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Review

Molecular Virology of Hepatitis C Virus

Nobuyuki Kato*

Department of Molecular Biology, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558, Japan

Hepatitis C virus (HCV), discovered in 1989, is the major causative agent of parenteral non-A, non-B hepatitis worldwide. Following the development of a method of diagnosing HCV infection, it became apparent that HCV frequently causes chronic hepatitis. Persistent infection with HCV is implicated in liver cirrhosis and hepatocellular carcinoma. Current worldwide estimations suggest that more than 170 million people have been infected with HCV, an enveloped positive single-stranded RNA (9.6-kilobases) virus belonging to the *Flaviviridae*. The HCV genome shows remarkable sequence variation, especially in the hypervariable region 1 of the E2 protein-encoding region, and globally, HCV appears to be distributed with more than 30 genotypes. Complicated "quasispecies" and frequent mutations of viral genomes have also emerged. The HCV genome encodes a large polyprotein precursor of about 3,000 amino acid residues, and this precursor protein is cleaved by the host and viral proteinases to generate at least 10 proteins in the following order: NH_2-core-envelope (E1)-E2-p7-nonstructural protein 2 (NS2)-NS3-NS4A-NS4B-NS5A-NS5B-COOH. These viral proteins not only function in viral replication but also affect a variety of cellular functions. Although several explanations have been proposed, the mechanisms of HCV infection and replication in targeted cells, the mechanism of persistent viral infection, and the pathogenesis of hepatic diseases (hepatitis or hepatocellular carcinoma) are all poorly understood. A major reason why these mechanisms remain unclear is the lack of a good experimental HCV replication system. Although several classical trials using cultured cells have been reported, several new, more promising experimental strategies (generations of infectious cDNA clone, replicon, animal models, *etc.*) are currently being designed and tested, in order to resolve these problems. In addition, new therapies for chronic hepatitis have also been developed. The enormous body of information collected thus far in the field of HCV research is summarized below, and an overview of the current status of HCV molecular virology of HCV is provided.

Key words: HCV genome, genotype, hypervariable region, quasispecies, HCV proteins, core protein, envelope protein, HCV proliferating system, persistent infection, chronic hepatitis, extrahepatic infection, hepatocellular carcinoma, interferon

For many years, hepatitis A and hepatitis B virus (HBV) were thought to be the major causative agents of hepatitis, including cases of transfusion-associated hepatitis. However, even after the establishment of prevention and diagnostic methods for these hepatitis viruses, transfusion-associated hepatitis continued to occur, and these cases were recognized as non-A, non-B hepatitis. Although the causative agent of

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*Corresponding author. Phone: +81-86-235-7385; Fax: +81-86-235-7392
E-mail: nkato@md.okayama-u.ac.jp (N. Kato)

parenteral non-A, non-B hepatitis was considered to be an unknown virus, this virus proved to be very difficult to identify. In 1989, after a large number of trials were conducted in order to find this new virus, Chiron's group (USA) finally succeeded in cloning part of the genome of the virus that came to be known as the hepatitis C virus (HCV) [1]. Moreover, this group developed an anti-HCV antibody detection system for the diagnosis of HCV infection [2]. This finding clarified that HCV is a major causative agent of parenteral non-A, non-B hepatitis. Persistent infection with HCV appears to be a major factor in the development of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).

To date, a large number of papers have been published regarding the HCV; issues including viral genetic analysis and functional analysis of viral proteins have been addressed at length. A large amount of important data has thus accumulated during the 1990s. We are therefore currently in a good position to develop landmark HCV research. However, the inability to quickly and reliably replicate HCV in cell cultures has been a serious handicap to fighting this disease. Due to this handicap, the mechanisms of HCV infection and replication in target cells, and the pathogenesis of hepatic diseases associated with HCV are still poorly understood. Furthermore, the mechanism of persistent viral infection also remains to be clarified. Numerous trials using new experimental strategies are currently making great strides in the effort to resolve these issues.

The present report provides a summary of the enormous body of information from HCV research to date and provides an overview of the current status of the molecular virology of HCV.

Abbreviations

ALT, alanine aminotransferase; CTL, cytotoxic T lymphocytes; ER, endoplasmic reticulum, E1, envelope 1; E2, envelope 2; ELISA, enzyme-linked immunosorbent assay; EBV, Epstein-Barr virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; HIV-1, human immunodeficiency virus type 1; HVR, hypervariable region; IFN, interferon; PKR, IFN-induced double-stranded RNA-activated protein kinase; ISDR, interferon sensitivity determining region; IRES, internal ribosome entry site; kb, kilobases; LTR, long terminal repeat; LDLR, low density lipoprotein receptor; NS, non-structural; ORF, open reading frame; PB-DC, peripheral blood-dendritic cells; PBMC, peripheral blood mononuclear cells; PML, polymorphonuclear leukocytes; PTB, polypyrimidine tract binding protein; RT-PCR, reverse transcription-polymerase chain reaction; RdRp, RNA-dependent RNA polymerase; SV40, simian virus 40; TNF, tumor necrosis factor; 5' UTR, 5' untranslated region; 3' UTR, 3' untranslated region.

I. Discovery of HCV

In 1989, Choo *et al.* first isolated part of the HCV genome by immunoscreening a cDNA library derived from the plasma of a chimpanzee chronically infected with HCV; the HCV genome was considered to be a positive-stranded RNA molecule of about 10 kilobases (kb) [1]. An anti-HCV enzyme-linked immunosorbent assay (ELISA) using the recombinant HCV antigen, which was expressed in yeast, was also simultaneously developed by Chiron [2]. Patients with post-transfusion non-A, non-B hepatitis in Japan show a high prevalence of antibody against the HCV antigen (78% of patients with chronic hepatitis and 15% of those with acute hepatitis) [2]. Among blood donors in the Tokyo district, 1.2% were estimated to be positive for this antibody [3]. This estimate indicates an abnormally high incidence of the antibody among patients with hepatitis. Similar results have been reported in other countries [2-8]. These results suggest that HCV is a major causative agent of parenteral non-A, non-B chronic hepatitis throughout the world. Since such non-A, non-B hepatitis (termed hepatitis C) was frequently linked to liver cirrhosis that could develop into HCC 20 to 30 years later, the incidence of the antibody in patients with HCC was examined by several groups [9-13]. Patients with HCC that was not associated with HBV showed a high incidence (more than 70%) of the antibody. These results suggest that HCV is closely associated with HCC.

Although the HCV genome has provided a better understanding of the natural history, immunology, and epidemiology of HCV, the morphology of HCV was not definitively characterized until 1994. Spherical virus-like particles, 50 to 70 nm in diameter, taken from human serum and liver tissue, were identified as hepatitis C virion by immunoelectron microscopy using the antibodies against the HCV envelope protein [14-16]. The nucleocapsid of the particles, which were found to be 50 nm in diameter, appeared to be icosahedral in structure and surrounded by an envelope covered with surface projections [15]. The morphological features of the HCV particle were shown to be consistent with the characteristics of flaviviruses and pestiviruses of the *Flaviviridae* family.

II. Epidemiology

In 1989, Kuo *et al.* developed an anti-HCV ELISA

using recombinant HCV non-structural (NS) antigen (C100) as a first-generation assay system for the detection of HCV antibody [2]. Afterwards, second and third generation assay systems using 3 HCV recombinant proteins, including the core protein, were established in order to increase the specificity and sensitivity of the detection of antibodies [17-19]. Currently, these second and third generation assay systems are widely used for the diagnosis of HCV infection. As powerful means for the diagnosis of HCV infection, reverse transcription-polymerase chain reaction (RT-PCR) methods (competitive PCR method, Amplicor method, *etc.*) and a branched chain DNA amplification method were developed for the detection of the HCV RNA genome and for the quantitative analysis of virus load using the primers derived from the 5' untranslated region (5'UTR) [20-22]. These DNA amplification systems are currently used in combination with the antibody detection systems in order to achieve a diagnosis of HCV infection. Using these established assay systems for the antibody and HCV genome, a number of epidemiological data have been reported to date [for a review, see 23]; it is currently estimated that there are more than 170 million persons infected with HCV worldwide [24]. In developed nations, the general population HCV prevalence rates are generally less than 3% (*e.g.*, 1.2% in Japan, 1.8% in the United States). Among volunteer blood donors, the rate tends to be less than 1%. However, in a few nations and in distinct regions within some nations, HCV prevalence rates in the general population exceed 10%. In Egypt, the HCV prevalence rate appears to be between 10% to 30% [25]. The major reason for the spread of HCV infection is speculated to be a national campaign to treat schistosomiasis infections. Similarly, in several areas in Japan and Italy, a high prevalence of HCV among older persons is linked to the receipt of medical care [26, 27]. High rates of HCV infection also have been reported in urban areas of the United States; these cases are thought to be drugs, and not by medical procedures [28].

III. Gene organization of HCV genome

1) Total traits of viral genome

After the nucleotide sequence of the NS region of the HCV RNA genome (about 7 kb) was made available by Chiron's group [29], many researchers including ourselves rushed to obtain a full-length cDNA clone containing the structural region in order to elucidate the complete structure of the HCV genome. In Aug of 1990, 2 Japanese groups independently succeeded in cloning the structural region of the HCV genome [30, 31], and in Dec of 1990, our group finally cloned almost the entire HCV-J viral genome (9,413 nucleotides) derived from Japanese patients with chronic hepatitis [32].

Since the elucidation of the HCV-J genome [32], the complete structures of dozens of HCV genomes belonging to many genotypes (described later) have been reported. In 1995, 98 highly conserved additional nucleotides were found downstream of the oligo (U) stretch and were considered to be the 3' end structure of the HCV genome [33]. The HCV genome is a positive, single-stranded RNA of about 9.6 kb, although the length varies a little between the genotypes, and a large precursor protein (3,008-3,037 amino acids) is produced from an open reading frame (ORF) of more than 9,024 nucleotides. Schematic representation of HCV genome structure (strain HCV-J) is shown in Fig. 1.

Processing of the HCV precursor protein (described later) occurs co-translationally as well as post-translationally by a cellular signal peptidase and viral NS2-NS3 and NS3 proteinases [34-41] (Fig. 1). The structural proteins, which form viral particles, are located on the amino-terminal side of the precursor protein in the following order: core, envelope 1 (E1), and E2. Downstream of the structural proteins, NS proteins, which may be not included in the viral particle, are located in the order: NS2, NS3, NS4A, NS4B, NS5A, and NS5B. It is not clear whether or not a small protein, p7, which is produced by the cleavage of the E2 protein [42, 43], is a component of the viral particle.

The 5'UTR consists of 341 nucleotides, and its nucleotide sequence is highly conserved (with a homology of more than 92%) between virus strains, suggesting that the 5'UTR plays important roles in key processes such as the replication of the viral genome and the translation of viral proteins. It has been found that the 5'UTR possesses an internal ribosome entry site (IRES) [44, 45]. Although most of the 5'UTR is required for the full activity of IRES [46-51], an RNA pseudoknot structure upstream of the initiation AUG appears to be an essential structural element of the IRES [47]. In addition, sequence heterogeneity between IRES elements has been shown to lead to important changes in the efficiency of their translation [52]. Cellular factors required for HCV IRES-mediated translation, namely, polypyrimidine tract-

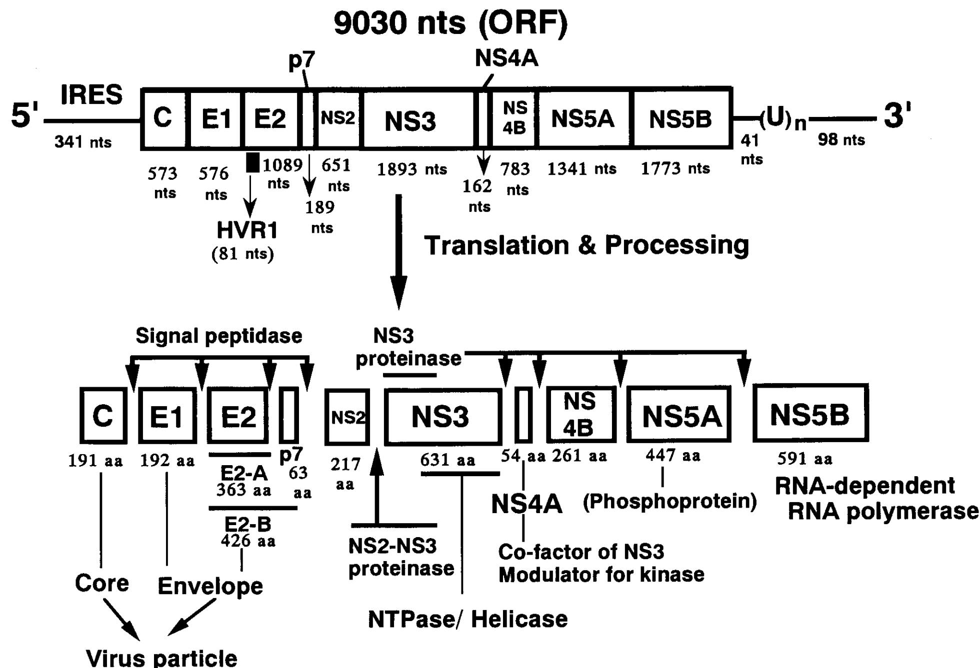

Fig. 1 Structure of the HCV genome and summary of HCV polyprotein processing. Schematic representation of HCV genome structure (strain HCV-J) was shown in the upper portion. nts, nucleotides; HVR1, hypervariable region 1; IRES, internal ribosome entry site. Processing of the HCV precursor polyprotein occurs with cellular signal peptidase and 2 virus-encoded NS2-NS3 and NS3 proteinases. Cleavage sites for these proteinases are indicated by arrows. Processed proteins are indicated by name.

binding protein (PTB) and La autoantigen, have recently been identified [53-56]. The functional roles of 4 highly structured domains between nucleotides 40 to 355 during HCV gene expression are detailed in recent reviews [57, 58]. Since the 5'UTR is the most conserved region of the HCV genome, a variety of primers derived from the 5'UTR are used for the detection and quantitative analysis of the HCV genome by RT-PCR [for a review, see 59].

The 3' untranslated region (3'UTR) consists of a poorly conserved sequence of approximately 40 nucleotides and an internal poly (U)/polypyrimidine tract, followed by a unique 98 nucleotide sequence [33, 60-62]. This latter tail sequence was highly conserved among virus strains and even between the 2 most genetically distant HCV types, 1b and 2a [60]; this sequence can fold into a conserved stem-and-loop structure [60, 63]. These results suggest that the novel 3' tail is a structure common to the HCV genome and that it plays an important role in the initiation of replication of the viral genome. PTB and La autoantigen that bind to the 5'UTR of the HCV genome have also been identified as cellular factors which bind to the 3'UTR [64, 65], suggesting that PTB and La autoantigen are involved not only in IRES-mediated translation, but also in viral replication.

The gene organization of HCV is similar to that of the *Flaviviridae* family, and several portions of the HCV precursor protein also show similar hydropathy profiles to that of the *Flaviviridae* [31, 66]. However, because of the low sequence homology of HCV genomes with those of flaviviruses and pestiviruses, HCV is now classified into a new genus (hepacivirus), distinct from the flaviviruses and pestiviruses of the *Flaviviridae* family [67].

2) Diversity of viral genome

In the early stage of the study, we noticed that the viral genome of Japanese HCV isolates (HCV-J) from a patient with HCC showed sequence variations from the original isolate (HCV-1) identified in the United States [68]. Two amplified regions of the HCV-J genome, encoding the NS5A and NS5B proteins, showed 17 to 19 % variation in nucleotide sequence from that of HCV-1 [68, 69]. The clarification of full-length HCV-J and

HCV-1 genomes [32, 70] revealed that HCV-J and HCV-1 showed 23% variation in nucleotide sequence and 15% variation in amino acid sequence. These results indicate that HCV-J and HCV-1 belong to different HCV genotypes. These findings also suggest that at least 2 genotypes of HCV exist throughout the world. The more recent comprehensive sequence analyses of HCV genomes indicate that HCV is distributed with a number of genotypes worldwide. Furthermore, the complicated "quasispecies" nature [71] of HCV have been discussed, and frequent mutations of HCV genomes *in vivo* have also been identified.

In fact, more than 30 HCV genotypes, which are classified into 11 groups, have thus far been identified in the world [72, 73]. Each genotype regularly shows a difference of more than 20% at the nucleotide level and more than 15% at the amino acid level, although the 5' UTR and core protein-encoding region showed high homology, *i.e.*, > 90%. It has been proposed that all HCV genotypes should be reclassified into 6 groups [74], and that the nomenclature of HCV viral genotypes should follow the following format: HCV-1a, HCV-1b, HCV-2a, *etc.* [75]; this nomenclature is now widely used. Although the frequency of HCV genotypes is notably different between countries [72, 76], 6 HCV genotypes (HCV-1a, -1b, -2a, -2b, -3a and -3b) are distributed worldwide [72]. To date, the complete nucleotide sequences of HCV genomes belonging to 18 different genotypes have been reported. The representative sequenced HCV genomes are summarized as follows: HCV-1[70], HCV-H[77] and HCV-J1[78] for HCV-1a; HCV-J[32], HCV-BK [79] and HCV-JT [80] for HCV-1b; HC-G9 [81] for HCV-1c; HC-J6[82] for HCV-2a; HC-J8 [83] for HCV-2b; BEBE1 [84] for HCV-2c; VAT96 [85] for HCV-2k; NZL1 [86] and HCV-K3a/650 [87] for HCV-3a; HCV-Tr [88] for HCV-3b; ED43 [89] for HCV-4a; EUH1480 [90] for HCV-5a; EVHK2 [91] for HCV-6a; Th580 [92] for HCV-6b; VN235 [92] for HCV-7b; VN405 [92] for HCV-8b; VN004 [92] for HCV-9a; JK049 [73] for HCV-10a; JK046 [73] for HCV-11a. Intensive sequence analyses of HCV genomes are currently being conducted. However, the relationship between HCV genotypes and liver disease status is still not clear.

The sequence diversity within a single genotype was also clarified by comparisons of HCV genomes. Our results [69, 93] showed 5-8% variation in the nucleotide sequence and 4-5% variation in amino acid sequence, although the degree of genetic diversity is not homogeneous throughout the HCV genome.

An analysis of sequence variation between the HCV genomes in an individual revealed that the variation in nucleotide sequence averaged 0.9%, indicating the presence of HCV genomes as a quasispecies. The quasispecies nature was distributed throughout the genome except in the 5'UTR, where no variation was observed. The sequence diversity was higher (1.6%) in the envelope proteins-encoding region than in other regions [80]. Martell *et al.* (1992) [94] similarly was able to demonstrated the quasispecies nature of the HCV genome distribution in a single patient.

The most characteristic region in the HCV genome that shows a quasispecies nature is the hypervariable region 1 (HVR1), which is 27 amino acids in length, and begins from the N-terminal amino acid of the E2 envelope protein [93, 95, 96]. Much of the data on the structure of HVR1 has already been reported using clinical specimens from patients with hepatitis and specimens from HCV-infected chimpanzees [93, 95-105]. The characteristic features of HVR1 are summarized as follows. (1) HVR1 is located at the surface of the E2 envelope protein and has B-cell epitopes. (2) HVR1 shows a maximum of 80% amino acid sequence diversity, but several positions within HVR1 are relatively conserved, suggesting that a common secondary structure of HVR1 is required for the viability of the virus. (3) HVR1 complexity does not correlate with the severity of liver disease, HCV genotype, or viral load. (4) HVR1 is rich in serine and threonine residues (average, 27%). (5) Cysteine residues and N-linked glycosylation sites are rare in HVR1. The remarkable sequence heterogeneity of HVR1 allowed us to use it as a good molecular marker for distinguishing HCV species [106-109]. Although HVR1 has been identified in all HCV genotypes, the hypervariable region 2 (HVR2) is only found in the HCV-1b genotype [93]. HVR2 consists of 7 amino acids and is located at positions 91 to 97 of the E2 envelope protein. HVR2 shows a maximum of 100% amino acid sequence diversity and has no B-cell epitopes.

A comparison of complete genome sequences of HCV strains reveals that synonymous changes in coding regions at both the 5' and 3' ends of the genome are suppressed by the probably RNA secondary structures identified within the core and NS5B-encoding regions. Non-synonymous substitutions are less frequent than synonymous ones, except in HVR, and transitions are

more frequent than transversions, particularly at the third position of the codon [110].

3) Variations in the viral genome

Since the HCV genome shows remarkable sequence diversity, it is assumed that HCV frequently causes the mutations of the viral genome. In 1991, the mutation rate of the HCV-H (genotype 1a) was estimated to be approximately 1.92×10^{-3} base substitutions per site per year, in comparison to the HCV genomes obtained from plasma collected in 1977 and 1990 [111]. One experimental model, a chimpanzee experimentally infected with HCV, has been used to compare 2 full-length HCV genomes (one during the early acute phase and another during the chronic phase 8.2 years later). The mutation rate of the HCV genome was estimated to be approximately 1.44×10^{-3} base substitutions per site per year [112]. The mutation rate varied among the domains of the HCV genome, and the envelope proteins-encoding region showed a several-fold higher mutation rate than the other regions [112]. These data support the above mentioned hypothesis that sequence diversity among HCV genomes is the result of accumulation of mutations. However, it is difficult to determine the actual mutation rate of the HCV genome, because HCV always exists as complicated quasispecies *in vivo*, and also because experiments on HCV replication in humans are ethically problematic. Recently, 2 chimpanzees were infected with HCV following intrahepatic inoculation with RNA transcript from a full-length HCV cDNA clone (described later); the mutation rate of the HCV genome was estimated to be 1.48 to 1.57×10^{-3} base substitutions per site per year, as determined by a sequence analysis of the full-length HCV genome at week 60 postinoculation [113]. However, a recent report using acute-phase plasma samples from a human (the inoculum) and 6 serially infected chimpanzees showed that the genetic mutation rates of HCV were significantly lower in the chimpanzees than in the human [114].

From the genetic analysis of the envelope proteins (E1 and E2)-encoding region of HCV genomes isolated during the progression of hepatitis, we found that HVR1 was a major site for genetic mutations in HCV after the onset of hepatitis [106, 115]. This result is consistent with the observation that the sequence diversity of HCV is most marked in HVR1. To date, although the mechanism of frequent genetic mutations in HVR1 is still not fully understood, the mode of genetic mutation in HVR1 is thought to be via the following process [98, 100, 106-108, 115-122]. (1) HVR1 quasispecies continuously change in patients with chronic hepatitis and asymptomatic carriers. (2) The mutation rate of HVR1 is positively related to the level of serum alanine aminotransferase (ALT). (3) The conditions of HVR1 quasispecies depend on the intensity of the host's immunological response. The mutation rate of HVR1 decreases when the host is immunodeficient or immunosuppressed. (4) In some cases, the status of the HVR1 quasispecies is drastically changed by therapy with IFN.

IV. Structure and function of HCV proteins

1) Processing of viral precursor polyprotein

Individual viral proteins are produced by cleavage of the precursor polyprotein produced from the largest ORF of the HCV genome. Processing of HCV-1b precursor polyprotein (strain HCV-J) is summarized in Fig. 1. Proteolytic cleavage of the viral precursor polyprotein is mediated by at least 3 enzymes, namely, a cellular signal peptidase and viral NS2-NS3 and NS3 proteinases [34-41]. Another small viral protein, NS4A, is required for proteolytic processing as a cofactor of NS3 proteinase [123, 124]. HCV structural proteins, a core and 3 envelope proteins, E1, E2 (E2-A), and E2-p7 (E2-B), are produced by cleavage by the signal peptidase(s) [34, 42, 43, 125]. Since the cleavage efficiency at the C-terminal position of E2 (E2-A) is poor, E2-p7 (E2-B) is generated [42, 43]. Initially, the core protein was considered to have a total of 191 amino acids, as determined by *in vitro* transcription/translation analysis [34]. However, several studies have reported that the second processing in the C-terminal hydrophobic region of the core protein occurred during core protein maturation, and resulted in 21 kDa, 19 kDa, or 16 kDa [126-131]. These cleavages in the C-terminal region of the core protein suggest that processing by an eukaryotic signal peptide peptidase takes place [128]. However, the precise cleavage sites have not yet been determined, although amino acids 173, 179, and 182 have been identified as candidates of the C-terminal residues of mature core protein *in vivo* [128-130].

Two virus-coded proteinases (NS2-NS3 and NS3 proteinases) are responsible for the cleavage of the latter half of the precursor polyprotein [35, 37, 39-41, 132-135]. NS2-NS3 proteinase is a zinc-dependent enzyme and cleaves the site between NS2 and NS3 [35, 38], and therefore this proteinase activity is lost by self-

cleavage between NS2 and NS3. The amino acid sequence surrounding the cleavage site, (NH_2) Leu-Leu/Ala-Pro-Ile-Thr (COOH), is conserved among HCV strains. NS3 serine proteinase cleaves the site between NS3 and NS4A in a *cis*-acting manner, whereas this enzyme cleaves the other junctions (NS4A/4B, NS4B/5A and NS5A/5B) in a *trans*-acting manner [136-140]. The amino acid sequences of the NS3 serine proteinase-dependent cleavage sites have also been identified [36, 37]. It has been noted that the Cys residue at the P1 position is conserved in all of the *trans*-cleavage sites. Substitution of this amino acid by others impaired the cleavage [137]. Kinetic study of HCV polyprotein processing revealed the sequential production of NS proteins. Production of NS3 and NS5B was rapid, but an intermediate processing protein (NS4B-NS5A) was observed, suggesting that the cleavage rate between NS4B and NS5A is relatively slow [139]. To date, at least 6 NS proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) have been shown to be produced from the NS region of the HCV genome by NS2-NS3/NS3 proteinases [36, 38-42, 140]. Therefore, processing of the HCV precursor protein yields at least 10 distinct cleavage products, in the following order: NH_2-Core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH.

2) Core protein

The core protein is produced from the N-terminal portion of the precursor protein by host cell signal peptidase(s) [34, 39-41, 142, 143]. As regards the subcellular localization of the core protein, several controversial results have been reported [39, 127, 129]. The results show that the core protein is distributed as a cytoplasmic or nuclear protein, depending on the size of the protein and the genotype analyzed; however, the core protein possesses a putative nuclear localization signal (NLS, amino acid 38-43) [144, 145]. By subcellular fractionation and confocal laser scanning microscopy, it has recently been shown that the core protein resides predominantly in the cytoplasm and is localized on the endoplasmic reticulum (ER) and on lipid droplets; but a portion of the core protein also appeared to be localized in the nucleus [130, 146].

The core protein is rich in basic amino acids and proline residues, and can bind to the 5'UTR of the HCV RNA genome [147, 148]. Further analysis has indicated that the core protein bound most efficiently to the 31-nucleotide-long sequence of the loop IIId domain, and that the secondary structure of which is highly conserved among the various HCV genotypes [148]. In addition, homotypic interaction and multimerization of the core protein, and the interaction between the core protein and E1 envelope protein have been demonstrated *in vitro* and *in vivo* [149, 150]. These findings suggest that the core protein functions as a nucleocapsid in virus particles.

Recently, the HCV core protein has been found to affect a variety of cellular functions described below. As regards the oncogenic potential of the core protein, it has been shown that the core protein cooperates with H-ras and transforms primary rat embryo fibroblasts [151], Rat-1 cells [152], and BALB/3T3 cells [153] into a tumorigenic phenotype. Furthermore, Moriya *et al.* demonstrated that 2 independent lines of transgenic mice showing constitutive expression of the core protein developed hepatic steatosis early in life as a histological feature characteristic of chronic hepatitis C; this resulted in the development of HCC after the age of 16 months [154]. These results suggest that the core protein plays an important role in the regulation of HCV-infected cell growth, in the transformation to a tumorigenic phenotype and in the development of HCC.

As regards the effect of HCV core protein on apoptosis, controversial results have been reported. Ray *et al.* showed that HCV core protein inhibited cisplatin-mediated apoptosis in human cervical epithelial cells [155], tumor necrosis factor (TNF)-α mediated apoptosis in human breast carcinoma cells (MCF-7) [156], and apoptosis induced by the overexpression of c-myc in Chinese hamster ovarian cells [155]. In contrast, 2 different groups reported sensitization to apoptosis by the core protein. Ruggieri *et al.* showed that the core protein made human hepatoma cells (HepG2) prone to Fas-mediated apoptotic death [157]. Zhu *et al.* also showed that the core protein could enhance TNF-induced apoptosis in a mouse cell line (BC10ME) and in 2 human cell lines (HepG2 and HeLa) via TNF signaling pathways; this type of apoptosis is possibly the result of an interaction with the cytoplasmic tail of TNF receptor 1 [158]. The inconsistency of these results may be due to the possibility that the different functions of core protein depend on the kind of cells used or the kind of inducers for apoptosis. Since these results were obtained by using the stable core expressing clones which were selected with antibiotics, Marusawa *et al.* recently carried out a similar experiment using the transient expression system of core protein in HepG2 and MCF-7 cells. The results showed that the core protein inhibited

Fas- and TNF-α mediated apoptosis via NF-kappaB activation [159]. This anti-apoptotic effect introduced by the core protein might be advantageous for HCV by allowing the host hepatocytes to survive apoptosis, which would then result in sustained infection.

An additional functional feature of the core protein has been suggested as well. Several reports using reporter assay systems have shown that the core protein played a role in the transcriptional regulation of cellular and viral genes. The core protein activated the promoters of c-myc, the Rous sarcoma virus long terminal repeat (LTR), simian virus 40 (SV40) early gene [160] and interleukin 2 [161]. In contrast, the core protein suppressed the promoters of HBV (162), human immunodeficiency virus type 1 (HIV-1) LTR [160], c-fos [160], p21^{Waf1} [163], and p53 [164]. In addition, several groups recently discovered that the core protein activated NF-kappaB, AP-1, and SRE-associated signal pathways [157, 164-167]. However, opposing effects of the core protein have also been reported. For example, Shrivastava *et al.* observed that the core protein suppressed the NF-kappaB signal pathway [168], and 2 independent groups demonstrated that the core protein activated p53 function [169, 170]. In these studies, there are 2 problems that remain to be resolved. First, the core proteins used in these studies possess sequence variations of several amino acids. Second, a variety of non-human cells or non-hepatocyte cells have been used in these studies. To elucidate the primary function of the core protein, we recently obtained the core protein having the consensus amino acid sequence from HCV-replicating human non-neoplastic PH5CH hepatocytes (described later), and we examined whether this core protein affects signal transduction pathways (NF-kappaB, AP-1, CRE, SRE, and p53) in PH5CH cells. The results revealed that no signal transduction pathways were significantly affected by the core protein in the cells [171]. However, during the course of this study, we found that the core protein activated the promoters of both exogenous and endogenous IFN-inducible 2'-5' oligoadenylate synthetase (2'-5' OAS) genes, and that this activation by the core protein was mediated through the IFN-stimulated response element [171]. This result suggests that the core protein can activate the 2'-5' OAS/RNase L pathway, thereby decreasing the viral RNA dose.

As an alternative approach to clarify the functions of the core protein, core protein-binding cellular proteins have been screened using a yeast two-hybrid system. To date, lymphotoxin-β receptor [172, 173], TNF receptor 1 [158], heterogeneous nuclear ribonucleoprotein K [174], and RNA helicase [175] were obtained as putative candidates. In addition, it has been demonstrated that the core protein formed a complex with p21^{Waf1} cell cycle regulator [176]. However, there is still no direct evidence that the physical interactions between these candidates with the core protein affect the important functions of the cells.

Comparative sequence analysis of HCV genomes derived from non-cancerous and cancerous HCC lesions revealed that deletions and mutations in the core-encoding region occurred more frequently in cancerous lesions [177, 178], although the biological significance of these findings is still not clear.

3) Envelope proteins

The HCV E1 and E2 envelope proteins are encoded downstream of the core protein-encoding region, and are heavily glycosylated proteins of about 35 kDa and 70 kDa, respectively [34, 179-182]. N-linked glycosylation sites (5 or 6 in the E1 protein and 9 or 10 in the E2 protein) are well conserved among HCV strains, and E1 and E2 proteins are glycosylated with high-mannose type and complex-type oligosaccharides containing sialic acid [34, 183]. The N-terminus of the E1 and E2 proteins are amino acid positions 192 and 384, respectively, of the precursor protein, and amino acid positions 746 and 809 were identified as the 2 C-termini of the E2 protein, namely, E2 (E2-A) and E2-p7 (E2-B) [42, 43, 125]. These results revealed the production of p7 from the envelope proteins-encoding region. However, it remains unclear whether or not p7 is a component of the virus particle.

E1 and E2 proteins form a non-covalently linked heterodimer, which probably represents the native prebudding complex [184], although these proteins form a disulfide-linked heterodimer representing misfolded aggregates [185-187]. Non-covalently associated E1-E2 complexes localize predominantly to the endoplasmic reticular network and at least 2 signals (transmembrane domains of E1 and E2 proteins) are involved in ER retention of the HCV glycoprotein complex [185, 188-190].

CD81 or low density lipoprotein receptor (LDLR) was recently identified as a putative HCV receptor [191-194]. Recent characterization of these candidates for HCV receptor indicates that CD81 binds to the E2 protein, but that CD81 is not involved in cell fusion

caused by HCV [195]; in contrast, HCV particles utilize LDLR for binding and entry into the cells [196]. However, since the expression of both CD81 and LDLR is not restricted in hepatocytes, both proteins may not be general HCV receptors or an additional, second receptor may be present for cell specificity.

It has been shown that E2 protein activates the promoters of 2 intraluminal chaperone proteins, GRP78 and GRP94; the E2 protein is tightly bound to GRP78 [197]. Since overexpression of GRP78, which is a sensor of stress in the ER, has been shown to decrease the sensitivity of cells to apoptosis by cytotoxic T lymphocytes (CTL), this activity of E2 protein may be involved in the pathogenesis of HCV-induced diseases.

In addition, the E2 protein was shown to interact with the IFN-induced double-stranded RNA-activated protein kinase (PKR). The PKR-eIF2 alpha (translation initiation factor) phosphorylation homology domain (12 amino acids) in the E2 protein appeared to be involved in this interaction [198]. This domain was essential for blocking the virus replication inhibitory ability of PKR, suggesting that the interaction of E2 and PKR may be related to viral resistance to IFN [198, 199]. However, a recent report by Cochrane *et al.* showed that there was no evidence that IFN therapy exerts selection pressure in this domain [200].

4) Nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B)

The latter half of the HCV genome encodes several NS proteins that may not be components of the virus particle. As described in the previous section, 2 distinct proteinases (NS2-NS3 zinc-dependent metalloproteinase and NS3 serine proteinase) are encoded downstream of the envelope protein-encoding region. Although NS2 protein (217 amino acid residues for HCV-1b) is rich in hydrophobic amino acids and is produced by self-cleavage between NS2 and NS3 with NS2-NS3 proteinase, the biological function of NS2 protein itself has not yet been clarified. In addition to serine proteinase activity, which is located in the one-third of the NS3 protein at the N-terminal end, helicase and nucleoside triphosphatase activities were identified in the C-terminal half of the NS3 protein [201-206]. These results indicate that the NS3 protein (631 amino acid residues for HCV-1b) is a multi-functional viral protein. Furthermore, to date, NS3 protein has been found to affect several cellular functions. In 1995, it was reported that NS3 protein transforms NIH3T3 cells [207], and then suppression of actinomycin D-induced apoptosis [208] and inhibition of cAMP-dependent protein kinase [209-211] by the NS3 protein have been reported. In addition, wild-type p53-mediated nuclear accumulation of NS3 protein [212, 213] and complex formation of wild-type p53 with NS3 protein [214] have also been demonstrated. However, the mechanisms and biological significance of these phenomena are still not clear.

NS4A, which is a small protein of 54 amino acid residues, forms a stable complex with the NS3 protein as an essential cofactor of the NS3 proteinase and this complex is required for the efficient processing of NS proteins [123, 124, 215-220]. Stable NS3-NS4A complex formation requires the 22 amino acid residues at the N-terminal of the NS3 protein, suggesting that the interaction between NS3 and NS4A is primarily important for the NS4A-dependent processing of NS proteins [221, 222].

NS4B protein (261 amino acid residues for HCV-1b) is rich in hydrophobic amino acid residues and has been detected primarily in the membrane fraction [36, 39]. The function of the NS4B protein remains unknown, although it has been recently demonstrated that the NS4B protein in association with the Ha-ras gene played an important role in the malignant transformation of NIH3T3 cells [223].

NS5A protein (447 amino acid residues for HCV-1b) is phosphorylated at its serine residues for basal phosphorylation in the C-terminal region. In an NS4A protein-dependent manner, NS5A protein is hyperphosphorylated in serine residues in the central region [217, 224-226]. NS4A protein also associates with and directs hyperphosphorylation of the NS5A protein [227]. Recently, 2 groups [228, 229] demonstrated that hyperphosphorylation occurs when NS5A protein is expressed as part of a continuous NS3-NS5A polyprotein. This finding suggests the formation of a highly ordered NS3-NS5A multisubunit complex responsible for the differential phosphorylation of the NS5A protein. Although the role of NS5A in the HCV life cycle is not yet known, an interesting genetic feature has been noted, namely, a region (amino acid 2,209 to 2,248) of NS5A is associated with sensitivity to IFN. This genetic feature was discovered by comparing pairs of IFN-resistant and IFN-sensitive Japanese HCV-1b isolates [230]. Furthermore, it has been demonstrated that this region (40 amino acids), designated as the IFN-sensitivity determining region (ISDR), is useful for predicting the response to IFN

[231]. However, no studies in Europe or in the United States have supported this finding [232-235]. This discrepancy suggests that there is a genetic difference between HCV isolates in Japan, Europe, and the United States [236]. On the other hand, it has been observed that NS5A protein repressed PKR through a direct interaction with the protein catalytic domain [237], and that NS5A protein disrupted PKR dimerization *in vivo*, which resulted in the inhibition of PKR-mediated eIF-2 alpha phosphorylation [238]. However, it remains unclear whether or not NS5A protein is involved in the resistance of infected cells to the antiviral effects of IFN, because thus far, only controversial results have been reported [239-241].

Three independent groups have demonstrated that NS5A protein functions as a potent transcriptional activator when 129-146 amino acid residues at the N-terminal of the NS5A protein are deleted [242-244]. In contrast, it has also been shown that NS5A protein physically associates with p53 and suppresses the transcription of the cell cycle regulatory gene $p21^{Waf1}$ in a p53-dependent manner [246]. Furthermore, NS5A protein induced anchorage-independent growth in NIH3T3 cells and tumor formation in nude mice [245]. These results suggest that NS5A protein also possesses oncogenic potential. In addition, a functional NLS was recently found near the N-terminus of the NS5A protein (amino acids 27-38), although this NLS is usually masked, and the N- (amino acids 1-21) or C-terminal region (amino acids 353-447) were responsible for targeting NS5A protein to the cytoplasm [247]. This NLS may be involved in functioning as a transcriptional regulator of the NS5A protein.

On the other hand, several NS5A-binding cellular proteins, growth factor receptor-bound protein 2 adaptor protein [248], transcription factor SRCAP [249], karyopherin β3 [250], and hVAP-33 (a vesicle-associated membrane protein-associated protein of 33kDa) [251] have been identified using a yeast two-hybrid screening system. Further analysis will be necessary for clarifying the biological significance of the interaction between NS5A protein and these other previously identified proteins.

NS5B protein (591 amino acid residues for HCV-1b) was identified as the RNA-dependent RNA polymerase (RdRp). Recombinant NS5B protein expressed in insect cells using a baculovirus vector [252, 253] or in *E. coli* [254-257] showed RdRp activity *in vitro*. By using a mutational analysis, 4 amino acid sequence motifs crucial for RdRp activity were identified. Most substitutions of conserved residues with these motifs severely reduced enzymatic activity [253]. Ribavirin triphosphates had no inhibitory effect on this polymerase activity, suggesting that the therapeutic effect observed among patients is not related to a direct inhibition of the viral polymerase [258]. Interestingly, hVAP-33 has been also shown to interact with NS5B protein, and it has been demonstrated that NS5A binds to the C-terminus of hVAP-33, whereas NS5B protein binds to the N-terminus [251]. Since hVAP-33 is predominantly associated with the ER, the Golgi complex, and the prelysosomal membrane, these results suggest that NS5A protein is a part of the viral RNA replication complex.

The crystal structures of the NS3 proteinase [259, 260], NS3 helicase [261], NS3/4A complex [262], and NS5B RdRp [263, 264] have also been determined.

V. Systems to proliferate HCV

1) Cell culture system

Although our understanding of the molecular biology of HCV has progressed rapidly as described above, most of the data regarding the functions of HCV proteins have been obtained in artificial systems, either *in vitro* or *in vivo*, using established mammalian cell expression systems. Therefore, these data need to be confirmed in HCV replication systems. In the last several years, many trials for the establishment of HCV replication and multiplication systems using cultured mammalian cells have been performed. To date, 3 different approaches have been used to achieve this goal.

First, the susceptibility of various cultured cell lines to HCV infection was examined by inoculation with HCV-positive sera. At the early stage of using this strategy, the human T cell lines MOLT-4Ma [265], HPB-Ma [266], H9 [267], MT-2 [268], human bone marrow-derived lymphoid cell lines CE and TOFE (269, 270), and human B cell line Daudi [271], were found to be susceptible to HCV infection. Among HCV- susceptible lymphocyte cell lines, HCV culture systems using the retrovirus-infected human T cell lines HPB-Ma and MT-2 have been well characterized. An amphotropic murine retrovirus-infected human T cell line, HPB-Ma, was found to be able to support replication of HCV [266]. Cloning of HPB-Ma cells revealed a clonal variation in susceptibility to HCV infection. Using one of the clones,

HPB-Ma clone 10–2, it was found that the *in vitro* infectivity titers correlated with the reported infectivity titers of the inocula in chimpanzees, and that IFN-α and IFN-β were shown to effectively inhibit HCV replication [266]. Using this HCV culture system, HCV was successfully cultured for more than 1 year, although intracellular HCV genome titers were not determined [271]. Characterization of HCV virion produced from HPB-Ma cells revealed that the virion density was around 1.12 g/ml [271]. This value was in the range of those reported for pestiviruses [272]. Furthermore, virus-like particles with a diameter of approximately 50 nm were detected by immunoelectron microscopy in cytoplasmic vesicles of HPB-Ma cells [16]. Our group also found that MT-2, a human T cell leukemia virus type 1 (HTLV-I)-infected cell line, was susceptible to HCV infection [268]. By the cloning of MT-2 cells, 5 MT-2 clones in which HCV replicated more persistently than in parental MT-2 cells were obtained [273]. Using a semi-quantitative analysis of HCV RNA by RT-PCR, it was found that the level of viral RNA synthesis in the MT-2C (one of five clones) cells increased after inoculation and reached a maximum level at 4 days post-infection [273]. The 2 different methods determined that negative-stranded HCV RNA, an intermediate in HCV replication, was also detected, with strand specificity, in the infected MT-2C cells [273]. By shifting the temperature after virus inoculation from 37 °C to 32 °C, HCV was successfully cultured for several months [274]. This observation suggests that cells cultured at 32 °C become more susceptible to HCV infection or replication, or that the infectivity or stability of HCV produced by cells cultured at 32 °C is greater than at 37 °C. IFN-α or an antisense oligonucleotide complementary to the region containing the initiator AUG codon of the core-encoding region also produced inhibitory effects on HCV replication in MT-2C cells [273, 275]. Since both HPB-Ma and MT-2 cells are infected with a mouse retrovirus and HTLV-I, respectively, retrovirus infection may be favorable for HCV infection or replication. However, to date, there is no evidence that these retroviruses cause the enhancement of cell susceptibility to HCV infection or HCV replication. In these cell culture systems, both cell-to-cell [276] and cell-free [274] viral transmissions were also demonstrated. Furthermore, we recently determined the entire nucleotide sequence of the HCV genome (HCV-JS, 9,535 nucleotides) obtained from HCV-infected MT-2C cells [104]. Thus, these human lymphocyte cell lines were initially selected and characterized as HCV-susceptible cell lines. However, since hepatocytes are thought to be the major natural target cells for HCV replication, screening of HCV-susceptible cell lines has progressed toward hepatocyte cell lines.

To date, primary chimpanzee [277] and human [278–281] hepatocytes, WRL68 [282], HepG2 [282, 283], non-neoplastic immortalized PH5CH [284], and Huh-7 [283] cells have been reported as HCV-susceptible hepatocyte cells. Among these hepatocyte cells, an HCV culture system using PH5CH clones obtained by our group has been well characterized [107, 284, 285]. The following evidence for HCV replication in PH5CH cells was obtained. (1) intracellular HCV RNA was detected up to 100 days post-infection. (2) HCV RNA disappeared in infected cells treated with IFN-α. (3) A strong selection of HCV variants in HVR1 occurred during the course of culture post-infection. Similar evidence for HCV replication was obtained from the HCV-infected human T-cell lines HPB-Ma clone 10–2 and MT-2C. However, to date, the level of HCV replication in these culture systems appears to be far from satisfactory, because RT-PCR was still required for the detection of HCV RNA in all reports describing HCV replication.

To resolve this difficult problem, several groups have tried to isolate infectious HCV cDNA clones as a second strategy for the establishment of an HCV replication system. Recently, some infectious cDNA clones were obtained by direct intrahepatic injection of chimpanzees with RNA transcripts [286–291]. In these studies, it appeared that repair to the consensus sequence of HCV genome was necessary to obtain infectious cDNA clones. Increase of viral titer and antibodies against HCV were also detected in these chimpanzees, but in some cases, the chimpanzee did not develop hepatitis [288, 290]. Mutation analysis of these infectious cDNA clones in the chimpanzees clarified that HCV-encoded enzymatic activity (NS2-NS3 proteinase, NS3 serine proteinase, NS3 NTPase/helicase and NS5B RdRp), and the poly (U) stretch and the conserved region (98 nucleotides), but not the variable region, of the 3'UTR were essential for replication *in vivo* [292, 293]. However, this approach also turned out to be somewhat problematic for the study of HCV in humans, because infectious HCV cDNA clones have not proliferated at the level of human cell culture.

As a third strategy for the development of a cell

culture propagation system of HCV, HCV subgenomic selectable replicons containing the NS2-NS5B or the NS3-NS5B regions were recently produced using a human hepatoma cell line Huh-7 [294]. To allow for selection of only those cells in which HCV will efficiently replicate, neo gene was introduced downstream of the HCV IRES. A second encephalomyocarditis virus IRES was also used to allow for production of the HCV NS proteins. Upon transfection into the cells, both the NS2-NS5B and the NS3-NS5B replicons were found to replicate in the cells. In this system, replicated HCV RNAs were detectable by Northern blot analysis and the HCV proteins that were produced were detected by immunoprecipitation [294]. Recently, more improved replication systems were established by the identification of multiple independent adaptive mutations that cluster in the NS5A- or NS5B-encoding region [295, 296]. Although this system cannot produce HCV virion itself, it will be useful for future molecular studies of HCV, including the screening of antiviral reagents.

2) Animal model system

As the susceptible animal model to HCV, only the chimpanzee is used for HCV research [for a review, see 297]. HCV can infect and multiply in the chimpanzee and infection leads to acute and chronic hepatitis. In fact, chimpanzees were used as an animal model in many of the HCV studies described above. However, the use of chimpanzees is limited for ethical reasons, as well as because of the scarcity value and high maintenance costs of keeping the animals. The attempts of many researchers, including our group, to establish a small animal model have thus far proven fruitless, although it has been found that the Tupaias, a species shown to be susceptible to infection with HBV, sometimes became infected with HCV and developed viremia with rather low titers [298]. Recently, 2 alternative animal models have been reported for the multiplication of HBV or woodchuck hepatitis virus. Both systems are based on the engraftment of human or woodchuck liver tissue into immunocompromised mice [299, 300]. In particular, in the so-called trimera system [299], viremia was induced by transplantation of *ex vivo* HBV-infected human liver fragments. These methodologies will be useful for the multiplication of HCV. In fact, recently, HCV-trimera mice have also been developed by the same group (Ilan *et al*., personal communication).

VI. Mechanisms of persistent infection

1) Escape from host immune defense

HCV frequently establishes a persistent infection in humans and chimpanzees. When serum ALT is elevated for a duration of longer than 6 months, then ALT levels are used as a marker of chronicity. About 70% of the patients with transfusion-associated HCV is estimated to have chronic hepatitis. Furthermore, when the presence of serum HCV RNA is used to detect persistent infection, the rate of persistent infection increases more than 80% [301]. Although the mechanisms underlying persistent HCV infection *in vivo* are still ambiguous, several possible mechanisms have been proposed, as described below.

As the first potential possibility, escape from the host immunosurveillance system is considered. Our group, as well as other groups, has proposed the hypothesis that genetic drift of HVR1 might be involved in escape from neutralizing antibodies [97, 99, 101]. It was clearly shown that shifts in B-cell epitopes, which were found in HVR1, were frequently driven by immunological pressure during the clinical course of hepatitis [106]. Using an experimental chimpanzee model, it has been shown that HVR1 is a critical neutralization domain of HCV [302, 303]. These results suggest that anti-HVR1 antibody functions as a neutralizing antibody, and that frequent mutations in HVR1 are involved in persistent HCV infection. According to this hypothesis, during chronic hepatitis, clearance of HCV by anti-HVR1 antibodies is slower than the rate of escape from the antibodies that is caused by amino acid substitution. Recent sequence analysis of the E1 and E2 envelope genes derived from 12 patients with different clinical outcomes showed data consistent with the hypothesis of selective pressure by the host immune system [304]. As an alternative suggestion, it has been proposed that mutations in immunological T-cell epitopes are involved in escape from cellular immunity [305, 306]. These results also provide stronger evidence in support of the hypothesis that the genetic variability of HCV is driven by immune selection pressure, although contradictory views have also been reported [307, 308].

An additional possible mechanism, namely, the low antigenecity of HCV *in vivo*, has been considered. This hypothesis is supported by the observation that HCV-specific CTL is rather inactive in comparison to HBV or HIV-specific CTL [310]. On the other hand, suppres-

sion of the immune response by HCV proteins may also explain this phenomenon. Recently, Large *et al.* examined the influence of specific HCV gene products on the host immune response to vaccinia virus in a murine model, and found that core protein was sufficient for immunosuppression in the generation of HCV-specific CTLs and prolonged viremia [311]. Further analysis revealed that the core protein could inhibit T-cell proliferative responses *in vitro*; such inhibition would be effected by the binding of core protein to a complement receptor gC1qR involved in the early host defense against infection [312]. In addition, Lee *et al.* showed that the production of interleukin 12 and nitric oxide was significantly suppressed in both HCV core-expressing macrophage cell lines and in mouse peritoneal macrophages treated with core protein [313]. These results suggest that the core protein could play a role in suppressing the induction of Th1 immunity via the inhibition of interleukin 12 and nitric oxide production. The possibility of suppression of HCV proliferation by viral proteins has also been considered, because the amount of HCV *in vivo* is generally somewhat lower than that of HBV. As described in the previous section, we recently found that core protein could activate the 2'-5' OAS/RNase L pathway and probably thereby decreases the dose of HCV in infected cells [170]. Therefore, core protein is probably involved in the maintenance of a low steady state of HCV *in vivo*, enabling HCV to escape from the antigen presentation in cases of host immunity. An additional possibility would be the limited expression of HCV proteins; however, no evidence for this hypothesis has thus far been reported.

2) Extrahepatic infection

Another factor that facilitates persistent infection is extrahepatic HCV infection. Several epidemiological groups have indicated an association between HCV and certain lymphoproliferative diseases such as cryoglobulinemia and B-cell non-Hodgkin's lymphoma [314-318]. These epidemiological findings suggest that HCV can infect and replicate in both hepatocytes and lymphocytes. In order to provide supportive data for this suggestion, we demonstrated *in vitro* replication of HCV in cultured human lymphocytes and hepatocytes, namely, in MT-2 and PH5CH cells [268, 284]. Another group also showed high susceptibility to HCV infection of human T and B cells *i.e.*, in HPB-Ma and Daudi cells [266, 271]. Using clinical specimens, it was shown that peripheral blood mono nuclear cells (PBMC) and lymph nodes were also natural targets for HCV infection and replication, because negative-stranded HCV RNA, an intermediate form in HCV replication, was also found in some of these lymphoid tissues [319-322]. Furthermore, HCV infection of extrahepatic organs (ovary, uterus, heart, *etc.*) other than lymphoid tissue has also been reported [320, 323, 324]. To further characterize HCV infection in PBMC, Crovatto *et al.* fractionated B cells, T cells, monocytes, and polymorphonuclear leukocytes (PML). They examined the presence of positive and negative-stranded HCV RNAs in these cell populations. The results showed that B cells and PML were replication sites of HCV, because both stranded-HCV RNAs were frequently detected in these cell populations [325]. From these results, HCV infection of human lymphoid cells may cause a decline in the immunofunction of these lymphocytes. Actually, it has been recently demonstrated that peripheral blood-dendritic cells (PB-DC) are also targets for HCV infection and replication (Goutagny *et al.*, personal communication), and that the allostimulatory capacity of PB-DC from HCV-infected individuals was impaired [326]. Together with these results, HCV infection of extrahepatic tissues, particularly of lymphoid tissue, could help establish a persistent infection with HCV. To clarify this point, further immunological characterization of HCV-infected lymphoid cells such as B cells, PML, and PB-DC is necessary. Furthermore, co-operation of several host and viral factors may be involved in the establishment of persistent HCV infections.

VII. Mechanisms of viral hepatocarcinogenesis

HCV replication and viral protein expression have been observed in HCCs, but the molecular mechanism of HCV-associated hepatocarcinogenesis is poorly understood. In general, immortalization is an initial step in malignant cell transformation and a number of genetic alterations are required for the multistep development of a liver tumor. Disregulation of cell proliferation by HCV multiplication is probably essential for the immortalization of an HCV-infected cell population. In HCV-related hepatocarcinogenesis, it has been speculated that repeated hepatocytic regeneration processes also occur in HCV-infected individuals, in order to maintain sufficient liver function previously damaged by the multiplication of HCV; this process of damage and regeneration is probably important for increasing the chances of ensuing

genetic alteration [327]. Recently, Tornillo *et al.* examined the genetic differences between HBV- and HCV-induced HCC, but no significant difference was found in the number and type of chromosomal imbalances between HBV- and HCV-infected tumors [328]. This finding is consistent with models suggesting that HBV and HCV cause cancer through nonspecific inflammatory and regenerative processes. On the other hand, several other views have also been proposed. Although 90% of patients with HCC have cirrhosis [329], some patients with chronic HCV infection without cirrhosis have developed HCC [330]. The clinical data suggest that HCV may be directly involved in hepatocarcinogenesis, with viral proteins involved in regulating hepatocyte proliferation. The HCV core protein is a likely oncogenic candidate, because it may co-operate with H-ras in the process of transforming the cells into malignant phenotypes [151-153]. HCC developed in transgenic mice demonstrated constitutive expression of HCV core protein [154]. Furthermore, core protein was revealed to have anti-apoptotic effects, although these effects depend on the kind of cells used, or the substances used for inducing apoptosis. Therefore, we may speculate that HCV core protein helps to establish persistent infection and that it contributes to the pathogenesis of HCC by inhibiting apoptosis, which is an important defensive mechanism of hepatocytes against malignant transformation. In addition, the HCV core protein could act as the transcriptional regulator of several cellular genes, including p53, and several signal pathways, including NF-kappaB. This function of core protein also may prove advantageous for cellular transformation, although the biological significance of each affected gene or signal pathway for transformation has not yet been identified. In contrast to such speculation, it has also been reported that transgenic mice expressing core protein showed no histologic evidence of HCC, suggesting that the core protein may not be cytopathic for hepatocytes [331, 332]. Furthermore, it has recently been reported that other HCV proteins (NS3, NS4B, and NS5A) also have oncogenic potential [207, 223, 245]. Thus, in order to clarify the molecular mechanism of the development of HCC as caused by persistent infection with HCV, comprehensive functional analyses of virus proteins involved in the process of cellular transformation will be important.

One of the factors that may be involved in hepatocarcinogenesis may be co-infection with HCV and other human viruses. Recently, Sugawara *et al.* found that 30-40% of HCV-positive patients with HCC had a high Epstein-Barr virus (EBV) load [333, 334], and they demonstrated that an enhancement of HCV replication was mediated by EBV-encoded nuclear antigen 1 [335]. These findings suggest that EBV acts as a helper virus for HCV replication, and plays some role in the development of HCV-related HCC. HBV is also frequently found in liver biopsy samples from HCV-positive patients with chronic hepatitis [336] and HCC [337, 338]. These findings suggest that HBV also may play some role in the development of HCC in patients with chronic hepatitis C, although there is still no direct evidence supporting an essential role of HBV in HCV replication. In addition, hepatitis G virus or HIV, whose co-infection with HCV is observed in some populations, may affect HCV-related hepatocarcinogenesis, although direct evidence of the co-operation of both viruses with HCV has not yet been reported.

VIII. Strategies for chronic hepatitis therapy

In 1986 before the discovery of HCV, Hoofnagle *et al.* reported that IFN-α was effective for the treatment of chronic non-A, non-B hepatitis [339]. After the development of a method of diagnosing HCV infection, it has become apparent that HCV RNA in the blood of patients with chronic hepatitis C became undetectable following treatment with IFN-α [340]. Therefore, antiviral therapy using IFN-α or IFN-β has been of great clinical interest and has been used in many patients with hepatitis C as an anti-HCV reagent. However, it should be noted that the effectiveness of IFN is limited to 20-30% of cases [341, 342]. More promising studies [343-345] have shown that a combination therapy with IFN-α and rivavirin induces a sustained virological response in about 40% of patients with chronic hepatitis C. The sustained response rates were mainly dependent on the viral genotype (about 60% in genotype 2 or 3 and 30% in genotype 1). In these studies, it has also been observed that patients with higher initial HCV RNA levels (more than 2×10^6 copies/ml) had a lower response rate than those with lower initial HCV RNA levels, independent of genotype. As a novel version of IFN therapy, an IFN-α-2a (peginterferon α-2a) was modified by the attachment of a 40-kd branched-chain polyethylene glycol moiety and this substance has recently been used to improve the pharmacodynamics and hence the efficacy of IFN [346, 347].

As a new candidate anti-HCV reagent, our group recently suggested bovine and human lactoferrins (LF), milk glycoproteins belonging to the iron transporter family; these glycoproteins prevented HCV infection in normally susceptible human hepatocyte PH5CH8 cells [348]. Further characterization revealed that the HCV-inhibiting activity of LF was due to a direct interaction between LF and HCV; furthermore, this phenomenon was observed regardless of the HCV strain or genotype used [348-350]. Since LF is a natural milk protein and has the major clinical advantage of a low risk of severe side effects, we assumed that LF would be a good candidate as an anti-HCV reagent. We tested the hypothesis that LF inhibits HCV viremia in patients with chronic hepatitis C. Eleven Japanese patients with chronic hepatitis C received an 8-week course of bovine LF (1.8 or 3.6 g/day *per os*). At the end of LF treatment, serum ALT and HCV RNA concentrations were decreased in 3 of 4 patients with low pretreatment serum concentrations (less than 100 kcopy/ml) of HCV RNA [351]. However, 7 patients with high pretreatment serum concentrations showed no significant changes in these indices. It has been reported that rats fed a 2% bovine LF diet displayed no significant side effects [352]. Thus, the findings in our pilot study may encourage the clinical use of LF in patients with chronic hepatitis C. Furthermore, combination therapy using both IFN and LF may potentially provide more effective treatment of HCV infection, because IFN and LF exhibit different kinds of antiviral activity, in the intra- and extracellular spaces, respectively.

Development of an effective vaccine against this virus is also of great importance. However, the following problems render this challenge difficult. First, as described above, HCV exists as a quasispecies because of high frequent mutations in HVR1, which is a major B-cell epitope in the virus envelope protein and because it contains a principal neutralizing epitope. Second, immunoresponses to the envelope proteins develop slowly and do not reach high titer levels [353]. Third, there is no convenient infectious tissue culture system for the assay of neutralizing antibodies or for the passage of attenuated HCV strains. Initial experiments using chimpanzees have demonstrated that presenting a reinfectious challenge to recovered chimpanzees via a homologous or heterologous HCV strain resulted in re-infection, suggesting a lack of protective immunity after natural infection [354, 355]. Although such problems hinder progress, several approaches have been used to develop an HCV vaccine. As the first effort, a recombinant envelope (E1/E2) protein was used as a subunit-based vaccine. This vaccine could induce a sterilizing immunity against a homologous viral challenge, but not against a heterologous viral challenge [356]. However, Weiner *et al.* recently succeed at inducing protective immunity against heterologous viral strains in chimpanzees by intrahepatic genetic inoculation with *in vitro* transcribed HCV RNA [Weiner *et al.*, personal communication]. An alternative approach that has been suggested/tested relies on the synthesis and production of HCV-like particles [357]. Since the envelope proteins of HCV-like particles are presented in their native conformation, virus-like particles may therefore be superior in eliciting a protective immune response. In addition, a CTL response also may be elicited by virus-like particles. In spite of the complexity of immune responses against HCV infection and the lack of a convenient model system to proliferate HCV, development of an effective HCV vaccine is still required to prevent the spread of HCV infection worldwide and to control hepatic diseases due to HCV infection.

IX. Perspective

In this review, I have summarized the present state of basic HCV research. Recent extensive study has provided much information about HCV at the molecular level and has suggested excellent preventive methods against HCV infection, as well as novel diagnostic techniques. However, we have not yet obtained an efficient and convenient cell culture or animal model system for viral proliferation, which is the most important experimental means for molecular virology. Several current trials were introduced in this review. The lack of a system that can propagate HCV obstructs the clarification of mechanisms of viral replication and persistence; the development of antiviral reagents and vaccine is also hindered by our inability to induce proliferation *in vitro*. The clarification of the mechanism of hepatocarcinogenesis remains remote. However, 2 recent promising experimental results have been announced. First, Bartenschlager's group has successfully constructed a replicon system using a full-length HCV genome. They observed a tight linkage of replicon RNA translation and replication with host cell growth, suggesting that certain cellular factors that are important for HCV replication and/or translation fluctuate during the cell cycle [358, Pietschmann *et al.*,

personal communication]. Second, Lai's group has succeeded in establishing a B-cell line derived from the spleen tissues of an HCV patient with cryoglobulinemia and monocytoid B-cell lymphoma. Positive and negative-stranded HCV RNAs and viral structural and NS proteins have continuously been detected by Northern and Western blot analyses, respectively, in this cell line for at least 10 months in culture. The quasispecies of HCV RNA in the cells appears to be identical to that in the spleen but is different from that observed in serum, indicating an efficient extrahepatic replication of HCV [Sung *et al.*, personal communication]. Thus, these data show that both replicon-bearing cells and HCV-proliferating B-cells are closely associated with persistent infection. These systems will become valuable means to study various aspects of the HCV life cycle. Furthermore, these results are potentially very useful for future HCV research, including the clarification of the mechanisms of hepatocarcinogenesis and the development of effective new anti-HCV reagents. Recently developed DNA microarray technology will be also useful for understanding the status of viral persistence and disease progression. This technique may be a potential tool for identifying new therapies. Emerging new therapies for patients with chronic hepatitis C will include inhibitors of viral enzymes (proteinase, helicase, and polymerase), cytokines (*ex.* IL-10 and IL-12), antisense oligonucleotides, and ribozymes. In addition, a new LF-derived moleclule designed at the molecular level may be also produced as an effective antiviral reagent with no side effects. The first powerful candidate will be available in the next several years.

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