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Construction and characterization of infectious inter-genotypic Hepatitis C Virus chimeras.

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RIASSUNTO

Il maggior ostacolo allo sviluppo di terapie anti HCV è rappresentato dalla mancanza di culture cellulari che supportano efficacemente la produzione di virus. Questo ostacolo è stato recentemente superato grazie all'isolamento di un clone, denominato JFH1 (genotipo 2a), il cui genoma porta alla produzione di particelle virali infettive sia in *vitro* che in *vivo*. Anche se tale sistema cellulare (HCVcc) rappresenta un'importante conquista, presenta il grosso limite della dipendenza dal subtipo JFH1 e da una chimera intra-genotipica in cui la regione strutturale deriva dal subtipo J6, comunque di genotipo 2a. Per effettuare studi comparativi su diversi genotipi, sono stati quindi ingegnerizzati virus chimerici inter-genotipici in cui la prima metà del genoma di JFH1 (dal 5'- UTR alla proteina NS2) è stata sostituita con l'analoga regione proveniente da altri genotipi.

Nel presente lavoro sono state costruite due chimere inter-genotipiche con una nuova strategia. A differenza delle precedenti, la regione strutturale è stata mantenuta interamente di JFH1, ad eccezione della porzione solubile (ectodominio) di una o entrambe le proteine dell'envelope E1 e E2. In questo modo è stato possibile analizzare il ruolo di tale regione, ed in particolare dell'ectodominio di E2, nella produzione di particelle infettive di HCV.

I risultati riportati dimostrano per la prima volta che proteine dell'envelope derivanti da genotipi diversi (nello specifico E1 di genotipo 2a ed E2 di genotipo 1b) possono correttamente interagire a formare dimeri E1E2, rappresentanti la forma funzionale delle proteine dell'envelope. Ciononostante nessuno dei due costrutti chimerici prodotti ha portato all'ottenimento di particelle virali infettive, indicando la presenza di determinanti all'interno della porzione sostituita importanti per la produzione di HCV. In particolare, grazie ad esperimenti di microscopia confocale, analisi della presenza di precursori virali all'interno delle cellule trasfettate ed esperimenti di *trans*-complementazione, per la prima volta dimostriamo che la regione ectodominica della proteina dell'envelope E2 è fortemente implicata nell'assemblaggio di nuovi virioni, probabilmente attraverso interazioni genotipo-specifiche con altre proteine strutturali. Di conseguenza, nel nostro sistema, la produzione di particelle virali risulta drasticamente alterata a causa dell'incompatibilità genetica tra il backbone JFH1 e la porzione ectodominica di E2 derivante da genotipo 1b. Dato che recentemente è stato riportato un ruolo chiave per NS2 nel processo di assemblaggio, la nostra ipotesi è che una delle interazioni genotipo-specifiche richieste coinvolga per l'appunto E2 nella sua regione solubile e la proteina non strutturale NS2. Quest'ipotesi rimane comunque

da approfondire con ulteriori esperimenti atti a meglio definire il tipo di relazione tra proteine virali e il ruolo di tali interazioni nell'assemblaggio di nuovi virioni.

ABSTRACT

A major impediment in Hepatitis C Virus (HCV) research and drug development has been the lack of a culture system supporting virus production. This obstacle was recently overcome by using JFH1-based full-length genomes that allow the production of infectious viruses both *in vitro* and *in vivo*. Despite such improvement, the system was restricted to two structural gene sequences (JFH1 and J6), both derived from the genotype 2a, limiting comparative studies between different HCV strains. The system was thus extended by the creation of a series of inter-genotypic HCV chimeras that allow the production of infectious genotypes 1a, 1b and 3a particles, even if with a less efficiency in terms of productivity compared with the wild type JFH1 strain.

In the present study, based on the JFH1 strain, we generated two novel chimeric HCV constructs in which only the sequences encoding for the E1 and/or E2 glycoprotein ectodomains are substituted from JFH1 to the Con1 strain (genotype 1b). The entire structural region is maintained of the infectious strain JFH1, with the exception of the soluble portion of E1 and/or E2, allowing comparative analysis of the impact of such regions on virus morphogenesis.

Both JFH1/Con1E1E2 and JFH/Con1E2 chimeric constructs are able to replicate in hepatic cells. Importantly, we demonstrate for the first time that the E1E2 heterodimer formation is not hampered by the ectodomain swapping since glycoproteins from genotypes 2a and 1b can correctly interact each other. Nevertheless, none of the chimeric constructs allow the production of infectious viral particles. This evidence first suggests a specific role of E1 and E2 glycoproteins in HCV particle morphogenesis, and it is corroborated by several experiments. In particular we examined the JFH/Con1E2 construct, in which only the E2 ectodomain region is swapped from genotype 2a to 1b, by confocal microscopy analysis, *trans*-complementation experiments and by evaluating the presence of HCV infectious particle precursor within transfected cells. Overall our data provide strong evidences that the E2 ectodomain is involved in the HCV assembly through a genotype-specific interplay with the remaining viral structural proteins. As consequence, in the case of inter-genotypic chimeras, genetic incompatibility between JFH1 backbone and the E2 structural protein of genotype 1b dramatically affects the production of viral particles in our system. One of the most likely involved protein in such interactions seems to be NS2, the role of which was recently demonstrated as crucial in the HCV assembly/release processes. However this

remains a hypothesis that needs to be verified in order to define the relationships among HCV proteins.

§ 1. INTRODUCTION

Hepatitis C virus (HCV) is a major cause of acute and chronic hepatitis worldwide being about 170 million people currently infected as witnessed by the world Health Organization (WHO) reports. This number is probably underestimated since the acute infection is usually asymptomatic, making the early diagnosis difficult to achieve. There is a general agreement on the fact that 20-30% of acutely infected individuals undergo a spontaneous viral clearance that results in a resolution of the infection without any health complications. However, in the main percentage of infected individuals, the acute infections turn into persistent. When individuals become chronically infected by HCV, they have a very high risk of developing serious liver disease that may begin with liver steatosis, cirrhosis and progress to hepatocellular carcinoma (1, 2). The liver is the HCV primary target, although it has been shown that the viral RNA is able to replicate also in hematopoietic cells, as dendritic cells and B lymphocytes (3, 4). The primary source of HCV infection has not been determined in up to 40% of cases, but it is in generally thought to occur trough parental routes. Since most Hepatitis C infections become chronic, the transmission of the virus by parental exposure to blood from a persistently infected individual is thought to be the most common means of infection. In addition to the parental exposure, the most important mode of transmission is the illicit use of injecting drugs, in particular the re-use of needles and syringes that have not been adequately sterilized. Sexual transmission is also a major cause of infection (171).

Although many efforts have been made to develop an efficacious vaccine against HCV, both therapeutic and prophylactic, the target of these studies has still to be achieved. So far, the only way to cure the disease is based on the combined use of the antiviral drugs interferon-α and ribavirin, which unfortunately are effective only in less then 50% of the infected population, depending on individuals and mostly on the HCV genotype. Hepatitis C virus, in fact, is present around the world in six main genotypes and a larger number of subtypes, some of which are mainly distributed in certain areas compared to the others. Genotypes 1 is prevalent in Northern Europe and USA (subtype 1a), as well as in Chorea, Japan, Southern and East Europe (subtype 1b), whilst genotype 5 is only present in South Africa and genotypes 4 and 6 are more frequent in Asia and Australia (5, 6). Genotypes 2 and 3 are widely distributed throughout the world, and it is against them that treatments with antiviral drug cocktails achieve the best results being successful in about 80 % of patients (7, 8). Unfortunately, genotype 1 causes approximately 70%–80% of chronic HCV infections in the United States and more than 60% in Europe and Asia, making the current standard of care

unsatisfactory for many patients. Thus, more effective, more tolerable, and/or more tailored therapies are required.

1.1 HCV life cycle.

Hepatitis C virus (HCV) is a small enveloped positive stranded RNA virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family. The HCV particle is thought to have a diameter of about 50 nm. It is composed by a nucleocapsid surrounded by a host cell-derived membrane envelope that contains the viral glycoproteins E1 and E2 (9-11). HCV glycoproteins are type I membrane proteins with a C-terminal transmembrane domain anchored in the lipid envelope. The transmembrane regions of the two proteins are essential for E1E2 heterodimerization, being the E1 and E2 non-covalently linked heterodimer recognized as viral spike. The ectodomain of HCV envelope glycoproteins is highly glycosylated, and these glycans have been shown to play a major role in protein folding, in virus entry and in protection against neutralizing antibodies (11-14).

The HCV genome comprises about 9600 nucleotides that encode a single polyprotein of about 3000 amino acids residues. This precursor is co-translationally processed by cellular and viral encoded proteases into at least 10 different structural and non structural proteins (Figure 1) (15).

The HCV life cycle and host-virus interactions that determine the outcome of infection have been difficult to investigate due to the lack of an efficient cell culture able to support the virus production *in vitro* and the lack of small animal models. However, a big breakthrough arose recently with the propagation of the virus in a human liver hepatoma cell line, Huh7, by transfecting these cells with RNA transcribed from a full-length cDNA isolated from a patient with a fulminant hepatitis. The patient was infected with a genotype 2a isolate (10, 16). Although this result represents a great step forward in the study of HCV life cycle, the virus yield from this cell culture system (HCVcc) is still too low to be broadly applied. Consequently, mechanisms of viral entry, uncoating, trafficking, assembly and egress, as well as the host functions involved, remain to be elucidated. In conclusion, whereas several data have been generated by using the HCVcc system and much more are coming, the bulk of knowledge on HCV functions and life cycle are still based on old and new data obtained using "viral surrogates" and alternative methods comprising the replicon system (17, 18) and the HCV pseudoparticles (19, 106, 107).

To initiate its life cycle, a virus must bind to the host cell and crosses the plasma membrane to gain access to the inner contents of the cell. Attachment is mediated by the binding of a virion

surface protein to a molecule on the cell surface acting as receptor. Viral attachment can occur as a multistep process, involving more than one type of receptor or co-receptor. After binding to the host cell, enveloped viruses gain entry to the cytoplasm by fusing their lipid envelope with the plasma membrane or an endosomal membrane (Figure 1).

Plasma-derived HCV have been reported to be in complex with low-density and very-low-density lipoproteins (LDL and VLDL). As a result of this association between HCV and lipoproteins, the LDL receptor (LDL-R) has been proposed as potential attachment factor for HCV (21, 173). After the initial attachment to the host cell, a virus binds to high affinity receptor(s) or specific entry factor(s), which are responsible for initiating a series of events leading to the release of the viral genome into the cytosol. With the development of tools to study HCV, several cell surface molecules have been described as specific entry factors for this virus. The first identified and best characterized is the tetraspanin CD81, which was initially shown to interact with HCV glycoprotein E2. To date, CD81 remains the only identified putative receptor proved to be essential, although not sufficient, for viral infection (22, 23). After the identification and characterization of CD81, the human scavenger receptor class B type I (SR-BI, also called CLA-1) has been proposed as another potential entry factor for this virus (24). However, the expression of CD81 and SR-BI is not sufficient to support HCV entry. This observation led to the hypothesis that at least one additional factor might be required (20, 25).

By screening a complementary DNA library derived from HCV permissive cells for genes that render cells susceptible to HCVpp infection, the tight junction protein Claudin-1 (CLDN1) has recently been identified as a new entry factor for HCV. Kinetics of inhibition with antibodies suggests that CLDNs might play a role in a late step of the entry process, probably after virus binding and interaction with CD81, but the precise role of CLDN proteins in HCV entry remains to be determined (26). Furthermore the observation that expression of CD81, SR-BI and CLDN1 is still not sufficient to support HCV entry into some cell lines suggests that other cellular molecules are involved in such process.

The binding of enveloped viruses to cell surface molecules is followed by fusion of the lipid envelope with a cellular membrane. For HCV, it has been shown that the particle enters target cells by clathrin-mediated endocytosis and fusion has been proposed to occur in the early endosomes due to the low pH of such compartment (27, 28). Based on its classification in the *Flaviviridae* family, it is currently thought that HCV envelope proteins have a behavior similar to

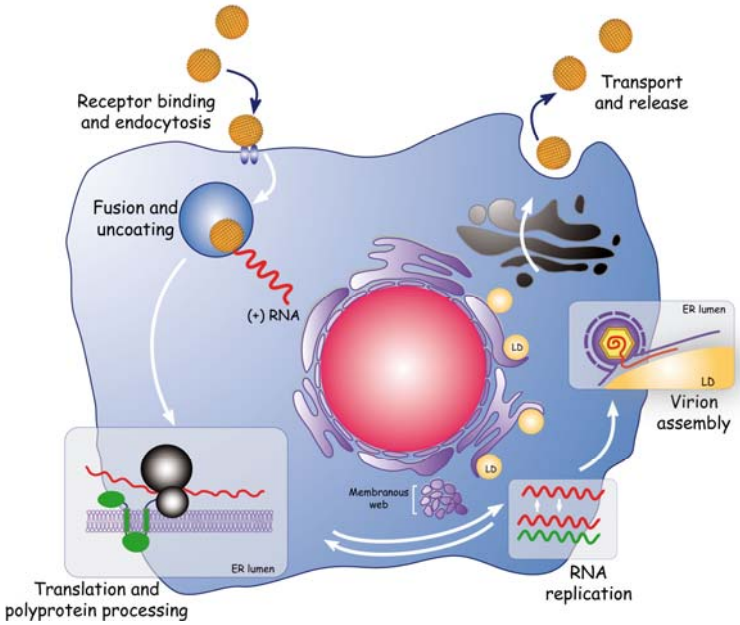


Figure 1. Schematic representation of the major steps of HCV life cycle. The virus binds to a receptor at the cell surface, which leads to endocytosis of the particle. Fusion between the viral envelope and an endosomal membrane leads to the release of HCV genome into the cytosol. HCV genome is a positive strand RNA, which is directly translated and all the viral proteins are simultaneously produced. Expression of HCV proteins induces intracellular membrane alterations (the membranous web), which is the site of RNA replication. The nonstructural proteins NS3 to NS5B assemble in association with cellular factors to form a replication complex, which is responsible for RNA replication. Accumulation of HCV genomic RNA and the structural proteins leads to the assembly of a nucleocapsid, which acquires its envelope within an intracellular compartment. The viral particle is then secreted by following the classical secretory pathway.

class II fusion proteins (29). Both envelope glycoproteins seem to have a chaperone activity for their partner and they both seem to play a role in the fusion process. Finally, the transmembrane domains of both proteins have been recently suggested to play an important role in fusion (11, 30, 31). After fusion between the viral envelope and the endosomal membrane, the positive strand genome is released into the cytosol. The HCV nucleic acid can then function as both mRNA and viral genome. The plus-strand RNA is in fact translated into viral proteins and transcribed into its negative-strand that acts as template for the serial production of RNA positive-stranded genomes.

The HCV positive strand RNA molecule encodes for a single polyprotein of about 3000 amino acids. Apart for the unique open reading frame, the about 9,600 nucleotides of the genome include two untranslated regions (UTRs) positioned at the 5' and 3' ends (Figure 2).
The **HCV 5′ untranslated region** (5′UTR), the most conserved region of the genome, contains 4 domains, numbered I to IV, that are highly structured in numerous stem loops and a pseudoknot (32, 33). Domains II, III, and IV together with the first 12 to 30 nucleotides of the core-coding region constitute the internal ribosome entry site (IRES) (34). HCV genome translation is under the control of the IRES. Domain I of the 5′UTR does not belong to the

IRES but plays an important role by modulating IRES-dependent translation (35, 36). The IRES mediates cap-independent internal initiation of HCV polyprotein translation by recruiting both viral and cellular proteins, including eukaryotic initiation factors (eIF) 2 and 3 (37, 38)

Translation of the polyprotein proceeds into the cytoplasm until the C-terminal region of core is reached. At this point, the nascent peptide is targeted to the ER membrane, a process mediated by the internal signal sequence located at the end of the core protein. The synthesis continues into the lumen of the ER, starting from E1 until another translocation event from the luminal to the cytoplasm side of the ER occurs. This event is still a matter of debate since it is unknown on which site of the ER the protein NS2 is located. The remaining nonstructural proteins, form NS3 through NS5b, are synthesizes into the cytoplasm although they remain ER membrane associated. Cleavage of the precursor occurs co- and post-translationally by concerted action of two cellular and two viral proteases into at least 10 different products (39) (Figure 2).

A synthetic more detailed picture of the chronological series of events and players is the follow. The cellular signal peptidase (SP) is responsible for the cleavage between Core-E1, E1-E2, E2-p7 and p7-NS2 but with differential efficiency and timing. Besides the presence of uncleaved precursors (see below), the mainstream of the process allows co-translational separation of core and E1, whereas E2 is primarily found as a short-living E2-p7-NS2 precursor that gives rise to a more stable E2-p7 species. The first cleavage separate NS2 from NS3 and it is efficiently carried out by the first-acting viral protease, namely NS2/3 protease. Shortly after, SP separates efficiently the p7 from NS2, meanwhile the E2p7 subsequent cleavage is slow and inefficient since a certain percentage of the precursor is found also when folding is completed. It is still unknown whether p7 is part of the viral particles, either as E2p7 species or alone (119, 174, and 175). Apart from E2-p7, the presence of other different precursors have been reported in literature, although their characterization has never been systematically approached and their relative abundance and kinetic depend on the expression system used (134). The other proteases involved in the generation of the distinct species are the cellular signal peptide peptidase (SPP) and the viral NS3/4A. The first one removes about 15 residues C-terminus of the core within the transmembrane region of the protein, a reaction required for the correct localization of the protein. The NS3/4 protease is responsible for the processing of all the downstream proteins of the polypeptide.

To most of the resulting proteins distinct functions have been described. The structural proteins core (C), and the envelope glycoproteins E1 and E2 are the major constituents of the virus particle (see below) (40, 41, 148).

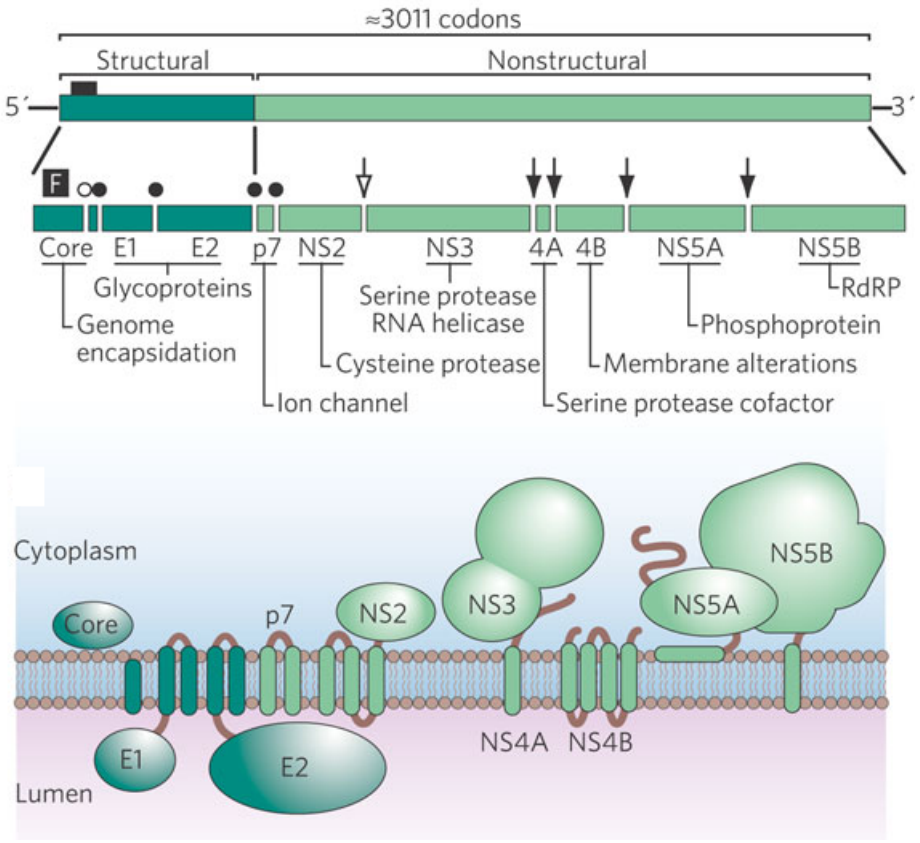


Figure 2. HCV genome organization (top) and polyprotein processing (bottom). Structural and non structural regions are shown, as well as the 5' and 3' non-coding regions. The polyprotein processing and the location of the 10 proteins relative to the endoplasmic reticulum membrane are schematically represented on the lower part of the figure. Closed circles indicate cleavage by the ER signal peptidase, the white arrow the self-cleavage by the viral NS2-NS3 protease, whilst the black arrows indicate the cleavage site of the NS3 protease.

The **core** protein is an RNA-binding protein that is supposed to form the viral nucleocapsid. When expressed in the context of heterologous expression systems or HCV replicons, core is found both attached to the Endoplasmatic Reticulum (ER) and at the surface of lipid droplets (LDs) (42, 43). However in the context of infectious virus, core is only found in association with the latter one, playing the major role in viral particle assembly. To support this conclusion, recent works (44, 45) demonstrated the interaction of core with the non-structural protein NS5A as determinant for the viral assembly process, and with the ectodomain of E1 protein. The latter interaction has been claimed to be important for the envelope proteins incorporation in the nascent viral particles (46).

The small hydrophobic polypeptide **p7** has recently been shown to be an ion channel that is sensitive to inhibition by amantadine, a drug that blocks ion channel function in influenza viruses (47). It is speculated that the p7 protein is a viroporin and responsible for the flow of calcium ions from the endoplasmic reticulum into the cytoplasm. However, recently it was demonstrated that p7 is primarily involved in the late phase of the HCV replication cycle, in particular in the assembly and release steps (48). Similarly, the function of **NS2** in its mature

form is still under investigation, even if recent publications indicate its involvement in the virus assembly and release as well as p7 protein (49, 50). The only well established function of NS2 regards an enzymatic activity effective before its own cleavage from the polyprotein. In fact, the NS2 C-terminus together with the amino terminal domain of NS3 constitute the **NS2-3** proteinase that mediates cleavage between NS2 and NS3 (51, 52). Although the exact mode of action of this enzyme has not been elucidated, recent evidence suggests that it is a cysteine-proteinase presumably activated via interaction with the cellular chaperone Hsp90 (53-55). The NS3 protein displays two different functions in a single polypeptide. The N-terminus region shows a helicase activity involved in both translation and replication of the virus. The C-terminal two thirds domain of **NS3** carries a serine-type proteinase activity that forms a stable heterodimeric complex with **NS4A** (56-59). The central region of NS4A, in particular, acts as co-factor of NS3 proteinase activity, allowing its stabilization, localization at the ER-membrane and activation at cleavage site (60, 61). **NS4B** is a highly hydrophobic protein predicted to harbor at least 4 transmembrane domains and an N-terminal amphipatic helix that are responsible for the ER-membrane association (62-64). In fact one of the functions of NS4B is to serve as a membrane anchor for the replication complex (65, 66).
Controversially discussed is the role of **NS5A** in the HCV life cycle. It is a 56 to 58-kDa phosphorylated zinc-metalloprotein that plays an important role in virus replication. The N-terminal region of NS5A contains an amphipatic α-helix that is necessary and sufficient for its association with the perinuclear membranes as well as for assembly of the replication complex (67-69). Although the mechanism by which NS5A regulates HCV replication is not entirely clear, very recently it was demonstrated that it is a major determinant for infectious virus production (45).
NS5B is a membrane-associated protein essential for RNA replication in cell culture. Like most HCV proteins, NS5B is detected in association with ER or ER-derived membranes (70-72). NS5B is an RNA-dependent RNA polymerase, which is the catalytic component of the HCV RNA replication machinery. This enzyme synthesizes RNA using an RNA template. NS5B can initiate RNA synthesis de novo, at least *in vitro*, and it is assumed that de novo initiation is also operating *in vivo* (73).

The precise mechanism of HCV replication is still poorly understood, with few invariant steps relatively well defined. A rearrangement of intracellular membranes is a prerequisite for the formation of the replication complex that includes viral proteins, cellular components and the nascent RNA strands. The HCV NS4B protein seems to be sufficient to induce the formation of a “membranous web”, recruiting membranes derive from the ER compartment (73).

Overall, the membranous web consists of small vesicles embedded in a membranous matrix, forming a membrane-associated multiprotein complex that contains all of the nonstructural HCV proteins (65). In that context, the positive strand genome RNA is used by NS5B RdRp as template for the synthesis of a negative-strand intermediate of replication. In a second step, negative-strand RNA serves as a template to produce numerous strands of positive polarity that will subsequently be used for polyprotein translation, synthesis of new intermediates of replication, or packaging into new virus particles (74).

Due to the lack of appropriate study models, little is known about the mechanisms of HCV nucleocapsid assembly, formation of intracellular particles and their exit from the cells. HCV core proteins can efficiently self-assemble to yield nucleocapsid-like particles with a spherical morphology and a diameter of 60 nm. Considering that this process requires the presence of a nucleic acid, viral particle formation is probably initiated by the interaction of the core protein with genomic RNA. Thus, the core-RNA interaction could play a major role in the switch from replication to packaging (75, 76).

The mechanism by which mature, infectious HCV progeny finally leaves the cell is still enigmatic. One possibility is that the formed nucleocaspid acquires its envelope proteins E1 and E2 by budding into the lumen of the ER (39). Nevertheless, in a recent work Miyanari et al. highlight the important role that core and lipid droplets (LDs) have in producing infectious viruses (see below). Once the virions have assembled they are likely released from cells through the exocitic pathway (77, 176).

1.2 HCV envelope glycoproteins.

The two glycoproteins, E1 and E2, are essential components of the HCV virion envelope and are necessary for viral entry and fusion (19, 78). E1 and E2 have molecular weights of 33–35 and 70–72 kDa respectively, and assemble as non covalent heterodimers. They have been classified as type I transmembrane glycoproteins, with N-terminal ectodomains of 160 and 334/337 amino acids respectively, and a short C-terminal transmembrane domain of approximately 30 amino acids (79).

The ectodomains of HCV envelope glycoproteins are highly modified by N-linked glycans. Indeed, E1 and E2 possess up to 6 and 11 potential glycosylation sites, respectively, and most of them are well conserved and used (80, 81). It is worth noting that some glycans have been shown to play a role in HCV glycoprotein folding, in virus entry or in modulating the immune response (82, 83). Hypervariable regions have been identified in the E2 glycoprotein ectodomain. Hypervariable region 1 (HVR1) contains 27 amino acids and is a major (but not

the only) HCV neutralizing epitope (84-86). HVR1 sequence variability, the physicochemical properties of the residues at each position and the overall conformation of HVR1 are highly conserved among all known HCV genotypes, suggesting an important role in the virus life cycle (87). Another hypervariable region, HVR2, has also been described in E2, and this region has been proposed to modulate E2 receptor binding (88).

Although HCV glycoproteins can be detected at the plasma membrane when they are over-expressed, the E1E2 heterodimer is mainly retained in the ER (97, 107). The determinants for ER retention of HCV envelope glycoproteins have been mapped in the transmembrane domains (TMDs) of E1 and E2 (89, 91, 178). The E1 and E2 anchor domains are composed of two stretches of hydrophobic residues separated by a short segment containing at least one fully conserved charged residue. It is reported that the TMD of E1 spans position 353 throughout 383, while the TMD of E2 protein is 29 aminoacids in length, extending from residue 718 to 746 of the polyprotein (89). However the native structure of E1 and E2 proteins is still under investigation since crystallography studies are not been achieved. As consequence, the topology deducted for both envelope proteins mainly derives from structural prediction analysis based on their primary sequence. The TMDs of HCV envelope proteins are examples of multifunctionality. Indeed these regions are involved in membrane anchoring, ER retention, recently confirmed in HCVcc infected cells, and they also play a major role in the assembly of E1E2 heterodimer (90-93). Analyses of the E1 and E2 maturation shows that folding kinetics is slow and proceeds to completion after the formation of the dimers (94-96). Interestingly, the folding of E1 has been shown to be dependent on the co-expression of E2, and similarly, the folding of E2 is also dependent on the co-expression of E1 (94, 97, 98). Altogether, these observations indicate that HCV envelope glycoproteins cooperate for the formation of a functional complex. Importantly, the E1E2 heterodimers assembly is a prerequisite for virus formation (10).

1.3 Hepatitis C virus infectious particle assembly.

As mentioned above, there is not a broad consensus on a single model for viral particle assembly. The one recently proposed by Miyanari and colleagues (77) takes advantage on the HCVcc system to introduce a role for lipid droplets in the assembly of nascent HCV particles. A link between lipid droplets and HCV was first suggested 10 years ago, when the HCV core protein was produced *in vitro* in various cell types using heterologous expression systems. In these studies core protein was found as cytoplasmic, and either associated with the ER membrane or on the surface of lipid droplets (102, 103).

Lipid droplets are intracellular organelles present in all eukaryotic organisms that function as storage place of neutral lipids. They consist of a core of neutral lipid, comprising mainly triacylglycerols and/or cholesterol esters, surrounded by a monolayer of phospholipids (99). On electron microscopy, lipid droplets appear as grey amorphous circular masses, usually surrounded by a darker rim. The LD biogenesis is not completely determined, it has been suggested that neutral lipid accumulation within the lipid bilayer of the ER membrane induces the budding of an independent organelle, surrounded by the phospholipid monolayer originating from the cytoplasmic leaflet of the ER membrane (100). However, rather than inert inclusion, LDs dynamically move through the cytoplasm, interacting with other organelles, including the endoplasmic reticulum. These interactions are thought to facilitate the transport of lipids and proteins to other organelles (101).

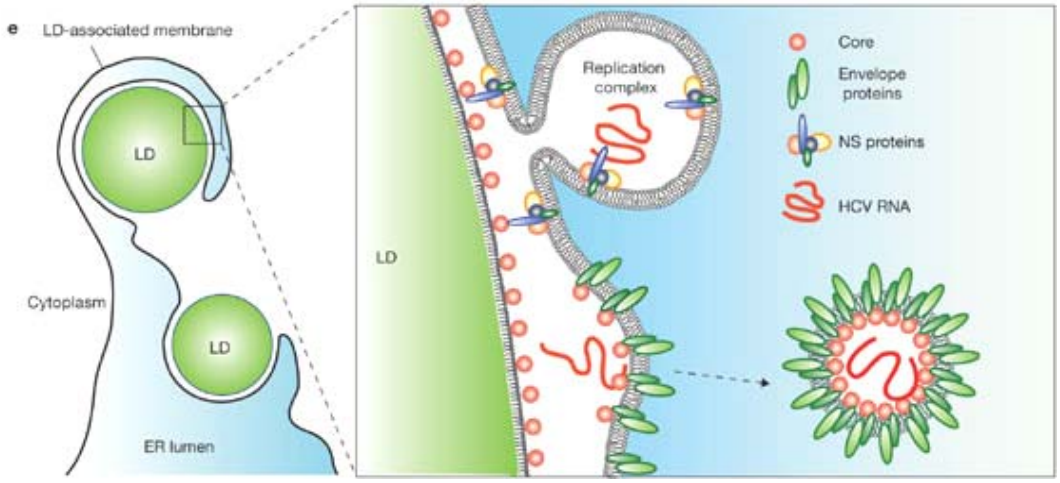


Figure 3. A model for the production of infectious hepatitis C virus (HCV). Core mainly localizes on the monolayer membrane that surrounds the LD. HCV induces the apposition of the LD to the endoplasmic reticulum (ER)-derived bilayer membranes (LD-associated membrane). Core recruits NS proteins, as well as replication complexes, to the LD-associated membrane. NS proteins around the LD can then participate in infectious virus production. E2 also localizes around the LD.

The model of particle assembly proposed by Miyanari starts from the observation that core mainly localizes on the monolayer membrane that surrounds the LD. They hypothesize that HCV induces the apposition of the LD to the endoplasmic reticulum (ER)-derived bilayer membranes (LD-associated membrane). The ER from infected cells, and such LD-associated membranes, is loaded with structural and non-structural HCV proteins. Thus, core recruits first NS proteins, as well as the replication complexes, to the LD-associated membrane and, through particularly NS5a, realizes its association with the nucleic acid. The recognition of the HCV RNA by core is then sufficient to trigger the particle assembly. At some point during nucleocapsid formation, the complex interacts with the E1E2 dimers spanning the membrane,

probably via core-E1 interaction. It is still unclear where exactly envelopment of the nucleocapsid occurs. However, it is possible that LDs tightly surrounded by ER membranes are the sites where nucleocapsid form, which could then acquire their envelope via budding into the ER lumen at sites in close proximity of LDs (Figure 3).

1.4 Hepatitis C virus model systems.

Soon after the discovery of the hepatitis C virus (HCV) in 1989, attention turned to the development of models whereby replication of the virus could be investigated. Unfortunately the possibility to study numerous aspects of the hepatitis C virus life cycle was strongly hampered by the lack of a cell culture that efficiently supported the *in vitro* viral production. As consequence, the knowledge mainly derives by the use of HCV surrogates and heterologous expression systems, comprising the replicon system (17, 18), the HCV pseudoparticles (20, 104) and the recombinant proteins E2 or E1E2 complex (105, 106).

The big breakthrough arose in 2005 when Wakita and colleagues published the discovery of a subgenomic replicon of the JFH1 genotype 2a strain able to efficiently replicate in cell culture and, above all, supporting the secretion of viral particles. Nevertheless most of the knowledge on Hepatitis C Virus was achieved by using the other tools just mentioned. It is worth noting that such models are still used in the HCV research as useful tools to support the HCVcc system.

The soluble, truncated forms of the E2 glycoprotein (sE2: $E_{348\text{-}661}$, $E_{348\text{-}715}$) have been used as a tool to search for cell-surface proteins potentially involved in HCV attachment and entry. Indeed it is through the sE2 use that Pileri et al. (22) were able to identify the CD81 molecule as potential receptor for HCV. The following availability of both E1 and E2 glycoproteins, thanks to the co-purification method developed by Choo et al. permitted to perform the experiments with a more physiological ligand respect to the sE2 protein (106).

Another tool applied to investigate the virus-host interaction is based on the HCV pseudoparticle (HCVpp) system in which the HCV envelope proteins are incorporated into the envelope of retroviral particles (20, 104, 107). HCVpp mimic the early steps of HCV life cycle in terms of attachment, receptor binding and cell entry pathways. In fact they show a particular tropism for liver-derived cells and are neutralized by anti-E2 specific antibodies. All together these characteristics made HCVpp useful for the characterization of some candidate receptors for HCV (108), but it is important to note that HCV pseudoparticles are basically different from the natural HCV virions. As consequence they are not applying in the study of the subsequent steps of the HCV life cycle.

In conclusion, among the HCV replication models developed, the HCV RNA replicon and the newly discovered infectious cell culture system have had an immediate impact on the study of HCV replication, and will continue to lead to important advances in our understanding of HCV replication.

HCV replicon system.

The description of an autonomously replicating subgenomic HCV replicon is the first demonstration of truly robust *in vitro* HCV replication. Development of subgenomic HCV replicons in fact have offered the opportunity to study HCV expression of both RNA and proteins and their effects on intracellular events (17).

The HCV replicon is a bicistronic RNA molecule which contains an HCV IRES in front of the neomycin phosphotransferase gene. The second cistron in the RNA molecule contains an encephalomyocarditis virus (EMCV) IRES, followed by the HCV non structural proteins (NS3 trough NS5B) and terminating with the HCV 3' UTR (Figure 4) (17, 18). Importantly, HCV replicons are self-amplifying elements. They are maintained during numerous passages after Huh7 cells are transfected with the *in vitro*-synthesized RNAs. However during this period, a number of adaptative mutations accumulate in the subgenomic replicon. Certain of such tissue culture-adapted replicons thus acquire the ability to replicate at higher efficiency than the parental strain. In this way, they disguise their original sequence identity that once represented HCV isolates extracted from infected patients.

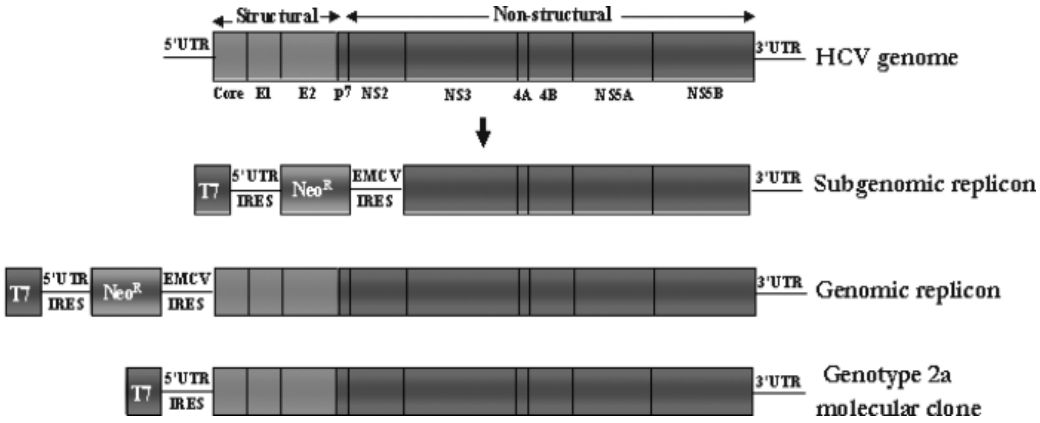


Figure 4. The structure and organization of the (a) HCV genome, (b) subgenomic HCV replicon, (c) the genomic HCV RNA replicon, (d) genotype 2a molecular clone used for production of infectious hepatitis C virions. Core, core protein; E1, envelope 1 glycoprotein; E2, envelope 2 glycoprotein; NS2, nonstructural protein 2; NS3, nonstructural protein 3; NS4A, nonstructural protein 4A; NS4B, nonstructural protein 4B; NS5A, nonstructural protein 5A; NS5B, nonstructural protein 5B; 3'-UTR, 3'-untranslated region; 5'-UTR, 5'-untranslated region; IRES, internal ribosome entry site; EMCV, encephalomyocarditis virus; T7, the promoter region of the T7 RNA polymerase.

An important expansion of the sub-genomic replicon system is the development of full-length genomic HCV replicon as potential tool to generate viral particles in cell culture. In fact, the entire HCV genome is maintained in this RNA molecule, including the structural proteins (Figure 4). However, despite the high level of viral RNA replication and protein expression, HCV full-length replicon does not allow the production of infectious viral particles (109-111). To overcome this deficiency, a large panel of different replicons has been engineered. Although these systems present a superior replication capability thanks to the development of adaptive mutations within NS coding region, they are not able again to produce infectious viral particles (112, 113).

HCV cell culture system (HCVcc).

In spite of the accumulating number of cell culture adaptive mutations described for a number of HCV RNA replicons, the production of infectious hepatitis C virions was never observed (113-115). The major breakthrough that overcome this obstacle in the study of the molecular biology of HCV was the isolation of a peculiar HCV genotype 2a strain, designated as JFH1 (Japanese Fulminant Hepatitis), from a patient with fulminant hepatitis. The subgenomic HCV replicon derived from this genotype 2a isolate is in fact able to replicate at high levels in human hepatoma cells Huh7 without the need for adaptive mutations. In particular, its ability to replicate is 58-fold higher than that of the genotype 1b replicon harboring cell culture-adapted mutations (113). Importantly, Wakita *et al.* demonstrated that the full-length JFH1 genome supports secretion of viral particles post RNA transfection of Huh-7 cells. In addition, the cell culture-generated HCV is infectious in chimpanzees and could be subpassaged to naïve Huh-7 cells (10). Next, two research groups combined the JFH1 molecular clone with an highly permissive Huh 7 cell line clone (Huh 7.5) to describe an efficient *in vitro* model that allows the production of detectable quantities of infectious hepatitis C virions (10, 15, 29, 117). This HCV genome replicates in cell culture producing ~10^3 infectious units per milliliter within 48 hours in Huh-7.5 cells. Despite this improvement, a yield of virus title of about 10^3 pfu/ml (particle forming unit) is definitely low. In support of the JFH1 strain-based system, Lindenbach *et al.* (2005) then constructed a chimeric full-length HCV genome composing of the JFH1 NS3–5B and the core–NS2 region from another genotype 2a strain (J6). This intra-genotypic chimera, designed Jc1, yields infectious titers 100 to 1000-fold higher than the parental isolate.

The achievement is clearly important to study complete HCV replication cycle in cell culture. Nevertheless the system is limited by the dependence on two structural gene sequence (JFH1 and J6), both derived from the genotype 2a. Therefore, comparative studies, about the impact

of variability in the structural genes on neutralization in an authentic infection system, or evaluation of antiviral compounds targeting different steps of the HCV life cycle of multiple genotypes, cannot be performed on a broad scale. In fact, as previously described, HCV displays a marked genetic heterogeneity, which justifies its classification in six major genotypes. Among these genotypes, genotypes 1 and 2 have a worldwide distribution and are known to be associated with different clinical profiles and therapeutic responses (5). These differences in clinical features are likely a result of viral characteristics. To study the molecular mechanism underlying such differences the scope of the genotype 2a-based cell culture system was extended by the construction of inter-genotypic JFH1-based chimeras consisting of the 3'-half genome of JFH1 and the 5'-moiety, including part of NS2, taken from genotype 1a, 1b, 3a and 4a (49, 118) (Figure 5). When these novel constructs are transfected in human hepatoma cells Huh7 and Huh7.5, infectious viral particles are released in all cases, with the exception of the genotype 3a. However, the low efficiencies of these systems, compared with the original JFH1, do not allow the development of satisfactory cell culture HCV production. Moreover, a tremendous difference in the kinetics of virus release with the different chimeras is observed, arguing for a genotype or isolate-specific determinants in the structural genes that govern virus assembly and release (49). Thus, although the availability of a spectrum of chimeric HCV genomes differing in their structural region represents an important improvement, there remains a need for a cell culture system to investigate the specific role of each structural protein in morphogenesis of HCV particles.

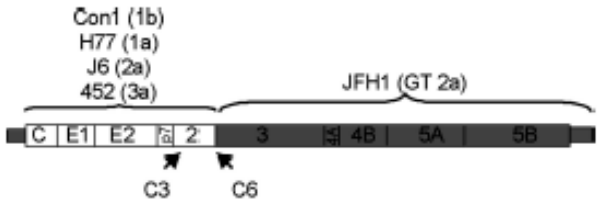


Figure 5. A schematic representation of the chimeric genome design is shown. Sites used for fusion of the genome segments are indicated by arrows and localize to a position right after the first TMD of NS2 (C3) or exactly at the NS2–3 cleavage site (C6).

In this study we describe the construction and characterization of novel inter-genotypic JFH1-based chimeras, in which only the sequences encoding for the E1 and/or E2 glycoprotein ectodomain are swapped from JFH1 to the Con1 strain (genotype 1b). Thus, the entire structural region of the infectious strain JFH1 is maintained, with the exception of parts of the envelope proteins coding sequence, allowing comparative analysis of the impact of E1 and E2 on virus morphogenesis.

§ 2. AIM

HCV is a major cause of chronic liver diseases, but development of selectable drugs and efficient vaccines has been hampered by poor virus growth in cell culture, beside this it was difficult to unravel the HCV life cycle in absence of adequate cell culture system.

Recently, a full length genotype 2a HCV genome, designed JFH1, has been isolated. Genomic replicon of this clone and of its derived intra-genotypic chimera J6, are able to replicate in Huh7 cells and allow the production of infectious viral particles. In the present study we intended to exploit the ability of the JFH1 system to develop a new type of inter-genotypic chimera in which only the encoding sequence for the ectodomain of the envelope proteins E1 and/or E2 of JFH1 was substituted with the relative sequence belonging to the genotype 1b. In such way, our chimeras would represent a valid tool to define the role of the envelope protein ectodomains in the HCV morphogenesis. In fact, maintaining almost all the sequence from the infectious strain JFH1, the relationships among HCV envelope proteins and the other structural proteins, and their dependence by the genotypic-specific sequence, could be evaluate.

§ 3. MATERIALS AND METHODS

3.1 Cell culture and antibodies.

Experiments were performed by using human hepatoma cells Huh.7 and S6.1. Cells were maintained in complete DMEM supplemented with 10% FCS/2mM L-glutamine/ 100 U/ml penicillin/ 100µg/ml streptomycin at 5% CO_2. S6.1 cells were derived from a Huh.7 HCV replicon cell line that was cultured in presence of INF-γ to eradicate the HCV replicon. Clearance of such replicon bearing the neomycin resistance gene was confirmed and the resulting S6.1 cell line was highly permissive for HCV replication.

S6.1 cell line served also for the construction of HCV packaging cells S6.1/E1E2:2a and S6.1/E1E2:1b. The first one stably expresses E1 and E2 envelope proteins from genotype 2a, the second E1 and E2 genotype 1b-derived. S6.1/E1E2:2a and S6.1/E1E2:1b packaging cell lines were obtained in our laboratory by using the lentiviral system. Briefly, lentiviral particles expressing the envelope proteins of JFH1 or Con1 strain were used to trasduce S6.1 cells. The stable integration of E1E2 dsDNA into the genome of S6.1 cells led to the creation of the two packaging cell lines constitutively expressing the envelope proteins of HCV genotype 2a or 1b.

The three specific anti-HCV glycoproteins antibodies used in this study consist on the polyclonal chimpanzee antiserum Ch-L559, and the human monoclonal antibodies anti-E2 CBH2 and CBH5. Ch-L559 is derived from the serum of a chimpanzee immunized with recombinant E1E2 purified from HeLa cells infected with HCV glycoproteins, whose expression was driven by vaccinia virus (106). This polyclonal antiserum is able to recognize all forms of E1 and E2 in Western blot analysis, neutralize binding of E2 and recognize HCV glycoproteins in immunofluorescence studies on permeabilized cells (119, 120). The human monoclonal anti-E2 antibodies CBH2 and CBH5 are two of a panel of nine conformational sensitive antibodies generated from peripheral B-cells of an asymptomatic HCV infected individual. These antibodies do not recognize denatured E2, inhibits binding of E2 to the putative receptor CD81, and specifically recognizes and immunoprecipitates the properly folded E2 (98, 121). The anti-core 3G1-1 monoclonal antibody was generated in mice immunized with aa 143 of the core protein purified from yeast and was provided by Michael Houghton (Chiron Corporation, Emeryville, USA). The following commercially available antibodies were used: 7-D4 anti-NS5A (Biodesign International), MMM33 anti-NS3

(Novocastra Lab. Ltd.). All the secondary antibodies conjugates to Alexa Fluor dyes are from Invitrogen Molecular Probes.

3.2 Plasmids.

pUC.JFH1 and pUC.neo-con1 encode for the full length HCV genotype 2a (strain JFH1) and genotype 1b (strain Con1) respectively. The plasmid pUC.JFH1ΔE1E2 encodes for the full-length JFH1 genome deleted of the entire E1-E2 coding region, spanning from the aa 192 (Ala) to the aa 750 (Ala). As consequence the C-terminus of core protein is directly fused to the N-terminus of p7.

To design pUC.JFHCon1E1E2 plasmid, we studied two in frame deletions in the JFH1 genome corresponding to the E1 and E2 proteins ectodomain region, from aa 192 to aa 329 and from aa 384 to aa 683 for E1 and E2 respectively. These two fragments were then substituted with the corresponding fragments taken from the Con1 genome encoded by pUC.neo-con1 vector. Following the primers used for this cloning.

PCR 1 (fig. 6)

Forward: 5' TCACCGTTCCGGTCTCTGCT TATGAAGTGCGCAAC 3'

(3' core (GT 2a) + 5' E1 (GT 1b))

This forward primer was used in combination with four different reverse primers as shown in figure 6.

Reverse:

- 5' TACGCCAGGATCATGGTGGC TGTAGGTGACCAGTT 3'
  (20 nt 5'TM E1 (GT 2a) + 15 nt 3'ect E1 (GT1b))
- 5'CTCGGGGACGCGCATCACGTACGCCAGGATCATGG 3'
  (E1 TM (GT2a) + last portion of previous rev primer)
- 5' CTAACGATGTCTATGATGACCTCGGGGACGCGCAT 3'
  (E1 TM (GT2a) + last portion of previous rev primer)
- 5' ATGACGCCCCAGTGAGCCCCGCTAACGATGTCTATG 3'
  (E1 TM (GT2a) + last portion of previous rev primer)

Reverse: 5' AGACCAGTTGACAAAGCGGG TAGGGTGGTGAAGGA 3'

(5'E2 TM (GT2a) + 3'E2 ect (GT1b))

This reverse primer was used in combination with four different forward primers as shown in figure 6.

Forward:

- 5’ TCATCCTTCTGCTGGCCGCT GGCGTTGACGGGGGA 3’
  (3’E1 TM (GT2a) + 5’E2 ect (GT1b))
- 5’ AGCGTGGGCGAAGGTCATTGTCATCCTTCTGCTGG 3’
  (E1 TM (GT2a) + last portion of previous fwd primer)
- 5’ GCCTACTTCTCTATGCAGGGAGCGTGGGCGAAGGT 3’
  (E1 TM (GT2a) + last portion of previous fwd primer)
- 5’ GGGGCGTCATGTTCGGCTTGGCCTACTTCTCTATG 3’
  (E1 TM (GT2a) + last portion of previous fwd primer)

PCR 2 (fig.7)

Forward: 5’ CCATAGTGGTCTGCGG 3’
(5’ UTR)

Reverse: 5’ GATACGTTGCGCACTTCATA AGCAGAGACCGGAAC 3’
(E1 ect (GT1b) + core (GT2a))

PCR 3 (fig. 7)

Forward: 5’ CCCTGTTCCTTCACCACCCTA CCCGCTTTGTCAACT 3’
(5’ E2 ect (GT1b) + E2 TM (GT2a))

Reverse: 5’ TGA CGG CCC ACG CGA TGC 3’
(NS2)

PCR 4 (fig.8)

Forward: 5’ TTC CCA AAC GCG TTA ATA C 3’
(T7 promoter)

Reverse: 5’ TGT CAA ACA CCA CAC CCG 3’
(NS2)

Chimeric JFH1/Con1E1E2 fragment so obtained was inserted into the vector pUC.JFH1 between the restriction sites AgeI and NotI (Roche).

The plasmid pUC.Con1E2 encodes for the second HCV inter-genotypic chimera in which the entire coding sequence derived from the JFH1 sequence with the exception of the E2 ectodomain region. This plasmid was obtained by digestion of the previous pUC.JFH-Con1E1E2 vector to delete the region between EcoRI (Roche) restriction site in the 5’NCR and the BsiwI (Roche) sites that maps in the E1 transmembrane region (fig. 6). The JFH1/Con1E2 chimeric fragment was then inserted into the pUC.JFH1 vector between the same restriction sites.

To permit generation of *in vitro* transcripts, the HCV wild type and chimeric genomes are flanked at 5'-end by the T7 promoter sequence.

3.3 In vitro transcription and RNA electroporation.

In *vitro* transcripts of the individual constructs were generated by linearizing 5 to 10 µg of the respective plasmid by digestion over night with XbaI followed by Mung Bean nuclease treatment to eliminate any additional nucleotide that does not correspond to the viral genome. Plasmid DNA was precipitated with ethanol and dissolved in RNase-free water. In vitro transcription reaction mixtures contained 80 mM HEPES (pH 7.5), 12 mM MgCl2, 2 mM spermidine, 40 mM dithiothreitol (DTT), a 3.125 mM concentration of each ribonucleoside triphosphate, 1 U of RNasin (Promega, Mannheim, Germany) per µl, 0.1 µg of plasmid DNA/µl, and 0.6 U of T7 RNA polymerase (Promega) per µl. After incubation for 4 h at 37°C, transcription was terminated by the addition of 1.2 U of RNase-free DNase (Promega) per µg of plasmid DNA and a 30 min incubation at 37°C. The RNA was extracted with phenol and chloroform, precipitated with isopropanol, and dissolved in RNase-free water. The concentration was determined by measurement of the optical density at 260 nm. Denaturing agarose gel electrophoresis was used to check RNA integrity.

For electroporation of HCV RNA into S6.1 cells, single-cell suspensions were prepared by trypsinization of monolayer and subsequent resuspension with DMEM complete. S6.1 cells were washed with phosphate buffered saline (PBS), counted, and resuspended at $6x10^6$cells per ml l. 5 µg of in vitro transcribed RNA was mixed with 400 µl of cell suspension by pipetting and then electroporated with a Gene Pulser system (Bio-Rad, Munich, Germany) in a cuvette with a gap width of 0.4 cm (Bio-Rad) at 975 µF and 270 V. Cells were immediately transferred to 6 ml of DMEM complete, and 150 µl of the cell suspension was seeded per well of a 48-well plate.

3.4 Production of cell-culture HCV (HCVcc).

S6.1 cells were seeded into 25 cm^2 flasks immediately after transfection and fed with DMEM complete medium containing 10% FCS. Cells were passaged at 3 to 4-day intervals post-transfection by trypsinization and reseeding with a 1:3 to 1:4 split into fresh culture vessels. Supernatants collected were clarified by centrifugation at 4500 x g for 5 min to remove the cell debris. Viral particles were than maintained at 4°C until titration or concentrated by ultracentrifugation at 52000 x g (26000 rpm, type JA 25.50 rotor. Beckman Coulter) for 3

hours at 4°C. pelleted viral particles were resuspended (100-fold concentration) in PBS/0.1%BSA and stored at -80°C before being titrated.

3.5 Immunofluorescence staining and virus titration.

S6.1 cells were seeded in 96-well immediately after transfection. After 3 days, cells were washed with PBS, fixed for 30 min with cold 4% paraformaldehyde, washed with PBS, permeabilized with PBS containing 0.1% triton X-100 (Invitrogen), and then blocked for 1 h with PBS containing 0.5% bovine serum albumin. After wash with PBS, core protein was detected with a 1:1,000 dilution of the anti-core 3G1-1. Cells were washed with PBS and bound 3G1-1 was detect by incubation with goat antibodies specific to mouse immunoglobulin G (IgG) conjugated with Alexa Fluor 568 (Invitrogen) at a dilution of 1:200 in PBS/0.5% BSA for 1 hour in the dark. Replication positive cells were determined by core detection under a fluorescence microscope (Zeiss, Observer.A1).

The infectivity titer was determined on Huh 7 naïve cells by end-point dilution and immunofluorescence staining. Tipically, 15 µl of supernatant or cell lysate was serially diluted 10-fold in DMEM 10% FCS according to the Spearman and Kaerber fit. Cells were seeded in 96-well plates at a density of $1x10^4$ cells per well 24 h prior to inoculation with 100 µl of the diluted supernatant or cell lysate (at least eight wells were used per dilution). Cells were fixed 72 h post-infection, permeabilized, and core-stained as described above. Virus titer was expressed in particles forming units per ml (pfu/ml) enumerating the positive cells by use of a fluorescent microscope.

3.6 Cell labeling and pulse-chase experiments.

Subconfluent 35 mm diameter dishes of S6.1/JFH1 and S6.1/JFHCon1E2 transfected cells (or not transfected as negative control) were starved for 1 hour at 37°C in cysteine- and methionine-free medium. Cells were pulse-labeled in presence of methionine- and cysteine free medium supplemented with 350 µCi ml^{-1} of [^{35}S]-labeled methionine and cysteine (ProMix, Amersham Pharmacia Biotech) for 20 min at 37 °C. The pulse period was stopped by washing cells twice with PBS. For metabolic labeling, the starving step was skipped and cells were incubated for 6 hours to over night in presence of 50 µCi ml^{-1} of [^{35}S]-labeled methionine and cysteine (ProMix). Chase period was initiated by adding complete DMEM supplemented with 2.5 mM unlabeled methionine and cysteine, and incubating cells for different chase times at 37 °C. For dithiothreitol (DTT) resistance experiments, the regular chase was followed by 5 min incubation at 37 °C in presence of 5 mM DTT. After chase time,

cells were placed on ice; medium was removed and replaced with ice-cold PBS containing 20 mM NEM. This alkylating agent blocks free sulphydrilic groups and prevents further oxidation of the cysteines (122). After 5 min of PBS-NEM incubation, cells were lysed in 900 µl lyses buffer, containing 1% Triton X-100 in HEPES-buffered saline (HBS, pH 7.4) including protease inhibitors cocktail (Complete, EDTA free, Roche), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM NEM. After 30 min of incubation at 4 °C under agitation, total lysates were centrifuged 20 min at 13,000 x g to pellet nuclei and cellular debris. PNSs were used for immunoprecipitation with specific anti-E2 antibodies.

For the proteasomal inhibition experiments, Lactacystein and MG132 inhibitors (Boston Biochem.) were added directly to the medium from the starving to the end of the chase period at a final concentration of 25 µM and 20 µM respectively.

3.7 Immunoprecipitation and SDS-PAGE.

The two specific antibodies used in this study consist of human monoclonal conformational anti-E2, the CBH-2 and CBH-5. Both of them are conformation-sensitive antibodies that do not recognize denatured E2, inhibits binding of E2 to the putative receptor CD81 and specifically recognizes and immunoprecipitates properly folded E2. CBH-2 was chose to immunoprecipitate E2 genotype 2a, CBH-5 to immunoprecipitate E2 from genotype 1b. PNSs were precleared on protein G/protein A coupled to Sepharose Fast Flow (Amersham Pharmacia Biotech) for 1 h at 4 °C and were subsequently immunoprecipitated with 0.5 µg of CBH-2 or CBH-5 (121) at 4°C overnight in presence of 20 µl Dynabeads Protein G (Dynal Biotech ASA). The radioactive counts were determined on 2 µl of each precleared lysate. Equivalent amounts of total labeled proteins were used for CBH-2 and CBH-5 anti-E2 immunoprecipitations. The immunocomplexes were collected with the proper magnetic device, washed three times with lyses buffer and solubilized at 95 °C for 5 min in 20 µl of SDS loading buffer and separated on SDS-PAGE, followed by autoradiography. For SDS-PAGE in reducing condition DTT 100 mM final was added to the samples.

3.8 Endoglycosidase H treatment.

Endoglycosidase H (endoH) cleaves ER type N-linked high mannose sugar chains between the two N-acetylglucosamine residues, thus eliminating most of the contribution of the glycan on the apparent molecular weight in SDS electrophoresis (123). Immunoprecipitated proteins recovered from beads were incubated with 50 mU endoH in 250 mM sodium acetate pH 5.5,

in a final volume of 0.25 ml. The reaction was performed for 6 hours at 37 °C in presence of 1 mM PMSF. Digested proteins were added of loading buffer with (reducing) or without (non-reducing) 100 mM DTT for SDS-PAGE analysis.

3.9 Confocal analysis.

S6.1/JFH1 and S6.1/JFHCon1E2 cells (or S6.1 not transfected cells as negative control) were plated on 30 mm coverslips in 24-well plates at a density of 5 x 10^4 cells per well. One day after seeding, cells were fixed in 4% formaldehyde, permeabilized with 0.1% Triton X-100 in PBS, pretreated with blocking solution (0.5% bovine serum albumin in PBS) and incubated at room temperature with the primary antibodies diluted in blocking solution. After three washes in PBS, AlexaFluor-conjugated secondary antibodies were added to the cells. Lipid droplets were stained in paraformaldehyde-fixed cells by briefly rinsing coverslips in 60% propan-2-ol followed by incubation with 0·5 ml 60% propan-2-ol containing oil red O (final concentration approximately 0·6%) for 1.5 to 2 min at room temperature. Coverslips were briefly rinsed with 60% propan-2-ol, washed with PBS and H_2O. The oil red O staining solution was prepared from a saturated stock of approximately 1% oil red O (Sigma) dissolved in propan-2-ol. Before staining, the stock was diluted with H_2O and then filtered (124). Coverslips were mounted in aqueous mounting medium Vectashield. Confocal microscopy was performed with a Bio-Rad 2100 Confocal Microscope using 60 x oil or 100 x oil objectives. Image analysis was performed using the standard operating software provided with the microscopes.

3.10 Cell lysate preparation, sedimentation equilibrium gradient and western blot analysis.

S6.1/JFH1 and S6.1/JFHCon1E2 cells were washed once with phosphate buffered saline and incubated with trypsin-EDTA for 2 min at 37°C. Cells were resuspended inPBS and collected by centrifugation at 1,500 rpm for 3 min. The cell pellet was resuspended in DMEM-10% FCS, and cells were lysed by four freeze-thaw cycles in liquid nitrogen and a 37°C water bath, respectively. Cell debris was pelleted by centrifugation for 5 min at 4,000 rpm. The supernatant was collected and overlaid onto a discontinuous sucrose gradient. Gradients were formed by equal-volume (750 µl) steps of 20, 30, 40, 50, and 60% sucrose solutions in TNE buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM EDTA). 250 µl of each supernatant were overlaid on the gradients, and equilibrium was reached by ultracentrifugation for 16 hours at 36,000 rpm (135,000 x *g*) in an SW60Ti rotor at 4°C in a Beckman L8-80 M preparative ultracentrifuge. After ultracentrifugation, gradient fractions were collected from

the top and titrated for virus infectivity as previously described. The density of the fractions was determined by measuring the refractive index of 10-µl aliquots of each sample.

Western blot analysis was finally performed on each fraction. Samples added of the SDS-loading buffer were heated for 5 min at 95°C, and loaded onto a 10% polyacrylamide-SDS gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane using a semidry blotter (Bio-Rad) according to the manufacturer's instructions. The membrane was blocked in PBS supplemented with 0.5% Tween (PBS-T) and 5% milk for at least 1 h. Upon blocking, the membrane was incubated with primary antibodies diluted in PBS-T supplemented with 1% milk and, after extensive washing with PBS-T, incubated with horseradish peroxidase-conjugated secondary antibodies diluted in PBS-T supplemented with 1% milk. As primary antibodies were used core-specific mouse monoclonal antibody (3G1-1) at a 1:500 concentration, polyclonal anti-serum Ch-L559 against E1 and E2 proteins at a 1:1000 dilution and the mouse monoclonal anti-NS3 antibody at a 1:200 dilution. Bound antibodies were detected after a washing step with the ECL Plus Western Blotting Detection System (GE Healthcare).

§ 4. RESULTS

In the case of HCV, the system that mimics closely a viral production in cell culture is an infection system based on the JFH-1 strain (genotype 2a) (10, 29). Although these virus culture system was an important achievement permitting studies of the HCV life cycle *in vitro*, right from the beginning, two major warnings have occupied the researcher involved**: a)** its dependence on genotype 2a, limiting comparative studies such as the impact of variability in the structural genes on neutralization in an authentic infection system or the evaluation of antiviral compounds targeting multiple genotypes and b) the low yield of particles production, being limited to a titer of 10^3-10^4 per ml of cell culture medium. Partial progresses have been made on both sides. Viral yield was increased of about one log by an intra-genotypic HCV chimera, named JFH1/C6, composed of the core to NS2 region from the J6 strain (genotype 2a) substituting the analogous region in the JFH1 genome (125, 126). Althought 10^4-10^5 pfu/ml is still the highest viral production achievable and only by using genotype 2a scaffolds, the approach itself indicated the direction to solve the inter-genotypic problem. It was in fact extended by the construction of additional JFH-1-based chimeras consisting of the 3'-half genome of JFH1 and the 5'-moiety, including part of NS2, taken from genotype 1a, 1b, 3a and 4a (48, 125).

While the construction of the intra-genotypic chimera was highly successful, it was soon patent that the virus yield obtained using inter-genotypic chimeras was dramatically reduced. At the same time, the data obtained strongly suggested that determinants within the structural proteins govern kinetics and efficiency of virus assembly and release (125).

To improve the productivity of the inter-genotypic HCVcc system and to better define to what extent the HCV envelope proteins E1 and E2 influence the former processes, we designed two 2a/1b inter-genotypic chimeras exploiting a new strategy. In our approach only the sequences encoding for the E1 and/or E2 glycoprotein are swapped from JFH1 to the Con1 strain (genotype 1b). More precisely, we substitute the regions of the two proteins supposed to be exposed on the surface of the virion, the so called ectodomain. Our rationale is that, leaving the transmembrane domain of the 2a strain, the correct interactions with the other proteins of the scaffold necessary for the particle assembly are maintained. At the same time, the region carrying the HCV epitopes would have appeared as component of the 1b strain, allowing comparative analysis of the impact of E1 and E2 surface-exposed domain on virus morphogenesis.

The envelope proteins E1 and E2 are type I transmembrane proteins with a highly glycosylated N-terminal ectodomain and a C-terminal hydrophobic anchor, that is inserted into the envelope membranes. The latter domain, the transmembrane one, is crucial for the non covalent association of E1 and E2 in heterodimers, a prerequisite for virus formation (89, 93), HCV entry (127, 128), and for the interaction with the core protein, the key component of the viral capside (91, 129). The transmembrane anchor domains are composed of two stretches of hydrophobic residues separated by a short segment containing at least one fully conserved charged residue .It is reported that the TMD of E1 start at position 353 to stop at 383 residue, while the TMD of E2 protein is 29 amino acids in length, from residue 718 to 746 of the polyprotein (position numbers based on 1a strain sequence) (89). However the native structure of E1 and E2 proteins is still under investigation since crystallography studies are not been achieved yet. As consequence the topology deducted for both envelope proteins mainly derives from structural prediction analysis based on the E1 and E2 primary sequences.
To better define the portion of E1 and E2 that spams the membrane we decided to perform a secondary structure prediction analysis based on two algorithmics, namely j-pred and phy-pred. As reported in figure 5, we found that for both proteins, the region with hydrophobic characteristic is wider than those reported by others. In particular, the TMD of E1 start at position 330 instead of 353 and it is also preceded by and amphipatic α-helix. Concerning E2, based on our prediction analysis, the region that spam the membrane initiates at the residue 683 and it is flanked by other two hydrophobic portions of about 10-12 amino acids.

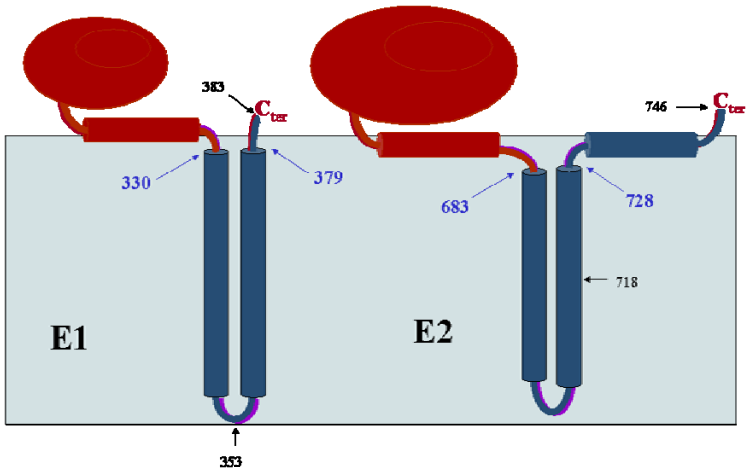


Figure 5: schematic representation of E1 and E2 protein anchored to the membrane.
E1 and E2 proteins are shown as anchored to the membrane through their transmembrane domains. Amino acid positions of the putative portions that spam the membrane are reported in blue. Transmembrane domain positions reported in literature are indicated in black. For either E1 and E2 proteins the portions encoded by JFH1 genome (the TMDs) are shown in blue, while those Con1-derived in red.

While these structure studies are still under investigation for E2 protein, the tendency of this region of E1 to form α-helix in a membrane context has been already confirmed by circular dicroism and NMR analysis (data not shown). Since such results corroborate the prediction analysis here showed, we decided to study a PCR strategy based on this novel configuration.

We designed two chimeric constructs, JFH/Con1E1E2 and JFH/Con1E2, in which only the E1 and/or E2 ectodomains are swapped from the original JFH1 genome to genotype 1b, Con1 strain. Wild type JFH1 was then used as control for comparative analysis of assembly and infection.

We found that both JFH/Con1E1E2 and JFH/Con1E2 constructs are able to replicate at the same level of the JFH1 wild type genome in hepatoma cells in culture. We also demonstrate for the first time that the E1E2 heterodimer formation is not hampered by the ectodomain swapping and that glycoproteins from genotypes 2a and 1b can correctly interact. In addition, the endo H sensitivity of E1E2 heterodimers indicates that in both wild type and chimeric species the sugar moiety of E1 and E2 do not undergo post-ER modifications as widely reported in literature (80, 91, 131).

Nevertheless, none of the chimeric constructs allow the production of infectious viral particles, suggesting that determinants within the structural E1 and, in particular, E2 protein govern the kinetics and efficiency of virus assembly/release/infection. Importantly, it is the first report that demonstrates the influence of the envelope protein E2 in the viral assembly process. Indeed, although we cannot explain the precise molecular mechanism, we show that the interchange of E2 ectodomain profoundly alters the efficiency of the former step of the viral life cycle. Overall our results support the hypothesis that the substitution of the E2 ectodomain leads to an abortive particle assembly process, most likely caused by a genetic incompatibility between genotype 1b E2 protein and the other viral structural proteins derived from genotype 2a.

4.1 Synthesis of pUC.Con1E1E2 chimera construct.

JFH1 sequence derived from the pUC-JFH1 plasmid in which the JFH1 entire genome is under the control of a T7 RNA polymerase promoter. This organization is necessary for the *in vitro* transcription of the entire viral genome as RNA, the transfection of which into hepatoma cells allows viral life cycle to proceed. To obtain the plasmid pUC.JFHCon1E1E2 we designed two in frame deletions in the JFH1 genome corresponding to the E1 and E2 ectodomain regions, from aa 192 to aa 329 and from aa 384 to aa 683 for E1 and E2 respectively. These two fragments were then substituted with the corresponding fragments taken from the Con1 genome (genotype 1b) cloned originally into the pUC.neo-con1 vector.

PCR 1.

To swap the E1 glycoprotein ectodomain we used pUC.neo-con1 as template. The strategy adopted is showed in figure 6, where the Con1 sequence is represented in red, and the JFH1 in blue. We used a forward primer consisting of a 5'-tail of 15 nt homologous to the end of JFH core protein coding region, and the 3'-portion complementary to the beginning of E1 ectodomain taken from Con1 sequence. This oligo was used in combination with three different reverse primers that were spanning the extremity of the E1 ectodomain region of Con1 and the entire E1 transmembrane of JFH1. This strategy allowed us to progressively swap the E1 ectodomain from genotype 2a to 1b.

In the case of E2 glycoprotein, we used a reverse primer consisting on 20nt complementary to the end of E2 ectodomain Con1-derived plus a tail homologous to the beginning of the E2 transmembrane region from JFH1 sequence. This primer was used in combination with three forward primers complementary to the genotype 2a E1 transmembrane region in order to complete the substitution of such portion. In this way from PCR 1 we obtained an E1 chimeric glycoprotein, in sense that the ectodomain region was maintained from Con1 genome, while the entire transmembrane portions turned into genotype 2a. In addition, at the 3' and 5'-end of the fragment we added a tail complementary to JFH1 genome necessary for the following fusion.

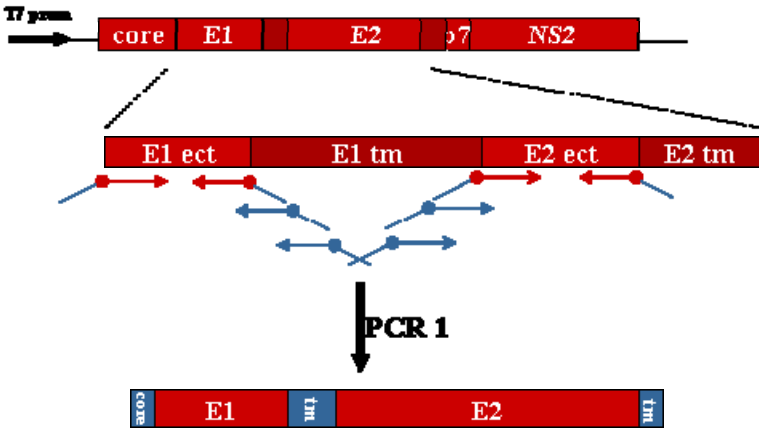


Figure 6: Synthesis of the construct pUC.Con1E1E2. PCR1.
Using the Con1 genome as template, three reverse and three forward primers were designed to progressively swap the transmembrane region of E1 protein from genotype 1b to genotype 2a. In addition, at the 5' and 3'-end of the final product, a tail complementary to JFH-1 sequence was added for the subsequent fusion PCRs.
Con1 sequence is represented in red, JFH-1 sequence in blue

PCR 2.

The second sets of PCRs were performed to amplify the 5'NTR-core and the E2 trasmembrane-NS2 regions of JFH1 genome using pUC.JFH-1 plasmid as template (figure 7). To this purpose, we used a T7 forward primer in combination with a reverse primer whose 3'-end was complementary to the end of JFH1 core coding region, while the 5'-end was homologous to the origin of E1 protein Con1-derived. Therefore, from PCR 2 we obtained a fragment consisting on the T7 promoter sequence, the JFH-1 5'-NCR (non coding region) and

the entire core sequence plus a 15 nt tail complementary to the beginning of the E1 protein Con1-derived.

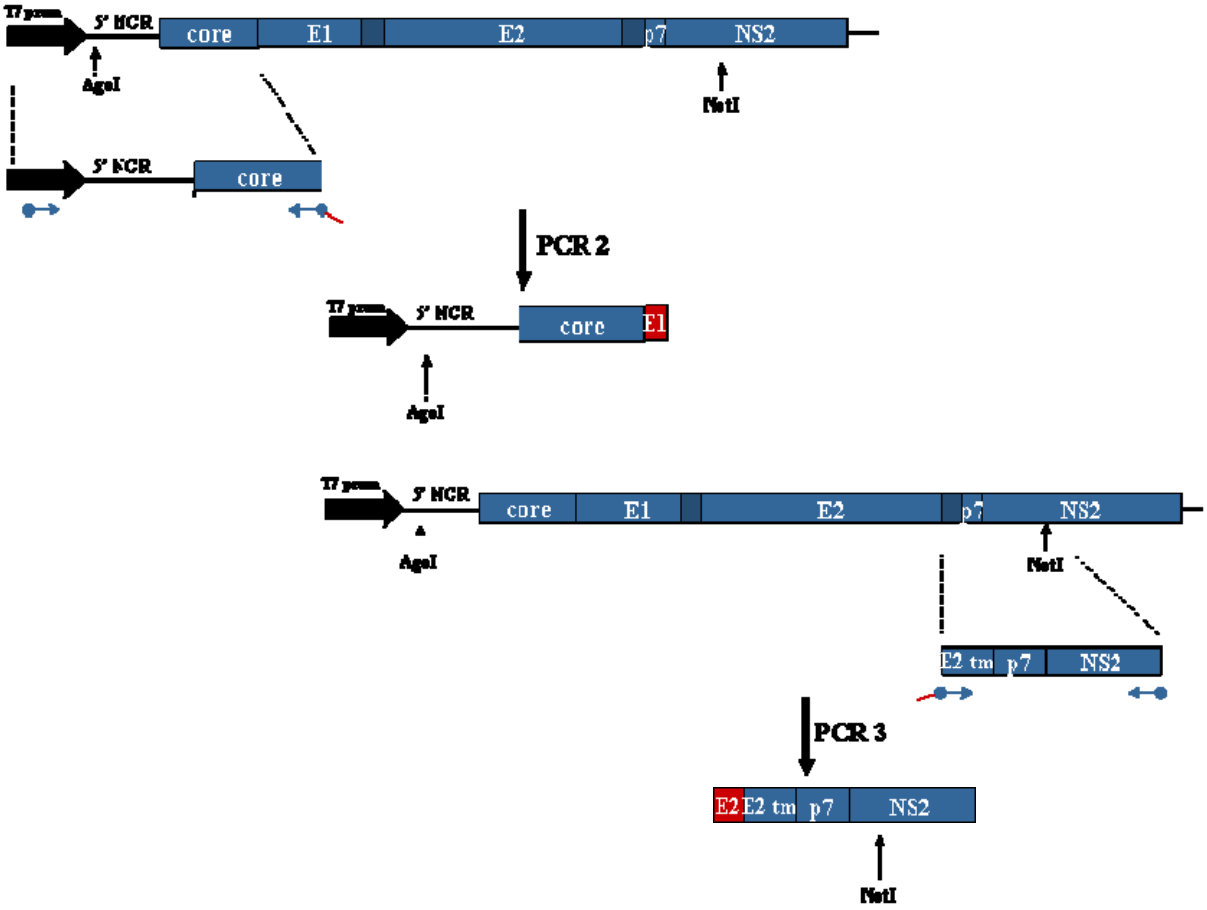


Figure 7: Synthesis of the construct pUC.Con1E1E2. PCR 2 and PCR3.
Using the JFH-1 genome as template, the 5'UTR-core (PCR2) and the E2 trasmembrane-NS2 regions (PCR3) were amplified. In addition, at the 3'-end of the PCR2 final product was added a tail complementary to the Con1 sequence. Similarly at the 5' end of the PCR3 product was added a tail complementary to the Con1 sequence.JFH-1 sequence is represented in blue. Con1 sequence in red.

PCR 3.

Similarly, for the PCR 3 we used the pUC.JFH-1 plasmid as template. As showed in figure 7, the forward primer in part annealed to the beginning of the E2 transmembrane region JFH1-derived, while the first 15 nt were homologous to the end of E2 ectodomain from Con1 sequence. As reverse primer we used an oligo complementary to NS2 protein from JFH1 genome. From PCR 3 we then obtained a fragment consisting of the E2 transmembrane region, the p7 sequence, and part of NS2 coding region taken from JFH1 sequence. In addition, it included at 5'-end of such fragment 15 nucleotides homologous to the end of the E2 ectodomain Con1-derived.

PCR 4.

Finally, using the products obtained from the previous amplification as template and two external primers, one homologous to the T7 promoter and the second one to NS2 region of JFH1, we were able to fuse the PCR fragments in a single DNA molecule (figure 8).

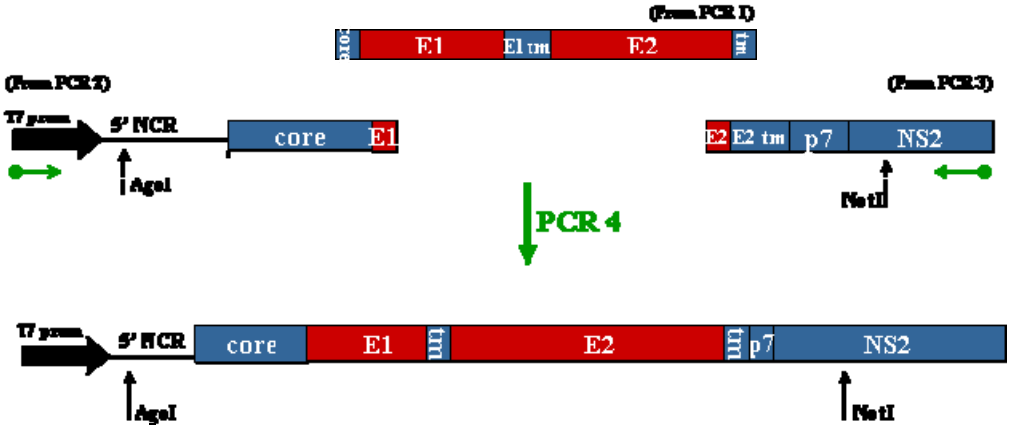


Figure 8: Synthesis of the construct pUC.Con1E1E2. PCR 4.
A couple of primers designed on the JFH-1 external regions, namely the 5'-NTR and at the C-term of NS2 protein, were used to fuse the products obtained from PCR1, PCR2 and PCR3. JFH-1 sequence is represented in blue. Con1 sequence in red.

The chimeric JFH1/Con1E1E2 product obtained in the PCR 4 was then cloned into pUC.JFH-1 plasmid between the Age I and Not I restriction sites replacing the same T7 promoter-NS2 protein region (figure 9).

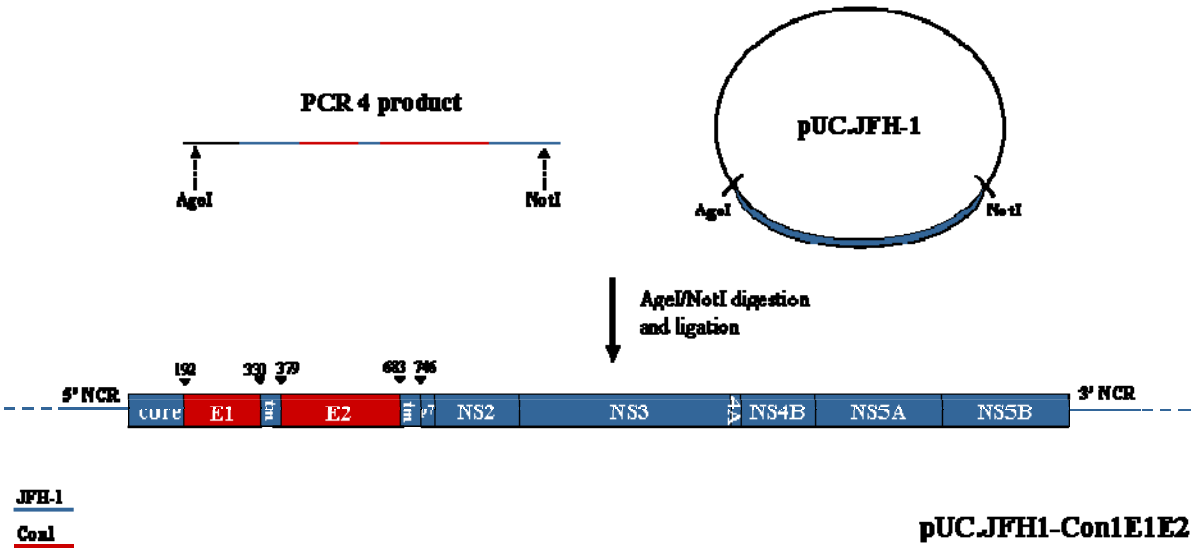


Figure 9: Synthesis of the construct pUC.Con1E1E2. Cloning into pUC.JFH1 plasmid.
The chimeric product obtained in PCR 4 was cloned into pUC.JFH-1 plasmid between the Age I and Not I resctriction sites. JFH-1 sequence is represented in blue. Con1 sequence in red.

4.2 Synthesis of pUC.JFHCon1E2 chimera construct.

To generate the plasmid pUC.JFHCon1E2 we studied an in frame deletion in the pUC.JFH-Con1E1E2 plasmid, spanning the region between EcoRI restriction site in the 5'NCR and the BsiwI sites that maps in the E1 transmembrane region.

This digestion product was finally replaced with the corresponding fragment derived from the JFH1 full length genome in order to obtain a chimeric construct in which only the E2 ectodomain region is swapped from JFH1 to Con1 sequence (figure 10).

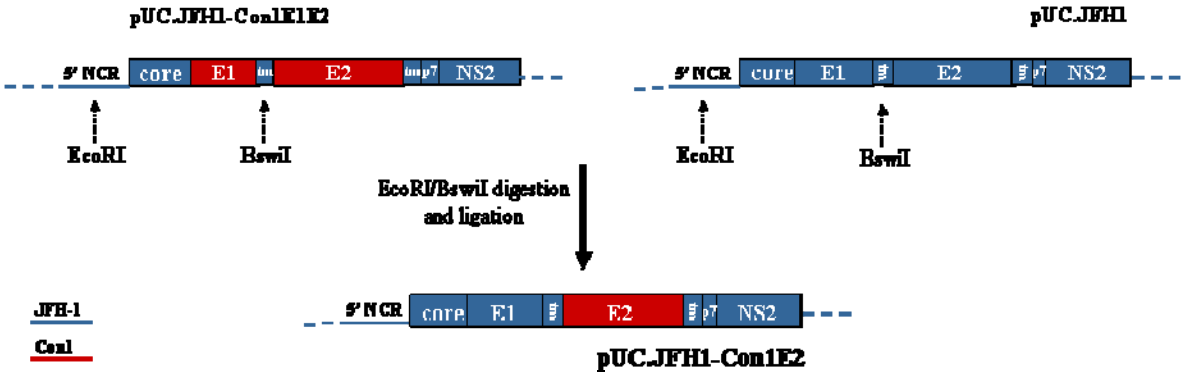


Figure 10: Synthesis of the construct pUC.Con1E2.
The pUC.JFH1-Con1E1E2 was digested with EcoRI and BswI resctriction enzymes: Both of them have unic restriction sites within the JFH-1 and Con1 genomes. EcoRI site maps in the 5' UTR, while BswI site in the E1 transmembrane region. The digestion product obtained from pUC.JFH1-Con1E1E2 was replaced by ligation with that obtained from the EcoRI/BswI digestion of pUC:JFH-1 plasmid.

4.3 The JFH/Con1E1E2 and JFH/Con1E2 constructs are replication-competent.

To allow viral life cycle to proceed, we need to transfect the viral genome as RNA. Thus, first step was the *in vitro* transcription of all the vectors assembled as described. To generate genomic HCV RNA, the three plasmids (pUC.JFH-Con1E1E2, pUC.JFH-Con1E2 and pUC.JFH-1) were linearized at the 3' end of the cDNA with the restriction enzyme XbaI and treated with Mung Bean nuclease to eliminate any additional nucleotide that do not correspond to the viral genome. The linearized DNAs were then extracted by phenol-chloroform, precipitated, resuspended in RNase-free water, and used as template for the *in vitro* transcription using T7 RNA polymerase. To degrade the DNA template, DNaseI was added to the mixture. Finally, the transcripts were extracted by phenol-chloroform and precipitated by the addition of ammonium acetate.

In all experiments 5 µg of viral RNAs was delivered into S6.1 cells by electroporation. This cell line was derived from Huh7 cells selected for their ability to support the replication of the HCV replicon, that were cultured in presence of human INF-α in order to eradicate the replicon.

Transfected cells were then passaged as needed to maintain subconfluent cultures throughout the experiments. At the indicated times, cell culture supernatants were collected, clarified from cellular debris and checked for the presence of infectious viral particles (figure 11).

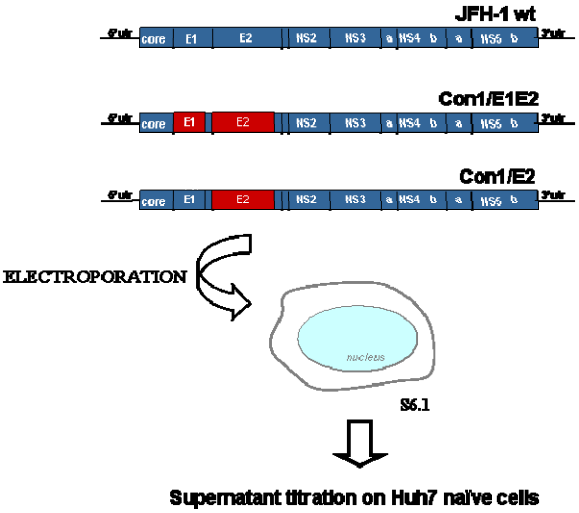


Figure 11: schematic representation of viral particles production.
In vitro transcribed genomic JFH-1, Con1/E1E2 and Con1/E2 RNA are delivered to S6.1 hepatoma cells by electroporation. After 72 hrs and for the following indicates time points, cell supernatant was collected, clarify and tested for the presence of infectious particles by inoculation onto Huh7 naïve cells.

To asses the ability of the chimeric constructs JFH/Con1E1E2 and JFH/Con1E2 to replicate, 72hrs post transfection each cell type was checked for the presence of viral core and E1E2 proteins by immunofluorescence staining. In parallel, cell lysates were submitted to immunoblot analysis (figure 12).

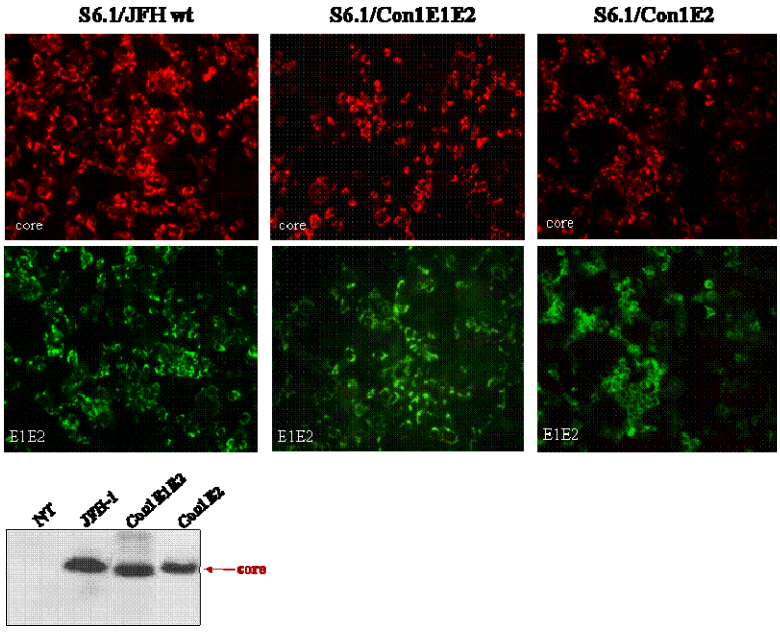


Figure 12. Replication of wild type and chimeric HCV constructs.
S6.1 cells were seeded in 48 well/plate immediately after viral RNAs electroporation and incubated for 72 hours. Intracellular expression of HCV core protein (red) was assayed by indirect immunofluorescence with α-core antibody (3GI-1). Similarly, E1/E2 (green) expression was detected by immunofluorescence using the anti-E1E2 chimpanzee antisera L559. Cell type is reported above the panels.
Western blot analysis of PNSs from JFH-1, Con1/E1E2, Con1/E2 transfected cells and from non-transfected cells (NT) is reported in the lower part of the figure. The band that appears in each transfected cell type has an approximate size of 20 KDa and corresponds to core protein

The positive cells show core-specific and E1E2-specific cytoplasmic staining patterns as previously described (132). In all cases, transfection appears to be efficient in about 80-90%

of cells. The immunoblot obtained on cell lysates using the anti-core antibody result in a single band with an approximate size of 20 KDa, consistent with the molecular weight of core protein, that is present in both the wild type and chimeric genomes samples.

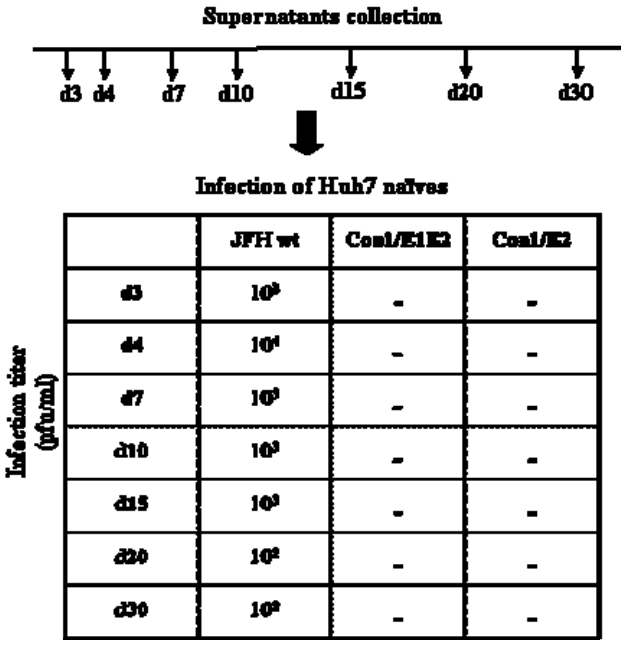
4.4 The chimeric genomes do not allow the production of infectious particles.

Althought the previous data clearly indicate that the JFH/Con1E1E2 and JFH/Con1E2 genomes are able to replicate upon transfection in hepatoma cells, they are not directly linked to the ability to produce infectious viral particles. To verify the release of such particles by S6.1 transfected cells, supernatant fluids were collected at different time point, from 72 hours until 30 days post-transfection, and tested for the presence of infectious particles by inoculation onto Huh7 naïve cells. After 72 hours, infected cells were fixed, permeabilized, and examined for foci of cells expressing HCV core protein by using indirect immunofluorescence as described in materials and methods. A proper titration was possible solely for the S6.1/wt cells since none of the chimeric constructs allowed the production of infectious particles. (figure 13).

In the case of inter-genotypic chimeras in which the complete HCV structural region, from core to NS2 protein, is substituted, other groups reported that the infectious particles release increase between day 9 to day 15 from transfection. This kinetics of virus release is due to the accumulation of compensatory mutations in the chimeric RNA that enhance the yield of infectious particles. Since the cellular environment in which the chimera replicate is constant, these mutations likely emerge because they correct incompatibilities between the proteins coming from different HCV genotypes (133).

Figure 13. Determination of S6.1/JFH1, S6.1/Con1E1E2 and S6.1/Con1E2 cells infectivity.
Infection of naïve Huh7 cells by cell culture-generated JFH-1 virus and JFH1/Con1 chimeras. Naïve Huh7 cells were inoculated with cell supernatants harvested 72 and 96 hours after transfection with JFH-1 wt, JFH1/Con1E1E2 or JFH1/Con1E2 genomes. After 72 hours, expression of HCV core protein was detected by indirect immunofluorescence using an α-core antibody (3GI-1).TCID50 was determined by serial dilution only for JFH-1 wt since no infection was observed for cells inoculated with supernatants from chimeras. Cell types are reported in the bottom of each figure.

To assess if this phenomenon happened in our system, we monitored the infectivity of S6.1/Con1E1E2 and S6.1/Con1E2 cell supernatants until day 30 post transfection in replicate experiments, but none of the chimeras produced detectable viral particles (figure 14).



	JFH wt	Con1/E1E2	Con1/E2
d3	10^3	-	-
d4	10^4	-	-
d7	10^3	-	-
d10	10^3	-	-
d15	10^3	-	-
d20	10^2	-	-
d30	10^2	-	-

Figure 14. Viral particles collection and titration. Supernatants collected at the indicated time points from S6.1/JFH1, S6.1/Con1E1E2 and S6.1/Con1E2 cells were clarified by brief centrigugation. Each supernatant was then titrated on Huh7 naïve cells following the Speraman and Kaerber fit. Positive Huh7 cells were counted under a fluorescent microscope and the infectious titer was expressed as particles forming unit per ml (pfu/ml).

As the ability of the viral RNA to replicate doesn't seem to be affected by either exchange, we thought that the ectodomain swapping affects one or more of the steps downstream of RNA replication, in particular:

- E1E2 heterodimers formation
- Viral particle assembly process
- Budding process
- Particles release

Based on this consideration we performed a series of experiments aiming at evaluating which process is influenced by the substitution of the E1 and E2 ectodomain coding region in the JFH1 scaffold.

A recent paper indicates the importance of the E1-core protein interaction for a proper viral particle assembly process. In particular, the authors claim that E1 associates with the core protein through a cytoplasmic loop containing five aminoacids critical for this role (from 312 to 315) (46). Alignment of the aminoacids sequence shows a high variability between Con1 and JFH1 genotypes in the entire E1 coding region. In order to exclude the possibility that E1 protein Con1-derived was unable to interact with the capside protein, we thus decided to focus our attention on the JFH/ConE2 chimera in which the original JFH1 E1 sequence is maintained. Regarding E2 protein, it is worth noting that its transmembrane region is reported

to play a key role in the coordinated assembly and reorganization of the E1E2 heterodimer (93). However, in our chimeric JFH1/Con1E2 construct the E2 transmembrane region derives from the genotype 2a, as well as the E1 protein. In this way the ability of the chimeric E2 to dimerize with E1 protein JFH1-derived should be maintained. Thus, the subsequent experiments have been performed with the JFH1/Con1E2 chimera that allowed us to i) better define the importance of the E2 ectodomain in the virus production and ii) exclude the possibility that E1 and core proteins cannot correctly interact each other.

4.5 E1 protein encoded by JFH1 sequence interacts with E2 ectodomain from Con1 subtype to form E1E2 non covalent heterodimers.

E1 and E2 proteins are targeted to the endoplasmic reticulum by signal sequence and co-translationally separated from each other by host signal peptidase cleavages. In this site they acquire several N-linked oligosaccharides chains and assemble in non-covalently bound E1E2 heterodimers that represent the functional units of the HCV spike (80). To analyze the formation of the heterodimer in our system, we tested different anti-E2 conformational mAbs in immunoprecipitation of metabolic labeled S6.1/JFH1 wt and S6.1/JFHCon1E2 cell lysates, and checked for the presence of co-immunoprecipitated E1. The figure 15 shows the results obtained using the human mAbs CBH2, CBH5, CBH7, that are three of a panel of nine conformation-sensitive antibodies; H111, a human conformational antibody anti-E1 protein, and the polyclonal sera Ch-L559, that is able to recognize all forms of genotype 1a E1 and E2 proteins.

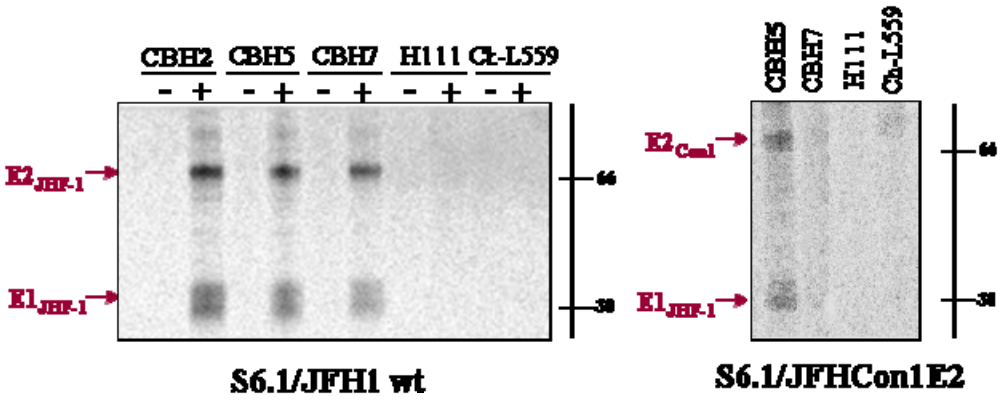


Figure 15 Metabolic labeling of HCV glycoproteins E1 and E2.
S6.1 cells transfected with JFH-1 wt (left panel) or JFH-1/Con1E2 (right panel) RNA were labeled over night with [^{35}S] met and cys. PNS were immunoprecipitated with several conformational monoclonal anti-E2 antibodies (CBH2, CBH5, CBH7), a conformational monoclonal anti-E1 antibody (H111) and an anti E1/E2 antiserum (Ch-L599) and analyzed on 10% SDS-PAGE under non-reducing. The symbols refer to the E1 and E2 coding sequence, namely JFH-1 or Con1 strain. Position on gels of pre-stained molecular weight marker is reported on the right side.

In the left panel of figure 15 is reported the result for the JFH1-derived heterodimer, in which the properly folded E2 is recognized by CBH2, CBH5 and CBH7 antibodies. In such immunoprecipitations, E2 protein always carries down E1, indicating that the E1E2 heterodimers can form. The same experiment performed on the lysate from cells transfected with the chimeric species show that only CBH5 antibody immunoprecipitates genotype 1b E2 (figure 15, right panel). In addition, this anti-E2 antibody co-immunoprecipitates also E1 protein JFH1-derived, indicating that glycoproteins from genotypes 2a and 1b are able to interact and form heterodimers. This data suggest that E1E2 heterodimer assembly is not hampered by the ectodomain swapping. However more specific analysis were performed to compare the assembly process of both wild type and chimeric species.

To study the behavior of newly synthesized E1 and E2 in S6.1/JFH1 wild type and S6.1/JFHCon1E2 cells we performed pulse-chase experiments followed by immunoprecipitation. In this kind of experiments, cells are exposed to radio-labeled aminoacids (methionine and cysteine in our case) for a brief period, called "pulse", as long as the system requires. In our replication process, 20-30 minutes of pulse are needed to label enough *de novo* synthesized proteins. To stop incorporation of the isotopes in newly synthesize proteins, cell cultures are washed and incubated with an excess of non-labeled aminoacids in the medium for different time period (chase), at the end of which the cells are lysated with a detergent. In this way, only those proteins synthesized during the pulse period will be detectable, giving us the possibility to follow their time-dependent folding process (figure 16, upper part). The centrifuged lysates, called post-nuclear supernatants (PNS), are then used for the immunoprecipitation and immunocomplexes are solubilized at 95°C in SDS loading buffer prior to be separated on SDS-PAGE in reducing or non reducing conditions (figure16). Oxidative status of intra-chain disulfide-bound proteins can be evaluated as electrophoretic mobility shift between "unoxidized" and fully or partially oxidized proteins on SDS-PAGE under non reducing conditions. Indeed, in absence of reducing agent treatment, the progressive formation of intra-chain disulfide bonds results in an increase in protein mobility, meaning that the protein is progressively assuming the native, more compact, conformation.

Treatment with the reducing agent leads to the complete reduction of disulfides and to thus only one band will appear on the gel, corresponding to the slower migrating conformer. It represents the unoxidized (or "reduced" in this case) form of the protein, whose migration depends only on the molecular weight and not on its conformational status (illustrated in figure 16).

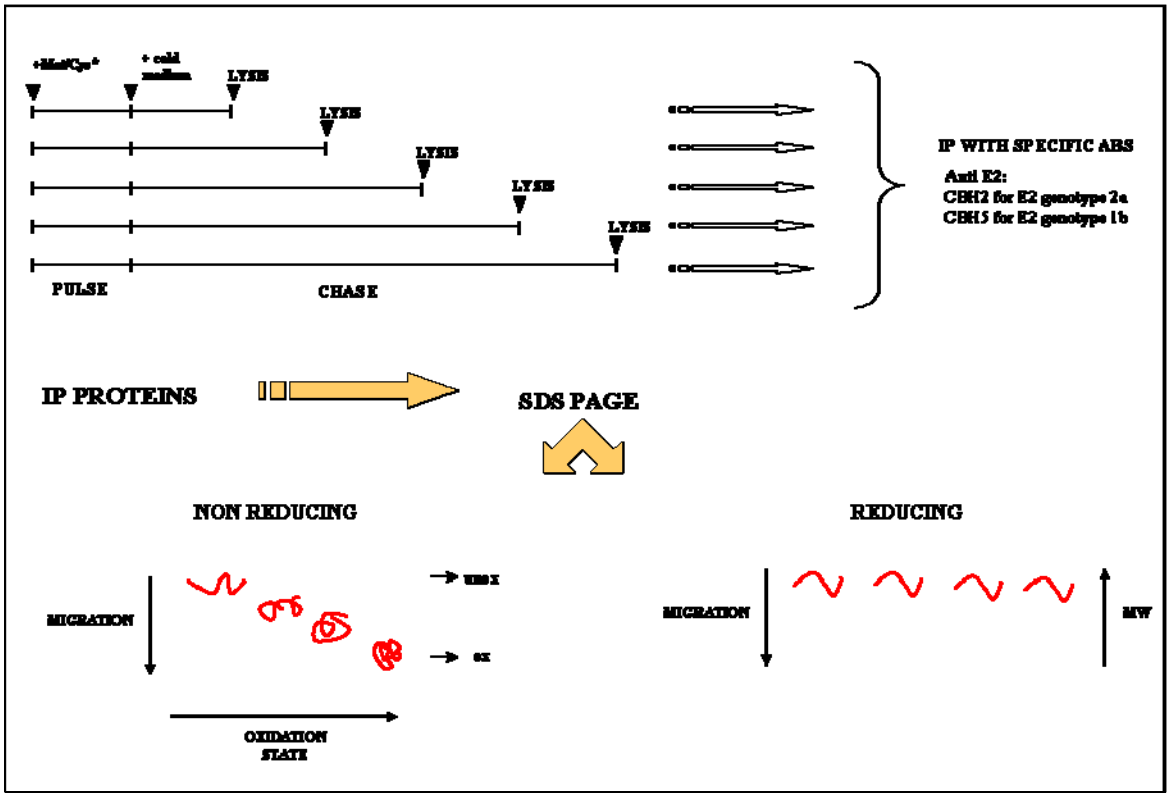


Figure 16. Pulse-chase experiment.
Cells are exposed to a radio-labeled compound (Met or Cys) for a period called "pulse": After washing with cold medium, the cells are incubated with a non-labeled aminoacids for different time period (chase). Cells are then lysated and the post-nuclear supernatants (PNS) are used for the immunoprecipitation with specific antibodies. Immunocomplexes are solubilized at 95°C in SDS loading buffer prior to be separated on SDS-PAGE in reducing or non reducing conditions.

It is reported that between 2 and 4 hours post-synthesis, E2 completes its oxidation process and is fully included in heterodimers. However 2 additional hours are required to achieve the DTT-resistant conformation, since the reshuffling of disulfide bonds is completed only when proteins are included in the final detergent-resistant E1E2 heterodimer (94).

The panel A of figure 17 shows a representative SDS-PAGE in non reducing and reducing conditions of an anti-E2 conformational mAb (CBH2) immunoprecipitated sample from JFH1 infected cell lysate. In this case, both glycoproteins belong to genotype 2a and E1 would be revealed only if it co-immunoprecipitates along with E2.

The formation and recognition of the conformational epitope on E2 is weakly revealed 1 hour after synthesis but it is unequivocally evident 2 hours after synthesis. Immunoprecipitation of the E2 protein with the conformational Ab always carries down E1, indicating that the formation of the conformational epitope on E2 is contemporary, or follows, the genesis of E1E2 heterodimers. As indicate by the intensity of the band corresponding to E2, the number of folding molecules increases progressively from 1 to 6 hours post synthesis. In parallel, the

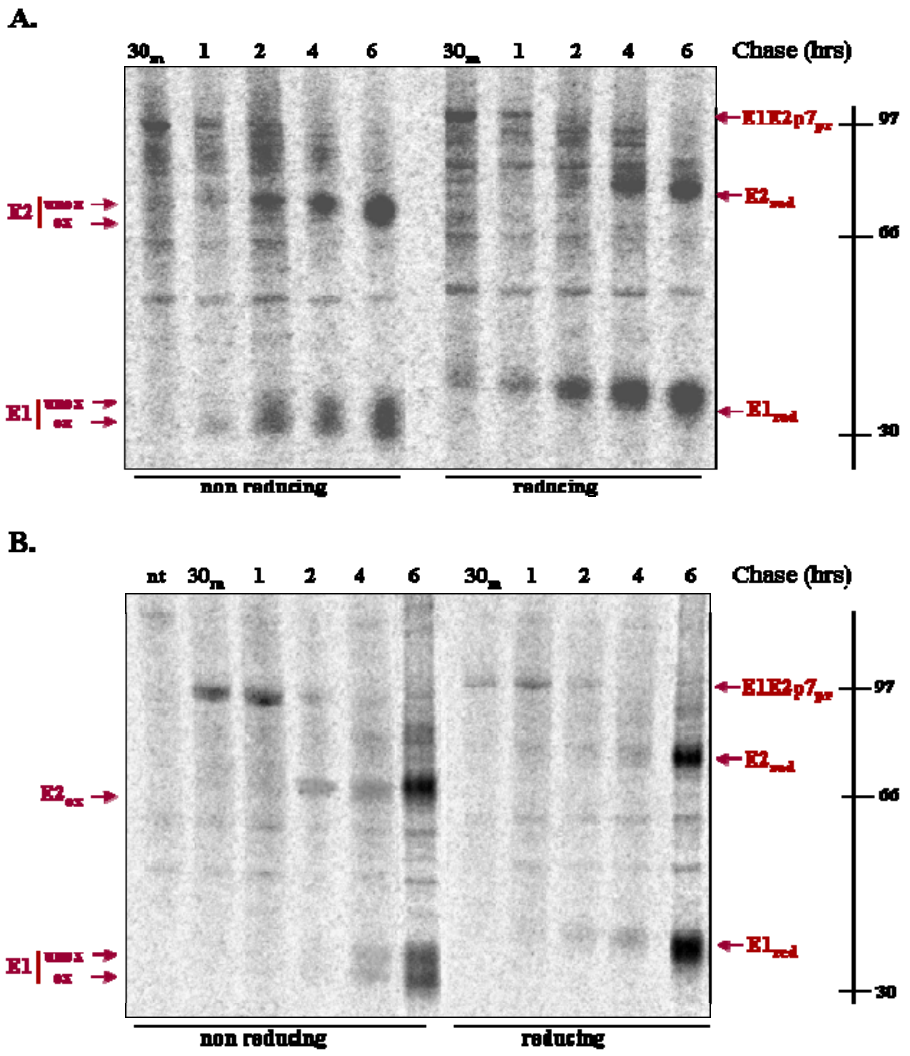


Figure 17. Oxidation kinetics of HCV glycoproteins E1 and E2. (A) S6.1 cells transfected with JFH-1 wt or (B) with JFH-1/Con1E2 RNA were pulse labeled with [35S] methionine and cysteine for 20 min and chased for different time periods, from 30 min until 6 hrs. Cellular lysates were immunoprecipitated with a conformational monoclonal anti-E2 antibody (CBH2 or CBH5 respectively for genotype 2a and 1b) and analyzed on SDS-PAGE. Chase periods are reported above the lanes. Samples were analyzed on 10% SDS-PAGE under non-reducing and reducing conditions (DTT 200mM). Symbols refer to different form of E1 and E2: ox for oxidized; red for reduced; pr for protein precursor; NT for not transfected. Position on gel of prestained Molecular Weight marker (Amersham) is reported on the right side.

amount of E1 increases in the same time-dependent manner, indicating that the E2 maturation proceeds contemporary to E1E2 heterodimer formation.The considerations gather from the wild type heterodimer are extendible to the chimeric E1E2 species (see panel B of fig. 17), confirming that the Con1-derived E2 portion interacts with the E1 glycoprotein encoded by JFH1. However, the kinetics of the precursor processing appears to be slower. Indeed, in the case of JFH1 wt, the formation and recognition of the conformational epitopes requires at least 1 hour of chase, while for the chimeric species, at the same time point the majority of E1 and E2 are still found as protein precursor. This observation will be discussed further (see below). Althought the time-dependent kinetics in slightly different, for both wild type and chimeric dimers we can observe a similar oxidation pattern. The E1 protein shows a single

broad signal that is clearly split in two bands in the chimeric sample (compare 6 hrs of chase in the panel A and B). This observation indicates that the protein is simultaneously present in different oxidized and unoxidized forms. Following reduction, in fact, only one marked band is visible on the gel, meaning that the non-reducing conditions have separated different E1 conformers. This result is different from what reported in the literature in which only the oxidized E1 has been found to be co-immunoprecipitated with anti-E2 conformational antibodies (94). It is most likely due to the system used, since it is the first time that folding analysis has been performed using the infectious viral system (HCVcc).

Similarly to E1, the oxidation process of E2 is also revealed by a time dependent increased migration (compare non reducing and reducing conditions of both panel A and B). In this case, the contemporary presence of several E2 species, including differently trimmed and oxidized conformers of both E2 and un-cleaved E2p7 precursor (see below), renders diffuse the signal. All of these species are similar in terms of apparent molecular weight, resulting in a difficult separation on SDS-PAGE. Finally, the gradual decrease in the apparent molecular weight of both glycoproteins under reducing conditions is due to the trimming of N-linked oligosaccharides that occurs in the ER compartment (see below).

In both gels of the figure there are several high molecular weight bands. In agreement with other reports (94, 134), the one of about 100 Kda represents the unprocessed E1E2 precursor, possibly containing also p7 protein. The half time of this species, correlate with the E2, makes the most remarkable differences between the wt and the chimeric samples.

From these data appear an important difference between wild type and chimeric E1E2 heterodimers folding process. Indeed, in the case of JFH1 wt, the formation and recognition of the conformational epitopes require 1 hour from protein synthesis, while for the chimeric species, at the same time point the majority of E1 and E2 are still present as protein precursor. This evidence lead us to suppose a more difficult process of association between E1 and E2 deriving from different genotypes. As consequence, the protein precursor could accumulate in the ER-compartment, where, if not correctly processed, could be targeted to the proteasomal pathway.

4.6 The envelope proteins encoded by the chimeric construct are not targeted prematurely to the degradation pathway.

Overall the folding analyses have confirmed that the Con1-derived E2 portion interacts with the E1 glycoprotein encoded by JFH1 allowing heterodimers formation, even though the kinetics of the precursor processing seems to be slower. As consequence, it is might be possible that the viral precursor accumulation leads to an overload of the ER-resident

machinery that is responsible for the correct processing and folding of newly synthesized proteins. As a consequence, the HCV E1E2 heterodimers could be targeted to presecretory proteasomal degradation instead of being incorporated into mature viral particles.

The ER-compartment plays a mayor role in protein processing and folding control. It is known that in the ER the nascent polypeptides fold into their functional conformation under the control of a multi-enzymatic machinery that facilitates the selective elimination of incompletely folded proteins. In practice, the role of such conformation-based quality control is to eliminate misfolded polypeptides and unassembled multimeric protein complexes from the endoplasmic reticulum, ensuring the deployment of only functional molecules. Proteins that fail to reach their native conformation in the ER are selectively eliminated by ER-associated degradation (ERAD) that targets such proteins to the proteasomal pathway. However, elimination of misfolded proteins takes place after certain delay from synthesis, giving them enough time to reach the "native" conformation (135-136). By using bovine viral diarrhea virus (BVDV) as a surrogate model of HCV, it was recently demonstrated that an incorrect folding/assembly processes leads to an accelerated degradation of the E1E2 heterodimers and a decrease in the availability of these complexes for viral budding and morphogenesis (137). In addition, several HCV proteins posses a turnover tightly regulated by the proteasomal pathway. For example, binding of HCV RNA-dependent RNA polymerase (NS5B) to an ubiquitin-like protein was reported to decrease the NS5B quantity in the cell. Moreover, an unglycosylated cytosolic form of E2 protein was described as sensitive to proteasome degradation (138, 139).

To assess the possibility that the HCV polyprotein precursor is degradated by the cellular proteasome, we first verified whether the E1E2 heterodimers are still present in the transfect cells at later time points, prolonging the chase period to 48 hours. As showed in figure 18, E1E2 heterodimers are recovered in both wild type and chimeric species 48 hours from protein synthesis. Since it is unlikely that in both wt and chimeric transfected cells the envelope proteins are targeted to the proteasome, this result suggests that chimeric E1E2 are not prematurely degradated.

The absence of smeared E1 and E2 bands at later time points is coherent with a successfully folding process that reduces the heterogeneity of the trimmed species. Moreover, during the chase period, a decrease of E1 and E2 signals intensity can be noted, which is consistent with the progressive incorporations of the heterodimers in the nascent particle.

To further confirm this observation, we determined whether proteasomal inhibitors influence the cellular turn-over of the viral protein precursor. To such purpose we used Lactacystein and

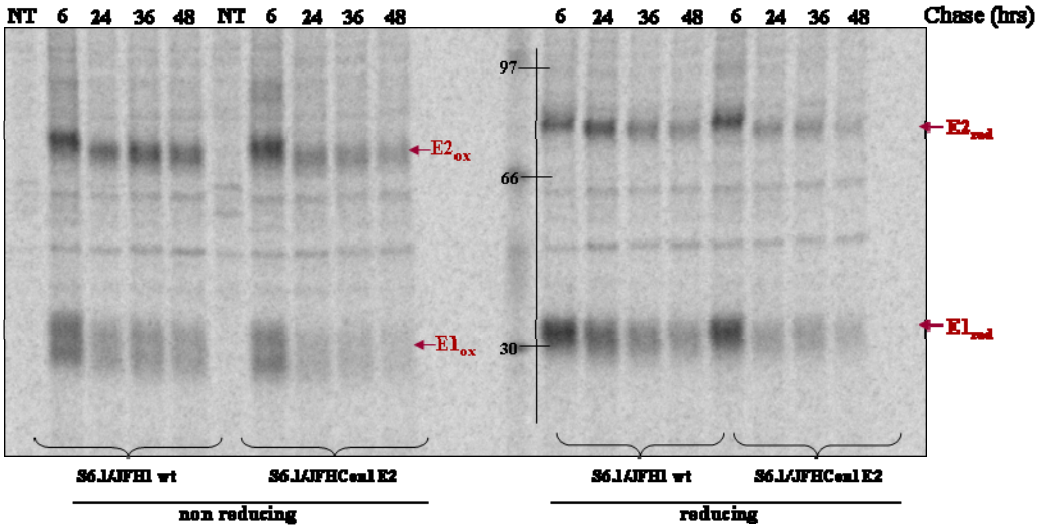


Figure 18. Oxidation kinetics of HCV glycoproteins E1 and E2.
S6.1 cells transfected with JFH-1 wt (left panel) or with JFH-1/Con1E2 (right panel) RNA were pulse labeled with [35S] methionine and cysteine for 20 min and chased for different time periods, from 6hrs until 48 hrs, to verify their permanence into the transfected cells. Cellular lysates were immunoprecipitated with a conformational monoclonal anti-E2 antibody (CBH2 or CBH5 respectively for genotype 2a and 1b) and analyzed on SDS-PAGE. Chase periods are reported above the lanes. Samples were analyzed on 10% SDS-PAGE under non-reducing and reducing conditions (DTT 200mM). Symbols refer to different form of E1 and E2: ox for oxidized; red for reduced; NT for not transfected. Position on gel of prestained Molecular Weight

MG132, two potent inhibitors of the proteasomal degradation pathway, irreversible and reversible respectively (figure 19). S6.1/JFH wild type and S6.1/JFHCon1E2 cells were metabolically pulse labeled with [^{35}S]cysteine for 20 min and chased for 30 min and 6 hours in the absence or presence of Lactacystein and MG132. After each chase time, cell lysates were immunoprecipitated with CBH2 or CBH5 for the wild type or chimeric species respectively.

As showed in the figure 19, in both S6.1/JFH1 wild type and S6.1/JFHCon1E2 cells, we do not find any impact of Lactacystein and MG132 on the protein precursor. In fact the E1E2/E1E2p7 precursor of 100 Kda can be recovered with the same intensity in both treated and untreated samples. In addition, at later times of chase, the 100 Kda band, is completely absent, indicating that the disappearance of the precursor at later time points is due to its processing and not to the proteasome-dependent degradation. In agreement with this interpretation, the signals corresponding to mature E1 and E2 proteins are comparable in treated and not treated samples, indicating that neither Lactacystein nor MG132 have any effect on their half-life. These results argue against a proteasomal degradation mechanism, excluding the possibility that E1 and E2 glycoproteins are not available for their incorporation in the nascent chimeric particles.

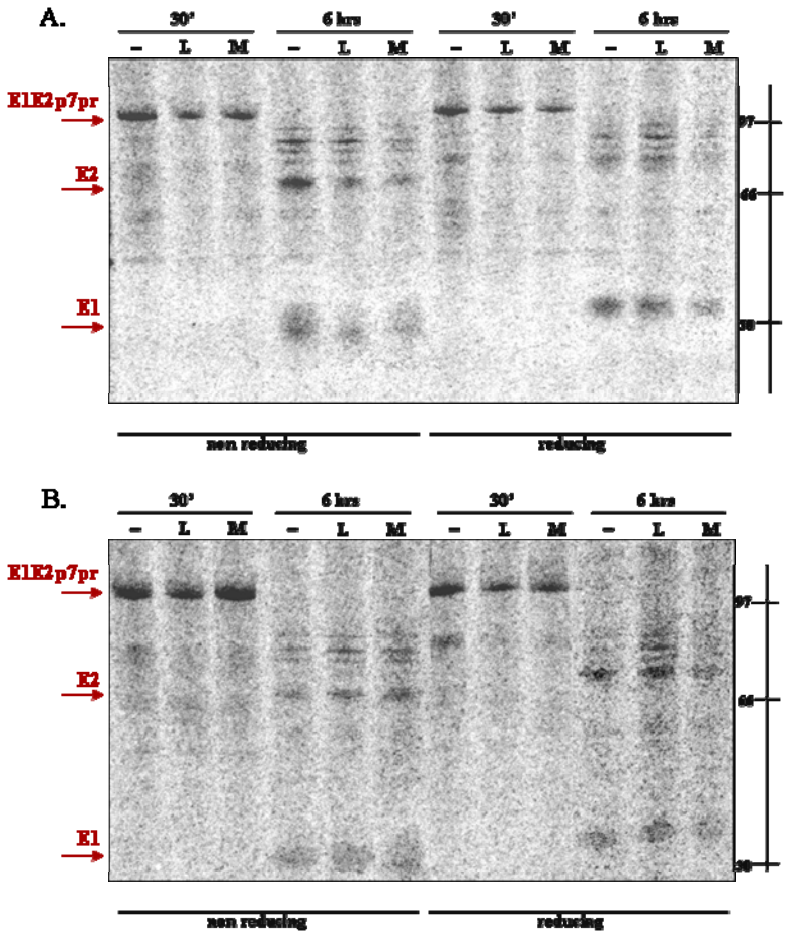


Figure 19. Effect of proteasomal inhibitors on E1E2 heterodimers. S6.1 cells transfected with JFH-1 wt (panel A) or with JFH-1/Con1E2 (panel B) RNA were pulse labeled with [35S] methionine and cysteine for 20 min and chased for 30min or 6 hrs. Proteasomal inhibitor Lactacysteine (L) or MG132 (M) were (or not) added directly to the starving medium, and also maintained during the pulse and chase medium, at the final concentration of 25µM and 20µM respectively. After lyses, PNSs were immunoprecipitated with a conformational monoclonal anti-E2 antibody (CBH2 or CBH5 respectively for genotype 2a and 1b) and analyzed on 10% SDS-PAGE under non-reducing and reducing conditions. Position on gel of prestained Molecular Weight marker (Amersham) is reported on the right.

4.7 Wild type and chimeric E1E2 heterodimers undergo to the same maturation process.

Oxidative status.

Comparative migration of the same protein in SDS-PAGE under both non reducing and reducing conditions has been used to monitor oxidative folding, a reflex of intra-chain disulfide bond formation, in viral glycoproteins (140). This method takes advantage on the faster migration of more compact conformations acquired by the protein through the formation of "native" disulfide bonds. With 8 cysteine residues for E1 and 20 for E2, the native HCV glycoproteins are known to form intra-chain disulfide bonds. It has also been proved that once the protein has completed its folding, such intra-chain bonds are not anymore accessible to exogenously added reducing agent (95). Vice versa, folding intermediates containing disulfide bonds are partially or totally reduced by the DTT treatment. The folding status is thus tested by conducting pulse-chase experiments adding a membrane permeable reducing agent, such as DTT, to cell culture medium 5 minute before the end of each chase period. When added to the cells, in fact, DTT blocks disulfide bond formation in the ER compartment and disrupts them when accessible, for example in incompletely folded

proteins (141-144). If disulfide bonds are not accessible, the protein would migrate at the same position as the oxidized one. Conversely, migrations of the DTT-sensitive species collapse upon reduction to the same position as the reduced or partially oxidized proteins.

The pulse-chase experiment was performed as described in the Materials and Method using two plates for each chase point. On one plate of the pair, medium was added with 5 mM DTT and further incubated for additional 5 min. Following pulse-chase, S6.1/JFH1 and S6.1/Con1E2 cells were lysed in the presence of NEM to prevent further disulfide bond formation, and the viral proteins were precipitated with the anti-E2 mAb CBH2 or CBH5 for the wild type or chimeric species respectively. Each immunoprecipitate was further split into two equal portions, one added with DTT for the analysis in reducing conditions. Results from this experiment are shown in figure 20.

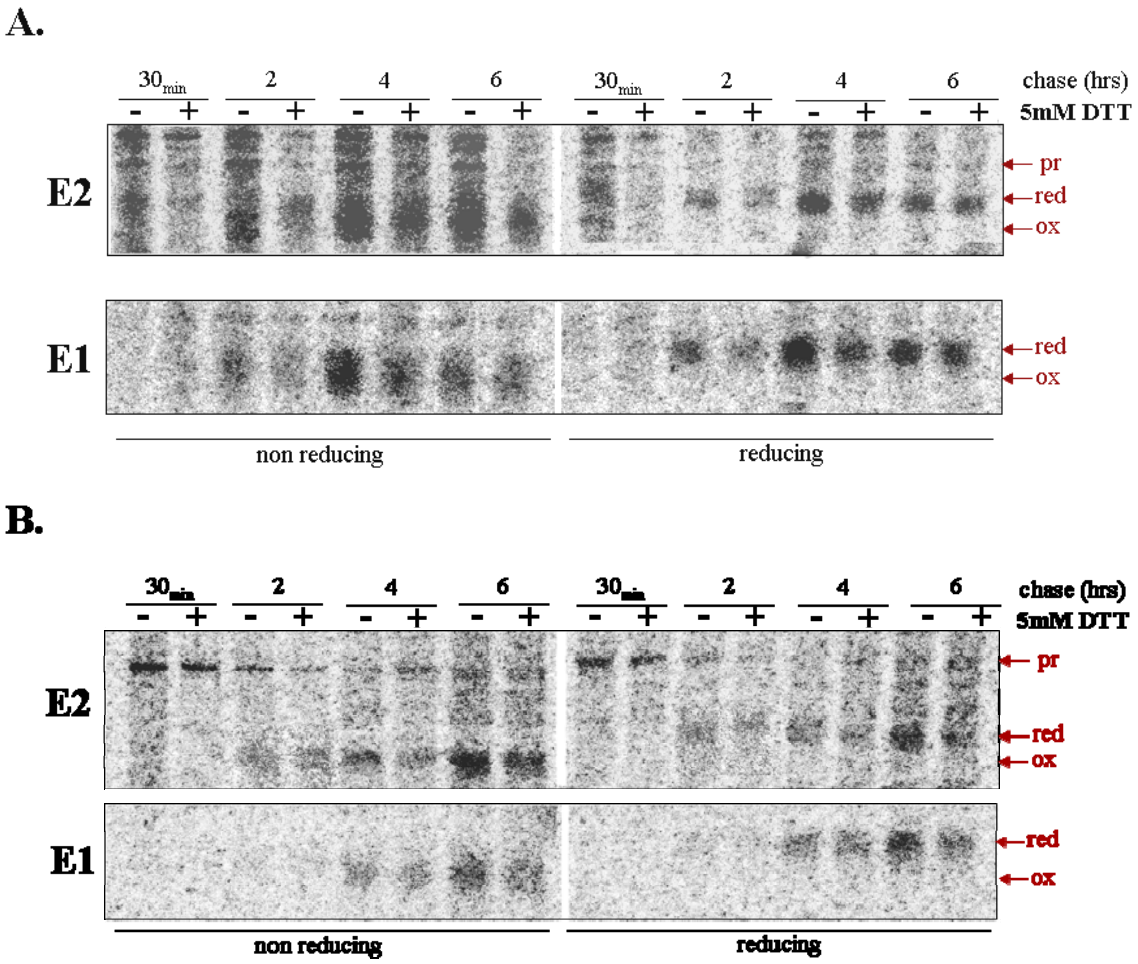


Figure 20. DTT-resistance analysis of E1 and E2 proteins in S6.1/JFH1 and JFH1/Con1E2 cells.
S6.1 cells transfected with JFH-1 wt (panel A) or with JFH-1/Con1E2 (panel B) RNA were pulse labeled with [35S] Met and Cys for 20 min and chased for different time periods, from 30min to 6 hrs, in duplicates. One of the dishes in each pair was chased for 5 min in presence of 5mM DTT (+) in the culture media before proceeding with lysis PNSs were immunoprecipitated with a conformational monoclonal anti-E2 antibody (CBH2 or CBH5 respectively for genotype 2a and 1b) and analyzed on 10% SDS-PAGE under non-reducing and reducing conditions (DTT 200mM). Symbols refer to different form of E1 and E2. ox for oxidized; red for reduced; pr for precursor. Position on gel of prestained Molecular Weight marker (Amersham) is reported in the middle.

To establish the fraction of the oxidized species that has progressed towards the native state, it is important to compare the intensity and position of the band in samples of the same time point treated and not treated with exogenous DTT. Analyzing the wild type species (fig. 20, panel A) we could notice that until 2 hours from the synthesis the entire populations of E1 and E2 are almost completely DTT-sensitive. In fact the signal corresponding to the oxidized E1 and E2 strongly decreases in the DTT-treated samples (fig. 20, compare lanes with and without 5mM DTT). Following 2 hours of chase, however, an important rearrangement of the HCV glycoproteins takes place since at 4 hours from the synthesis the majority of E1 and E2 became DTT resistant. At 6 hours of chase, almost all the E2 protein, as well as the E1, acquires full DTT-resistance, indicating that the oxidation process is completed. The chimeric species shows a different behavior in the same conditions (panel B). As for the wild type, 2 hours of chase is the shortest time point at which E2 is revealed, but with two important differences: i) the oxidized species is already DTT resistant, as witnessed by the unchanged position and intensity of the exogenously treated band, and ii) E1 still does not co-precipitate. At 4 and 6 hours chase, both a DTT-sensitive and -resistant fractions appeared for both HCV glycoproteins. The former population increases over time, but it never reaches the totality since at 6 hours of chase it is still present a minor amount of the DTT-sensitive conformers. Finally, it is worth to note that the E1E2/E1E2p7 precursors are notable 6 hours after protein synthesis only in those samples derived from the chimeric transfected cells (fig. 20, panel B). In other words, the situation pictured for the chimeric species at 4 and 6 hours post-synthesis mirrors the folding process observed at 2 and 4 hours respectively for the wild type virus.

All together these results point out several aspects: i) the conformational anti-E2 mAbs CBH2 and CBH5 are able to recognize both DTT-sensitive and DTT-resistant populations of oxidized E2, but not the early synthesized conformers. This observation indicates that generation of the conformational epitope requires a certain degree of oxidation but that, once formed, it is not reverted by mild reduction conditions. ii) The E1E2 wild type and chimeric heterodimers include both DTT-sensitive and DTT-resistant E2 and E1 proteins. iii) The E2, from both JFH1 and Con1 sequences achieves the DTT-resistance while already associated with E1. Furthermore, this experiment confirms that the kinetics of the chimeric precursor processing and heterodimer formation is slower than the wild type since i) the E1E2/E1E2p7 precursor is still present after 6 hours from protein synthesis; ii) the E2 protein Con1-derived recognizes E1 at 4 hours of chase, namely 2 hours later then the wild JFH1 E2 and iii) at 6 hours of chase a fraction of both E1 and E2 proteins are still present as non-oxidized species.

We did not perform the same experiment of DTT resistance at longer times post-synthesis. As consequence we do not know if, for the chimeric species, the process is efficient as for the wt.

Glycosylation status.

Deglycosilation studies of HCV envelope proteins have shown that these proteins are highly modified by N-linked glycans. In particular E2 protein contains 11 N-glycosylation sites in its sequence, while for E1 5 (or 6) potential sites are reported, strongly conserved along the different genotypes. The sugar moieties are added post-translationally on nascent E1 and E2 proteins in the ER-compartment. This modification is a common step in the maturation of viral envelope proteins and it is often necessary for their proper folding, indeed elimination of just a single site can abrogate the normal processing (145-147).

Analysis of the glycans bound to HCV envelope glycoprotein has indicated that only high-mannose type oligosaccharides are associated with these proteins (79). The lack of complex-type glycosylation excludes transit through the medial Golgi and immunofluorescence studies have confirmed that HCV glycoprotein heterodimer is located in the ER or an ER-like compartment (148).

A common method to analyze the glycosylation is based on the treatment with the Endoglycosydase H enzyme, that cleaves only the high-mannose structure and does not recognized oligosaccharides that have been modified in the Golgi network (149). Metabolically labeled S6.1/JFH1 and S6.1/Con1E2 cell lysates were immunoprecipitated and the proteins recovered from the beads were incubated or not with Endo H enzyme or submitted to the same treatment in absence of the enzyme. The digested proteins were then analyzed in SDS-PAGE in reducing conditions.

As shown in figure 21, both E1 and E2 proteins migrate considerably faster after Endo H digestion. This is consistent with a complete deglycosylation upon Endo H treatment that can be observed equally in both samples. These data confirm not only that the newly synthesized envelope proteins are retained in a pre-Golgi compartment, but also that both the wt and the chimeric species undergo a process of native saccharides trimming, a crucial event in the heterodimers folding. Upon Endo H treatment, we could visualize a splitting of the band corresponding to E2 protein. It is consistent with the simultaneous presence of two species: the mature E2 and the E2p7 precursor. This latter species has been reported previously (134), and it is assessable following carbohydrates digestion since only the deglycosylated forms can be separated on SDS-PAGE gels. An additional slightly slower migrating band was sometimes detected for both samples. Such a partial resistance to Endo H treatment has

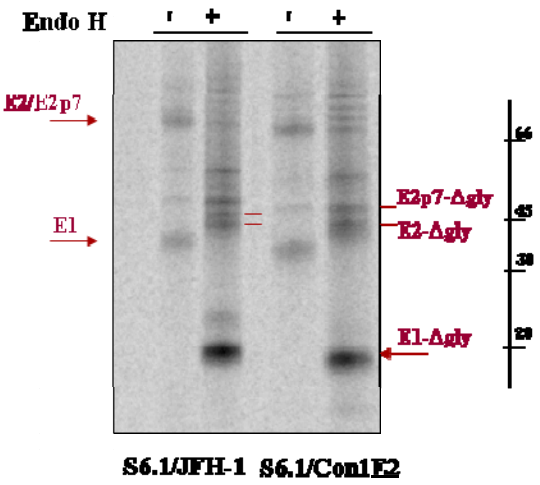


Figure 21. S6.1 cells transfected with JFH1 or JFH-1/Con1E2.
RNA were metabolically labeled for 6 hours in presence of [35S] Met and Cys. E1 and E2 proteins were immunoprecipitated from the PNS with CBH2 or CBH5 respectively for wt and chimeric species and treated (+) or not (-) with endo H and analyzed on 10% SDS-PAGE under non reducing conditions. Note that Con1-derived proteins display an higher electrophoretic mobility than E1E2 proteins from JFH1 transfected cells. Symbol Δgly is for deglycosylated proteins.

already been observed for truncated form of E1 glycoprotein , as well as for E2 expressed in HCV pseudotype particles (150).

Taken together, the DTT-resistance experiments and the deglycosylation study support the idea that the chimeric E1E2 undergo a correct maturation and association processes leading to the formation of functional heterodimers, crucial for the infection ability of the nascent viral particles. In addition, the DTT-resistance data support the delayed kinetics of chimeric precursor processing and heterodimer formation already observed in previous pulse-chase experiments. Therefore, our next attempt was to clarify subsequent steps in the viral particles generation, namely the assembly process. To this purpose we first analyzed the subcellular localization of the viral structural and non structural proteins trough confocal immunofluorescence analysis and then we performed sucrose gradient experiments in order to verify viral proteins association with cellular membraneous structures.

4.8 Subcellular localization of viral structural and non-structural proteins in S6.1/JFH-1 and S6.1/Con1E2 transfected cells.

There is still little understanding of the mechanism underlying the assembly process of hepatitis C virus in cultured cells. However, recent studies have assessed the role as a potential assembly site to the cellular lipid droplets (LDs). Lipid droplets are intracellular organelles that function as storage of neutral lipids. As mentioned, their interest in the HCV fields date about 10 year ago when the core protein was found as associated with the ER membrane and either on the surface of lipid droplets (99-101). Next, taking advantage of the JFH1 culture model, the investigation of the subcellular localization of HCV core and NS5a proteins was possible in the context of an infectious cycle. Confocal microscopy analysis have confirmed that core protein co-localize perfectly with LDs, showing a ring-like pattern that

corresponds to the surface of LDs (44). Because core protein is targeted to LDs whereas RNA replication most likely is mediated by a replicase complex associated with ER or ER-derived membranes, in some stage during virus assembly the RNA genome is transferred from the replicase complex to the core protein. This requires interactions between core and the replicase complex, presumably with involvement of NS5a, which is an RNA-binding protein assumed to play a role in virus assembly. Indeed, NS5a was found to co-localize with core in a similar ring-like pattern, indicating that this protein also associates with LDs (44). More in details, NS5a mainly localized around the core-positive area, resulting in a doughnut-shaped signal with a diameter slightly larger than that core. These data support the idea that non structural proteins may directly participate to the assembly process providing the RNA genome to the assembling nucleocapside (77).

In this study we investigate the subcellular localization of HCV E1 and E2 envelope proteins in the context of the JFH1-replicating system. To evaluate potential differences in the viral protein distribution that might be the cause of a deficient assembly process, we analyzed the localization of E1E2, core and NS5a proteins in both wild type and chimeric genomes transfected cells by using confocal microscopy analysis.

Intracellular localization of viral structural proteins.

The subcellular distribution of HCV structural proteins was examined in S6.1/JFH1 and S6.1/Con1E2 replicating cells by confocal immunofluorescence microscopy. Briefly, transfected cells were grown on glass coverslips, fixed with paraformaldehyde and processed for indirect immunofluorescence with different antibodies. In particular, to detect the capsid protein we used the 3G1-1 mouse monoclonal antibody that recognized the core protein. Concerning E1 and E2, the Ch-L559 polyclonal chimpanzee antiserum was found to specifically recover either wild type or chimeric E1E2 heterodimers and thus chosen for the immunofluorescent staining. Finally, the lipid droplets were identified by using oil red O, that specifically stains triacylglycerols and/or cholesterol, the main content of such organelles (124). It is known that there is no change in the subcellular localization of HCV glycoproteins when analyzed at different time post-transfection, therefore we performed our experiments independently from such parameter (92).

As shown in figure 22, **E1E2 heterodimers** are distributed in a comparable way in both JFH1 and JHF1/Con1E2 transfected cells. In particular, they show a pattern of specific fluorescence in a network of cytoplasmic membranes and at the nuclear periphery. This is in agreement with most of previous reports in which, due to the absence of a robust cell culture model, the E1/E2 subcellular localization analysis have been performed with heterologous expression

system or in the context of HCV replicon (43, 71). In particular, we published the establishment of a replicon system in which the production of the HCV glycoproteins occur independently from the viral RNA by *trans*-complementation of their expression. This system overcame the low level of glycoprotein expression commonly obtained with the full length replicon, permitting the study of the E1/E2 intracellular localization (152). In such paper we have demonstrated that E1/E2 have an ER-like distribution, in agreement with their retention in the ER compartment reported by most of the studies, and with their co-localization with ER proteins, also previously reported (153, 154). In the current study, we confirm the ER-like distribution of E1E2 heterodimers by using the HCVcc system, for both the wild type and chimeric species.

It has been show that, in some transient expression systems, a fraction of HCV envelope proteins can also be detected at the plasma membrane (20, 104, 107). In contrast with them, we never recover E1E2 heterodimers at the cell surface. It is worth noting that there is a correlation between the level of expression and cell surface detection. Therefore, the presence of HCV envelope proteins at plasma membrane observed in the context of heterologous systems is likely due to protein over expression, or at least to the absence of particle formation.

Concerning the **capsid** protein staining obtained in both S6.1/JFH1 and S6.1/Con1E2 cells, we can observe an organization in ring-like structure, never detected by using the replicon system, but amply reported in the context of cells transfected with the infectious JFH1 genome (44, 77, 92). When lipid droplets are labeled with oil red O, an association between core protein and LDs is clearly notable (see merge panel of fig. 22). This evidence is in agreement with confocal analysis performed by other groups and it is also supported by the co-localization of core protein with ADRP, a protein resident on the surface of LDs (44, 77). Although a fraction of the capsid protein is found as not directly associated with the lipid droplets, but rather to a membranous compartment associated with them, in both wild type and chimeric species, most of core localizes on the LDs surface (see zoomed in the merge panel fig.22). The cellular distribution of such complexes, however, shows differences in the two cell lines. In the case of S6.1 transfected with the JFH/Con1E2 genome, in fact, LDs-core associated tend to accumulate in the periphery of the cell. By contrast, in almost all cells transfected with the wild type genome, LDs-core coated do not cluster but rather are distributed throughout in the cytoplasm. The accumulation of the capsid protein on the putative assembly site, not observed in cells expressing the JFH1 genome, might prevent the release of virus particles. Indeed, a rapid assembly process should result in a rapid liberation

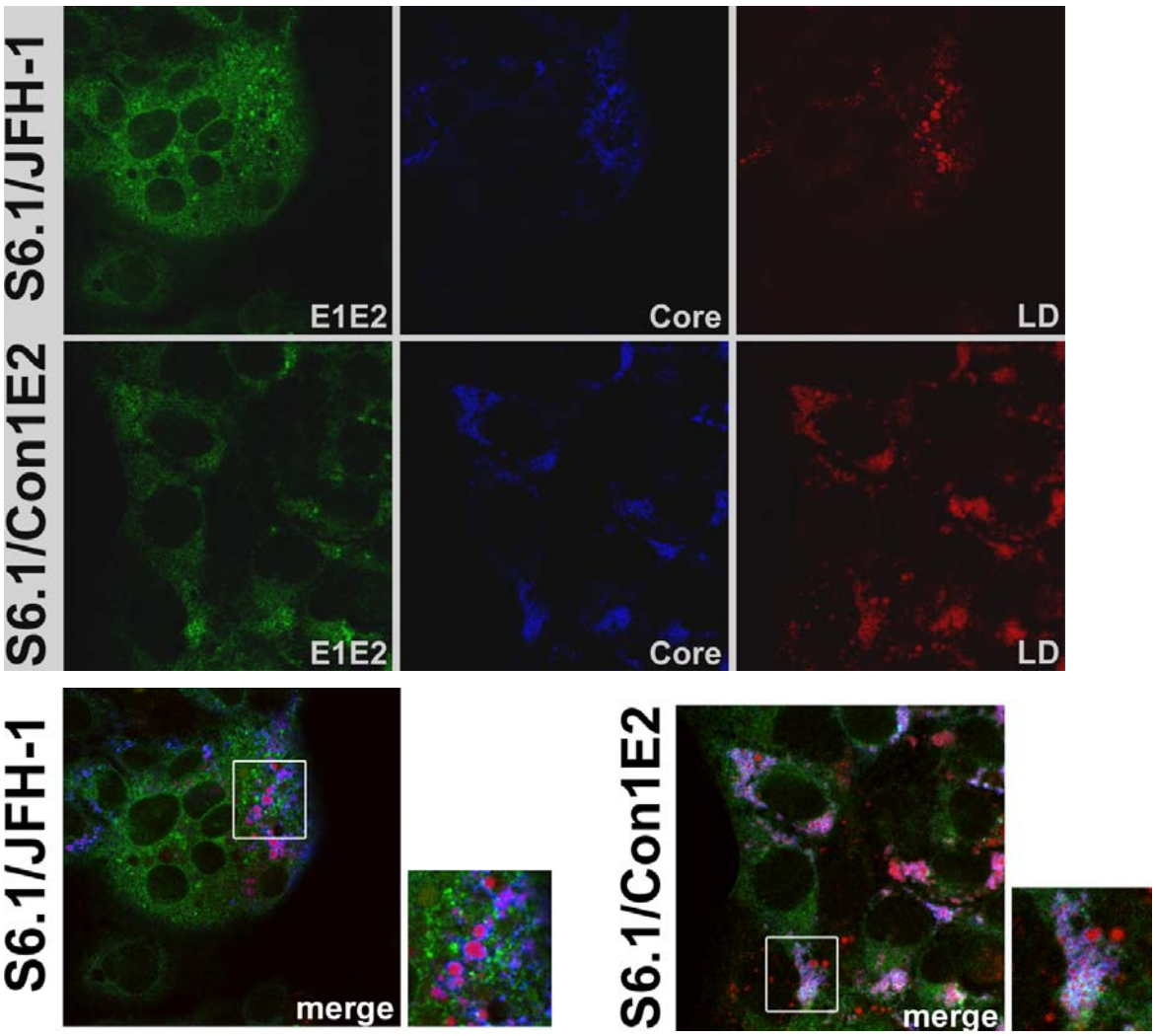


Figure 22. Intracellular distribution of HCV structural proteins.
Transfected S6.1/JFH-1 and S6.1/Con1E2 grown on coverslips were fixed and stained with the anti-E1/E2 chimpanzee antisera L559 (green) and the mouse monoclonal antibody 3GI-I (blue). Lipid droplets were stained with oil red O. Merge panel and the correspondent magnification are reported in the third row.

of HCV proteins from LDs, which should not accumulate in the periphery of the cell, as observed in S6.1/Con1E2 cells.

To gain more accurate information about the relative localization of structural proteins, in the next figure (figure 23) we report the immunofluorescence staining of E1E2 heterodimer and core, underlining their co-localization, and above all the presence of two different "populations" in the S6.1/Con1E2 cell context.

Again, E1E2 heterodimers in S6.1/JFH-1 cells display an ER-like distribution, while core protein shows the ring-like pattern. In the merge panel (see top of the right column and the corresponded magnification) we report for the first time the co-localization between all the viral structural proteins, that clearly associate in correspondence of the potential assembly sites. By contrast, in cells expressing the chimeric genome, we could not evidence such a

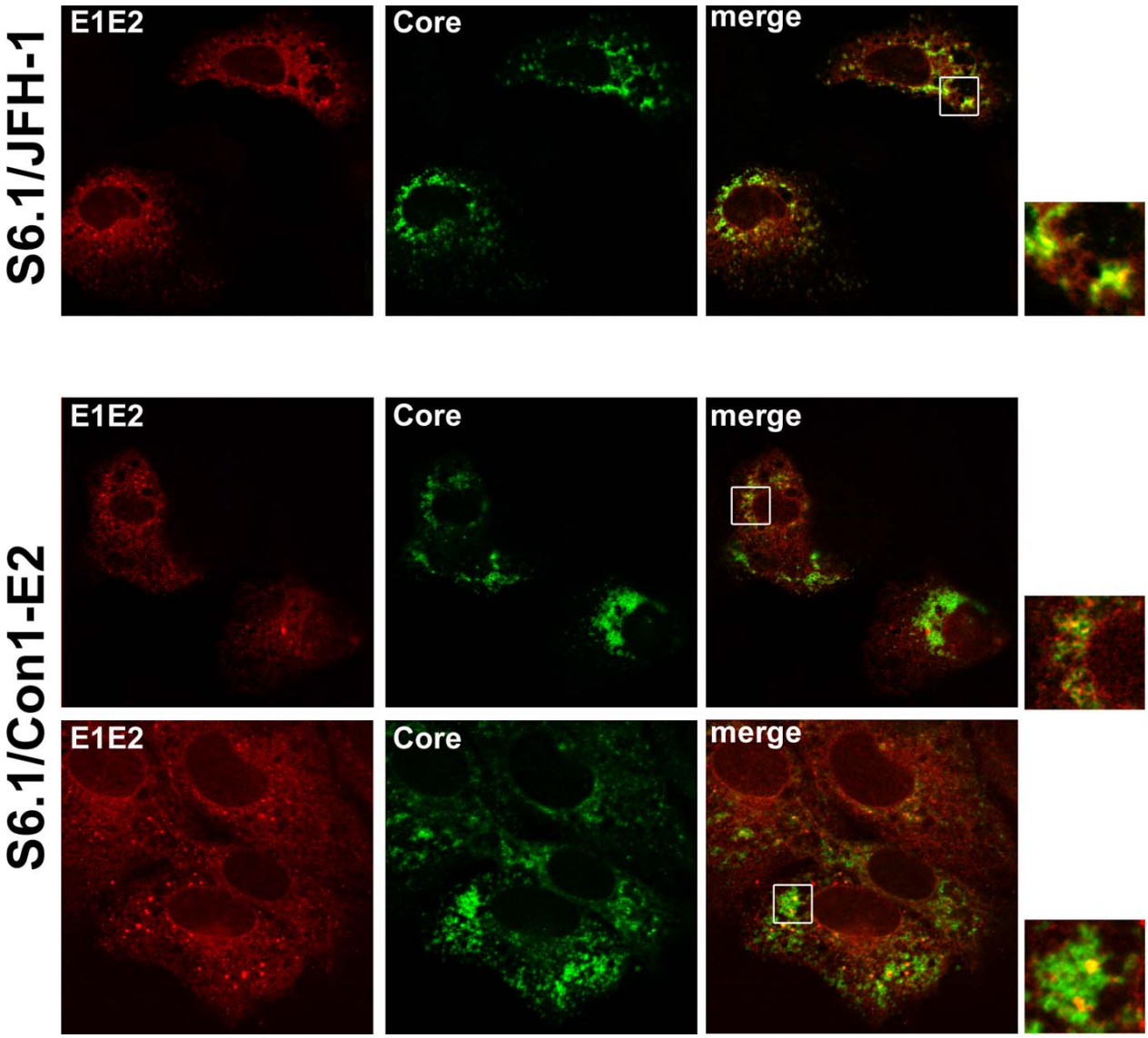


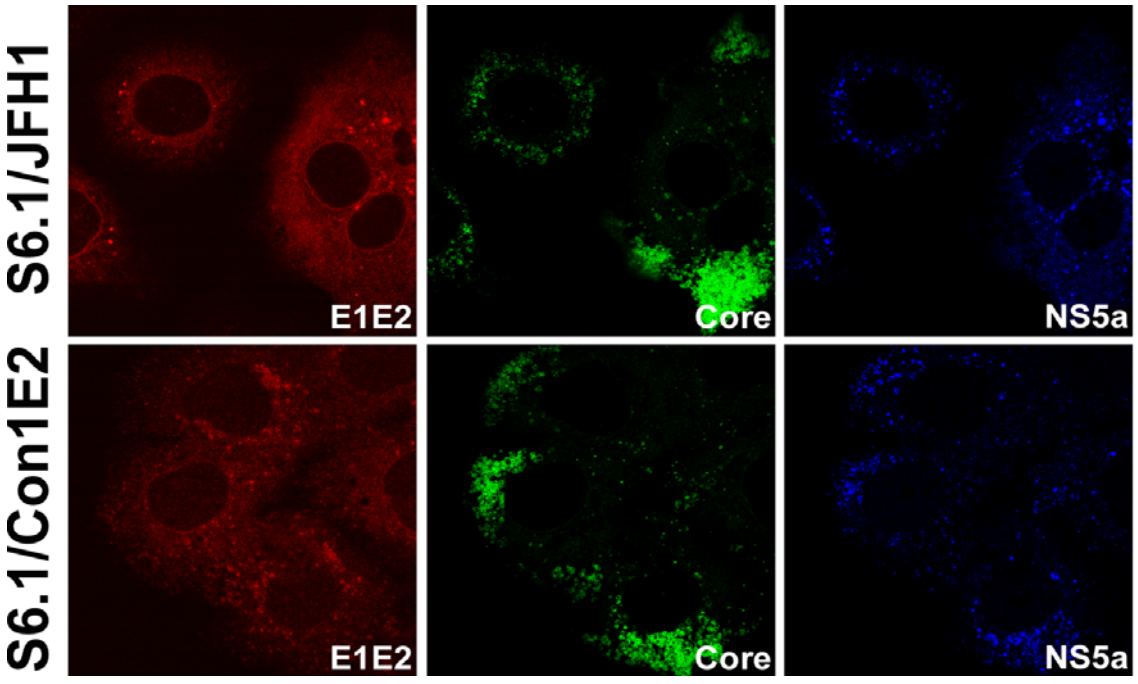
Figure 23. Intracellular co-localization of HCV core with E1/E2 proteins.
Transfected S6.1/JFH-1 and S6.1/Con1E2 grown on coverslips were fixed and double-stained with the anti-E1/E2 chimpanzee antisera L559 (red) and the mouse monoclonal antibody 3GI-I (green). The merge images and the correspondent magnification are showed in the right column. Two distribution patterns are reported for S6.1/Con1E2. Second row is representative of the major fraction of cells. Third row is representative of a subset of S6.1/Con1E2 (roughly 10%) in which the core, E1/E2 structural proteins co-localize showed in dots.

clear co-localization of the three structural proteins, even though core, E1 and E2 have a similar distribution in the two cell types (compare first and second row). In some S6.1/Con1E2 cells we could detect a different pattern of E1E2 distribution, as shown in the third row of figure. In fact, in a minor subset of those cells (roughly 20%) we found a concentration of the glycoproteins in dot-like structures. Importantly, as evident in the merge panel (see bottom of the right column), core protein co-localizes with E1E2 in these dot structures. In our previous work, based on the replicon system, we have described similar dense accumulations of the viral structural and non structural proteins that associated to generate dot-like structures (152). It is important to stress here that such structures accumulating all the viral components are typical of a system, the replicon one, which cannot

support the production of infectious particles. On the contrary, in a system that allows the generation of infectious particles such as the HCV cell culture model, a similar patch-distribution is not observed. It is therefore conceivable that these dots represent sites of storage of viral proteins rather then pre-budding site.

Relative intracellular localization of E1E2 heterodimer, core protein and NS5a.

In the recently proposed mechanism for HCV assembly process, core protein accumulates on the surface of LDs and recruits the replication complex in order to trigger particle formation (77, 155). NS5a, in addition to core, seems to be a key element in that process, probably providing the RNA genome to the assembling nucleocapsid (45). We therefore analyzed whether the absence of infectious particles in the supernatant of S6.1/Con1E2 cells was due to a lack of association of the viral structural proteins with the replication complex. To this end we compared the co-localization of E1E2, core and NS5a in S6.1 cells transfected with the wild type and chimeric genomes. In addition to the antibodies previous described to detect E1E2 heterodimers and core protein, we used the commercially available anti-NS5a antibody to label such non structural protein.



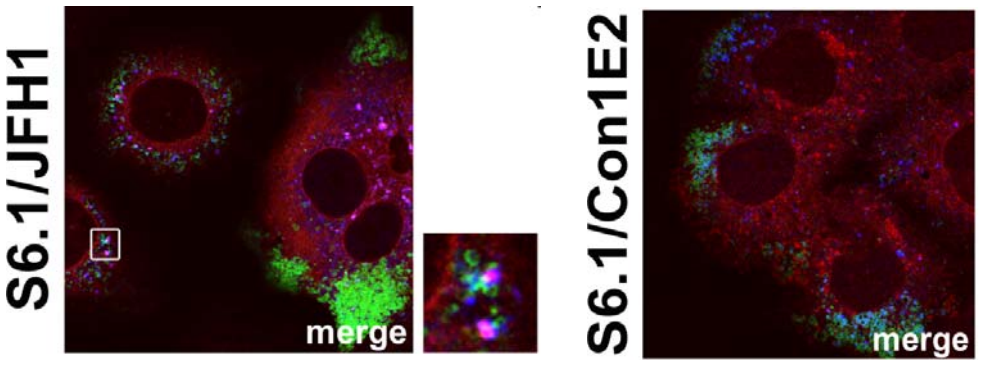


Figure 24. Relative intracellular localization of HCV E1/E2, core and NS5a proteins.
Transfected S6.1/JFH-1 and S6.1/Con1E2 grown on coverslips were fixed and stained with the anti-E1/E2 chimpanzee antisera L559 (red), the mouse monoclonal antibody 3GI-I (green), and the rabbit monoclonal antibody against NS5a (blue). The merge images and the correspondent magnification are showed in the third

As shown in figure 24, in both cases the majority of NS5a displays a ring-like pattern; similar to the one displayed by core, indicating that NS5A also associates with LDs. Importantly, NS5a partially co-localized with core also in S6.1/Con1E2 transfected cells, suggesting that there is no difference in core-NS5a interaction. The E1E2 heterodimer display the same staining-pattern in both wild type and chimeric species. However, we can detect an overlap of distribution of all the three viral proteins only in S6.1/JFH1 cells.

The relative localization of E1E2 heterodimer and NS5a protein is better shown in figure 25. HCV E1 and E2 and the non structural proteins have been shown to co-localize in the membranous web, hypothetical site of the replication complex, confirming that the replication and the assembly factories might be located within the same region. In this figure the clearest co-localization between E1E2 and NS5a corresponds to the dot-like structures previous described. Again, these punctuated sites of protein accumulation are found mainly in S6.1/Con1E2 rather than in S6.1/JFH1 cells. Since the association between structural and non structural proteins is not affected by the exchange of the E2 ectodomain, we can speculate that chimeric viruses are hampered in their assembly process at very later stages.

Overall our confocal microscopy data indicate first that both the wt and the E1E2 chimeric heterodimers have an ER-like distribution, in agreement with their retention in the ER-compartment, as indicated by the Endo-H treatment. Secondly, double-label immunofluorescence experiments with anti-core and anti-E1E2 antibodies show that the structural proteins do not co-localize in S6.1/Con1E2 cells, whereas they can be found to associate in JFH1-transfected cells.

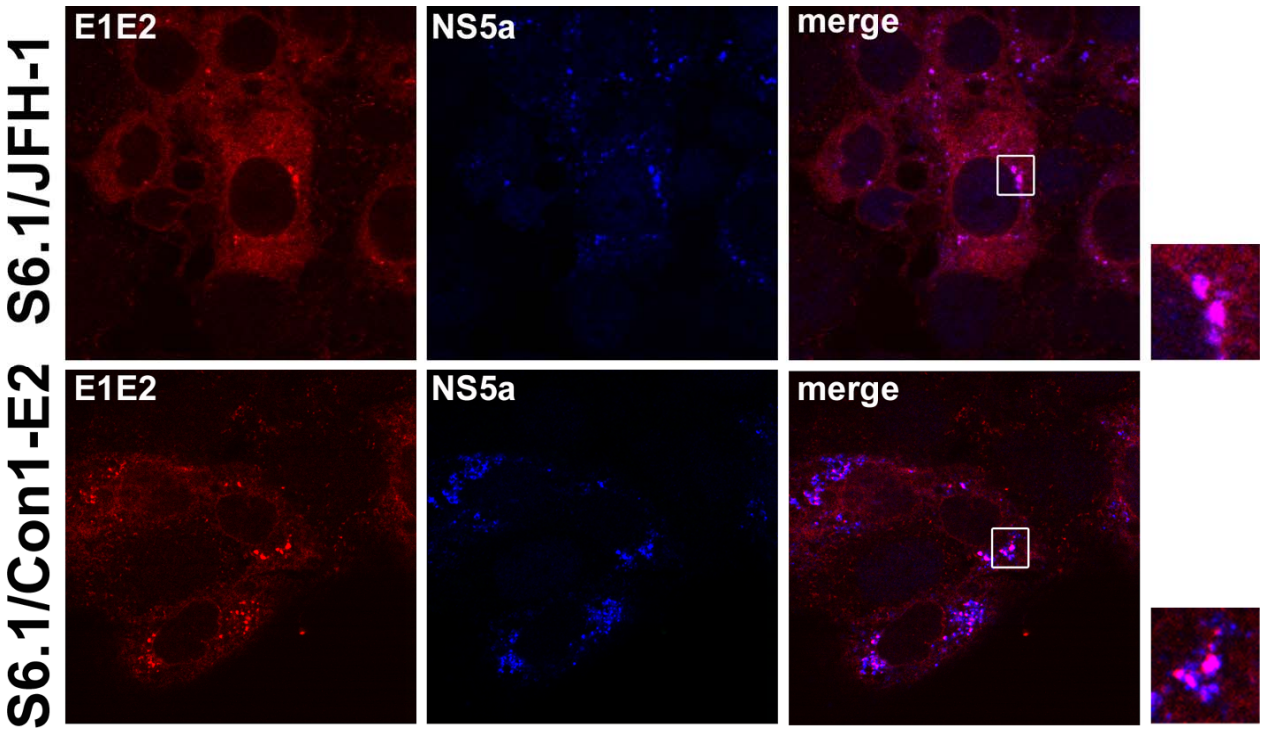


Figure 25. Intracellular co-localization of HCV E1/E2 with the non structural protein NS5a.
Transfected S6.1/JFH-1 and S6.1/Con1E2 grown on coverslips were fixed and double-stained with the anti-E1/E2 chimpanzee antisera L559 (red) and the anti-NS5 rabbit monoclonal antibody (blue). The merge images and the correspondent magnification are showed in the right column. Two distribution patterns are reported for S6.1/Con1E2. Second row is representative of the major fraction of cells.

Importantly, in S6.1/Con1E2 cells, core together with LDs accumulate in the peripheral area, suggesting a delay in the assembly/release process of chimeric particles respect on wild type particles that might be the cause of an inefficient virus production. Another difference between S6.1/JFH1 and S6.1/Con1E2 cells is the presence in the latter one of dot-like structures in which E1E2, core and also NS5a accumulate. The co-localization of viral structural and non structural proteins in such structures was already found in cells expressing the full-length replicon. In the latter context it was thought that dots represented HCV pre-budding areas where all the viral proteins and RNA accumulated on ER-derived membranes. We do not detect any co-localization of E1E2, core and NS5a proteins in dot-like structures in S6.1/JFH1 cells. Thus, we might speculate that the accumulation of all viral proteins in dots structures is the results of an inefficient assembly process rather then a pre-budding area. This idea is also supported by the recently reported role of LDs as HCV assembly site, established by using the HCVcc infectious system, and by the recover of the capsid protein on the surface of such organelles.

Therefore, taken together our data suggest that chimeric viruses are impaired in their assembly process at later stages. On the other hand, mature chimeric particles could be generate and then accumulate into the cells, instead of transit the secretory pathway to be

release in the supernatant. This hypothesis is enhanced by recent studies reporting the existence of intracellular infectious particles in JFH1 genome-transfected cells. In fact it has been shown that the liberation of infectious viral particles is preceded by the accumulation of intracellular infectious virions, that reach the final configuration along their egress (156, 157). Based on this consideration we sought to investigate the presence of intracellular infectious particles in the S6.1/Con1E2 cells to verify if the E2 ectodomain exchange affects the assembly process or the egress progression.

4.9 S6.1/Con1E2 cells do not contain infectious viral particle.

Confocal microscopy analyses suggest that in S6.1/Con1E2 cells the chimeric particle assembly is in some way hampered by the E2 ectodomain exchange. Although the subcellular localization of core, E1E2 and NS5a proteins mainly displays the same pattern in S6.1/JFH1 and S6.1/Con1E2 cells, we note an accumulation of lipid droplets coated by the capsid protein in almost all the cells transfected with the chimeric genome. By contrast, this phenomenon is only rarely observed in the case of S6.1/JFH1 cells, suggesting that there is an inverse correlation between the efficiency of virus production and LDs-core protein clustering within the cells. Therefore, to further investigate if these observations reflect a real functional difference in terms of particle production, we verified whether chimeric particles are correctly assembled and then accumulated in S6.1/Con1E2 cells. In other words, our attempt was to elucidate at which stage the virus production process is impaired. Indeed, if intra-cellular immature virions are formed, the defect could be ascribed to the release step. By contrast, if infectious particles could be detected inside the cells, our conclusion would have been that the assembly process cannot proceed to maturation.

To design this experiment, we took advantage of some recent studies reporting the existence of infectious HCV particles within hepatoma cells transfected with the JFH1 genome (156, 157). In such studies it was demonstrated that the intracellular particle, considered as viral precursor, exhibits a different buoyant density respect to the particle secreted in the milieu, but it is already infectious when inoculate onto Huh7 naïve cells. The density profile, determined by sedimentation equilibrium gradient, indicated that the biochemical composition of the intracellular infectious particles differs from that of the particles present in the supernatant. More in detail, the intracellular particles showed a density range located between 1.15 to 1.20 g/ml, while the supernatant particles density is lower, ranging from 1.03 to 1.16 g/ml. Based on the different density, the authors proposed that infectious viral particles are assembled in intracellular compartments as high density precursor, and than they acquire elements, probably lipids, that confer the low density configuration along their egress.

Taking advantage from such different biophysical characteristic, we checked the presence of infectious particles within S6.1/Con1E2 cells by using a sedimentation sucrose gradient analysis. The separation of membrane-associated proteins according to rate of sedimentation through sucrose density gradient is in fact a widely used technique. Typically, larger (in size) and heavier (in density) particles travel through a gradient faster and settle further down the gradient. In the case of membrane vesicle separation by a density gradient, at equilibrium, a particular vesicle species remain in a specific fraction, with the density of this fraction equal to the density of the vesicles. Thus, density gradient ultracentrifugation technique provides an accurate way to separate both protein complexes and subcellular membrane vesicles based on their size and density.

Briefly, S6.1/JFH1 and S6.1/Con1E2 cells were lysed by short incubation in hypotonic buffer followed by four freeze-thaw cycles in liquid nitrogen and a 37°C water bath respectively. We did not use any membrane detergent for the lyses in order to maintain unaltered the potentially immature virions. Cell debris was then pelleted by centrifugation and the collected supernatant collected was overlaid onto a discontinuous sucrose gradient (20 to 60% in TNE buffer) as detailed in Material e Methods. After ultracentrifugation, gradient fractions were collected from the top and titrated for virus infectivity as previously described. The density of the fractions was determined by measuring the refractive index of 10-µl aliquots of each sample.

As reported in figure 26, the density profile of the collected fractions is identical for both S6.1/JFH1 and S6.1/Con1E2 cell lysates. In terms of infectivity of the density-separated samples, three of the fractions obtained from JFH1 transfected cells result infectious. In agreement with data reported in literature (156, 157), the infectivity is observed in fractions corresponding to densities ranging from 1.15 to 1.21 g/ml, consistently with intracellular infectious particles. By contrast, none of the fractions collected from S6.1/Con1E2 cell lysates show any infectivity. The lack of infectious particles inside the cells transfected with the chimeric genome confirms the idea that the E2 ectodomain exchange affects the assembly process rather then the following release step.

To further support this interpretation, we analyzed each sucrose fraction by Western blot in order to determine whether structural and non structural viral proteins co-sediment in the same membranous fractions. Indeed, as explained above, a density gradient ultracentrifugation allows separation of membrane and non-membrane, namely cytosolic, fractions according to the rate of sedimentation through the sucrose gradient. In particular, cytosolic fractions reach the equilibrium at higher sucrose concentrations (about 50-60%), while the membrane-containing materials remain in the lighter fractions. Intracellular

infectious particles are commonly reported in the middle of the gradient, corresponding to fractions 5, 6 and 7 in our hands.

Before to proceed to Western blot analysis, membranes contained in each fraction were solubilized by treatment with the nonionic detergent NP-40 on ice, a condition that achieves a better protein resolution on SDS-PAGE. Previous studies on the HCV replicon cells have shown that the majority of HCV non structural proteins are detected in membranous fractions, namely light fractions (39, 154). Shi et al. (2003) also suggested that the newly synthesized HCV RNA is present in the same fractions, providing strong evidence that HCV replication occurs through membrane-associated replication complexes (RCs). Therefore we checked the presence of NS3, core, E1 and E2 proteins in each sucrose fraction obtained from both S6.1/JFH1 and S6.1/Con1E2 cells in order to: i) confirm the presence of the non structural protein NS3 in light fractions, where is typically found the viral replication complex; ii) evaluate whether the viral structural proteins are associated with membranous structures; iii) verify the co-sedimentation of all the viral structural proteins in the same light fractions, that is an index of their co-presence in a membranous structure, such as viral particle. The result of Western blot analysis is reported in the bottom on figure 26.

According to the studies just mentioned, **NS3** protein is recovered in light fractions in the S6.1/JFH1 cell lysate, mostly in fractions 4 and 5 but residual amounts also in fraction 6. As expected, NS3 protein is not detected in the cytosolic fractions. In the S6.1/Con1E2 cell sample, the density profile is slightly shifted since **NS3** is mostly recovered in fractions 5 and 6.

Concerning the viral structural proteins, **core** is detectable in almost all fractions, consistent with its presence in both soluble and membrane-associated forms. Importantly, comparing the pattern of core distribution along the gradient, we remark an accumulation of this protein in the heaviest fraction (number 9) in the S6.1/Con1E2 cell lysate, not observed in the wild type sample. It is worth noting that, as indicate by the signal corresponding to the total lysate (TL), there is no significant difference in the total amount of core protein initially loaded onto the gradient between wild type and chimeric samples. Thus, this result indicates that a consistent portion of the capsid protein is present as high molecular weight complexes that sediment at high concentration of sucrose. These complexes likely represent non functional aggregates, probably composed mainly by proteins (core and other viral and cellular proteins), although we cannot exclude the presence of membranous structure that contribute to the aggregation. Importantly, such abundance of aggregates is not noticed in the S6.1/JFH1 case, as indicate by the fact that the signal corresponding to core is weaker in lanes 8 and 9 than in lanes 6 and 7.

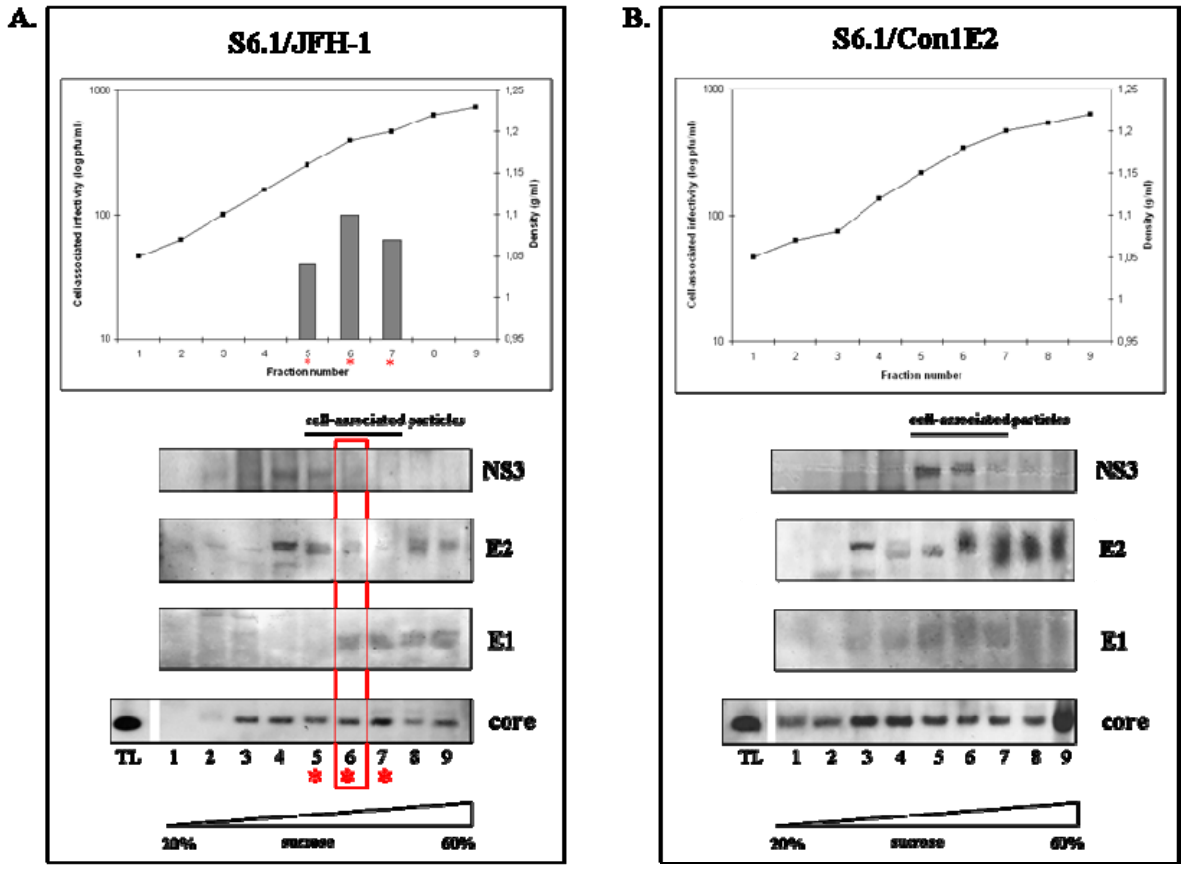


Figure 26. Sedimentation equilibrium analysis.
Buoyant density profile of S6.1/JFH-1 (panel A) and S6.1/Con1E2 (panel B) cell lysates determined by equilibrium ultracentrifugation in sucrose gradient is reported on the top of each panel. Fractions of the 20-60% gradient were collected from the top, and infectivity was determined by serial dilution and immunofluorescence staining anti core protein. The infectivity (pfu/ml) is shown as bar chart. The density (g/ml) of each fraction is shown as a dotted line. HCV nonstructural protein NS3 and E1, E2 and core structural proteins were detected by Western blot analysis of each sucrose fraction (bottom of each panel).

The sedimentation profiles of **E1** and **E2** proteins, as well as their SDS-PAGE appearance, show remarkable differences between wild type and chimeric intracellular samples **E2** recovered from the S6.1/JFH1 cells is mostly present in fractions 4 and 5; still detectable in fraction 6 and 7; and again evident at the bottom of the gradient (fraction 8 and 9). By contrast, the majority of **E2** protein inside S6.1/Con1E2 cells is found in the heavier fractions, suggesting that, similarly to core, it is prevalently present as high molecular weight aggregates. Furthermore, regarding the chimeric **E2** protein, the higher is the sucrose concentration, the more diffuse is the band. About **E1** protein, in the case of S6.1/JFH1 cell lysate, we detect a signal in correspondence of fractions 6, 7, 8 and 9, indicating that it is found both associates to membranes and in an aggregated form. Unfortunately, even if we repeated this experiments several times, we never clearly detected the E1 protein band in the chimeric sample. In fact, only a very weak signal, distributed in the middle of the gradient, can be noted, but it is not enough intense to be correctly interpreted.

Finally, from Western blot analysis appears another important difference between S6.1/JFH1 and S6.1/Con1E2 cell lysates. As mark in figure 26, core, E1 and E2 proteins co-fractionate in the wild type gradient, but never in the chimeric case. Importantly, one of the fraction in which all the viral structural proteins are co-present is also the most infectious one, as reported in the upper graph (see fraction 6). This clearly indicate that only in S6.1/JFH1 cells, core, E1 and E2 proteins associate in membranous structures that most likely correspond to the intracellular infectious particles. The presence of a minor amount of NS3 protein in fraction 6 indicate that also the HCV replication complex, already reported as membrane-associate, sediment at the same sucrose concentration (39).

Taking together the infectivity assay with the sedimentation equilibrium gradient demonstrates that only S6.1/JFH1 cells contain intracellular infectious particles that, based on the density profile, represent the high density viral precursor described by other groups (77, 156). This result is confirmed by the Western blot analysis since all the viral structural proteins co-sediment in the light fraction that corresponds to the most infectious one.
By contrast, in S6.1/Con1E2 cells we do not recover any infectious particles. In addition, looking at the Western blot analysis, the majority of the viral structural proteins sediment in the bottom of the gradient, indicating that they are present as high molecular weigh complexes. Although we cannot define the nature of such aggregates, their appearance is consistent with the dot like structures pointed out by the confocal analysis. We in fact might speculate that in S6.1/Con1E2 cells all viral proteins accumulate in dots as non functional aggregates, as results of an inefficient assembly process.
Summarizing the data shown so far, we have demonstrated that the E2 protein ectodomain exchange does not impaired the HCV replication as well as the E1E2 heterodimer formation, but completely abolish the infectious particles production. To distinguish between defective assembly and impaired release of virions, we have checked the presence of intracellular infectious chimeric viruses, since their accumulation precedes the release in the supernatant. As last shown, the substitution of the E2 ectodomain from genotype 2a to genotype 1b suppresses the formation of intracellular immature viruses, strongly suggesting that the hampered process is the assembly of chimeric infectious particles rather then their release in the milieu. Western blot on density separated samples encourage us to make a correlation with the confocal microscopy analysis, in which only in S6.1/Con1E2 cells we note an accumulation of the viral proteins in dot-like structures. We could hypothesize that the impairment in the assembly process leads to an accumulation of high density aggregates, revealed as spots in the confocal analysis.

It is well known that both HCV envelope proteins are essential for the production of infectious viral particles, as demonstrate by the abolishment of virion release when the E1-E2 coding region is deleted (10). It is worth reminding that in our chimeric construct the entire structural protein sequence belongs almost completely to JFH1, with the exception of the E2 protein ectodomain (from aa 683 to aa 728) that derives from the Con1 sequence (genotype 1b). Nevertheless, although the chimeric genome is replication-competent, our data demonstrate that the interchange of E2 ectodomain profoundly alters the efficiency of virus production. Thus, the data lead us to suppose a genetic incompatibility between genotype 1b E2 protein and genotype 2a structural proteins.

We investigated further in this direction designing an experiment that takes advantage from the "flexibility" of the HCV RNA genome. More precisely, for HCV, as well as for a number of other *Flavivirus*, the assembly of progeny viruses can be achieved when structural proteins are expressed in *trans*, namely independently, from the RNA molecule that encodes the replicase proteins (159, 160). Based on such peculiarity, we sought to verify if, expressing the viral E1 and E2 proteins in *trans*, we could rescue the defect of our chimeric construct, and obtain infectious chimeric particles in the S6.1/Con1E2 cell supernatant.

4.10 Genotype dependence of *trans*-packaging of JFH-1 and JFH/Con1 constructs.

The possibility to rescue a defective genome by providing the necessary functions in *trans*, that means independently from the genome itself, is a known feature of several plus-strand RNA virus called *trans*-complementation. As consequence of such flexibility, genomes with defects inactivating viral functions essential for autonomous assembly and release can be rescue by expression of the lacking functions in *trans* (161-164).

Regarding Hepatitis C virus, it is now known that all viral proteins involved in RNA replication, with the exception of NS5a, cannot be complemented in *trans*, presumably as consequence of the secluded architecture of the membrane-associated replication machinery (165). In contrast, very recently it was demonstrated that the entire HCV structural region, between core to the N-term of NS2 protein, can be *trans*-complemented, providing new insights into the HCV particle assembly (166, 167). Similarly, it is reported that HCV genomes with lethal mutations in core protein can be rescue by ectopic expression of the functional core protein (77). Therefore, considering our chimeric genome as defective for some assembly functions, we sought to rescue the production of infectious particles providing the lacking functions in *trans*. The defect is clearly due to the swapping of the E2 ectodomain in the chimeric construct, from genotype 2a to genotype 1b. As consequence, we decided to transfect the JFH1/Con1E2 RNA genome into S6.1 cells stably expressing the genotype 2a E1

and E2 proteins, and then verify the presence of infectious particles in the supernatant. The *trans*-complementation could be in fact achieved with two modalities. A defective viral genome, capable of RNA replication but unable to produce progeny viruses, can be rescue firstly by expression of the structural proteins via helper virus (163, 164). Alternatively, as in our case, E1 and E2 proteins can be stably expressed by a packaging cell line under the control of an eukaryotic promoter (166, 167).

Thus, our next attempt was to verify whether JFH1/Con1E2 genome allowed the infectious particle production once supplied in *trans* the E2 protein genotype 2a-derived, namely of the same genotype of the other structural proteins. This evidence would be in agreement with our previous results and would support the idea of genotypic incompatibility between genotype 1b E2 protein and genotype 2a core, E1, p7 and NS2 proteins.

The *trans*-complementation experiments were performed exploiting as tool two different S6.1 packaging cell lines obtained in our laboratory. Once stably expressing the HCV genotype 2a envelope proteins, the second one expressing E1 and E2 proteins of genotype 1b, designed S6.1/E1E2:2a and S6.1/E1E2:1b respectively. As reported in figure 27, we confirm the expression of the envelope proteins by Western blot analysis in both S6.1/E1E2:2a and S6.1/E1E2:1b cell lines.

Next, we transfected each cell line with the chimeric JFH1/Con1E2 RNA as already described. In addition to the latter one, we used the JFH1ΔE1E2 construct as control for the *trans*-complementation. In fact, lacking of both the envelope protein coding region, this genome JFH1-derived is unable to induce the infectious viral particles production, thus representing the best tool to evaluate the *trans*-complementation efficiency. Once transfected into S6.1/E1E2:2a or S6.1/E1E2:1b cells, we proceeded to verify whether the defect of the viral genome was rescued simply assessing the presence of infectious particles in the supernatant. In that case, in fact, the envelope proteins expressed in *trans* by the cells have provided the necessary functions for the assembly/release of viral particles. Infectious deficient supernatant, on the contrary, would grant that glycoproteins are not able to be successfully incorporated into the virus particle. To better explain the *trans*-complementation system we used, the figure 27 schematically reports our approach.

JFH1/Con1E2 and JFH1ΔE1E2 RNA constructs were both transfected into S6.1/E1E2:2a or S6.1/E1E2:1b packaging cells by electroporation. Three days post transfection each cell type was checked for the presence of viral core protein by immunofluorescence to confirm their replication-competence.

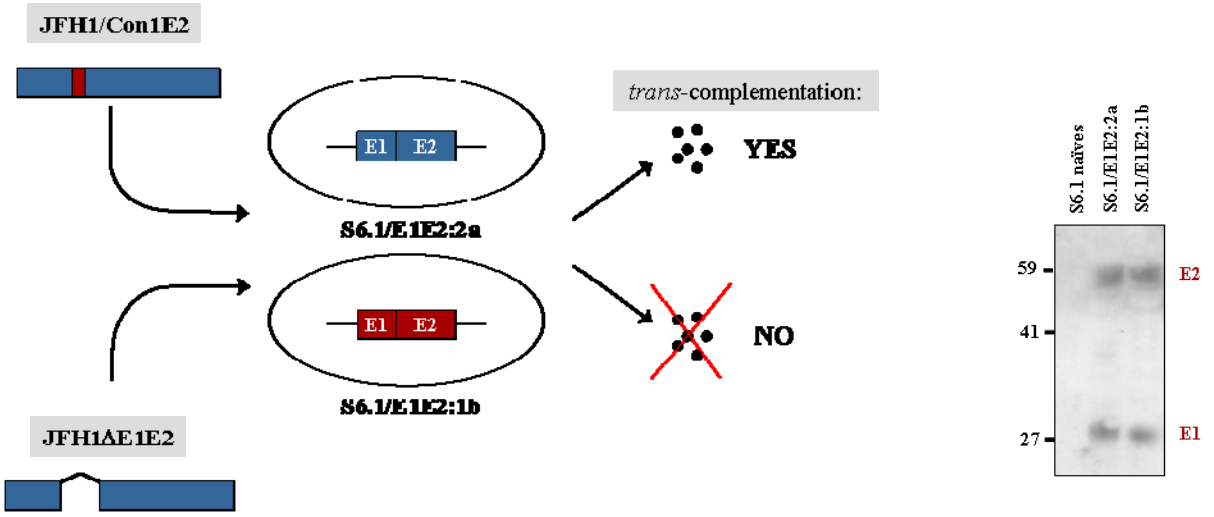


Figure 27: schematic representation of *trans*-complementation

In vitro transcribed genomic JFH1ΔE1E2 and Con1/E2 defective RNAs are delivered to S6.1/E1E2:2a and S6.1/E1E2:1b packaging cell lines that stably express E1 and E2 HCV proteins of genotype 2a and 1b respectively. After 72 hrs from transfection, cell supernatants are collected and tested for the presence of infectious viral particles. The recover of infectious particles indicates that the lacking function of the defective viral RNA has been *trans*-complemented by the cell. Conversely, when no infectious viral particles are recovered in the supernatant, the *trans*-complementation has not been achieved.
On the right of the panel is reported the Western blot result. The analysis was performed on S6.1/E1E2:2a and S6.1/E1E2:1b cell lysates using the polyclonal anti-serum Ch-L559. Two bands, relative to E1 and E2 proteins, are evident in S6.1/E1E2:2a and S6.1/E1E2:1b samples, whilst are absent in the S6.1 naive cells.

As expected, both JFH1/Con1E2 and JFH1ΔE1E2 genomes are able to efficiently replicate in S6.1/E1E2:2a and S6.1/E1E2:1b cell lines (figure 28). Next, we verified the release of infectious viral particles, namely the ability of the packaging cell lines to *trans*-complement the defects of JFH1/Con1E2 and JFH1ΔE1E2 genomes. Supernatant fluids were collected at different time point, from 72 hours until 7 days post-transfection, and tested for the presence of infectious particles by inoculation onto Huh7 naïve cells. After 72 hours, Huh7 naïve cells were fixed, permeabilized, and examined for foci of cells expressing HCV core protein by using indirect immunofluorescence as already described.

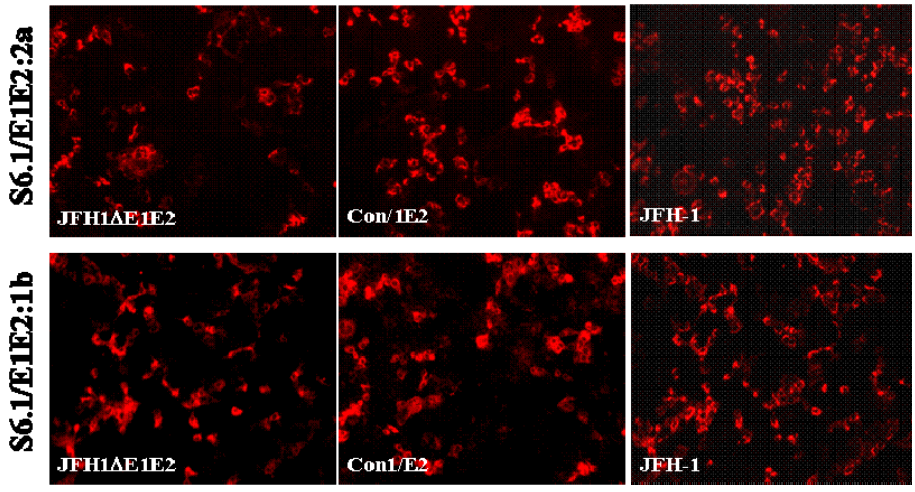


Figure 28. Replication of JFH-1, JFH1ΔE1E2 and Con1/E2 constructs.
S6.1/E1E2:2a and S6.1/E1E2:1b packaging cells were seeded in 48 well/plate immediately after viral RNAs electroporation and incubated for 72 hours. Intracellular expression of HCV core protein was assayed by indirect immunofluorescence with α-core antibody (3GI-1).

As further control, we used the full length JFH1 genome. It is worth noting that, transfecting this RNA, we cannot verify the *trans*-complementation, since this genome itself leads to the infectious viral particles production. Nevertheless, because the JFH1 full length is not a defective genome, it allows us to confirm the ability of S6.1/E1E2:2a and S6.1/E1E2:1b cells to generate infectious viral particles.

In figure 29 is reported the results of the *trans*-complementation experiment. Supernatants from of S6.1/E1E2:2a and S6.1/E1E2:1b cells transfected with the three different genomes were collected from day 3 to day 7 and each one titrated by inoculation onto Huh7 naïve cells. As expected, the JFH1 full length genome allows the production of infectious particles, confirming that both packaging cell lines support the HCV replication, assembly and release. Despite this conclusion, comparing the virus titles obtained from S6.1/E1E2:2a and S6.1/E1E2:1b cells clearly appears a substantially difference in terms of productivity. In fact the HCVcc title reached in the S6.1/E1E2:2a supernatants is approximately 10 fold higher than that obtained from S6.1/E1E2:1b. This will be discuss below, however, assuming as true our hypothesis of genotypic incompatibility, it might be possible that the interaction between genotype 1b E1E2 and all other structural proteins genotype 2a-derived leads to an abortive assembly process. As consequence, since the envelope proteins expressed by the packaging cell in part displace those of genotype 2a, the S6.1/E1E2:1b cell supernatant reaches a virus title much lower then that of S6.1/E1E2:2a cells.

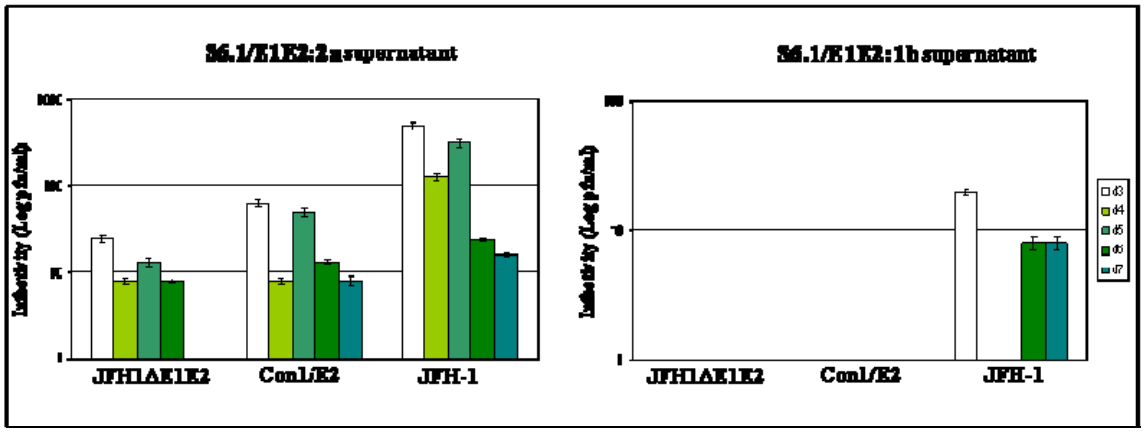


Figure 29: infectivity of *trans*-complemented JFH1ΔE1E2 and Con1/E2 viral genomes.
In vitro transcribed genomic JFH1ΔE1E2, Con1/E2 defective RNAs and the full length JFH-1 genome were delivered to S6.1/E1E2:2a (left chart) and S6.1/E1E2:1b (right chart) packaging cell lines that stably express E1 and E2 HCV proteins of genotype 2a and 1b respectively. From day 3 to day 7 after transfection, cell supernatants were collected and tested for the infectivity (pfu/ml) by serial dilution and immunofluorescence. The infectious title (pfu/ml) is shown by bar chart.

As evident in figure 29, the chimeric genome JFH1/Con1E2 shows the same behavior of the defective construct JFH1ΔE1E2, confirming that the swapping of the E2 ectodomain is equivalent to the deletion of the E2 envelope protein coding region.

Importantly, only in S6.1/E1E2:2a cell supernatants we recovered infectious particles upon transfection with both JFH1ΔE1E2 and JFH1/Con1E2 RNAs. This clearly indicates that the *trans*-complementation is possible, but solely when the packaging cell provides autologous envelope proteins. Indeed, as evident by the absence of infectious particles in the supernatant, S6.1/E1E2:1b cells are not able to rescue the defective assembly function neither of JFH1ΔE1E2, nor of the JFH1/Con1E2 genome.

Finally, it is worth to note that the virus titles reached with the defective genomes are lower then those obtained with JFH1 full length RNA. This is likely due to a different efficiency in the envelope proteins incorporation into nascent viruses depending if they are provided in *cis,* i.e. by the genome itself, or in *trans* by the packaging cell line S6.1/E1E2:2a. The former type of expression most likely proceeds with a delayed kinetics compared to the *cis*-active one of the JFH-1 genome.

Overall the *trans*-complementation experiments confirm that the lacking of chimeric infectious particles is caused by an impaired assembly process consequent to the E2 ectodomain exchange. In fact, the JFH1/Con1E2 genome is at least equivalent to the defective construct JFH1ΔE1E2, in which the deletion of the envelope protein coding region makes the viral RNA unable to induce the infectious viral particles production. Therefore, these data further confirm that the E2 protein genotype 1b-derived does not correctly interact with the other structural proteins from genotype 2a to leads to a functional assembly process.

4.11 In summary.

- Two HCV 2a/1b inter-genotypic chimeras have been designed exploiting a new strategy. Both JFH/Con1E1E2 and JFH/Con1E2 chimeric constructs are able to replicate at the same level of the JFH1 wild type genome in the S6.1 hepatoma cell line.
- None of the JFH/Con1E1E2 and JFH/Con1E2 genomes allow the production of infectious viral particles, suggesting that determinants within the structural E1 and in particular E2 protein govern the kinetics and efficiency of virus assembly/release/infection.
- E1 genotype JFH1-derived can correctly interact with the E2 Con1-derived to form E1E2 heterodimers. Wild type and chimeric heterodimers undergo the same maturation process as demonstrate by the pulse-chase and Endo-H treatment experiments. Nevertheless the folding kinetics and the DTT-resistance experiments show a delay in the chimeric precursor processing and heterodimers assemble.
- Chimeric E1E2 heterodimers are potentially available for their incorporation into the viral envelope since they are not targeted for the proteasomal pathway.
- Confocal microscopy analysis show that most of chimeric E1E2 localized in the ER or ER-derived compartment. No co-localization with the capside protein is clearly evident. Core is mainly distributed on the lipid droplets surface, but, in contrast with the wild type context, in S6.1/Con1E2 cells LDs core-coated accumulate on the cell periphery. The co-localization of E1E2 with the non structural protein NS5a is clear in correspondence of dot-like structures that are only rarely observed in the S6.1/JFH1 cells.
- Cell-associated infectivity assay together with the sedimentation equilibrium gradient demonstrate that only S6.1/JFH1 cells contain intracellular infectious particles. By contrast, in S6.1/Con1E2 cells we do not recover any infectious particles. *Trans*-complementation experiments finally confirm that the JFH1/Con1E2 construct is equivalent to an assembly-defective HCV genome as demonstrate by the possibility to rescue the infectious particles production by providing the E1E2 structural proteins in *trans*. Importantly, *trans*-complementation is possible solely when the packaging cell provides autologous envelope proteins.

Results are schematically reported in figure 30.

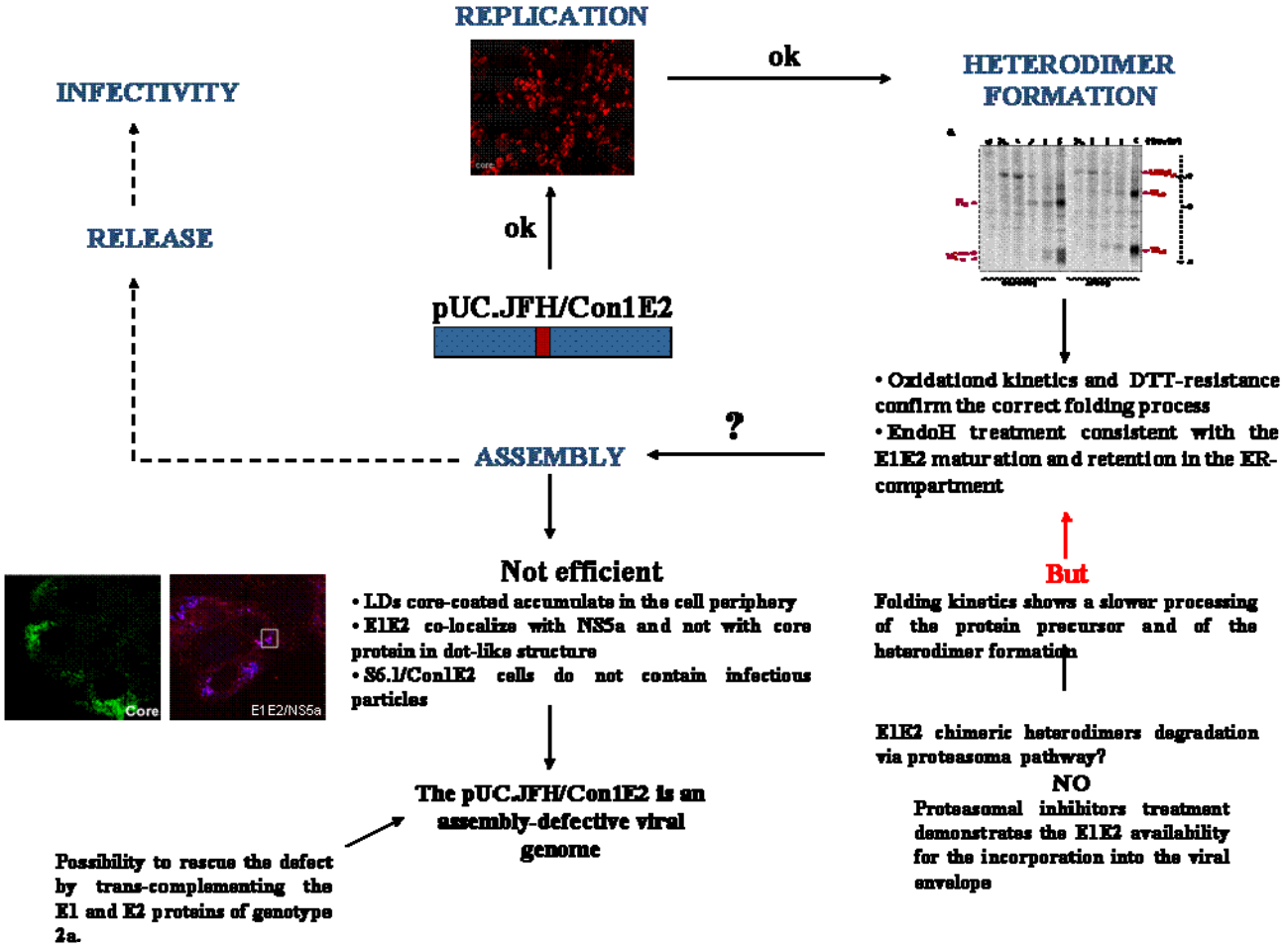


Figure 30. Schematically summary of the results.

§ 5. DISCUSSION

The recent development of the full HCV infectious system (HCVcc) by Wakita and colleagues represents a big breakthrough in the HCV research since it allows the complete life cycle of HCV to be studied for the first time and the roles of proteins not required for the viral RNA replication to be assigned (10). Despite the importance of such improvement, the system is limited by the dependence on two structural gene sequences, JFH1 and J6, both derived from the genotype 2a. Therefore, comparative studies, about the impact of variability in the structural genes on neutralization in an authentic infection system, or evaluation of antiviral compounds against all genotypes, cannot be performed on a broad scale (49,126). Hepatitis C virus is in fact present in six main genotypes and a larger number of subtypes. Among these, genotypes 1 and 2 have a worldwide distribution and are known to be associated with different clinical profiles and therapeutic responses. Such marked genetic heterogeneity, and the consequent differences in clinical features, are likely a result of viral characteristics. Therefore, in order to study the molecular mechanism underlying this variability, the scope of the genotype 2a-based cell culture system was extended by the construction of intra and inter-genotypic chimeras. Those commonly reported consist on the 3'-half genome of JFH1 and the 5'-moiety, including part of NS2, taken from genotype 1a, 1b, 3a and 4a. While the construction of the intra-genotypic chimera was highly successful, initial reports indicated that the virus yield obtained using inter-genotypic chimeras was dramatically hampered, suggesting that determinants within the structural proteins govern kinetics and efficiency of virus assembly and release (48, 49). Currently, the inefficiency of a cell culture system to support the production of all the HCV genotypes still remains a major obstacle to study different HCV genotypes and develop antiviral drugs that are effective against all genotypes.

In the present study we describe two novel inter-genotypic HCV chimeras based on the JFH1 strain, in which only the ectodomain of E1 and/or E2 envelope proteins is substituted, from JFH1 to Con1 strain (genotype 1b). The entire structural region is maintained of the infectious isolate, with the exception of the soluble portion of the envelope proteins, allowing comparative analysis of the impact of E1 and E2 on virus morphogenesis.

As expected, E1 and E2 envelope proteins are not involved in the HCV genome replication process since both chimeric constructs, JFH/Con1E1E2 and JFH1/Con1E2, as well as the defective genome JFH1ΔE1E2 (without the E1 and E2 coding sequence), are able to

efficiently replicate in S6.1 hepatoma cells. This first observation is in agreement with the common role assigned to E1 and E2 proteins. Indeed, it is amply reported that they associate in non covalent bound E1E2 heterodimers, assumed to be the viral spikes, and thus crucial for the HCV entry into the target cell (25, 78). Conversely, it is quite surprisingly that none of the chimeric HCV genomes allow the production of infectious viral particles. Although the precise mechanism is still unknown, the E1E2 formation was recently discovered as a prerequisite for the viral particle formation (10). Here we show that the heterodimers association is necessary but not a sufficient condition for the virus production. We in fact demonstrate that following the E1E2 heterodimerization, further interactions, involving the E2 ectodomain and the remaining structural proteins, are required to lead to an efficient viral particle assembly.

In order to demonstrate to what extent E2 ectodomain influences the assembly process in the HCV morphogenesis, we focused our attention on the JFH1/Con1E2 chimeric construct, in which only this portion is swapped. Overall our results indicate the need for homologous (same genotype) structural proteins sequence for efficient infectious particle production. This in turn suggests that E2 ectodomain is likely to interact with one or more of the viral structural proteins during virus assembly and that significant incompatibility exist in the E2 sequences of JFH1 and Con1 that prevent efficient interaction of Con1 E2 with the JFH1 structural proteins (core, E1, p7 and the non structural NS2).

The lack of infectious chimeric particles in the medium of S6.1 transfected cells was first attributed to a non functional interaction between genotype 2a E1 and genotype 1b E2, since it is the first steps that might be hampered by the E2 ectodomain exchange. The formation of chimeric E1E2 heterodimers was therefore evaluated, and the results demonstrate that E1 genotype 2a-derived can correctly associate with the ectodomain of Con1-E2 protein (to our knowledge, this is the first report showing the formation of chimeric E1E2 heterodimers). Nevertheless, even if the chimeric E1E2 complex undergo the same maturation process of wild type heterodimers, the folding kinetics analysis shows a delay in the protein precursor processing and E1E2 association. The possibility that E1 and E2 glycoproteins are not available for their incorporation in the nascent chimeric particles has been excluded by experiments of inhibition of the proteasomal pathway. Notwithstanding, it remains conceivable that this loss of folding/assembly efficiency in part explains why infectious particles are not produced in our system. It is simply a speculation, but supported by the fact that in the case of the intra-genotypic chimera Jc1, the most efficient in terms of virus

production, it was observed an enhanced protein precursor processing (49). However, the presence of chimeric heterodimers prompted us to evaluate the subsequent steps in the viral morphogenesis, in particular looking at the assembly process.

Confocal microscopy analysis firstly suggested that in S6.1/Con1E2 cells the chimeric particle assembly is in some way affected by the E2 ectodomain exchange. In fact, although the subcellular localization of core, E1E2 and NS5a proteins mainly display the same pattern in S6.1/JFH1 and S6.1/Con1E2 cells, we note an accumulation of lipid droplets (LDs) coated by the capsid protein in almost the cells transfected with the chimeric genome. Since LDs have been recently discovered as the site of HCV assembly (77), it might be possible that the accumulation of LDs-core coated on the S6.1/Con1E2 cell periphery causes a delay in the assembly/release processes, that are thus not enough efficient to allow the release of chimeric particles. This idea is also supported by the co-presence of viral structural and non structural proteins in dense pointed structures, noted as dots, in the chimeric RNA transfected cells. Presumably these structures represent the sites in which the viral proteins are stored instead of released as mature particles. In the replicon system, the co-localization of viral structural and non structural proteins in such structures was commonly notable. In that context it was suggested that dots represented HCV pre-budding areas where all the viral proteins and RNA accumulated on ER-derived membranes. So far, our results argue against this conclusion since we do not detect any similar co-localization of E1E2, core and NS5a proteins into virus-producing cells. As consequence, reminding that the replicon system does not allow the production of infectious viral particles, as well as the S6.1/Con1E2 cells, we can here hypothesize that the accumulation of all the viral proteins in dots is the results of an inefficient assembly process rather then a pre-budding area. We could also speculate that such accumulations are partially consequent to the delayed processing of the chimeric precursor that, as explained before, we noted in the folding analysis.

It is worth mentioning that the production of extracellular infectious virus is preceded by the accumulation of intracellular infectious particles, that reach the final configuration along their egress (156, 157). As consequence, if E2 has a function solely in the late stage of virus production, namely from the budding to the release process, infectious particles could form inside the cells and would be liberated from S6.1/Con1E2 cells by freeze-thaw. As we do not detect such immature infectious viral precursor, we conclude that E2 likely acts early in morphogenesis, namely during the particle assembly. On the other hand, we cannot completely exclude that the chimeric particle can assemble, but that, once produced, it is unable to infect the target cell. It worth noting that the occurrence of noninfectious particles is

a known phenomenon for many viruses, and interestingly, HCV noninfectious particles are recently found in samples from chronically infected patients (168). Given this observation, it is conceivable that in the chimeric context the ratio of infectious to noninfectious particles is so low to render impossible the detection of JFH1/Con1E2 particles from the cell milieu. Although it remains an opened possibility, the fact that we did not detect any infectious chimeric particle either in the concentrated supernatant or in the cell lysate of S6.1/Con1E2 cells argue for an hampered assembly process rather than for the production of noninfectious particles.

As deduced from the data presented, an interplay exist between the E2 ectodomain and the other viral structural proteins that depends on the genotypic sequence. To corroborate this hypothesis, we took advantage from a common feature of plus-strand RNA viruses, including HCV. They in fact show a sort of "flexibility" that allows the possibility to rescue a viral function essential for autonomous assembly and release by expression of this function in *trans* (161, 162). For HCV in particular, it is known that the entire structural region, from core to the N-term of NS2, can be *trans*-complemented, whilst all viral proteins involved in RNA replication, with the exception of NS5A, cannot be provided in *trans* (165, 166). As consequence, assuming that i) JFH1/Con1E2 construct is defective for same assembly functions and that ii) the defect clearly depends on the E2 ectodomain exchange; such defect could be rescued by providing the genotype 2a E2 ectodomain, namely from the same isolate as the remaining structural region.

As expected, we found infectious particles in the JFH1/Con1E2-transfected cells following *trans*-complementation of the genotype 2a E2 protein. Such infectious particles are most likely decorated with the wild type E1E2 heterodimer, meaning that they are not inter-genotypic chimeras. Indeed, we suppose that the formation of genotype 2a E1E2 complex leads to a functional assembly of nascent particles, while the association between Con1 E2-derived with E1 from JFH1 induces an abortive assembly process. Unfortunately, the lacking of genotype-specific anti-E2 antibodies does not permit to distinguish between wild type and chimeric HCV particles and thus to completely exclude the presence of inter-genotypic chimeras in the *trans*-complemented cell supernatant. However, our hypothesis is supported by two evidences. First, the *trans*-complementation is possible solely when the autologous envelope protein is provided. Second, the HCVcc title reached in the S6.1/E1E2:2a supernatant is approximately 10 fold higher than that obtained from S6.1/E1E2:1b. Bartenschlager and colleagues recently described a similar *trans*-complementation system in which a ΔE1E2 replicon was rescued by the JFH1 helper virus (166). Interesting, they

observed a very low efficiency in infectious virus production, explained in terms of competition between the defective RNA (replicon) and the helper virus (full length JFH1). More in detail, the two RNA species compete for limiting cellular factors essential for genome amplification, causing a decreased helper replication. This means lack of sufficient quantities of structural proteins for the following assembly step and therefore reduced virus production. Similarly, in our system it might be possible that the envelope proteins expressed by the packaging cells compete with those encoded by the JFH1 genome for the cellular factors involved in the viral assembly process and/or for the incorporation into the viral envelope. Furthermore, about the S6.1/E1E2:1b cell line, the envelope proteins that displaces those encoded by the viral genome derive from a different genotype, namely 1b. Assuming as true our hypothesis of genotypic incompatibility, it might be possible that the interaction between genotype 1b E1E2 and all other structural proteins genotype 2a-derived leads to an abortive assembly and thus to the strong decrease in virus production we detected.
Finally, virus title reached with the JFH1/Con1E2 defective genome complemented in *trans* is lower then that obtained with JFH1 wild type RNA. This observation is likely due to a different efficiency in the envelope proteins incorporation into nascent viruses. In the JFH1 case, in fact, the E1E2 heterodimers are provided firstly in *cis* by the genome itself, and secondly in *trans*. Instead, in the case of the chimeric construct, the functional E1E2 heterodimers are expressed only in *trans*. This kind of expression most likely proceeds with a delayed kinetics compared to the *cis*-active one, allowing a minor efficiency in terms of virus production.

In addition to the results here presented, our hypothesis is in agreement with several lines of evidence. Firstly, all the chimeric JFH1-based viruses successfully obtained contain the entire genotype 2a structural region, from core to part of NS2 protein, overcoming the incompatibility from structural proteins derived from different genotypes. Furthermore, it is worth mentioning that a genotypic incompatibility has been already observed for other HCV proteins, namely p7 and NS2 (48, 50, 125, 169). In particular, it was demonstrated that both p7 and NS2 proteins are essential for the infectious particles assembly and release, and that their functions most likely imply genotypic-specific interactions with the other structural proteins. Importantly, Pietschmann and coworkers also suggested an interaction between E2 and NS2 that might be important in HCV morphogenesis. Our results, not only confirm this observation, but also extend it, since we deduct that such interaction involves a precise region of E2 protein, namely the soluble portion.

One interpretation is that the loss of efficiency in terms of chimeric heterodimer folding interferes with the incorporation of the envelope proteins into the nucleocapsid. As consequence, lacking the driving force represented by the heterodimer formation, core protein accumulates on the surface of LDs. LDs-core coated are then moved to the periphery of the cells, as we found in S6.1/JFH1Con1 cells, instead of proceed to the secretory pathway. However, since our data show that the chimeric E1E2 heterodimer at least can form, we lean towards an impaired interaction between the E2 ectodomain and core, p7 or NS2 protein. Based on the topology model of each protein (Figure 2), the E2 ectodomain could interact with the small polypeptide p7 and NS2, while the interaction with core is highly underprivileged (E2 ectodomain is oriented versus the ER lumen whilst core protein reside in the cytoplasm). The protein relative position into the ER membrane further argues for an interaction between E2 ectodomain and NS2 protein. In fact the N terminus of NS2 consists of three transmembrane domains connected by two random loops, the second of which is juxtaposed to the ectodomain of E2 protein, and thus available the hypothetical interaction with it (170).

In conclusion, whatever the exact mechanism is, our data provide evidence that the E2 ectodomain plays an additional role to the one as receptor binding. It might be in fact involved in the HCV assembly, presumably through a genotype-specific interaction with NS2 protein, which is known to play a major role in the assembly of nascent viral particles. However, it remains to be determined the precise molecular mechanism by which E2 contributes to the process.

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