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REVIEW ARTICLE

Replication of hepatitis C virus

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Introduction

After the development of diagnostic tests for hepatitis A and hepatitis B viruses in the 1970s, an additional parenterally transmitted agent responsible for the majority of transfusion-associated non-A, non-B hepatitis cases was recognized. The identification of this agent turned out to be very difficult and only with the advent of recombinant DNA technology was it possible to clone the genome of the virus that was termed hepatitis C virus (HCV) (Choo *et al.*, 1989). Since then HCV has become a focus of intensive research for several reasons (Lavanchy *et al.*, 1999). First, most infections persist, leading in about 50% of all cases to chronic hepatitis, which can develop into chronic active hepatitis, liver cirrhosis and hepatocellular carcinoma. Second, HCV is distributed worldwide, with the number of infected individuals being estimated to be ~ 170 million. Third, the only therapy currently available is combination treatment with a high dose of interferon-α (IFN-α) and the nucleoside analogue ribavirin. However, only ~ 40% of all patients benefit from this treatment and develop a sustained response, demonstrating the urgent need for more effective antiviral therapeutics.

HCV genome organization

HCV has been classified as the sole member of a distinct genus called hepacivirus in the family *Flaviviridae*, which includes the flaviviruses, the animal pathogenic pestiviruses and, although this awaits official confirmation, the recently cloned GB virus A (GBV-A), GBV-B and GBV-C/hepatitis G viruses (Murphy *et al.*, 1995). These viruses have in common an enveloped particle harbouring a plus-strand RNA that, in the case of HCV, has a length of ~ 9600 nucleotides. The genome carries a single long open reading frame (ORF) encoding a polyprotein that is proteolytically cleaved into a set of distinct products (Fig. 1). Translation of the HCV ORF is directed via a ~ 340 nucleotide long 5′ non-translated region (NTR) functioning as an internal ribosome entry site (IRES) and permitting the direct binding of ribosomes in close proximity to the start codon of the ORF (Tsukiyama-Kohara *et al.*, 1992; Wang *et al.*, 1993). The first ~ 40 nucleotides of the RNA genome are not required for translation but, based on analogy with other plus-strand RNA viruses, are involved most likely in RNA replication (Boyer & Haenni, 1994). The 3′ NTR was only recently discovered (Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1995, 1996; Yamada *et al.*, 1996). It has a tripartite structure composed of a variable sequence following the stop codon of the ORF, a poly(U) tract of heterogeneous length and a highly conserved 98 nucleotide sequence essential for replication *in vivo* (Yanagi *et al.*, 1999; Kolykhalov *et al.*, 2000).

The HCV polyprotein is cleaved co- and post-translationally by cellular and viral proteinases into ten different products, with the structural proteins located in the amino-terminal one-third and the nonstructural replicative proteins in the remainder (Fig. 1) (for recent reviews see Bartenschlager, 1999; Reed & Rice, 1998). The first cleavage product of the polyprotein is the highly basic core protein, forming the major constituent of the nucleocapsid (Yasui *et al.*, 1998). In addition, a number of other functions like modulation of several cellular processes or induction of hepatocellular carcinoma in transgenic mice have been described (Chang *et al.*, 1998; Chen *et al.*, 1997; Matsumoto *et al.*, 1997; Moriya *et al.*, 1998). Envelope proteins (E1 and E2) are highly glycosylated type 1 transmembrane proteins, forming two types of stable heterodimeric complexes: a disulfide-linked form representing misfolded aggregates and a non-covalently linked heterodimer corresponding most likely to the pre-budding complex (Deleersnyder *et al.*, 1997). In addition, E2 was shown to interact with the IFN-induced double-stranded RNA-activated protein kinase PKR. Upon induction by IFN-α, this enzyme reduces protein synthesis via phosphorylation of translation initiation factor eIF2-α, but in cells containing E2, PKR is inhibited, allowing continuation of translation in the presence of IFN (Taylor *et al.*, 1999). Protein p7, located at the carboxy terminus of E2, is a highly hydrophobic polypeptide of unknown function. Most of the nonstructural (NS) proteins 2–5B (the term indicates that these proteins are not expected to be constituents of the virus particle) are required for replication of the viral RNA (Lohmann *et al.*, 1999*b*). NS2 and the amino-terminal domain of NS3 constitute the NS2–3 proteinase, catalysing cleavage at the NS2/3 site (Grakoui *et al.*, 1993*a*; Hijikata *et al.*, 1993*a*; Hirowatari *et al.*, 1993). NS3 is a bifunctional molecule carrying, in the amino-terminal ~ 180 residues, a serine-type proteinase responsible for cleavage at the NS3/4A, NS4A/B, NS4B/5A and NS5A/B sites and, in the carboxy-terminal remainder, NTPase/helicase activities

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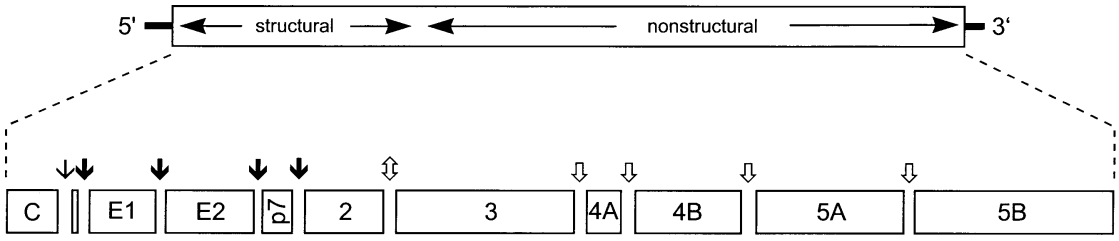

Fig. 1. HCV genome organization and polyprotein cleavage products. A schematic representation of the HCV genome indicating the positions of the structural and the nonstructural proteins within the polyprotein as well as the 5′ and 3′ NTRs (thick lines). The polyprotein cleavage products are drawn below. Cleavage sites for host cell signalases (↓), the NS2–3 proteinase (⇳), the NS3 proteinase (⇩) and an unknown cellular proteinase (↓) are marked.

essential for translation and replication of the HCV genome (Bartenschlager *et al.*, 1993; Eckart *et al.*, 1993; Grakoui *et al.*, 1993*b*; Gwack *et al.*, 1996; Hong *et al.*, 1996; Kim *et al.*, 1995; Suzich *et al.*, 1993; Tai *et al.*, 1996; Tomei *et al.*, 1993; Kolykhalov *et al.*, 2000). In addition, NS3 may have other properties involved in interference with host cell functions like inhibition of protein kinase A-mediated signal transduction or cell transformation (Borowski *et al.*, 1996; Sakamuro *et al.*, 1995). NS4A is an essential cofactor of the NS3 proteinase and is required for efficient polyprotein processing (Bartenschlager *et al.*, 1994; Failla *et al.*, 1994; Lin *et al.*, 1994 *b*; Tanji *et al.*, 1995*a*). The function of the hydrophobic NS4B is so far unknown. NS5A is a highly phosphorylated protein and, at least with some HCV isolates, the level of phosphorylation is influenced by NS4A via direct interaction with NS5A or it requires the expression of NS5A in the context of a NS3–5A polyprotein (Asabe *et al.*, 1997; Kaneko *et al.*, 1994; Koch & Bartenschlager, 1999; Neddermann *et al.*, 1999; Tanji *et al.*, 1995*b*). NS5A phosphorylation is mediated by an as yet unknown cellular kinase (Ide *et al.*, 1997; Reed *et al.*, 1997; Tanji *et al.*, 1995*b*). For the HCV-H isolate the major phosphorylation site has been mapped to serine residue 2321 of the polyprotein and the proline-rich nature of the flanking sequence suggests that a proline-directed kinase is responsible for NS5A phosphorylation (Reed & Rice, 1999). The role NS5A may play in RNA replication is so far not known, but based on analogy with other RNA viruses, where phosphoproteins are important regulators of replication, one could assume that NS5A plays a similar role. Apart from such a function, NS5A appears to be involved in resistance of the infected cell to the antiviral effect of IFN. At least for some HCV isolates NS5A is able to bind to PKR, blocking the translational reduction in the IFN-treated cell (Gale *et al.*, 1997, 1998). Interestingly, an alanine substitution for the major phosphorylation site at serine residue 2321 did not affect the NS5A:PKR interaction, showing that phosphorylation at this particular site is not required for complex formation with PKR (Reed & Rice, 1999). NS5B was identified as the RNA-dependent RNA polymerase (RdRp) (Al *et al.*, 1998; Behrens *et al.*, 1996; Lohmann *et al.*, 1997; Yamashita *et al.*, 1998; Yuan *et al.*, 1997).

Virus replication

HCV nonstructural proteins and viral RNA have been detected in livers of infected patients or experimentally inoculated chimpanzees, confirming that the liver is a site of HCV replication (for a review see Blight & Gowans, 1995). Unfortunately, the amounts of viral proteins and RNA in infected tissues are very low, necessitating the use of highly sensitive but also less reliable detection methods. This may in part explain why the reported number of HCV-positive cells detected in infected liver tissue is contradictory and estimates vary between less than 5% and up to 100% (Blight & Gowans, 1995). Apart from liver cells, there is strong evidence that HCV can also replicate in peripheral blood mononuclear cells (PBMCs) both *in vivo* and *ex vivo* or in experimentally infected B- and T-cell lines (see below). Such a lymphotropism may account for the numerous immunological disorders, in particular type II and type III cryoglobulinaemia, observed in more than 50% of chronic hepatitis C patients (Esteban *et al.*, 1998).

The dynamics of HCV replication can be deduced from the rapid rates of virus production and emergence of mutants. A careful analysis of viral dynamics during antiviral treatment of patients with IFN-α revealed a virion half-life of 3–5 h and a clearance and production rate of $\sim 10^{12}$ particles per day (Zeuzem *et al.*, 1998; Neumann *et al.*, 1998; Ramratnam *et al.*, 1999). Although in absolute amounts these numbers are high, they are not with respect to a single cell. Assuming that $\sim$ 10% of the hepatocytes of a liver are infected and that a liver contains $\sim 2 \times 10^{11}$ hepatocytes, this would correspond to a virion production rate of 50 particles per hepatocyte per day (Neumann *et al.*, 1998).

Another feature of HCV replication is the rapid generation of virus variants. In fact, based on the genomic variability in a small region of NS5B, HCV has been classified into at least six genotypes each with several subtypes (Simmonds *et al.*, 1993). Even within a patient HCV does not exist as a single entity but rather as a swarm of microvariants of a predominant 'master sequence', a phenomenon that has been referred to as quasispecies (for a review see Holland *et al.*, 1992). The production of such a large number of variants is primarily due

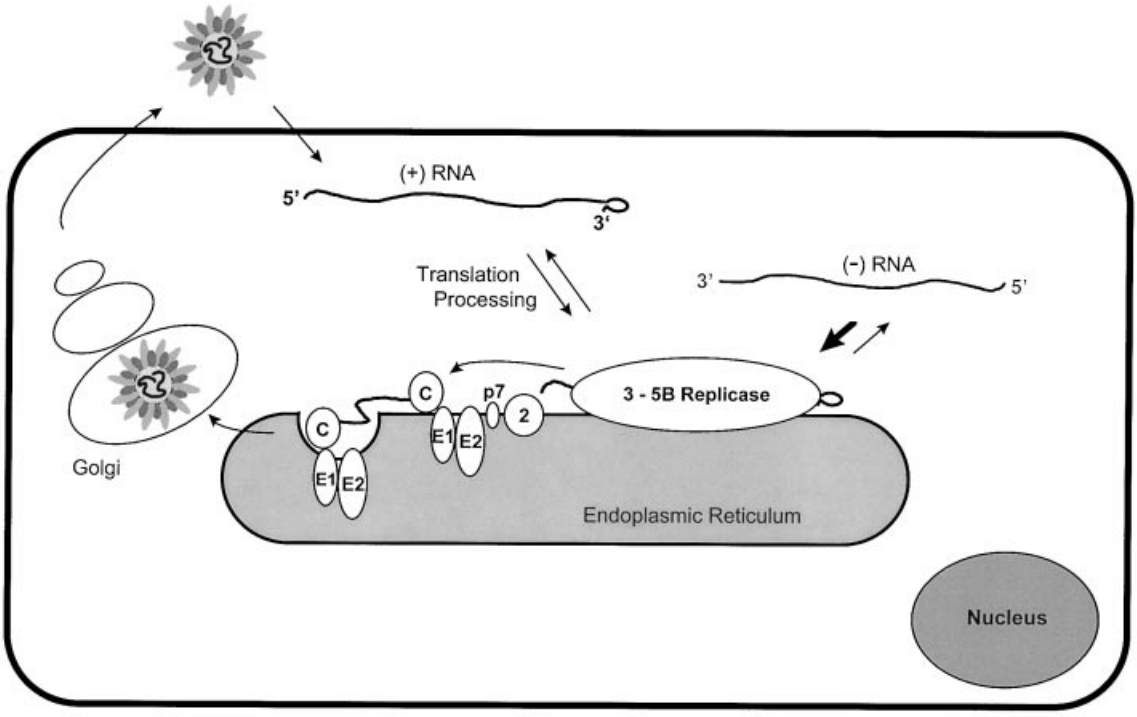

Fig. 2. Hypothetical model of the HCV replication cycle. Upon infection of the host cell (large rectangle) the plus-strand RNA genome (+RNA) is liberated into the cytoplasm and translated. The polyprotein is processed and viral proteins remain tightly associated with membranes of the ER. Minus-strand RNA (–RNA) is synthesized by the replicase composed of NS3–5B and serves as template for production of excess amounts of plus strand. Via interaction with the structural proteins plus-strand RNA is encapsidated. Particles are enveloped by budding into the lumen of the ER and virus particles are exported via transit through the Golgi complex.

to the high error rate of the viral RdRp that, based on analogies with RdRps of other plus-strand RNA viruses, is expected to be in the range of 10^{-4}. This high error rate is reflected by the high mutation rate observed in patients or experimentally inoculated chimpanzees. Using comparative sequence analyses of HCV genomes isolated over intervals of 8 or 13 years a mutation rate of $1{\cdot}44 \times 10^{-3}$ or $1{\cdot}92 \times 10^{-3}$ base substitutions per site per year was found, respectively (Ogata *et al.*, 1991; Okamoto *et al.*, 1992). The high variation observed with HCV replication may also account for the fact that a significant fraction of virus genomes appear to be defective (Martell *et al.*, 1992).

Owing to the lack of a convenient animal model and an efficient cell culture system our current understanding of the molecular mechanisms of HCV replication is based primarily on analogies to the closely related flavi- and pestiviruses and on the characterization of recombinant HCV proteins. Using this limited information the HCV replication cycle can be summarized as follows (Fig. 2): (1) penetration of the host cell and liberation of the genomic RNA from the virus particle into the cytoplasm; (2) translation of the input RNA, processing of the polyprotein and formation of a replicase complex associated with intracellular membranes; (3) utilization of the input plus-strand for synthesis of a minus-strand RNA intermediate; (4) production of new plus-strand RNA molecules which in turn can be used for synthesis of new minus strands, for polyprotein expression or packaging into progeny virions; (5) release of virus from the infected cell.

Attachment and entry

The first step in a virus life-cycle is the attachment of the infectious particle to the host cell, for which a specific interaction between a receptor on the cell surface and a viral attachment protein on the surface of the particle is required. Recently, CD81 was identified as a putative HCV receptor based on its strong interaction with E2 as well as with virus particles *in vitro* (Pileri *et al.*, 1998). Furthermore, preincubation of the HCV-containing plasma used for the binding studies with sera from chimpanzees that were protected from HCV challenge by vaccination with recombinant E1 and E2 also blocked *in vitro* binding of HCV to CD81 (Pileri *et al.*, 1998). However, whether virus binding to CD81 is followed by internalization of the virus particle is not known.

Apart from this route, HCV as well as other members of the *Flaviviridae* family may enter the cell by binding to low-density lipoprotein (LDL) receptors. Based on the observation that HCV particles are associated with beta-lipoproteins (Thomssen *et al.*, 1992), Agnello *et al.* (1999) analysed whether endo-

cytosis of HCV is mediated by LDL receptors. Using *in situ* hybridization to determine HCV-RNA-positive cells, a direct correlation between the level of cell surface-expressed LDL receptor and the number of positive cells was found. This result and the finding that HCV does not bind to COS-7 cells unless they have been transfected with the LDL receptor gene (Monazahian *et al.*, 1999) suggest that HCV particles associated with LDL bind to this receptor. Whether interaction with the LDL receptor or CD81 leads to a productive infection remains to be determined.

While the nature of the HCV receptor is not known currently, the major envelope glycoprotein E2 is thought to be responsible for initiating virus attachment to the host cell because E2-specific antisera can block binding to cells (Rosa *et al.*, 1996; Zibert *et al.*, 1995; Farci *et al.*, 1996). The role of E1 is less clear but the presence of a stretch of hydrophobic amino acids tentatively called the E1 fusion peptide, displaying similarities to the fusion peptides of paramyxovirus and flavivirus suggests that E1 is involved in membrane fusion (Flint *et al.*, 1999). To study the early steps in the HCV life-cycle, Lagging *et al.* (1998) generated vesicular stomatitis virus (VSV) pseudotypes. They expressed chimeric envelope proteins composed of the HCV E1 or E2 ectodomains fused to the transmembrane and cytoplasmic domains of the VSV G protein. As indicated by the formation of plaques, a baby hamster kidney cell line (BHK-21), the human T-cell line MOLT4 and the human hepatoma cell line HepG2 were susceptible to infection with the pseudotypes, but the human cervical carcinoma cell line HeLa and the human embryonic lung cell line L-132 were not. However, only pseudotypes carrying either the E1 or the E2 ectodomain were used, not pseudotypes with both chimeric HCV proteins. This is surprising, because E2 acts as a chaperone for E1, which in the absence of E2 forms misfolded aggregates (Michalak *et al.*, 1997). Furthermore, E1 and E2 form stable heterodimers that most likely represent the native form found in the HCV envelope (Dubuisson *et al.*, 1994). Finally, studies with engineered cell surface-expressed E2 glycoproteins folded in a manner comparable to authentic E2 demonstrated that this protein alone did not enable cell fusion (Flint *et al.*, 1999).

Polyprotein translation and processing

Once inside the cytoplasm the genomic RNA is directly translated. Since HCV most likely does not encode a methyl transferase activity and replicates in the cytoplasm where such cellular enzymes are missing, the genome is not capped. Therefore, translation of the viral RNA is not mediated by a cap-dependent mechanism with ribosomes scanning along the RNA up to the first initiator AUG codon (depending on the isolate there are 4–5 start codons upstream of the polyprotein translation start site), but rather by an IRES (Tsukiyama-Kohara *et al.*, 1992; Wang *et al.*, 1993). This RNA element residing approximately between nucleotides 40 and 355 forms four highly structured domains. With the exception of one report (Fukushi *et al.*, 1994) the small domain I, forming a single stem–loop between nucleotides 5 and 20, was shown to be dispensable for translation activity and the 5′ border of the IRES was mapped between nucleotides 38 and 46 (Honda *et al.*, 1996; Rijnbrand *et al.*, 1995; Yoo *et al.*, 1992). The fact that deletion of the region encompassing domain I stimulates translation suggests a regulatory role of this sequence for polyprotein translation. Because of its 5′ proximal position it may also be involved in RNA replication. Two oligopyrimidine tracts are present in the IRES, one in the apical loop of domain III and one between domain III and the small domain II. Domain IV consists of a small stem–loop containing the polyprotein start codon at nucleotide position 342 and forms a pseudoknot via base-pairing with a loop in domain III. Since mutagenesis or insertion of AUG initiator codons upstream from the polyprotein start codon have little effect on IRES activity, ribosomes appear to bind in close proximity to the polyprotein initiator AUG with little or no scanning (Reynolds *et al.*, 1996; Rijnbrand *et al.*, 1996). The mapping of the actual border of the 3′ end of the IRES is still a matter of debate. Most experimental evidence suggests that sequences of the core coding sequence, but not the core protein itself, are required for full IRES activity (Honda *et al.*, 1996; Reynolds *et al.*, 1995; Zhao *et al.*, 1999). However, whether the core-coding sequence is a real component of the IRES or is required to prevent unfavourable base-pairings of the IRES with downstream sequences, disturbing the structure of the 5′ NTR, is not known. In agreement with the latter assumption purified 40S ribosome subunits were found to bind to an IRES lacking core-coding sequences (Pestova *et al.*, 1998). In the same report it was also shown that the HCV IRES binds specifically to the 40S ribosome subunit and does not require any additional translation factors. This property is unique among all eukaryotic RNAs and resembles the interaction of the prokaryotic 30S ribosome subunit with the Shine–Dalgarno sequence (Pestova *et al.*, 1998). The IRES elements of picornaviruses require additional cellular factors for binding of the 40S ribosome subunit.

Activity of the HCV IRES is influenced by several factors. First, the X-tail at the very 3′ end of the HCV genome appears to enhance IRES-dependent translation by an as yet unidentified mechanism (Ito *et al.*, 1998). Second, several cellular factors have been demonstrated to bind to the HCV IRES and, in most cases, stimulate translation. These include polypyrimidine-tract-binding protein (PTB) (Ali & Siddiqui, 1995; Kaminski *et al.*, 1995), the La antigen (Ali & Siddiqui, 1997), heterogeneous nuclear ribonucleoprotein L (Hahm *et al.*, 1998) and as yet unidentified proteins with apparent molecular masses of 120, 87 and 25 kDa (Fukushi *et al.*, 1997; Yen *et al.*, 1995). The requirements for cellular factors for IRES activity may also explain the dependence on the cell cycle. Using cell lines stably expressing bicistronic reporter constructs with a cap-dependently expressed upstream reporter and a down-

stream reporter translated from the HCV IRES it was found that IRES-dependent translation was greatest in mitotic and lowest in quiescent (G_0) cells (Honda *et al.*, 2000). One possible explanation would be that HCV translation is regulated by cellular proteins that vary in abundance or activity during the cell cycle.

Directed by the IRES, the polyprotein is translated at the rough endoplasmic reticulum (ER) and cleaved co- and post-translationally by host cell signalases and two viral proteinases. In the last few years a wealth of information regarding the mechanisms of polyprotein processing has been published. It is beyond the scope of this review to summarize all of them and the interested reader is referred to recently published reviews (Bartenschlager, 1999; De Francesco *et al.*, 1998; Reed & Rice, 1998). As deduced from hydrophobic sequences preceding the cleavage sites and the dependence on microsomal membranes, the C–NS2 region is processed by host signal peptidases cleaving at the C/E1, E1/E2, E2/p7, p7/NS2 junctions (Fig. 1) (Grakoui *et al.*, 1993*b*; Hijikata *et al.*, 1991; Lin *et al.*, 1994*a*; Mizushima *et al.*, 1994). The production of processing intermediates, most notably an E2–p7–NS2 protein, indicates that not all cleavages within the structural region are cotranslational. Furthermore, a second post-translational cleavage close to the carboxy terminus of the core protein takes place, removing the E1 signal sequence by an as yet unidentified cellular enzyme (Santolini *et al.*, 1994; Hüssy *et al.*, 1996). Processing between NS2 and NS3 is a rapid intramolecular reaction and is accomplished by the NS2–3 proteinase (Grakoui *et al.*, 1993*a*; Hijikata *et al.*, 1993*a*; Santolini *et al.*, 1995). Efficient cleavage at this site requires the 130 carboxy-terminal residues of NS2 and the first 180 amino acids of NS3. Within the NS2 sequence His-952 and Cys-993 are essential for enzymatic activity (Grakoui *et al.*, 1993*a*; Hijikata *et al.*, 1993*a*) and it has been proposed that NS2–3 is a cysteine proteinase with His-952 and Cys-993 forming a catalytic dyad or, together with Glu-972, a catalytic triad (Gorbalenya & Snijder, 1998). Alternatively, owing to the activation by zinc and the inhibition by chelating agents like EDTA, the NS2–3 proteinase might be a zinc-dependent metalloproteinase (Hijikata *et al.*, 1993*a*). However, since zinc is required for proper folding of the NS3 proteinase domain it is not known whether zinc is bound by the NS2 domain too or whether the activating effect is due to proper folding of the NS3 domain. Alternatively, the zinc bound by the NS3 domain might play an essential role in catalysis (Wu *et al.*, 1998).

Processing of the NS3–5B region is mediated by the NS3 proteinase with the following preferred but not obligatory order of cleavages: NS3/4A → NS5A/B → NS4A/B → NS4B/5A (Bartenschlager *et al.*, 1994; Failla *et al.*, 1995; Lin *et al.*, 1994*b*; Tanji *et al.*, 1994). Processing at the NS3/4A site is a cotranslational intramolecular reaction whereas cleavage at the other sites can be mediated intermolecularly. However, the HCV proteins most likely form a stable higher-order complex associated with intracellular membranes (Hijikata *et al.*, 1993*b*; Ishido *et al.*, 1998; Koch & Bartenschlager, 1999; Lin *et al.*, 1997; Neddermann *et al.*, 1999). Therefore, even after liberation of NS3 from the polyprotein, the enzyme and its substrate are in very close proximity.

Although enzymatically active on its own, proteolytic activity of NS3 is greatly stimulated by NS4A both in transfected cells and in various *in vitro* assay systems. Coprecipitation studies demonstrated the formation of a detergent-stable complex and the interaction domains have been mapped to the ~ 30 amino-terminal residues of NS3 and a 12 residue sequence in the centre of NS4A (Bartenschlager *et al.*, 1995; Failla *et al.*, 1995; Lin *et al.*, 1994*b*, 1995; Satoh *et al.*, 1995; Tanji *et al.*, 1995*a*), which can be supplied as a synthetic peptide without loss of activation function (Butkiewicz *et al.*, 1996; Koch *et al.*, 1996; Shimizu *et al.*, 1996*a*; Steinkühler *et al.*, 1996; Tomei *et al.*, 1996). Since mutational ablation of complex formation drastically reduces proteinase activity, it was assumed that interaction of NS4A with NS3 induces conformational changes within the proteinase domain, enhancing enzymatic activity. This hypothesis was confirmed by the resolution of the three-dimensional X-ray structure of the NS3 proteinase (Love *et al.*, 1996) or NS3 complexed with a synthetic NS4A peptide (Kim *et al.*, 1996; Yan *et al.*, 1998). Overall, the enzyme adopts a chymotrypsin-like fold and consists of two β-barrel domains separated by a deep cleft where the amino acid residues forming the catalytic triad are located. The carboxy-terminal domain forms a six-stranded β-barrel and its structural integrity requires a tetrahedrally coordinated zinc ion (De Francesco *et al.*, 1996*b*; Stempniak *et al.*, 1997). In the absence of NS4A the ~ 30 amino-terminal NS3 residues are flexible and extend away from the protein, whereas in the presence of the cofactor, this region is highly structured and participates in an eight-stranded β-barrel with one strand contributed by NS4A tightly intercalated via several side chains into the NS3 domain. A second structural rearrangement upon NS4A binding leads to an optimization of the geometry of the catalytic triad, accounting most likely for the enhancement of proteinase activity (Love *et al.*, 1998; Yan *et al.*, 1998). In addition to serving as a proteinase cofactor, NS4A has two further functions that might contribute to efficient polyprotein cleavage and replication. First, increasing the metabolic stability of NS3 that in the absence of NS4A is degraded very rapidly, and second, anchoring NS3 to intracellular membranes where most of the HCV proteins are located, thereby increasing the local enzyme:substrate concentration and facilitating the formation of a membrane-associated replicase complex (Tanji *et al.*, 1995*a*; Wölk *et al.*, 2000).

A feature conserved in many plus-strand RNA virus families is that proteinase and NTPase/helicase activities reside in a single polypeptide, and the same is found with NS3 of HCV. In fact, two lines of evidence suggest an interdomain coupling.

(1) Although the catalytic efficiencies of the isolated proteinase domain and full-length NS3 are very similar this is

not the case with the helicase. A comparison of RNA unwinding activities of a recombinant single chain NS4A–full-length NS3, a full-length NS3 without NS4A and the isolated helicase domain suggests that the presence of the proteinase domain and NS4A enhance helicase activity (Howe *et al*., 1999). This enhancement may be due to, first, a stabilization of the helicase fold in the full-length complex (Yao *et al*., 1999) and, second, RNA binding sites in the proteinase domain contributing to helicase substrate binding (Gallinari *et al*., 1998).

(2) The resolution of the three-dimensional X-ray crystal structure of a single chain fusion protein of full-length NS3 and the NS4A activator domain revealed that the proteinase and the helicase/NTPase domains are segregated and connected by a flexible single strand (Yao *et al*., 1999). Interestingly, in this molecule the proteinase active site is occupied by the carboxy terminus of NS3. This observation suggests an autoinhibition of the proteinase that may be overcome by interaction with the polyprotein substrate, inducing structural changes and displacing the inhibitor from the active site (Barbato *et al*., 1999; Yao *et al*., 1999).

RNA replication

As deduced from several coprecipitation studies (Hijikata *et al*., 1993*b*; Ishido *et al*., 1998; Lin *et al*., 1997), most or all of the HCV polyprotein cleavage products, in particular NS3–5B, form a replicase complex associated with intracellular membranes that most likely contains cellular proteins too. The formation of such a complex is a feature typical of plus-strand RNA viruses like poliovirus or flaviviruses (Bolten *et al*., 1998; Westaway *et al*., 1997) and it allows the production of viral proteins and RNA in a distinct compartment. In addition, complex formation permits the tight coupling of functions residing in different polypeptide chains.

The individual steps underlying RNA replication are largely unknown. It is obvious that the NS5B RdRp is the key player catalysing the synthesis of minus- and plus-strand RNA. *In vitro* the enzyme prefers a primer-dependent initiation of RNA synthesis, either by elongation of a primer hybridized to an RNA homopolymer or via a 'copy-back' mechanism when using heteropolymeric templates (Al *et al*., 1998; Behrens *et al*., 1996; Ferrari *et al*., 1999; Lohmann *et al*., 1997; Yamashita *et al*., 1998; Yuan *et al*., 1997). In the latter case, sequences at the 3′ end fold back intramolecularly and hybridize, generating a 3′ end that can be used for elongation, resulting in a product approximately twice the length of the input template. However, at least under certain experimental conditions, HCV NS5B, as well as the RdRp of the closely related pestivirus bovine viral diarrhoea virus (BVDV), can initiate RNA synthesis *de novo* and it is plausible that this mechanism also operates *in vivo* (Kao *et al*., 1999; Oh *et al*., 1999; Luo *et al*., 2000; Zhong *et al*., 2000). Interestingly, when using high concentrations of GTP or ATP, HCV NS5B can synthesize RNA primer independently from the homopolymeric templates poly(C) and poly(U), respectively, whereas RNA synthesis from poly(I) or poly(A) templates is primer-dependent irrespective of the NTP concentration (Luo *et al*., 2000). These results suggest that the enzyme can probably only use GTP or ATP for *de novo* initiation. It is interesting to note that the 5′-terminal nucleotides of plus- or minus-strand RNA are guanosine or adenine, respectively. The dependence of *de novo* initiation on high GTP or ATP concentration and the inability of the enzyme to initiate RNA synthesis from poly(A) templates also explains why some groups only observed primer-dependent RNA synthesis with their *in vitro* assays (Al *et al*., 1998; De Francesco *et al*., 1996*a*; Ferrari *et al*., 1999; Lohmann *et al*., 1999*a*; Yamashita *et al*., 1998).

A still unresolved question is how template specificity is achieved. In most studies NS5B was found to utilize and bind to virtually every RNA (and even DNA) template, albeit with different efficiencies. However, using electrophoretic mobility shift assays and competition experiments, Cheng *et al*. (1999) provided evidence for a preferential binding of recombinant NS5B to a sequence in the 3′ coding region of NS5B. Alternatively, template specificity may be accomplished by the high local concentration of NS5B and the RNA genome from which it is translated, within the replicase complex. A preferential *cis*-activity of NS5B also would discriminate against RNA genomes containing stop codons or frame-shift mutations introduced by errors of the viral replicase, reminiscent of what has been described for poliovirus (Novak & Kirkegaard, 1994). While *in vitro* NS5B is able to copy even a complete full-length HCV genome (Lohmann *et al*., 1997), it is very likely that *in vivo* additional viral or cellular factors are required. Possible viral candidates are the NS3 helicase, by unwinding stable structures in the RNA template and facilitating replication, or the phosphoprotein NS5A. Although the replication function of this protein could not be studied thus far, by analogy to other RNA viruses it is tempting to speculate that NS5A is involved in regulation of RNA replication. For example, in the case of VSV, RNA synthesis occurs only after phosphorylation of the P protein (Barik & Banerjee, 1992). In the case of the Dengue virus NS5 replicase, subcellular localization and interaction with NS3 are altered by the level of phosphorylation (Kapoor *et al*., 1995). Phosphorylation of NS5 or NS5A is a biochemical property conserved in all members of the flaviviruses studied thus far, suggesting that this modification plays an important role in the life-cycle of these viruses (Reed *et al*., 1998).

In addition to viral proteins, cellular components are probably involved in RNA synthesis, too. One candidate is PTB, found to specifically interact with sequences at the 3′ NTR (Chung & Kaplan, 1999; Ito & Lai, 1997; Tsuchihara *et al*., 1997). Another candidate is glyceraldehyde-3-phosphate dehydrogenase, binding to the poly(U)-sequence in the 3′ NTR (Petrik *et al*., 1999). Finally, cellular proteins provisionally called p87 and p130 were identified by UV-cross linking

experiments with the X-tail sequence, but the nature of these proteins remains to be determined (Inoue *et al*., 1998).

Proteins from other viruses may also affect HCV replication. It is interesting to note that the cell lines supporting HCV replication the best are, in most cases, (co-)infected with other viruses like human T-lymphotropic virus type I (in the case of the MT2 T-cell line), a murine retrovirus (in the case of the MOLT4-Ma T-cell line) or Epstein–Barr (EBV) (in the case of the Daudi B-cell line). Furthermore, Sugawara *et al*. (1999) observed that HCV-positive patients with a hepatocellular carcinoma frequently have a high EBV load, and presented evidence that the enhancement of HCV replication is mediated by EBV nuclear antigen 1. Since this protein is localized in the nucleus, whereas HCV replication occurs in the cytoplasm, the enhancement may be due to an indirect effect, e.g. via activation of transcription of cellular genes, although there is no cellular promoter with an EBV nuclear antigen 1 binding motif known.

Virion assembly and release

In the absence of systems allowing the production of biochemical amounts of virus particles, the assembly of HCV cannot be studied in detail. One potential approach to overcome this limitation is the production of virus-like particles (VLPs) by expression of the structural proteins in heterologous systems, but for HCV this turned out to be very difficult. Thus far there are two reports describing the formation of VLPs. In one report the full-length HCV coding region was transiently expressed with the vaccinia virus T7 hybrid system in HeLa G cells (Mizuno *et al*., 1995). Particles with diameters of ~ 30 and 45 nm, assumed to correspond to non-enveloped core-like particles and enveloped particles, respectively, were found. In another report, Baumert *et al*. (1998) used recombinant baculoviruses to express a region of the HCV genome corresponding to a part of the 5′ NTR and the C–E2 region in insect cells. They observed VLPs containing selectively encapsidated HCV RNA. Although this report is promising, the efficiency of VLP formation still appears rather low, because the majority of the HCV proteins form aggregates and only a minority assemble to produce VLPs. Furthermore, these particles reside in intracellular membrane vesicles and are not transported out of the cell.

Particle formation may be initiated by core protein interacting with the RNA genome. Although *in vitro* core protein binds to RNA without detectable specificity, recent evidence indicates a preferential intracellular binding to RNA sequences in the 5′ half of the HCV genome (Shimoike *et al*., 1999). Such binding may not only accomplish a selective packaging of the plus-stranded genome but also appears to repress translation from the IRES, suggesting a potential mechanism to switch from translation/replication to assembly (Shimoike *et al*., 1999). Whether the core protein forms a distinct nucleocapsid structure or a rather non-structured ribonucleoprotein complex with the RNA genome is not known. Certainly, core protein interacts with itself and the sequences required for this interaction have been mapped to the amino-terminal 115 residues (Matsumoto *et al*., 1996). Within this region a tryptophan-rich primary interaction domain was identified between residues 82 and 102 that is masked in the full-length core protein and revealed only under certain experimental conditions (Nolandt *et al*., 1997).

A feature typical of the HCV E proteins is their retention in the ER compartment when expressed with various heterologous systems in cell culture (Dubuisson *et al*., 1994). The retention is achieved by signals in the transmembrane domains of E1 and E2 and it has been shown to be a true retention in the ER (Duvet *et al*., 1999; Cocquerel *et al*., 1999). This observation suggests that viral nucleocapsids acquire their envelope by budding through ER membranes. In this case the virus may be exported via the constitutive secretory pathway. In agreement with this assumption, complex N-linked glycans were found on the surface of partially purified virus particles, suggesting virus transit through the Golgi (Sato *et al*., 1993). However, since HCV particles tend to associate with cellular components it remains to be determined whether these glycans are present on the E proteins or on cellular proteins associated with HCV particles.

Model systems to study HCV replication

HCV-related viruses

As alluded to in a previous section, HCV belongs to the family *Flaviviridae* and is most closely related to pestiviruses and the GB-/hepatitis G viruses. Despite a low sequence identity between the genomes of these viruses and HCV their similar genomic organizations, in particular the superimposable delineation of the nonstructural proteins 2–5B and their functional homologies (proteinase, helicase, RdRp), make these viruses an attractive model. Although for GBV-B, infectious clones have recently been described (Bukh *et al*., 1999), pestiviruses provide the better model because of both the availability of cloned infectious genomes and efficient cell culture systems (Moormann *et al*., 1996; Ruggli *et al*., 1996; Meyers *et al*., 1996; Mendez *et al*., 1998). We can expect that the overall replication schemes of HCV and pestiviruses are similar and, therefore, studies performed with pestiviruses should help to clarify the HCV replication cycle. To give one example, it was shown for BVDV that a subgenomic RNA, originally identified as a defective interfering particle and lacking all the structural proteins can replicate autonomously in cells (Behrens *et al*., 1998). This observation could be confirmed with HCV and led to the construction of RNAs capable of replicating in a human hepatoma cell line (Lohmann *et al*., 1999*b*; see below). However, apart from these overall similarities, distinct differences exist. For example, a hallmark of pestiviruses is the formation of cytopathogenic variants

characterized by the formation of NS3 that in the case of non-cytopathogenic isolates is produced as an NS2–3 fusion protein. In the case of all HCV isolates analysed thus far there is no evidence for the formation of such a protein but rather an obligatory cleavage at the NS2/3 junction (see above). Another difference relates to host cell factors required for pestivirus or HCV replication. For example, the BVDV replicon described by Behrens *et al*. (1998) was shown to replicate in several different cell lines of bovine and even human origin, including the human hepatoma cell lines HepG2 and Huh-7. In contrast, the subgenomic HCV replicon developed by Lohmann *et al*. (1999*b*) thus far only replicates in Huh-7 cells, suggesting a dependence on distinct cellular factors.

Animal models

The only animal that can be infected with HCV reproducibly is the chimpanzee, but its use is limited by ethical reasons, its scarcity and high maintenance costs. In an attempt to establish a small animal model Xie *et al*. (1998) experimentally inoculated *Tupaias* (*T. belangeri chinensis*), a species shown to be susceptible to infection with the hepatitis B virus (Walter *et al*., 1996; Yan *et al*., 1996). However, only about one-quarter of the animals became infected with HCV and developed either transient or intermittent viraemia with rather low titres. Recently, two potential alternatives have been described for the propagation of hepatitis B viruses that might be used for HCV as well. Both systems are based on the engraftment of human liver tissue into immunocompromised mice. In the so-called trimera system lethally irradiated mice are rescued with SCID mouse bone marrow cells and used as transplant recipients for human liver pieces infected *ex vivo* (Ilan *et al*., 1999). The other system uses transgenic mice containing the urokinase-type plasminogen activator gene whose expression under control of the liver-specific albumin promoter is hepatotoxic and destroys a high proportion of liver cells. These mice were crossed to mice that due to a recombination activation gene 2 knockout lack mature B and T lymphocytes. Upon transfer of liver cells from an animal susceptible to infection with a hepadnavirus into these mice, the parenchymas of the regenerating mouse livers were composed of up to 90% of the transplanted cells and after infection with the hepadnavirus these mice became highly viraemic (Petersen *et al*., 1998). Although both mouse systems have thus far only been used for the propagation of hepadnaviruses, the methodologies might be used for HCV as well.

Infection of cultured cells with HCV

In the last few years several cell culture propagation systems for HCV have been described that are based on the infection of primary cell cultures or cell lines or the cultivation of primary cells from chronically infected patients. However, thus far these systems suffer from poor reproducibility and a low level of HCV replication that can be measured only with highly sensitive techniques. In many cases the minus-strand RNA intermediate that is only formed upon productive replication was measured by strand-specific RT–PCR, but owing to false priming during the RT–PCR process this assay alone is not really reliable. Several modifications of this method have been developed (Gunji *et al*., 1994; Lanford *et al*., 1994), but scepticism remains. Therefore, in addition to minus-strand RNA detection, several other indicators for productive replication have been used, such as an increase of plus-strand RNA during the cultivation period (determined either by b-DNA assay or quantitative RT–PCR), the inhibition of replication upon incubation of the cells with IFN-α or antisense oligonucleotides, the transmission of cell-culture-grown HCV to naive cells, the sequence analysis of HCV genomes or genome fragments to demonstrate genomic variability and selection of variants upon infection and cultivation, the detection of viral antigens by immunofluorescence or flow cytometry and the long-term propagation of HCV. A representative selection of these reports is given in Table 1 and only a few will be described in some detail here.

The infection of primary hepatocytes from humans or chimpanzees with high-titre HCV-containing serum has been described by several groups (Fournier *et al*., 1998; Iacovacci *et al*., 1993; Lanford *et al*., 1994; Rumin *et al*., 1999). Although discussed controversially (Lanford *et al*., 1995; Laskus *et al*., 1997), infection of PBMCs seems possible, too, indicating that HCV can also replicate in extrahepatic cells (Cribier *et al*., 1995; Lerat *et al*., 1996). Consistent with this notion, HCV replication was reported in PBMCs isolated from chronically infected patients (Bouffard *et al*., 1992; Müller *et al*., 1993; Zignego *et al*., 1992).

Since primary cell cultures are not routinely available, most attempts have been undertaken with cell lines of human origin. With respect to hepatoma cell lines, the most detailed results are available for the non-neoplastic cell line PH5CH (Ikeda *et al*., 1998; Kato *et al*., 1996). Evidence for HCV replication in these cells is based on (1) the detection of HCV RNA up to 100 days post-infection, (2) the loss of plus-strand RNA upon incubation of the infected cells with IFN-α, and (3) a strong selection for HCV variants in the hypervariable region 1 (HVR1) of the E2 protein, suggesting that only certain variants can bind to or replicate in these cells. A similar decrease of complexity of viral quasispecies has been described for HCV propagated in primary human hepatocytes for up to 3 months (Rumin *et al*., 1999).

T- and B-cells selected for high HCV replication have been derived from the T-cell lines MT-2 and MOLT-4 and the B-cell line Daudi. For example, an MT-2C cell clone was described that supports HCV replication for up to 198 days post-infection (Mizutani *et al*., 1996*b*). By comparing the complete sequence of the HCV genome replicating in these cells with the sequences present in the inoculum it was found that only a

Table 1. Examples of *in vitro* infection systems for HCV

Cell type*	Detection method†	Time‡	References
Cultivation of infected PHH	(+); (−); IF; HVR sequence; transmission	28	Ito *et al.* (1996)
Cultivation of infected PBMC	(+); (−); antigen	9	Bouffard *et al.* (1992); Müller *et al.* (1993); Zignego *et al.* (1992)
PHH (foetal)	(+); (−)	24	Iacovacci *et al.* (1993)
PCH	(+); (−); IFN-α	25	Lanford *et al.* (1994)
PBMC	(+); (−); ISH; transmission	26	Cribier *et al.* (1995)
PHH	(+); (−)	14	Fournier *et al.* (1998)
PHH	(+); (−)	90	Rumin *et al.* (1999)
HepG2; WRL68 hepatocytes	(+); (−)	< 20; 62	Tagawa *et al.* (1995)
CE B-cell	(+); IF	65	Bertolini *et al.* (1993)
TOFE B-cell	(+); 5′ NTR sequence	~ 180	Valli *et al.* (1995)
HepG2, Huh-7 hepatocytes and others	(+); (−); transmission	130	Seipp *et al.* (1997)
PH5CH hepatocytes	(+); HVR sequence	30	Kato *et al.* (1996)
PH5CH clones	(+); IFN-α	100	Ikeda *et al.* (1998)
MT-2 T-cell	(+); (−); HVR sequence	15	Kato *et al.* (1995)
MT-2C cell clone	(+); (−); IFN-α; antisense	30/80	Mizutani *et al.* (1996*b*)
MT-2C cell clone	(+); transmission; HVR sequence	198	Mizutani *et al.* (1996*a*)
MT-2C cell clone	Complete genome sequence		Sugiyama *et al.* (1997)
MOLT-4 Ma T-cell	(+); (−); ISH; IF	25	Shimizu *et al.* (1992)
MOLT-4 Ma T-cell	Transmission		Shimizu & Yoshikura (1994)
HPB-Ma T-cell	(+); (−); IF	76	Shimizu *et al.* (1993)
HPBMa10-2 clone; Daudi B-cell	Genome-sequences; transmission; EM	> 365	Nakajima *et al.* (1996); Shimizu *et al.* (1996*b*)
Daudi B-cell	Infection of chimpanzee		Shimizu *et al.* (1998)

* Primary cell culture or cell line used for infection. PHH, Primary human hepatocytes; PCH, primary chimpanzee hepatocytes.
† Method used to monitor HCV replication. (+), Plus-strand RNA; (−), minus-strand RNA; IFN-α and antisense, inhibition of HCV replication by interferon-α or antisense oligonucleotides; transmission, transfer of HCV to naive cells by cocultivation with infected cells or incubation with medium from infected cells; IF, immunofluorescence; ISH, *in situ* hybridization; EM, detection of VLPs by electron microscopy; HVR sequence, determination of the nucleotide sequence of the hypervariable region.
‡ Number of days between infection and the last day of detection of HCV plus-strand RNA.

limited virus population replicates in MT-2C cells (Sugiyama *et al.*, 1997), suggesting the selection of certain variants particularly adapted to these cells. Long-term propagation of HCV for more than 1 year has been described for the human T- and B-cell lines HPBMa10-2 and Daudi (Nakajima *et al.*, 1996), and virus could be transmitted several times to naive cells by cocultivation (Shimizu & Yoshikura, 1994). To demonstrate infectivity of cell-culture-grown HCV in an animal, Shimizu *et al.* (1998) inoculated a chimpanzee with $\sim 10^3$ genome equivalents present in the culture supernatants from Daudi cells that had been infected with HCV for 58 days. After ~ 5 weeks HCV RNA became detectable in the serum of the animal and vanished after week 25. Interestingly, the major HVR1 sequence in the serum of the animal corresponded to the predominant variant in the patient serum used for infection of Daudi cells. However, in PBMCs of the chimpanzee the major variant corresponded to the dominant variant found in Daudi cells and this variant was not found in the inoculum patient serum. These results suggest the selection of a lymphotropic HCV variant during cell culture passage.

Transfection of cell lines with cloned HCV sequences

Compared to the infection of cell lines with HCV-containing patient material, the introduction of cloned virus genomes is superior because the inoculum is well defined and can be generated in high quantities. Most importantly, the genome can be manipulated at will, permitting a detailed genetic analysis of viral functions. Although the production of infectious virus from cells transfected with cRNA (RNA generated by *in vitro* transcription of a cloned DNA copy of the viral genome) has been described for several plus-strand RNA viruses (Boyer & Haenni, 1994), this approach turned out to be very difficult for HCV. Up to now only two reports have been published describing the replication of a transfected HCV genome in the human hepatoma cell lines Huh-7 or HepG2 (Dash *et al.*, 1997; Yoo *et al.*, 1995). However, in both studies

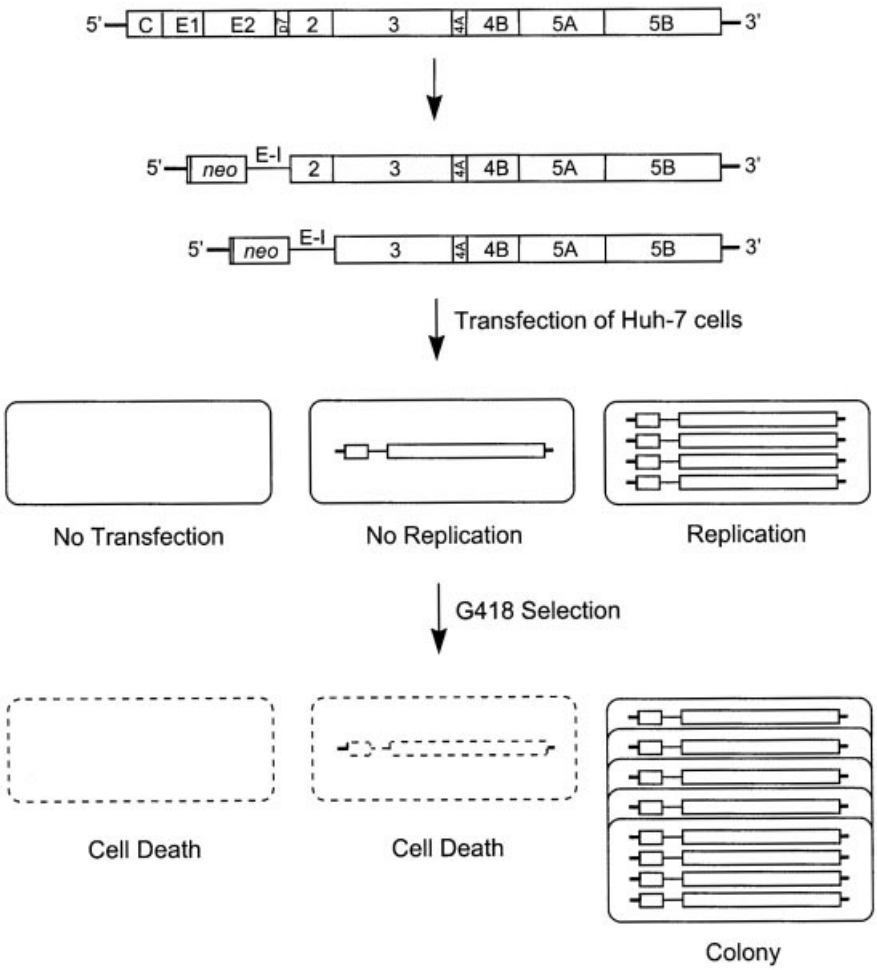

Fig. 3. Schematic representation of the method used to establish HCV-replicon-containing cell lines. The structure of the HCV genome is given at the top. The subgenomic RNAs derived therefrom, composed of the HCV 5′ NTR plus a small fragment of the core-coding region (thin box), the *neo* gene, the encephalomyocarditis virus IRES (E-I), HCV NS2–5B or NS3–5B and the 3′ NTR, are drawn below. Since core-coding sequences are required for full IRES activity, the ~ 20 amino-terminal residues of the core protein are fused to the amino terminus of the neomycin phosphotransferase. Upon transfection of Huh-7 cells, only those supporting replication of the HCV RNAs amplify the *neo* gene and develop resistance against the drug G418. Therefore, only these cells will form colonies, whereas untransfected cells and cells that do not support replication of these RNAs will be eliminated during the selection.

truncated HCV genomes were used that lacked the authentic 3′ NTR. The replication competence of such genomes contradicts the finding that the highly conserved sequence at the very 3′ end of the genome is essential for replication *in vivo* (Yanagi *et al.*, 1999; Kolykhalov *et al.*, 2000) and in cells transfected with selectable HCV replicons (unpublished results; see below). A clarification of this issue awaits the sequence analysis of the recloned 3′ truncated RNAs and the successful infection of chimpanzees with culture medium from these cells.

Recently we have developed selectable subgenomic HCV RNA molecules replicating to high levels after transfection into the human hepatoma cell line Huh-7 (Lohmann *et al.*, 1999*b*). Based on the assumption that high expression levels of the structural proteins might be cytotoxic (Moradpour *et al.*, 1998) and the observation that for several plus-strand RNA viruses like alpha-, flavi- and pestiviruses the structural proteins are not required for RNA replication (Behrens *et al.*, 1998; Khromykh & Westaway, 1997; Liljestrom & Garoff, 1991), the sequences of the structural proteins were deleted. To allow selection for only those cells in which HCV will efficiently replicate, the gene encoding the neomycin phosphotransferase, conferring resistance to the antibiotic G418, was introduced downstream of the HCV IRES (Fig. 3). A second IRES element was included to allow translation of the HCV NS proteins. Since we did not know whether NS2 was required for replication, two variants were generated spanning the NS2–5B or the NS3–5B region. Upon transfection of these bicistronic RNAs and selection of the cells with G418, only those

supporting replication of these RNAs amplified the *neo* gene and developed resistance, whereas non-transfected cells and cells unable to support replication died. With this approach cell clones could be established with both the NS2–5B and the NS3–5B replicon, albeit with low efficiency. Surprisingly, these selected cells carried large amounts of HCV RNAs detectable by Northern blot, or after metabolic radiolabelling with ^{3}[H]uridine, providing formal proof that these RNAs were actively replicating in the cells. Using these techniques we detected 1000–5000 RNA molecules per cell, which is several orders of magnitude higher compared to infection systems (Blight & Gowans, 1995). As expected for a replicative intermediate, minus-strand RNA was present in ~ 10-fold molar lower amounts compared to plus-strand RNA. HCV proteins could be detected by immunoprecipitation after metabolic radiolabelling with ^{35}[S]methionine (Lohmann *et al.*, 1999 *b*) or Western blot (K. Kurpanek & R. Bartenschlager, unpublished results) and were confined to the cytoplasm.

A still unresolved question is why we only obtained colonies with the cell line Huh-7. Although intensive tests still have to be done, this result suggests that specific host cell factors present in only a few cell lines are required for HCV replication and possibly determine a narrow host range.

Concluding remarks

Since the molecular cloning of the HCV genome only ca. 10 years have elapsed, but in this short period our knowledge about this insidious pathogen has increased tremendously. This rapid progress is best reflected by the resolution of the three-dimensional structures of the viral enzymes participating in polyprotein processing and replication: the NS3/4A proteinase, the NS3 helicase and the NS5B RdRp (Ago *et al.*, 1999; Bressanelli *et al.*, 1999; Kim *et al.*, 1996, 1998; Lesburg *et al.*, 1999; Love *et al.*, 1996; Yan *et al.*, 1998; Yao *et al.*, 1997). However, from what the field suffers most is the lack of a reproducible and efficient cell culture system. The recent demonstration of HCV replication and disease development after intrahepatic inoculation of chimpanzees with cloned virus genomes (Beard *et al.*, 1999; Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997, 1998) has opened some new avenues to study HCV replication and pathogenesis, but even for these genomes so far no cell culture system has been described. A first step in this direction is the development of selectable HCV replicons. Although this system still does not allow the production of infectious virus particles, it probably provides the framework to proceed towards this goal.

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