REVIEW

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Structure and Functions of Hepatitis C Virus Proteins: 15 Years After

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ABSTRACT. Since its discovery in 1988, the hepatitis C virus (HCV) has become a hot topic of research by many groups around the world. This globally spread infectious agent is responsible for a large proportion of chronic viral hepatitides. The clue to halting the hepatitis C pandemic may be the detailed understanding of the virus structure, its replication mechanism, and the exact functions of the various proteins. Such understanding could enable the development of new antivirals targeted against hepatitis C virus and possibly an effective vaccine. This review recaps the current knowledge about the HCV genome 15 years after its discovery. The structure and function of particular viral structural (core, E1, E2) and nonstructural (NS2, NS3, NS4, NS5) proteins and noncoding regions known to date are described. With respect to frequent conflicting reports from different research groups, results reproducibly demonstrated by independent investigators are emphasized. Owing to many obstacles and limitations inherent in doing research on this noteworthy virus, the current knowledge is incomplete and the answers to many important questions are to be expected in the future.

Abbreviations

ER	endoplasmic reticulum
Fas	a cell-surface protein receptor expressed on essentially all cells of the body that when bound to its ligand (FasL) signals a caspase cascade, ultimately resulting in apoptosis (programmed cell death)
HBV	hepatitis B virus
HCV	hepatitis C virus
HVR1	hypervariable region (1)
IRES	internal ribosomal entry site
ISDR	interferon sensitivity-determining region
LTβR	receptors for lymphotoxin β
NC	noncoding region
NF-κB	nuclear factor κB
NS	nonstructural (protein)
ORF	open reading frame
PKR	protein kinase, dsRNA-dependent
RT-PCR	reverse transcription polymerase chain reaction
dsRNA	double-stranded RNA
ssRNA	single-stranded RNA
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor (family)

CONTENTS

1 INTRODUCTION

The advancement in molecular biology diagnostic methods in the 1980s has made possible a great advance in the identification of new viral entities. Viral infectious disease research of mechanisms at the subcellular level has facilitated elucidation of pathogenesis as well as the development of new diagnostic methods. Hepatitis C virus can be considered a prototype for that research period. Its existence had been suspected from indirect evidence during the whole 1980s and even earlier. The clinical picture of chronic hepatitis caused by a presumed virus was known but the evidence of its existence was missing. Finally in 1988

the Houghton and Bradley's groups, using advanced cloning methods, succeeded in identifying and describing a virus that came to be known as hepatitis C virus (HCV). This important discovery was published the following year (Choo *et al.* 1989). However, despite the fifteen years of intense research since, HCV genome organization and mechanisms of its replication are not fully elucidated. The major obstacles, of course, to research progress have been the inability to cultivate HCV *in vitro* and the absence of a small animal model susceptible to HCV infection.

Current data on HCV epidemics warrant serious concern. The number of people infected with HCV worldwide approaches 200 million and it is increasing in many regions of the world. HCV causes chronic infection in approximately 70–80 % of those who become infected.

Long-term sequelae of HCV infection are currently the single most frequent reason for liver transplant.

In addition to the characterization of the virus, there has also been important research progress related to therapeutics. Introduction of interferons in the treatment of viral hepatitis in 1986 (without the knowledge of non-A non-B hepatitis etiology at that time) has been a major advance in the treatment of chronic hepatitis. Subsequent introduction of combined antiviral therapy and most recently the improved pharmacokinetics of interferons (pegylated interferons) increased the response to HCV treatment considerably (Fried *et al.* 2002). On the one hand, we can consider current treatment algorithms with the possibility of sustained elimination of the virus in more than half of the treated patients with chronic HCV infection as a major achievement. Particularly, infection with HCV genotype 2 or 3 is becoming a practically fully curable infectious disease. On the other hand, even the successful treatment course is accompanied by serious adverse events and decreased quality of life while on medication. There still remains significant proportion of patients where the current therapy fails. The majority of them are infected with widespread but therapeutically unfavorable HCV genotype 1. Differences in biological properties of the particular viral variants and their clinical significance have become an important issue at the end of the twentieth century and they currently remain the target of intense research. Concurrently with the identification of the novel structures and properties of the viral proteins the applied research of prospective new antivirals takes place. Finally, the development of an effective vaccine against HCV remains elusive. These and other reasons are of considerable impetus for ongoing scientific efforts.

This review does not address the issues of HCV host immune response, HCV diagnostics and treatment as well as the prospects of HCV vaccine development.

2 HCV CLASSIFICATION

Hepatitis C virus is an enveloped single stranded RNA virus with positive strand (Fig. 1). It is classified in the family *Flaviviridae* (Lindenbach *et al.* 2001). *Flaviviridae* comprise 4 genera Flavivirus, Pestivirius, Hepacivirus and unclassified viruses (genera Flavivirus and Pestivirius were originally classified as a *Togaviridae* family). Besides the HCV, the *Flaviviridae* family includes yellow fever virus, tick encephalitis virus, Japanese encephalis virus, dengue virus, *etc*. HCV, together with the newly discovered HGV/GBV-A, B and HGV/GBV-C viruses, are the only members of the Hepacivirus genus so far (Simmonds 2001).

HCV strains exhibit a great degree of genetic heterogeneity. At the *2nd International Conference of HCV and Related Viruses*, a consensus classification scheme was proposed for HCV. According to this system, HCV is classified into six major genotypes, which are further divided into subtypes (Simmonds *et al.* 1993, 1994). Such strain differences influence the natural history and clinical outcome of the disease.

By analogy with flaviviruses, the replication of HCV RNA occurs entirely in cytoplasm, mainly at the membranes of ER, *via* a minus-strand RNA intermediate. HCV RNA strategy can be briefly described in the following steps:

(*1*) entry, uncoating of the incoming viral particle and release of the genomic RNA (positive polarity ssRNA);
(*2*) translation and production of a single large polyprotein;
(*3*) co- and posttranslational splicing of the large polyprotein: production of mature viral proteins;
(*4*) synthesis of complementary antigenomic (minus stranded) RNA strands, which serve as templates for the synthesis of progeny-positive RNA strands;
(*5*) packaging into new virions, additional translation;
(*6*) release of mature virions.

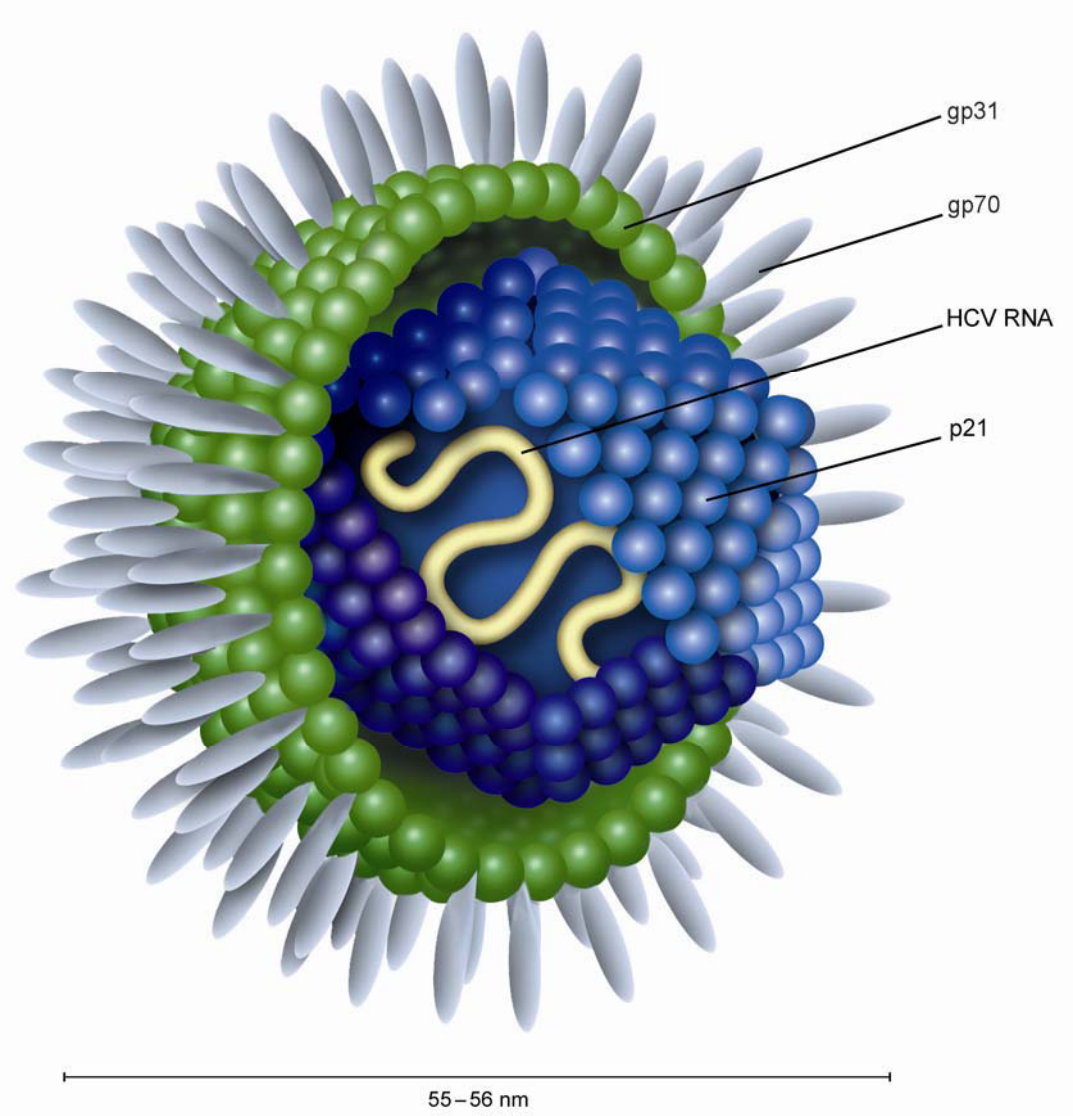

Fig. 1. Model of HCV particle; for further details *see* the text.

3 HCV GENOME

The prototype HCV genome is comprised of ≈9600 nucleotides, and HCV isolates from different parts of the world differ in their length (Choo *et al.* 1991). The genome consists of 5′ and 3′ terminal non-coding regions (5′- or 3′-NC), and of a central coding region (*see* Fig. 2; Choo *et al.* 1991; Lindenbach *et al.* 2001). The coding region comprises the part encoding structural proteins (region C, E1 and E2) and that for nonstructural proteins-regions NS2, NS3, NS4A, NS4B, NS5A and NS5B.

Table I summarizes the main functions of the viral proteins. The function of small viral protein p7 has not been fully elucidated to date, so it is not strictly classified into any of these subgroups. It is probably a nonstructural protein (Lin *et al.* 1994; Lindenbach *et al.* 2001).

Translation occurs immediately after the naked genomic RNA has entered the cytoplasm of host cell. The genomic RNA is directly translated; the translation is mediated by a cap-independent mechanism directed by an IRES (*see below* 5′-NC section). Ribosomes appear to bind in close proximity to the polyprotein initiator AUG with little or no scanning.

The whole coding region is translated from a single ORF, in a manner similar to that in other members of *Flaviviridae* family. One resulting polyprotein with the length of 3010–3033 amino acids is formed according to the particular genotype or isolate of the virus (Kato e*t al.* 1990; Choo e*t al.* 1991). This polyprotein is subsequently proteolytically co-translationally and postranslationally cleaved by cellular enzymes as well as by viral proteinases (Fig. 2). Consecutive cleavage forms final viral structural and nonstructural

proteins (Lindenbach *et al.* 2001). Recent publications show evidence of possible existence of overlapping reading frames in HCV coding regions (Matsufuji *et al.* 1996; Malarkannan *et al.* 1999; Walewski *et al.* 2001; Xu *et al.* 2001). That is, a particular sequence may contain two overlapping genes and is read twice, always from another ORF. A similar complex exploitation of the genome is noted in HBV, where the coding regions overlap and each HBV DNA nucleotide is read 1.5 times on average (Ganem *et al.* 2001).

Table I. The HCV proteins

Viral protein	Main function of the protein	Viral protein	Main function of the protein
C	nucleocapsid	NS4A	NS3/4 proteinase co-factor
	RNA binding	NS4B	NS5A phosphorylation
E1	envelope protein		induction of ER "membranous web"
E2	envelope protein	NS5A	inhibition of IFN-α ??
p7	ion channel ??		inhibition of apoptosis ??
NS2	component of NS2/3 proteinase	NS5B	RNA-dependent RNA polymerase
NS3	NS2/3 proteinase		
	NS3/4 proteinase		
	NTPase		
	RNA helicase		
	RNA binding		

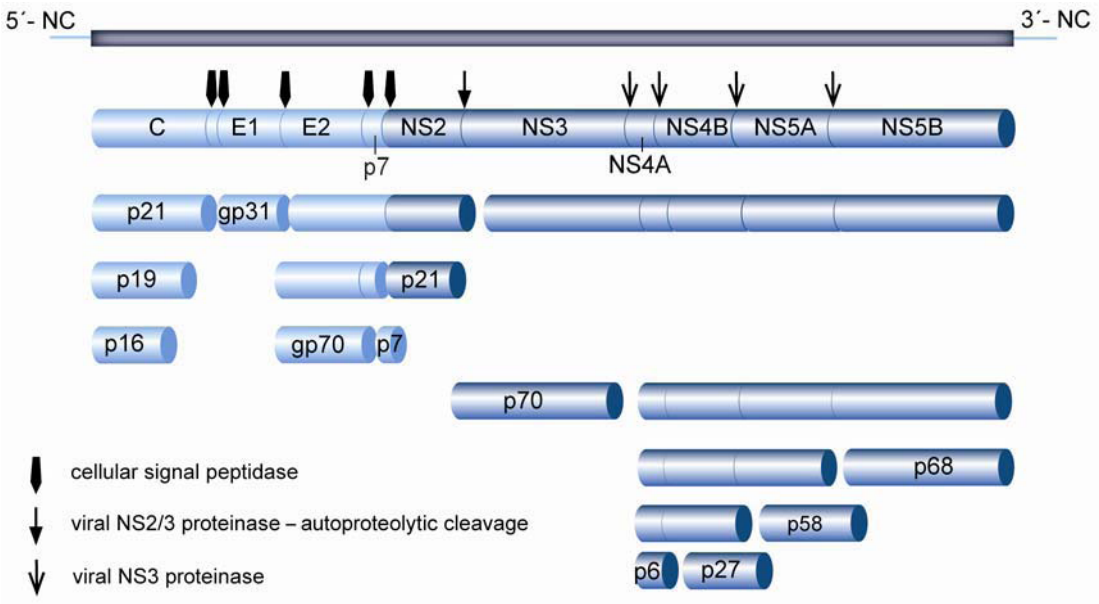

Fig. 2. Scheme of HCV genome (*dark blue* – coding region), post-translational splicing and resulting final viral proteins (according to Bartenschlager *et al.* 2000; Lindenbach *et al*. 2001).

4 HCV GENOME NONCODING REGIONS

4.1 5′-Noncoding region (5′-NC region)

The 5′-NC region is a highly conserved RNA sequence containing 341–344 nucleotides, which directly follows the methionine-initiating codon, the AUG codon of the open reading frame of HCV RNA coding region. This is the only part of HCV genome, which shows at least partial similarity with other known viral genomes; as an example, the sequence similarity of 5′-NC of animal pestiviruses is ≈50 %. 5′-NC region is highly conserved among particular HCV strains, subtypes and genotypes; the minimal sequence matching is >90 % (Fig. 3). Thus the 5′-NC region serves as an ideal target for primers in HCV RNA RT-PCR tests (Bukh *et al.* 1992). Among the other functions, this region initiates the attachment of HCV RNA on the host cell ribosomes. The HCV genome does not contain 5′-methyl cap. This finding is suppor-

ted by the fact that HCV genome does not contain sequences coding for methyltransferase and/or RNA triphosphatase of flaviviruses and also by the partial knowledge of HCV replication strategy (HCV RNA replicates in the cytoplasm, where the host cell methyltransferase is not present).

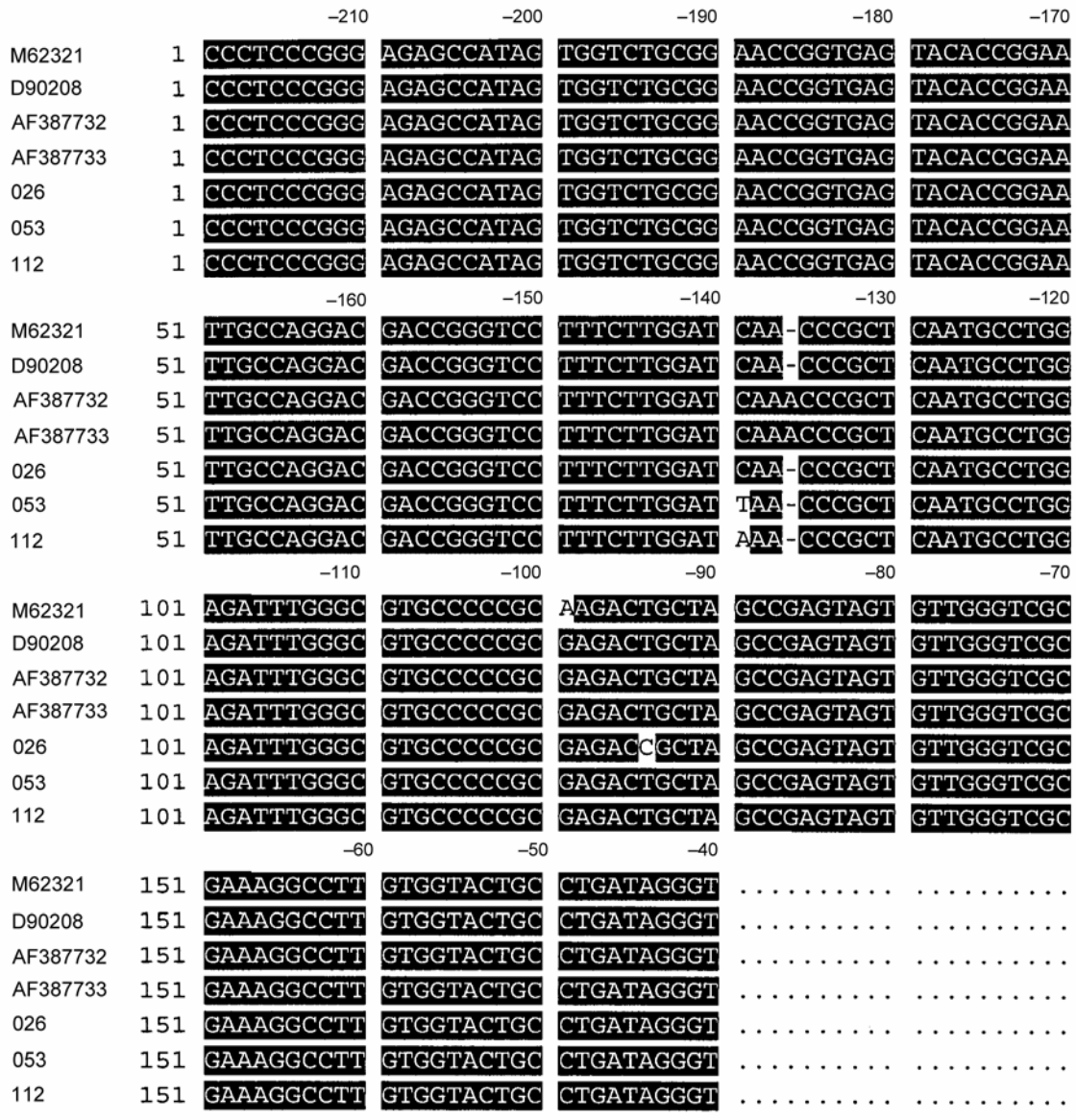

Fig. 3. Multiple alignment of 5′-NC sequences of clinical samples illustrates the high level of compatibility and only minimal changes among different HCV strains within this noncoding region; prototype sequences M62321 (HCV 1a) and D90208 (HCV-J 1b) – *GenBank*, and sequences of clinical samples (AF387732, AF387732, 026, 053 and 112) (Krekulová *et al.* 2005); the nucleotide positions are numbered according to Choo e*t al.* (1991).

Relatively complicated secondary and tertiary structure of 5′-NC region was experimentally shown (Fig. 4). It consists of four major domains (I–IV) and a pseudoknot (Ψ).

Approximately 40 initial nucleotides of the 5′-end are not essential for viral replication and translation but, similar to other viruses with positive ssRNA, the initial element of viral RNA is active in the process of viral replication (Boyer *et al.* 1994). The deletion of the first loop of 5′-NC leads to a significant increase of the efficacy of the viral mRNA translation (Honda *et al.* 1996).

5′-NC region contains internal ribosomal entry site (IRES); major part of this functional element is probably located between the nucleotides 40 and 355 (Tsukiyama-Kohara *et al.* 1992; Wang *et al.* 1993; Honda *et al.* 1996). HCV IRES likely spans through nearly the entire 5′-NC region, and recent studies bring convincing evidence about the position of 5′-end of HCV IRES, placing it between the nucleotides at positions 29–69 at loop IIa of 5′-NC region (Wang *et al.* 1993; Rijnbrand *et al.* 1995; Honda *et al.* 1996) (Fig. 4).

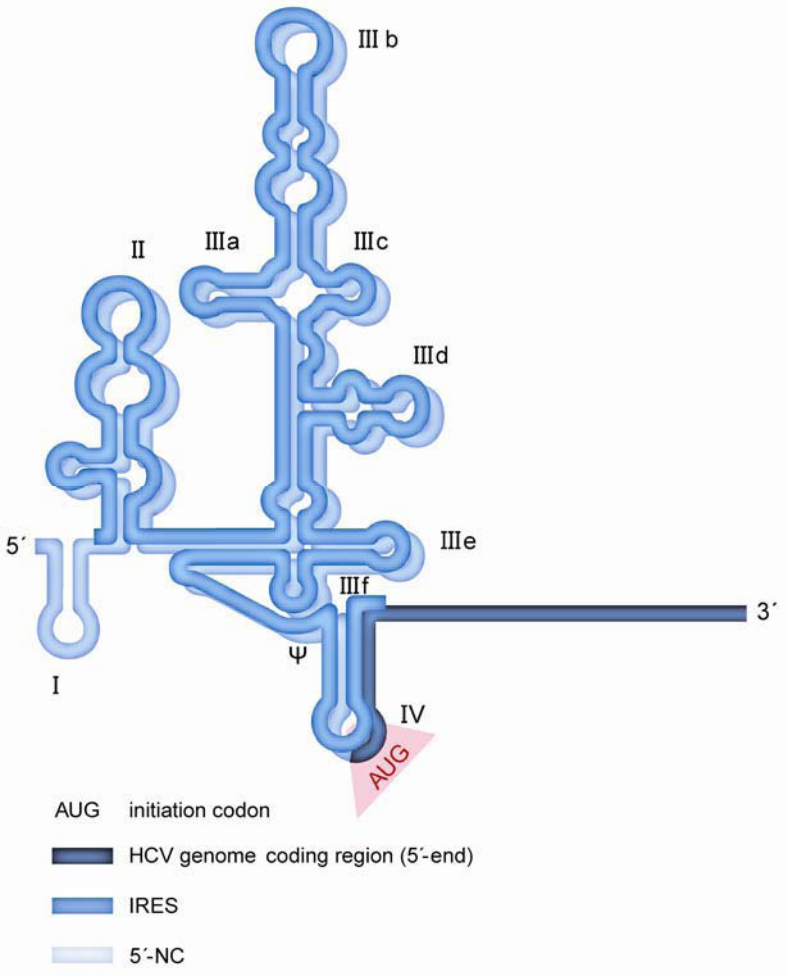

Fig. 4. Secondary structure of 5′-NC region; IRES (*blue-grey*) is positioned within the nearly entire noncoding region and overlaps to the core protein coding region (*dark blue*) (adapted from Lindenbach *et al.* 2001, Pestova *et al.* 1998).

IRES facilitates the methyl cap-independent translation initiation of the HCV RNA coding region. By means of multiple intermolecular contacts, direct binding of 40S ribosomal subunit and translational initiating factor eIF3 takes place at the specified IRES loci (Spahn *et al.* 2001). That leads to exact placement of AUG codon on the ribosome and to the initiation of translation without the participation of other cellular translational initiating factors (*e.g.*, eIF4A, eIF4B, eIF4F) and also without searching the initiatinon AUG codon by the RNA bound ribosomes (Tsukiyama-Kohara *et al.* 1992; Wang *et al.* 1993).

Ribosomes enter contact with IRES in the vicinity of initiation AUG codon at position 342, and they bind directly to or very close to the initiation codon. In the situation when RNA scanning and searching for the initiating point for translation occurs, this scanning is limited to a minimal segment of 6 nucleotides upstream and 9 nucleotides downstream of the initiation codon (Rijnbrand *et al.* 1996).

Multiple interactions between IRES and ribosome lead to the generation of stable binary complexes (quite unique for eukaryotic cells) without the involvement of initiating factors. This feature is unique within the RNA biological systems, and does not have any equivalent among eukaryotic cells. Similar binary complexes were observed only in prokaryotes (Pestova *et al.* 1998). It resembles the interaction between 30S ribosomal subunit and Shine–Dalgarno sequence of prokaryotes. Therefore, some of the atypical attributes of HCV IRES are similar to the initiation of prokaryotic protein synthesis (McCarthy *et al.* 1994; Pestova *et al.* 1998).

The complex structure of IRES is responsible for correct location of the initiation codon on peptidyl position ("P" position) of the ribosome. During binding of HCV IRES RNA to 40S subunit, the ribosome subunit undergoes conformational changes. This is the first example of active manipulation of host replication apparatus by a viral RNA (Spahn *et al.* 2001). Structural changes are apparently essential for activation, translation initiation and translation itself without the involvement of other initiating factors. Translation initiation mediated by IRES is unexpectedly much simpler than initiation dependent on methyl cap.

As mentioned *above*, the 5′-NC region where IRES is located has a complex secondary and tertiary structure (*see* Fig. 4). One part of the HCV IRES pseudoknot is a hairpin with internal loop of domain IV, where located on the 342 position is the initiation codon (AUG) and a short segment of coding sequence from the 5′-end of core-coding region (Honda *et al.* 1996). Exact position of IRES 3′-end is currently a matter of ongoing research. Some studies show that IRES 3′-end outreaches the 5′-NC region and overlaps a part of the core-coding region, where it occupies the initial 12–30 nucleotides (Reynolds *et al.*1995). Thus HCV IRES is different from IRES of *Picornaviridae* where the initiating codon is not the part of IRES.

Coding sequence by itself is apparently important for initiation of translation, although the exact mechanism of mutual interaction is not known. It was confirmed in *in vitro* experiments that HCV IRES function is dependent on the presence of sequences located distally from initiating codon. The presence of coding sequences (not their products, *i.e*. core protein) is essential (Zhao *et al.* 1999).

It is not clear whether the core-coding region is a functional component of IRES or whether its presence is essential only for blocking of undesirable bonds between IRES and coding sequences. Pseudoknot formed by nucleotide matching between the loop IIIf and region just upstream of the loop of IV domain is essential for adequate IRES functions (Wang *et al*. 1995; Honda *et al.* 1996). We expect that the coding region is essential for interaction with 5′-NC IRES region and leads to establishment and stabilization of essential secondary structures.

IIa, IIIb and IIIc hairpins are also important for IRES correct function (Rijnbrand *et al.* 1995; Honda *et al*. 1996). Deletion of these hairpins considerably limits 48S initiation-complex assembly. Domains II and III 5′-NC are essential for IRES activity. Highly conserved regions downstream of the initiation codon at the 5′-end of core protein-coding region are essential for optimal IRES-controlled translation (Honda *et al.* 1996). IRES is a critical region for viral replication and HCV infectivity (Bartenschlager *et al.* 2000; Lindenbach *et al.* 2001).

The 5′-NC region probably contains also the reverse components of the information, that can be recognized by the replication complex (*i.e*. in reverse from the 3′-end of the negative ssRNA), and direct the initiation of the positive RNA strand.

4.2 3′-Noncoding region (3′-NC region)

The 3′-end of the NC region was discovered as late as 1996 (Tanaka *et al.* 1995; Kolykhalov *et al.* 1996). According to our current knowledge, this region consists of a short (≈40 nucleotides) variable domain, polyuridine–polypyrimidine region and the last, highly conserved sequence (≈98 nucleotides). The two latter domains probably play a key role in the infectivity of HCV as it was experimentally shown with animal (chimpanzee) models. The 3′-NC increases the efficiency of the HCV genome translation, and it likely serves as a primer for the RNA synthesis (for the NS5B protein description *see below*).

The secondary structure of 3′-NC has not yet been fully elucidated (Fig. 5). HCV 3′-NC interacts with the spectrum of host cell proteins, including polypyrimidine tract binding protein (PTB). This interaction leads to increased translational activity observed in genomic RNA, *viz*. for those comprising 5′-IRES and the 3′-NC regions.

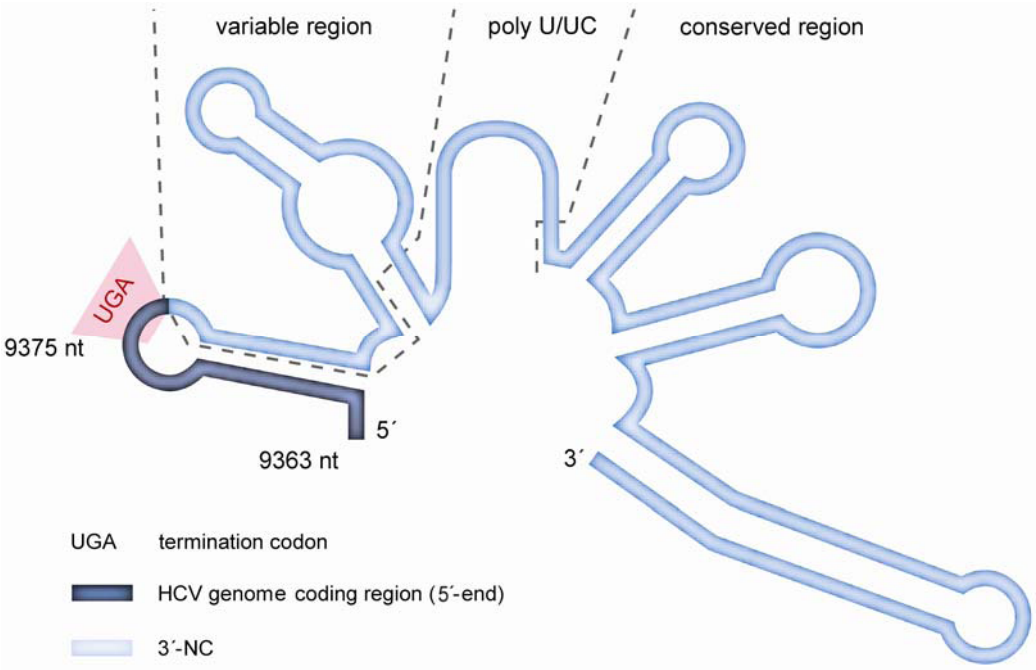

Fig. 5. Secondary structure of 3′-NC region (adapted from Lindenbach *et al.* 2001); nt – nucleotide.

5 STRUCTURAL HCV PROTEINS

5.1 Core protein

Core protein is a highly conserved basic protein, which forms the main component of HCV nucleocapsid. During viral replication it is cleaved from the large polyprotein first. The cellular signal peptidase localized in ER of host cells is responsible for the cleavage in the core E1 junction area. Core protein exists in 2, or alternatively 3 forms with the molar mass of 21 kDa (191 amino acids), 19 kDa (173 amino acids) and 16 kDa (Lo *et al.* 1995). The smaller forms (16 and 19 kDa) result from cleavage at a second site near residue 173, where the signal sequence of E1 protein is cleaved off by a so far unspecified enzyme and the "mature" form of C protein is created.

The 19 and 21 kDa proteins are bound on membranes of ER, while the 16 kDa truncated form is localized preferentially to the perinuclear space (Lo *et al.* 1995). According to more recent observations, the smallest form of core protein is not generated by post-translational splicing, but by translation from the second (alternative) reading frame (Xu *et al.* 2001). Thus, based on the observation of variable location of these forms of core protein, one can estimate multiple roles for the core protein in the HCV replication cycle and mechanism of the infection. A small proportion of 19 kDa protein is translocated into the nucleus. Earlier publications assumed that this form of the core protein has a regulatory function in the nucleus and affects the expression of host cell genome; later papers showed that the ratio of particular core protein subtypes (21, 19 and 16 kDa) as well as their location within the host cell is important for cell transformation (Chang *et al.* 1998).

As mentioned *above*, the genome sequence coding for the core protein is highly conserved even within the different HCV genotypes. The core protein coding region can be divided into three primary domains. The first (amino acids 1–122) contains a high proportion of basic residues; the second domain (123–174) is more hydrophobic than the first domain, and its C-terminus is identical to the C-terminus of a 19-kDa protein. The third domain (amino acids 175–191) is highly hydrophobic and serves as a signal sequence for E1 (McLauchlan *et al.* 2000). Core protein has pleiotropic functions; it is a structural protein of HCV nucleocapsid, and additionally it has the capability of influencing the apoptosis, lipid metabolism, transcription, host cell transformation and immune response of the infected host (McLauchlan *et al.* 2000).

Structural function – forming the major component of the HCV nucleocapsid – is the primary function of the core protein. According to the majority of authors, the N-terminus hydrophilic portion (the initial 115 amino acids of core protein) is essential for mutual interaction and multimerization (McLauchlan *et al.* 2000). The interaction between the core and E1 proteins is likely to be necessary during the assembly of viral particles.

The specific interaction between the core protein and viral HCV RNA has also been experimentally demonstrated. Shimoike *et al.* (1999) have documented that the above interaction takes place at the RNA strand of positive polarity within a span of initial 2327 nucleotides, *i.e.* from 5′-NC to the E2 coding region. HCV core protein expression and subsequent interaction with 5′-NC region down-regulates the translation of viral RNA. Based on this knowledge we can assume that the core protein plays an important role not only in encapsidation but also during the regulation of expression of particular viral proteins and establishment of chronic HCV infection.

Recent publications provide evidence that the core protein, particularly the short sequence located at the N-terminal part of the first domain, influences the translational potential of IRES (*see above* in the text – 5′-NC region) localized at 5′-end of the viral genome within the 5′-NC (Wang *et al.* 1995; Honda *et al.* 1996) (*see* Fig. 4).

The observation that core protein can induce steatosis in transgenic mice can be explained by the ability of the core protein to bind to fat droplets and apolipoprotein II (Barba *et al.* 1997). Core protein bound to apolipoprotein ousts from the original connection the second protein commonly associated with HDL particles, adipophyllin.

Core protein also interacts with LTβR, TNF and Fas. These interactions most probably influence the efficacy of the host antiviral immune response and very likely function as one of the key elements for the development of chronic infection. They are the likely explanation for the relation between the core protein expression and changes in the host cell sensitivity to apoptosis (Nagata *et al.* 1995; Ray *et al.* 1996; Chen *et al.* 1997; Marusawa *et al.* 1999). Activation of NF-κB by HCV core protein, independently described by several authors, also influences apoptosis. Anti-apoptotic effect of this interaction protects the infected cell from anti-Fas- and TNF-α-induced cell death (Marusawa *et al.* 1999). LTβR receptor functions have not yet been fully elucidated. This receptor is expressed on the surface of several cell types with the exception of T- and B-lymphocytes. We can speculate that in chronic HCV infection, the effect of the HCV core-protein

binding on LTβR augments the immune response, which subsequently leads to extrahepatic manifestations of HCV infection (Lunel *et al.* 1994; Matsumoto *et al.* 1997). The core protein ability to interact with several members of TNFR family determines its key role in the pathogenesis of HCV infection.

The binding of core protein to so-called "death domain" of the TNFR1 has been shown (Zhu *et al.* 1998). (Cytoplasmic side of the TNF receptor 1 bears the sequence called the "death domain"; it is indispensable for the main effect of the TNF induction, *i.e.* cell death signaling and NF-κB activation.) The cells are more susceptible to TNF-induced apoptosis not due to up-regulation of TNFR1 expression, as originally assumed, but through the effect of the above interaction (Zhu *et al.* 1998).

Besides apoptosis, there are several actions influenced by the TNFR1 pathway, including inflammation and tumor necrosis, as well as cell proliferation and differentiation. TNF level in the sera of the persons with chronic HCV infection is significantly higher (Tilg *et al.* 1992). The increased sensitivity of the core-protein-expressing cells towards the action of TNF together with the enhanced secretion of TNF renders the infected hepatocytes more vulnerable. What evolutionary advantage is there for the virus to express this protein that potentiates apoptosis of infected host cells? One possible explanation can be that the apoptosis of the infected cell containing mature viral particles leads to a more efficient dissemination of the infectious agent. The dissemination of newly synthesized HCV particles embedded within the apoptotic bodies lead to only limited inflammation, and immune cells and antibody reaction of the host (Teodoro *et al.* 1997).

Discrepancies regarding the core-protein effects on apoptotic mechanisms are apparent from the above cited works. One group of investigators demonstrated anti-apoptotic effects, while another group showed an opposite – pro-apoptotic effect; the difference in these observations has not yet been explained. It cannot be ruled out that core protein has an ambiguous role in such a complex mechanism as cell death.

Many authors documented core-protein occurrence (especially the small 16 kDa form) in the nucleus and nucleolus. The significance of this observation has not yet been adequately explained. Several possible functions are presumed, including the *trans* modulation of the cellular gene transcription, and also, for example, repression of the viral transcription of HIV and HBV during co-infection with HCV. The latter effect is regulated by phosphorylation of the core protein on the serine residues at the 99 and 116 positions (Shih *et al.* 1995). Experiments describe the ability of the core protein to transform rat embryonic fibroblasts and immortalize some cell lines (Chang *et al.* 1998). In one study (Moriya *et al*. 1998), the core-protein expression induced hepatocellular carcinoma in transgenic mice. Another, newly observed mechanisms, by which the core protein controls gene expression on the splicing level in infected cells, is its binding to human "DEAD box" protein DDX3, a putative RNA helicase (Owsianka *et al.* 1999).

(Helicases are involved in unwinding nucleic acids. The DEAD box helicases are involved in various aspects of RNA metabolism, including nuclear transcription, pre-mRNA splicing, nucleocytoplasmic transport, translation, *etc*. The DEAD-box RNA helicase family has six specific conserved sequence motifs of amino acids; the name of the family comes from one of the motifs, which contains four amino acids (D, E, A and D) in characteristic location.)

5.2 Glycoproteins E1 and E2

Structural proteins E1 (30–35 kDa) and E2 (70–72 kDa) are extensively modified by glycosylation. They bind to other proteins on ER membranes, *viz*. with calnexin, calreticulin and BiP (heavy immunoglobulin chain binding protein). E1 and E2 proteins are localized in the lumen of the ER. Noncovalent interaction of both proteins has been described; they form so-called natural heterodimers. Simultaneously the E1–E2 aggregates, connected with a disulfide bond, have been documented (Flint *et al.* 1999). Heterodimers are resistant to proteinases. Substantial for viral particle assembly and establishment of natural heterodimers are mainly the intramolecular disulfide bonds in the E1 protein and its glycosylation.

For the above reasons, the co-expression of E2 protein is essential for adequate heterodimer creation. Viral glycoproteins E1 and E2 are likely to play an important role in host cell interaction. The fact that HCV glycoproteins are localized solely within the membranes of endoplasmic reticulum and are practically not detectable on the plasma membrane of infected cells supports the hypothesis that HCV (similar to other members of *Flaviviridae* family) is released from host cells by budding from ER and subsequent exocytosis (Lindenbach *et al.* 2001). Absence of the viral proteins on the cell membrane of the infected cells also limits the host immune response potential and could contribute to the establishment and maintenance of chronic infection.

E2 ectodomain binds to the extracellular domain of the human surface molecule CD81 (amino acids 113–201) (Pileri *et al.* 1998). Despite the absence of any direct evidence, this interaction is apparently essential for the attachment and entry of HCV into cells. However, with respect to CD81 ubiquity, it does not

explain HCV tropism. This is why it is believed that other factors are needed for membrane fusion and subsequent HCV entry into host cells (Bartosch *et al.* 2003).

The so-called hypervariable region (HVR1) is located at the N-terminus of E2 protein (Kurosaki *et al.* 1993; Bartenschlager *et al.* 2000; Lindenbach *et al.* 2001). Neutralizing antibodies produced by the immune system of the infected host are aimed specifically to the E2 HVR1 region (Weiner *et al.* 1992). This region, or more precisely its sequence variability is part of viral host evasion strategy. It is the way how HCV copes with the host immune defense and potentiates viral "quasispecies" selection capable of escaping the host immune system (Weiner *et al.* 1992; Shimizu *et al.* 1996). However, HVR1 region, despite its undeniable importance, is not essential for HCV replication. The E2 glycoprotein expression inhibits interferon-induced dsRNA-activated PKR protein kinase. The precise mechanism of how this important pathway of antiviral defense is evaded is not known (Lo *et al.* 1995). Several viruses, including HCV, possess the strategy to overcome PKR antiviral effect (Taylor *et al.* 1999).

6 NONSTRUCTURAL HCV PROTEINS

6.1 Protein p7

P7 protein is a highly hydrophobic polyprotein located at the C-terminus of the E2 locus. Host signal peptidase is responsible for cleavage in this area. The exact function of the p7 protein remains unknown. It is not even clear whether it is a small structural protein or whether it has the functions of nonstructural proteins. Analogously to pestivirus p7 protein, we can assume that also this protein is not the structural part of the virion (Elbers *et al.* 1996). We can speculate that its function could be the interaction with the host cells. p7 also appears to be essential for infection (Sakai *et al.* 2003). *In vitro* studies have shown that p7 acts as a calcium ion channel; it can be inhibited by amantadine (Griffin *et al.* 2003, 2004).

6.2 NS2 protein

This protein of a molar mass of ≈23 kDa contains a domain, predicted to interact with the N-terminus of the adjacent NS3 protein. The resulting NS2/3 proteinase is specific for the NS2/NS3 cleavage site. Cleavage at the NS2/NS3 junction is the first posttranslational autocatalytic cleavage of the large polyprotein (Hijikata *et al.* 1993).

Except for the above described proteinase activity, no other function of NS2 protein has yet been described.

6.3 NS3 protein

HCV NS3 protein with a molar mass of 70 kDa similar to that of other *Flaviviridae* consists of two domains. Proteinase domain at the N-terminus extends through one-third of the locus (approximately initial 180 residues) while the NTPase–helicase domain occupies the remaining two-thirds of the locus at the C-terminus (Kim *et al.* 1995).

Serine proteinase is necessary for HCV infectivity as well as for co- and post-translational cleavage at NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B sites (Fig. 2). The cleavage at NS3/4A site is *cis*, and all remaining cleavages are *trans*. NS4A (*see below*) is a serine proteinase co-factor essential for all above mentioned cleavages with the exception of NS5A/5B.

The structure of HCV NS3 proteinase is similar to the structure of other members of trypsin proteinase superfamily. Unlike the others NS4A cofactor is an integral part of its structure and interacts with N-terminus residues (Tai *et al.* 1996).

HCV NS3 helicase is a member of Asp-Glu-Cys-His subgroup of so-called "DEAD-box" helicases (for details *see Chapter 5.1*). Its ATPase activity is stimulated by ssRNA.

This helicase unwinds RNA and DNA homo- and heteroduplexes in $3' \rightarrow 5'$ direction. Mg^{2+} or Mn^{2+} cations and ATP are essential for its activity and so the helicase activity and ATP hydrolysis connection can be assumed.

The exact role of RNA helicase in the replication process is not known. Nevertheless NS3 mutations, which alter helicase activity, affect HCV infectivity *in vitro*. Helicase is essential for viral translation and replication.

Besides the above-described role in HCV replication and posttranslational editing, the NS3 protein presumably has also other functions that interfere with host cell functions. As an example, NS3 inhibits phos-

phorylation mediated by cAMP-dependent protein kinase (*i.e.* protein kinase A, PKA) thus induces cell signal transduction.

NS3 proteinase domain has a weak transforming capacity shown experimentally on NIH-3T3 cells. It weakens actinomycin D induced apoptosis. It is not yet clear, whether this effect has a causal relation to the observed shared localization of NS3 and p53 products in cells (Muramatsu *et al.* 1997).

6.4 NS4A protein

This protein (8 kDa) contains a hydrophobic domain at the N-terminus, which is likely to interact with the membranes and with other replicase components. NS4A protein is also an essential cofactor of NS3 proteinase and its presence is vital for posttranslational cleavage of the primary large polyprotein (Tanji *et al.* 1995*a*; Tomei *et al.* 1996). The serine proteinase cofactor activity of this protein is located in the central portion of NS4A.

6.5 NS4B protein

NS4B is a hydrophobic protein (≈30 kDa) of unknown function (Bartenschlager *et al.* 2000; Lindenbach *et al.* 2001). The expression of NS4B can induce the formation of a "membranous web" derived from the ER with which all the genomic HCV RNA and all the viral proteins are associated (Gosert *et al.* 2003). As in the case of other members of the *Flaviviridae* family, this is probably the site of RNA replication in HCV-infected cells.

6.6 NS5A protein

Despite belonging to hydrophilic proteins, NS5A is bound to membranes and exists in at least 2 forms with a molar mass of 56 and 58 kDa. Originally, it has been assumed that these forms originate by different proteolysis, but according to the latest findings they are products of different phosphorylation. NS5A is phosphorylated particularly on the serine residues and to a minor extent on threonine.

The sites of product 58 "hyperphosphorylation" were mapped by means of deletion analysis of HCV-J viral isolate (HCV subtype 1b). They are located in the central part of NS5A polyprotein between amino acid residues 2200 and 2500 in the positions Ser 2197, Ser 2201 and Ser 2204 (Tanji *et al.* 1995*b*). NS5A protein phosphorylation is a feature common to a broader spectrum of HCV isolates.

The major site of both *in vitro* and *in vivo* phosphorylation of HCV subtype 1a isolates was amino acid residue Ser 2321 (Reed *et al.* 1999). This serine residue is not present in all HCV genotypes since the C-terminal part of the coding region is considerably variable and varies among particular isolates. Recent studies demonstrate that the influence on NS5A phosphorylation is multifactorial, and virtually all NS proteins localized upstream of NS5A (towards the 5′-end of viral genome) are involved (Koch *et al.* 1999). It is assumed that NS5A is a component of the HCV replication complex.

The kinase responsible for NS5A phosphorylation is probably of cellular origin. This hypothesis is corroborated by several observations. The first, NS5A does not contain any known kinase motif, and the second, phosphorylation takes place even within the tissue culture where NS5A is produced in isolated fashion in the absence of other viral proteins; and, finally, NS5A expression by *E. coli* requires the addition of eukaryotic cell extracts for phosphorylation to occur (Reed *et al.* 1997). This kinase is Mn^{2+} dependent, at least in an *in vitro* experiment, and Ca^{2+} ions have an inhibitory effect at a concentration of >0.25 mmol/L.

Detailed understanding of the role of NS5A protein and NS5A phosphorylation in the process of viral replication is a matter of further research.

Within the NS5A coding region, particularly between the 2209 and 2248 amino acid residues, is a region called interferon sensitivity-determining region (ISDR). The existence of such region was confirmed only by studies performed in Japan in HCV subtype 1b infected patients (Enomoto *et al.* 1996).

Besides the connection of ISDR and interferon-treatment response, which has not yet been confirmed in non-Japanese and non-1b subtype Japanese patients, NS5A inhibits interferon-induced dsRNA-dependent kinase PKR activity. (PKR is the main effector of host antiviral defense; it reduces viral translation by phosphorylation of the α-subunit of initiation translational factor eIF2.)

NS5A probably interacts with PKR and inhibits dimerization, which is essential for PKR activation. NS5A region, which is likely responsible for this interaction, comprises putative ISDR locus and another residue downstream of this locus (Gale *et al.* 1997). The observation confirmed by several other investigations showed that cells producing NS5A are capable of partial resistance to interferon antiviral effect. It has been further learned that mutations that enable more effective initiation of subgenomic HCV replicon replication in tissue cultures occur in the NS5A region, especially in the region upstream of ISDR.

The above observations demonstrate an important and very active role that NS5A plays during HCV replication. Modulation of NS5A function by phosphorylation could be one of the regulatory mechanisms for one or more steps in HCV replication.

NS5A interacts with a wide spectrum of cellular proteins (*e.g.*, snare-like protein, hVAp-33, SRCAP, *etc.*), and the significance of these interactions is further studied (Ghosh *et al.* 2000).

6.7 NS5B protein

NS5B coding region is highly heterogeneous among particular HCV strains. This variability was used to establish the classification system of HCV strains into genotypes and subtypes (Simmonds *et al.* 1993) (Fig. 6).

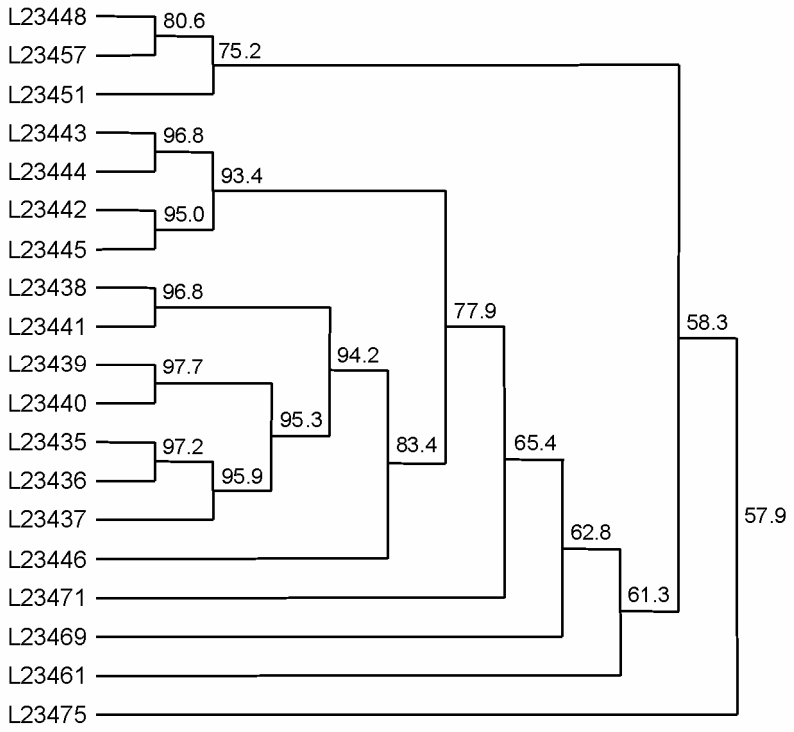

Fig. 6. Phylogenic tree of HCV genotypes and subtypes according to NS5B region (DNASIS software); comparison of the 222-bp fragments of NS5B coding regions (Simmonds *et al.* 1993). The reference strain sequences according to the *GenBank*: HCV subtypes: 1a – L23435-41, 1b – L23442-5, 1c – L23446, 2a – L23448, 2b – L23551, 2c – L23457, 3a – L23461, 4a – L23469, 5a – L23471, 6a – L23475; *numbers* at lines – % similarity.

This hydrophilic protein (molar mass ≈68 kDa) contains the GDD motif, which is common to all viral RNA dependent RNA polymerases. The biochemical activity of this viral RNA-dependent RNA polymerase (RdRp) is dependent on divalent cations (Mn^{2+} or Mg^{2+}), pH near to neutral and a very low concentration of salts. The estimated rate of HCV RNA template elongation is 150–200 nucleotides per min and is not dependent on the NS5B concentration. As mentioned above the presence of Mn^{2+} or Mg^{2+} cations is essential for the optimum RdRp activity, and these cations have inhibitory functions (Ferrari *et al.* 1999). In the presence of Mn^{2+} the RdRp activity is ≈20 times higher than in the presence of Mg^{2+} ions (Luo *et al.* 2000).

Mutations in the RdRp polymerase active site halt the replication of subgenomic HCV RNA in the culture as well as the *in vivo* HCV infectivity. The experiments with recombinant NS5B protein confirmed the polymerase activity *in vitro*; these reactions have not shown the specificity of RdRp towards the HCV templates.

Oligonucleotide primers can be extended by the NS5B polymerase on homopolymeric templates or cellular RNAs. The 3′-end of HCV genomic RNA contains noncoding sequences (*see below*), which include a stable hairpin (which is formed from the RNA chain, where the amino acid residues are interconnected in complementary fashion according to the Watson–Crick rules, similarly as in dsDNA). Particularly this part of HCV genome can also be utilized as a self-priming template for RNA extension (Lohmann *et al.* 1997).

NS5B catalyzes not only the RNA elongation; recently the ability of *de novo*, primer-independent RNA synthesis has been discovered *in vitro*, usually when increasing the nucleotide concentration (Luo *et al.* 2000). High concentration of GTP stimulates *in vitro* RNA synthesis by nearly 100-fold (Lohmann *et al.* 1999).

The C-terminus hydrophobic domain within the NS5B coding region is responsible for the connection with the nuclear membrane, where the HCV replication probably takes place. It also contains motifs for the interaction with the NS5A. The deletion analysis has shown the hydrophobic nature of C-terminus 21 amino acid residues of NS5B. The truncated form of NS5B RdRp with the C′-terminal deletion is soluble, while the complete RdRp is located in the perinuclear space anchored to the membrane. These findings are consistent with the hypothesis that the C′-terminus of RdRp is not necessary for the polymerase activity but probably plays an important role in the replication cycle of viral RNA *in vivo*. Even though the bond between RNA and NS5B is nonspecific, some preference in the binding with the RNA containing the 3′-NC region has been documented. NS5B interacts directly with the NS3 and NS4A HCV viral proteins, and these proteins further form complex with the NS4B and NS5A. The above interactions among the viral proteins are likely to be essential for the function of the HCV RNA replication complex.

7 CONCLUSIONS

Despite considerable efforts exerted during the recent 15 years to elucidate the basic structure and function of viral proteins as well as the mechanism of HCV replication, our current knowledge is not complete. Basic research of this infectious agent is limited by the inability to cultivate the virus by common methods and also by the lack of small animal models.

In vitro experiments performed by utilizing the best of the most recent advances in molecular biology are limited by the fact that HCV replication or transcription of particular viral products is done under the artificial conditions detached from interactions with host cell and macroorganism. Thus, it is often very tricky to read correctly the obtained results. Sometimes we observe contradictory results from particular laboratories.

Detailed understanding of viral protein function and exact mechanisms of HCV replication is actually the prerequisite for antiviral treatment development. To achieve a potent control of this infection requires further research with the identification of the new targets for antivirals. In the initial phases of clinical trials several new agents appear promising, for example the inhibitors of helicase activity, caspase inhibitors and plenty of others.

In the light of clinical and economic burden of HCV late complications, the outcomes of basic virological and immunological research are impatiently awaited by both physicians and their patients.

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