III, and 66.4% homology to type IV. The putative amino acid sequence homologies to types I, II, III, and IV were 82.8~84.7%, 92.5~95.1%, 72.5%, and 71.1%, respectively. This data strongly suggests that HCV-S should be classified as type II. Significant similarities of hydrophobicity profiles and putative transmembranous domains were found in HCV-S and four major prototypes, indicating that the protein structure is similar in spite of the heterogeneities of intertype homologies at the level of the primary nucleotide and amino acid sequences.

Key words: HCV, RT-PCR, sequence homology, NS4

Hepatitis C virus (HCV) is a major causative agent of post-transfusion non-A, non-B hepatitis, which frequently develops into chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. The cDNA for HCV genomic RNA has been molecularly cloned by using a recombinant immunoscreening approach (4). Since then, considerable progress has been made in characterization of this virus. The similarity of the genome organization and the hydrophobicity profiles of the polyproteins of HCV to those of flaviviruses and pestiviruses (17) have resulted in the proposal that HCV should be classified as a separate genus within the family Flaviviridae (13).

The single-stranded positive sense RNA of HCV (9.4 kb in length) contains a large translational open reading frame (ORF) which spans almost the entire genome and encodes a polyprotein of about 3,000 amino acids (5, 14, 24). The viral genome consists of a 324~341 nt long 5′ untranslated region (UTR), a core (C), an envelope protein coding region (E1, E2), a nonstructural protein coding region (NS), and a variable length of 3′ untranslated region (UTR) (5). Secondary structure of the 5′ UTR revealed the presence of a large conserved stem-loop structure which serves as a putative cis-acting internal ribosomal entry site (IRES) (27). On the basis of current knowledge, HCV polyprotein translation initiates at an IRES within the 5′ UTR, proximal to the initiator AUG codon of the polyprotein.

Three putative structural proteins, the core protein and two envelope glycoproteins (E1, E2), are produced from cleavage of polyprotein by cellular signal peptidase. At least two virus-encoded proteinases are required for additional processing of the polyprotein. A zinc-dependent metalloproteinase activity (11, 12), which encompassed NS2 and the N-terminal part of NS3, is essential for cleavage at the NS2/NS3 boundary (9, 10). A virus-encoded serine proteinase located in the N-terminal portion of the NS3 protein (8, 9), cleaves the NS3/NS4a boundary *in cis* (26) and the NS4b/NS5a boundary, as well as internal sites in the NS4 and NS5 regions (NS4a/NS4b and NS5a/NS5b), *in trans* (8, 9, 26). Kinetic studies on the protease activity of NS3 and NS4a with various protease inhibitors led to classify NS3 as a chymotrypsin-like protease (10).

There are indications that different types of HCV may

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have different clinical relevance, such as response to interferon treatment (19, 21, 28) and the risk of developing cirrhosis or hepatocellular carcinoma. Different HCV genotypes may also evoke different serological responses (1). Sequence diversity may result in the production of type-specific antibody responses in infected hosts and the NS4 protein has been found to react with genotype-specific antibody (16).

Recent studies on NS4a revealed that NS4a acts as an effector of NS3 proteolytic activity when supplied *in trans* (7), but the function of NS4b remains to be clarified. The NS4b protein contains highly immunogenic epitopes and several predicted transmembrane regions. The first cloned C100-3 antigen, which is used in most antibody detection assays, is an antigen, spanning the C-terminus of NS3, NS4a, and the N-terminal part of NS4b (4). But the sensitivity and specificity of the assay is dependent on the genotype of HCV (6).

In this study, the cDNA of NS4 region of a Korean HCV carrier was synthesized by RT-PCR and the nucleotide sequence was determined. Based on our data and those of others, we have concluded that the Korean HCV isolate belongs to type II. Using computer-assisted protein analysis to search for sequences with significant motif of the Korean HCV isolates, we also predicted the primary structure and putative functional role of NS4b protein.

Materials and Methods

HCV-infected plasma

HCV-infected plasma was obtained from a blood donor at Yonsei University, School of Medicine, who was positive for anti-HCV c100-3 antibody (Ortho Diagnostic Systems).

Isolation of HCV RNA

HCV RNA was isolated by the modified version of the single-step method with acid guanidium thiocyanate-phenol-chloroform extraction described in Chomczynsky *et al.* (3). 200 μl of serum sample was homogenized by shaking vigorously for 15 seconds after the addition of 800 μl of RNAzol™ B (Cinna/Biotecx) and 80 μl of chloroform, and stood on ice for 5 min. The mixture was centrifuged at 10,000×g (4℃) for 15 min and the aqueous phase was transferred into a fresh tube. The RNA was precipitated by addition of an equal volume of isopropanol and centrifugation at 10,000×g (4℃). The RNA pellet was washed twice with 75% ethanol, dried, and then dissolved in 10 μl of diethylpyrocarbonate (DEPC)-treated deionized water.

Table 1. Sequences and locations of oligonucleotide primers used for RT-PCR and cDNA sequencing.

Primer	Sequence (5′ to 3′)	size	Nucleotide positions
p12 (antisence)	CCATGCCACGTATTGCTA-CAGGT	23nt	6480-6502
p1 (sense)	ATGTGGAAGTGTCTCATA-CGG	21nt	5148-5168
p2 (antisense)	ACTTGGACTCCACCACGGG	19nt	5571-5589
p11 (sense)	CAGGAGTTCGATGAAATG-GAAGAGTG	26nt	5436-5461
p4 (antisense)	TCGCCGCTCATGACCT-TAAAGGC	23nt	5922-5944
p5 (sense)	CTTCGGCTTTCGTGGGCG	18nt	5806-5823
p8 (antisense)	CCACATGGGCAGGTGGTTT-GCAT	23nt	6402-6424
p6 (antisense)	GTGATGGTAAGGCTGGAGA-GAGGAT	25nt	6168-6192
p7 (sense)	GCCTGTGCAGTGGAT-GAACC	20nt	6062-6081

Primers

Computer-assisted nucleotide sequence analysis (DNASIS™ program) was used to look for cDNA sequence similarity among major four types of HCV (HCV-1, HCV-J, HC-J4, and HC-J6). After aligning the cDNA sequences, highly conserved sequences were selected as primers for RT-PCR and cDNA sequencing (Table 1). Primer 12 was used for the synthesis of the first strand of cDNA. Primers 1 and 2, primers 11 and 4, and primers 5 and 8 were used for PCR and sequencing. Primers 6 and 7 were used for sequencing as internal primers to clarify the sequence ambiguities. Nucleotide positions were numbered by a system starting from the first base of the HCV-J isolate (14). Each primer was purchased from Korea Biotech.

Synthesis of HCV cDNA

The mixture of 10 μl HCV RNA and 1 μl antisense primer 12 (20 pmol) was heated at 95℃ for 5 min and quenched on ice. The mixture was adjusted to a volume of 20 μl with 1× reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 80 mM sodium pyrophosphate, 1.25 mM of each deoxynucleotide triphosphate (dNTP), 20 units of RNasin (Promega), and 20 units of avian myeloblastosis virus reverse transcriptase (Promega). The reaction mixture was incubated at 42℃ for 60 min, and then heated at 95℃ for 5 min in order to inactivate reverse transcriptase.

PCR

The single-stranded cDNA was subjected to PCR for amplification. Five μl of HCV cDNA were added to the reaction mixture containing 1×PCR reaction buffer (20 mM Tris-HCl, pH 8.7, 40 mM KCl, 1 mM $MgCl_2$), 0.2 mM of each dNTP, 20 pmol of each specific sense and antisense primers and 1 unit of Tca polymerase (Korea Biotech) (20) in a volume of 50 μl. The mixture was amplified in a thermal cycler (Daehan Medical System) for 30 cycles. Each cycle was initiated by denaturation at 95℃ for 20 seconds followed by primer annealing for at 55℃ for 20 seconds and extension at 74℃ for 40 seconds. Amplified cDNAs were separated on agarose gel, recovered from gel slices (22), and cloned into pT7 Blue T-vector (NovaGen).

DNA sequencing

Single stranded phagemid DNA was rescued from *E. coli* NovaBlue containing recombinant pT7Blue T-vector and the nucleotide sequence was determined by the dideoxy chain termination method (23) with T7 promoter primer. Sequence determination was performed using the Sequenase 2.0 DNA Sequencing Kit (USB) according to the manufacturer's recommended procedure. Nucleotide sequence and protein data bases were obtained from the GenBank and European Molecular Biology Organization. The DNASIS™ (Pharmacia) and the PROSIS™ (Pharmacia) programs were used to determine nucleotide and deduced amino acid sequence homologies between Korean and foreign HCV isolates. The program PROSIS™ also allowed determination of the hydrophobicity profile and putative protein molecular weight. The program MEMSAT (Bionet) was used to search for the transmembrane domain.

Results and Discussion

Cloning and sequence determination of NS4 region of Korean HCV

Analysis of the genome structure of the HCV isolated from the Korean patient is important for typing Korean HCV and facilitates investigation of the function of HCV-coded protein. It will also be useful for making efficient immunological diagnostic tools. In order to clone cDNA for the NS4 region of Korean HCV, RNA was extracted from plasma obtained from an anti-HCV c100-3 antibody positive patient. The cDNA spanning the entire NS4 region was synthesized by using primer 12. The 440 bp, 510 bp, and 620 bp-long DNA fragments were amplified by PCR using primers 1 and 2, primers 11 and 4, and primers 5 and 8 (Table 1). Each RT-PCR product spanning 5148-5589, 5436-5944, and 5806-6424 nucleotide positions (deduced from the HCV-J sequence (14)) was named PCR12, PCR114, and PCR58 (Fig. 1).

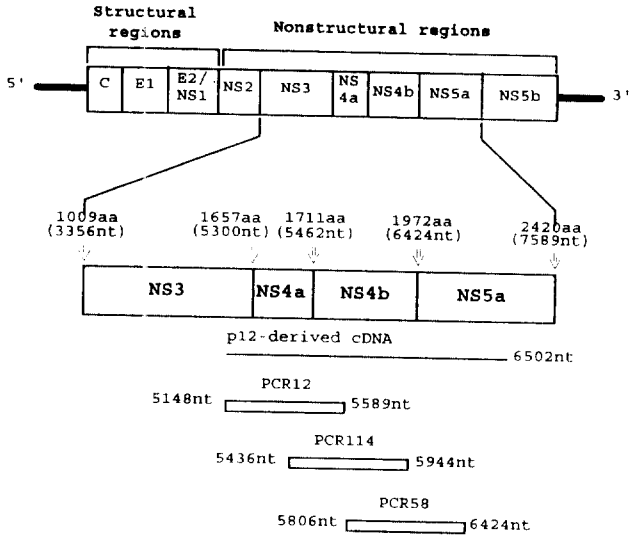

Fig. 1. HCV genome structure and RT-PCR strategy. A schematic presentation of the HCV genome encoding the structural and nonstructural proteins is shown. The 5′ and 3′ nontranslated regions are indicated as thick lines. A detailed view of the nonstructural protein region (NS3/NS4a/NS4b/NS5a), including the cleavage sites for the NS3 proteinase (↓), is drawn below. Numbers above the arrows refer to the amino acids at P1 positions. Regions of the RT-PCR products are drawn below. Nucleotide positions in RT-PCR products were numbered by a system starting from the first base of the HCV-J isolate (14).

The amplified PCR products were cloned into pT7Blue vector. Each plasmid containing cDNA of PCR12, PCR 114, PCR58 was named pHCV-K12, pHCV-K114, pHCV-K58, respectively and the entire clone was named HCV-S. The cDNA sequence of HCV-S was determined on both plus and minus strands by the dideoxy chain termination method (23) with T7 promoter primer and primers used for cDNA synthesis (Table 1). The entire nucleotide sequence shown in Fig. 2 is 1,274 base pairs in length and has a base composition of 19.39% adenine, 20.33% thymine, 30.14% cysteine, and 30.14% guanine. The genomic organization of the cloned cDNA is part of NS3 (153 nt), NS4a (162 nt), NS4b (780 nt), and part of NS5a (179 nt), based on the assumed cleavage site of the precursor polyprotein for processing (9).

Sequence comparison of HCV-S with other HCV isolates

Previous reports (18) indicated that all HCV isolates could be classified as four prototypes represented by HCV-1, HCV-J, HC-J6, and HC-J8. By this classification, the intratype homology was greater than 90% and the intertype homology varied from 57~86%, depending upon the isolates compared. The nucleotide sequence

Table 2. Homology for nucleotide (nt) and deduced amino acid (aa) sequences of HCV-S nonstructural regions with type specific HCV isolates.

Type	Each isolates	Overall		NS3		NS4a		NS4b		NS5a	
		nt*	aa*	nt	aa	nt	aa	nt	aa	nt	aa
I	HCV-1	78.5	84.7	82.2	96.1	85.1	83.3	77.1	85.7	78.8	74.6
	HCV-H	77.4	82.8	69.7	90.2	88.2	87.0	75.3	83.0	76.5	74.6
II	HCV-BK	92.1	94.6	89.5	90.2	92.5	94.4	93.1	96.2	90.5	91.5
	HC-C2	91.8	93.2	91.5	90.2	92.5	94.4	92.4	94.3	89.9	89.8
	HC-J4	92.6	95.1	92.2	90.2	93.2	96.3	92.8	95.8	91.6	94.9
	HCV-JT'	92.2	94.1	93.5	94.6	92.5	96.3	92.1	94.3	91.6	91.5
	HCV-J	91.9	94.8	89.5	90.2	92.6	96.3	92.6	95.4	91.1	94.9
	HCV-T	90.4	92.5	87.5	88.2	93.2	96.3	89.9	92.3	92.7	93.2
III	HC-J6	67.2	72.5	69.5	80.4	66.9	66.7	67.6	72.4	65.0	71.2
IV	HC-J8	66.4	71.1	72.4	80.4	65.2	63.0	66.3	71.6	63.1	67.8

* % homology of nt or aa=(number of identical nt or aa×100)/total number of nt or aa.

of HCV-S showed high homology (90.4~92.6%) to HCV-BK, HC-C2, HC-J4, HCV-JT', HCV-J, HCV-K (2) and HCV-T which belong to type II (Table 2). Only 77.4% and 78.5% homology was observed in HCV-H and HCV-1, which belong to type I. Similarly, there was 67.2% nucleotide sequence homology to HC-J6 of type III and 66.4% to HC-J8 of type IV.

Amino acid sequence homology of HCV-S to that of foreign isolates also suggests that it is closely related to type II. The putative amino acid sequence homologies with type I, II, III, and IV were 82.8~84.7%, 92.5~95.1 %, 72.5%, and 71.1%, respectively. This data indicates that HCV-S is related to type II isolates with 90.4~92.2 % nucleotide and 92.5~95.1% amino acid sequence homologies and deserves to be classified as type II.

Deduced amino acid sequences of HCV-S cDNA

The HCV-S genome, composed of 1,274 nucleotides, has an open reading frame (ORF) capable of encoding a 424-amino acid protein. The ORF is organized as the C-terminal part of NS3, NS4a, NS4b, and the N-terminal part of NS5a (Fig. 2). The truncated C-terminal part of the putative NS3 protein is 51 amino acids long and has a 5,756-dalton molecular weight. It has been proposed that the C-terminal half of the NS3 protein possesses NTPase and helicase activities (8, 9, 17). The C-terminal domain is belived to interact with the replication complex during HCV genomic RNA replication, whereas the N-terminal domain acts as a proteinase.

Analysis of the NS4a (54 aa, 5,781 M.W.) suggests that the NS4a, possessing an N-terminal hydrophobic domain and a C-terminal hydrophilic domain (Fig. 3), might have an N-terminal membrane anchoring domain. Previous data (7) indicated that NS4a acts as an effector of the NS3 proteolytic activity when supplied *in trans*. Furthermore the central region of NS4a, particularly hydrophobic residue Ile-29, seems to play a key role in NS4a activity (15). The C-terminal 33-amino-acid region of the NS4a spans a hydrophilic C-terminal domain (Fig. 3) and this region may be responsible for interacting with NS3 proteinase.

Immunoprecipitation and deletion analysis (11, 12) indicated that NS4a protein is closely associated with NS3 proteinase and is responsible for the membrane association of NS3 protein. *In vitro* translation-processing analysis (25) of the HCV nonstructural polyprotein shows that NS4a helps to anchor the NS3 protein to the surface of the endoplasmic reticulum. These results are consistent with the fact that one aspect of the function of NS4a may be to anchor NS3 proteinase on the surface of the endoplasmic reticulum through its N-terminal hydrophobic and putative transmembranous domain. The NS4a protein is thus thought to function in the holding of NS3 protease on the endoplasmic reticulum and could act as a virus-encoded chaperone aiding the correct folding of the protease domain in NS3 protein. The hydrophobicity profiles and the presence of transmembranous regions deduced from this study (Figures 2 and 3) are generally consistent with previous studies.

The function of the NS4b (260 aa, 27,079 M.W.) protein remains unknown. NS4b protein contains highly immunogenic epitopes and several predicted transmembrane regions which could associate with nonstructural proteins in a complex to form an active heteromultimeric replicatory complex. In fact a stable commplex between NS4b and NS3 and NS4a proteins has been observed *in vitro* (25).

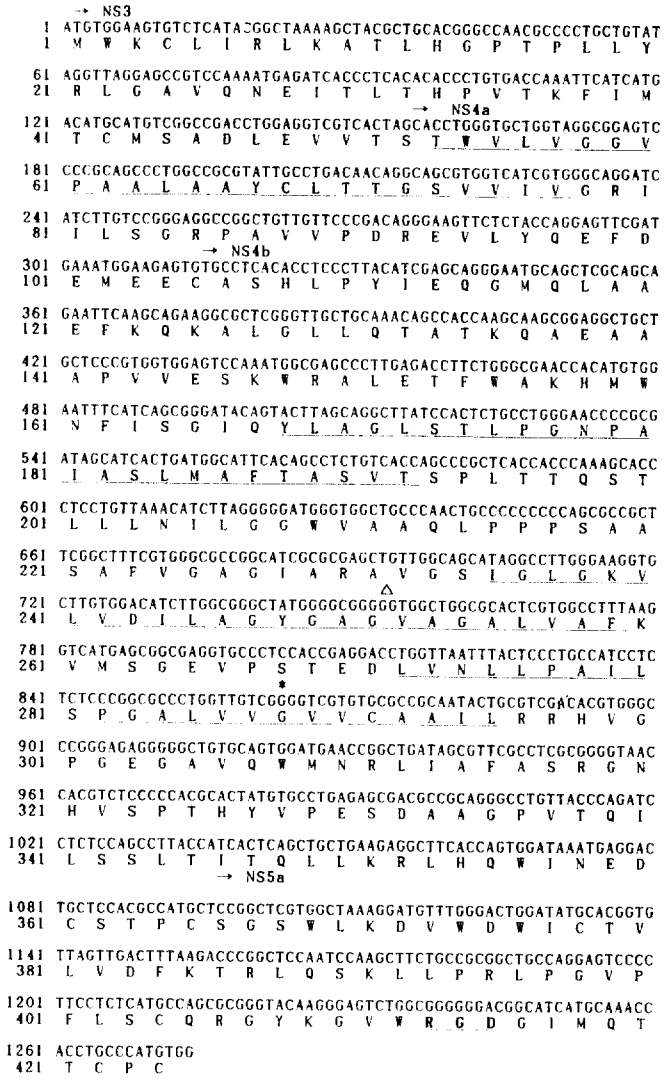

Fig. 2. Nucleotide and deduced amino acid sequences of HCV cDNA from Korean isolate (HCV-S). The nucleotide sequence was determined from overlapping clones, HCV-K12, HCV-K114, and HCV-K58 as described in the text. Nucleotides are numbered from the 5′ end of cDNA and amino acid residues (presented in one-letter code) are numbered from the first Met of polyprotein. The triangle (△) indicates the position of the amino acid deletion compared with the sequence of other HCV isolates. Potential casein kinaseII phosphorylation site (STED) is indicated by an asterisk (*). The sequence Arg-Gly-Asp commonly found in cell adhesion molecule is double-underlined. Putative transmembranous domains are underlined. The different structural domains are denoted by arrows (→) according to Grakoui *et al.* (8, 9).

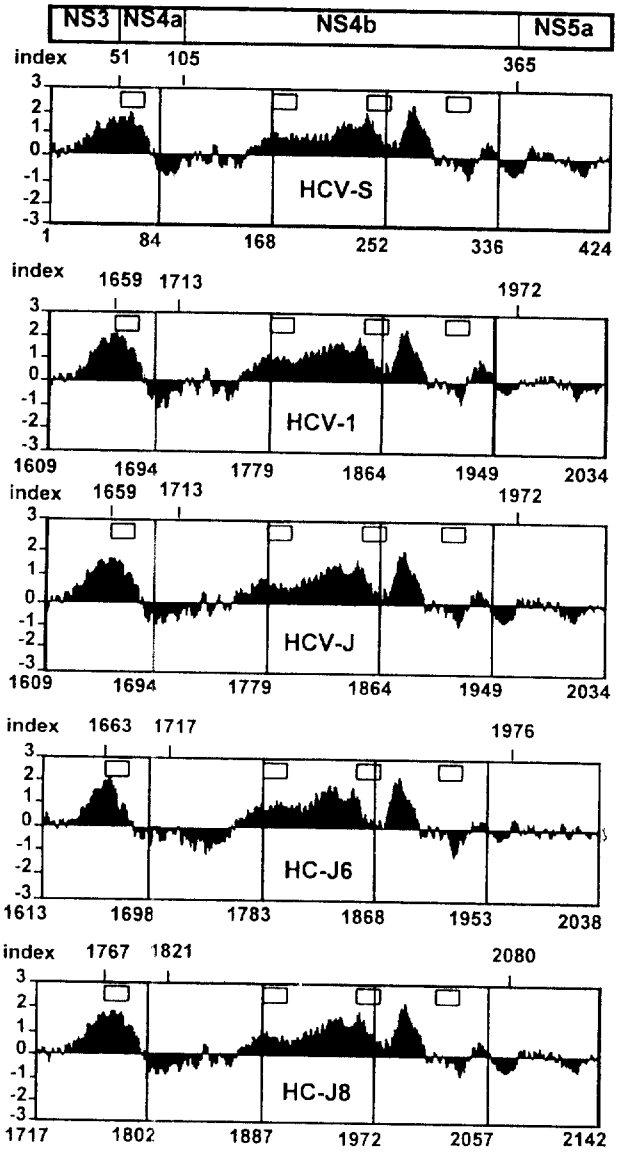

Fig. 3. Comparison of the hydrophobicity profiles of the polyproteins (NS3/NS4a/NS4b/NS5a) encoded by HCV-S, HCV-1, HCV-J, HC-J6, and HC-J8. The average hydrophobicity index with a search length of 20 amino acids was obtained with the program PROSIS™. Hydrophobic regions are above the horizontal line, whereas hydrophilic regions are below the horizontal line. The putative cleavage sites of the nonstructural proteins are indicated above the hydrophobicity profiles as open blocks. Each of amino acid residues at P1 sites was numbered. The putative transmembranous domains are marked by open boxes.

Comparison of the hydrophobicity profiles and putative transmembranous domains of HCV-S, HCV-1, HCV-J, HC-J6, and HC-J8

On the basis of sequence homologies between HCV-S and other HCV isolates, hydrophobicity profiles and putative transmembranous domains of HCV-S, HCV-1, HCV-J, HC-J6, and HC-J8 nonstructural proteins (NS3/NS4a/NS4b/NS5a) were tentatively assigned (Fig. 3). When the hydrophobicity profiles of the encoded proteins were compared based on provisional cleavage sites, significant similarities were found between HCV-S and four major prototypes represented by HCV-1, HCV-J, HC-J6, and HC-J8. This indicated a general similarity in protein structure and organization despite the hetero-

geneities of intertype homologies at the level of the primary nucleotide and amino acid sequences. Judging from the extensive similarities in the hydrophobicity profiles of the polyproteins (NS3/NS4a/NS4b/NS5a) of HCV-S and four major prototypes of HCV-1, HCV-J, HC-J6, and HC-J8, the distribution of mutations along the genome may not abolish proper functioning of resulting polyproteins.

The availability of nucleotide sequences of the NS4 region from HCV-S cDNA should facilitate virological studies of Korean HCV isolates and of the putative functional role of NS4b protein. It may also allow for the production of diagnostically useful antigens and vaccines to prevent the majority of post-transfusion non-A, non-B hepatitis cases as well as HCV-associated malignant liver diseases.

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