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HEPATITIS C VIRUS AND ITS GENE STRUCTURE AND FUNCTION

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Abstract: Hepatitis C virus (HCV) is a member of the family *Flaviviridae*, and the principal cause of parenteral non A non B hepatitis. HCV is a single stranded, positive sense RNA virus whose genome contains a single open reading frame of about 10 kb in length which encodes a polyprotein of about 3000 amino acids. HCV is responsible for majority of the transfusion associated cases of hepatitis and a significant proportion of community-acquired hepatitis which leads to a range of clinical manifestations from an apparent carrier state to severe chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Cell-free translation and cell culture transient expression studies revealed that HCV genome initially transcribed as a polyprotein is processed by cellular and viral proteases to produce the putative viral structural and nonstructural proteins. The preliminary map of the gene order for HCV was established: 5′ untranslated region (UTR)-core (C)-envelope 1 (E1)-E2-nonstructural 2(NS2)-NS3-NS4A-NS4B-NS5A-NS5B-UTR3′. Nonstructural proteins, NS2, NS3 and NS5, are believed to be a component of viral coded enzymes. Mutations in hypervariable region 1 (HVR1) of the E2 protein was considered to be a possible mechanism of chronicity of HCV infection. HCV infection can be monitored either by serodiagnosis or by genodiagnosis. Interferon seems to be considered as the sole drug for HCV treatment until now. In the history of virology, HCV was the first example that was identified by molecular cloning of its genome from infectious materials.

Key words: Hepatitis C virus, chronic hepatitis, genotypes, hepatocellular carcinoma, hypervariability, quasispecies, untranslated region

INTRODUCTION

Hepatitis caused by virus(es) other than hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis delta virus (HDV) or other known hepatotropic viruses, such as cytomegalovirus, Epstein-Barr virus and human parvovirus (B19), have been referred to as non-A, non-B (NANB) hepatitis. There are at least two types of NANB hepatitis reported, namely water-borne and blood-borne NANB hepatitis (Alter *et al.*, 1982; Sherlock, 1986). The causative agent for water-borne hepatitis has recently been cloned, and it's genome was identified as a

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single stranded, positive sense polyadenylated RNA of about 7.6kb (Reyes *et al.*, 1990). It is now designated as hepatitis E virus. Hepatitis E virus is endemic to the Indian subcontinent and its clinical manifestations resemble to those by hepatitis A virus.

There are two types of aetiological agents for blood-borne NANB hepatitis as described by cross-challenge and physicochemical studies (Yoshizawa *et al.*, 1981, 1982; Bradley *et al.*, 1983, 1985). One of them, the so-called tubule-forming agent, is chloroform sensitive and is specifically associated with the formation of characteristic hepatocyte cytoplasomic alterations in chimpanzees (Shimizu *et al.*, 1979; Bradley *et al.*, 1980). The genome of the tubule-forming agent has recently been cloned and is composed of a single-stranded, positive sense RNA of about 10000 bases (Choo *et al* ., 1989). It is now designated as hepatitis C virus. HCV was the first example in the history of virus which was identified by molecular cloning of the viral genome. Another one, the so-called non-tubule forming agent, is not chloroform sensitive and does not induce tubule formation when injected into chimpanzees. The causative agent is still unidentified.

Here we report an overview of genomic structure of HCV and the role and function of its different gene products in pathogenesis in liver diseases.

Importance as disease agent

HCV is responsible for the vast majority of transfusion-associated cases of hepatitis and a significant proportion of community-acquired hepatitis worldwide which account for over 90% of all cases of non A non B hepatitis and 25% of sporadic acute viral hepatitis (Choo *et al.*, 1989; Kuo *et al.*, 1989; Alter, 1989). By transient infection, HCV causes acute hepatitis, certain proportion of which leads to chronic hepatitis followed by the development of liver cirrhosis, hepatocellular carcinoma and establishment of an asymtomatic carrier state which may persist for life (Hollinger, 1990). HCV infection is estimated to affect around 50 million people around the world. Approximately 50% of the affected individuals gradually develop into chronic infection of which about 20% eventually suffered from liver cirrhosis and hepatocellular carcinoma (Mast and Alter, 1993). HCV infection is estimated to infect 100,000 to 300,000 new cases per year in the United States alone (Weiner *et al.*, 1991). Therefore, HCV is considered as one of the biggest healthcare problem throughout the world.

Biological status of HCV

Extensive computer analysis of the nucleotide sequences and encoded polypeptides of the first isolated cDNA clone obtained from a experimentally chimpanzee indicated that HCV is unique in its genome structure which shows very little overall homology with known viral sequences (Choo *et al.*, 1989, 1991a). However, several lines of evidence indicate that HCV is a akin of human flaviviruses and animal pestiviruses. Firstly, the length of the polyprotein encoded by the genome of these viruses is similar i.e. 3010, 3400 and 4000 respectively (Rice *et al.*, 1985; Choo *et al.*, 1989; Collet *et al.*, 1988a. b). Secondly, the organization of the genome of these three viruses is almost similar i.e. 5′ untranslated region (UTR) followed by struc-

tural, nonstructural and 3′ UTR (Choo *et al.*, 1991a, b). Thirdly, all these viruses contain several common motifs in the same region of the genome i.e. protease, NTP-binding and helicase domains in the NS3 protein, and replicase domain in the NS5 protein (Bazan and Fletterick, 1989; Gorbalenya *et al.*, 1989; Miller and Purcell, 1990; Wengler and Wengler, 1993; Tamura *et al.*, 1993; Suzich *et al.*, 1993). Finally, the hydrophobicity profiles of the polyproteins from these viruses are remarkably similar (Choo *et al.*, 1991a; Takamizawa *et al.*, 1991). Considering all of these points HCV has been placed as the third genus in the family Flaviviridae together with other two genus, *flavivirus* and *pestivirus* (Choo *et al.*, 1989; Francki *et al.*, 1991). Again HCV shows relatively closer relationship to pestiviruses than flaviviruses in terms of the genetic organization of their envelope and NS5 proteins (Hijikata *et al.*, 1991a; Manabe *et al.*, 1994). Flaviviruses contain a prM protein gene in between core and envelope genes which is not present in pestiviruses and HCV.

Morphology of the HCV virion

Very little information has been accumulated on the morphological properties of the HCV viral particles. Several attempts heve been initiated for the determination of the buoyant density of the virion by gradient ultracentrifugation. The average buoyant density of the virus particles was determined to be 1.08g/ml, which is similar to that of pestiviruses (1.09−1.15 g/ml) and slightly lower than that of flaviviruses (1.20 g/ml) (Bradley *et al.*, 1991; Miyamoto *et al.*, 1992, Hijikata *et al.*, 1993c).

The particle size of HCV was determined as 30−38 nm by microfiltration (Yuasa *et al.*, 1991). HCV particles were appeared as spherical with membranous envelope containing surface projections which morphologically resemble flaviviruses. Abe *et al.* (1991) reported 37−40 nm particles from the hepatocytes.

Genome structure of HCV

Since the initial molecular cloning of this virus, the genome structure of several independent HCV isolates have heen elucidated and our knowledge of HCV molecular biology is progressing rapidly. HCV is an enveloped, single-stranded, positive-sense RNA virus whose genome is approximately 9,030 to 9,099 nucleotides in length and contains a highly conserved 5′ UTR followed by a long open reading frame encoding a polyprotein of 3,010 to 3,033 amino acids (Choo *et al.*, 1989; Kato *et al.*, 1990; Takamizawa *et al.*, 1991; Houghton *et al.*, 1991) which is processed by a combination of host and viral proteases (Hijikata *et al.*, 1991a; Grakoui *et al.*, 1993b). Due to lack of an efficient system for cell culture replication of HCV, no definite study is possible to observe how the authentic polypeptides are produced in HCV-infected cells. The map of the HCV polyprotein has been obtained by cell-free translation and cell culture transient expression studies (Hijikata *et al.*, 1991a, 1993b; Grakoui *et al.*, 1993b; Selby *et al.*, 1993; Manabe *et al.*, 1994). The gene map that has been obtained from these studies is 5′ UTR-C-E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′ UTR. C, E1 and E2 are viral putative structural genes wherease NS2 through NS5 are putative nonstructural genes. A UTR of 324 to 341 nucleotides at the 5′ terminus, and a variable length of UTR

with either a poly(U) or a poly (A) tail at the 3′ end, have been reported (Choo *et al.*, 1989; Han *et al.*, 1991; Takamizawa *et al.*, 1991).

Genomic Heterogeneity

Many HCV strains from different parts of the world have been entirely and partially sequenced and the data revealed that the nucleotide sequence between types (groups) as well as various isolates of the same type vary in different regions of the HCV genome (Esumi and Shikata, 1994). Some regions of the genome, especially 5′ terminal UTR, are highly conserved, showing 93−99% homology among different isolates. The core regions of different isolates exhibit identity of 81−96% in the nucleotide sequence and 90−98% in the amino acid sequence, respectively. But some regions of the genome show very low homology, for instance, envelope coding region (E1 and E2) and the NS2. These regions showed very low nucleotide sequence homology between type II and III or IV, with a identity of 55−69% and between type I and II, with a identity of 71−75%, NS3, NS4 and NS5 also showed considerable variation both at nucleotide and putative amino acid sequences between different types. Marked genetic variation was noted in the 3′ UTR.

Proteolytic processing of the viral polyprotein

Due to lack of a laboratory experimental animal except chimpanzee, and an efficient cell culture system, the study of the exact processing mechanism of the HCV polyprotein *in vitro* has not been possible since the discovery of this virus. Several attempts have been made to study the processing of the HCV polyprotein *in vitro* using cell-free translation and cell culture transient expression systems (Hijikata *et al.*, 1991a, 1993b; Harada *et al.*, 1991; Grakoui *et al.*, 1993b; Selby *et al.*, 1993; Manabe *et al.*, 1994). These studies established that HCV polyprotein is processed by cellular and viral encoded proteases to produce putative structural and nonstructural proteins. The order and nomenclature of the cleavage products are as follows: NH2-C-E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. C is viral nucleocapsid protein; E1 and E2 are virion envelope glycoprotein; NS2 through NS5B are putative nonstructural proteins, at least some of which may be the functional equivalent of the homologous proteins encoded by flaviviruses and pestiviruses. Studies on the processing of structural proteins of HCV revealed that host signal peptidase localized in the lumen of endoplasomic reticulum is responsible for catalytic cleavages at the C/E1, E1/E2 and E2/NS2 junctions, which are reminiscent of the flaviviruses and pestiviruses. Predictive amino acid sequence data showed that N-termini of E1, E2, and NS2 contain predicted signalase cleavage sites and C-termini of C, E1 and E2 contain hydrophobic regions that are essential prerequisite for signalase cleavages.

A Zn^{2+}-dependent metalloprotease comprising NS2 and the N-terminal one-third of the NS3 is responsible for the autocatalytic cleavage at the NS2/3 junction (Grakoui *et al.*, 1993c; Hijikata *et al.*, 1993a). But the proteolytic cleavage at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions is catalyzed by serine protease, encoded by N-terminal one-third of the NS3 gene (Grakoui *et al.*, 1993a; Hijikata *et al.*, 1993b; Tomei *et al.*, 1993). For its efficient

function as protease, NS3 needs NS4A as a co-factor. NS4A is required for the cleavage of NS3/NS4A and NS4B/NS5A sites and enhances cleavage at the NS5A/5B site (Bartenschlager *et al.*, 1993; Failla *et al.*, 1994). Cleavages at these sites occur either in *cis* or in *trans*. Cleavage at the NS2/3 and NS3/4 sites normally occur in *cis*, whereas at the other sites normally occur in *trans* (Grakoui *et al.*, 1993b; Tomei *et al.*, 1993; Bartenschlager *et al.*, 1995).

Properties of viral proteins

Separate gene regions are identified on the HCV genome from the 5′ to the 3′ end. The location of each region is shown in Figure 1 and putative function of each gene product is discussed.

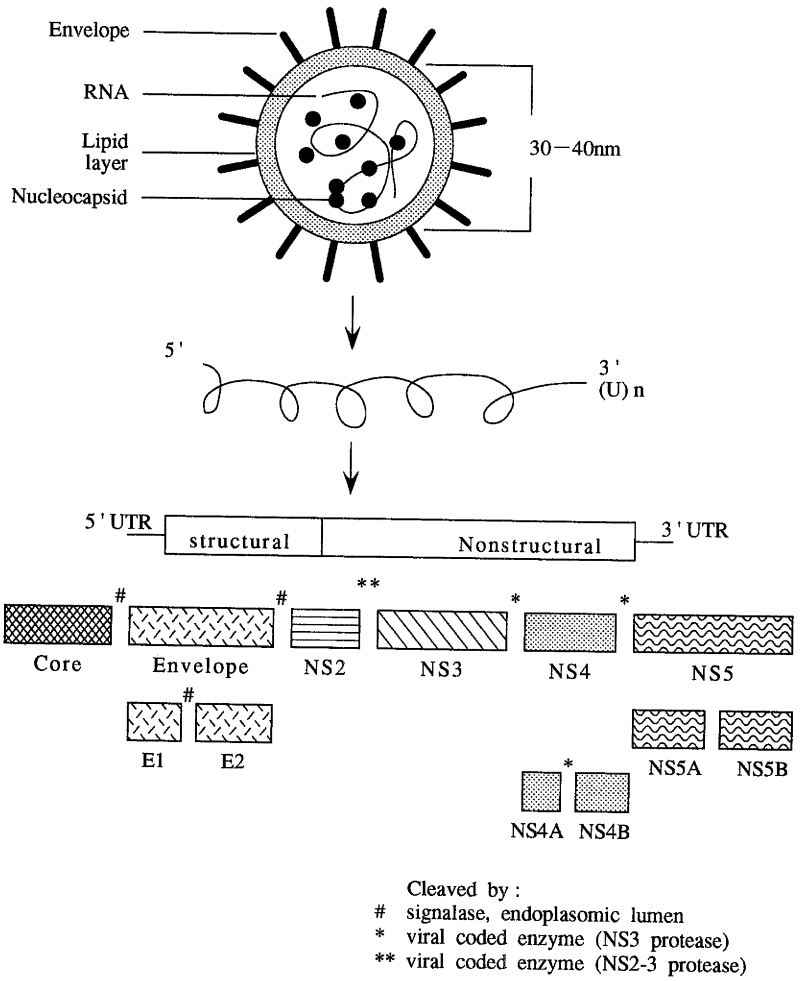

Fig. 1. Schematic presentation of a HCV particle, its genomic organization and processing events of polyprotein.

Structural proteins

The Core protein The core protein (C), molecular weight 22 kDa (p22), is located in the amino terminal region of polyprotein and is followed by the envelope and nonstructural proteins. The first 190 amino acids of the N-terminal end of the polyprotein are identified as core protein (Hijikata *et al.*, 1991a). The C-protein contains high amount of basic amino acids, arginine and lysine residues, (about 20%) like the nucleocapsid protein of flaviviruses and pestiviruses, and many proline residues (about 12%) (Houghton *et al.*, 1991; Choo *et al.*, 1991a). It lacks N-glycosylation sites. The C-terminal domain contains 20 highly hydrophobic amino acids and believed to act as a signal sequence which is recognized by a cellular signalase, thereby cleaving the C-protein from the precursor polyprotein (Hijikata *et al.*, 1991a; Kumar *et al.*, 1992). P22 has an RNA binding capacity and can specifically bind to the genomic RNA for the formation of nucleocapsid particles (Takahashi *et al.*, 1992; Santolini *et al.*, 1994). P22 also contains several highly immunoreactive linear epitopes and can be used for detection of early antibodies in most of the patients (Katayama *et al.*, 1992; Okamoto *et al.*, 1992d; Isida *et al.*, 1993).

The envelope proteins, E1 and E2 Two viral envelope glycoproteins, E1 and E2, have been identified by cell-free translation and transient expression systems. The exact cleavage site between C and E1 proteins catalyzed by the cellular signalase is considered to be between Ala191 and Tyr192 which occurs in the lumen of the endoplasomic reticulum (Hijikata *et al.*, 1991a). The E1 is a glycoprotein of approximately 35 kDa (p35) and contains 5 or 6 potential N-glycosylation sites that has been demonstrated by expressing the protein in cell-free translation system, by N-glyconase treatment and by *in vivo* labeling using glucosamine (Hijikata *et al.*, 1991a; Matsuura *et al.*, 1992). This region is equivalent to the E region of flavi- or pestivirus but it is much shorter and there is no significant sequence homology with them. The C-terminal of E1 glycoprotein contains a stretch of hydrophobic amino acids (residues 348−370), which could act as a membrane anchor. E1 protein is synthesized during the course of HCV infection (Matsuura *et al.*, 1992; Kohara *et al.*, 1992). However, the antibody to the gp35 does not seem to be directly associated with the neutralization of the virion.

The second envelope glycoprotein E2, molecular weight 70 kDa (p70), is coded by the E2 region of the genome. The precise location of cleavage was determined at Gly383/His384 for E1/E2 (Hijikata *et al.*, 1991a). The E2 protein is highly glycosylated and contains 9 or 11 potential N-linked glycosylation sites which have been identified by *in vitro* expression system and by direct labeling of the expressed protein with ^{3}H glucosamine (Hijikata *et al.*, 1991a; Spaete *et al.*, 1992). The N-terminus of E2 protein starts with residue His384 and its C-terminus ends with amino acid residue Asn755 (Hijikata *et al.*, 1991a). A conserved repeated amino acid sequence motif has been identified between residues 471−511, and contains 6 cystiene residues, indicating the possible complex secondary or tertiary structure of the E2 glycoproteins.

When full-length E2 region was expressed in various animal cells by several expression

systems, the expressed protein was membrane associated and was not secreted into the medium. Presumably this was caused by the presence of a single membrane anchor at the C-terminal, since C-terminal truncated E2 is rapidly secreted (Spaete *et al.*, 1992). The N-terminus of E2 is located at the outside of the virion and would allow surveillance by the immune system. However, baculovirus expression system showed that E2 protein could be efficiently secreted in to the medium (Matsuura and Miyamura 1993). Moreover, the secreted E2 protein was suggested to form a heterodimer with E1 protein that is co-immunoprecipitable with E2 (Grakoui *et al.*, 1993b; Ralston *et al.*, 1993). Conflicting results have been reported regarding the interaction between E1 and E2 proteins. Ralston *et al* (1993) and Matsuura *et al.* (1994) reported that E1 and E2 proteins are associated with each other in their N-terminal regions by disulfide bonds, whereas Grakoui *et al.* (1993b) reported that they are linked by noncovalent interaction. Recent studies have suggested that E1 and E2 glycoproteins can also be bound with NS2 and NS3 proteins, whereas association of E2 and NS4B was also reported (Grakoui *et al.*, 1993b; Selby *et al.*, 1994; Dubuission *et al.*, 1994). Such complex processing of HCV encoded proteins and protein: protein interaction suggested their possible involvement in the morphogenesis of HCV particle.

The E2 glycoprotein corresponds to the NS1 glycoprotein of the flavivirus and the major envelope protein gp53/gp55 (E2) of the pestivirus (Collet *et al.*, 1988b; Spaepe *et al.*, 1992). Both NS1 protein of flavivirus and E2 protein of pestivirus have been shown to develop protective immunity in the immunized animals (Schlesinger *et al.*, 1985; Hulst *et al.*, 1993). Therefore, the mature form of E2 protein may have an important role in the infection of HCV. However, the direct evidence suggesting the involvement of anti-E2 antibody for the protection of HCV has not yet been obtained. Recently, Choo *et al.* (1994) reported that heterodimer complex of E1 and E2 proteins which were purified from the HeLa cells infected with recombinant vaccinia viruses induced protective immunity in chimpanzees. Thus, the result indicates that not only linear epitopes of the envelope proteins but also conformation-dependent epitopes on the complex of E1 and E2 proteins might be important for induction of protective immunity against HCV infection. Since flavivirus E protein contains a number of biologically important epitopes including hemagglutination, neutralization and cell attachment, the possibility that such reactive epitopes may exist in the E proteins of HCV still remain to be determined.

There are two hypervariable regions (HVR1 and HVR2, Fig. 2) identified in gp70 of HCV (Hijikata *et al.*, 1991b; Weiner *et al.*, 1991; Kato *et al.*, 1992). One of them (HVR1) is the region from the N-terminus of gp70 to amino acid residue number 27 and the another (HVR2) is the region from amino acid 90 to 97 from the N-terminus. HVR1 lacks a conserved secondary structure and resembles the V3 loop of human immunodeficiency virus 1 gp120 (Weiner *et al.*, 1992). HVR1 contains a B-cell epitope and induces anti-HVR1 antibody (Kato *et al.*, 1993). Amino acid substitution in the HVR1 could escape recognition by preexisting anti-HVR1 antibodies leading to escaped mutants that might play an important role for establishing and maintenance of persistent infection by HCV (Kato *et al.*, 1994).

The variability of gp70 may have significant implications for the development of a pro-

tective immune response and is important in vaccine strategies. However, it would be difficult to develop an effective vaccine to control HCV infection if the HVRs contain neutralizing epitope(s).

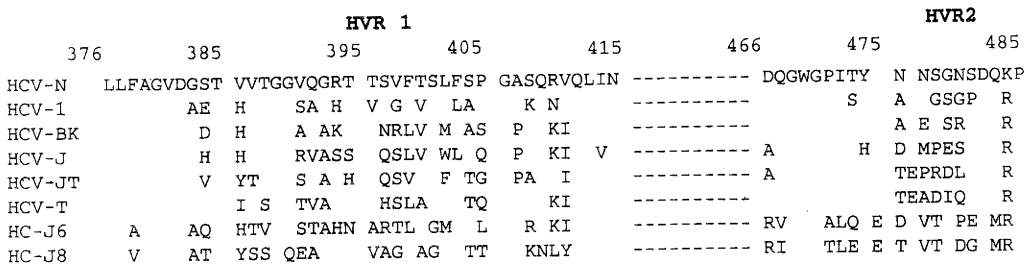

Fig. 2. Variation in amino acid sequence of the hypervariable regions (HVR1 and HVR2) of the E2 protein of 8 HCV isolates. HVR1 (amino acid 384−411) and HVR2 (amino acid 475−480) are indecated (data reproduced from Zheng, 1994).

Nonstructural proteins

The NS2 protein The nonstructural proteins appear to start with the NS2 region encoding a hydrophobic protein of about 23 kDa whose function is unknown. The biological function of the gene product of the equivalent region of the flavivirus and pestivirus is also unknown. However, in flavivirus NS2B acts as a co-factor for NS3 protease for the cleavage of downstream sites of NS3 protein (Chambers *et al.*, 1991; Falgout *et al.*, 1991; Arias *et al.*, 1993; Lin *et al.*, 1993). But in HCV, NS2 in conjunction with NS3 protein acts as a viral metalloprotease, encompassing the NS2 region and the N-terminal portion of the NS3, which is required for the autoproteolytic cleavage at the NS2/3 site (Hijikata *et al.*, 1993a; Grakoui *et al.*, 1993c). The region between amino acid residues 898−1233 was shown to be essential for the detection of this second viral Zn^{+2} requiring metalloprotease.

The NS3 protein The nonstructural protein NS3, molecular weight 70 kDa, shows limited amino acid homology with corresponding flavivirus and pestivirus protein. NS3 protein contains two distinct functional domains essential for virus replication. The N-terminal one third of the NS3 contains a protease domain similar to that found in flavi- and pestiviruses (Bartenschager *et al.*, 1993, 1995; Grakoui *et al.*, 1993a; Tomei *et al.*, 1993; Pizzi *et al.*, 1994). Amino acid sequence analysis of the putative NS3 protein shows conservation of residues His-1083, Asp-1107 and Ser-1165 among all HCV strains so far sequenced. The nature and position of these residues resemble to the catalytic triad of chymotrypsin-like serine protease. If the Ser-1165 in the proposed catalytic site is replaced with another amino acid, the proteolytic activity of the NS3 protein is abolished. All four cleavages that occur at C-terminal of the protease domain (NS3/4A, 4A/4B, 4B/5A and 5A/5B) were abolished by modification of His 1083 and Ser 1165. These cleavage sites shows several common features, which probably determine the substrate specificity of the NS3 protease. NS3 acts in *cis* on its own N- and C-terminals but the remaining three C-terminal sites can only be processed by

NS3 in *trans*.

Predicted amino acid sequence analysis and experimental data have also revealed that the C-terminal of NS3 protein contains a nucleoside triphosphate binding helicase domain (Miller and Purcell, 1990; Suzich *et al.*, 1993) that are presumably involved in the unwinding of the binding dsRNA replicative intermediate, which is necessary for genome replication.

The NS4 proteins The NS4 protein, which is hydrophobic and contains highly immunogenic epitopes, is processed into NS4A (MW 4 kDA) and NS4B (MW 27 kDa), whose exact functions are still unknown. Some experimental data have shown that NS4A binds with NS3 and make a heterodimer complex which in turn binds to the NS4B, NS5A and NS5B to form a complex structure and thereby helps virus replication (Hijikata *et al.*, 1993b; Tanji *et al.*, 1995). In *vitro* transcription and translation of the HCV polyprotein revealed that NS4A helps to anchor NS3 protein to the surface of the microsomal membrane by their mutual association. Recent report showed that NS4A is necessary for cleavage at the NS3/4A and NS4B/5A sites and enhances the efficiency of the cleavages at the NS5A/5B and NS4A/4B sites (Bartenschlager *et al.*, 1994; Failla *et al.*, 1994, 1995). Very recently, Tanji *et al.* (1995) reported using a transient protein expression system that NS4A helps in stabilization and localization of the NS3 protein. In case of flaviviruses, NS2B is required as a co-factor to facilitate efficient proteolytic processing of the nonstructural region of the viral polyprotein by NS3. Despite their different topological arrangement on their respective genome, NS4 of HCV and NS2 of flaviviruses are believed to have some common functional properties, that is, both act as co-factor of NS3 protease, can function either in *cis* or in *trans*, and can liberate from their respective catalytic domains by an intramolecular cleavage.

The NS5 proteins The nonstructural protein NS5 is processed into NS5A (MW 56 kDa) and 5B (66 kDa). The NS5A protein contains important B-cell epitopes whose function is still unknown (DeLeys *et al.*, 1993). NS5B contains a sequence motif, Gly-Asp-Asp (residue 2737−2739), which is characteristic for RNA-dependent RNA polymerases of positive stranded RNA viruses (Miller and Purcell, 1990). Therefore, NS5B is considered to encode viral replicase essential for virus replication.

The noncoding regions

The 5′ UTR According to sequence data of Han *et al.* (1991), the 5′ UTR is 341 nucleotide in length but many shorter sequences have been reported. Moreover, it has been suggested that HCV genome can exist as a truncated RNA molecules *in vivo* (Han *et al.*, 1991), implying difficulty to determine the precise terminus of the native RNA genome. The 5′ UTR is highly conserved among HCV isolates, about 93% nucleotide sequence identity between groups and about 98% nucleotides identity within group (Matsuura and Miyamura, 1993). The 5′ UTR of HCV is very much similar to pestivirus bovine viral diarrhoeal virus (BVDV) and hog cholera virus (Houghton *et al.*, 1991). The HCV 5′ UTR is composed of a relatively long leader and contains five small open reading frames (ORF) that are comparable

to picornaviral 5′ UTR which is unusually long, ranging from 610−1200 nucleotides depending on the virus isolates, and contains many silent AUG codons. But it is still unknown whether these ORFs in HCV genome are actually translated and what are the functions of the gene products (Kitamura *et al.*, 1981; Yoo *et al.*, 1992). The secondary structure analysis of the 5′ UTR of HCV has indicated the presence of a large conserved stem-loop structure in the proximal part of the 5′ UTR, which serves as a putative internal ribosomal entry site (IRES). Other studies using the mono- and dicistronic RNA constructs suggested the existence of an IRES in the HCV 5′ UTR (Wang *et al.*, 1993). From the genetic organization of the 5′ UTR, it has been postulated that the process of translation initiation of the HCV genome may be similar to that of picornaviruses. The mechanism of translation initiation used by picornaviruses involves the binding of ribosomes to a complex secondary/tertiary structure, which is called as IRES, within the 5′ UTR of the viral genome. The IRES mechanism allows cap independent translation of the genome (Pelletier and Sonenberg, 1988). Thus, HCV polyprotein translation seems to be cap independent and initiates at an IRES within the 5′ UTR, proximal to the initiator AUG codon of the polyprotein (Tsukiyama-Kohara *et al.*, 1992; Wang *et al.*, 1993). The initiation of translation seems to be inhibited by the presence of a 5′ terminal 27 nucleotides sequence capable of forming a stable hairpin structure (Han *et al.*, 1991), which is highly conserved in picornavirus.

The contradictory report has also been published to explain the ribosome scanning mechanism for translation initiation of HCV polyprotein (Yoo *et al.*, 1992). Ribosome scanning mechanism for protein translation needs a cap structure at the 5′ end of the genomic RNA which binds to the ribosome with the help of cellular initiation factor elF-4F, and initiation occurs at the AUG codon nearest to the 5′ end. Unlike flaviviruses, HCV lacks a cap structure at the 5′ end of the RAN genome.

The 3′ UTR The reported 3′ UTR of HCV shows significant variation both in length (27 to 66 nucleotides) and in sequence (26 to 40% identity between groups). It is reported that HCV contains an homopolimer tail of 10 to 100 residues at the 3′ UTR, which is identified as a poly A tail for some isolates (Choo *et al.*, 1991a, b; Han *et al.*, 1991), whereas a poly U tail is present for other isolates (Kato *et al.*, 1990; Takamizawa *et al.*, 1991; Chen *et al.*, 1992; Okamoto *et al.*, 1992b; Tanaka *et al.*, 1992). More investigations are still necessary to clarify this issue whether 3′ terminus contains poly A or poly U tail. Another remaining issue need to be solved is whether the reported 3′-terminal sequences are completed or not. The 3′ terminus is very important for plus stranded RNA viruses for RNA replication because it provides a entry site for RNA polymerase to copy the minus strand, indicating that this region might be important for the RNA replication of HCV.

Genotyping of HCV

Since the isolation of the first full-length cDNA of HCV by Chiron group, to date the entire genome of at least 17 isolates are available and a numerous partial sequence of HCV isolates have been reported throughout the world. The nucleotide sequence analysis of the 17

isolates have revealed that they are identical in only 68.1 to 91.8% indicating the existence of more than one type of HCV in nature. It is believed that this degree of sequence variability is sufficient to alter the antigenic and biological properties of the virus significantly. Data from recent studies have shown that samples reactive with the second generation commercial test kit but non-reactive or 'intermediate' with the first generation test, suggest that these are presumably new types or variants of HCV (Cha *et al.*, 1992). Supporting this hypothesis is the recent discovery of HCV variants that differ markedly in nucleotide sequence from the original American and Japanese types (Enomoto *et al.*, 1990; Nakao *et al.*, 1991; Okamoto *et al.*, 1991). In addition, there is some evidence for variation in the course of infection associated with different HCV viriants and in response to treatment with interferon (Kanai *et al.*, 1992; Yoshioka *et al.*, 1992). Altogether these evidences stress the importance of HCV genotyping.

Since the nucleotide sequence variation is distributed throughout the viral genome, comparison of full-length genome sequences is the best way to identify the HCV isolates, but practically it is quite impossible due to many limitations. Some part of the genome especially regions encoding the putative envelope proteins (E1 and E2) are most variable, whereas the 5′ UTR is most conserved. For genotyping of HCV, 5′ UTR and some less variable part, for example C, E1, NS2, NS4, or NS5 can be used for this purpose.

Based on full-sequence information and nucleotide sequence similarity, 17 HCV isolates have been classified into 3 groups or types and 6 subgroups or subtypes (Sakamoto *et al.*, 1994; Zheng, 1994). A proposed classification for these 17 isolates is shown in the Table 1.

In Japan, HCV II/Ib is the main subtype followed by subtypes III/2a and IV/2b. In Japan, about 70% of patients with chronic hepatitis were infected by HCV II/Ib, while 20% of them by III/2b and 10% of them by IV/2b, respectively. Mixed infection with II+III, II+IV and I+V account only a small fraction of the HCV infected patients (see Okamoto *et al.*, 1992c).

Several classification systems have been proposed based on nucleotide sequence information of specific region of the HCV genome and found that there are some advantages

Table. 1 Classsification of 17 HCV strains based on entire nucleotide sequence information of their genome into group, subgroup and isolate as proposed by Sakamoto *et al.* (1994) and Zheng (1994).

Group	Subgroup	Isolates	Distribution
Group 1	1/1a	HCV-1, HCV-H, HC-J1	USA, Japan
	1c	HC-G9	Indonesia
	11/1b	HC-J4/83, HC-J4/91, HCV-J, HC-C2, HCV-JT, HCV-JT', HCV-BK, HCV-T, HCV-JK1, HCV-N	Japan, China, Taiwan
Group 2	111/2a	HC-J6	Japan
	1V/2b	HC-J8	Japan
Group 3	V/3a	NZL1	New Zealand

and disadvantages associated with the system regarding the establishment of new types or subtypes (Chan *et al.*, 1992; Stuyver *et al.*, 1993; Simmonds *et al.*, 1993a). Recently, Simmonds *et al.* (1993b) proposed a new classification system based on sequence alignments of NS5 of a large number of HCV sequences derived from a variety of geographical regions. They revealed that the proposed classification is more appropriated and new groups (types) or subgroups (subtypes) can easily be fitted into the new system.

Infection rate in Japan

The prevalences of anti-HCV measured by the first and the second generation assays have been reported in many countries in the world. All these surveys showed that the carrier rates were almost same in Europe and North America which is about 1%. The rate is slightly higher in Middle East and Asia about 1−3%, and in Africa and Egypt, the rate may exceed these ranges (see Nishioka, 1994).

The prevalence of HCV infection among Japanese blood donor has been documented in Japan using the first and the second generation anti-HCV assays, which indicated that HCV infection rate is slightly higher in the western part and high rates exist among the older people over 50. The percentages of anti-HCV positivities for each age group were 0.36% (16−20 years), 0.69% (21−30 years), 1.14% (31−40 years), 1.74% (41−50 years) and 2.78% (51−64 years). Concurrently, when 1,224 presumably healthy school children (5−15 years) were tested, none of them were anti-HCV positive.

It has been demonstrated that in Japan, almost 90% of all main liver diseases, i.e. chronic hepatitis, liver cirrhosis and hepatocellular carcinoma are associated with HBV or HCV (see Esumi and Shikata, 1994). In Japan, 70% chronic hepatitis cases are due to HCV infection, whereas only 30% due to HBV. In liver cirrhosis cases, about 65−70% were associated with HCV infection, less than 20% with HBV and less than 10% due to alcohol consumption. But in case of hepatocellular carcinoma, almost 80% were due to HCV infection, whereas less than 20% cases were due to HBV. So HCV alone is responsible for about 65 to 80% of liver diseases in Japan.

In the United States, HCV is considered as a major cause of both chronic liver diseases and persistent viremia which account for an average of 21% of acute viral hepatitis cases. The carrier rates of HCV in Japan and the United States are almost similar but the incidence of hepatocellular carcinoma in these two countries are not similar. It is speculated that this difference is either by different virus subtypes (subgroups) or different routes of infection. In Japan, most of the infections are due to HCV subtype II, whereas subtype I is most prevalent in the United States. These two virus types considerably vary both in the nucleotide (14−16%) and amino acid (11−22%) sequences (Choo *et al.*, 1989; Takamizawa *et al.*, 1990; Kato *et al.*, 1990). In Japan, the major route of HCV infection is considered to be blood transfusion. In the United States, HCV infection is much more predominant among drug abusers. Since the development of liver cirrhosis and hepatocellular carcinoma needs 20 to 30 years after HCV infection, these people die before the appearance of hepatocellular carcinoma. More investigations are still needed to identify the actual causes associated with the development of hepatocellular carcinoma.

Transmission of HCV

1) Parenteral

Transfusion of blood products from the HCV infected donors has been documented as a major route of HCV infection throughout the world. HCV infection at high rate was also reported among hemophiliac patients (Oguchi *et al.*, 1990; Watson et al., 1992; Laurian *et al.*, 1992). Moreover, several cases of HCV infection were also reported among intravenous immunoglobulin recipients in the United States, Sweden, Spain and England (Williams, 1988; Nishioka, 1994). Transmission of HCV infection from infected person to health care workers through needle stick injuries have also been reported in Japan (Kiyosawa *et al.*, 1991; Mitsui *et al.*, 1992). The intravenous drug abusers are the high risk group for HCV infection. The HCV infection rates among intravenous drug users range from 56 to 80% (Iwamura *et al.*, 1992; IARC, 1994).

2) Non-parenteral

Perinatal transmission of HCV has been documented in several studies and confirmed that vertical transmission of HCV can take place (Ohto *et al.*, 1994). Controversial results have been reported for sexual transmission of HCV (Melbye *et al.*, 1990; Gordon *et al.*, 1992; Bresters *et al.*, 1993; Kao *et al.*, 1993; Skidmore *et al.*, 1994). Careful epidemiological studies are required to resolve this issue. Intrafamilial transmission of HCV was also reported (Kamitukasa *et al.*, 1987).

Diagnosis of HCV infection

Two highly sensitive and specific virological methods are now available to diagnose HCV infection: 1) detection of circulating antibodies by the second generation ELISA assay and, 2) detection of HCV genome sequences from infectious materials by the reverse transcription-polymerase chain reaction (RT-PCR). The first one is referred as serodiagnosis and the second one as genodiagnosis. The routine method for detection of HCV infection is mainly performed on the basis of these two assays.

Serodiagnosis

The first generation assay for the detection of anti-HCV became available in 1989 utilizing a recombinant yeast expressed antigen, C100-3, which contained antigenic epitopes located in the NS3-NS4 region (Kuo *et al.*, 1989). Anti-C100-3 was used for diagnosis of HCV from patient serum and screening donor blood and prevented 60−80% post transfusion non A non B hepatitis in Japan (Watanabe *et al.*, 1993; Japanese Red Cross NANB Hepatitis Research Group, 1991).

In the improved second generation anti-HCV ELISA assay, recombinant core antigen was added to the previously used C100-3 antigen. This assay was found to be more sensitive than the first generation ELISA. In addition to the ELISA, agglutination test using gelatin particles or fixed erythrocytes coated with the second generation antigens have been used to measure anti-HCV antibodies. The second generation assay systems showed excellent performance in detecting anti-HCV in donor blood. In Japan, almost 99% donor blood can now be

screened by the second generation assay systems (Esumi and Shikata, 1994). Thus, in Japan post transfusion HCV is almost under control.

In the third generation assay, NS5 antigen has been added to the second generation test to make it more sensitive. These anti-HCV antibody assay systems detect mainly IgG type antibodies. Recently, attempts have been made to detect IgM type antibodies from clinical specimens and these assays were found to be quite useful in early diagnosis of HCV infection (Brillanti *et al.*, 1992; Clemens *et al.*, 1992).

Genodiagnosis

Since the isolation of a cDNA clone by Chiron group, diagnoses on HCV infections have been conducted by the detection of viral RNA by RT-PCR. HCV RNA is extracted from serum samples and cDNA is synthesized by reverse transcriptase and amplified by the PCR using HCV-specific primers. Primer selection is prerequisite to obtain accurate results. Generally primers are selected from the highly conserved region of the HCV genome, preferably from 5′ UTR and C-protein region. Other regions such as NS5 can also be used for this purpose. The nested RT-PCR has been performed in order to increase the specificity and sensitivity of the PCR (Garson *et al.*, 1990; Okamoto *et al.*, 1990, 1992c). Competitive RT-PCR has also been used for the quantative detection of HCV-RNA from infectious materials, providing a very useful quantative method (Kaneko *et al.*, 1992; Hagiwara *et al,.* 1993).

Markers useful for detecting acute hepatitis C

Detection of IgG antibodies is not a useful marker in the early diagnosis of acute hepatitis C. Since the second generation ELISA assay can only detect IgG type antibodies, half of the HCV positive patients become negative to this test within one month after the onset of the disease (Hino, 1994). But during this time, all HCV patients show RNA levels which can easily be detected by RT-PCR. So the best marker for diagnosis of acute HCV infection is the detection of HCV-RNA. Transient infection can also be diagnosed by detecting HCV RNA. In case of transient infection, acute phase is very important, because RNA level from patient serum decrease to undetectable level after three months of onset. So diagnosis becomes impossible if the acute phase is missed.

Quantitative assay for both second generation anti-HCV IgG and IgM can be applied to differentiate acute and chronic hepatitis C. At the early stage of infection, most of the patients show detectable level of serum IgM and undetectable level of IgG. After 3 month of onset, anti-HCV IgG can be detected at high rates, while IgM level may be absent or present at low level. So in the early stage of acute hepatitis C, IgM is positive while in chronic cases, IgG antibodies are always present in high levels.

Serum alanine amino transferase (ALT) levels

Serum ALT is not always a useful indication to detect hepatitis C virus infection. It has been found that some patients with HCV infection have normal ALT level. In such case,

determination of past and current infection is obligatory which might be difficult by determining the HCV RNA and anti-HCV antibodies. So in this case, quantative assay of HCV RNA, anti-HCV IgG and anti-HCV C-protein are very useful to differentiate past and current infection.

Liver biopsy is also an effective diagnosis to detect the HCV infection because healthy carrier is hardly found.

Persistent infection with HCV

The mechanisms by which acute HCV infection develops into persistent infection in the same patient have not been clearly understood. Most plausible explanation for chronicity was given by the genomic structure of the HCV. It has been noted that HCV genome undergoes frequent mutations during natural course of chronic infection. The overall mutation rate of the entire HCV genome in a chronically infected chimpanzee has been estimated between 0.9 and 1.92×10^{-3} base substitution per site per year (Ogata *et al.*, 1991; Okamoto *et al.*, 1992a; Abe *et al.*, 1992).

Recent molecular biological studies of HCV from chronically infected patients indicate that most of the individuals contain a large variety of related HCV sequences described as 'quasispecies' (Martell *et al.*, 1992). Different HCV sequences from a single patient at different time intervals were also reported (Murakawa *et al.*, 1992), indicating that some parts of the HCV genome undergo selection pressure and able to escape from the immune-surveillance system which might play a crucial role in the mechanism of persistent chronic infection (Enomoto *et al.*, 1993; Higashi *et al.*, 1993; Kato *et al.*, 1993; Sakamoto *et al.*, 1994; Kurosaki *et al.*, 1994). To clarify the potential mechanism of chronic HCV infection with acute hepatitis, the amino acid substitutions in the E1, E2, and NS3 regions of HCV strains have been investigated extensively in several studies (Ogata *et al.*, 1991; Okamoto *et al.*, 1992a, Kato *et al.*, 1992). It has been reported that after the IFN treatment, the HCV RNA in serum decreases significantly, but subsequently reappears. During this period, the amino acid substitution rate in the HVR I of E2 increased significantly. Thus it is predicted that the rapid amino acid substitution in the HVR I might be one of the mechanism of chronicity in HCV infection.

Hypervariability and 'Quasispecies'

Comparison of the amino acid sequences of the envelope region, E1 and E2, of multiple HCV isolates have identified two hypervariable regions (HVR, Fig. 2). HVR 1 near the N-terminus of E2 consists of 27 amino acids and the HVR2 consists of 8 amino acids, position 90 to 97 from the N-terminus of E2 region (Hijikata *et al.*, 1991b; Kato *et al.*, 1992). The importance of HVR1 in the disease mechanism associated with HCV has been analyzed in a few studies which indicated that HVR1 was directly associated with the development of chronic infection. The alteration of amino acids in HVR1 occurs sequentially during the natural course of chronic infection at a rate of 0.5 to 1.7 amino acids per month (Kato *et al.*, 1992). HVR1 was able to induce anti-HVR1 which could only neutralize the homologous virus

isolate and HCV with an amino acid substitution in its HVR1 could escape from recognition by preexisting anti-HVR1 antibodies, indicating the possible importance of the HVR1 in chronic infection by HCV (Kato *et al.*, 1993).

The appearance of amino acid substitution in HVR during chronic HCV infection has been observed in many studies. These substitutions have been explained either by viral coded RNA-dependent RNA polymerase needed for genome replication or by selective immune pressure on viral particles imposed by infected host. Errors may accumulate as mutation during replication of viral genome by RNA-dependent RNA polymerase, due to lack of proofreading activity of this enzyme encoded by all RNA viruses. As a result, variant RNA genomes evolve *in vivo* that form a quasispecies population which contain one or more master sequence and a large spectrum of closely related variants (Holland *et al.*, 1992). Several closely related sequences have been isolated from a single HCV infected patient (Murakawa *et al.*, 1992). Of course in such case, the possibility of infection by more than one virus strain cannot be ruled out. Since the HVR is thought to contain neutralizing epitopes, selective immune pressure of the infected host may give rise to the mutation in the viral genome which can escape from the host immunity.

Moreover, phylogenetic analysis have suggested that there are two different genetic mechanisms underlying the change in HVR sequences, either by continuous accumulation of mutations *de novo* and/or selective overgrowth of preexisting minor variants from the large spectrum of quasispecies populations (Kurosaki *et al.*, 1994).

Treatment of HCV infection

Until now, there is no known chemotherapeutic agents available for the treatment of HCV infection. Despite unusual side effects , interferon (IFN) is the only drug which has been used for the treatment of acute and chronic hepatitis C virus infection. IFN is believed to induce 2′-5′ oligo-adenylate synthetase which in turn inhibit the viral protein synthesis and thereby inhibit the viral replication (McNair and Kerr, 1992). It has been reported that most of the HCV patients effectively respond to the IFN and show normal ALT level and negative HCV RNA within six months of treatment and remain normal at least six months after completion of therapy (Shindo *et al.*, 1991). However, subsequent to the withdrawing of IFN treatment, HCV RNA and ALT reappear and become worse even than pretreatment level and the disease turns into chronic state (Davis *et al.*, 1990; Lindsay *et al.*, 1990). The reason of why all the patients do not respond equally to IFN treatment and why IFN cannot clear the virus from the system are still unknown. The outcome of the IFN treatment appears to depend on 1) the condition of the liver histology, 2) the HCV-RNA level of the serum and 3) the presence of HCV genotype.

Concluding remarks

The new information obtained about the structure and function of the HCV genes and their proteins since the initial characterization of the HCV is really impressive. Due to the lack of an *in vitro* replication system for HCV, investigation of the many biological functions

such as mechanism of replication of the viral genome, host-virus interaction, morphogenesis of virus particles, function of individual viral coded protein, immunology, viral inhibition assays etc have been greatly impeded. Therefore the establishment of an *in vitro* propagation system for HCV is imperative. The development of an effective chemotherapeutic agent is obligatory which can be used to cure HCV infected patients. In this purpose, viral coded enzymes, such as protease, helicase, replicase etc could be attractive targets. Therefore, it is necessary to obtain more information about the molecular biology and replicative strategy of HCV. The role of cellular and humoral immune responses should be investigated extensively in order to provide insight into the chronicity associated with HCV infection. The construction of infectious cDNA of HCV should provide useful approaches for studying molecular biology and pathogenesis of HCV infection and help to prevent and control of HCV infection. With the advent of the first and the second generation ELISA, most of the post-transfusion non A non B hepatitis is now under control. So we greatly hope that HCV and HCV-associated diseases can be controlled effectively in the near future.

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