CMLS, Cell. Mol. Life Sci. 58 (2001) 1276–1295
1420-682X/01/091276-20 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Hepatitis C viral RNA: challenges and promises

S. T. Shi[b] and M. M. C. Lai[a, b, *]

[a] Howard Hughes Medical Institute, and
[b] Keck School of Medicine, Department of Molecular Microbiology and Immunology, University of Southern California, 2011 Zonal Avenue, Los Angeles, California 90033 (USA), Fax +1 323 342 9555,
e-mail: michlai@hsc.usc.edu

Received 15 January 2001; received after revision 2 March 2001; accepted 18 April 2001

Abstract. Hepatitis C virus (HCV), a positive-sense, single-stranded RNA virus of the Flaviviridae family, is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide. Its RNA is difficult to study because biological materials are scarce and RNA replication is of low efficiency. This review focuses on the structure and functions of HCV RNA along with their biological and clinical significance. Despite the challenging characteristics of HCV, significant progress has been made in understanding the properties of HCV RNA and developing viral replication systems toward the improvement of antiviral therapies.

Key words. HCV; RNA; structure; heterogeneity; replication; translation; detection.

Introduction

Hepatitis C virus (HCV) has emerged in recent years as the leading cause worldwide of blood-transmitted chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1]. The infection becomes persistent in about 85% of infected individuals, despite the presence of a strong humoral and cellular immune response [2]. Currently, about 1–2% of the world population are chronic carriers of HCV, which represents an important public health problem. The mechanism whereby HCV establishes persistent infection is still largely unknown. Viral factors such as the extreme genetic plasticity of the viral genome and the defense-evasive properties of the viral proteins have been suggested to contribute to these characteristics. Interferon (IFN)-α, alone or in combination with a nucleoside analogue, ribavirin, is the only therapy available against HCV. However, most HCV patients, especially those infected with viral genotypes 1 and 4, do not respond to these therapies [3, 4].

The HCV genome was initially cloned by immunoscreening of the cDNA derived from the RNA materials of pooled patients' sera without the benefit of virus isolation [5]. This was the first time that a virus was identified by characterization of the genomic sequence prior to understanding the biochemical properties of the virus, a milestone in modern virology. HCV is classified as the sole member of the Hepacivirus genus of the Flaviviridae family, which includes two other genera, Pestivirus and Flavivirus [6]. Although viruses of different genera have different biological properties and do not show serological cross-reactivity, they share significant similarities in virion morphology, genome organization and, probably, replication strategy. Viruses of the Flaviviridae family are enveloped and each contains a single-stranded, positive-sense RNA genome, which has a single large open reading frame (ORF) flanked by an untranslated region (UTR) at each end (fig. 1). This ORF encodes a polyprotein with an N-terminal part composed of viral structural proteins and a C-terminal part of nonstructural (NS) proteins, with one exception that the NS protein N^{pro} of pestivirus is located at the N terminus of the polyprotein. Variable extents of sequence homology have been noted

* Corresponding author.

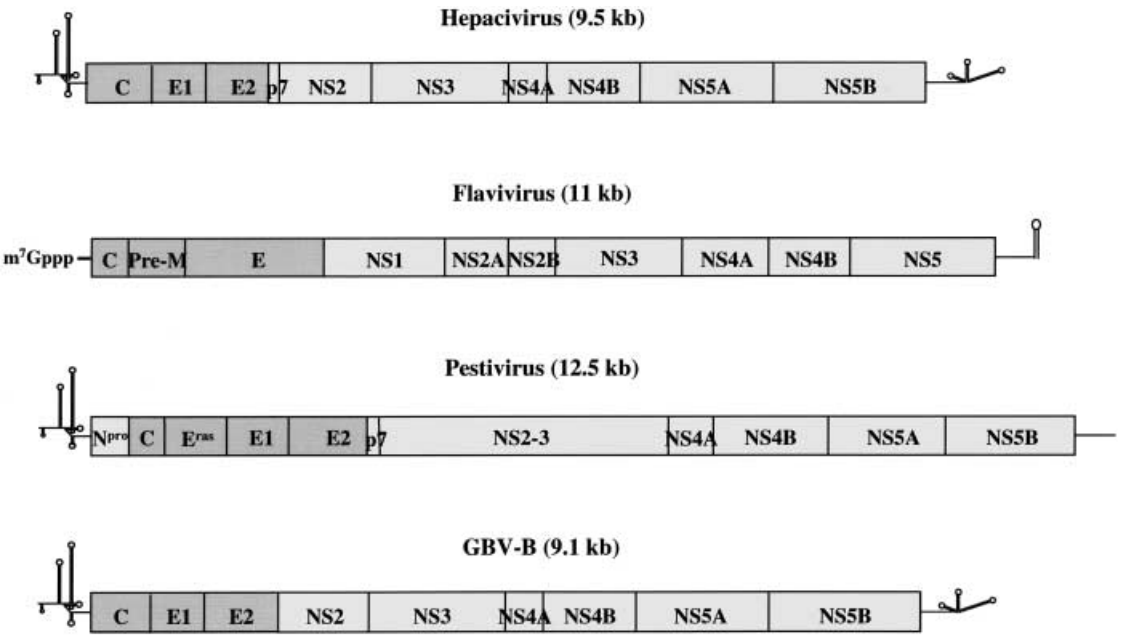

Figure 1. Genome organization of the representative viruses from the Flaviviridae family. Hepaciviral and pestiviral genomic RNAs share similar internal ribosomal entry site structures in the 5′-UTR, whereas the genome of flaviviruses contains a cap structure at the 5′ end of its RNA. The closest relative to HCV is the unclassified GBV-B, sharing extensive sequence and structure homology, particularly in the 5′ and 3′ ends, with the HCV genome. The structural proteins are represented by dark gray-colored boxes. The nonstructural proteins are represented by light gray-colored boxes.

in the colinear regions of the flaviviral genomes [7–9]. Many of the homologous flaviviral proteins are also functionally equivalent. The genome organization, nucleotide (nt) sequence, and polyprotein processing of HCV appear to be most similar to those of pestiviruses and a group of unclassified viruses, GB viruses, particularly GB virus B (GBV-B). The nucleotide sequences within the NS3 and NS5B regions are particularly conserved between HCV and GBV-B [9]. Furthermore, the 5′-UTRs of HCV, GBV-B [9], and pestiviruses, such as bovine viral diarrhea virus (BVDV) and classical swine fever virus, share extensive homology in primary sequence and secondary structure [10–13], signifying GBV-B and pestiviruses as the closest relatives to HCV [14]. Because they can be grown relatively easily in culture, GBV-B and BVDV have been used as surrogate models for various aspects of HCV study. Furthermore, GBV-B infects and causes hepatitis in a small primate, the tamarin [15], which provides a valuable model to evaluate the pathogenesis, antiviral therapy, and immune clearance of the virus.

Genomic organization of HCV RNA

The HCV genome is about 9.5 kb long, slightly shorter than the genomic RNAs of pestiviruses and flaviviruses. It encodes a large polyprotein of 3010–3033 amino acids (aa) [5, 16], which is cleaved by both host- and virus-specific proteases into at least ten structural and nonstructural proteins. The N-terminal quarter of the polyprotein contains the virion structural proteins, the core protein, and two envelope proteins, E1 and E2, all of which are released from the viral polyprotein by host signal peptidases. The core protein constitutes the viral nucleocapsid and also possesses other activities that can modulate various cellular functions, including potential oncogenic properties [17]. The E1 and E2 proteins are type I transmembrane glycoproteins, which are localized predominantly to the endoplasmic reticulum (ER) in cells. They form a noncovalently linked heterodimer, which is presumed to be the functional subunit of the viral envelope [18–20]. As the virion envelope proteins, E1 and E2 are thought to be responsible for virus binding to target cells. Indeed, some E2-specific antibodies exhibit virus-neutralizing activity [21]. The E2 protein has also been shown to inhibit double-stranded (ds) RNA-activated protein kinase (PKR) [22], a key mediator of the antiviral and antiproliferative effects of IFN-α [23]. The remainder of the polyprotein is processed by viral proteases into the viral NS proteins, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [24], although a structural role has been implicated for p7 [25, 26]. NS2 and NS3 are the viral proteases responsible for the cleavages at different boundaries between the different NS proteins. Cleavage at the NS2/ NS3 junction is accomplished by a zinc-dependent metalloprotease encoded within NS2 and the N terminus of NS3 [27–29]. The remaining cleavages downstream from this site are carried out by a serine protease con-

tained within the N-terminal region of NS3 [30–32]. Besides its protease function, NS3 also contains RNA helicase and RNA-stimulated NTPase activities at its C terminus, which are likely involved in HCV RNA replication [33, 34]. NS4A forms a heterodimeric complex with NS3 and serves as an essential cofactor for efficient proteolytic processing by the NS3 protease [35, 36]. The function of the hydrophobic NS4B has not been defined, but it is required for the hyperphosphorylation of NS5A [37, 38]. A recent study has reported that NS4B is capable of transforming NIH3T3 cells in cooperation with the Ha-ras oncogene [39]. NS5A is a phosphoprotein, which appears to possess multiple functions. In particular, it was the first HCV protein identified to bind and inhibit PKR [40] and may be one of the determinants of the degree of response of HCV patients to IFN-α treatment [41, 42]. The N-terminus-truncated NS5A possesses a transactivation activity [43, 44]. More recently, it has also been implicated in viral RNA replication [45]. NS5B, a membrane-associated RNA-dependent RNA polymerase (RdRp), is the key enzyme responsible for HCV RNA replication.

The HCV genomic RNA has an UTR at each end of the coding region; the 5′-UTR and the extreme end of the 3′-UTR are the most conserved regions of the viral RNA and contain signals for RNA replication and translation (fig. 2). The binding of viral or cellular proteins to these regions may modulate the secondary and/or tertiary structure of the viral RNA to facilitate its recognition by the viral replicase complex and/or the translation machinery. These proteins may recruit additional cellular factors and mediate crosstalks between the 5′-UTR and 3′-UTR of HCV RNA.

5′-UTR

The 5′-UTR of the HCV genome is 341 nt long in most viral isolates. The precise structure of the 5′ end is not yet clear, but it contains a highly structured internal ribosomal entry site (IRES) [11], which can mediate translation of the HCV ORF in the absence of a cap structure [46]. The 5′-UTR sequences generally share more than 90% sequence identity among different HCV genotypes, with some segments nearly identical among different strains [47]. Identical or very similar segments have also been found within the 5′-UTRs of GBV-B and, to a lesser extent, pestiviruses [7, 12]. A combination of computational, phylogenetic, and mutational analyses of the HCV 5′-UTR has identified four major structural domains (fig. 2), most of which are conserved among HCV genotypes, GBV-B, and pestiviruses [11, 13, 48, 49]. Common features include a large stem-loop III and a pseudoknot. The 5′-UTR of HCV and GBV-B also has two smaller stem-loops, stem-loop I near the extreme 5′ end and stem-loop IV containing the translation initiation codon [13]. According to a structure-based classification scheme originally designed for picornaviral IRESs [50], the HCV IRES, together with the IRESs of the closely related pestiviruses and GBV-B, is classified into type 3 of four existing types [51]. The IRESs of types 1, 2, and 4 are represented by poliovirus, encephalomyocarditis virus (EMCV), and GBV-C (also known as HGV), respectively. The picornaviral and flaviviral IRESs are significantly different in a number of aspects, suggesting distinct mechanisms of translation initiation for these two virus families [52]. The HCV IRES has three stem-loops with a pseudoknot at the 3′ border of the major stem-loop (stem-loop III) (fig. 2). The picornaviral type 1 and 2 IRESs contain more stem-loops and are distinct from those in the HCV IRES in size, structure, and distance between each other. In addition, there is a Yn-Xm-AUG motif consisting of a pyrimidine-rich tract (Yn) separated from a downstream AUG codon by a spacer (Xm) at the 3′ border, which is important for IRES function. The type 4 GBV-C IRES also contains structurally and spatially unique stem-loops, with a pseudoknot present in one of the smaller stem-loops [51]. The picornaviral IRESs are more efficient in directing translation than the HCV IRES [53]. In contrast, flaviviruses (e.g., yellow fever virus) have relatively short 5′-UTRs with a cap structure, $m^7GpppN_{1m}pN_2$ [54], and are translated by a cap-dependent process.

The first 40 nts of the HCV RNA genome, including the first stem-loop domain, are not required for translation [55, 56], but are most likely involved in RNA replication [57]. Nevertheless, a dinucleotide sequence at nt 34–35 has been shown to contribute to the differential translation efficiencies between genotype 1a and 1b isolates [58]. The remaining three stem-loop domains, which are essential for the translation of HCV RNA, form the IRES. Domains II and III are relatively more complex than domain IV and contain a number of stems and loops [49, 51]. The base of domain III forms a highly conserved pseudoknot, which is critical for IRES activity [59]. Remarkably, similar pseudoknots with almost identical primary sequences also exist in the pestiviral and GBV-B IRESs [51]. In light of the functions of pseudoknot structures present in other RNAs, the pseudoknot in the HCV IRES may serve as an efficient binding site for ribosomes or other initiation factors. Indeed, Kolupaeva et al. [60] identified the pseudoknot as part of the binding site for the 40S ribosome subunit. Domain IV is composed of a small stem-loop (stem-loop IV) in which the initiator codon AUG is located within the single-stranded loop region [13]. Stem-loop IV is not required for internal entry of ribosomes. In fact, the stability of this stem-loop structure has been negatively correlated with the translation efficiency of the viral RNA [13].

The 5′ boundary of IRES has been mapped to nt 44, which coincides with the 5′ terminus of domain II [49, 55, 56, 61]. However, the precise distal border of the IRES

Figure 2. The structures of the 5′-UTR [52] and 3′-UTR [71, 75] of HCV RNA (represented by the HCV-H strain). The structural diagram of the 5′-UTR was kindly provided by Drs René Rijnbrand and Stanley Lemon. psk, pseudoknot. The start codon in the 5′-UTR (nt 342–344) and the stop codon in the 3′-UTR are shown in bold.

has yet to be determined, due to the controversies regarding the role of the RNA sequence downstream of the start codon AUG (nt 342–344) in HCV translation. Although stem-loop IV of the IRES extends into the coding region to include the first 10 nts (nt 345–354) of the core gene, efficient translation has been observed with reporter genes fused immediately after the start codon [46, 62]. However, more recent studies have reported the requirement for a short sequence (up to 30 nts) in the core-coding region for optimal IRES function [13, 63–65]. So far, nt 354 is generally regarded as the consensus 3′ boundary of the IRES [49], but the sequence immediately downstream of the IRES (up to nt 371) may have a stimulating effect on IRES-directed translation. Interestingly, the core-coding sequences further downstream have been shown to play a negative regulatory role in HCV translation [66, 67]. This will be discussed later in this review.

The HCV IRES is responsible for directing the 40S ribosomal subunit in close contact with the start codon for translation initiation [51, 62]. The precise 40S subunit-binding sites have recently been identified to be the GGG triplet (nt 266–268) of the hexanucleotide (UUGGGU) apical loop of stem-loop IIId and the pseudoknot [60]. Mutagenesis studies also confirmed that the GGG triplet is essential for IRES activity both in vitro and in vivo [68]. As shown by sequence comparison, the hexanucleotide is absolutely conserved across HCV genotypes, whereas the GGG triplet is strongly conserved among the IRES sequences of related flaviviruses and pestiviruses.

3′-UTR

Sequence analysis of the 3′ end of HCV RNA from different genotypes has identified a 3′-UTR of between 200 and 235 nt, which consists of three distinct regions, a variable region, a poly(U/UC) stretch, and a highly conserved 98-nt X region [69–73]. Computer analysis of the 3′-UTR has identified extensive secondary structures, including two possible stem-loop structures present in the variable region with the first stem-loop extending into the 3′ end of the NS5B-coding sequence [71, 74]. The X region forms three stem-loop structures and is highly conserved across all genotypes [71, 75] (fig. 2). Some of the features of the HCV 3′-UTR are conserved among members of the Flaviviridae. None of these viruses have poly(A) tails. The sequences immediately following the ORF tend to be poorly conserved and are often variable in length. This region is typically followed by highly conserved RNA sequences and structures. The pestiviral 3′-UTRs are about 225 nts in length and terminate with a stretch of three to five C residues [76]. Multiple secondary structures at or near the 3′ terminus have been predicted, but are yet to be confirmed. The 3′-UTRs of the members of the Flavivirus genus are more variable in length, but all contain a predicted 3′-terminal secondary structure of about 100 bases [77–81]. The 3′-UTR of the GBV-B consists of a 27-nt sequence downstream of the termination codon of ORF, a subsequent poly(U) stretch, and a 309-nt sequence at the 3′ terminus [82, 83]. The extreme 3′ end of the GBV-B genome can also be arranged into a secondary structure resembling that of the HCV X region [82, 83]. These conserved terminal sequences or structures of viral RNA have been shown in many viruses to play critical roles in replication, translation, stabilization, and/or packaging of the RNA.

Within the 3′-UTR of HCV, the sequence that immediately follows the termination codon is a genotype-specific variable region, which is variable in length (ranging from 27 to 70 nts) and composition among different genotypes. However, it is highly conserved among clones obtained from the same viral genotype [71, 84, 85]. The highly type-specific nature of this region suggests that it may confer HCV-genotype-specific differences in the biological properties of HCV. It may also provide an ideal site for genotyping clinical viral isolates. However, the variable region has not been incorporated into clinical practice, probably because its secondary structure prevents reliable RT-PCR detection. It does not seem to be required for the infectivity of HCV in chimpanzees, as shown by mutagenesis studies of an infectious cDNA clone [86]. Studies of other flaviviruses also reported that the variable sequences between the ORF and the conserved region of the 3′-UTR are not critical for viral replication [87–89]. In contrast, the poly(U/UC) tract and the X region are critical elements for HCV replication in vivo [86, 90].

The poly(U/UC) tract consists of a poly(U) stretch and a C(U)n-repeat region (designated as the transitional region) and varies greatly in length and slightly in sequence among different genotypes [70]. The transitional regions of genotypes 2a, 3a, and 3b have several conserved A residues, which are not present in the same regions of genotypes 1b and 2b [70, 72, 91]. The length of the poly(U/UC) region may influence viral replication since HCV RNA transcripts from an infectious clone with a poly(U/UC) region of 133 nt exhibit a replicative advantage in chimpanzees over those with a poly(U/UC) region of 75 nt [92]. Conceivably, the poly(U/UC)-rich sequence may serve as an attenuator of RNA replication, as shown in an in vitro RNA polymerase reaction, in which HCV RNA polymerase stutters at this region [93]. The poly(U/UC)-rich region is a hot spot in the HCV genome for binding cellular proteins (fig. 3), two of which are the *Drosophila melanogaster* embryonic lethal, abnormal visual system (ELAV)-like RNA-binding protein, HuR, and hnRNP C [94, 95]. Interestingly, HuR and hnRNP C interact with the 3′ ends of both the positive- and negative-strand HCV RNA, suggesting that HuR and hnRNP C may be involved in the replication of the HCV RNA genome. Because it is pyrimidine-rich, the poly(U/UC)-

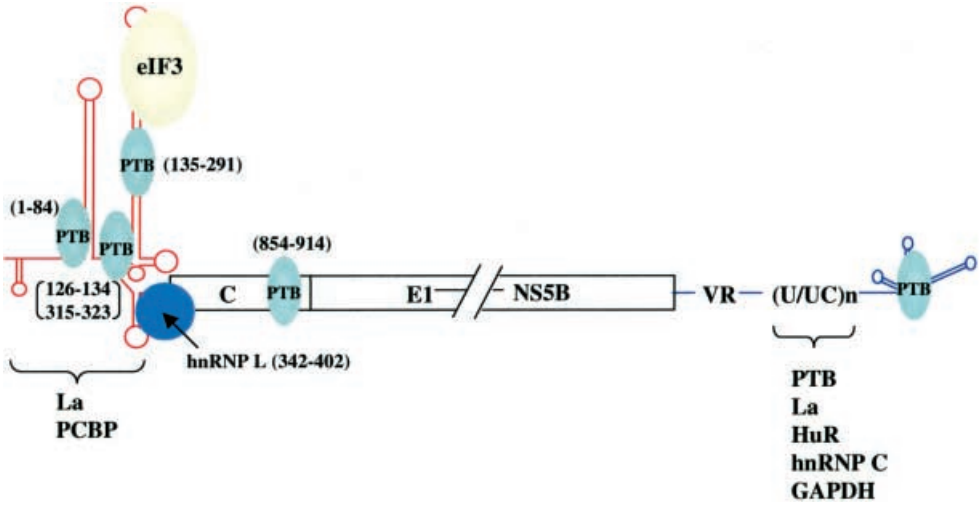

Figure 3. Cellular proteins that interact with HCV RNA. The 5′-UTR interacts with a basal translation factor (eIF3), noncanonical translation factors (PTB and La), and other cellular proteins that may regulate translation (hnRNP L and PCBP). The numbers in parentheses represent the nucleotides sequence in the HCV genome at which the proteins bind. PTB has three distinct binding sites in the 5′-UTR, whereas hnRNP L interacts with a region immediately downstream of the AUG codon. Both La autoantigen and PCBP recognize the entire 5′-UTR. There is a PTB-binding site in the core-coding region, which plays a negative regulatory role in HCV translation. The 3′-UTR is bound by a variety of proteins, all of which interact with the poly(U/UC) region. PTB also binds the conserved X region. These 5′-UTR- and 3′-UTR-binding proteins may affect viral replication (HuR, hnRNP C and GAPDH), translation (PTB), or RNA stability (La). VR, variable region.

rich region, interacts with the polypyrimidine-tract-binding protein (PTB) as expected [94, 96]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) also interacts with the poly(U/UC) tract [97], but the functional relevance of this interaction has yet to be determined. Based on studies of hepatitis A virus (HAV), the binding of GAPDH to the 5′-UTR of HAV may directly influence IRES-dependent translation and/or replication of viral RNA, by destabilizing the folded structure of stem-loop IIIa of the HAV IRES and competing with PTB for binding to this structure [98, 99]. The 3′-UTR also binds La autoantigen, which protects the HCV RNA from rapid degradation [100].

The highly conserved X region has been implicated in both HCV RNA replication and translation. It interacts specifically with the recombinant HCV RNA polymerase [93, 101], although other parts of the 3′ end of the HCV genome may contain additional NS5B-binding sites [101]. The NS5B-binding domain within the X region has been mapped to stem II and the single-stranded region connecting stem-loops I and II [93]. Consistent with the binding results, truncation of 40 nts or more from the 3′ end of the X region abolished its template activity in vitro, suggesting that the X region is essential for HCV RNA replication [93, 102]. The 5′ end of the X region also appears necessary for HCV RNA synthesis, suggesting that integrity of the entire structure is important [102]. In addition to replication, the X region may play a role in HCV RNA translation, since it has been shown to interact with PTB [75, 103] and to enhance HCV IRES-mediated translation [104]. The importance of the X region in HCV replication has been further confirmed by in vivo studies demonstrating that deletion of the X region abolished the infectivity of infectious clones in chimpanzees [86, 90], although the exact function of the X region cannot be deduced from these studies.

As a result of the stem-loop formation in the X region, the HCV genome is predicted to end with a double-stranded stem; its implications in the initiation of RNA replication will be discussed below. Examination of the 3′-terminal sequences of the HCV genome in infectious sera revealed that most RNAs contain identical 3′ ends with no extra sequence downstream of the X tail [70]. However, one particular cDNA clone derived from a patient's serum did contain two additional nucleotides (UU), thus generating a single-stranded tail [72]. Whether or not this structure is present in most native RNA molecules is not clear.

Other structural components of the HCV RNA

In addition to the 5′-UTR and 3′-UTR, RNA secondary structures have been proposed for the core- and NS5B-coding regions [105], which may be important for the translation and replication of the viral RNA, respectively. In addition to the first 14 nts of the core gene being part of the IRES stem-loop IV [51], there are two more stem-loops between nt 47 and 167 of the core-coding sequence (nt 391–511 of the genome), which are conserved among all six HCV genotypes [105]. This region coincides with the nucleotide sequence (nt 408–929) shown by Honda et

al. [58] to be involved in RNA-RNA interactions with the 5′-UTR. The C terminus of the NS5B gene in most genotypes also contains potential stem-loop structures [70, 71, 74, 105]. Consistent with the constraint of the secondary structures, synonymous changes are suppressed in the structured core and NS5B regions and compensatory mutations are frequently observed within the stems [105, 106]. Due to the fact that these structured domains are located near the 5′ and 3′ ends of the genome, they conceivably play a role in viral replication or translation. As suggested by a recent analysis based on a method for detecting conserved structures in a family of related RNA sequences, additional secondary structures may exist in other parts of the HCV genome [107]. Identification and characterization of these conserved structural domains should reveal additional functions of HCV RNA.

Genetic heterogeneity of HCV RNA

Similar to other RNA viruses, one of the most important features of HCV RNA is its high degree of genetic variability, as a result of mutations that occur during viral replication. Due to the absence of an intrinsic repair mechanism associated with RNA-dependent RNA polymerases, the HCV RNA has an estimated mutation rate of $1-2 \times 10^{-3}$ base substitutions per genome site per year [108, 109]. The mutation rate varies significantly in the different regions of the HCV genome, with the lowest in the 5′-UTR and sequences encoding the core, NS3, and NS5B proteins, and the highest in genes encoding the two envelope proteins, E1 and E2. The high rate of mutations likely contributes to the evolution of HCV sequences into genotypes and subtypes as well as the diversification of HCV sequences into quasispecies.

Genotypes and subtypes

HCV variants obtained from different geographical regions around the world show considerable nucleotide sequence heterogeneity. Based on phylogenetic analysis of the HCV NS5B region, an evolutionary tree has been constructed, categorizing HCV isolates into at least six genotypes and more than 50 subtypes [110–115]. The distinction between genotypes and subtypes holds even when other regions of the HCV RNA are used as a basis for classification [114, 116]. The major and minor branches of the phylogenetic tree correspond to genotypes and subtypes, respectively. HCV genotypes differ from each other by more than 30% over the complete viral genome. Within the same genotype, different subtypes may vary by more than 20%, while within each subtype variation is less than 10% [112]. Currently, there are a total of 88 complete sequences available in GenBank. In addition to the nucleotide sequence heterogeneity, the genotypes and subtypes exhibit distinct geographical and epidemiological distribution [112, 114, 115]. Genotypes 1, 2, and, to a lesser extent, 3 are widely distributed around the world and constitute a majority of HCV isolates in many countries.

There are still controversies regarding whether there is a correlation between HCV genotypes and viral pathogenesis. However, in the studies that support such a correlation, HCV genotype 1b has always been associated with more severe liver disease in HCV-infected individuals [114, 117]. In addition, HCV genotypes 1 and 4, in general, exhibit a higher level of resistance to IFN-α therapy than other genotypes [118]. An association between HCV genotype and response to treatment has also been observed with the IFN-α and ribavirin combination therapy [3, 119].

Quasispecies

As a consequence of its high genetic variability, HCV exists in vivo as a population of heterogeneous, albeit closely related, genomes known as quasispecies, which contain a quantitatively predominant ‘master’ genome and a multitude of minor genomes, representing variable proportions of the total population. The quasispecies heterogeneity of the HCV RNA has been observed throughout the genome [84, 85], but the extent of sequence variation is not uniform in different regions of the genome [114]. The genes encoding the E1 and E2 proteins exhibit the highest nucleotide and amino acid sequence variability, particularly in the hypervariable region (HVR)1 at the N terminus of E2 [120, 121]. The HVR1 undergoes frequent nonsynonymous nucleotide substitutions during persistent infection and IFN-α treatment [122–125] and has been used extensively to characterize the quasispecies distribution of HCV in infected individuals.

The quasispecies nature of HCV has significant biological consequences and clinical implications, which may include the appearance of neutralization-escape mutants and possible alterations in cell tropism, viral transmission, and virulence [117, 126]. Most notably, HCV quasispecies heterogeneity, especially within HVR1, has been considered as a potential mechanism for the establishment of viral persistence, mainly by allowing the virus to escape host immune surveillance [127, 128]. Indeed, quasispecies are significantly less pronounced in immune-compromised patients [128]. However, results from other studies do not support immune escape as the cause of persistence of HCV infection [129–132]. Furthermore, long-term follow-up studies of chimpanzees infected with homogeneous viral populations have revealed the development of chronic infections in the absence of amino acids changes within the envelope proteins [131, 132]. A recent study reported the establishment of persis-

tent infection with an infectious clone lacking the HVR1, indicating that HVR1 is not essential for the viability of HCV, the resolution of infection, or the progression to chronicity, although the virus appears to be attenuated [133]. The existence of extensive quasispecies can also strongly influence HCV pathogenesis and the response of patients to IFN-α therapy, but the results are still largely controversial [117, 128, 134]. HCV quasispecies heterogeneity may also be a critical obstacle for the development of a broadly active vaccine for HCV infection [134].

HCV replication

Due to the lack of efficient cell culture or small-animal models for HCV and the generally very low virus titers in clinical samples, the mechanisms of HCV replication and pathogenesis remain largely unknown. Current understanding of the HCV life cycle has been mostly inferred from analysis of its nucleotide sequence and analogies to other RNA viruses closely related to HCV, such as pestiviruses, flaviviruses, and picornaviruses.

Systems for studying RNA replication

HCV infection in cultured cells

Primary hepatocytes from humans and chimpanzees can be infected with serum containing high-titer HCV [135–139]. However, the viral RNA can only be detected by sensitive methods based on RT-PCR, indicating that viral replication is very limited. The production of viral proteins can, at best, be detected by immunofluorescent staining, but not by biochemical methods, such as Western blotting. Furthermore, the viral RNA usually persists for only a short period of time. Persistent infection of a nonneoplastic human hepatocyte line PH5CH by HCV has been observed and shown to be sensitive to IFN-α treatment [140, 141]. Studies of some of these infected cell lines have reported a decrease in HCV quasispecies complexity over time, possibly due to the lack of immune pressure in culture [139, 140]. Clearly, HCV can, at least transiently, infect cultured hepatocytes, but the low efficiency of infection has limited the utility of these cell lines for studying HCV replication.

HCV has also been demonstrated to infect B cell [142] and T cell lines [143–145] in vitro. A large number of reports suggest that peripheral blood mononuclear cells (PBMCs) either infected in culture [146, 147] or isolated from chronically infected patients [148–150] can support HCV replication. Again, the viral RNA could be detected only by RT-PCR. Analysis of the HCV RNA quasispecies revealed the possible evolution and selection of lymphotropic HCV variants during passage of virus in lymphoid cells [142, 151]. The lymphotropic nature of HCV may account for the various immunological disorders associated with HCV infection [152]. For example, type II and type III cryoglobulinemia, which are characterized by the oligoclonal expansion of B cells, have been observed in more than 50% of chronic HCV patients [153].

Because of the low efficiency of viral replication, this approach has, so far, not yielded significant insight into the mechanism of the HCV life cycle.

Infectious clones

The full-length HCV RNA was first cloned from the sera of patients infected with HCV-1a, strain H77 [5, 84, 92]. However, the RNA transcript from this cDNA clone could not replicate when introduced into either chimpanzees or tissue culture. Only after extensive engineering of the recombinant cDNA to arrive at a consensus cDNA clone did the RNA transcript turn out to be infectious when inoculated intrahepatically into chimpanzees [84, 92]. This fact illustrates the heterogeneity of HCV quasispecies and suggests that most of the viral RNA molecules are not infectious, which may explain the relatively low titer of HCV in most clinical samples. Subsequently, the infectious clones of genotypes 1b [154], 2a [91], and a 1a/1b chimera [85] have also been constructed. When inoculated intrahepatically, the RNA transcripts of these cDNA clones caused viremia and HCV-associated hepatitis, although the hepatitis tended to be mild [92]. These infectious clones provided the first tools for molecular genetic analysis of HCV RNA. Using this approach, the poly(U/UC)-rich sequence and the conserved X region, but not the variable region, have been shown to be required for infectivity in chimpanzees [86]. Since the RNA transcribed from each infectious clone consists of a pure population, these clones are particularly useful for the study of immune escape mutants, quasispecies development, and their biological consequences during HCV infection. However, for still unknown reasons, these infectious clones cannot replicate in cultured cells, perhaps because the cells lack certain factors or contain certain inhibitors of HCV replication.

Subgenomic replicons

The development of subgenomic HCV replicons by Lohmann et al. [155] was another breakthrough for the study of HCV replication. The replicon contains a neomycin resistance gene under the control of the HCV IRES and the sequences encoding the HCV NS proteins under the control of the EMCV IRES. When transfected into the human hepatoma cell line Huh-7, the neomycin-resistant cells produce large amounts of viral RNA (approximately 1000–5000 RNA molecules per cell) and proteins, which can readily be detected by Northern and Western blotting, respectively. The negative-strand RNA intermediate can also be detected at a level tenfold less

than the positive-strand RNA, consistent with the reported ratio between positive- and negative-strand RNA detected during natural HCV infection [156]. This approach provides the first recombinant subgenomic HCV RNA capable of replicating in tissue culture. Since the minimum replicon contains only the region encoding NS3 to NS5B, NS2 is apparently not required for HCV RNA replication. The lack of the structural protein gene in this subgenomic replicon prevents the studies of viral particle assembly. This approach also has several puzzling limitations. (i) It is only applicable to a particular HCV genotype 1b isolate. Attempts to use other infectious HCV clones, i.e., HCV-H of genotype 1a, for constructing a subgenomic replicon based on the same approach have so far failed [45]. Thus, this particular HCV clone may have acquired some mutations that allow it to bypass the requirement for cellular factors or to increase its efficiency of replication. (ii) The efficiency of colony formation is extremely low (0.0005% or only one to four clones per microgram of RNA transfected). Nevertheless, each positive clone supports a level of HCV RNA replication that is much higher than that seen in the infected liver. Further analysis of the replicons isolated from the cell colonies has identified adaptive mutations in several different regions of the NS proteins [157]. When engineered back into the replicon, these mutations, particularly that in the NS5B gene, significantly increased the efficiency of colony formation (up to 10%). Another recent study using exactly the same approach obtained G418-resistant HCV RNA clones that contain adaptive mutations clustered in a distinct region of NS5A, which also greatly increased the efficiency of colony formation [45]. However, when some of the mutations were introduced into the HCV-H-derived replicon, no detectable replication was observed in Huh-7 cells, suggesting that different or additional adaptive mutations may be required for RNA replication of other HCV isolates [45]. (iii) These replicons can replicate only in Huh-7 cells, but not in other cell lines. (iv) It is not clear whether the EMCV IRES, which is known to have a higher efficiency of translation than the HCV IRES, contributed to the success of this subgenomic replicon.

Despite such limitations, this approach, together with the recent improvements, promises to be a powerful tool for studying HCV RNA replication, particularly the functional roles of the viral NS proteins. In fact, insights into the possible cytotoxicity of HCV replication, the kinetics of polyprotein processing, and some of the biochemical properties of the NS proteins have recently been gained from the cell lines carrying the self-replicating HCV RNA replicon [158].

Animal models

As the only animal model available for studying HCV, the chimpanzee has provided valuable insights into various aspects of HCV infection. However, its use is limited by animal welfare concerns. Recently, two mouse models have been developed for hepatitis B virus (HBV), which are based on the transplantation of liver tissues into immunocompromised mice [159, 160]. In the first mouse model, woodchuck hepatocytes were transplanted into the liver of mice that contain the urokinase-type plasminogen activator transgene and lack mature B and T lymphocytes [159]. The mice containing woodchuck hepatocytes were susceptible to woodchuck hepatitis virus and developed persistent infection. In the so-called Trimera mouse model, ex vivo HBV-infected human liver fragments were engrafted into lethally irradiated mice radioprotected with SCID mouse bone marrow cells [160]. HBV infection was observed in 85% of the transplanted animals. Both models have been used to evaluate potential anti-HBV therapeutic agents, including IFN-α and lamivudine. A similar approach can also be applied for constructing the HCV-Trimera mouse model. However, the utilization of these models is also limited by inefficient and short-term viral infection.

Overview of the replication cycle of HCV

The HCV replication cycle (fig. 4) starts with the attachment of the virus to target cells through receptor binding, a task thought to be mediated mainly by E2 [21, 127, 161]. The virus then enters by either virus-cell membrane fusion, which may require both E1 and E2, or endocytosis [162]. A number of studies have suggested CD81 or the low-density lipoprotein (LDL) receptor as putative HCV receptors [163–165]. The E2 protein can bind CD81, and this binding is blocked by neutralizing antibody against E2. However, virus-CD81 binding has not been demonstrated to lead to virus entry. On the other hand, the potential role of the LDL receptor (LDLR) as an HCV receptor is consistent with the fact that HCV is complexed with serum lipid and lipoproteins. The LDLR has also been shown to enable HCV to enter cells, although the assay used in these studies was not unequivocal [164]. Furthermore, circumstantial evidence has indicated that only the viruses complexed with very low density lipoprotein (VLDL) or LDL can enter cells containing LDLR [164, 166]. Once the virus is in the cell, the viral positive-sense genomic RNA is released from the virus particle and translated into a polyprotein, which is subsequently processed into a number of functional proteins. Most of the viral NS proteins (NS3–5B) form a membrane-associated replicase complex with cellular proteins to initiate viral RNA replication [167–171]. The genomic RNA serves as a template to synthesize a negative-strand RNA intermediate, which is then copied into a new positive-strand RNA. The positive-strand viral RNA is used for translation and packaged with the viral structural proteins into progeny virions. The virus parti-

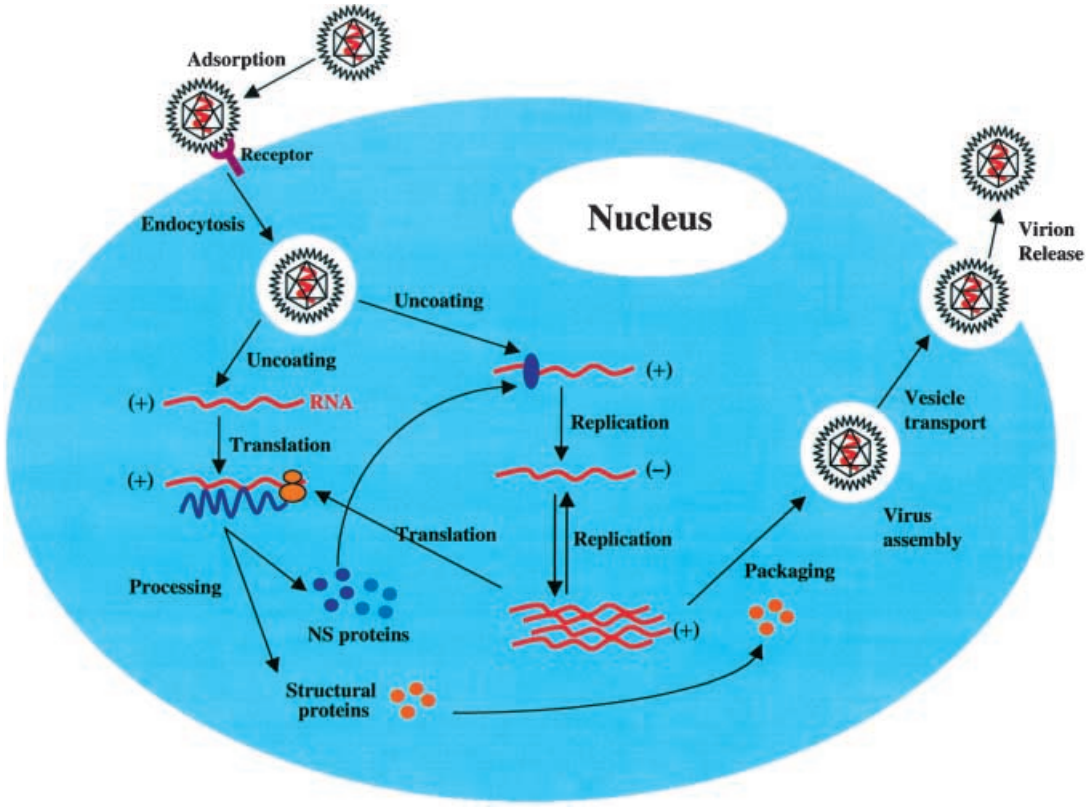

Figure 4. The HCV replication cycle. The HCV particle first binds to the cell surface receptors and enters the cell via endocytosis. The genomic RNA is released from the virus and used for viral protein synthesis. Some or all of the viral NS proteins form the replicase complex (dark blue), which initiates viral RNA replication to synthesize negative-strand RNA. Once synthesized, negative-strand RNAs serve as templates for the synthesis of an excess amount of positive-strand RNAs. These RNA products are used for viral protein synthesis and packaging into progeny virus particles. The progeny viruses are formed in the ER and Golgi compartments, exported to the cell surface, and released via cellular vesicle transport.

cle assembly probably occurs at the ER or Golgi complex. The particles accumulate in the intracellular vesicles and are eventually released from the host cells through secretory pathways.

Replication of HCV RNA

HCV RNA replication is believed to occur in the cytoplasm of virus-infected cells based on the cytoplasmic localization of viral RNA, shown by a majority of in situ hybridization (ISH) studies [156], and viral polymerase, shown by immunofluorescent staining [172, 173]. However, HCV RNA has also been localized to the nucleus by ISH [174, 175], and so has the HCV helicase NS3 [176–178]. Thus, the site of HCV RNA replication needs to be further examined in cell culture systems or animal models that support efficient HCV replication. RNA replication requires at least NS5B, the RdRp, and probably most of the other NS proteins as well.

NS5B

NS5B is a membrane-associated phosphoprotein [173], which contains signature motifs, such as GDD, shared by other viral RdRps [179]. The C-terminal 21 aa of NS5B plays a role in anchoring the protein to the membrane [180]. NS5B also interacts with a SNARE-like cellular membrane protein, human vesicle-associated membrane protein (VAMP)-associated protein of 33 kDa (hVAP-33), which may target the polymerase to specific cellular membrane structures, such as the ER and Golgi, for RNA replication [167]. Although multiple potential phosphorylation sites exist within the NS5B amino acid sequence, no site is conserved among all HCV isolates examined [181], suggesting that phosphorylation of NS5B may vary among different isolates.

The crystal structure of NS5B has recently been determined; it shares significant similarity to other polymerases, but also displays certain striking differences [182–184]. The domain organization in NS5B can be subdivided into the fingers, palm, and thumb, similar to other polymerases. However, other polymerases, such as the poliovirus 3D polymerase, are distinctly U-shaped, while the finger and thumb domains of NS5B exhibit extensive contacts between each other, resulting in a globular-shaped molecule. The encircled active site is relatively inflexible and can accommodate only a template: primer

duplex without global conformational changes. The C terminus of NS5B (excluding the hydrophobic tail) is present in the active site of the protein and has been hypothesized to play a role in the regulation of RdRp activity and template discrimination [184].

So far, the biochemical and enzymatic properties of the HCV RdRp have been studied using recombinant NS5B expressed in *Escherichia coli* or insect cells in in vitro RdRp assays. Most of these assays used homo-polyribonucleotides or artificial RNA as templates [180, 185–190]. In these assays, NS5B usually uses the 3′ end of the template RNA or an artificial oligonucleotide as a primer. However, in more recent studies, NS5B has been shown to initiate de novo RNA synthesis in a primer-independent manner [93, 102, 191–193]. Furthermore, NS5B is able to copy a full-length HCV genome, although it also utilizes many other RNA templates, indicating a lack of template specificity in vitro [102, 187]. In vitro NS5B binds preferentially to several regions in the 3′ end of HCV RNA, including the 3′ coding region of NS5B, the U/UC-rich sequence, and part of the X region (in stem I and II) (fig. 2) [93, 101]. Partial deletion of the 3′-UTR of HCV RNA abolished the template activity of the RNA [101, 102]. Thus, NS5B apparently recognizes some specific sequence or structural elements at the 3′ end of HCV RNA [93, 101]. Once it has bound the stem structure of the 3′-UTR, however, NS5B initiates RNA synthesis only from the single-stranded RNA region closest to the 3′ end of the template [93]. This conclusion is supported by a recent study showing that the RdRp reaction mediated by NS5B requires a stable secondary structure and a single-stranded sequence with at least one 3′-end cytidylate in the RNA template [194].

Since the 3′ end of HCV RNA ends with a near-perfect double-stranded stem (stem I) (fig. 2), how does HCV RNA synthesis initiate in vivo? There are several potential mechanisms whereby the 3′-end sequence of the viral RNA is retained during RNA replication: (i) The 3′ end of HCV RNA may be extended by a terminal transferase so that there is a single-stranded tail at the 3′ end to allow NS5B to initiate from the precise 3′ end. Indeed, an HCV cDNA clone containing two additional nucleotides (UU) at the 3′ end of HCV RNA has been detected [72]. (ii) RNA helicase or unwinding proteins may be present in the HCV replicative complex to unwind the 3′-end stem structure into the single-stranded region. (iii) RNA synthesis may initiate internally in the single-stranded region within the 3′-UTR; the 3′-end sequence may be recovered during positive-strand RNA synthesis since the complementary sequence can be made by fold-back RNA synthesis.

So far, HCV RdRp activity has not been studied in the context of virus-infected cells or using the recombinant RdRp expressed in the mammalian cells. In mammalian cells, NS5B is likely associated with other viral and cellular proteins, and anchored to the cellular membrane, which may alter the enzymatic properties or template specificity of NS5B.

Other viral proteins

The RNA helicase function is presumed to be necessary to separate the positive- and negative-strand HCV RNA during replication. The HCV helicase lies within the C-terminal half of NS3, which has been shown to possess NTPase, single-stranded (ss) polynucleotide binding, and duplex-unwinding activities [33, 34]. It is capable of unwinding dsRNA, dsDNA, and RNA/DNA heteroduplexes in the 3′ to 5′ direction [34]. However, the role of the unwinding activity of NS3 in the replication cycle remains elusive.

The HCV replication complex is most likely membrane-associated [195] and consists of cellular or other viral NS proteins in addition to NS5B and NS3. NS5B is capable of anchoring on the membrane structures by itself or through its binding to hVAP-33 [167]. Interestingly, while NS5B interacts with the N terminus of hVAP-33, NS5A binds the C terminus of hVAP-33. The importance of NS5A in HCV replication has been further suggested by the detection of a number of adaptive mutations clustered in a defined region of NS5A in a subgenomic HCV replicon [45]. This region may mediate the interaction of NS5A with a cellular protein that inhibits HCV replication. Mutations within this region may disrupt the interaction, thereby allowing the establishment of HCV replication in vitro. In addition, most of the HCV NS proteins, including NS3, NS4A, NS4B, NS5A, and NS5B, have been shown to interact with each other either directly or indirectly [167–171]. For example, a protein complex consisting of NS5B, NS3, and NS4A has been observed in mammalian cells [171]. These proteins may have remained associated after cleavage from the polyprotein to form a functional replication complex. Further evidence supporting the existence of a replication complex consisting of multiple HCV NS proteins came from an analysis of the adaptive mutations derived from a subgenomic HCV replicon [157]. An adaptive mutation in NS5B was found incompatible with those in NS5A or NS4B when introduced back into the same replicon. These mutations may affect contact sites between these proteins in the replication complex, dramatically reducing replication efficiency.

Detection of negative-strand RNA

The ultimate proof of HCV RNA replication is the detection of the negative-strand HCV RNA, which is usually present in infected cells in such a low quantity that it can only be detected by RT-PCR. The detection of the negative-strand RNA is further complicated by the large excess of positive-strand RNA [156]. Furthermore, all of the negative-strand RNA is likely present in the double-stranded form by complexing with the positive-strand

RNA in the virus-infected cells. These limitations entail the requirements of very high stringency for strand specificity of RT-PCR, which may have sacrificed the sensitivity of detection. A number of strand-specific RT-PCR protocols have been devised using various primers and polymerases to detect the negative-strand HCV RNA, usually targeting the 5′-UTR. However, this region contains multiple hairpin structures, which often cause self-priming events that lower the specificity of detection of negative-strand RNA molecules. Lanford et al. [136] have developed a 'tagged PCR' technique proven to significantly enhance the sensitivity and reduce self-priming events. The method employs a primer, which contains non-HCV sequences at the 5′ end as a tag, during cDNA synthesis. The subsequent PCR amplification of the tagged cDNA is performed using only the tag portion of the cDNA primer as one of the primers and an HCV-specific primer. This tagged PCR assay permits the detection of 1 fg of negative-strand RNA, which is 10,000-fold lower than the detection limit for the positive-strand RNA. A later study, however, has found that the 5′-UTR-based RT-PCR still cannot completely eliminate mis-priming or artificial priming and recommended the use of the core-coding sequence for the detection of the negative-strand RNA [147]. The size and species of HCV negative-strand RNA are still not clear.

Translation of HCV RNA

The 5′-UTR of HCV RNA contains a typical IRES structure, which allows cap-independent translation. Several translation initiation factors have been reported to be involved in HCV IRES-mediated translation. The eukaryotic initiation factor (eIF)3 (fig. 3), alone or together with the 40S ribosome subunit and the eIF2-GTP-initiator tRNA complex, can specifically interact with HCV IRES stem-loop III in the absence of eIF4A, eIF4B, and eIF4F, which are required for ribosomal binding during cap- or EMCV IRES-dependent translation [60, 196, 197]. Furthermore, eIF2Bγ and eIF2γ have also been identified as cofactors of HCV IRES-mediated translation by a functional genomics approach [198]. These findings suggest that HCV may employ a modified mechanism of IRES-dependent translation. Indeed, recent studies showed that rabbit reticulocyte lysates depleted of certain translation factors, such as eIF4G, cannot support foot-and-mouth disease virus IRES-dependent, but still can support HCV IRES-dependent translation [199].

As a translation initiation site, the HCV IRES also recruits noncanonical cellular translation factors, such as La autoantigen [200] and PTB [201] (fig. 3). The La antigen recognizes the intact HCV IRES structure and significantly augments the IRES-directed translation in vitro [200]. The nucleic acid-dependent ATPase activity of La has been suggested to promote the transformation of stem-loop IV into single-stranded conformation, which is favorable for 40S ribosome binding [51]. PTB interacts with three distinct pyrimidine-rich sequences within the HCV IRES [201]. Immunodepletion of PTB results in the loss of IRES-directed translation, which cannot be restored with the addition of purified PTB, suggesting that additional factors tightly associated with PTB are also required to enhance IRES activity [201]. The functional requirement of PTB in IRES-mediated translation has been further confirmed by the inhibition of translation with PTB-binding RNAs selected with the systematic evolution of ligand by exponential enrichment (SELEX) method [202]. Heterogeneous nuclear ribonucleoprotein (hnRNP) L has been identified to specifically interact with the 3′ border of the HCV IRES in the core-coding sequence; the binding correlates with the translation efficiency from the IRES [203]. In addition, the entire IRES is required for the association of HCV RNA with poly(C)-binding proteins (PCBPs), PCBP-1 and PCBP-2, but the roles of these proteins in translation have not been determined [204].

Due to the absence of a poly(A) tail in HCV RNA, the mechanism of regulation of HCV RNA translation is predicted to differ from those for eukaryotic mRNAs and other viral RNAs. The conserved X region of the 3′-UTR has been shown to enhance translation of HCV RNA [104], suggesting that the functions of the X region may be similar to that of poly(A) in eukaryotic mRNA translation [205, 206]. PTB interacts with the X region [75, 103, 104, 207] and enhance HCV IRES-directed translation [104], possibly through the PTB-mediated cross-talk between the 5′- and 3′-ends of HCV RNA. Thus, the mechanism of translation enhancement by PTB may be similar to that of the poly(A)-binding protein in mRNA translation [205, 206].

HCV IRES-mediated translation is relatively inefficient compared to that of other viruses [53]. The core protein has been found to interact with the viral genomic RNA at several sites within the 5′-UTR and to suppress the translation directed by the HCV IRES [208]. However, a separate study showed that the core-coding sequence, but not the core protein, caused the suppression of HCV IRES-dependent translation [67]. Furthermore, a PTB-binding site has been identified in the core-coding region, which serves as a negative regulator for HCV translation [66]. Direct RNA-RNA interaction, involving the 5′-UTR and the core-coding sequences, or indirect interaction mediated through PTB may be responsible for the reduced IRES activity [58, 66]. The stem-loop IV of the IRES may be one of the candidates for feedback control, since the stabilization of this structure can reduce IRES activity and the primary sequence within this stem-loop is conserved in nearly all HCV strains [13]. Two other HCV proteins, E2 [22] and NS5A [209], may have an indirect effect on HCV translation by inhibiting PKR, but the biological significance of this effect is not clear.

Despite its highly conserved nature, the HCV IRES has been found to contain minor sequence modifications among quasispecies, which result in different IRES activities both in in vitro translation using rabbit reticulocyte lysates [210] and when expressed in cells in a cell type-specific manner [210, 211]. Remarkably, the appearance of new virus variants containing identical substitutions at three sites within the IRES has been observed in cultured lymphoblastoid cells inoculated with HCV-infected serum [142]. These new virus variants confer enhancement of HCV IRES-directed translation in lymphoid cells, suggesting a possible selection of lymphotropic HCV strains via translational regulation [212].

Clinical detection and genotyping of HCV RNA

Detection of HCV RNA is the most definitive diagnostic test for acute and chronic HCV infection. Unfortunately, it is often complicated by the low levels of HCV replication or small numbers of infected cells in hepatitis C patients. Nevertheless, the techniques for detecting and quantifying HCV RNA have improved dramatically over the past decade.

Most qualitative and quantitative diagnostic tests are RT-PCR aiming at the 5′-UTR, the most conserved region of the HCV genome. Primers or probes deduced from these sequences can be utilized for universal detection of HCV RNA. However, the sensitivity of this method is accompanied by problems of false priming, presumably in areas of RNA secondary structure. This has been partially solved by the use of tagged primers or a thermostable reverse transcriptase [136]. Since the core and NS5B genes are relatively conserved and do not contain extensive secondary structures, they have also been employed in various detection methods. The X region of the 3′-UTR is another highly conserved region in the HCV genome, but it is highly structured. It has not been used in the detection of HCV RNA due to its lack of practical advantages over the well-established tests based on the 5′-UTR sequence.

HCV genotyping in patients is essential for diagnostic and epidemiological studies, as well as in studies of the natural history and treatment of HCV infection. Early studies relied on the core, E1, and NS5B regions for genotyping by direct sequencing of RT-PCR products [110, 213], RT-PCR using type-specific primers [214–217], or hybridization of PCR products with type-specific probes [218–221]. The 5′-UTR has also been widely used for HCV genotyping because it contains type-specific sequence differences in an otherwise conserved region (maximum 10% sequence divergence) [112, 222], which is convenient for the initial RNA amplification step. Furthermore, genotyping can be achieved by simplified procedures, such as restriction fragment length polymorphism analysis [223–225] and line probe assay [226]. However, the 5′-UTR cannot be used to differentiate among certain subtypes, especially within genotypes 1 (1a and 1b), 2 (2a and 2c), and 4 (4c and 4d) [47, 213]. Combined methods involving two separate regions of the HCV genome are used for accurate determination of genotypes and subtypes [227, 228]. Genotyping by heteroduplex mobility analysis of the 5′-UTR or NS5B region has recently been developed, which suggests the NS5B region to be more accurate for genotyping [229].

Perspectives

Since the cloning of HCV RNA 11 years ago, there has been an explosion of molecular and clinical studies on HCV RNA. These studies have revealed many important insights into the biological properties of the virus, and will form the basis for future development of more effective therapies against HCV. However, they also present many challenges that are yet to be overcome. Chief among these is the development of robust replication systems for studying HCV replication, since our current understanding of HCV RNA and its replication and expression has, so far, been largely based on predictions from the cloned sequences or inference from other viruses. The improved subgenomic replicon systems [45, 157] are beginning to fulfill this expectation. However, this replicon technology still cannot be applied to studying all HCV RNA sequences. Furthermore, it does not allow the study of the complete viral life cycle. Establishing a cell culture system capable of supporting viral infection and production remains a formidable challenge. The extensive genetic heterogeneity of HCV RNA also presents a substantial challenge for understanding viral pathogenesis and for the development of novel antiviral drugs against HCV. The highly conserved 5′-UTR and 3′-UTR of HCV RNA currently offer the best targets for novel antiviral strategies, such as the use of antisense RNAs or ribozymes. The inhibition of HCV RNA translation has been observed with ribozymes or antisense oligonucleotides that target the 5′-UTR alone or together with the core-coding sequence of HCV [230–234]. However, there are also substantial limitations caused by the inherent structures of these UTR sequences and associated with the ribozyme and antisense technologies [234–236]. With the increasing knowledge of HCV replication and viral pathogenesis, new targets and strategies for anti-HCV drugs will likely emerge, portending the future treatment of chronic HCV infection and its associated diseases.

1 Seeff L. B. (1995) Natural history of viral hepatitis, type C. Semin. Gastrointest. Dis. **6:** 20–27

2 Cerny A. and Chisari F. V. (1999) Pathogenesis of chronic hepatitis C: immunological features of hepatic injury and viral persistence. Hepatology **30:** 595–601

3 McHutchison J. G., Gordon S. C., Schiff E. R., Shiffman M. L., Lee W. M., Rustgi V. K. et al. (1998) Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N. Engl. J. Med. **339:** 1485–1492

4 Poynard T., Marcellin P., Lee S. S., Niederau C., Minuk G. S., Ideo G. et al. (1998) Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet. **352:** 1426–1432

5 Choo Q. L., Kuo G., Weiner A. J., Overby L. R., Bradley D. W. and Houghton M. (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science **244:** 359–362

6 Murphy F. A., Fauquet C. M., Bishop D. H. L., Ghabrial S. A., Jarvis A. W., Martelli G. P. et al. (1995) Classification and Nomenclature of Viruses: Sixth Report of the International Committee on Taxonomy of Viruses, pp. 424–426, Springer, Vienna

7 Miller R. H. and Purcell R. H. (1990) Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. Proc. Natl. Acad. Sci. USA **87:** 2057–2061

8 Choo Q. L., Richman K. H., Han J. H., Berger K., Lee C., Dong C. et al. (1991) Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:** 2451–2455

9 Muerhoff A. S., Leary T. P., Simons J. N., Pilot-Matias T. J., Dawson G. J., Erker J. C. et al. (1995) Genomic organization of GB viruses A and B: two new members of the Flaviviridae associated with GB agent hepatitis. J. Virol. **69:** 5621–5630

10 Han J. H., Shyamala V., Richman K. H., Brauer M. J., Irvine B., Urdea M. S. et al. (1991) Characterization of the terminal regions of hepatitis C viral RNA: identification of conserved sequences in the 5′ untranslated region and poly(A) tails at the 3′ end. Proc. Natl. Acad. Sci. USA **88:** 1711–1715

11 Brown E. A., Zhang H., Ping L. H. and Lemon S. M. (1992) Secondary structure of the 5′ nontranslated regions of hepatitis C virus and pestivirus genomic RNAs. Nucleic Acids Res. **20:** 5041–5045

12 Simons J. N., Pilot-Matias T. J., Leary T. P., Dawson G. J., Desai S. M., Schlauder G. G. et al. (1995) Identification of two flavivirus-like genomes in the GB hepatitis agent. Proc. Natl. Acad. Sci. USA **92:** 3401–3405

13 Honda M., Brown E. A. and Lemon S. M. (1996) Stability of a stem-loop involving the initiator AUG controls the efficiency of internal initiation of translation on hepatitis C virus RNA. RNA **2:** 955–968

14 Ohba K., Mizokami M., Lau J. Y., Orito E., Ikeo K. and Gojobori T. (1996) Evolutionary relationship of hepatitis C, pesti-, flavi-, plantviruses, and newly discovered GB hepatitis agents. FEBS Lett. **378:** 232–234

15 Deinhardt F., Holmes A. W., Capps R. B. and Popper H. (1967) Studies on the transmission of human viral hepatitis to marmoset monkeys. I. Transmission of disease, serial passages, and description of liver lesions. J. Exp. Med. **125:** 673–688

16 Kato N., Hijikata M., Ootsuyama Y., Nakagawa M., Ohkoshi S., Sugimura T. et al. (1990) Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. USA **87:** 9524–9528

17 Lai M. M. C. and Ware C. F. (2000) Hepatitis C virus core protein: possible roles in viral pathogenesis. Curr Top Microbiol Immunol **242:** 117–134

18 Lanford R. E., Notvall L., Chavez D., White R., Frenzel G., Simonsen C. et al. (1993) Analysis of hepatitis C virus capsid, E1, and E2/NS1 proteins expressed in insect cells. Virology **197:** 225–235

19 Ralston R., Thudium K., Berger K., Kuo C., Gervase B., Hall J. et al. (1993) Characterization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia viruses. J. Virol. **67:** 6753–6761

20 Dubuisson J., Hsu H. H., Cheung R. C., Greenberg H. B., Russell D. G. and Rice C. M. (1994) Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. J. Virol. **68:** 6147–6160

21 Rosa D., Campagnoli S., Moretto C., Guenzi E., Cousens L., Chin M. et al. (1996) A quantitative test to estimate neutralizing antibodies to the hepatitis C virus: cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells. Proc. Natl. Acad. Sci. USA **93:** 1759–1763

22 Taylor D. R., Shi S. T., Romano P. R., Barber G. N. and Lai M. M. C. (1999) Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science **285:** 107–110

23 Barber G. N., Wambach M., Thompson S., Jagus R. and Katze M. G. (1995) Mutants of the RNA-dependent protein kinase (PKR) lacking double- stranded RNA binding domain I can act as transdominant inhibitors and induce malignant transformation. Mol. Cell. Biol. **15:** 3138–3146

24 De Francesco R., Neddermann P., Tomei L., Steinkuhler C., Gallinari P. and Folgori A. (2000) Biochemical and immunologic properties of the nonstructural proteins of the hepatitis C virus: implications for development of antiviral agents and vaccines. Semin. Liver Dis. **20:** 69–83

25 Lin C., Lindenbach B. D., Pragai B. M., McCourt D. W. and Rice C. M. (1994) Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini. J. Virol. **68:** 5063–5073

26 Mizushima H., Hijikata M., Asabe S., Hirota M., Kimura K. and Shimotohno K. (1994) Two hepatitis C virus glycoprotein E2 products with different C termini. J. Virol. **68:** 6215–6222

27 Grakoui A., McCourt D. W., Wychowski C., Feinstone S. M. and Rice C. M. (1993) A second hepatitis C virus-encoded proteinase. Proc. Natl. Acad. Sci. USA **90:** 10583–10587

28 Hijikata M., Mizushima H., Akagi T., Mori S., Kakiuchi N., Kato N. et al. (1993) Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J. Virol. **67:** 4665–4675

29 Reed K. E., Grakoui A. and Rice C. M. (1995) Hepatitis C virus-encoded NS2-3 protease: cleavage-site mutagenesis and requirements for bimolecular cleavage. J. Virol. **69:** 4127–4136

30 Bartenschlager R., Ahlborn-Laake L., Mous J. and Jacobsen H. (1994) Kinetic and structural analyses of hepatitis C virus polyprotein processing. J. Virol. **68:** 5045–5055

31 Tanji Y., Hijikata M., Hirowatari Y. and Shimotohno K. (1994) Hepatitis C virus polyprotein processing: kinetics and mutagenic analysis of serine proteinase-dependent cleavage. J. Virol. **68:** 8418–8422

32 Han D. S., Hahm B., Rho H. M. and Jang S. K. (1995) Identification of the protease domain in NS3 of hepatitis C virus. J. Gen. Virol. **76:** 985–993

33 Kim D. W., Gwack Y., Han J. H. and Choe J. (1995) C-terminal domain of the hepatitis C virus NS3 protein contains an RNA helicase activity. Biochem. Biophys. Res. Commun. **215:** 160–166

34 Tai C. L., Chi W. K., Chen D. S. and Hwang L. H. (1996) The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). J. Virol. **70:** 8477–8484

35 Failla C., Tomei L. and De Francesco R. (1995) An amino-terminal domain of the hepatitis C virus NS3 protease is essential for interaction with NS4A. J. Virol. **69:** 1769–1777

36 Kim J. L., Morgenstern K. A., Lin C., Fox T., Dwyer M. D., Landro J. A. et al. (1996) Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. Cell **87:** 343–355

37 Koch J. O. and Bartenschlager R. (1999) Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3, NS4A, and NS4B. J. Virol. **73:** 7138–7146

38 Neddermann P., Clementi A. and De Francesco R. (1999) Hyperphosphorylation of the hepatitis C virus NS5A protein requires an active NS3 protease, NS4A, NS4B, and NS5A encoded on the same polyprotein. J. Virol. **73:** 9984–9991

39 Park J. S., Yang J. M. and Min M. K. (2000) Hepatitis C virus nonstructural protein NS4B transforms NIH3T3 cells in cooperation with the Ha-ras oncogene. Biochem. Biophys. Res. Commun. **267:** 581–587

40 Gale M. J. Jr, Korth M. J. and Katze M. G. (1998) Repression of the PKR protein kinase by the hepatitis C virus NS5A protein: a potential mechanism of interferon resistance. Clin. Diagn. Virol. **10:** 157–162

41 Enomoto N., Sakuma I., Asahina Y., Kurosaki M., Murakami T., Yamamoto C. et al. (1995) Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b: sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J. Clin. Invest. **96:** 224–230

42 Taylor D. R., Shi S. T. and Lai M. M. C. (2000) Hepatitis C virus and interferon resistance. Microbes Inf. **2:** 1743–1756

43 Kato N., Lan K. H., Ono-Nita S. K., Shiratori Y. and Omata M. (1997) Hepatitis C virus nonstructural region 5A protein is a potent transcriptional activator. J. Virol. **71:** 8856–8859

44 Tanimoto A., Ide Y., Arima N., Sasaguri Y. and Padmanabhan R. (1997) The amino terminal deletion mutants of hepatitis C virus nonstructural protein NS5A function as transcriptional activators in yeast. Biochem. Biophys. Res. Commun. **236:** 360–364

45 Blight K. J., Kolykhalov A. A. and Rice C. M. (2000) Efficient initiation of HCV RNA replication in cell culture. Science **290:** 1972–1975

46 Tsukiyama-Kohara K., Iizuka N., Kohara M. and Nomoto A. (1992) Internal ribosome entry site within hepatitis C virus RNA. J. Virol. **66:** 1476–1483

47 Bukh J., Purcell R. H. and Miller R. H. (1992) Sequence analysis of the 5′ noncoding region of hepatitis C virus. Proc Natl. Acad. Sci. USA **89:** 4942–4946

48 Smith D. B., Mellor J., Jarvis L. M., Davidson F., Kolberg J., Urdea M. et al. (1995) Variation of the hepatitis C virus 5′ non-coding region: implications for secondary structure, virus detection and typing. The International HCV Collaborative Study Group. J. Gen. Virol. **76:** 1749–1761

49 Honda M., Beard M. R., Ping L. H. and Lemon S. M. (1999) A phylogenetically conserved stem-loop structure at the 5′ border of the internal ribosome entry site of hepatitis C virus is required for cap-independent viral translation. J. Virol. **73:** 1165–1174

50 Wimmer E., Hellen C. U. and Cao X. (1993) Genetics of poliovirus. Annu. Rev. Genet. **27:** 353–436

51 Lemon S. and Honda M. (1997) Internal ribosome entry sites within the RNA genomes of hepatitis C virus and other flaviviruses. Semin. Virol. **8:** 274–288

52 Rijnbrand R. C. and Lemon S. M. (2000) Internal ribosome entry site-mediated translation in hepatitis C virus replication. Curr. Top Microbiol. Immunol. **242:** 85–116

53 Borman A. M., Bailly J. L., Girard M. and Kean K. M. (1995) Picornavirus internal ribosome entry segments: comparison of translation efficiency and the requirements for optimal internal initiation of translation in vitro. Nucleic Acids Res. **23:** 3656–3663

54 Westaway E. G. (1987) Flavivirus replication strategy. Adv. Virus Res. **33:** 45–90

55 Rijnbrand R., Bredenbeek P., Straaten T. van der, Whetter L., Inchauspe G., Lemon S. et al. (1995) Almost the entire 5′ nontranslated region of hepatitis C virus is required for cap-independent translation. FEBS Lett. **365:** 115–119

56 Honda M., Ping L. H., Rijnbrand R. C., Amphlett E., Clarke B., Rowlands D. et al. (1996) Structural requirements for initiation of translation by internal ribosome entry within genome-length hepatitis C virus RNA. Virology **222:** 31–42

57 Boyer J. C. and Haenni A. L. (1994) Infectious transcripts and cDNA clones of RNA viruses. Virology **198:** 415–426

58 Honda M., Rijnbrand R., Abell G., Kim D. and Lemon S. M. (1999) Natural variation in translational activities of the 5′ nontranslated RNAs of hepatitis C virus genotypes 1a and 1b: evidence for a long-range RNA-RNA interaction outside of the internal ribosomal entry site. J. Virol. **73:** 4941–4951

59 Wang C., Le S. Y., Ali N. and Siddiqui A. (1995) An RNA pseudoknot is an essential structural element of the internal ribosome entry site located within the hepatitis C virus 5′ noncoding region. RNA **1:** 526–537

60 Kolupaeva V. G., Pestova T. V. and Hellen C. U. (2000) An enzymatic footprinting analysis of the interaction of 40S ribosomal subunits with the internal ribosomal entry site of hepatitis C virus. J. Virol. **74:** 6242–6250

61 Reynolds J. E., Kaminski A., Kettinen H. J., Grace K., Clarke B. E., Carroll A. R. et al. (1995) Unique features of internal initiation of hepatitis C virus RNA translation. EMBO J. **14:** 6010–6020

62 Wang C., Sarnow P. and Siddiqui A. (1993) Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. J. Virol. **67:** 3338–3344

63 Lu H. H. and Wimmer E. (1996) Poliovirus chimeras replicating under the translational control of genetic elements of hepatitis C virus reveal unusual properties of the internal ribosomal entry site of hepatitis C virus. Proc. Natl. Acad. Sci. USA **93:** 1412–1417

64 Reynolds J. E., Kaminski A., Carroll A. R., Clarke B. E., Rowlands D. J. and Jackson R. J. (1996) Internal initiation of translation of hepatitis C virus RNA: the ribosome entry site is at the authentic initiation codon. RNA **2:** 867–878

65 Hwang L. H., Hsieh C. L., Yen A., Chung Y. L. and Chen D. S. (1998) Involvement of the 5′ proximal coding sequences of hepatitis C virus with internal initiation of viral translation. Biochem. Biophys. Res. Commun. **252:** 455–460

66 Ito T. and Lai M. M. C. (1999) An internal polypyrimidine-tract-binding protein-binding site in the hepatitis C virus RNA attenuates translation, which is relieved by the 3′-untranslated sequence. Virology **254:** 288–296

67 Wang T. H., Rijnbrand R. C. and Lemon S. M. (2000) Core protein-coding sequence, but not core protein, modulates the efficiency of cap-independent translation directed by the internal ribosome entry site of hepatitis C virus. J. Virol. **74:** 11347–11358

68 Jubin R., Vantuno N. E., Kieft J. S., Murray M. G., Doudna J. A., Lau J. Y. et al. (2000) Hepatitis C virus internal ribosome entry site (IRES) stem loop IIId contains a phylogenetically conserved GGG triplet essential for translation and IRES folding. J. Virol. **74:** 10430–10437

69 Tanaka T., Kato N., Cho M. J. and Shimotohno K. (1995) A novel sequence found at the 3′ terminus of hepatitis C virus genome. Biochem. Biophys. Res. Commun. **215:** 744–749

70 Tanaka T., Kato N., Cho M. J., Sugiyama K. and Shimotohno K. (1996) Structure of the 3′ terminus of the hepatitis C virus genome. J. Virol. **70:** 3307–3312

71 Kolykhalov A. A., Feinstone S. M. and Rice C. M. (1996) Identification of a highly conserved sequence element at the 3′ terminus of hepatitis C virus genome RNA. J. Virol. **70:** 3363–3371

72 Yamada N., Tanihara K., Takada A., Yorihuzi T., Tsutsumi M., Shimomura H. et al. (1996) Genetic organization and diversity of the 3′ noncoding region of the hepatitis C virus genome. Virology **223:** 255–261

73 Blight K. J. and Rice C. M. (1997) Secondary structure determination of the conserved 98-base sequence at the 3′ terminus of hepatitis C virus genome RNA. J. Virol. **71:** 7345–7352

74 Han J. H. and Houghton M. (1992) Group specific sequences and conserved secondary structures at the 3′ end of HCV

genome and its implication for viral replication. Nucleic Acids Res. **20:** 3520
75 Ito T. and Lai M. M. C. (1997) Determination of the secondary structure of and cellular protein binding to the 3′-untranslated region of the hepatitis C virus RNA genome. J. Virol. **71:** 8698–8706
76 Deng R. and Brock K. V. (1993) 5′ and 3′ untranslated regions of pestivirus genome: primary and secondary structure analyses. Nucleic Acids Res. **21:** 1949–1957
77 Chambers T. J., Hahn C. S., Galler R. and Rice C. M. (1990) Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. **44:** 649–688
78 Mohan P. M. and Padmanabhan R. (1991) Detection of stable secondary structure at the 3′ terminus of dengue virus type 2 RNA. Gene **108:** 185–191
79 Mandl C. W., Holzmann H., Kunz C. and Heinz F. X. (1993) Complete genomic sequence of Powassan virus: evaluation of genetic elements in tick-borne versus mosquito-borne flaviviruses. Virology **194:** 173–184
80 Rauscher S., Flamm C., Mandl C. W., Heinz F. X. and Stadler P. F. (1997) Secondary structure of the 3′-noncoding region of flavivirus genomes: comparative analysis of base pairing probabilities. RNA **3:** 779–791
81 Proutski V., Gaunt M. W., Gould E. A. and Holmes E. C. (1997) Secondary structure of the 3′-untranslated region of yellow fever virus: implications for virulence, attenuation and vaccine development. J. Gen. Virol. **78:** 1543–1549
82 Bukh J., Apgar C. L. and Yanagi M. (1999) Toward a surrogate model for hepatitis C virus: An infectious molecular clone of the GB virus-B hepatitis agent. Virology **262:** 470–478
83 Sbardellati A., Scarselli E., Tomei L., Kekule A. S. and Traboni C. (1999) Identification of a novel sequence at the 3′ end of the GB virus B genome. J. Virol. **73:** 10546–10550
84 Yanagi M., Purcell R. H., Emerson S. U. and Bukh J. (1997) Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. Proc. Natl. Acad. Sci. USA **94:** 8738–8743
85 Yanagi M., St. Claire M., Shapiro M., Emerson S. U., Purcell R. H. and Bukh J. (1998) Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious in vivo. Virology **244:** 161–172
86 Yanagi M., St Claire M., Emerson S. U., Purcell R. H. and Bukh J. (1999) In vivo analysis of the 3′ untranslated region of the hepatitis C virus after in vitro mutagenesis of an infectious cDNA clone. Proc. Natl. Acad. Sci. USA **96:** 2291–2295
87 Khromykh A. A. and Westaway E. G. (1997) Subgenomic replicons of the flavivirus Kunjin: construction and applications. J. Virol. **71:** 1497–1505
88 Mandl C. W., Holzmann H., Meixner T., Rauscher S., Stadler P. F., Allison S. L. et al. (1998) Spontaneous and engineered deletions in the 3′ noncoding region of tick-borne encephalitis virus: construction of highly attenuated mutants of a flavivirus. J. Virol. **72:** 2132–2140
89 Men R., Bray M., Clark D., Chanock R. M. and Lai C. J. (1996) Dengue type 4 virus mutants containing deletions in the 3' noncoding region of the RNA genome: analysis of growth restriction in cell culture and altered viremia pattern and immunogenicity in rhesus monkeys. J. Virol. **70:** 3930–3937
90 Kolykhalov A. A., Mihalik K., Feinstone S. M. and Rice C. M. (2000) Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3′ nontranslated region are essential for virus replication in vivo. J. Virol. **74:** 2046–2051
91 Yanagi M., Purcell R. H., Emerson S. U. and Bukh J. (1999) Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. Virology **262:** 250–263
92 Kolykhalov A. A., Agapov E. V., Blight K. J., Mihalik K., Feinstone S. M. and Rice C. M. (1997) Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science **277:** 570–574
93 Oh J. W., Sheu G. T. and Lai M. M. (2000) Template requirement and initiation site selection by hepatitis C virus polymerase on a minimal viral RNA template. J. Biol. Chem. **275:** 17710–17717
94 Gontarek R. R., Gutshall L. L., Herold K. M., Tsai J., Sathe G. M., Mao J. et al. (1999) hnRNP C and polypyrimidine tract-binding protein specifically interact with the pyrimidine-rich region within the 3′NTR of the HCV RNA genome. Nucleic Acids Res. **27:** 1457–1463
95 Spångberg K., Wiklund L. and Schwartz S. (2000) HuR, a protein implicated in oncogene and growth factor mRNA decay, binds to the 3′ ends of hepatitis C virus RNA of both polarities. Virology **274:** 378–390
96 Luo G. (1999) Cellular proteins bind to the poly(U) tract of the 3′ untranslated region of hepatitis C virus RNA genome. Virology **256:** 105–118
97 Petrik J., Parker H. and Alexander G. J. (1999) Human hepatic glyceraldehyde-3-phosphate dehydrogenase binds to the poly(U) tract of the 3′ non-coding region of hepatitis C virus genomic RNA. J. Gen. Virol. **80:** 3109–3113
98 Schultz D. E., Hardin C. C. and Lemon S. M. (1996) Specific interaction of glyceraldehyde 3-phosphate dehydrogenase with the 5′-nontranslated RNA of hepatitis A virus. J. Biol. Chem. **271:** 14134–14142
99 Yi M., Schultz D. E. and Lemon S. M. (2000) Functional significance of the interaction of hepatitis A virus RNA with glyceraldehyde 3-phosphate dehydrogenase (GAPDH): opposing effects of GAPDH and polypyrimidine tract binding protein on internal ribosome entry site function. J. Virol. **74:** 6459–6468
100 Spångberg K., Wiklund L. and Schwartz S. (2001) Binding of the La autoantigen to the hepatitis C virus 3′ untranslated region protects the RNA from rapid degradation in vitro. J. Gen. Virol. **82:** 113–120
101 Cheng J. C., Chang M. F. and Chang S. C. (1999) Specific interaction between the hepatitis C virus NS5B RNA polymerase and the 3′ end of the viral RNA. J. Virol. **73:** 7044–7049
102 Oh J. W., Ito T. and Lai M. M. C. (1999) A recombinant hepatitis C virus RNA-dependent RNA polymerase capable of copying the full-length viral RNA. J. Virol. **73:** 7694–7702
103 Tsuchihara K., Tanaka T., Hijikata M., Kuge S., Toyoda H., Nomoto A. et al. (1997) Specific interaction of polypyrimidine tract-binding protein with the extreme 3′-terminal structure of the hepatitis C virus genome, the 3′X. J. Virol. **71:** 6720–6726
104 Ito T., Tahara S. M. and Lai M. M. C. (1998) The 3′-untranslated region of hepatitis C virus RNA enhances translation from an internal ribosomal entry site. J. Virol. **72:** 8789–8796
105 Smith D. B. and Simmonds P. (1997) Characteristics of nucleotide substitution in the hepatitis C virus genome: constraints on sequence change in coding regions at both ends of the genome. J. Mol. Evol. **45:** 238–246
106 Ina Y., Mizokami M., Ohba K. and Gojobori T. (1994) Reduction of synonymous substitutions in the core protein gene of hepatitis C virus. J. Mol. Evol. **38:** 50–56
107 Hofacker I. L., Fekete M., Flamm C., Huynen M. A., Rauscher S., Stolorz P. E. et al. (1998) Automatic detection of conserved RNA structure elements in complete RNA virus genomes. Nucleic Acids Res. **26:** 3825–3836
108 Okamoto H., Kojima M., Okada S., Yoshizawa H., Iizuka H., Tanaka T. et al. (1992) Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. Virology **190:** 894–899
109 Ogata N., Alter H. J., Miller R. H. and Purcell R. H. (1991) Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:** 3392–3396

110 Simmonds P., Holmes E. C., Cha T. A., Chan S. W., McOmish F., Irvine B. et al. (1993) Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J. Gen. Virol. **74:** 2391–2399
111 Simmonds P., Smith D. B., McOmish F., Yap P. L., Kolberg J., Urdea M. S. et al. (1994) Identification of genotypes of hepatitis C virus by sequence comparisons in the core, E1 and NS-5 regions. J. Gen. Virol. **75:** 1053–1061
112 Simmonds P. (1994) Variability of hepatitis C virus genome. Curr. Stud. Hematol. Blood Transfus. **61:** 12–35
113 Bukh J. and Miller R. H. (1994) Diagnostic and clinical implications of the different genotypes of hepatitis C virus. Hepatology **20:** 256–259
114 Bukh J., Miller R. H. and Purcell R. H. (1995) Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. Semin. Liver Dis. **15:** 41–63
115 Simmonds P. (1995) Variability of hepatitis C virus. Hepatology **21:** 570–583
116 Bukh J., Purcell R. H. and Miller R. H. (1994) Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc. Natl. Acad. Sci. USA **91:** 8239–8243
117 Farci P. and Purcell R. H. (2000) Clinical significance of hepatitis C virus genotypes and quasispecies. Semin. Liver. Dis. **20:** 103–126
118 Fried M. W. and Hoofnagle J. H. (1995) Therapy of hepatitis C. Semin. Liver Dis. **15:** 82–91
119 Davis G. L., Esteban-Mur R., Rustgi V., Hoefs J., Gordon S. C., Trepo C. et al. (1998) Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. N. Engl. J. Med. **339:** 1493–1499
120 Hijikata M., Kato N., Ootsuyama Y., Nakagawa M., Ohkoshi S. and Shimotohno K. (1991) Hypervariable regions in the putative glycoprotein of hepatitis C virus. Biochem. Biophys. Res. Commun. **175:** 220–228
121 Weiner A. J., Brauer M. J., Rosenblatt J., Richman K. H., Tung J., Crawford K. et al. (1991) Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. Virology **180:** 842–848
122 Kato N., Ootsuyama Y., Tanaka T., Nakagawa M., Nakazawa T., Muraiso K. et al. (1992) Marked sequence diversity in the putative envelope proteins of hepatitis C viruses. Virus. Res. **22:** 107–123
123 Weiner A. J., Geysen H. M., Christopherson C., Hall J. E., Mason T. J., Saracco G. et al. (1992) Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: potential role in chronic HCV infections. Proc. Natl. Acad. Sci. USA **89:** 3468–3472
124 Enomoto N., Sakamoto N., Kurosaki M., Marumo F. and Sato C. (1993) The hypervariable region of the HCV genome changes sequentially during the progression of acute HCV infection to chronic hepatitis. J. Hepatol. **17:** 415–416
125 Enomoto N., Kurosaki M., Tanaka Y., Marumo F. and Sato C. (1994) Fluctuation of hepatitis C virus quasispecies in persistent infection and interferon treatment revealed by single-strand conformation polymorphism analysis. J. Gen. Virol. **75:** 1361–1369
126 Forns X., Purcell R. H. and Bukh J. (1999) Quasispecies in viral persistence and pathogenesis of hepatitis C virus. Trends Microbiol. **7:** 402–410
127 Farci P., Shimoda A., Wong D., Cabezon T., De Gioannis D., Strazzera A. et al. (1996) Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. Proc. Natl. Acad. Sci. USA **93:** 15394–15399
128 Farci P., Shimoda A., Coiana A., Diaz G., Peddis G., Melpolder J. C. et al. (2000) The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. Science **288:** 339–344
129 McAllister J., Casino C., Davidson F., Power J., Lawlor E., Yap P. L. et al. (1998) Long-term evolution of the hypervariable region of hepatitis C virus in a common-source-infected cohort. J. Virol. **72:** 4893–4905
130 Bassett S. E., Thomas D. L., Brasky K. M. and Lanford R. E. (1999) Viral persistence, antibody to E1 and E2, and hypervariable region 1 sequence stability in hepatitis C virus-inoculated chimpanzees. J. Virol. **73:** 1118–1126
131 Major M. E., Mihalik K., Fernandez J., Seidman J., Kleiner D., Kolykhalov A. A. et al. (1999) Long-term follow-up of chimpanzees inoculated with the first infectious clone for hepatitis C virus. J. Virol. **73:** 3317–3325
132 Hong Z., Beaudet-Miller M., Lanford R. E., Guerra B., Wright-Minogue J., Skelton A. et al. (1999) Generation of transmissible hepatitis C virions from a molecular clone in chimpanzees. Virology **256:** 36–44
133 Forns X., Thimme R., Govindarajan S., Emerson S. U., Purcell R. H., Chisari F. V. et al. (2000) Hepatitis C virus lacking the hypervariable region 1 of the second envelope protein is infectious and causes acute resolving or persistent infection in chimpanzees. Proc. Natl. Acad. Sci. USA **97:** 13318–13323
134 Farci P., Bukh J. and Purcell R. H. (1997) The quasispecies of hepatitis C virus and the host immune response. Springer Semin. Immunopathol. **19:** 5–26
135 Iacovacci S., Sargiacomo M., Parolini I., Ponzetto A., Peschle C. and Carloni G. (1993) Replication and multiplication of hepatitis C virus genome in human foetal liver cells. Res. Virol. **144:** 275–279
136 Lanford R. E., Sureau C., Jacob J. R., White R. and Fuerst T. R. (1994) Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT/PCR. Virology **202:** 606–614
137 Ito T., Mukaigawa J., Zuo J., Hirabayashi Y., Mitamura K. and Yasui K. (1996) Cultivation of hepatitis C virus in primary hepatocyte culture from patients with chronic hepatitis C results in release of high titre infectious virus. J. Gen. Virol. **77:** 1043–1054
138 Fournier C., Sureau C., Coste J., Ducos J., Pageaux G., Larrey D. et al. (1998) In vitro infection of adult normal human hepatocytes in primary culture by hepatitis C virus. J. Gen. Virol. **79:** 2367–2374
139 Rumin S., Berthillon P., Tanaka E., Kiyosawa K., Trabaud M. A., Bizollon T. et al. (1999) Dynamic analysis of hepatitis C virus replication and quasispecies selection in long-term cultures of adult human hepatocytes infected in vitro. J. Gen. Virol. **80:** 3007–3018
140 Kato N., Ikeda M., Mizutani T., Sugiyama K., Noguchi M., Hirohashi S. et al. (1996) Replication of hepatitis C virus in cultured non-neoplastic human hepatocytes. Jpn. J. Cancer Res. **87:** 787–792
141 Ikeda M., Sugiyama K., Mizutani T., Tanaka T., Tanaka K., Sekihara H. et al. (1998) Human hepatocyte clonal cell lines that support persistent replication of hepatitis C virus. Virus Res. **56:** 157–167
142 Nakajima N., Hijikata M., Yoshikura H. and Shimizu Y. K. (1996) Characterization of long-term cultures of hepatitis C virus. J. Virol. **70:** 3325–3329
143 Shimizu Y. K., Iwamoto A., Hijikata M., Purcell R. H. and Yoshikura H. (1992) Evidence for in vitro replication of hepatitis C virus genome in a human T-cell line. Proc. Natl. Acad. Sci. USA **89:** 5477–5481
144 Mizutani T., Kato N., Saito S., Ikeda M., Sugiyama K. and Shimotohno K. (1996) Characterization of hepatitis C virus replication in cloned cells obtained from a human T-cell leukemia virus type 1-infected cell line, MT-2. J Virol. **70:** 7219–7223
145 Sugiyama K., Kato N., Mizutani T., Ikeda M., Tanaka T. and Shimotohno K. (1997) Genetic analysis of the hepatitis C

virus (HCV) genome from HCV- infected human T cells. J. Gen. Virol. **78:** 329–336
146 Cribier B., Schmitt C., Bingen A., Kirn A. and Keller F. (1995) In vitro infection of peripheral blood mononuclear cells by hepatitis C virus. J. Gen. Virol. **76:** 2485–2491
147 Lerat H., Berby F., Trabaud M. A., Vidalin O., Major M., Trepo C. et al. (1996) Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. J. Clin. Invest. **97:** 845–851
148 Bouffard P., Hayashi P. H., Acevedo R., Levy N. and Zeldis J. B. (1992) Hepatitis C virus is detected in a monocyte/macrophage subpopulation of peripheral blood mononuclear cells of infected patients. J. Infect. Dis. **166:** 1276–1280
149 Müller H. M., Pfaff E., Goeser T., Kallinowski B., Solbach C. and Theilmann L. (1993) Peripheral blood leukocytes serve as a possible extrahepatic site for hepatitis C virus replication. J. Gen. Virol. **74:** 669–676
150 Zignego A. L., Macchia D., Monti M., Thiers V., Mazzetti M., Foschi M. et al. (1992) Infection of peripheral mononuclear blood cells by hepatitis C virus. J. Hepatol. **15:** 382–386
151 Shimizu Y. K., Igarashi H., Kiyohara T., Shapiro M., Wong D. C., Purcell R. H. et al. (1998) Infection of a chimpanzee with hepatitis C virus grown in cell culture. J. Gen. Virol. **79:** 1383–1386
152 Killenberg P. G. (2000) Extrahepatic manifestations of chronic hepatitis C. Semin. Gastrointest. Dis. **11:** 62–68
153 Lunel F. and Musset L. (1998) Hepatitis C virus infection and cryoglobulinemia. J. Hepatol. **29:** 848–855
154 Beard M. R., Abell G., Honda M., Carroll A., Gartland M., Clarke B. et al. (1999) An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. Hepatology **30:** 316–324
155 Lohmann V., Korner F., Koch J., Herian U., Theilmann L. and Bartenschlager R. (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **285:** 110–113
156 Gowans E. J. (2000) Distribution of markers of hepatitis C virus infection throughout the body. Semin. Liver Dis. **20:** 85–102
157 Lohmann V., Korner F., Dobierzewska A. and Bartenschlager R. (2001) Mutations in hepatitis C virus RNAs conferring cell culture adaptation. J. Virol. **75:** 1437–1449
158 Pietschmann T., Lohmann V., Rutter G., Kurpanek K. and Bartenschlager R. (2001) Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J. Virol. **75:** 1252–1264
159 Petersen J., Dandri M., Gupta S. and Rogler C. E. (1998) Liver repopulation with xenogenic hepatocytes in B and T cell-deficient mice leads to chronic hepadnavirus infection and clonal growth of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA **95:** 310–315
160 Ilan E., Burakova T., Dagan S., Nussbaum O., Lubin I., Eren R. et al. (1999) The hepatitis B virus-trimera mouse: a model for human HBV infection and evaluation of anti-HBV therapeutic agents. Hepatology **29:** 553–562
161 Zibert A., Schreier E. and Roggendorf M. (1995) Antibodies in human sera specific to hypervariable region 1 of hepatitis C virus can block viral attachment. Virology **208:** 653–661
162 Takikawa S., Ishii K., Aizaki H., Suzuki T., Asakura H., Matsuura Y. et al. (2000) Cell fusion activity of hepatitis C virus envelope proteins. J. Virol. **74:** 5066–5074
163 Pileri P., Uematsu Y., Campagnoli S., Galli G., Falugi F., Petracca R. et al. (1998) Binding of hepatitis C virus to CD81. Science **282:** 93–941
164 Agnello V., Abel G., Elfahal M., Knight G. B. and Zhang Q. X. (1999) Hepatitis C virus and other Flaviviridae viruses enter cells via low density lipoprotein receptor. Proc. Natl. Acad. Sci. USA **96:** 12766–12771
165 Monazahian M., Bohme I., Bonk S., Koch A., Scholz C., Grethe S. et al. (1999) Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. J. Med. Virol. **57:** 223–229
166 Wunschmann S., Medh J. D., Klinzmann D., Schmidt W. N. and Stapleton J. T. (2000) Characterization of hepatitis C virus (HCV) and HCV E2 interactions with CD81 and the low-density lipoprotein receptor. J. Virol. **74:** 10055–10062
167 Tu H., Gao L., Shi S. T., Taylor D. R., Yang T., Mircheff A. K. et al. (1999) Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. Virology **263:** 30–41
168 Bartenschlager R., Lohmann V., Wilkinson T. and Koch J. O. (1995) Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation. J. Virol. **69:** 7519–7528
169 Lin C., Wu J. W., Hsiao K. and Su M. S. (1997) The hepatitis C virus NS4A protein: interactions with the NS4B and NS5A proteins. J. Virol. **71:** 6465–6471
170 Asabe S. I., Tanji Y., Satoh S., Kaneko T., Kimura K. and Shimotohno K. (1997) The N-terminal region of hepatitis C virus-encoded NS5A is important for NS4A-dependent phosphorylation. J. Virol. **71:** 790–796
171 Ishido S., Fujita T. and Hotta H. (1998) Complex formation of NS5B with NS3 and NS4A proteins of hepatitis C virus. Biochem. Biophys. Res. Commun. **244:** 35–40
172 Selby M. J., Choo Q. L., Berger K., Kuo G., Glazer E., Eckart M. et al. (1993) Expression, identification and subcellular localization of the proteins encoded by the hepatitis C viral genome. J. Gen. Virol. **74:** 1103–1113
173 Hwang S. B., Park K. J., Kim Y. S., Sung Y. C. and Lai M. M. (1997) Hepatitis C virus NS5B protein is a membrane-associated phosphoprotein with a predominantly perinuclear localization. Virology **227:** 439–446
174 Nouri Aria K. T., Sallie R., Sangar D., Alexander G. J., Smith H., Byrne J. et al. (1993) Detection of genomic and intermediate replicative strands of hepatitis C virus in liver tissue by in situ hybridization. J. Clin. Invest. **91:** 2226–2234
175 Haruna Y., Hayashi N., Hiramatsu N., Takehara T., Hagiwara H., Sasaki Y. et al. (1993) Detection of hepatitis C virus RNA in liver tissues by an in situ hybridization technique. J. Hepatol. **18:** 96–100
176 Ishido S., Muramatsu S., Fujita T., Iwanaga Y., Tong W. Y., Katayama Y. et al. (1997) Wild-type, but not mutant-type, p53 enhances nuclear accumulation of the NS3 protein of hepatitis C virus. Biochem. Biophys. Res. Commun. **230:** 431–436
177 Muramatsu S., Ishido S., Fujita T., Itoh M. and Hotta H. (1997) Nuclear localization of the NS3 protein of hepatitis C virus and factors affecting the localization. J. Virol. **71:** 4954–4961
178 Errington W., Wardell A. D., McDonald S., Goldin R. D. and McGarvey M. J. (1999) Subcellular localisation of NS3 in HCV-infected hepatocytes. J. Med. Virol. **59:** 456–462
179 Koonin E. V. (1991) The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J. Gen. Virol. **72:** 2197–2206
180 Yamashita T., Kaneko S., Shirota Y., Qin W., Nomura T., Kobayashi K. et al. (1998) RNA-dependent RNA polymerase activity of the soluble recombinant hepatitis C virus NS5B protein truncated at the C-terminal region. J. Biol. Chem. **273:** 15479–15486
181 Altschul S. F., Madden T. L., Schaffer A. A., Zhang J., Zhang Z., Miller W. et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:** 3389–3402
182 Lesburg C. A., Cable M. B., Ferrari E., Hong Z., Mannarino A. F. and Weber P. C. (1999) Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. Nat. Struct. Biol. **6:** 937–943
183 Bressanelli S., Tomei L., Roussel A., Incitti I., Vitale R. L., Mathieu M. et al. (1999) Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Proc. Natl. Acad. Sci. USA 96: 13034–13039

184 Ago H., Adachi T., Yoshida A., Yamamoto M., Habuka N., Yatsunami K. et al. (1999) Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Struct. Fold Des. **7:** 1417–1426
185 Behrens S. E., Tomei L. and De Francesco R. (1996) Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J. **15:** 12–22
186 De Francesco R., Behrens S. E., Tomei L., Altamura S. and Jiricny J. (1996) RNA-dependent RNA polymerase of hepatitis C virus. Methods Enzymol. **275:** 58–67
187 Lohmann V., Korner F., Herian U. and Bartenschlager R. (1997) Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. J. Virol. **71:** 8416–8428
188 Yuan Z. H., Kumar U., Thomas H. C., Wen Y. M. and Monjardino J. (1997) Expression, purification, and partial characterization of HCV RNA polymerase. Biochem. Biophys. Res. Commun. **232:** 231–235
189 Al R. H., Xie Y., Wang Y. and Hagedorn C. H. (1998) Expression of recombinant hepatitis C virus non-structural protein 5B in *Escherichia coli*. Virus Res. **53:** 141–149
190 Ferrari E., Wright-Minogue J., Fang J. W., Baroudy B. M., Lau J. Y. and Hong Z. (1999) Characterization of soluble hepatitis C virus RNA-dependent RNA polymerase expressed in *Escherichia coli*. J. Virol. **73:** 1649–1654
191 Luo G., Hamatake R. K., Mathis D. M., Racela J., Rigat K. L., Lemm J. et al. (2000) De novo initiation of RNA synthesis by the RNA-dependent RNA polymerase (NS5B) of hepatitis C virus. J. Virol. **74:** 851–863
192 Sun X. L., Johnson R. B., Hockman M. A. and Wang Q. M. (2000) De novo RNA synthesis catalyzed by HCV RNA-dependent RNA polymerase. Biochem. Biophys. Res. Commun. **268:** 798–803
193 Zhong W., Uss A. S., Ferrari E., Lau J. Y. and Hong Z. (2000) De novo initiation of RNA synthesis by hepatitis C virus nonstructural protein 5B polymerase. J. Virol. **74:** 2017–2022
194 Kao C. C., Yang X., Kline A., Wang Q. M., Barket D. and Heinz B. A. (2000) Template requirements for RNA synthesis by a recombinant hepatitis C virus RNA-dependent RNA polymerase. J. Virol. **74:** 11121–11128
195 Hijikata M., Mizushima H., Tanji Y., Komoda Y., Hirowatari Y., Akagi T. et al. (1993) Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proc. Natl. Acad. Sci. USA **90:** 10773–10777
196 Pestova T. V., Shatsky I. N., Fletcher S. P., Jackson R. J. and Hellen C. U. (1998) A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. Genes Dev. **12:** 67–83
197 Sizova D. V., Kolupaeva V. G., Pestova T. V., Shatsky I. N. and Hellen C. U. (1998) Specific interaction of eukaryotic translation initiation factor 3 with the 5′ nontranslated regions of hepatitis C virus and classical swine fever virus RNAs. J. Virol. **72:** 4775–4782
198 Krüger M., Beger C., Li Q. X., Welch P. J., Tritz R., Leavitt M. et al. (2000) Identification of eIF2Bgamma and eIF2gamma as cofactors of hepatitis C virus internal ribosome entry site-mediated translation using a functional genomics approach. Proc. Natl. Acad. Sci. USA **97:** 8566–8571
199 Stassinopoulos I. A. and Belsham G. J. (2001) A novel protein-RNA binding assay: functional interactions of the foot-and-mouth disease virus internal ribosome entry site with cellular proteins. RNA **7:** 114–122
200 Ali N. and Siddiqui A. (1997) The La antigen binds 5′ noncoding region of the hepatitis C virus RNA in the context of the initiator AUG codon and stimulates internal ribosome entry site-mediated translation. Proc. Natl. Acad. Sci. USA **94:** 2249–2254
201 Ali N. and Siddiqui A. (1995) Interaction of polypyrimidine tract-binding protein with the 5′ noncoding region of the hepatitis C virus RNA genome and its functional requirement in internal initiation of translation. J. Virol. **69:** 6367–6375
202 Anwar A., Ali N., Tanveer R. and Siddiqui A. (2000) Demonstration of functional requirement of polypyrimidine tract-binding protein by SELEX RNA during hepatitis C virus internal ribosome entry site-mediated translation initiation. J. Biol. Chem. **275:** 3423–34235
203 Hahm B., Kim Y. K., Kim J. H., Kim T. Y. and Jang S. K. (1998) Heterogeneous nuclear ribonucleoprotein L interacts with the 3′ border of the internal ribosomal entry site of hepatitis C virus. J. Virol. **72**: 8782–8788
204 Spångberg K. and Schwartz S. (1999) Poly(C)-binding protein interacts with the hepatitis C virus 5′ untranslated region. J. Gen. Virol. **80:** 1371–1376
205 Tarun S. Z. and Sachs A. B. (1996) Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G. EMBO J. **15:** 7168–7177
206 Craig A. W., Haghighat A., Yu A. T. and Sonenberg N. (1998) Interaction of polyadenylate-binding protein with the eIF4G homologue PAIP enhances translation. Nature **392:** 520–523
207 Chung R. T. and Kaplan L. M. (1999) Heterogeneous nuclear ribonucleoprotein I (hnRNP-I/PTB) selectively binds the conserved 3′ terminus of hepatitis C viral RNA. Biochem Biophys Res. Commun. **254:** 351–362
208 Shimoike T., Mimori S., Tani H., Matsuura Y. and Miyamura T. (1999) Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. J Virol **73:** 9718–9725
209 Gale M. J. Jr, Korth M. J., Tang N. M., Tan S. L., Hopkins D. A., Dever T. E. et al. (1997) Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology **230:** 217–227
210 Laporte J., Malet I., Andrieu T., Thibault V., Toulme J. J., Wychowski C. et al. (2000) Comparative analysis of translation efficiencies of hepatitis C virus 5′ untranslated regions among intraindividual quasispecies present in chronic infection: opposite behaviors depending on cell type. J. Virol. **74:** 10827–10833
211 Collier A. J., Tang S. and Elliott R. M. (1998) Translation efficiencies of the 5′ untranslated region from representatives of the six major genotypes of hepatitis C virus using a novel bicistronic reporter assay system. J. Gen. Virol. **79:** 2359–2366
212 Lerat H., Shimizu Y. K. and Lemon S. M. (2000) Cell type-specific enhancement of hepatitis C virus internal ribosome entry site-directed translation due to 5′ nontranslated region substitutions selected during passage of virus in lymphoblastoid cells. J. Virol. **74:** 7024–7031
213 Bukh J., Purcell R. H. and Miller R. H. (1993) At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. Proc. Natl. Acad. Sci. USA **90:** 8234–8238
214 Okamoto H., Sugiyama Y., Okada S., Kurai K., Akahane Y., Sugai Y. et al. (1992) Typing hepatitis C virus by polymerase chain reaction with type- specific primers: application to clinical surveys and tracing infectious sources. J. Gen. Virol. **73:** 673–679
215 Okamoto H., Tokita H., Sakamoto M., Horikita M., Kojima M., Iizuka H. et al. (1993) Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. J. Gen. Virol. **74:** 2385–2390
216 Chayama K., Tsubota A., Arase Y., Saitoh S., Koida I., Ikeda K. et al. (1993) Genotypic subtyping of hepatitis C virus. J. Gastroenterol Hepatol. **8:** 150–156
217 Widell A., Shev S., Mansson S., Zhang Y. Y., Foberg U., Norkrans G. et al. (1994) Genotyping of hepatitis C virus isolates by a modified polymerase chain reaction assay using

type specific primers: epidemiological applications. J. Med. Virol. **44:** 272–279
218 Viazov S., Zibert A., Ramakrishnan K., Widell A., Cavicchini A., Schreier E. et al. (1994) Typing of hepatitis C virus isolates by DNA enzyme immunoassay. J. Virol. Methods **48:** 81–91
219 Ravaggi A., Zonaro A., Marin M. G., Puoti M., Albertini A. and Cariani E. (1994) Distribution of viral genotypes in Italy determined by hepatitis C virus typing by DNA immunoassay. J. Clin. Microbiol. **32:** 2280–2284
220 Takada N., Takase S., Enomoto N., Takada A. and Date T. (1992) Clinical backgrounds of the patients having different types of hepatitis C virus genomes. J. Hepatol. **14:** 35–40
221 Takada N., Takase S., Takada A. and Date T. (1993) Differences in the hepatitis C virus genotypes in different countries. J. Hepatol. **17:** 277–283
222 Simmonds P., McOmish F., Yap P. L., Chan S. W., Lin C. K., Dusheiko G. et al. (1993) Sequence variability in the 5′ noncoding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. J. Gen. Virol. **74:** 661–668
223 McOmish F., Yap P. L., Dow B. C., Follett E. A., Seed C., Keller A. J. et al. (1994) Geographical distribution of hepatitis C virus genotypes in blood donors: an international collaborative survey. J. Clin. Microbiol. **32:** 884–892
224 Dusheiko G., Schmilovitz-Weiss H., Brown D., McOmish F., Yap P. L., Sherlock S. et al. (1994) Hepatitis C virus genotypes: an investigation of type-specific differences in geographic origin and disease. Hepatology **19:** 13–18
225 Murphy D., Willems B. and Delage G. (1994) Use of the 5′ noncoding region for genotyping hepatitis C virus. J. Infect. Dis. **169:** 473–475
226 Stuyver L., Rossau R., Wyseur A., Duhamel M., Vanderborght B., Van Heuverswyn H. et al. (1993) Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. J. Gen. Virol. **74:** 1093–1102
227 Stuyver L., Arnhem W. van, Wyseur A., Hernandez F., Delaporte E. and Maertens G. (1994) Classification of hepatitis C viruses based on phylogenetic analysis of the envelope 1 and nonstructural 5B regions and identification of five additional subtypes. Proc. Natl. Acad. Sci. USA **91:** 10134–10138
228 Stuyver L., Wyseur A., Arnhem W. van, Lunel F., Laurent-Puig P., Pawlotsky J. M. et al. (1995) Hepatitis C virus genotyping by means of 5′-UR/core line probe assays and molecular analysis of untypeable samples. Virus Res. **38:** 137–157
229 White P. A., Zhai X., Carter I., Zhao Y. and Rawlinson W. D. (2000) Simplified hepatitis C virus genotyping by heteroduplex mobility analysis. J. Clin. Microbiol. **38:** 477–482
230 Welch P. J., Tritz R., Yei S., Leavitt M., Yu M. and Barber J. (1996) A potential therapeutic application of hairpin ribozymes: in vitro and in vivo studies of gene therapy for hepatitis C virus infection. Gene Ther. **3:** 994–1001
231 Sakamoto N., Wu C. H. and Wu G. Y. (1996) Intracellular cleavage of hepatitis C virus RNA and inhibition of viral protein translation by hammerhead ribozymes. J. Clin. Invest. **98:** 2720–2728
232 Ohkawa K., Yuki N., Kanazawa Y., Ueda K., Mita E., Sasaki Y. et al. (1997) Cleavage of viral RNA and inhibition of viral translation by hepatitis C virus RNA-specific hammerhead ribozyme in vitro. J. Hepatol. **27:** 78–84
233 Macejak D. G., Jensen K. L., Jamison S. F., Domenico K., Roberts E. C., Chaudhary N. et al. (2000) Inhibition of hepatitis C virus (HCV)-RNA-dependent translation and replication of a chimeric HCV poliovirus using synthetic stabilized ribozymes. Hepatology. **31:** 769–776
234 Branch A. D. (2000) Hepatitis C virus RNA codes for proteins and replicates: does it also trigger the interferon response? Semin. Liver Dis. **20:** 57–68
235 Crooke S. T. and Bennett C. F. (1996) Progress in antisense oligonucleotide therapeutics. Annu. Rev. Pharmacol. Toxicol. **36:** 107–129
236 Branch A. D. (1998) A good antisense molecule is hard to find. Trends Biochem. Sci. **23:** 45–50