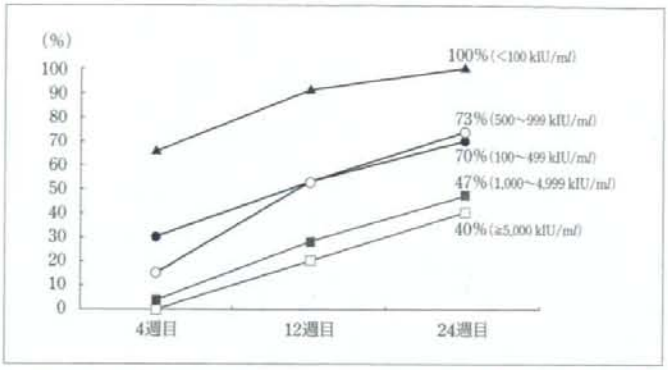

図5　1型・高ウイルス高齢者の治療前のウイルス量別経時的HCV RNA陰性化率

Ⅵ. 1型・高ウイルス症例の減量投与，通常投与別の経時的HCV RNA陰性化率

年齢別（65歳未満，65歳以上）の減量投与群と通常投与群の経時的HCV RNAの陰性化率は**図7**に示す．65歳未満では，12週目・24週目で減量投与群のHCV RNA陰性化率が通常投与群に比べ有意に低かった（$p < 0.01$），65歳以上群では12週目，24週目のHCV-RNA陰性化率に減量投与群と通常投与群間での差はみられなかった．

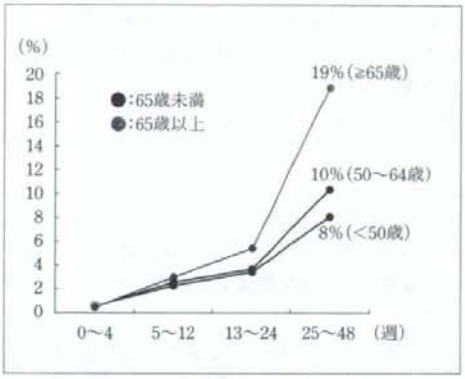

図6　年齢別経時的治療中止率

Ⅶ. 考　察

現在（2007年1月31日現在）のPeg-IFN/ribavirin治療はPeg-IFN α-2bとribavirinの併用治療のみであり，この治療における治療効果および副作用のデータが現在集積されている．今のところ高齢者への重篤な副作用はあまりみられていない．今後はPeg-IFN α-2aとribavirinによる併用治療が開始されるが，十分な治療経験がない．とくにPeg-IFN α-2aは単剤投与においても血小板減少や間質性肺炎などの重篤な副作用の報告がPEG-IFN α-2b/ribavirinより高率に認められているので，厳重な観察が必要である．血小板減少については必ず直前の検査が義務づけられているので遵守すべきである．また高齢者では治療後期に副作用が出やすいので，間質性肺炎などの副作用などについても十分な観察が必要である．高齢者は血小板減少や間質性肺炎などが出現した場合より重篤になりやすいので，注意を要する．欧米でも高齢者のC型慢性肝炎へのPeg-IFN/ribavirin治療とその効果における経済効果について検討があるが，軽症の高齢者のC型慢性肝炎へのPeg-IFN/ribavirinは控えるように述べている[4]．

高齢者では治療早期のHCV RNA陰性化（4週目，12週目）やHCV量の低下率が低いので，Peg-IFN/ribavirin治療の治療効果は低くなる．

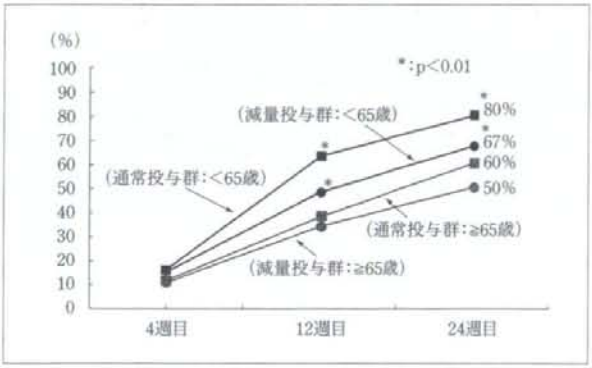

図7　1型・高ウイルス症例の投与群別経時的 HCV RNA 陰性化率

治療前のウイルス量が 1,000 KIU/ml 以上ではより低くなる．24 週後に HCV RNA が陰性化する症例はきわめて少なく，24 週後に副作用の出現も多くみられるので，治療中（とくに 12 週目の HCV RNA の陰性化またはウイルス量の減少）の経過をみながら，治療効果が低い場合や副作用の出現時は躊躇することなく中止すべきである．このため，治療前に患者へ十分な説明が必要である．中止後は，肝発癌予防目的で ALT 値や AFP 値の高値症例へは，IFN 単独少量治療も検討すべきである[5]．

血球減少による副作用で治療中に Peg-IFN を減量する場合は半量への減量となる．高齢者への治療の場合，約 20μg 程度の減量投与では治療中の HCV RNA の陰性化では差がみられなかった．治療開始時期から通常投与量より約 20μg 程度の減量投与が推奨される．このように高齢者へは，Peg-IFN α量の微調整なども必要となる．Peg-IFN α-2b では開始からの減量投与は容易であり，治療中主治医により 20μg 減量は可能であるが，Peg-IFN α-2a では 180μg または 90μg の固定量投与となる．減量投与では 90μg よりの開始となるので，治療中に血球減少がみられた場合は一時休薬となる．このため通常投与量の 60％以上を確保することが困難となり，減量投与の点からみれば微調整が必要な高齢者への減量投与開始や投与中の少量減量などができず不適かと思われる[6]．

ribavirin は高齢者では，投与量を体重のみで決定するのでなく，全身クリアランス（CL/F）などを用いて適用量を決定したり，通常投与量より 1 カプセル（200mg）の減量などを考慮すべきである[3]．投与中に貧血などの副作用の出現が軽い場合は，通常量への増量も可能である．

おわりに

C 型慢性肝炎への IFN 治療は，Peg-IFN/ribavirin により治療効果が向上した．また Peg-IFN の使用により発熱や全身倦怠感などの副作用が軽減化され高齢者の治療も行われているが，治療期間が 48 週間と長期間となったので中止例も増加した．高齢者（65 歳以上）では SVR 率は低く，24 週後の中止率が高いので，合併症（高血圧，糖尿病など）を有する患者，クレアチニンクリアランス（CCr）が低値の患者，体重が 40kg 以下の患者，貧血（Hb 値が 12.0g/dl 以下）の患者への積極的治療は控えるべきである．治療前のウイルス量が 1,000 KIU/ml 以上であれば，より治療効果が低下する．65 歳以上で Peg-IFN/ribavirin を希望する合併症を有しない患者へは，SVR 率が低いこと，副作用の出現率が高いこと，途中中止もありうることなどを十分に説明し，患者の承諾を得たうえで，治療を開始するべきである．高齢者で

は副作用が重篤になりやすいので，治療中は血小板減少や間質性肺炎などの重篤な副作用に十分考慮し，治療するべきである．

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Chapter 3

Assemble and Interact: Pleiotropic Functions of the HCV Core Protein

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ABSTRACT

While surrogate capsid assembly model systems are currently the best tools for studying HCV core assembly, bona fide HCV culture systems are being developed. The time will soon come when HCV culture systems and small animal models will be the norm, rather than the exception (see Chapters 12 and 16). It is now clear that HCV core protein interacts with many cellular proteins and signal transduction pathways, that HCV quasispecies influence biologic responses, and HCV proteins such as core can have different effects depending on whether the protein is encountered inside or outside the cell. The studies discussed herein have enhanced the understanding of HCV capsid assembly and the role(s) of HCV core and host cell interactions in the establishment of persistent infection and the pathogenesis of HCV liver disease. Continued studies of this nature will also provide a basis for the rational design of vaccines and novel therapeutics against HCV infection in humans.

INTRODUCTION

As covered elsewhere in this book, HCV infection is a serious global health problem, which accounts for billions of dollars in medical expenses in the US alone (Kim, 2002). Clinically, acute HCV infection is frequently anicteric and asymptomatic. The situation is compounded given the natural tendency for acute HCV infection to progress to chronic infection. Thus, more effective strategies to successfully cure patients of their infection are urgently needed. This chapter focuses on a key HCV molecule, the HCV core or nucleocapsid protein.

THE CORE OF THE PROBLEM

The HCV core protein has been reported to have many functions. With respect to the virus, the main function of the core protein is to form the capsid shell that will house and protect the HCV genomic RNA while the virus passes from one cell to another, or from one person to another. However, the HCV core protein also modulates many different host pathways by interacting with a variety of cellular factors. In the following sections, we will highlight important new developments in HCV capsid assembly and HCV core-host interactions.

THE ROLE OF HCV CORE IN CAPSID ASSEMBLY

WHAT IS A CAPSID?

A viral capsid is the protein shell that encapsidates and protects the viral genome. Viral capsids can be composed of one or more virus-encoded proteins. In the case of enveloped viruses, after assembling and encapsidating the genomic RNA, a viral capsid then facilitates virion formation by interacting with the viral envelope glycoproteins and budding. The budding process is sometimes, but not always, mediated by the viral capsid. For example, the capsid proteins of Ebola and HIV contain domains that regulate budding, while in the case of tick borne encephalitis (TBE) virus, it is the envelope glycoproteins that mediate budding. These events (capsid assembly, encapsidation, and budding) are typically referred to as late events in the viral life cycle. For HCV, as will be discussed below, many details of the late events in the HCV life cycle are unclear.

In the case of HCV, as is true for all members of the *Flaviviridae*, the core protein is the only viral protein present in the capsid. The final nucleocapsid contains genomic RNA, coated and protected by the capsid. HCV, being an enveloped virus, has a lipid envelope, containing the viral envelope glycoproteins as well as host membrane proteins, surrounding the nucleocapsid. The late events of the HCV life cycle, including capsid and virion assembly, are shown schematically in Fig. 1. In this section, we will focus on HCV core, its characteristics, what is known about its assembly into a bona fide HCV capsid, and the blocks to HCV capsid assembly that exist in mammalian cell culture systems.

PROPERTIES OF HCV CORE

The HCV genomic RNA is approximately 9.6 kilobases in length and encodes a single, large polyprotein of about 3000 amino acids (aa). The polyprotein is cleaved by viral and cellular proteases to generate at least 10 viral proteins (Suzuki et al., 1999). The core protein represents the first protein in the polyprotein, followed by two glycoproteins, E1 and E2. The immature form of HCV core contains 191 aa. These 191 aa have been separated into three general domains (McLauchlan, 2000). The first domain (domain I), encompassing aa 1 - ~122, is highly basic and very hydrophilic. This domain is thought to be responsible for binding RNA and mediating capsid assembly, and has been reported to interact with many cellular proteins. The second domain encompasses the majority of the C-terminus of HCV core. In contrast to domain I, domain II is hydrophobic. Thus, domain II mediates interactions with lipids and membrane proteins and is not present in capsid proteins of most other viruses in the *Flaviviridae*. The final domain (III), which is very hydrophobic and is predicted to form an alpha helix, is at the extreme C-terminus of the immature core protein, and corresponds to the signal sequence for E1 (aa 175 - 191). This domain is cleaved soon after core is translated and is absent from the

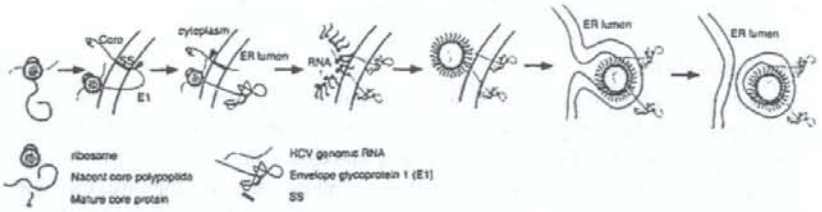

Fig. 1. Overview of capsid/virion assembly. Genomic RNA is translated by a host ribosome. HCV core is the first polypeptide encoded in the polyprotein. Just proximal to core is the membrane envelope glycoprotein E1. The signal sequence (SS) for E1 (distal to core) targets the polyprotein to the ER. Signal peptidase cleaves the immature form of core from the growing polypeptide. Signal peptide peptidase then cleaves the E1 SS releasing the mature form of core. Core then multimerizes and encapsidates HCV RNA at the cytoplasmic face of the ER. Capsids that are formed in the cytoplasm then interact with E1 and bud into the ER lumen. Enveloped virions are then released, presumably via the secretory pathway.

mature form of HCV core. Nevertheless, domain III appears to be very important in terms of HCV core stability, targeting, and function. Two major forms of core protein, corresponding to 21- and 23-kDa (p21 and p23), are generated *in vitro* and in cultured cells (Yasui et al., 1998), corresponding to the mature (signal cleaved) and immature (signal uncleaved) forms of the protein.

HCV CORE BIOGENESIS

Synthesis of HCV core in the same polyprotein as the HCV envelope proteins creates an interesting predicament in that core, the capsid protein, needs to be soluble and cytoplasmic, while the envelope glycoproteins are transmembrane and anchored into the host membrane. Therefore, like other flaviviruses, HCV has evolved an internal signal sequence for E1, the first envelope glycoprotein (referred to as E1 SS or domain III, as described above). The E1SS is encoded between HCV core and E1. Thus, after core is translated, the nascent polyprotein is targeted to the ER translocation channel by the E1 SS (Fig. 1). A host enzyme located in the ER, signal peptidase, cleaves just proximal to the E1 SS, releasing the immature form of core from the polypeptide (Hijikata et al., 1991; Santolini et al., 1994). A different endoplasmic reticulum (ER) enzyme, signal peptide peptidase (SPP), subsequently cleaves just before the E1 SS liberating the mature form of HCV core at the cytoplasmic face of the ER (McLauchlan, 2000; McLauchlan et al., 2002). SPP is a presenilin-type aspartic protease that catalyses intramembrane proteolysis of signal sequences and membrane proteins within the ER (Weihofen et al., 2002). Precise mutational analyses have shown that intramembrane cleavage by SPP is abolished when helix-breaking and -bending residues in the C-terminal signal sequence are replaced by basic residues. Furthermore, the signal sequence itself and three hydrophobic aa Leu-139, Val-140, and Leu-144 of the core protein

are required for SPP cleavage, although none of these residues are essential for cleavage at the core-E1 junction by signal peptidase, or for translocation of E1 into the ER (Okamoto et al., 2004). The exact cleavage site for producing mature core (p21) is still controversial, since Leu-179 (Hussy et al., 1996; McLauchlan et al., 2002), Leu-182 (Hussy et al., 1996), Ser-173 (Santolini et al., 1994), and Phe-177 (Okamoto et al., 2004) have all been reported as potential sites of cleavage. After being cleaved into the mature form at the ER, core can undergo a number of possible fates, including assembly into capsids, targeting to other organelles, and interaction with host proteins resulting in modulation of various cellular processes, as will be discussed in more detail below.

HCV CAPSID STRUCTURE AND PROPERTIES

The main role for HCV core in the viral life cycle is to form a nucleocapsid to protect the viral genome. Once cleaved from the polyprotein, the mature core protein presumably assembles into HCV capsids, most likely at the cytoplasmic face of the ER (Mizuno, 1995; Blanchard, 2002; Blanchard, 2003). Unfortunately, no cellular system robustly recapitulates late events in the viral life cycle, although there may be hope with the recent development of an infectious HCV system (see Chapter 16). For this reason, mechanistic details of this process are lacking. HCV replicon systems (see Chapter 11), first developed in 1999, represented a major breakthrough because they allowed replication of HCV RNA in mammalian cells (Blight et al., 2000; Lohmann et al., 1999). However, even when HCV core is synthesized to high levels, late events in the HCV life cycle do not occur in most replicon systems, as judged by electron microscopy (Pietschmann et al., 2002). Therefore a number of model systems have been developed to study the structure of HCV capsids and HCV capsid assembly.

Knowledge of HCV capsid appearance *in vivo* has come from examining particles in serum or in infected liver biopsies. Non-enveloped capsids have been observed in the cytoplasm of liver cells, while enveloped particles have been seen in the cisternae of the ER, as judged by transmission electron microscopy (TEM) (Bosman et al., 1998; Shimizu et al., 1996). The presence of capsids at or in the ER by TEM in numerous studies implicates the ER as the site of HCV capsid assembly (Blanchard, 2002; Maillard, 2001; Mizuno, 1995; Shimizu, 1996). More recently, a careful TEM analysis of HCV virions and non-enveloped nucleocapsids from serum of HCV infected patients was performed (Maillard et al., 2001). This study revealed that non-enveloped HCV nucleocapids can be found in significant quantities in serum. These capsids, as well as those obtained by detergent treatment of enveloped virions, are spherical but heterogeneous in size, with a bimodal distribution of capsid diameters corresponding to ~38 - 43 nm and ~54 – 62 nm. It remains unclear what governs capsid size and whether the size differences are biologically significant. Unfortunately, unlike with other flaviviruses, visualization of HCV virions or capsids at atomic resolution has not yet been achieved.

Biochemical analyses have determined that enveloped HCV virions have a density 1.08 to 1.16 g/ml (Bradley et al., 1991; Kaito et al., 1994; Kanto et al., 1994; Miyamoto et al., 1992). Similar studies on non-enveloped HCV capsids have yielded conflicting results. HCV capsids with envelopes removed using detergent have densities of approximately 1.25 g/ml (Kaito et al., 1994; Kanto et al., 1994; Miyamoto et al., 1992) or 1.32 - 1.34 g/ml (Maillard et al., 2001; Shindo et al., 1994), with the electron microscopic appearance of capsids of both densities being otherwise very similar (Maillard et al., 2001). An explanation has been proposed to explain the finding of two different buoyant densities: capsids that band at the lower density (~1.25 g/ml) appear to be associated with fragments of membranes, while those banding at the higher density (~1.32 g/ml) appear to be free of membranes (Maillard et al., 2001). However, this hypothesis remains to be tested. Additionally, it appears that both the immature and mature form of core can assemble and be incorporated into capsids, although, not surprisingly, the mature form is the main species in virions (Yasui et al., 1998).

MODEL SYSTEMS FOR CAPSID ASSEMBLY

While electron micrographs of infected serum and hepatocytes give a literal snapshot of what is occurring *in vivo*, at the other extreme are minimal systems that can be used as surrogates for understanding the process of capsid assembly. In these minimal systems, purified recombinant core is incubated with RNA in the absence of other cellular factors. In the presence of RNAs containing a high degree of secondary structure (*e.g.* tRNA or the HCV 5' untranslated region), C-terminal truncation mutants were found to assemble into regularly shaped capsids that resemble HCV capsids from infected individuals (Kunkel et al., 2001). Similar results were obtained by expressing truncated core constructs in *E. coli* (Lorenzo et al., 2001). In contrast, full-length (wild-type) recombinant core assembles into particles with irregular shapes (Kunkel et al., 2001), raising the possibility that host factors or co-ordination of assembly with core synthesis may be required to assemble proper capsids from full-length HCV core. These studies also demonstrated that domain I is sufficient for core assembly. Furthermore, removal of domain II appeared to facilitate capsid assembly, allowing the purified core protein to assemble into more regular-shaped capsids. Together, these systems show that HCV core contains all of the information to assemble into capsid-like structures (in the presence of RNA) (Kunkel and Watowich, 2002). However, because of the minimalist nature of these systems, other systems will be required to determine the mechanism by which wild-type core assembles into capsids within cells, where assembly is likely to be influenced by other events including *de novo* core translation, host factors, and targeting of core to specific organelles.

A cell-free system, a virtual hybrid between *in vitro* systems and cellular systems, has recently been devèloped to study HCV assembly. In these systems, cellular

extracts are used to reconstitute and link translation to post-translational events, such as capsid assembly. Thus, these systems combine the benefits of being able to manipulate the assembly reaction in a test tube while maintaining a cellular context. Cell-free systems faithfully reconstituted HCV capsid assembly when full-length core, either the immature or mature form, was expressed *de novo* in either wheat germ extracts or rabbit reticulocyte lysate (Klein et al., 2004). Moreover, TEM analysis revealed that capsids formed from full-length core in the cell-free system were morphologically very similar to capsids produced in infected patient serum, both in size and structure (Klein et al., 2004), thereby validating the cell-free system for mechanistic and mutational studies. In addition, cell-free HCV capsid assembly is very efficient, with over 60% of newly-synthesized core polypeptides assembling into immature capsids (Klein et al., 2004).

Some cellular systems have also been used to study capsid assembly. When over-expressed in insect cells, core assembles into 30 – 60 nm particles at the ER (Baumert et al., 1998; Baumert et al., 1999; Maillard et al., 2001) that closely resemble capsids produced *in vivo*. When the envelope proteins E1 and E2 are also expressed, capsids can be seen budding into the ER and cytoplasmic vesicles (Baumert et al., 1998); however, unfortunately no virus-like particles are released (Baumert et al., 1998; Baumert et al., 1999; Maillard et al., 2001). Therefore, this system recapitulates much of what is seen in hepatocytes and supports the notion that capsids assemble at the ER, although virion production is still blocked at a later step in the viral life cycle. Nucleocapsid-like particles have also been observed upon expression of HCV core in yeast (Majeau, 2004).

In contrast to these model systems, in general, mammalian cell lines do not support HCV capsid assembly. There have been isolated reports of capsids being produced in cultured mammalian cells (Blanchard, 2002; Ezelle, 20026; Mizuno, 1995); however, the extent of HCV assembly in these cells is unclear. As noted above, even in replicon cells with high levels of HCV core synthesis, HCV assembly is not supported (Pietschmann et al., 2002; Bukh et al., 2002), similar to most cultured mammalian cells (Hope and McLauchlan, 2000). These findings suggest that mammalian cell lines either lack a necessary cellular factor(s) or contain inhibitory factor(s) that cause the majority of core to be targeted away from the ER, as discussed below. This alternate localization of core (Pietschmann et al., 2002), possibly in conjunction with other negative regulatory influences, correlates with failure to assemble HCV capsids or virions in cultured cell lines. Consistent with this, when crude hepatocyte extracts containing membrane-bound organelles are added to the highly permissive cell-free capsid assembly system, efficiency of assembly is reduced (Klein et al., 2004).

HCV ASSEMBLY: REQUIREMENTS AND MECHANISTIC ANALYSIS

Although an ideal model system for HCV capsid assembly does not exist, much has been elucidated about the requirements and process of capsid assembly from the various systems mentioned above. *In vitro* studies have been useful for structural analyses, having revealed that HCV core undergoes a conformational change upon assembling into capsid like structures (Kunkel and Watowich, 2002). Meanwhile, the cell-free system for HCV capsid assembly has allowed the process of core assembly to be analyzed mechanistically (Klein et al., 2005; Klein et al., 2004). Pulse chase analyses in the cell-free system have revealed that assembly occurs very quickly, with very little delay between completion of translation and completion of assembly (Klein et al., 2004). Additionally, capsid assembly was not highly dependent on protein concentration or membranes, unlike many other viruses. When HCV core expression was decreased 200 fold, only a 2.3 fold decrease in amount of assembly was observed (Klein et al., 2004). Both the speed of HCV capsid assembly and its relative concentration independence differ from what has been seen with assembly of other types of viral capsids (*i.e.* lentiviruses and hepadnaviruses) in analogous cell-free systems (Lingappa et al., 1997; Lingappa et al., 1994; Lingappa et al., 2005). Thus, the basic assembly mechanism of HCV capsids may differ from that of many other viral capsids that assemble at the cytoplasmic face of membranes. Assembly may occur in microenvironments, for example on polysomes that contain a high concentration of core protein translating off a single mRNA. The presence of high local concentrations of newly-synthesized HCV core polypeptides, possibly in conjunction with cellular factors, could promote rapid and efficient HCV assembly in permissive cellular extracts, although future studies will be required to test this hypothesis.

Model systems for HCV assembly have also been used to define regions of HCV core that are important for HCV capsid assembly. Studies using recombinant HCV core truncation mutants have revealed that domains II and III are dispensable for assembly (Kunkel et al., 2001; Lorenzo et al., 2001). In fact, truncation mutants lacking these domains assemble better than full-length constructs *in vitro* (Kunkel et al., 2001). Systematic analysis of HCV capsid truncation, deletion, and point mutants in the cell-free HCV capsid assembly system have confirmed that the C-terminus is dispensable for assembly, and also demonstrated that the N-terminal 68 aa are required for capsid assembly (Klein et al., 2005; Klein et al., 2004). This region of HCV core contains numerous basic residues organized into two clusters. Removing either cluster of basic residues, or mutating as few as 4 basic residues to alanines in either cluster, significantly reduces assembly of capsids in wheat germ extracts (Klein et al., 2005). Conversely, when neutral aa were deleted from the same region, no effect on cell-free HCV capsid assembly was observed, suggesting that the critical determinant for assembly is the overall basic charge of the N-terminus. Likewise, deletions or mutations in other regions of HCV core

did not affect assembly (Klein et al., 2005). While these studies indicate that basic residues in the N-terminus are critical for assembly, it remains unclear whether the N-terminal 68 residues are sufficient for assembly. It should be noted that other domains of core are clearly important for interaction of core with cellular factors and for trafficking of HCV core to distinct cellular locations, as discussed below. Domains involved in core trafficking and cellular protein interactions are likely to influence or even regulate HCV capsid assembly in intact cells, but these events have not been studied together due to lack of cell lines that recapitulate HCV capsid assembly in a robust manner.

RNA BINDING AND ENCAPSIDATION BY CORE

Besides multimerization to form the capsid, the other major function performed by core during assembly is RNA encapsidation. Many viruses will encapsidate non-specific cellular RNA if viral genomic RNA is not present. Moreover, many viruses use RNA as a scaffold for assembly, and/or to nucleate the assembly process. HCV core appears to act similarly. Domain I of HCV core is extremely hydrophilic, largely due to the many basic residues clustered in this region. Basic residues are frequently involved in nucleic acid binding because the positive charge can interact with the negative phosphate backbone of nucleic acids. Indeed, HCV core binds RNA (Fan et al., 1999; Santolini et al., 1994; Shimoike et al., 1999) and this association is dependent on the basic N-terminus (Santolini et al., 1994). Consistent with this, and supporting the notion that RNA acts as a scaffold for assembly, RNA was required for *in vitro* assembly (Kunkel et al., 2001). Additionally HCV core has RNA chaperone capabilities, suggesting that core may also help restructure RNA, which may have implications for specific genomic encapsidation (Cristofari et al., 2004).

While the notion that HCV core binds to RNA is well established, it is unclear whether HCV core preferentially binds HCV genomic RNA over cellular RNAs. Core has been shown to bind ribosomal RNA (Santolini et al., 1994), tRNA (Kunkel et al., 2001), and HCV genomic RNA (Cristofari et al., 2004; Fan et al., 1999; Kunkel et al., 2001; Shimoike et al., 1999). It appears that the only requirement is that the RNA should contain significant amounts of secondary structure. When recombinant core was incubated with denatured, or unstructured, RNA, it failed to assemble into capsids suggesting that it could not interact with unstructured RNA. Conversely, when highly structured tRNA or the HCV UTR was used, core assembly was promoted (Kunkel et al., 2001).

If core binds to any structured RNA, how does genomic RNA get specifically packaged? Many viral capsid proteins have a higher affinity for specific structures in their cognate genomic RNA, allowing them to preferentially bind the proper RNA. It is unclear whether HCV core has higher affinity for HCV genomic RNA. One

study demonstrated that the HCV core protein binds specifically to a radiolabeled probe containing the 5' UTR of the genomic RNA. This interaction was abolished by excess unlabeled probe, but not by unlabeled, non-specific RNA, suggesting that core preferentially binds genomic RNA (Fan et al., 1999). This could explain how genomic RNA gets selectively packaged into virions over other cellular RNAs. Conversely, Santolini et al. reported that core fusion proteins bind equally well to HCV genomic RNA and heterologous RNA, suggesting that HCV core does not have enough specificity in its binding to promote genomic RNA encapsidation (Santolini et al., 1994). If HCV core does not specifically bind genomic RNA, then some other mechanism must exist to promote encapsidation of the genome. One possibility is that assembly occurs in microenvironments that contain only a single species of mRNA (*i.e.* HCV genomic RNA), as discussed above. Unfortunately, RNA encapsidation has not yet been analyzed in conjunction with capsid assembly in any system, so it remains unclear exactly what RNAs are encapsidated and how HCV core selects RNA for encapsidation during synthesis and assembly.

CAPSID ASSEMBLY: LIGHT AT THE END OF THE TUNNEL?

As mentioned, for the most part cell culture systems do not support virion production, or even capsid assembly. However, isolated reports have identified infectious virus propagated in special cell culture systems and at low levels. One group infected hepatocytes that were cultured in a radial-flow bioreactor and found that HCV is able to replicate to very low titers (Aizaki et al., 2003). Additionally, at the 11th International Meeting on Hepatitis C and Related Viruses in Heidelberg in October 2004, there were three reports of very low titer infectious virus particle formation in cells transfected with HCV genomic RNA (Murakami et al., 2004b; Pietschmann et al., 2004; Wakita et al., 2004). These initial studies have been confirmed by independent groups (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005) (See Chapter 16). Use of 3-dimensional cultures (Murakami et al., 2004b) or transfection with the JFH strain (Pietschmann et al., 2004; Wakita et al., 2004) resulted in production of infectious particles. In one case infection was receptor mediated, as antibodies to the putative HCV receptor, CD81, blocked infection (Pietschmann et al., 2004). Unfortunately, in all cases, levels of virus production were too low to result in measurable titers or any ultrastructural evidence of virus formation (Aizaki et al., 2003; Murakami et al., 2004b; Pietschmann et al., 2004; Wakita et al., 2004). Most recently, Heller et al. also isolated virus like particles from cell culture after transfecting RNA corresponding to the exact genomic sequence (Heller et al., 2005). This study also showed morphologic data, which suggests that the particles produced are, indeed, virions. Thus, a new wave of data shows evidence that HCV can assemble into capsids and, subsequently, into virions in mammalian cell culture. However, it is unclear whether assembly in these systems is just a stochastic event, what amount of virus or virus like particles are produced, how assembly is regulated in these systems, or what cellular subpopulation, if

any, is producing the limited number of viruses. By using a combination of all of the current model systems, as well as, newly described cellular systems, new insights into the mechanism by which HCV assembly is regulated in cells should be elucidated. This could allow for enhancements of current cell culture systems that could in turn facilitate the study of late events of the HCV life cycle in cells.

SUB-CELLULAR TARGETING OF HCV CORE

IF CORE DOES NOT ASSEMBLE, WHERE DOES IT GO?

While much information has been elucidated from the various model systems outlined above, surprisingly, mammalian cell lines including human liver-derived cell lines fail to produce quantifiable levels of HCV capsids, or virions. For reasons that remain unclear, in these cell lines HCV core polypeptides can be directed to alternate cellular locations upon release from the nascent HCV polyprotein. The fact that core assembles efficiently in infected humans and chimpanzees, but not in intact cultured cell-lines, suggests that HCV assembly can be negatively regulated. Trafficking of core to alternate sites is one possible mechanism for negative regulation of capsid assembly.

One approach to studying the subcellular localization of core involves immunostaining liver biopsy specimens from infected patients. This has revealed that the core protein predominantly localizes within the cytoplasm of infected hepatocytes, and often shows a punctate granular distribution within cells (Gonzalez-Peralta et al., 1994; Gowans, 2000; Sansonno et al., 2004; Yap et al., 1994). However, when the core protein alone or the entire viral polyprotein are expressed in mammalian cells, the majority of core has been observed at the ER membrane (Lo et al., 1995), on the surface of lipid droplets (Barba et al., 1997; Hope et al., 2002; McLauchlan et al., 2002; Pietschmann et al., 2002; Shi et al., 2002), and on mitochondrial and mitochondrial-associated membranes (Schwer et al., 2004; Suzuki et al., 2005). In addition, core is also known to target to the nucleus (Lo et al., 1995; Matsuura et al., 1994; Moriishi et al., 2003; Moriya et al., 1998; Yasui et al., 1998), where it can be a substrate for proteasomal degradation.

What governs whether core stays at the ER to assemble or traffics to other areas of the cell is not completely understood. Nevertheless, it is clear that such regulation exists and is quite complex. The finding that core targets to lipid droplets and mitochondria, but E1 and E2 do not (Pietschmann et al., 2002; Schwer et al., 2004), raises the possibility that targeting of core away from the ER occurs at a very early time after core synthesis, before core has had time to interact with the envelope glycoproteins. Furthermore, a number of studies suggest that aa in domains II and III direct the post-translational trafficking of core, although agreement is lacking as to which residues are critical. Okamoto et al. has shown that not only the C-

terminal signal sequence but also aa 128-151 are required for ER retention of the core protein by using a series of N-terminally truncated core protein constructs (Okamoto et al., 2004). Suzuki et al. has reported that a region of aa 112-152 mediates association of the core protein with the ER in the absence of the C-terminal signal sequence (Suzuki et al., 2005). McLauchlan et al. have proposed that a large part of the core protein remains within the cytoplasmic leaflet of the ER membrane after SPP cleavage (McLauchlan et al., 2002). Upon intramembrane cleavage of the transmembrane signal peptide, the processed core protein may traffic along the lipid bilayer from the site of biosynthesis to zones at the ER, where lipid droplets are produced (McLauchlan et al., 2002).

Deletion analyses have revealed that domain II (in particular residues between aa 125 - 144) plays a critical role in targeting core to lipid droplets (Hope and McLauchlan, 2000; Hope et al., 2002). Notably, no domain homologous to domain II is present in the core proteins of related pesti- and flavi-viruses. In contrast, the core protein of GB Virus B, from the GB virus group within the *Flaviviridae*, does contain a homologous domain that also appears to mediate targeting to lipid droplets (Hope et al., 2002). Domain II contains two closely spaced prolines that form a proline knot and appear to be required for targeting core to lipid droplets. The region containing this proline knot can be replaced with a proline knot domain from lipid-associated plant proteins called oleosins (Hope et al., 2002), with preservation of lipid targeting. Lipid targeting of HCV core can also be altered by mutations that affect SPP cleavage. Helix-breaking point mutations within the signal sequence (domain III) eliminate SPP cleavage, but also eliminate trafficking to lipid droplets, leaving core protein on the cytoplasmic face of the ER (McLauchlan et al., 2002; Okamoto et al., 2004). While these alternate pathways for core trafficking are beginning to be defined, the downstream consequences of different post-translational trafficking pathways on core function have not yet been explored. This is in part because using core mutants to study these cellular fates has proven to be relatively tricky. Studies have shown that C-terminally truncated versions of the core protein are localized exclusively to the nucleus (Suzuki et al., 1995). A fraction of the core protein was detected in the nucleus even when full-length HCV core gene was expressed, suggesting that the mature core protein also localizes to the nucleus (Moriya et al., 1997a; Yasui et al., 1998). The N-terminal domain of the core protein contains three stretches of arginine- and lysine- rich sequences. These basic-residue stretches function as nuclear localization signals (NLSs) for translocation of the core protein to the nucleus (Chang et al., 1994; Suzuki et al., 1995). Each of the NLS motifs of the core protein is able to bind importin-α. At least two of them are required for efficient nuclear distribution of the core protein in cells, suggesting that they constitute a bipartite NLS (Suzuki et al., 2005).

The major fate of core that is targeted to the nucleus is degradation by the nuclear proteasome (Hope et al., 2002; McLauchlan et al., 2002; Moriishi et al., 2003). Whether this is a cellular protein "quality control" mechanism, a normal pathway for core, or a pathway with other functional consequences is unclear. Nevertheless, it appears that constructs encoding mutations in the C terminus of core are less stable in cells than is wild-type core (Moriishi et al., 2003). McLauchlan and colleagues have proposed that the ability of domain II to mediate attachment of core to lipid droplets also protects core from degradation. Furthermore, they demonstrated that core constructs encoding a deletion in domain II are protected from degradation when they also encode a mutation that blocks cleavage of domain III by SPP (McLauchlan et al., 2002). Related to this observation, the mature form of core is much less stable when expressed as such than when expressed as the immature form of core which transiently contains domain III (E1 SS) before undergoing processing (Suzuki et al., 1995; Suzuki et al., 1999; Suzuki et al., 2001). Therefore, while the final product is the same, the presence of domain III during core biogenesis greatly influences core stability. Domain III, while not present in the mature wild-type core protein, plays a complex and important role in core stability. Like domain II, domain III and its cleavage may be involved in linking HCV core to cellular pathways that target it to other regions of the cell and protect it from degradation. Interestingly, although truncations and deletions in domain II lead to rapid degradation in mammalian cells, this phenomenon is not seen in cell-free capsid assembly systems, even when mammalian cell extracts are used (Klein et al., 2005; Klein et al., 2004). This is likely due to the absence of the nucleus in these systems, which prevents targeting to the nuclear proteasome, and allows such mutants to be expressed and analyzed.

Core appears to be peripherally associated with mitochondria, since it is accessible to protease digestion and carbonate extraction (Schwer et al., 2004) as is the case at the ER (McLauchlan et al., 2002). Most likely, core traffics from the ER to both the mitochondria and lipid droplets via membrane bridges, since both of these compartments are likely derived from the ER. Mitochondrial targeting appears to be governed by an aa sequence in core. Schwer et al. demonstrated that a short stretch extending from aa 149-158 located in domain II governs mitochondrial targeting (Schwer et al., 2004). Suzuki et al. reported that a region of 41 residues from aa 112-152 is responsible for association between the core protein and mitochondria (Suzuki et al., 2005). This discrepancy may be due to the differences of HCV clones and experimental settings.

POST-TRANSLATIONAL MODIFICATIONS OF HCV CORE

Post-translational modification plays crucial roles for regulating the function of the proteins. Several studies have shown post-translational modification of HCV core protein. Phosphorylation of the core protein in insects cells (Lanford et al., 1993),

reticulocyte lysates (Shih et al., 1995), and mammalian cells (Lu and Ou, 2002) have been reported. Cellular protein kinase A (PKA) and protein kinase C (PKC) were identified as possible protein kinases responsible for phosphorylation of HCV core protein. Phosphorylation at Ser-116 may regulate nuclear localization of the core protein (Lu and Ou, 2002).

Post-translational modification of the core protein by tissue transglutaminase has been reported (Lu et al., 2001). Tissue transglutaminase catalyzes the formation of a γ-carboxyl-ε-lysine isopeptide bond by joining the γ-carboxamide group of glutamine to the amino group of lysine. A small fraction of the core protein has been shown to form a dimer that is highly stable and resistant to denaturation and reduction by SDS and β-mercaptoethanol. A potential role for tissue transglutaminase in core dimer formation has been proposed (Lu et al., 2001). The ubiquitin-proteasome pathway is the major route by which selective protein degradation occurs in eukaryotic cells and is now emerging as an essential mechanism of cellular regulation (Finley et al., 2004; Hershko and Ciechanover, 1998). As mentioned above, the core protein is targeted for ubiquitination and degradation by an unknown ubiquitin ligase. The C-terminus of the core protein is important for regulating stability of the protein (Kato et al., 2003; Suzuki et al., 2001). When the core protein is expressed as the C-terminal truncated forms such as aa 1-173 (21kDa) and 1-152 (17kDa), the core protein is unstable (Kato et al., 2003; Moriishi et al., 2003; Suzuki et al., 2001). Specific proteasome inhibitors stabilize these short-lived forms of the core protein, suggesting that the proteasome machinery is responsible for their degradation (Fig. 2). By contrast, the full-length form of the core protein (aa 1-191) is long-lived. Only the C-terminal truncated form of the core protein can be multi-ubiquitinated, and the predominant stable form of the core protein links to a single or only a few ubiquitin moieties (Suzuki et al., 2001). To understand the mechanism of ubiquitination of the core protein, the specific E3 ubiquitin ligase that acts on HCV core has to be identified.

A proteasome activator, PA28γ, has been identified as a core-binding protein by yeast two-hybrid screening (Moriishi et al., 2003). PA28γ can interact with the core protein in cultured cells, as well as in the liver of transgenic mice and chronic hepatitis C patients. PA28γ predominates in the nucleus and forms a homopolymer, which associates with the 20S proteasome (Tanahashi et al., 1997), thereby enhancing proteasome activity (Realini et al., 1997). Over-expression of PA28γ enhanced proteolysis of the core protein, suggesting that PA28γ affects proteasomal activity and regulates stability of the core protein (Moriishi et al., 2003) (Fig. 2). Evidence has been accumulating that ubiquitin-proteasome pathway plays a crucial role in the viral life cycle and in pathogenesis (Banks et al., 2003; Scheffner et al., 1993). However, the biological significance of ubiquitin-dependent degradation of the core protein remains to be elucidated.

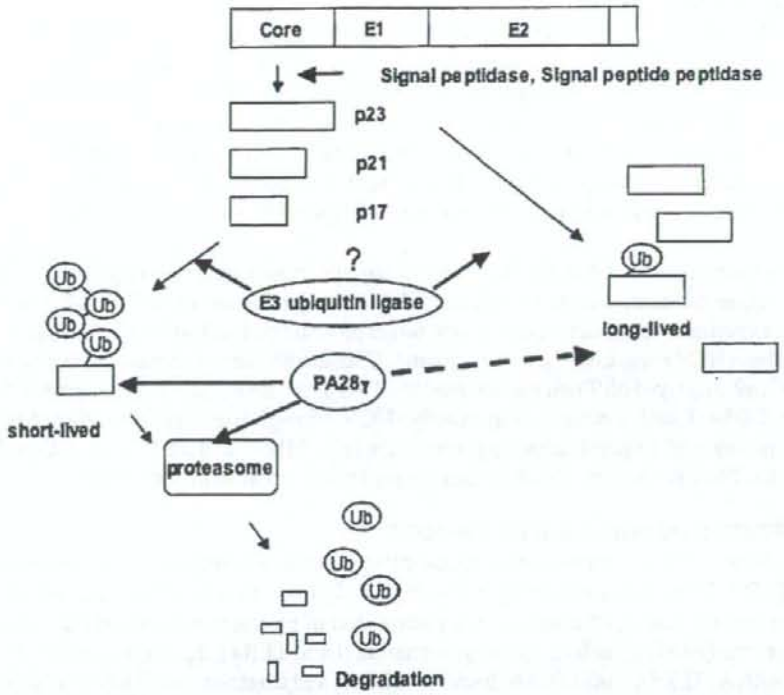

Fig. 2. A model for the processing of HCV precursor and degradation of the core protein by the Ubiquitin-proteasome pathway. The junction between core and E1 is cleaved by the signal peptidase, resulting in production of p23 form of the core protein. Additional cleavage of the core protein by signal peptide peptidase produces p21 form of the core protein. Further processed forms of the core protein, such as p17, are produced by unknown mechanisms. The C-terminal truncated form of the core protein is poly- ubiquitinated by an unidentified E3 ubiquitin ligase and targeted for proteasomal degradation. The immature core protein links to a single or a few ubiquitin moieties and is long-lived. A proteasome activator, PA28γ, enhances proteasomal degradation of the core protein.

HCV CORE-HOST INTERACTIONS

Core-host interactions will be discussed in terms of their affects on host antiviral and immune responses, and HCV pathogenesis. The recent finding of core protein in the serum on infected patients has forced one to think that HCV host interactions not only occur within infected cells, but they can also occur extracellularly.

EFFECTS ON T CELL FUNCTION

HCV infection in humans is almost invariably associated with viral persistence leading to chronic hepatitis – predisposing the host to development of cirrhosis

and hepatocellular carcinoma. CD8+ T cells play a pivotal role in controlling HCV infection; but, in chronic HCV patients, severe CD4+ and CD8+ T cell dysfunction has been observed (Shoukry et al., 2004). This suggests that HCV may employ mechanisms to evade or possibly suppress the host T cell response. In exploring the possible evasion mechanism(s) in order to design strategies for therapeutics and improved immunization, the HCV core protein was identified as an immunomodulatory molecule suppressing T lymphocyte responsiveness through its interaction with complement receptor (gC1qR) (Kittlesen et al., 2000).

It was demonstrated that the HCV core protein suppresses an *in vivo* anti-viral CD8+ T cell response to vaccinia virus, and inhibits the production of IFN-γ and IL-2 in an experimental murine model. A host target protein (gC1qR) on T cells was shown to bind HCV core. Like the natural ligand, C1q, the binding of extracellular core to gC1qR displayed on T cell surface lead to CD4+ T cell deregulation and suppression of CD8+ T cell function. Importantly, HCV core-gC1qR ligation induced the expression of negative signaling molecules (*e.g.* SHP-1 and SOCS1) in CD4+ T cells. The data suggest that core has potent immunomodulatory functions.

EFFECTS ON TOLL-LIKE RECEPTORS

Cells sense the presence of extracellular pathogens via cell surface toll-like receptors (TLRs). There are approximately 10-15 TLRs in mammals, which are responsible for sensing microbial infection, via recognition of pathogen associated molecular patterns (PAMP), such as lipopolysaccharide (LPS; TLR4), double-stranded RNA (dsRNA; TLR3), CpG DNA of bacteria (TLR9), and single-stranded RNA (ssRNA; TLR7) (Iwasaki and Medzhitov, 2004). After binding pathogens, TLR signaling involves coupling of toll-IL-1 receptor (TIR) containing adapter proteins such as TIRAP, TRIF, TIRAP and MAL, and activation of signaling molecules IL-1 receptor associated kinase (IRAK), MyD88, and TNF receptor-associated factor 6 (TRAF-6). Ultimately, transcription factors such as mitogen activated protein kinases (MAPK), NF-κB, and IRF-3 become activated, leading to production of IFN-α/β (Hertzog et al., 2003). Interestingly, DC maturation *in vitro* is impaired in chronic HCV infection when compared to those subjects with spontaneously resolved infection and normal controls (Anthony et al., 2004; Dolganiuc et al., 2003; Kanto et al., 2004; Murakami et al., 2004a; Sarobe et al., 2003; Tsubouchi et al., 2004a; Tsubouchi et al., 2004b; Wertheimer et al., 2004). Recent studies have provided mechanistic insights into these events.

In a study of the effect on the immunostimulatory effects of lipopeptides, 10 of 14 and 9 of 14 HCV core lipopeptides stimulated a reporter gene in TLR2-expressing and TLR4-expressing cells but not in mock-transfected control cells (Duesberg et al., 2002). However, activation was dependent on the lipid moiety since the same free peptides had no stimulatory effect on the TLR2 or TLR4 transfected

cells. A study by a different group found that addition of recombinant HCV core protein to human monocytes, and human embryonic kidney cells transfected with TLR2 triggered inflammatory cell activation and failed to activate macrophages from TLR2 or MyD88-deficient mice (Dolganiuc et al., 2004). HCV core induced interleukin (IL)-1 receptor-associated kinase (IRAK) activity, phosphorylation of p38, extracellular regulated (ERK), and c-jun N-terminal (JNK) kinases and induced AP-1 activation. Cell activation required core aa 2-122. Interestingly, HCV core protein was also taken up by macrophages, but this was independent of TLR2 expression. These data indicate that the HCV core protein can trigger innate immune responses.

EFFECTS OF HCV CORE ON THE INTERFERON SYSTEM

Several studies have documented that the HCV core protein can activate the interferon (IFN) system. For example, core activates the IFN stimulated genes (ISG) 2-5 OAS (Naganuma et al., 2000) and PKR (Delhem et al., 2001). PKR and 2-5 OAS are two major ISGs that mediate the IFN antiviral response against many viruses. It was also recently shown that HCV core protein activates the innate antiviral cellular response involving interferon regulatory factors (Miller et al., 2004). Core induced IRF-1 transcription and mRNA expression, and caused dose-dependent induction of the IFN-β promoter and IFN-β mRNA expression. In the presence of IFN-α, core expression caused increased IFN-stimulated gene factor 3 (ISGF3) binding to the IFN-stimulated response element (ISRE) and tyrosine phosphorylation of Stat1. Core expression also activated IFN-γ signaling (Miller et al., 2004).

The effects of core on innate cellular antiviral responses including TLR and IFN pathways may be critically important during acute infection. Following binding, internalization, and uncoating of HCV virions, core, in the form of nucleocapsid, is the first viral protein to interact with the intercellular milieu of cellular proteins and signaling pathways. Because core mutates during virus replication, HCV core is present as a quasispecies in infected patients (Pawlotsky, 2003). What is not clear at present is whether HCV core's inherent variability influences innate antiviral responses such as TLR signaling and IRF-Jak-Stat activation. Fig. 3 suggests that there is indeed heterogeneity in innate antiviral responses to genetically different HCV core isolates. Fig. 3A depicts the sequence of 2 core proteins (named Core 1 and Core 2) derived from 2 different genotype 1b infected patients. As shown in the figure, the two isolates differed by 7 aa. The 2 core genes were engineered into a tetracycline regulated expression vector, such that in the absence of tetracycline in the medium, both Core 1 and Core 2 proteins were expressed in HeLa cells. Addition of tetracycline to the medium blocked core expression. Fig. 3C presents the effects of Core 1 and Core 2 expression on transcription of an IFN responsive promoter, the ISRE. In the absence of IFN, expression of Core 1 was associated with a 3-fold increase in activation of the ISRE, compared to when

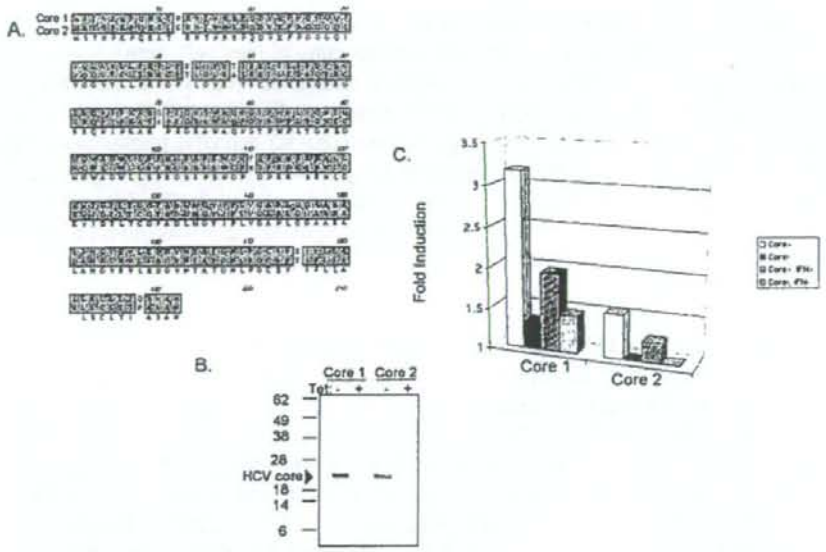

Fig. 3. Effects of HCV Core Protein Expression on Type I IFN Signal Transduction. A. the sequence of the Core 1 and Core 2 genes are aligned. B, Tetracycline regulated expression of the Core 1 and Core 2 proteins in HeLa cells. Plasmids were transfected into HeLa tet-off cells, grown in the absence and presence of tetracycline to induce and repress core protein expression, respectively, and protein lysates were subjected to Western blot analysis at 48 hours post-transfection. C, Differential effects of Core 1 and Core 2 proteins on ISRE activation. pTRE-Core 1 and pTRE-Core 2 plasmids were cotransfected with an ISRE-luciferase reporter plasmid into HeLa tet-off cells, incubated in the presence or absence of tetracycline for 40 hours, and treated with or without 500 U/ml of IFN-α for 6 hours. Luciferase activity was determined on equal amounts of protein lysates.

gene expression was repressed. In the presence of IFN, Core 1 induced a 2-fold increase in luciferase activity. Expression of Core 2 resulted in only marginal ISRE stimulation. These data demonstrate that 2 genetically different HCV core proteins activate a canonical IFN promoter to varying degrees. The data suggest that HCV quasispecies differentially modulate host cell responses. Indeed, other studies have demonstrated that NS5A mediated transcriptional activation varies among clinical quasispecies isolates (Pellerin et al., 2004). Thus, future studies should take into account genetic and structural heterogeneity of HCV isolates as being important factors in host responsiveness to HCV infection.

This concept may have clinical implications. It can be hypothesized that genetic and structural variants of HCV proteins such core could differentially trigger