

Experimental models for hepatitis C virus (HCV): New opportunities for combating hepatitis C

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Abstract

Infection with hepatitis C virus (HCV) is a global public health issue. More than 200 million people in the world are infected with HCV. Hepatitis C is considered one of the main causes of chronic hepatitis, cirrhosis, hepatocellular carcinoma and liver transplantation. The identification of the viral genome quickly allowed delineation of genomic organization, and the structure and biochemical characterization of the proteins of HCV. However, it has been difficult to study its life cycle, as well as the development of antiviral agents due to the lack of a system of permissible culture. Numerous attempts have been reported to establish an *in vitro* system for the study of HCV. Recently, a system of efficient culture was established that allows replication of subgenomic molecules of HCV in a cell line of human hepatoma. In this revision, after a brief description of the molecular biology, means of transmission and clinical characteristics of hepatitis C, some of the experimental models are described that have been developed to date, focusing mainly on the subgenomic replicon system and their use in the development of new antiviral treatments.

Key words: Hepatitis C, hepatitis C virus (HCV), subgenomic replicon, IRES, translation of proteins, ribavirin, IFN-alfa.

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Introduction

Hepatitis C virus (HCV) is a very important public health problem that surpasses even the problem with human immunodeficiency type 1 (VIH-1) virus on a worldwide basis. Since its discovery a little more than a decade ago, HCV has recently gained importance as one of the main ethiological agents of chronic hepatic illness worldwide. The World Health Organization (WHO) estimates that more than 200 million people are infected with this virus in the world and every year approximately 3 and 4 million infected people are added.¹⁻³

The high genetic heterogeneity of HCV allows it to reduce the effectiveness of antiviral treatments and to avoid the action of the immune system, which causes a high rate of chronic infection. This is one of the reasons that causes about 75 to 85% of the patients with acute illness to progress to chronic hepatitis, from 10 to 20% progress to cirrhosis and of these 1 to 5% develop hepatocellular carcinoma (HCC).⁴⁻⁶ In Mexico hepatitis C and alcoholism are the main causes of hepatic cirrhosis which represents the second cause of death of people between 35 and 55 years of age.⁷ The time necessary for progression to a more serious hepatic illness, is extremely variable, it can be as short as 2 years or as long as 10 to 30 years after infection.⁸ Although the illness can progress more rapidly in patients of advanced age and in those with alcoholic hepatic illness, it is difficult to predict which patients will present progression because the natural course of the illness is very variable.⁹

In recent years significant advances have been generated in the understanding of the genomic organization, mechanisms of persistence, replication mechanisms, synthesis and function of the proteins of HCV. However, there are still aspects that need to be clarified about the pathogenesis of this infection. In this revision we focus on the advances that have been carried out in study systems for HCV that have served as a basis for the design of new antiviral strategies to combat hepatitis C.

Epidemiology

The WHO estimates that more than 3% of the world's population is infected with this virus. In North America,

Europe, the Middle East and southern Asia the prevalence of HCV infection is 1 to 2.5%, while in countries of South-east Asia and central Africa the prevalence is 2.5 to 10%. In some countries of the African continent (Egypt, Cameroon and Guinea), Asia and South America (particularly Bolivia) the prevalence of HCV infection surpasses 10%.¹⁰

Modes of transmission

Diverse pathways exist by which Hepatitis C can be contracted and transmitted, these pathways include: transmission by the parenteral route by means of blood transfusions and hemoderivatives, percutaneous transmission by exposure to contaminated sharp objects and the use of intravenous drugs, mainly. Sexual transmission is associated to promiscuity and in vertical transmission (mother-son) the risk of infection can increase up to 20-30%, when the mother, besides having a high viral load of HCV, is positive for VIH-1.^{1,6,11} In a study carried out in Mexico from 1994 to 1998, it was found that the most important mode of transmission was blood transfusion, followed by the parenteral route through the use of contaminated needles and unsterilized syringes by drug addicts, and in organ transplant.¹² It was also found that the possibility of transmission by sexual contact and by perinatal infection is considerably smaller for HCV than for hepatitis B virus (VHB).¹²

Current treatments to combat hepatitis C

In recent years, the treatment options to combat chronic infection by HCV have improved vastly with the combination of ribavirin and pegylated interferon alpha (PEG-INF- α), reaching elimination rates of the virus with response rates greater than 50% (54 to 56%), which is why, at the present time, it is considered the therapeutic scheme of choice.¹³ In spite of this therapeutic option, about 50% of the patients do not respond after twelve months of treatment.¹³ Therefore, it is necessary to develop more effective antiviral treatments. Reports exist that patients with genotypes 2 and 3 respond more favorably to treatment than patients with genotype 1 and 4. This fact has an important impact in our population, since in Mexico genotype 1b is the most frequent (> 60%).¹² For this reason the identification of HCV genotypes has become a valuable tool for designing treatment schemes to follow in infected patients.

Molecular structure of the HCV genome

In 1989, Choo and cols.¹¹ identified the complete sequence of the viral genome of the causal agent known, until then, as Non A non B Hepatitis (NANB). With recombinant DNA technology, it was possible to clone the genome of this agent which would later be called Hepatitis C Virus (HCV).

HCV is the only member of the hepacivirus genus of the family *Flaviviridae*.¹⁴⁻¹⁶ In this family hepatitis G virus and the causal agents of dengue and yellow fever are also found, as well as Western Nile Virus and the virus that causes bovine diarrhea. These viruses have in common that they are particles of approximately 40-60 nanometers in diameter and their genome consists of a single strand of RNA of positive sense with a single open reading frame (ORF).^{3,17,18}

The HCV genome has a length of approximately 9,600 nucleotides.^{17,19,20} This RNA has a single ORF that codifies for a viral polyprotein of approximately 3,010 to 3,033 amino acids.^{7,21} Contrary to messenger RNA (RNAm), the viral genome does not possess cap-structure nor a poly (A) in the 5' and 3' ends, respectively.¹⁷ Instead of these structures, the HCV genome is flanked by two non-translated regions (NTRs) which are essential in the replication and synthesis of viral proteins.²² The first of these sequences is known as 5'NTR, because it is located at the beginning of the nucleotide sequence and it contains highly conserved sequences. Within this region (nucleotides 44 to 354), there is an area known as the internal ribosome entry site (IRES) which is an element of RNA that directly attaches to ribosomes to begin the translation of viral RNA and produce the viral proteins that allow it to cause infection to take place.²²⁻²⁴ Approximately, the first 45 nucleotides of the HCV genome are not required in translation, but based on analogies with other RNA viruses of positive sense, this sequence is probably involved in the replication of the virus.¹⁷ The second sequence is called 3'NTR, because it is located toward the end of the viral genome and is formed by three regions: a variable region, a poly(U)-tract that is heterogeneous in length, and a highly conserved region of 98 nucleotides known as the X region which is essential for viral replication.²⁵⁻²⁷

The processing of the polyprotein mediated by a combination of host and viral proteases, generates 10 proteins; the structural proteins (core, E1, E2 and p7) and the non-structural proteins (NS2, NS3, NS4a, NS4b, NS5a and NS5b) (*Figure 1*).¹⁷ The structural proteins participate in the assembly of viral particles of the new offspring, while the non-structural proteins are involved in viral replication and processing of polyprotein.²⁸ The protein core (C) has a highly conserved sequence of amino acids and is the main component of the nucleocapsid.²² Also, it seems to be involved in cell growth, apoptosis and carcinogenesis in cells infected with HCV.²⁹⁻³¹ Reports also exist that the core protein causes changes in the metabolism of triglycerides. One of the characteristics of patients infected with HCV is hepatic steatosis (accumulation of fat in the hepatocytes) which is very possibly due to the direct effect of this protein on fat metabolism.^{21,32} The envelope proteins (E1 and E2) are highly glycosylated type I transmembrane proteins forming stable heterodimers.¹⁷ The amino terminal domain of the E2 protein contains two hypervariable regions called HVR1

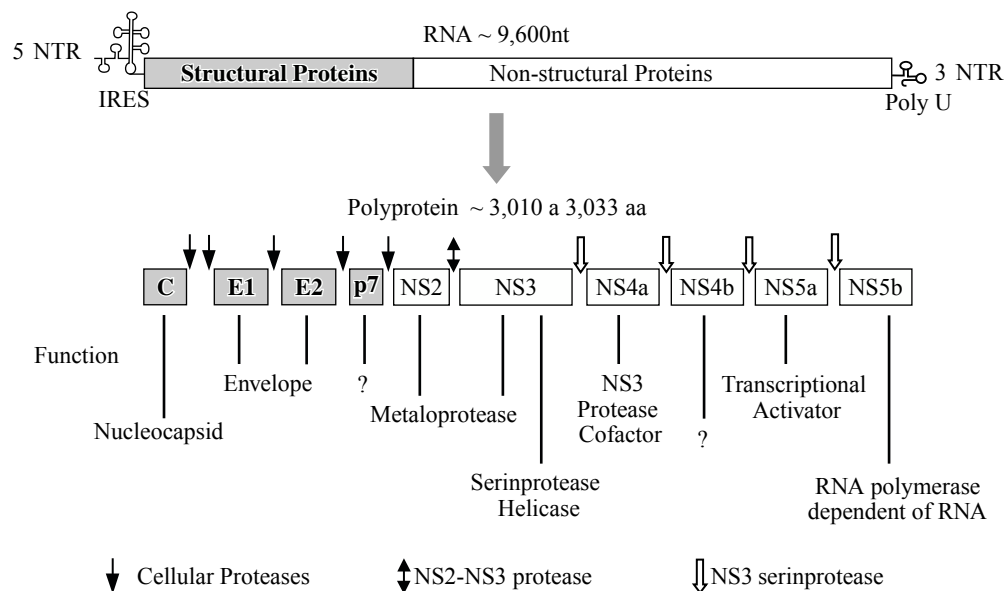


Figure 1. The HCV Genome. The cleavage sites of the cell proteases and the viral proteases are shown (NS2-NS3 protease and NS3 serinprotease), along the length of the polyprotein.

and HVR2 that allow HCV to avoid the immune response and to maintain active infection for many years.³³ Reports exist that the E2 protein is capable of interacting efficiently with the larger extracellular domain (LEL or EC2) of the CD81 protein, located on the surface of hepatocytes and lymphocytes, which is why the possibility has been considered that the CD81 protein is a receptor for HCV that facilitates access of the virus into the host cell.^{34,35} In the same way, the cell receptor of low density lipoproteins (LDL) has been proposed as a probable receptor for HCV. It is believed that HCV is associated with LDL in blood and through endocytosis of these proteins, mediated by its receptors, can enter the host cell.³⁶ Reports also exist that the E2 protein interacts and blocks the function of the kinase protein dependent of RNA known as PKR, which represents one of the natural antiviral mechanisms induced by interferon.³⁷ The protein p7, is a small hydrophobic peptide whose function is still unknown.¹⁷ Within the non-structural proteins (NS) are found: the NS2 protein which, together with the amino terminal domain of the NS3 protein, functions as a viral protease responsible for the rupture of the polyprotein in the union NS2/3. Also, the NS3 protein uses the NS4a protein as a co-factor to carry out its serinprotease function, and the carboxy terminal domain carries helicase activities.¹⁷ NS4b is a hydrophobic protein of unknown function. The function of the NS5a protein is uncertain, however, reports exist that relate this protein with sensitivity to therapy with INF- α . It has been proposed that within the sequence of the NS5a protein exists a region determinant of sensitivity to interferon known as ISDR. This discovery is still controversial, due to the fact that other reports have not been able to correlate the sequence of this region with the response to INF- α .^{38,39} As with the E2 protein, NS5a also interacts with the kinase protein

(PKR), but a lot of controversy still exists in this respect. The NS5b protein has a very important function as an RNA polymerase dependent of RNA, in charge of viral replication.⁴⁰⁻⁴³

One of the characteristics of HCV, is that it possesses a high mutational index that produces considerable genetic heterogeneity. This phenomenon is generated during viral replication, because the RNA polymerase lacks 3'-5' exonuclease activity, therefore, it cannot eliminate the nucleotides added erroneously.⁴⁴ As a result, the genetic heterogeneity has led to the appearance of six genotypes of HCV and numerous subtypes, which apparently present different evolutionary and pathogenic characteristics, and responses to antiviral treatment.⁴⁴

Experimental models for the study of HCV

Due to the clinical importance of this illness and to the need of identifying new therapeutic targets, it is important to know the structural characteristics of the HCV genome, the molecular mechanisms in which the viral proteins intervene, the interactions among viral and cell proteins, as well as the mechanisms of persistence of this virus in infected cells.

HCV is an obligated intracellular parasite, and to study its replication and also to obtain large quantities of the virus, the most useful tool for such purposes would be to have a permissive cell system.¹⁴ The most practical thing would be to use an already established cell line and infect it with particles of HCV and, in this way, study its replication as has been done with other viruses. However, for reasons unknown, this has not been possible. Unfortunately, the lack of a cell culture or animal models capable of maintaining replication of the complete HCV genome has enormously hindered the development of new alternatives for the prevention and treatment of hepatitis C.

With technology available at this moment, advances have been made that have allowed us to begin to understand the mechanisms of viral replication, genomic organization, persistence mechanisms, synthesis and function of proteins of HCV, however, there is still a long way to go in the knowledge of the pathogenesis of this infection. In the following, the advances that have been achieved in the models that have been proposed to study HCV are described.

Infection of primary cell cultures and cell lines

The first attempts to establish a study system for HCV, consisted of infecting primary cell cultures or cell lines already established with HCV *in vitro*, as well as culturing isolated cells from chronically infected patients (*Table I*).⁴⁵⁻⁴⁷ Unfortunately, these systems have shown low reproducibility and a low level of replication of HCV, what has meant the development of other more sensitive detection methods. Several groups have used primary cells from human to propagate HCV in cell culture. For instance, Iacovacci et al.^{45,46} infected primary cultures of hepatocytes with serum from patients with hepatitis C. The infection of the hepatocytes with HCV was demonstrated through an increase (up to 20 times greater) in the copies of a minus-strand RNA (this strand serves as a

template for the synthesis of new positive strands of RNA during HCV replication) during a 24 day cultivation period. However, the total efficiency of the system was very low showing a maximum level of 20,000 copies of RNA of the virus for 10⁶ cells. Similar results were found by Landford et al.⁴⁸ after infecting of primary hepatocytes from chimpanzees.

In the last few years new experimental systems have continued being developed based on the infection of cell lines, however, they continue presenting the same limitations as the previous ones, although the survival times have increase considerably (up to 4 months) without morphological changes, as in the experiments reported by Rumin et al.⁴⁹ Infection of peripheral blood mononuclear cells (PBMCs) has also been reported indicating that HCV can replicate in extrahepatic cells.⁴⁸ Cribier et al.,⁵⁰ reported the replication of HCV in a mixture of white cells obtained from 10 donors that were infected *in vitro* with serum that contained high titers of HCV. In this study the viral RNA was detected for up to 28 days, however, the replication levels and the quantity of RNA detected at the maximum peak of replication was very low, compared with the data obtained in other systems that also use hepatocytes. The restricted availability of primary cell cultures, particularly of primary hepatocytes, therefore, many attempts have been

Table I. Systems of cell culture for the study of HCV.

Cell type	Species	Methods for detecting HCV replication ^a	Days of Persistence ^b	Reference
Primary Cell Cultures				
Hepatocytes	Human	RNA (+), (-); antigen (IF); sequence-HVR; Transmission	28	Ito et al., 1996
Fetal hepatocytes	Human	RNA (+), (-)	24	Iacovacci et al., 1993, 1997
Hepatocytes	Chimpanzee	RNA (+), (-); INF-•	25	Landford et al., 1994
Hepatocytes	Human	RNA (+), (-); antigen (EIA)	90	Rumin et al., 1999
Immortalized Lines				
PBMCs	Human	RNA (+), (-); ISH; Transmission	26	Cribier et al., 1995
PH5CH liver cells	Human	RNA (+); HVR-sequence	30	Kato et al., 1996
PH5CH Clones	Human	RNA (+); INF-•	100	Ikeda et al., 1997, 1998
HepG2 Hepatoma	Human	RNA (+), (-)	< 20	Tagawa et al., 1995
WRL68 Hepatoma				
HepG2 and Huh-7	Human	RNA (+), (-)	130	Seipp et al., 1997
HepG2 Hepatoma	Human	RNA (+); HVR-sequence	77	Clarysse et al., 2001
JHH-1, -4, -6 Hepatoma	Human	RNA (+), RNA (-) only in JHH-4	Continuous	Tsuboi et al., 1996
Daudi B-Cells	Human	RNA (+); HVR-sequence; Transmission to chimpanzee	> 2 years	Yoshikura et al., 1995 Nakajima et al., 1996 Shimizu et al., 1998
MT-2 T Cells	Human	RNA (+), (-); HVR-sequence	15	Kato et al., 1995 Ikeda et al., 1997
MT-2C Clone	Human	RNA (+); Transmission; HVR-sequence	198	Mizutani et al., 1996
MOLT-4 Ma Cells	Human	Strand (+), (-); ISH; antigen (IF)	25	Shimizu et al., 1992
HPB-Ma T-cells	Human	RNA (+), (-); antigen (IF)	76	Shimizu et al., 1993
HPBMA 10-2 Clone	Human	RNA (+), genomic sequence, transmission; EM	> 365	Shimizu y Yoshikura, 1994; Nakajima et al., 1996; Shimizu et al., 1996

^a Methods used for monitoring replication of HCV; Strand (+) and (-) of RNA; Inhibition of HCV replication in the presence of interferon-• (INF-•); Transmission; Detection of antigen by Immunofluorescence (IF), Enzymatic immunoassay (EIA); *In situ* hybridization (ISH); Detection of viral particles by electron microscopy (EM); Determination of the nucleotide sequence of the hypervariable region (HVR-sequence).

^b Number of days between infection and the last day of detection of HCV plus strand RNA.

made to generate cell lines supporting high-level HCV replication. Two approaches have been tried using either human liver cell lines HepG2, Huh-7 and PH5CH,⁵¹⁻⁵⁷ or human B and T-cell lines, in particular MOLT-4, MT-2 and Daudi (Table 1).⁵⁸⁻⁶⁶ Of these cell lines, those that have shown a greater susceptibility to be infected with HCV are the Huh-7.⁵⁵ The relatively unsatisfactory results reached so far have led to the development of alternative experimental models of study.

Transfection of cell lines with cloned HCV genomes

The obtention of clones from the HCV genome opened great expectations. The transfection of cells with cloned viral DNA or with *in vitro* transcripts generated from this template (cRNA) is superior for several reasons. First, the inoculum is homogenous and well defined; second, the genome can be synthesized in large quantities; and third, it can be manipulated at will permitting genetic analyses of a whole variety of different aspects of the HCV life cycle.¹⁷ Thus far there are only two reports indicating the replication of HCV after transfection of synthetic RNA into the human hepatoma cell lines Huh-7 and HepG2.^{67,68} An analysis of the genomic sequence of the virus generated in this system has not been shown. Another disadvantage of this method, is that it has not been possible to infect chimpanzees with these HCV producer cells, which is why the usefulness of these systems is still questionable.¹⁷

Animal models

So far the chimpanzee (*Pan troglodytes*) is the only animal model that has been infected with HCV, and is capable of developing persistent infections, even in the presence of humoral and cellular immune response.¹⁷ Also, this animal model presents clinical and histopathological manifestations that resemble those that appear in humans. However, because of ethical reasons and high costs, only a few studies have been performed in these animals.²⁰ The ideal animal model would be the murine, in fact, diverse experiments have been carried out to obtain transgenic mice or

mice in whom xenotransplants of human or chimpanzee hepatic cells can be carried out.²⁰ Recently, Mercer et al,⁶⁹ reported the first murine model of infection with HCV (Figure 2). This group designed a model of viral infection in mice that were immunodeficient (SCID) and at the same time transgenic for the production of a hepatotoxic protein (albumin urokinase Alb-uPA). These predetermined genetic characteristics, permitted the generation of mice with liver damage, after that, the damaged liver was repopulated with human hepatocytes capable of dividing in this environment (xenotransplant). Later, the chimeric liver was infected with serum of a patient with HCV. Two months later viral replication was evident by means of detection of genomic RNA and also, it was demonstrated that this model was able to transmit HCV from one infected animal to another. The potential of this new animal model of infection is still unknown, but it promises to be a very useful tool in the study of replication and infection mechanisms used by HCV.

Transfection of cell lines with subgenomic replicons

In 1999, a group of German researchers reported a study system for HCV that vastly revolutionized knowledge of this viral agent.^{19,28} It consists of subgenomic replicons of HCV that are capable of replicating autonomously in hepatic cell cultures. The strategy to obtain them, consisted of isolating the viral genome starting with the serum of infected patients, and then synthesizing the complementary DNA (cDNA) by means of Retrotranscription (RT) and amplifying the viral genome using the Polymerase Chain Reaction (PCR). After several stages, clones were selected that contained the complete consequent sequence of the HCV genome including its two regions 5' and 3' NTR. Later, the cDNA was introduced in an expression vector (pCR2.1) that contains the T7 promoter, so that, after its linearization, it can be transcribed *in vitro* and copies of the viral RNA can be obtained. In this way, the first subgenomic replicon was obtained, into which was later introduced a gene resistant to a drug (neomycin) and the region that encodes for structural proteins was eliminated (Figure 3).

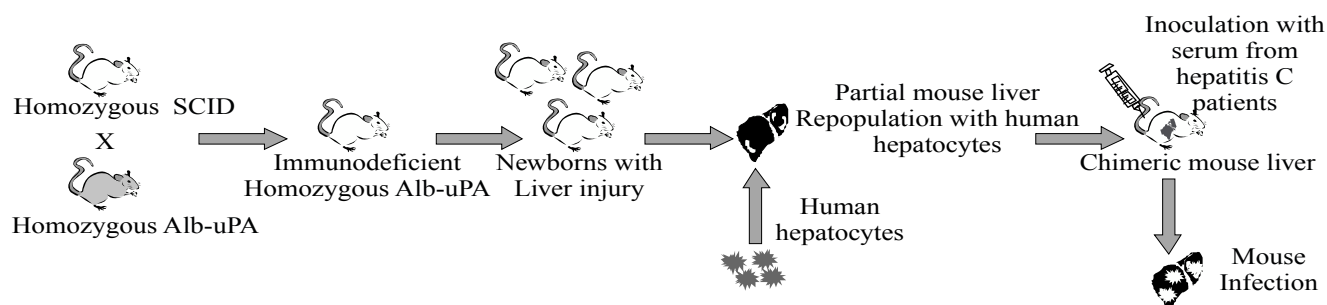


Figure 2. Murine model for HCV infection. In this model human hepatocytes were transplanted in mice that were immunosuppressed (SCID) and transgenic for the Alb-uPA protein. Later, the chimeric liver of these mice was infected with serum of a patient with HCV. Almost 75% of the mice developed hepatitis C that persisted for a period of approximately 15 to 17 weeks.

Therefore, the replicon of HCV contains the 5' and 3' NTR regions (including the IRES region of HCV), different regions that code for nonstructural proteins such as protease, helicase and RNA polymerase, a nucleotide sequence that codes for the enzyme neomycin phosphotransferase (NPT) that is used as an indicator of viral replication, and the IRES of encephalomyocarditis virus (EMCV). So, the subgenomic replicons of HCV, still lacking part of the information contained in the RNA of the virus, contains all the necessary elements for its replication and translation. Therefore, a replicon is the minimum genetic structure of the virus that is capable of replicating itself in the host's cells. In this way, they are capable of producing new subgenomic molecules that in turn, can continue being replicated, but they are not able to originate complete infectious virus.²⁰ To date, this is the most efficient and promissory experimental model for the study of HCV. The design and construction of these subgenomic replicons permitted the generation of cell lines capable of maintaining high levels of numbers of the replicon of HCV.²⁸ These replicons reproduce with high efficiency in the cell line of human hepatoma Huh-7. At the present time, it has already been possible to obtain cell lines capable of maintaining the *in vitro* replication of the replicons of HCV for more than one year.⁷⁰ Among the studies carried out with the replicons of HCV, have been the search for adaptative mutations that confer them advantages. Mutations have been found in the regions that code from the protein NS3 to the protein NS5b, most of them located in the NS5a region (Figure 4). Some mutations in the NS5a region (particularly Ser2204Ile or Ser2204Arg) and mutations in NS4b (particularly Lis1846Thr) increase replication more than 1,000 times.^{71,72}

A second generation of replicons of HCV was generated, with the purpose of producing complete viral particles. In these replicons a reporter gene was introduced

that allowed the measurement of replication of HCV in transitory assays, and that also contains the complete genome of the virus that codes for all the viral proteins required for the production of complete viral particles.⁷³ Unfortunately, in spite of an efficient expression of the structural proteins and high levels of replication, it has not been possible to produce viral particles in cell cultures. The forementioned is probably attributable to the characteristics of the host cells. Recently another replicon has been described (pFK-I₃₈₉/NS3-3'/5.1) that contains three adaptative mutations that increases the replication of HCV (E1202G, T1280I and S2197P).⁷¹ In this subgenomic replicon, the reporter gene luciferase and the sequence that encodes for ubiquitin were inserted downstream of the neomycin gene (neo) by recombination, which notably improved their replication efficiency.

Applications of experimental models for the study of HCV in the development of new antiviral therapies

The current treatments to combat HCV are not effective in many cases, because they are not able to eradicate viral replication in the organism.⁷⁴ On the other hand, an effective vaccine that helps to prevent or eradicate the illness is not available, however, the use of DNA vaccines (that encode for the protein core and nonstructural proteins of HCV) has been attempted to generate antigen presentation processes and to trigger an immunologic reaction.⁷⁵ Unfortunately, these DNA vaccines are still in the experimental phase in animals. Also, the effectiveness of standard immunoglobulin has not been demonstrated in the prevention of post-transfusional hepatitis C or after an accidental puncture.

The availability of the subgenomic replicon system of HCV represents an important alternative for clarifying the mechanisms of viral replication, as well as, for the devel-

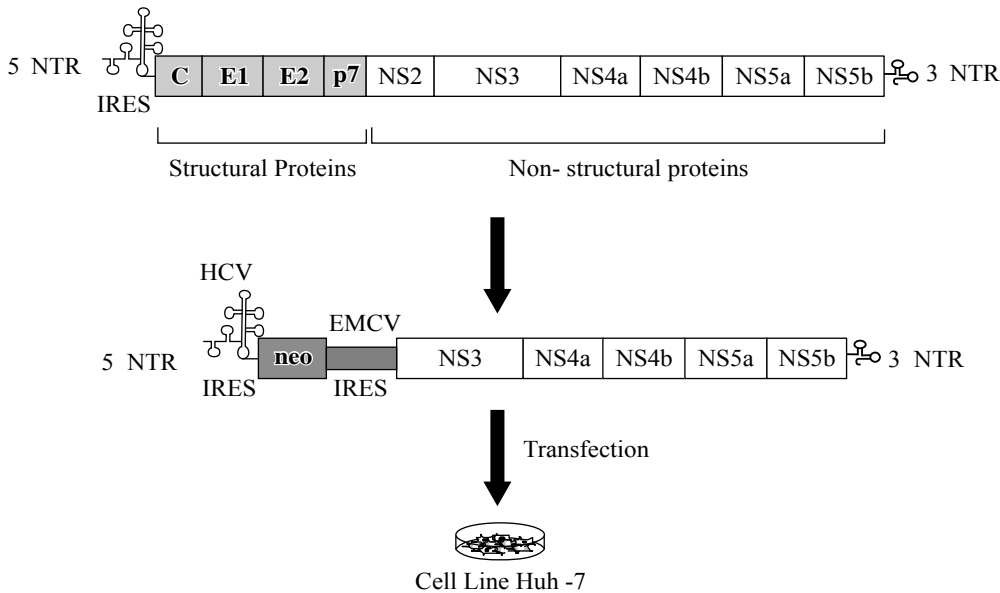


Figure 3. Structure of the subgenomic replicon of HCV. The subgenomic replicon of HCV was obtained by substituting the region that encodes the structural proteins for the neomycin gene (neo) and the IRES of the encephalomyocarditis virus (EMCV). The subgenomic replicon of HCV was cloned and transcribed *in vitro*, to later be transfected in the cell line of human hepatoma (Huh-7) that expresses the proteins of HCV.

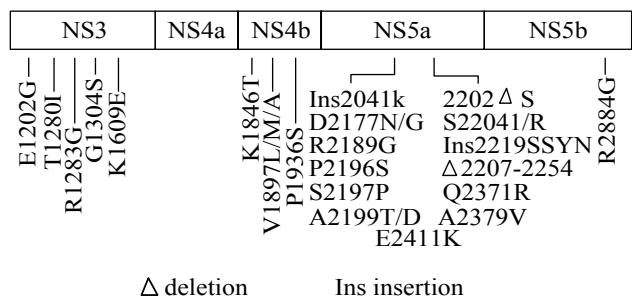


Figure 4. Localization of the adaptative mutations in the replicon of HCV. The adaptive mutations are located along the length of the region that encodes for nonstructural proteins.

opment of new antiviral therapies. The target enzymes in the development of antiviral drugs include the proteins NS2-NS3 protease, NS3 protease, NS3 helicase and NS5b RNA polymerase.²⁸ Using this replicon system, different drugs have begun to be evaluated, such as, protease and helicase inhibitors, RNA polymerase and ribozyme inhibitors. For example, the identification of the three-dimensional crystalline structure of the NS3 protease, has been very useful for the design of protease inhibitors with antiviral effects, as is the case of the drug BILN 2061.⁷⁶ The effect of this drug was evaluated initially in the model of replicons of HCV, later it was administered orally to patients infected with HCV. With this study it was shown that the drug BILN 2061 is able to significantly decrease viral replication in the human body without causing adverse effects, since it was seen that the blood levels of HCV decreased 100 to 1,000 times. For this reason, the drug BILN 2061, is the first of the drugs denominated serinprotease NS3 inhibitors which is tested in humans.⁷⁶ Other therapeutic options in development include the use of ribozymes and of complementary probes of regions with scarce genetic variability such as the 5' and 3' UTR regions. However, these strategies are still in very early stages before being tested in humans, but they promise to be of great usefulness for the development of new drugs.

As long as a study model is not obtained that is capable of maintaining the replication of the complete HCV genome, the availability of the subgenomic replicon system represents an important alternative in the advancement in the development of vaccines and in the search of new treatment options to combat hepatitis C. Regrettably, many questions still remain unsolved, however, with the vertiginous advancement of technology and enthusiasm of research groups in this area, in the near future, we hope to have a more optimistic view in the battle against hepatitis C virus.

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