Volume II: Basic Science

by Daniel Raymond

ACKNOWLEDGMENTS

I have relied on the generosity of several researchers who provided me copies of their work, answered questions, provided comments, or otherwise encouraged and stimulated my thinking on HCV research: Carol Brosgart, Kyong-Mi Chang, Nick Crispe, Gregory Dore, Rika Draenert, Brian Edlin, Bin Gao, Jay Hoofnagle, Lennox Jeffers, Leslye Johnson, Ronald Jubin, Margaret Koziel, Diane Lucas, John McHutchison, Shruti Mehta, Marion Peters, Barbara Rehermann, Charles Rice, Benedikt Sas, Kenneth Sherman, Sharon Stancliff, Mark Sulkowski, Andrew Talal, David Thomas, and Francesca Torriani.

I'd also like to thank my editors for their diligence, Lei Chou for his graphics and design work, Jules Levin for providing valuable information and perspective on HCV drug development, and Richard Jefferys for many helpful conversations about immune responses.

Finally, I am indebted to Tracy Swan for not only inviting me to work on this project, but also for the continued dialogue and feedback, the comments and insights into the structure and content of my chapters, and especially for her support and collaboration, which has enriched and inspired my work.

Introduction to Volume II: Basic Research and Drug Development

This is an exciting time for basic research and drug development in hepatitis C. Since 2003, a number of important papers and presentations have substantially added to our body of knowledge about the hepatitis C virus (HCV) and offered hope for the advent of new therapies. Key findings include:

- Proof-of-concept from human studies of an HCV NS3 protease inhibitor (BILN 2061), with other candidates moving into clinical trials;
- Initial clinical data on a new HCV polymerase inhibitor (NM283);
- A range of other HCV drugs entering phase II and III studies;
- Encouraging research on the prospects for developing a vaccine to prevent HCV, based on increased understanding of the correlates of protective immunity in chimpanzees and humans;
- New insights into the role of HCV proteins and the dynamics of viral replication;
- New data on the role of the HCV NS3 protease in regulating the interferon response within cells, offering clues to viral persistence;
- Advances in HCV replicon models used to study viral replication *in vitro* and test the activity of new drugs;
- A promising new *in vitro* model for studying HCV entry into target cells;
- Establishment of an HCV genetic database at the Los Alamos National Laboratory, with a database of T cell epitopes to follow.

These developments represent the culmination of 15 years of research since the discovery of HCV. But despite remarkable progress, much work remains. Critical aspects of HCV pathogenesis and the viral replication cycle are not fully understood, and further refinement of cell culture systems and animal models is an urgent priority. While the current HCV drug pipeline contains an ever-expanding roster of promising compounds, few have moved into advanced clinical trials.

These antiviral agents could bring about a paradigm shift in HCV treatment over the next decade, potentially supplanting current combination therapy with pegylated interferon and ribavirin. Yet dramatic improvements in the efficacy and tolerability of HCV treatment are unlikely to reach the market for several years. Furthermore, most observers anticipate that the development of an effective preventative vaccine for HCV will require decades of research.

HCV prevention, screening, care, and treatment have attracted a growing constituency of advocates, including people with hepatitis C and activists working on HIV, harm reduction and substance abuse, and prisoner health. This volume of the report aims to broaden the advocacy agenda to include advocacy for basic research and drug development. The following chapters provide a foundation for understanding current issues and research priorities in HCV virology, immunology, and drug and vaccine development.

Current changes in how research is conceived and conducted provide a range of opportunities for advocacy:

- The National Institutes of Health (NIH) unveiled a new initiative, the NIH Roadmap, intended to accelerate biomedical and translational research through new forms of collaboration. Yet NIH has entered a phase of relatively flat funding, following a five-year period during which Congress doubled the NIH budget. Funding for HCV research in fiscal year 2005 is estimated at $130 million. Flat funding will limit the pace of new advances and discoveries in HCV research.

- Within NIH, the National Institute of Diabetes and Digestive and Kidney Diseases created a Liver Disease Research Branch that encompasses research on HCV within the institute. The Liver Disease Research Branch is developing an Action Plan for Liver Disease Research that attempts to coordinate and prioritize liver disease research across NIH. The Action Plan has been soliciting comments from the research community and the public.

- The Food and Drugs Administration (FDA) has launched a 'Critical Path' initiative to accelerate drug development by stimulating research on scientific barriers to assessing the efficacy and safety of new drugs. FDA intends to identify and prioritize a list of opportunities for targeted research and collaboration between government and industry. FDA is soliciting comments on potential areas of focus.

- As new classes of HCV drugs—particularly protease and polymerase inhibitors—begin moving into clinical trials, FDA can also take a leadership role in providing guidance to industry on trial design, study populations, and conditions for accelerated approval. Advocates can work with FDA and industry to speed the development, testing, and approval of safe and effective new HCV drugs.

- New international research initiatives are stimulating novel research into liver disease and therapies. The Human Liver Proteome Project, spearheaded by China and coordinated by the Human Proteome Organization, aims to map the interactions of proteins in healthy and diseased livers. The Gates Foundation's 'Grand Challenges in Global Health' initiative will fund innovative research projects in areas including preventative and therapeutic vaccines, with HCV listed as a priority area. On a smaller scale, the Canadian Network for Vaccines and Immunotherapeutics (CANVAC) coordinates HCV vaccine research, and the European Commission funds the HepCVax collaborative project to research and develop preventative and therapeutic vaccines for HCV. The European Union has also funded the European Network for Hepatitis C Virus Envelope Glycoprotein Research consortium (ENHCV) to study the structure and function of HCV envelope proteins for the development of new HCV drugs. Finally, HIV vaccine research offers provocative models for effective collaboration and coordination of HCV vaccine research efforts, including the incipient Global HIV Vaccine Enterprise and the Neutralizing Antibody Consortium, led by the International AIDS Vaccine Initiative.

Future advances in HCV basic research and drug and vaccine development will require creative partnerships between government, industry, academia, and community. Basic and clinical research will mutually benefit from further integration, as occurs within the NIH-funded Hepatitis C Cooperative Research Centers. The science points to a new era of innovation and discovery; this vision can only be realized through increased leadership, resources, and advocacy.

Recommendations

- **Support and intensify research into the molecular biology of HCV.**
- **Support and intensify research into immune response, persistence and pathogenesis.**
- **Increase funding and coordination of research.**
- **Support refinement of in vitro and animal models.**
- **Promote drug development efforts that study safety and efficacy in real-world populations.**
- **Initiate partnerships between industry, government, academia, and community.**

VIII. The Molecular Virology of Hepatitis C

Introduction and Summary

The identification of HCV in the late 1980s was the culmination of a decade of work by several research groups and nearly half a century of investigation into the causes of post-transfusion hepatitis (Feitelson 2002). Every conventional method used to isolate viruses had failed until a group of researchers at the Chiron Corporation successfully identified HCV using the polymerase chain reaction (PCR), a new research tool developed in the mid-1980s that had not been previously applied to the discovery of a virus (Q. L. Choo 1989). Notably, the discovery of HCV was aided by the work of government researchers at the Centers for Disease Control and the National Institutes of Health.

These factors—the creative application of new technologies when conventional methods had failed, and the contribution of multiple researchers and groups, both public and private—have also characterized the best of subsequent research into HCV. The ongoing struggle to develop an adequate cell culture system and a small-animal model for studying hepatitis C continues to tax the resourcefulness of HCV researchers, although these efforts are finally beginning to bear fruit. Similarly, investments by government and industry have generated synergy between the respective domains of basic science and drug development. There are two other recurring themes in the field that should be noted: one is the strength of international contributions, ranging from U.S. based to East Asian and European research groups working independently and in collaboration. A second theme is the crerative utilization of technological innovations to advance the study of HCV.

The initial discovery of HCV in 1987–88 was rapidly followed by reports from the United States and Japan characterizing the entire HCV genome (Q. L. Choo 1991; N. Kato 1990a; Kuo 1989; Takamizawa 1991). Subsequent work by several research groups in Europe, Japan, and the United States successfully determined the viral proteins produced by HCV, the rudiments of viral replication, and the extent of viral diversity in different regions of the world. Just as the discovery of HCV might not have been possible before PCR technology was available, research into the dynamics of its replication has benefited from additions to the molecular biology toolbox. Similarly, over the last 15 years, the field of virology has embarked on new avenues of inquiry into viral protein synthesis, viral diversity, and viral–host cell interactions. The general insights yielded by this work have paved the way for understanding aspects of hepatitis C virology. Key findings from this substantial, and growing, body of research are highlighted in this chapter.

HCV is a small, enveloped RNA virus, consisting of nothing more than the viral genome (a single strand of RNA, or ribonucleic acid) enclosed in a capsid shell and surrounded by a viral envelope, or membrane. HCV's genome encodes at least ten viral proteins, including two envelope proteins, as well as protease, helicase, and polymerase enzymes (Bartenschlager 2000). HCV has been classified as a member of the Flaviviridae family of viruses based on these characteristics and similarities to other members of the Flaviviridae family (Büchen-Osmond 2003; Francki 1991). Most of the fundamental stages of HCV replication (entry, viral protein synthesis, cleavage, genome replication, and viral assembly and release) have been documented and analyzed; however, gaps in knowledge persist in important areas, particularly in the very earliest and latest stages of the viral replication cycle (how HCV gets inside cells, and how newly produced viral particles are

assembled and released). Two key related questions require further elucidation: how does the virus interact with cellular components at each point in the replication cycle? and What factors, viral and cellular, regulate the activities of each stage?

Despite substantial progress in addressing these questions, complete answers to the problems in the study of HCV replication may not be soon forthcoming. Research has been stymied by the lack of an efficient cell culture system, a way to study viral replication in laboratory-grown cells. For reasons not well understood, HCV does not replicate reliably or reproducibly in cell cultures, which has frustrated attempts to construct thorough maps of the dynamics of viral replication (Bartenschlager 2001; N. Kato 2000a). In the absence of cell cultures supporting HCV replication, synthetic genetic constructs called replicons have recently been developed as a model for the hepatitis C virus (Blight 2000; Lohmann 1999). These replicons contain some or all of the genes of HCV and can replicate autonomously in cell cultures (Blight 2002; M. Ikeda 2002; Pietschmann 2002).

The development of a replicon model was a watershed in HCV research, dramatically expanding the possibilities for studying viral replication, as well as for screening potential anti-HCV drugs for activity against viral enzymes (Bartenschlager 2002; Grakoui 2001; Lanford 2002; Randall 2001; V. Wu 2001). Replicons, however, cannot assemble into new viral particles capable of infecting other cells (Molenkamp 2003; Pietschmann 2002). Other limitations in the replicon model suggest that additional refinements will be necessary for the full simulation of HCV replication (Bartenschlager 2002; Blight 2002; Blight 2003; Bukh 2002; Lohmann 2003; Murray 2003; Pietschmann 2003).

The only other established model for hepatitis C is the chimpanzee, virtually the only species besides humans susceptible to HCV infection (Lanford 2001a). Chimpanzee research is prohibitively expensive; thus, there is an urgent need for a more broadly available and convenient small-animal model for HCV infection. The recent development of transgenic mice transplanted with cells from human livers may be a major step towards this goal (Dandri 2001; Ilan 2002; Mercer 2001). Currently, however, these transgenic mice are more an initial proof-of-concept than the final realization of a small-animal model for HCV (Brass 2002; Fausto 2001; Pietschmann 2003).

The immediate value of a cell culture system or small-animal model lies in its potential to illuminate the basic science and pathogenesis of hepatitis C, bringing into focus the full workings of viral replication and HCV's interactions with cells. Ideally, such research would begin to shed light on three urgent issues in the study of HCV:

- Persistence—How does HCV establish chronic infection and elude cellular and immune system defenses?
- Pathogenesis—How does HCV cause disease, and why does the course of disease vary in different people?
- Prospects for new therapies—How can drugs successfully target viral replication, offset liver damage, or prevent hepatitis C infection?

This chapter, focusing on viral replication, lays the foundation for beginning to answer these questions, which are more fully explored in subsequent chapters (see Chapter IX, Immune Response, Persistence, and Pathogenesis, and Chapter X, The Future of HCV Therapy).

Discovery of the Hepatitis Viruses

Hepatitis has a long history in humans. Jaundice, a common symptom of hepatitis, was first described by Hippocrates in the fifth century B.C., and documentation of hepatitis outbreaks extends back for centuries. Despite speculation in the early twentieth century that hepatitis might result from an infectious agent, it took several decades and the development of more sophisticated research methods and technology before scientists identified the hepatitis A, B, and C viruses. These discoveries were facilitated by the recognition that many blood transfusion recipients developed hepatitis. Post-transfusion hepatitis (PTH) (a syndrome associated with abnormal laboratory values on liver enzyme tests and symptoms which could include nausea, fever, and jaundice) was recognized as early as the 1940s, nearly 150 years after the first human blood transfusion was performed (Beeson 1943). Different clinical patterns suggested the existence of more than one form of hepatitis, originally described as "infectious" and "serum" hepatitis; the terms hepatitis A and hepatitis B were introduced in 1947 to distinguish between the two hypothesized forms of PTH (MacCallum 1947).

The concept of separate forms of hepatitis, with different epidemiological patterns and clinical presentations, had been firmly established by the 1960s (Krugman 1967). During this decade, a protein found in the blood of an Australian aborigine (thus designated Australia antigen) was linked to type B hepatitis (Blumberg 1967; Okochi 1968; Prince 1968). Despite initial controversy, the Australia antigen (later identified as hepatitis B surface antigen) formed the basis of the first test to screen blood for type B hepatitis, introduced in United States blood banks in 1971. A virus-like particle containing Australia antigen, initially called the Dane particle and later identified as the hepatitis B virus (HBV), was subsequently discovered in the early 1970s, spurring further research. A vaccine protecting against HBV was introduced in 1981 (CDC 1982; Dane 1970; Francis 1982; Szmuness 1980; Tobler 1997).

The viral nature of the infectious agent causing type A hepatitis was demonstrated in the late 1960s, through transmission by human serum to marmoset monkeys (Deinhardt 1967). A virus-like particle associated with type A hepatitis was also identified in the early 1970s, followed by a serological test for antibodies to the hepatitis A virus (HAV); by the end of the decade, researchers had developed methods to study viral replication in cells through an *in vitro* culture (Feinstone 1973; Miller 1975; Provost 1979).

The hepatitis C virus was more elusive and would not be identified until 1987–88 (Q. L. Choo 1989). The availability by the mid-1970s of serum tests and blood screening for hepatitis A and hepatitis B indicated, by process of exclusion, that at least one other unidentified infectious agent was responsible for a significant number of cases of post-transfusion hepatitis, designated non-A, non-B hepatitis, or NANBH (Alter 1975; Feinstone 1975; Prince 1974). A number of researchers would pursue the identification of NANBH's causative agent for nearly a decade, with various hypotheses—a retrovirus, a picornavirus, a relative of HBV—tentatively proposed and ultimately ruled out, though the agents ultimately named the hepatitis D (for hepatitis delta) and hepatitis E viruses were identified during this period (Balayan 1983; Bonino 1984; Rizzetto 1977). Unlike HAV and HBV, the hepatitis C virus proved refractory to conventional methods of serological testing for antibodies and direct visualization through electron microscopy, and was, therefore, equally refractory to identification. However, chimpanzees were susceptible to NANBH when

infused with serum from patients diagnosed with non-A, non-B hepatitis, establishing an animal model for infection. Further work suggested that NANBH was caused by a fairly small virus, surrounded by an envelope, and carrying its genetic information in the form of RNA (ribonucleic acid; see Viral Classification section below), though the search for viral particles, antigens, and antibodies was hindered by the relative paucity of circulating virus in NANBH patients and infected chimpanzees (Feitelson 2002).

To evaluate candidate pathogens, Harvey Alter of the National Institutes of Health had gathered blood samples from individuals with clinical and epidemiological profiles indicative of NANBH, and from chimpanzees infected with sera from NANBH patients. Additional blood samples were taken from control groups of healthy individuals and persons with other liver diseases. The Alter panel, as this sample collection was dubbed, provided a valuable screen to assess whether candidate pathogens were indeed associated with NANBH. If a proposed agent were truly the cause of NANBH, it would react only to antibodies in Alter panel samples that were from NANBH patients and infected chimpanzees, and exclude those samples from healthy or uninfected controls.

The identification of the hepatitis C virus in 1987–88, a landmark in the history of virology, ultimately resulted from a public-private collaboration, initiated in 1982, between the Chiron Corporation and the Centers for Disease Control (CDC). Dr. Daniel Bradley of CDC provided chimpanzee plasma containing a highly infectious, high-titer form of the NANBH agent produced through serial passage of infectious sera through chimpanzees. Michael Houghton, George Kuo, Qui-Lim Q. L. Choo, and colleagues at Chiron extracted plasma genetic material (RNA and DNA, or deoxyribonucleic acid) from an infectious chimpanzee. In theory, much of this genetic material should derive from chimpanzee genes, but a portion of the genetic material would be viral in origin; therefore, any genetic material not from chimpanzee genes could be from the NANBH agent. The Chiron team used a new molecular cloning technique, reverse transcriptase-polymerase chain reaction (RT-PCR), to search for this agent.

The researchers cloned from the chimpanzee plasma a genetic fragment that met the criteria for the NANBH agent. First, antibodies from NANBH patients recognized a section of a protein encoded by the genetic fragment. This suggested that the fragment was from an infectious agent that evoked an immune response. Also, the fragment did not correspond to chimpanzee (or human) DNA, further indicating that it was derived from or produced by a foreign agent, such as a virus. In addition, the fragment corresponded to RNA found in NANBH-infected (but not uninfected) chimpanzee blood and livers, suggesting an RNA virus. Finally, the relationship between the genetic fragment and NANBH was validated through the Alter panel, where the putative agent could successfully distinguish between blood from NANBH-infected patients and chimpanzees, and samples from uninfected controls. This research, published in 1989, provided the foundation for christening the agent hepatitis C, the causative agent of a majority of NANBH cases (Q. L. Choo 1989). The cloning of HCV represented the first time that a virus had been detected without first being visualized through electron microscopy or identified through serological techniques. The first HCV antibody test was quickly developed based on this work, and several researchers in the U.S. and Japan subsequently succeeded in cloning full-length HCV (Q. L. Choo 1991; N. Kato 1990a; Kuo 1989; Takamizawa 1991). The work of Michael Houghton and Harvey Alter in identifying HCV, developing sensitive screening methods, and virtually eliminating the risk of transfusion-associated hepatitis was recognized in 2000 when they received the prestigious

Albert Lasker Award for Clinical Medical Research.

The actual isolation of HCV particles from sera would not occur until the mid-1990s, which finally allowed viral particles to be studied under a microscope. Electron microscopy of HCV virions in the mid-1990s enabled rough descriptions of the size and structure of individual viral particles (de Vos 2002; Kaito 1994; X. Li 1995; Y. K. Shimizu 1996). The hepatitis C virus is a spherical virus with three major components: an external envelope, an internal shell, and a strand of RNA. The HCV genome is encoded by an RNA strand approximately 9,600 nucleotides in length (nucleotides being the basic units, or building blocks, of RNA). The single strand of genomic HCV RNA is surrounded by a shell, or protein coat, called the capsid. The capsid is structured as an icosahedron, a form consisting of 20 identical and symmetrical equilateral triangles that are made up of viral proteins. The combination of genomic RNA and capsid is referred to as the nucleocapsid. An envelope encompasses the HCV nucleocapsid and is composed of viral proteins and cellular membrane. The HCV envelope is a membrane composed of a lipid bilayer, derived from host cells, studded with viral envelope proteins. The diameter of an HCV virion is estimated at approximately 50–65 nanometers (nm), larger than hepatitis A (17–19 nm) and hepatitis B (42 nm) (Kaito 1994; Y. K. Shimizu 1996). By contrast, the diameter of HIV virions is at least twice as large, with estimates ranging from about 110 nm to 145 nm for mature HIV virions (Briggs 2003; Gentile 1994).

Viral Classification: HCV as an RNA Virus

The identification through molecular cloning of HCV was only the first step in understanding the virus. At first, virtually nothing was known about the genetic organization of the virus, the viral proteins that could be synthesized from the HCV genome, and the details of how HCV replicated. Such information would be crucial in order to understand how HCV caused liver disease, and how to develop drugs to treat HCV. Researchers in the early 1990s drew upon the knowledge of other viruses to construct tentative models for HCV's genetic structure and protein function; however, HCV bore little resemblance to the hepatitis A virus or the hepatitis B virus, even though all three viruses targeted the liver. Therefore, scientists turned to other viruses that had greater genetic similarities, using them to form testable hypotheses about HCV. These functional and structural comparisons were enabled by established systems of viral classification, called taxonomies. Current hepatitis C research continues to draw upon these systems in developing surrogate models for HCV from related viruses.

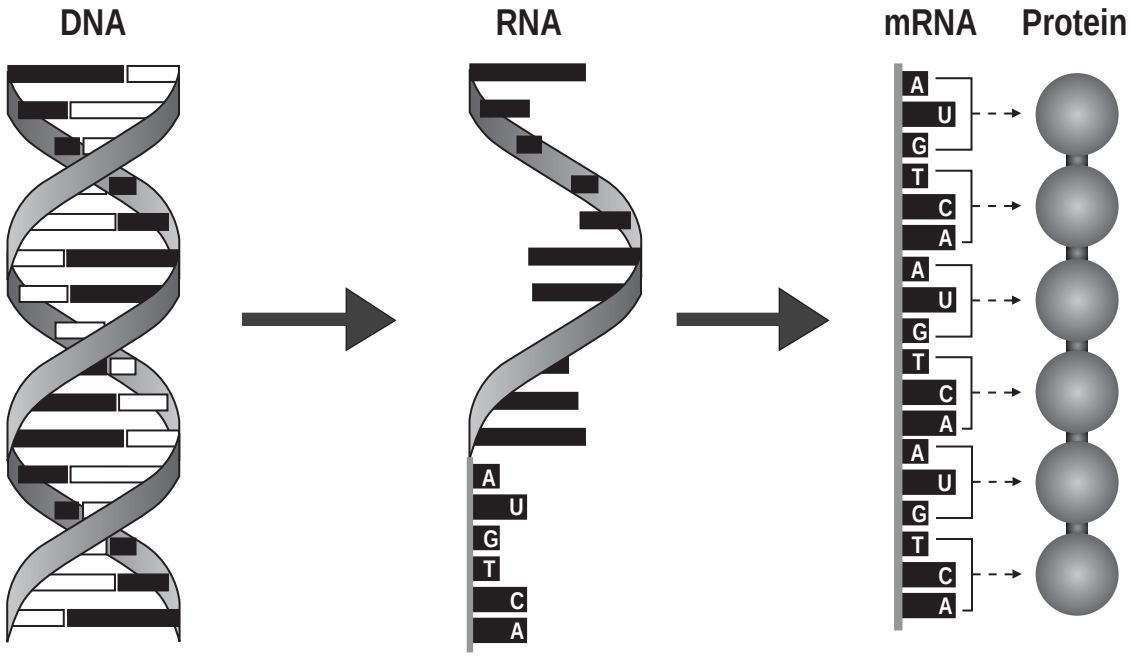
A major basis for distinction among viruses is how they encode their genetic information. Living organisms, including humans and other animals, bacteria, fungi, and plants, use DNA to store their genetic code. Some viruses also encode their genetic information as DNA, while others use RNA. Viruses are therefore classified as DNA viruses or RNA viruses. HCV is an RNA virus, and each viral particle contains a single RNA strand. HCV RNA has two functions:

1. Genomic RNA: The single RNA strand serves as HCV's genome, meaning that it contains all of HCV's genetic information. When a virus replicates, all new viral particles must contain a copy of the viral genome; therefore all viruses produce new copies of their genome during the replication process.

2. Messenger RNA (mRNA): All proteins (cellular and viral) are synthesized from an RNA template called messenger RNA. Messenger RNA is a single RNA strand that contains the genetic code or "message" describing the composition of the desired protein. The composition of mRNA provides the instruction set for protein synthesis. HCV uses the proteins encoded in its mRNA to replicate itself.

An RNA strand that can function as messenger RNA is also referred to as positive-sense RNA. Therefore, HCV is described as a positive-sense, single-stranded RNA virus. During replication, HCV synthesizes a negative-sense strand of RNA, a mirror-image copy of genomic RNA. This negative-sense RNA strand becomes the template for producing additional copies of positive-sense RNA. On this basis, HCV has been grouped with other viruses that have positive-sense RNA for genomes and have similar replication processes.

Figure 1. DNA, RNA, and Proteins



Genetic information in cells and many viruses is stored as DNA. DNA is transcribed into RNA. RNA, in the form of messenger RNA (mRNA), forms the template for synthesizing proteins. The HCV genome consists of a strand of RNA containing all of the virus' genetic information. The HCV genome also functions as mRNA, the template for synthesizing all HCV proteins.

Viruses are classified phylogenetically, that is, by presumed evolutionary history or genetic "family tree." This classification system considers several factors: similarities between viral genomes and proteins, as well as size and shape, physical and chemical properties, and type of host (i.e., whether viruses infect and replicate in plants, insects, or animals, etc.). In this system, viruses have been grouped hierarchically by:

1. Species (the most specific category—A species is a particular virus);
2. Genus (an intermediate category—A group of species make up a genus, the Latin word for "kind" or "class"); and
3. Family (the broadest category—There are 30 recognized families of viruses that infect vertebrates).

The hepatitis C virus is a species within the hepacivirus genus (Büchen-Osmond 2003). The hepacivirus genus belongs to the Flaviviridae family of viruses, which also includes flaviviruses and pestiviruses (Francki 1991). All Flaviviridae have positive-sense, single-stranded RNA genomes. The flavivirus genus includes viruses such as Dengue fever and yellow fever, as well as the Japanese and tick-borne encephalitis viruses. Pestiviruses are animal pathogens such as bovine viral diarrhea virus and classical swine fever virus. Research on these and other related viruses continues to inform the understanding of how HCV replicates and interacts with infected cells.

HCV was classified as a member of the Flaviviridae family based on its similarity to flaviviruses and pestiviruses, but categorized as the first member of a new genus designated hepacivirus. Because of significant genetic differences, other major hepatitis viruses are members of different viruses families, and are not directly related to HCV. The hepatitis A virus, like HCV, has a single-stranded, positive-sense genome, but is classified within the picornaviridae family. The hepatitis B virus (HBV) is a double-stranded DNA virus classified among the hepadnaviridae family. Three other viruses—GB viruses A, B, and C (the last previously misidentified as hepatitis G virus)—have been proposed for membership in the Flaviviridae family, but the GB viruses have not been assigned to a specific genus (see below for more on GB viruses).

Viral Diversity and the Origins of HCV

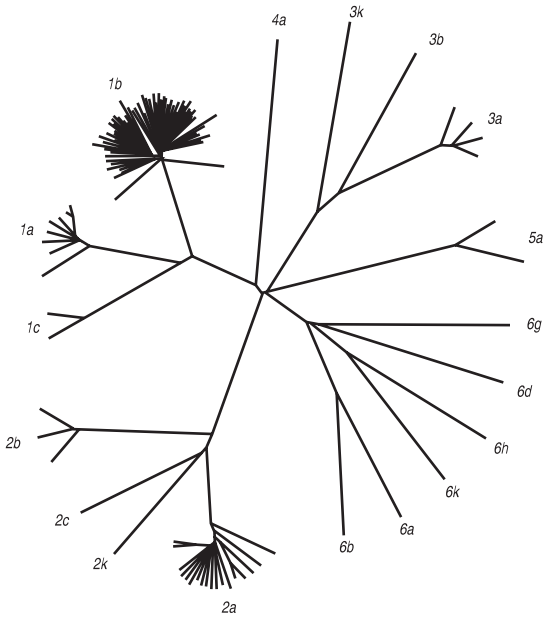
Hepatitis C isolates (individual samples of the virus) from around the world show significant genetic variability, as is common in RNA viruses. This variability results from the error-prone nature of HCV replication. RNA strands are composed of molecules called nucleotides. These nucleotides form the building blocks of RNA; the specific sequence of these nucleotides in the RNA strand determines the nature of the proteins encoded by the RNA. Each RNA nucleotide contains one of only four possible chemical bases—adenine, cytosine, guanine, and uracil. The synthesis of new strands of HCV RNA entails the assembly, in proper sequence, of a string of nearly 10,000 nucleotides. The synthesis of new strands of genomic RNA is an imperfect process, with no mechanism for "proof-reading" and correcting errors.

Mistakes during strand synthesis get incorporated into HCV RNA as mutations. When a new strand of genomic RNA is synthesized, these mutations are introduced more or less randomly; at any given position on the genome, there is a possibility that a "wrong" nucleotide may be added to the new RNA strand in place of the "right" one that would correspond to the original template. Mutations introduced into a prior round of replication will carry over every time the new RNA strand undergoes another round of replication. Over time, these and other mutations can accumulate with every new cycle of viral replication; therefore, a person chronically infected with hepatitis C will harbor a viral population that consists of a cloud or swarm of minor genetic variants. This viral ensemble is called a quasispecies, the term for a dynamic population of closely related but distinct genetic sequences.

The quasispecies nature of HCV was recognized during early research comparing sequences of various strains of the HCV genome (N. Kato 1990b). The extent of HCV replication provides ample opportunity for the introduction of mutations into the viral population within an infected individual. Viral production has been estimated at 10^{12} (one trillion) new HCV virions per day (Neumann 1998). In comparison with other RNA viruses, HCV has a relatively high mutation rate. The mutation rate of RNA viruses generally ranges between 10^3 and 10^5 nucleotide substitutions per genomic site per year. The mutation rate of HCV has been calculated at 0.816–1.92 x 10^3 (just under one or two per 1,000) substitutions per genomic site per year, based on studies of chronically infected humans and chimpanzees (W. Lu 2001; Ogata 1991; Okamoto 1992; Rispeter 2000). This relatively high rate would translate into the accumulation of between 8 and 18 mutations in genomic HCV RNA for each year of infection.

The extent of global HCV heterogeneity suggests that HCV has a complex evolutionary history with distinctive epidemiological patterns (Bukh 1995; Q. L. Choo 1991; Davidson 1995; Dusheiko 1994; Mellor 1995; D. B. Smith 1997). HCV variants have been classified into six major genotypes, or groupings, based on genetic similarities. Clusters of closely related variants within a genotype are categorized as subtypes (Robertson 1998; Simmonds 1994a; Simmonds 1994b). Genotypes are numbered, in order of their identification, while a letter denotes subtypes (for example, 1b refers to genotype 1, subtype b). The genetic sequences of HCV genomes differ by about one-third between genotypes; comparison of subtypes within a single genotype shows a lesser but still significant degree of variation. Some differences in pathogenicity between genotypes have been reported, though the nature and extent of these differences remain controversial (see Chapter II, Natural History of Hepatitis C); however, HCV genotype strongly influences the likelihood of responding to treatment (see Chapter V, Hepatitis C Treatment). The distribution of HCV genotypes and subtypes may shed light on the origins of hepatitis C.

Figure 2. HCV Phylogenetic Tree



A map of the genetic distances between HCV genotypes and subtypes (adapted from Los Alamos National Laboratory HCV sequence database, http://hcv.lanl.gov).

In theory, knowledge of the origins of a virus could help in understanding how it spreads, and how to develop strategies for prevention and disease control. This information might also shed light on how HCV causes disease and provide clues for designing effective drugs and vaccines. Unfortunately, little is known about the origin and history of the hepatitis C virus. Attempts to trace the origins of HCV have used the methods of molecular epidemiology. This approach combines epidemiology (the study of the global distribution of HCV genotypes and subtypes) with phylogenetic analysis (examining different viral strains for genetic similarities in order to construct a family tree of genotypes and subtypes). In the case of HCV, this form of analysis is highly speculative, depending on multiple unverifiable assumptions about transmission patterns and viral evolution (Simmonds 2001). A common obstacle in phylogenetic investigations of viruses, including HCV, is the absence of a historical archive of viral isolates suitable for study. Reconstructions of trends in viral evolution over time cannot be based on the kinds of evidence (such as fossils) available for studies of other forms of life. As a result, phylogenetic studies of HCV have relied on mathematical models, using recent estimates of the evolutionary rate of HCV.

Presumably, all HCV subtypes and genotypes have descended from a common ancestor, with genetic divergence occurring over time. The greatest diversity among variants is found in sub-Saharan Africa and Southeast Asia, suggesting that human populations in these regions have harbored HCV for longer periods. By analyzing calculated rates of sequence divergence in particular regions of the HCV genome and the geographical distribution of genotypes, initial estimates suggest that HCV genotypes diverged at least 500–2,000 years ago, while subtypes diverged over 300 years ago (D. B. Smith 1997). According to one model, subtypes 1a and 1b first emerged in the early twentieth century, while genotypes 4 and 6 date back hundreds of years (Pybus 2001). These models assume that viral heterogeneity (the extent of the differences between HCV isolates) has increased progressively over time. The evolutionary rate of HCV can be used to calculate when HCV subtypes and genotypes may have diverged from each other. This approach relies on a concept in evolutionary biology called the molecular clock, which assumes that genetic variation and divergence occurs at constant, ascertainable rates over time (Bromham 2003). Those rates vary by species, but known rates of variation can help to reconstruct evolutionary patterns and genetic divergence. The molecular clock has been applied to species evolution in general—the evolution of primates into various families and species, for example.

Some researchers, however, have noted that these calculations are at best conjectural and difficult to interpret due to uncertainties about the applicability of the molecular clock model to viral evolution (Holmes 2003; Simmonds 2001). In contrast to the evolution of animal species, viral evolution may not always occur at a steady rate, especially over the course of centuries. On the one hand, viruses mutate much more rapidly than other organisms; on the other hand, viral genetic constraints and trasmission bottlenecks may result in relative stability over long periods of time. Host immune pressure and genetic factors can also shape the evolutionary dynamics of viruses at a population level (Grenfell 2004; Moore 2002).

Furthermore, the historical record offers virtually no information about how HCV was transmitted during the periods when viral genotypes and subtypes supposedly diverged (Simmonds 2001). HCV transmission patterns in prior centuries must have differed substantially from those seen in recent decades. Most current HCV infections have occurred primarily through parenteral routes, such as drug injection and blood transfusions, factors which were non-existent until relatively

recently. Medical developments, and especially the invention of the hypodermic syringe in the mid-1800s, ushered in an era of widespread potential for exposure to pathogens through injection drug use, shots and vaccinations with unsterile syringes, and blood transfusions. The appearance of these new transmission risks coincides with the relatively recent global emergence of a hepatitis C pandemic during the middle decades of the twentieth century (Holmes 1995). The transmission patterns prior to this era, and the mechanisms for the apparent presence and persistence of HCV in human populations over several centuries, are unclear. HCV transmission is associated with direct blood-to-blood contact; sexual and perinatal transmission of HCV is fairly inefficient (see Chapter I, Epidemiology of Hepatitis C).

HCV most likely originated in non-human primates, but no clear viral antecedent or primate counterpart for HCV has been established. One candidate, GB virus B (GBV-B), bears the closest genetic relationship to HCV. GBV-B is infectious in tamarins, a species of small monkeys found in Central and South America. GBV-B was identified in 1995 from a tamarin infected with plasma derived from the blood of a surgeon (whose initials were G. B.) who had been diagnosed with acute hepatitis of unknown etiology in 1964 (Muerhoff 1995; Simons 1995a; Simons 1995b). Evidence suggests that GBV-B may be native to tamarins, though its prevalence in tamarins or other primates is unknown and warrants further investigation (Simons 1995b; N. D. Wolfe 1998). The value of such evolutionary studies can be seen in HIV research, where the search for viral ancestors in other primates has yielded important insights into the origins of HIV. These investigations have dated cross-species transmission events for HIV to the early decades of the twentieth century (B. H. Hahn 2000). Similar inquiries into HCV's origins may be more difficult. Phylogenetic analysis would indicate that cross-species transmission of HCV occurred well before the twentieth century. Subsequent primate population changes may have resulted in the extinction of a reservoir of antecedent virus. The ultimate origins of HCV, therefore, may never be known, but much can be learned from the virus in its current-day form.

Overview of HCV Research Methods

The innovation, ingenuity, and persistence required in the hunt for the hepatitis C virus in the 1980s also characterize subsequent research efforts aimed at unraveling the viral replication cycle. Such research is vital in order to understand the major challenges and questions posed by HCV infection:

- Persistence—How does HCV establish chronic infection and elude cellular and immune system defenses?
- Pathogenesis—How does HCV cause disease, and why does the course of disease vary in different people?
- Prospects for new therapies—How can drugs successfully target viral replication, offset liver damage, or prevent hepatitis C infection?

These issues require knowledge of the genetic organization of the virus, the structure and function of viral proteins, and how the virus interacts with the cell. Traditionally, researchers study the replication and pathogenesis of viruses through model systems—cell cultures (cells grown in laboratory conditions) and small animals such as mice—that are susceptible to infection and support viral replication. Unfortunately, for reasons that are not well understood, HCV does not

replicate efficiently in cell cultures or mouse livers (Bartenschlager 2001; Grakoui 2001; N. Kato 2000a). For most of the short history of HCV research, the only animal model for infection has been the chimpanzee, which shares 95–98% of its DNA with humans (Britten 2002; Bukh 2001a; Lanford 2001a). These constraints have inspired the creative use of both conventional and novel tools of molecular biology to study the hepatitis C virus.

Defining the genetic code of HCV RNA (the sequencing of the HCV genome) followed rapidly on the heels of the initial identification of HCV (Q. L. Choo 1991; N. Kato 1990a; Takamizawa 1991). The next major task was to identify and characterize the viral proteins encoded by HCV RNA. Researchers developed tools for protein expression (the production of individual HCV proteins) by using cloning vectors (DNA molecules that smuggle HCV genetic material inside a cell that would then synthesize viral proteins). This work led to determinations of the structure and function of the viral proteins, as well as to explorations of their location within infected cells and their interactions with cellular components and other HCV proteins (reviewed in Bartenschlager 2000; N. Kato 2001; Penin 2004). Viral protein research has been critical in developing strategies for designing drugs that can block HCV protein functions and interrupt viral replication (see Chapter X, The Future of HCV Therapy); however, approaches for studying individual viral proteins cannot fully substitute for systems capable of modeling the replication cycle of the entire hepatitis C virus.

Model systems for studying HCV replication

Studies of how viruses replicate inside hosts draw upon three main types of model systems:

Cell cultures use laboratory-grown cell lines derived from human or animal cells to study viral replication at the cellular level. This enables the examination of viral replication dynamics and viral-host cell interactions. Established cell lines, generated from the progeny of a single ancestor, are particularly useful because every cell in the line is genetically identical. This increases the consistency, reproducibility, and reliability of experimental results. The cells can be experimentally modified, and the virus mutated, to pinpoint the role of specific components in the replication process. Cell cultures may also use primary cells, derived directly from virus-infected individuals or animals. HCV primarily infects liver cells, called hepatocytes. Hepatocyte cell lines are thus the most relevant for HCV research. Recent technical advances have yielded hepatocyte cultures sustainable for several months, which will ultimately aid the study of HCV (Lázaro 2003).

Tissue cultures use actual tissue from human or animal organs (for example, liver tissues containing hepatocytes and surrounding structures). They can thus provide information on how viruses affect infected cells and the nearby environment, including other cell types. A tissue culture for HCV would enable better understanding of the mechanisms of fibrosis and liver injury. Currently no adequate tissue culture system is available that can model HCV pathogenesis in the liver (Lanford 2002; Pietschmann 2003).

Animal models examine viral infection in a living animal, studying the effects of a virus on the entire organism. Such research may be ethically unsound and logistically unfeasible in humans. Animal models allow researchers to look at overall viral-host dynamics beyond the cell and tissue level (for instance, the effects of the immune response on viral infection, and the distribution of the virus throughout the body). Primate models, such as chimpanzees, can provide particularly valuable information because of their genetic closeness to humans. However chimpanzees, an endangered species, are expensive and limited in availability for research. Small animals (particularly mice) offer several advantages in virology research, including their relative convenience and availability. The substantial genetic differences between mice and humans can be compensated for by introducing foreign genes into mice, producing transgenic mice. Human organ tissue, such as liver tissue, cannot be grown in mice, and must be grafted or transplanted into mice. However, engrafted human liver cells can divide after being transplanted into mice.

Success with cell and tissue cultures for HCV research has been limited until recently. Attempts to culture the hepatitis C virus itself in laboratory cell lines or primary cells have suffered from poor reproducibility and very low levels of viral replication (Bartenschlager 2001; N. Kato 2000a). For unknown reasons, introducing HCV into cell lines (even ones derived from hepatocytes, which should be an optimal environment for the virus) does not reliably result in sustained viral replication at detectable levels.

Subsequent efforts to develop cultures have attempted to use HCV genes, combined with the genetic material of other viruses or bacteria, as a surrogate for the virus itself. Initial progress in developing a cell culture system supporting HCV replication came with the construction of molecular clones based on full-length genomic HCV RNA (Kolykhalov 1997; Yanagi 1997). These clones have enabled studies of viral infectiousness in chimpanzees. Other researchers introduced specific HCV genes into other viruses, producing hybrid viral forms referred to as pseudotyped or chimeric viruses. Genetic material from HCV has been incorporated into several viruses for which cell culture systems exist, including bovine viral diarrhea virus, poliovirus, vesicular stomatitis virus, and yellow fever virus (Blanchard 2003a; Ezelle 2002; Macejak 2000; Matsuura 2001; Molenkamp 2003; Nam 2001; W. D. Zhao 2000; W. D. Zhao 2001). These chimeric viruses are used to study the role of various HCV proteins in viral replication and viral–host cell interactions, and to screen for potential anti-HCV drug candidates against select HCV proteins.

The most important development in HCV cell culture systems has been the advent of HCV replicons. Replicons are genetic material capable of autonomous replication and protein synthesis (Blight 2000; Lohmann 1999). HCV replicons are generated from synthetic DNA molecules that contain genetic material corresponding to some or all of the HCV genome. In cells, these molecules yield RNA transcripts containing HCV genes, along with other RNA sequences, that allow viral replication to occur and be measured. In lieu of a cell culture system for the virus itself, HCV replicons presented a major breakthrough for examining the molecular biology of HCV and developing screening methods for drug discovery (Bartenschlager 2002; Grakoui 2001; Lanford 2002; Randall 2001; V. Wu 2001). The first HCV replicons were described as subgenomic, since they only contain parts of the HCV genome (Blight 2000; Lohmann 1999). Recent efforts have led to the development of genomic replicons incorporating the full HCV genome (Blight 2002; M.

Ikeda 2002; Pietschmann 2002). A cell-free replication system derived from HCV replicon-bearing cells has also been described (N. Ali 2002); however, the assembly and release of infectious virions or HCV-like particles following replication still have not been observed in cell culture systems (Molenkamp 2003; Pietschmann 2002). Therefore even full-length genomic HCV replicons lack the capacity of actual hepatitis C virions to infect other cells, and cannot help elucidate the process of viral entry. Despite these limitations, replicon systems have opened up dramatic new possibilities for the study of viral replication, and will be particularly valuable in the preclinical assessment of potential antiviral drugs targeting HCV replication.

HCV replicons have other limitations:

- Replicons thus far only replicate efficiently in a particular cell line derived from human hepatocytes, designated Huh7. Replication of most HCV replicons has not been observed in other cell lines derived from human hepatocytes, though new research documents replication of the JFH-1 replicon in HepG2 and IMY-N9 hepatocyte-derived cell lines, albeit at lower efficiencies than those seen in Huh7 cells (Date 2004). Recent reports have also documented replication in a human embryonic kidney cell line, a human non-hepatic epithelial cell line, and a mouse hepatoma cell line (S. Ali 2004; Bartenschlager 2002; Q. Zhu 2003).
- Even among Huh7 cell lines, not all cells support replication; selection of specific lines of permissive "daughter" cells from the "parental" Huh7 cell line can increase replication efficiency 100-fold. Presumably, permissive cells either contain factors that support, or lack factors that inhibit, HCV replication, but these factors have not been identified (Blight 2002; Lohmann 2003; Murray 2003).
- Replicons have generally required adaptive HCV gene mutations to replicate at adequate levels within Huh7 cells; however, some of these adaptations are infrequently seen in naturally occurring HCV genomes, and may reduce the infectiousness of HCV. In genotype 1 replicons, the original HCV strains that replicate best *in vivo* seem to require more mutations to function efficiently as replicons (Bartenschlager 2002; Blight 2000; Bukh 2002; Evans 2003; Krieger 2001; Lohmann 2001; Lohmann 2003).
- Replication-competent replicons have been constructed from only certain strains of HCV, almost exclusively genotype 1 HCV RNA. Prior to recent reports describing the establishment of replicons derived from genotypes 1a and 2a, only genotype 1b replicons had succeeded in replicating in cell cultures; replication efficiency also varies among replicon strains (Bartenschlager 2002; Blight 2003; Date 2004; Evans 2003; Gu 2003a; T. Kato 2003b; Pietschmann 2003)

These challenges in refining HCV replicon models may derive from the characteristics of specific cell lines, which vary in levels of cellular gene expression and response to viral proteins. Even subtle variations in host factors between cell lines may produce divergent experimental outcomes (Borman 1997; Collier 1998; Koev 2002; Lagging 1998; Meng Soo 2002; Podevin 2001). Further refinements in HCV replicon systems will require more detailed knowledge of viral–host cell interactions. Recent major advances in extending host cell range and developing viable genotype

1a and 2a replicons provide crucial opportunities to study the role of host factors in HCV replication.

Aside from humans and chimpanzees, only one other species has been observed to have susceptibility to HCV infection. Two studies have reported that Tupaia belangeri, a primate-like species of Asian tree shrews, can be infected with HCV (Lanford 2001a; Lanford 2002; Xie 1998; X. Zhao 2002). Chimpanzees have had tremendous significance to hepatitis C research, beginning with the discovery of HCV and continuing with research into infectivity, viral clearance, immune responses, and gene expression; however, a cheaper and more widely available primate model for HCV infection would be extremely valuable for the testing of potential anti-HCV drugs and vaccines before they enter clinical trials in humans.

GB virus B, the virus perhaps most closely related to HCV, does infect tamarins, though HCV itself does not infect this family of small new world monkeys (Bukh 2001b). GBV-B may provide a surrogate animal model for certain studies exploring the dynamics of HCV infection. Chronic GBV-B infection has recently been observed in a tamarin, increasing the relevance of this model to HCV, with some close parallels to hepatitis C pathogenesis (A. Martin 2003). A tissue culture system using tamarin hepatocytes has been developed (Beames 2000; Beames 2001). Ideally, infectious molecular clones containing genomic elements of both GBV-B and HCV could be used in tamarins instead of chimpanzees (Bukh 1999; De Tomassi 2002; Sbardellati 2001). Marmosets, a related small New World primate, are also susceptible to GBV-B infection, and have been used to study the activity of antiviral drugs targeting HCV (Bright 2004); however, differences between the viruses can affect their relative susceptibility to inhibition by antiviral compounds (Ranjith-Kumar 2003).

Developing a mouse model for HCV infection is a high priority for drug discovery as well as for research on pathogenesis. Mice are not naturally susceptible to HCV infection, though transgenic mice can be genetically engineered to constitutively express HCV proteins (Feitelson 2001). These transgenic mice have been used to study the viral mechanisms underlying complications of HCV infection such as hepatocellular carcinoma (Ishikawa 2003; Koike 2002a; Koike 2002b; Moriya 1997; Moriya 1998; Moriya 2001a; Moriya 2001b). Other groups have developed more sophisticated approaches, transplanting human hepatocytes or HCV-infected human liver tissue into immune-deficient mice (Dandri 2001; Ilan 2002; Mercer 2001). Viral replication has been observed in these mice, suggesting that these models could be useful to screen potential anti-HCV drugs (Ilan 2002).

Despite their promise, these methods need refining. The transplantation process is difficult and time-consuming. About one third of these transgenic mice die shortly after birth. The limited availability of fresh human hepatocytes poses further difficulties, and techniques for the cryopreservation, or freezing, of hepatocytes, which would reduce the need for fresh cells, require further work (Brass 2002; Pietschmann 2003). These factors limit the utility and availability of a small-animal model. One commentator noted that, despite the importance of these models, he "suspect[s] that not many virologists will jump into hepatitis C research upon reading this [research]" (Fausto 2001). Furthermore, studies of viral pathogenesis and viral-host interactions would require that these mice be engineered to simulate the human immune system (Brass 2002; Pietschmann 2003). Nonetheless, this work constitutes a major advancement in HCV research and encourages the development of more refined mouse models of HCV infection and replication.

While the last five years have ushered in dramatic advances in HCV model systems (including replicons and small-animal models) further advances will require a sustained effort, ultimately bringing science closer to the holy grails of HCV research: a reliable, efficient cell culture system, a tissue culture system supporting viral infection and replication, and a small-animal model mimicking HCV pathogenesis in humans.

The Hepatitis C Viral Replication Cycle

Background: Viral Replication

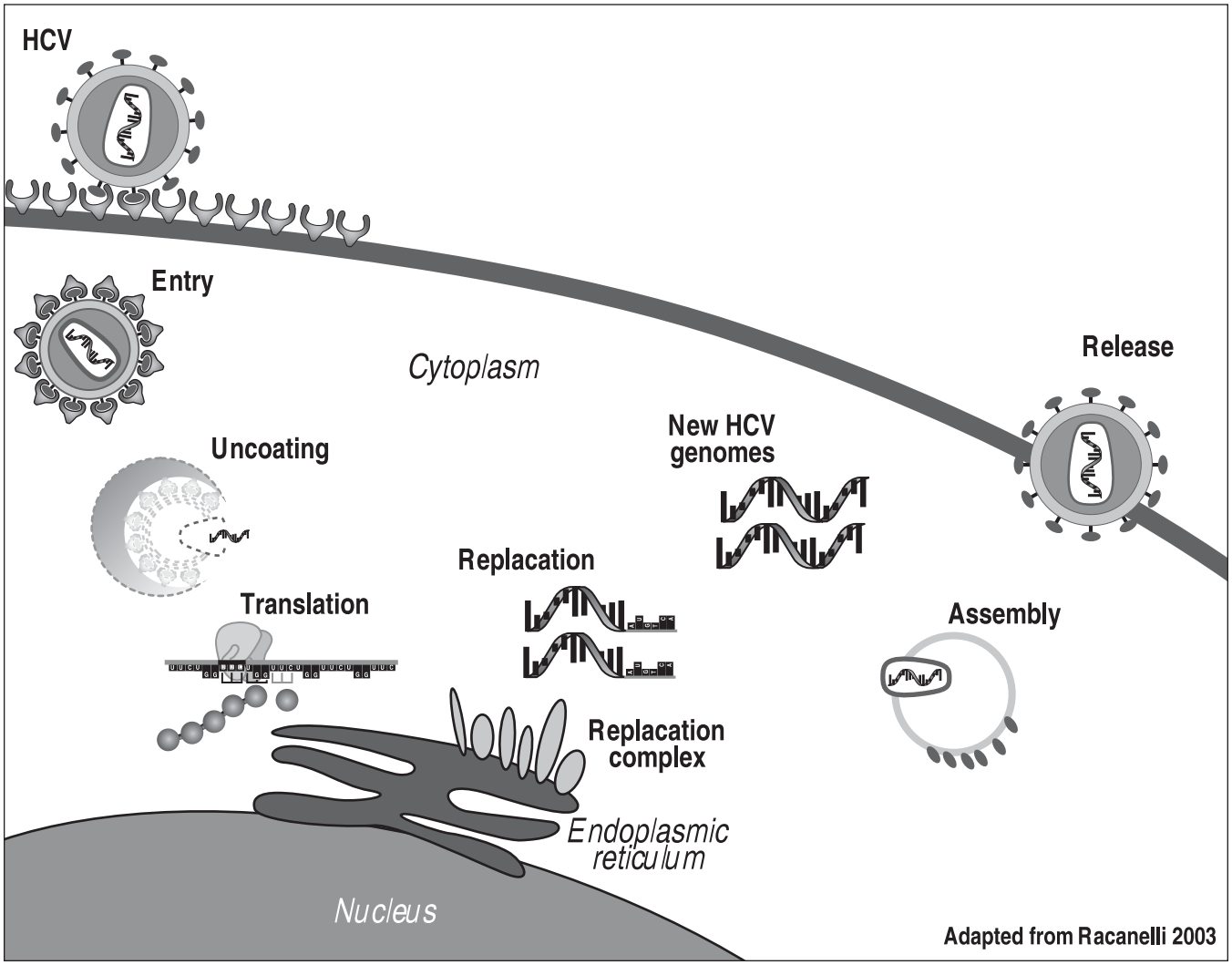
The primary goal of all viruses, including hepatitis C, is to replicate, to make new copies of itself. Unlike other organisms, a virus cannot replicate on its own; it simply doesn't possess all the tools necessary for the task. To replicate, HCV and other viruses must infect other cells and hijack the cellular apparatus, including enzymes and other proteins. An infected, or target, cell is called a host cell, because it "hosts" the virus. The term "host" is also used to describe the species susceptible to infection by a given virus. The hepatitis C virus primarily targets hepatocytes in the liver. Because HCV preferentially infects liver cells, the virus is considered hepatotropic (tropos is Greek for turning; hence, HCV "turns" toward the liver).

Once HCV enters the host cell, all subsequent events in the replication cycle occur in the cell's cytoplasm, the main area of the cell inside its membrane but outside of its nucleus. Both cellular proteins and viral proteins facilitate the progression of HCV through its replication cycle. The HCV genome encodes at least ten different viral proteins, including structural proteins (the envelope and core proteins) and nonstructural proteins. The structural proteins are incorporated into the capsid and envelope of new virions, while the nonstructural proteins are involved in the viral replication process. Research over the last decade has elucidated the functions of many of these proteins, but some proteins' roles in viral replication remain unclear, and many aspects of HCV replication and viral–host cell protein interactions are poorly understood (Ahlquist 2003).

HCV replicates through the following cycle (See Figure 3):

1. Host cell attachment, entry, and uncoating;
2. Translation of the HCV genome into viral proteins;
3. Cleavage and processing of viral proteins;
4. Replication of HCV genome; and
5. Assembly of new virions and release from host cell.

Figure 3. HCV Replication Cycle



The following sections will summarize current knowledge about the viral replication cycle and provide a foundation for discussion in subsequent chapters about HCV pathogenesis and drug development.

Stage 1: Host Cell Attachment, Entry, and Uncoating

The virus must enter a target cell to replicate. The events surrounding HCV's entry into cells are not known in any detail and have been difficult to research. In general, viruses in the Flaviviridae family enter cells in three stages:

1. Attachment. The viral envelope attaches to molecules on the surface of the target cell.
2. Entry. The virus passes through the cell's outer membrane (the plasma membrane), entering the target cell.
3. Uncoating. The virus sheds its envelope and releases the viral genome from the inner capsid shell into the cell's cytoplasm.

The study of viral entry attempts to address three broad questions:

- Which cells does HCV target?
- How does HCV attach to target cells?
- How does HCV enter cells and release its genome into the cell?

Cells targeted for infection by HCV

HCV, like other viruses, can infect and replicate only inside of certain cell types. Cell types that are susceptible to HCV infection are referred to as permissive cells. The range of cell types that a virus can enter is referred to as cell tropism (Baranowski 2001; Schneider-Schaulies 2000). HCV primarily infects hepatocytes, the main cell type in liver tissue; however, HCV has also been found in a range of other cells outside of the liver. These cells include white blood cells, components of the immune system. In particular, HCV has been found in certain peripheral blood mononuclear cells (PBMCs), specifically monocytes and macrophages, dendritic cells, T cells, and B cells (Caussin-Schwemling 2001; Goutagny 2003; Rodríguez-Iñigo 2000). HCV infection has also been observed in bone marrow cells (pluripotent hematopoietic CD34+ cells), the progenitors of these white blood cells (Radkowski 2000; Sansonno 1998). Many if not most viral particles circulating outside of cells are found in complexes bound to immunoglobulins (antibodies), lipoproteins, and platelets (André 2002; S. H. Choo 1995; Hamaia 2001; Hijikata 1993a; Kono 2003; Thomssen 1992; Thomssen 1993)

HCV replication in hepatocytes is well established. What is less clear is the extent of extrahepatic viral replication (whether HCV is capable of replicating outside of the liver) in PBMCs or bone marrow cells. In theory, HCV may be able to infect some cell types that do not themselves support viral replication. The ability of a particular cell type to support viral replication may depend on the requirements of later stages of the viral replication process, such as protein synthesis (Yanagiya 2003). Claims for replication in these other cell types have been controversial, though recent refinements in research methods may help clarify this issue (Laskus 1997; L. Lin 2002; Meier 2001).

The implications of HCV infection of PBMCs and bone marrow cells are also uncertain. These cells may constitute a viral reservoir (a pool of virus outside of the liver). In theory, successful treatment could clear HCV from the liver, while leaving viral reservoirs untouched. This could allow HCV to reestablish itself in the liver, making viral eradication through treatment more difficult. This scenario has not been documented in chronic HCV infection; however, such reservoirs may be partly responsible for HCV resurgence among liver transplant recipients (see Chapter V, Hepatitis C Treatment).

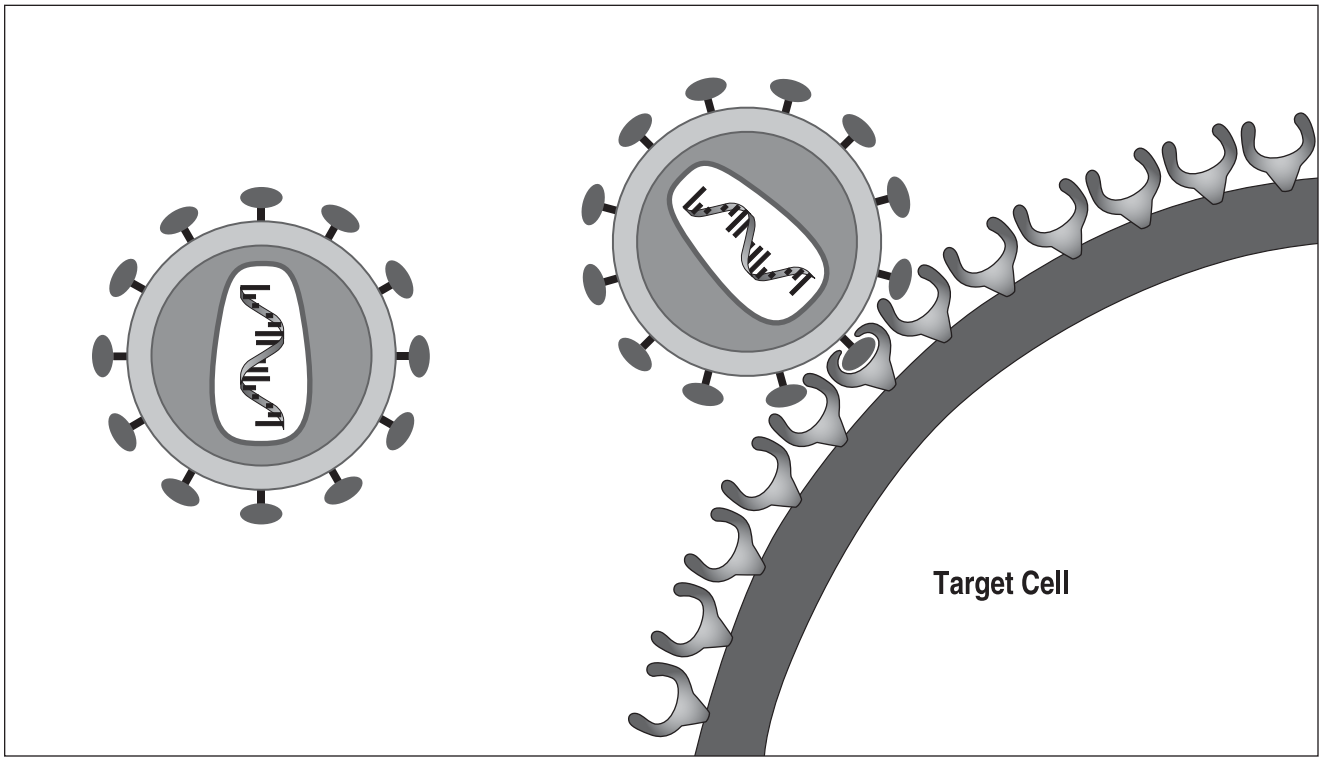
The extent of HCV infection of PBMCs may differ among HCV-infected individuals; it appears more common among people with a history of injection drug use (Resti 2002). Some evidence suggests that HCV replication in PBMCs is more likely to occur in people coinfected with HIV (Laskus 1998b; Laskus 2000; Laskus 2004). Even among HIV-negative persons, HCV infection of PBMCs and bone marrow cells may help impair immune responses to HCV (see Chapter IX, Immune Response, Persistence, and Pathogenesis). Infected PBMCs may also shuttle the virus to other parts of the body, and perhaps cause other extrahepatic manifestations of HCV disease (see

Chapter II, Natural History of Hepatitis C). For instance, HCV has been found in autopsied brain tissue, presumably carried by macrophages, perhaps contributing to the neurological complications sometimes associated with HCV (Radkowski 2002). Finally, HCV-infected PBMCs may facilitate viral transmission. A study of mother-to-child transmission found a strong association between detection of HCV in PBMCs and the likelihood of vertical transmission (Azzari 2000; see also Chapter I, Epidemiology of Hepatitis C). Further research on HCV cell tropism may shed light on viral pathogenesis, HIV coinfection, and HCV transmission.

Attachment of HCV to target cells

Attachment is believed (by analogy to other viruses) to occur through interactions between HCV's envelope and molecules on the surface of potential host cells. The HCV envelope includes two envelope proteins, E1 and E2, produced by the virus. E1 and E2 are bound together on the envelope, forming heterodimers (complexes formed by two different proteins) (Op de Beeck 2001). HCV, like other viruses, uses one or both of these envelope proteins to attach to molecules on target cells, whose outer membranes are studded with various molecules called cell surface receptors. These receptors are proteins that play a role in communication between cells. There are many kinds of cell surface receptors, each binding to one or more specific molecules or proteins. Receptor-binding proteins such as hormones and cytokines use the receptor to send signals to the cell's interior. The molecule that binds to a particular receptor is referred to as its ligand. While some receptors are ubiquitous, appearing on the surfaces of virtually all cell types, other receptors are specific to a particular type of cell. The receptors HCV can bind to, therefore, determine which kinds of cells the virus can enter.

Figure 4. Attachment of HCV to Target Cells



While several candidate HCV receptors have been proposed, their roles in viral entry have not been definitively established. HCV may require more than one type of receptor for attachment, for instance a primary receptor and a co-receptor, as with HIV infection (Schneider-Schaulies 2000). The search for cell surface receptors capable of binding to HCV virions has yielded three primary candidates: the CD81 receptor, the low-density lipoprotein (LDL) receptor, and the human scavenger receptor class B type I (SR-BI). HCV also binds to the molecules DC-SIGN (dendritic cell–specific intercellular adhesion molecule 3-grabbing non-integrin) and L-SIGN (liver/lymph node–specific intercellular adhesion molecule-3-grabbing integrin). Researchers have used a number of criteria to identify potential HCV receptors:

- Is the receptor expressed on the types of cells (hepatocytes, etc.) that HCV infects?
- Does the receptor bind to HCV particles, and can the binding site(s) for E1 or E2 be identified?
- Does blocking the receptor's binding site(s) prevent viral entry?
- Is the presence of the receptor sufficient for viral attachment and entry, or are co-receptors required?

CD81: CD81 is nearly ubiquitous on human cells, including hepatocytes and B cells (Maecker 1997). CD81 is involved in many cellular processes, including activation, adhesion, and differentiation, as well as the production of antibodies by B cells (S. Levy 1998; Maecker 1998). CD81's ability to bind to a region of the HCV E2 envelope protein is well documented, but CD81 appears to be insufficient for HCV entry into hepatocytes or binding to other cells (Hamaia 2001; M. Hsu 2003; Masciopinto 2002; Meola 2000; Roccasecca 2003; Sasaki 2003; Takikawa 2000; X. Zhao 2002). Some *in vitro* studies show that HCV-like particles containing E1 and E2 envelope proteins can still bind to cell lines lacking CD81 (Hamaia 2001; Roccasecca 2003; Sasaki 2003; Wellnitz 2002). Other studies have cast further doubt on CD81's specific role in viral entry, finding that viral binding to target cells is only partially inhibited by blocking the CD81 receptor. CD81 also appears to be relatively inefficient at internalizing E2 after binding (Germi 2002; Hamaia 2001; Petracca 2000; Roccasecca 2003; Triyatni 2002).

Some research, however, suggests that CD81 may function as a co-receptor aiding HCV in binding to another receptor (M. Hsu 2003; Pileri 1998). In particular, CD81 may facilitate the initial attachment of HCV E2 to the target cell as part of a receptor complex, with one or more other receptors responsible for binding and entry (Bartosch 2003b). Some studies that questioned CD81's role in viral entry used a truncated form of E2, which may not adequately model actual *in vivo* interactions. A recent report found that E1-E2 heterodimeric complexes bind to CD81 much more efficiently than truncated E2 alone (Cocquerel 2003b). Another recent study found that CD81 expression on target cells is necessary but not sufficient for viral entry (J. Zhang 2004).

LDL receptor (LDLR): Lipoproteins are proteins bound to fat molecules (cholesterol and triglycerides). Low-density lipoprotein receptors (LDLRs) help to regulate cellular cholesterol levels. LDLRs shuttle cholesterol-bearing lipoproteins into cells through receptor-mediated endocytosis. While all cells use cholesterol, the liver is a primary site for cholesterol metabolism. The HCV E1 and E2 envelope proteins both bind to low-density, very-low density, and high-density lipoproteins (Kono 2003; Monazahian 2000; Prince 1996; Thomssen 1992; Thomssen 1993). Indeed, many

circulating viral particles in HCV-infected people are bound in complexes with lipoproteins (Kono 2003; Thomssen 1992; Thomssen 1993). This suggests another potential mechanism of viral attachment, perhaps independent of the E1 and E2 envelope proteins, through binding of HCV-associated lipoproteins to cell surface receptors (Agnello 1999; André 2002; Germi 2002; Monazahian 1999). However, research on HCV attachment and entry has not yielded convincing evidence establishing a role for the LDL receptor (M. Hsu 2003; Wellnitz 2002; Wünschmann 2000).

SR-BI: SR-BI, like the LDL receptor, is a lipoprotein receptor involved in lipid metabolism. While the LDLR binds to low-density lipoproteins and is expressed on a wide range of cell types, SR-BI binds to high-density lipoproteins and is found in high concentrations primarily on hepatocytes. SR-BI was recently shown to bind to the HCV envelope protein E2 (Op de Beeck 2003; Scarselli 2002). A subsequent *in vitro* study found that SR-BI expression alone was not sufficient for infection of target cells (M. Hsu 2003). Most recently, another group found that on hepatic cells expressing CD81, SR-BI expression was essential for viral entry, which could be blocked by anti–SR-BI antibodies that prevented E2 binding (Bartosch 2003b). The latter study, however, indicated that one or more cellular factors in addition to SR-BI and CD81 were likely involved in viral entry.

DC-SIGN and L-SIGN: DC-SIGN and L-SIGN are closely related members of a class of carbohydrate-binding molecules called C-type lectins. DC-SIGN is predominantly expressed on dendritic cells, components of the immune system that patrol the body for foreign particles such as viruses and bacteria. Dendritic cells capture and process these particles, bringing them to lymph nodes (small tissue sites scattered throughout the body that serve as central meeting grounds for immune system cells). Dendritic cells then present the processed particles to CD4 T cells, which launch an immune response (see Chapter IX, Immune Response, Persistence, and Pathogenesis). L-SIGN is expressed on endothelial cells, a type of cell that lines blood vessels and separates blood from surrounding organ tissue. L-SIGN is primarily found on a type of endothelial cell found in the liver called liver sinusoidal endothelial cells (LSECs), as well as on the endothelial cells in lymph nodes. According to a recent report, LSECs can also express DC-SIGN (W. K. Lai 2004).

Both DC-SIGN and L-SIGN can bind to E2, though it is not clear whether this results in viral entry. HCV infection has been observed in dendritic cells, but no reports have yet documented the presence of HCV within endothelial cells (Goutagny 2003; Laporte 2003; Navas 2002). L-SIGN and DC-SIGN may act not primarily as viral entry receptors themselves, but rather as facilitators of HCV entry into hepatocytes and perhaps PBMCs (M. Hsu 2003). This form of cellular hijacking would mirror the role of DC-SIGN (and L-SIGN) in HIV infection, where these molecules bind to HIV and bring the virus into contact with its main target, CD4 T cells (Bashirova 2001; Geijtenbeek 2003; Pöhlmann 2001; Soilleux 2002; Soilleux 2003). For HCV, capture by L-SIGN could serve as a Trojan horse strategy, with liver sinusoidal endothelial cells inadvertently passing HCV on to hepatocytes (Feng 2004). Alternatively, viral binding to dendritic cells via DC-SIGN may function to disrupt immune responses, independent of any role in viral entry (see Chapter IX, Immune Response, Persistence, and Pathogenesis).

Other candidates: Viral entry may also be facilitated by other cell surface molecules that do not themselves function as receptors. Some researchers have suggested a role in HCV attachment and entry for glycosaminoglycans (GAGs), a group of polysaccharides such as heparin and heparan sulfate present on cell surfaces. In this model, GAGs would facilitate HCV's initial attachment to target cells, and support or strengthen the association between HCV and its receptor, as has been seen with other viral infections (Germi 2001; Germi 2002; Takikawa 2000). One group recently suggested that the E2 protein requires heparan sulfate proteoglycans, a type of GAG, for binding to target cells (Barth 2003). A section of E2 called the hypervariable region 1 (HVR1) can bind to cell surface GAGs, though this interaction is neither sufficient nor perhaps necessary for viral entry (Basu 2004).

Despite the range of candidates, there is good reason to believe that at least one other, as yet unidentified, HCV receptor is necessary for viral entry (M. Hsu 2003; Op de Beeck 2003; Pandya 2002; J. Zhang 2004). However, the ability of a particular viral envelope protein to bind to a given receptor does not constitute proof of that receptor's involvement in the infection of target cells. The binding properties of E2 may provide valuable clues for understanding HCV pathogenesis and immune response, but viral entry appears to require the presence of both E1 and E2 in their heterodimeric form (Bartosch 2003a; M. Hsu 2003; Takikawa 2000; Triyatni 2002). Similarly, the ability of an entire viral particle to bind to a particular receptor (such as L-SIGN) does not guarantee that HCV uses this receptor for cell entry.

Many of the challenges in studying binding and attachment stem from the lack of an efficient cell culture system that supports both viral replication and infection of new cells. Currently available HCV replicon models do not produce viral particles that are infectious to other cells in culture. Recent encouraging advances in the methods for researching viral entry will likely aid in identifying the actual receptors that HCV uses to infect cells (Bartosch 2003a; Cocquerel 2003b; M. Hsu 2003; Lambot 2002; Op de Beeck 2003). HCV pseudotype particles have envelopes that bear HCV E1 and E2 proteins on their surfaces; this envelope surrounds a core particle from a different type of virus, such as HIV. Theses infectious pseudotype particles have already begun to clarify HCV entry requirements (Bartosch 2003b; Castet 2003; Dumonceaux 2003; M. Hsu 2003; Op de Beeck 2004; J. Zhang 2004). The arrangement of HCV envelope proteins on retrovirus core particles appears to resemble the natural conformation and properties found on HCV virions, suggesting that HCV pseudotype particles are a valid model for studying viral entry (Op de Beeck 2004). Future research should elucidate the mechanisms of viral attachment leading to entry, and may provide clues for developing drugs that inhibit HCV attachment and entry (see Chapter X, The Future of HCV Therapy).

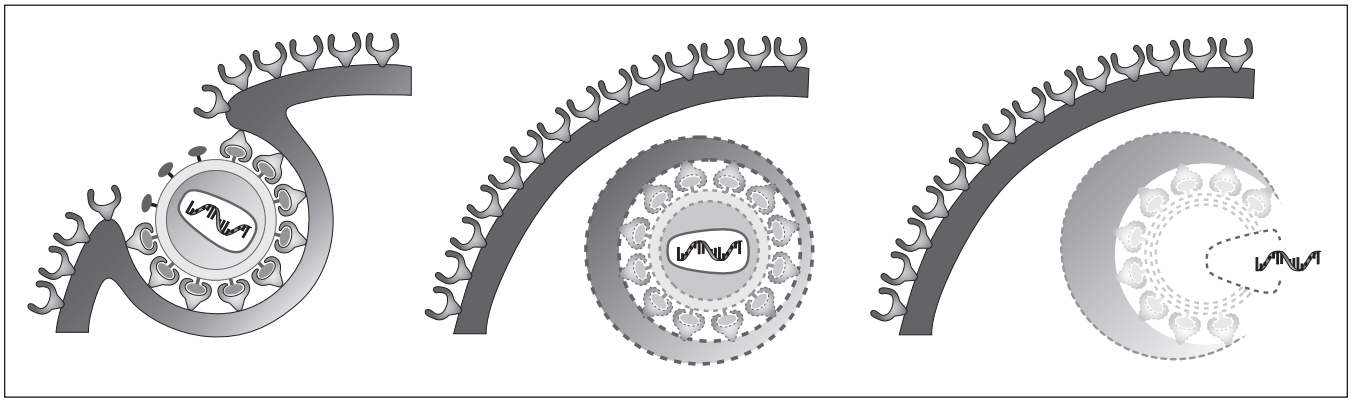
HCV entry into target cells and uncoating

The entry of HCV into target cells most likely occurs through receptor-mediated endocytosis, in which a cell internalizes a ligand-bound surface receptor. The receptor-ligand complex enters the cell encapsulated within a vesicle, a small pocket of fluid surrounded by a thin membrane. Cells use endocytosis to internalize particles involved in signaling and cell growth such as hormones, proteins, growth factors, and cholesterol. Receptor-mediated endocytosis is also used by flaviviruses closely related to HCV, though this mode of cell entry has not been conclusively demonstrated for HCV (Op de Beeck 2003). Other modes of viral entry, such as direct fusion with the cell

membrane (as with HIV's viral fusion protein, gp41) cannot be ruled out (Flint 2001; Hernandez 1996). Recent descriptions of the structure and mechanism of the envelope fusion proteins for the flaviviruses dengue virus and tick-borne encephalitis virus have spurred speculation that HCV may also enter host cells through fusion (Bressanelli 2004; Drummer 2004; Modis 2004). Recent evidence, however, indicates that hepatitis C viral entry is dependent on pH levels (the degree of acidity), a characteristic of receptor-mediated endocytosis but not of direct fusion (Bartosch 2003b; M. Hsu 2003).

Uncoating is the process that releases the viral genome into the cell's cytoplasm. Following entry, HCV must release its genome from its capsid shell and surrounding viral envelope in order to begin replication. Though the specific uncoating process for HCV has not been characterized, it likely follows a sequence of events common to other enveloped viruses entering through receptor-mediated endocytosis. In this model, the HCV envelope would fuse to the vesicle membrane once inside the cell. Fusion may depend on a change in pH levels within the vesicle. Envelope–vesicle membrane fusion results in the degradation of the viral capsid surrounding the viral genome, releasing the viral RNA into the cell's cytoplasm.

Figure 5. HCV Entry and Uncoating



The events surrounding HCV's entry into the cell are some of the least well-characterized aspects of the viral replication cycle. Improvements in research methods and model systems will clarify viral entry and uncoating, and could lead to therapies targeting these events in the viral life cycle.

Summary of HCV entry:

- HCV is an enveloped virus; the viral envelope contains two HCV proteins, E1 and E2, joined as heterodimers.
- HCV entry is thought, by analogy with other flaviviruses, to enter cells through receptor-mediated endocystosis.
- Candidate receptors include CD81, LDLR, and SR-BI.
- HCV entry may require more than one receptor, and the receptor(s) may differ according to cell type.
- Binding and entry may be mediated by cellular or endogenous proteins such as lipoproteins and glycosaminoglycans, as well as by HCV envelope proteins E1 and E2.
- There may be other HCV receptors that have not yet been identified.

Stage 2: Translation of the HCV Genome into Viral Proteins

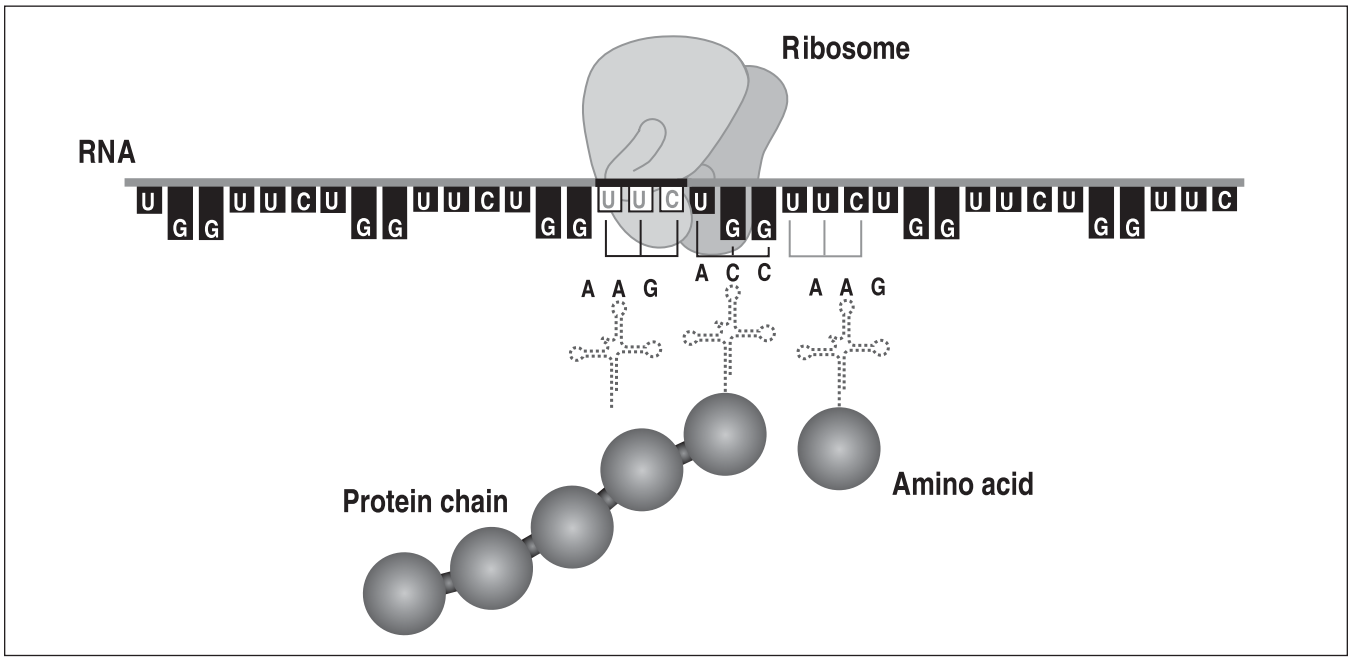
Once released inside the cell, HCV RNA is used as a blueprint for the production of viral proteins. The process of protein synthesis from RNA is called translation and uses cellular components (in particular, ribosomes) that are also employed in translating the cell's own mRNA into proteins. The primary product of HCV translation is a single polyprotein (a long protein chain) consisting of over 3,000 amino acids. The polyprotein contains all ten HCV proteins required for HCV replication.

Research into HCV translation falls into three overlapping areas:

- How does HCV initiate protein synthesis?
- How does the regulation of viral protein synthesis differ from that of cellular protein synthesis?
- What viral and cellular factors regulate this process?

All proteins, both viral and cellular, are assembled from molecules called amino acids. The structure and function of a protein is determined by its amino acid sequence and composition. Messenger RNA (mRNA) provides the genetic blueprint for protein assembly and determines the amino acid sequence of a given protein. In the translation process, large cellular complexes called ribosomes "read" this mRNA blueprint, translating the genetic code into instructions for protein synthesis. The ribosome works with other cellular components to assemble a chain of amino acids corresponding to the mRNA genetic sequence. These amino acids, or peptide chains, will form a new protein when translation is complete.

Figure 6. Translation



Translation is remarkably complex and exacting, requiring the choreography of multiple cellular components. Their availability and interactions with mRNA regulate translation, which is more

efficient when these components are more abundant. Cellular conditions determine the relative abundance of components required for translation, upregulating or downregulating protein translation depending on the cell's needs.

Protein synthesis is key to HCV replication, since new virions cannot be produced until key viral proteins have been synthesized. HCV translation requires some, but not all, of the translation components used by cells. This may allow HCV to initiate efficient translation even when cellular conditions do not favor protein synthesis. Indeed, conditions that limit the translation of cellular proteins may actually promote the translation of hepatitis C proteins.

Initiation of HCV protein synthesis

HCV RNA contains a complex structure called an internal ribosomal entry site (IRES) at the beginning of its genome (Tsukiyama-Kohara 1992; C. Wang 1993). The ribosome binds directly to the HCV IRES, which directs the ribosome to the mRNA site where translation is initiated. Virtually the entire HCV genome is translated, except for regions at either end of HCV RNA. These regions are therefore called the untranslated regions (UTRs), and designated the 5′ UTR (5-prime untranslated region) and the 3′ UTR (3-prime untranslated region). Translation of mRNA is directional, beginning at the RNA site immediately following the 5′ UTR and proceeding until reaching the 3′ UTR. The long region of RNA between the 5′ UTR and the 3′ UTR contains the genetic sequence encoding all HCV proteins. This region is called the open reading frame, because it frames the genetic sequences read by the ribosome.

HCV also appears to use an alternate form of translation, called frameshifting, in which the ribosome initiates translation from a slightly different site on HCV RNA, through which HCV synthesizes at least one other viral protein, ARFP (alternative reading frame protein) or F (frameshift) protein (Boulant 2003; Choi 2003; Roussel 2003; Varaklioti 2002; Vassilaki 2003; Xu 2001). The function of the F protein, and the conditions governing its expression, are currently unknown. Some evidence suggests that F protein expression may vary by genotype (Boulant 2003).

The IRES, comprising almost the entire 5′ UTR of HCV RNA, is folded into a three-dimensional scaffolding which contains various structural elements such as stem and hairpin loops, helices, and a pseudoknot (Beales 2001; Brown 1992; Fukushi 1994; M. Honda 1996; Kieft 1999; Rijnbrand 1995; C. Wang 1994; C. Wang 1995). These elements allow the IRES to bind to and assemble the ribosome and other cellular proteins required to initiate translation. Some of these proteins are called eukaryotic initiation factors (eIFs), cellular proteins that facilitate translation by coordinating ribosome assembly and its proper positioning at the mRNA site where translation begins. HCV uses two of these initiation factors, designated eIF2 and eIF3, to initiate translation.

Differences between HCV protein synthesis and cellular protein synthesis

Cellular mRNA, like HCV, uses ribosomes to assemble amino acids into proteins; however, internal ribosomal entry sites, though not uncommon in viral mRNA, are extremely rare in cellular mRNA, which therefore lacks a structure that can directly bind to the ribosome. Instead of an IRES, cellular mRNA has a chemical group at its 5′ end, called a methylated cap (because it "caps" the mRNA), which binds to another eukaryotic initiation factor, the eIF4 complex. This complex guides the

mRNA to the ribosome to initiate translation. This form of translation is called cap-dependent, because it relies on the interaction between the methylated cap and the eIF4 complex. HCV does not have a methylated cap, instead using its IRES to bind directly to the ribosome, a mechanism of translation initiation called cap-independent (synonymous with IRES-directed). Thus HCV does not require the eIF4 complex to begin translation. Another difference between cellular and HCV translation hinges on how the ribosome is positioned at the proper mRNA initiation site. HCV positions the ribosome directly at the initiation site, but the ribosome must scan cellular mRNA until it finds the initiation site. By replacing eukaryotic initiation factors and omitting key steps such as scanning required by cap-dependent translation initiation, HCV expedites viral protein synthesis (Kieft 2001; Kolupaeva 2000; Pestova 1998; Pestova 2001; Spahn 2001).

Some research has linked the regulation of HCV translation to the cell cycle, the series of events that a cell undergoes when it divides into two cells. HCV IRES activity seems to be relatively low in resting, nondividing cells, but viral protein synthesis increases in actively dividing cells (M. Honda 2000; Shimazaki 2002). This pattern contrasts with that observed in cap-dependent translation of most cellular mRNA, which dramatically decreases during cell division (Pyronnet 2001a; Pyronnet 2001b; Sachs 2000). Cell division disrupts the eIF4 complex that is essential for cap-dependent translation (Pyronnet 2001b). The relatively few cellular proteins synthesized through cap-independent translation tend to function during periods of cellular stress, such as cell death, cell division, and oxygen shortage (Lang 2002; Morrish 2002; Pyronnet 2000; Stein 1998; Stoneley 2000). Cells may use IRES-directed protein synthesis in emergency conditions when cap-dependent translation initiation is decreased or inhibited (Fernandez 2001).

These differences in translation efficiency suggest that the IRES benefits HCV RNA translation in actively dividing cells. By allowing HCV to bypass eIF4 to initiate translation, IRES permits HCV translation to occur during cell division, when most other protein synthesis shuts down. Conversely, HCV proteins would not be efficiently synthesized in resting cells—the state of most hepatocytes at any given time. Rather, resting cells would favor the synthesis of cellular proteins. These observations may prove to have important implications for developing new approaches to anti-HCV therapy that exploit differences in cellular and viral translation.

The cell cycle model of translation regulation also suggests a complex trade-off between factors promoting cellular and HCV protein synthesis. Viruses, including HCV, must ensure efficient synthesis of their proteins in order to replicate. This may entail competition with cellular mRNA for the host cell factors (including eIFs and ribosomes) required for translation (Sarnow 2003). Notably, both the 5′ UTR and the 3′ UTR of HCV RNA can bind to proteins within the ribosome, perhaps helping HCV to compete with cellular mRNA for ribosomes (Fukushi 2001b; Mauro 2002; Odreman-Macchioli 2001; Otto 2002; Pestova 1998; Wood 2001). Viewing the dynamics of protein synthesis in terms of competition for cellular resources may shed light on other factors involved in regulating HCV translation initiation.

Viral and cellular factors regulating HCV translation

The regulation of HCV translation can be understood as a balance between several requirements:

1. Efficient viral replication demands an adequate supply of viral proteins, and thus a minimum level of protein synthesis.
2. Viral replication also requires the preservation of the host cell environment, at least until new virions have been produced. Overproduction of viral proteins might damage or kill the host cell before the HCV replication cycle is complete.
3. RNA (both viral and cellular) does not persist indefinitely in cells; cellular enzymes target RNA for degradation or decay. HCV must either protect its RNA from degradation or complete protein synthesis before its RNA can be degraded.
4. Cellular defenses against viruses and other invaders can guard against replication by foreign (i.e., viral) RNA in part by depriving such RNA of cellular factors required for translation. HCV must block or overcome the triggering of host cell defenses in order to guarantee the synthesis of its proteins.
5. The same HCV RNA used for protein synthesis (translation) will also be used to make new copies of the HCV genome (transcription), but the RNA can be used for only one of these processes at a time. Therefore, HCV must devise a way to manage "traffic" (for example, ribosome movement) while ensuring that RNA is available for the execution of both translation and transcription.

Because of these multiple requirements, some factors stimulate HCV translation, while other factors repress it, depending on such conditions as cell cycle phase and viral replication stage.

The overall regulation of HCV protein synthesis remains poorly understood. Many factors are likely to regulate translation by increasing or decreasing its efficiency. Some cellular proteins may regulate translation by binding to various regions of HCV RNA, particularly the 5′ UTR and the 3′ UTR. These untranslated regions fold into complex structures that need to balance stability with flexibility. The IRES structure within the 5′ UTR contains several loops that bind components needed to start translation, such as the ribosome and eIF3. Other cellular proteins not directly involved in translation initiation can also bind to the HCV IRES. Some of these proteins may stabilize the IRES structure, increasing translation efficiency, while others may make the IRES structure too rigid, preventing it from functioning.

Among these cellular proteins, the most important regulatory factor may be the La antigen, which binds to the HCV IRES (N. Ali 1997; N. Ali 2000; Izumi 2004; Pudi 2003; Pudi 2004). The La antigen increases the efficiency of protein synthesis and may actually be required for translation initiation (N. Ali 2000; S. Das 1998a; Isoyama 1999; Pudi 2003; Shimazaki 2002). The La antigen also appears to bind to a region within the 3′ UTR, possibly protecting HCV RNA from degradation by cellular enzymes (McClaren 1997; Pannone 1998; Spångberg 2001). Some research suggests that the polypyrimidine tract binding protein (PTB) may also stimulate, or even be required for, HCV translation initiation (N. Ali 1995; Anwar 2000; Gosert 2000). Several other

cellular, RNA-binding proteins also bind to the HCV IRES, including polycytosine-binding proteins 1 and 2 (PCBP-1 and -2) and heterogeneous nuclear ribonucleoprotein L (hnRNP L), though their role in translation has not been fully established (Fukushi 2001a; Hahm 1998; Spångberg 1999). Similarly, some proteins bind to the 3′ UTR (including PTB, hnRNP C, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein L22) giving them a potential role in modifying translation (Blight 1997; Gontarek 1999; Y. Inoue 1998; Ito 1997; Ito 1998; Luo 1999; Petrik 1999; Tsuchihara 1997; Wood 2001); however, significant controversy remains about whether and how 3′ UTR is involved in regulating translation (Fang 2000; Friebe 2002; Imbert 2003; Kong 2002; Murakami 2001; Qi 2003; Wiklund 2002).

Determining which factors regulate HCV translation, and how, has been difficult in the absence of an efficient cell culture system, particularly in view of the potential interactions of multiple factors. In addition to cellular proteins, viral proteins synthesized during prior rounds of translation may also influence the efficiency of translation. Studies of the HCV core protein and its coding RNA region have reported conflicting results on its role in regulating translation (Fan 1999; M Honda 1999; Ito 1999; D. Li 2003; H. H. Lu 1996; Reynolds 1995; Shimoike 1999; Tanaka 2000; T. H. Wang 2000; J. Zhang 2002). Other reports suggest that HCV nonstructural proteins NS4A and NS4B may also regulate translation (Florese 2002; Y. He 2003; J. Kato 2002a). Part of the difficulty in defining the roles of these factors is that their observed effects in experimental systems may not reflect their role *in vivo*.

For example, some studies suggest that the HCV core protein inhibits HCV translation (D. Li 2003; Shimoike 1999); however, the core protein may take on different roles at other stages of the HCV replication cycle, such as regulating the switch between translation and transcription (J. Zhang 2002). Alternately, interactions between the core protein and the 5′ UTR may occur only during virion assembly rather than during translation regulation (Fan 1999; Tanaka 2000).

Other viral proteins (particularly NS5A) may indirectly promote viral translation by blocking cellular defenses. Cells defend against viral infection through an enzyme called PKR (double-stranded RNA-dependent protein kinase R), an important component of the antiviral effects of interferon alpha (see Chapter IX, Immune Response, Persistence, and Pathogenesis). PKR inactivates eIF2, a eukaryotic initiation factor required for protein synthesis. The HCV NS5A protein binds to PKR, preventing it from inactivating eIF2 and shutting down protein synthesis (Y. He 2001; Y. He 2003; C. Wang 2003); however, the degree to which eIF2 inactivation regulates HCV translation remains unclear (J. Kato 2002b; Koev 2002).

Directions for future research on translation regulation

The last five years have been enormously productive for the study of HCV protein synthesis and of internal ribosomal entry sites in general. While the broad outlines of HCV IRES–directed translation have been established, significant questions remain regarding the mechanisms of translation regulation. Researchers have identified a broad range of viral and cellular factors that may affect the efficiency of translation, though whether they are essential and how exactly they work have not been fully established. HCV translation regulation is a dynamic system involving the interaction of multiple factors operating synergistically, antagonistically, or redundantly, and may vary in significance depending on cellular conditions. Current knowledge of HCV protein

synthesis cannot yet provide a definitive model for translational control, but still permits the initial identification and prioritization of targets for anti-HCV drug development.

Summary of HCV translation:

- HCV initiates protein synthesis from its genome through an internal ribosomal entry site (IRES) contained within the 5′ UTR.
- HCV translation operates under a tightly regulated regime that results in sufficient protein synthesis but may also limit the production of viral proteins. This degree of regulation may be necessary in order to ensure successful viral replication while protecting the host cell from premature death.
- Several cellular proteins may play a role in regulating of HCV translation, including the La antigen, PTB, and hnRNP L, though it is unclear whether all of these proteins are essential or how they function.
- Several viral elements have also been implicated in regulating HCV translation, including the core protein and/or its RNA coding sequence. The NS5A protein may block cellular defenses that would otherwise shut down protein synthesis during viral infection.
- HCV translation may be regulated by the cell cycle, operating most efficiently in actively dividing cells.
- HCV may have a competitive advantage in ensuring its translation over cellular mRNA during conditions of cellular stress such as cell division.

Stage 3: Cleavage and processing of viral proteins

The product of HCV translation is a single, long polyprotein containing all viral structural and nonstructural proteins. The polyprotein must first be cleaved, or sliced up, into several individual proteins, in order for the viral proteins to function properly. Some viral proteins must then undergo further modifications. Cleavage of the HCV polyprotein uses both viral and cellular enzymes. These enzymes are called proteases; they catalyze reactions that separate the individual proteins. This stage of the viral replication cycle is an important target for HCV drug development; if cleavage or processing is blocked, viral proteins cannot produce new hepatitis C virions.

Studies of HCV cleavage and processing have addressed three key issues:

- Which viral proteins are produced as a result of cleavage and processing?
- Which cellular and viral enzymes are involved in the cleavage of HCV proteins?
- What further modifications following cleavage are required for viral protein function?

HCV viral proteins

The HCV polyprotein yields ten main viral proteins. These proteins are classified as either structural proteins, which are incorporated into new virions, or nonstructural proteins. The ten proteins are listed below, along with their functions in viral replication (some of which are described later in this chapter; see Stage 4 and Stage 5):

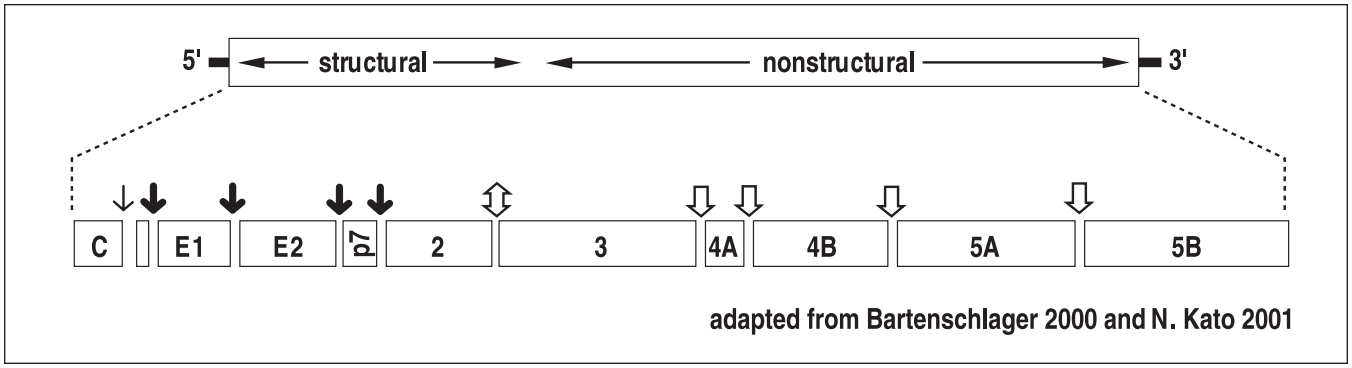
Structural proteins:

- Core protein—forms the capsid shell surrounding genomic HCV RNA
- E1 (envelope 1)—an envelope protein; probably involved in host cell attachment
- E2 (envelope 2)—an envelope protein; also involved in attachment to host cells
- p7—unknown function; may be involved in viral assembly and release; technically, not a known structural element of HCV virions, but generally grouped with the core, E1, and E2 structural proteins

Nonstructural proteins:

- NS2 (nonstructural protein 2)—part of the NS2-NS3 protease; possibly involved in the viral replication complex
- NS3 (nonstructural protein 3)—part of the NS2-NS3 protease; also contains the NS3 serine protease and a helicase/NTPase enzyme involved in HCV replication; probably involved in the viral replication complex
- NS4A (nonstructural protein 4A)—a cofactor for the NS3 serine protease, facilitating cleavage; probably involved in the viral replication complex
- NS4B (nonstructural protein 4B)—unknown function; probably involved in the viral replication complex
- NS5A (nonstructural protein 5A)—unknown function; probably involved in the viral replication complex
- NS5B (nonstructural protein 5B)—contains the RNA-dependent RNA polymerase enzyme, which copies the HCV genome via the viral replication complex

Figure 7. HCV Proteins and Cleavage Sites



Cellular and viral enzymes involved in cleavage of the HCV polyprotein

Cleavage of the HCV polyprotein occurs along the cell's endoplasmic reticulum (ER), a large internal network of membranes involved in protein synthesis, folding, and transport (Bartenschlager 2000; Reed 2000; J. Z. Wu 2001). Some cleavage occurs during translation (co-translational cleavage), but most cleavage occurs after the entire polyprotein has been translated (post-translational cleavage). HCV uses cellular proteases called signal peptidases to cleave its structural proteins. Two viral proteases, the NS2-NS3 protease and the NS3 serine protease, cleave HCV's nonstructural proteins:

- Signal peptidases cleave the core, E1, E2, and p7 proteins (Grakoui 1993a; Harada 1991; Hijikata 1991; C. Lin 1994a; Santolini 1994; Selby 1993). A full-length form of the core protein is further trimmed by signal peptidases to yield its mature, truncated form (Hedge 2002; Hussy 1996; T. Kato 2003a; Lemberg 2002; McLauchlan 2002; Weihofen 2002; M. S. Wolfe 2002).
- The cleavage of NS2 and NS3 is accomplished through the action of a protease comprised of the NS2 and NS3 proteins themselves, a process described as auto cleavage (Grakoui 1993c; Hijikata 1993b; Hirowatari 1993; Reed 1995). The NS2-NS3 protease has generally been described as a zinc protease, but some studies also suggest that it may belong to the cysteine protease family* (Pallaoro 2001; Pieroni 1997; Thibeault 2001).
- A separate NS3 protease enzyme, the NS3 serine protease, cleaves the remaining nonstructural proteins (Bartenschlager 1993; Bartenschlager 1999; Eckart 1993; Grakoui 1993b; Manabe 1994; Tomei 1993). The activity of the NS3 serine protease involves the HCV protein NS4A serving as a cofactor to facilitate cleavage and stabilize NS3 (Bartenschlager 1994; Failla 1994; Failla 1995; C. Lin 1994b; Tanji 1995).

Post-cleavage modifications to viral proteins

The HCV envelope proteins E1 and E2 must undergo further modifications before their assembly into the envelope of new HCV virions (Op de Beeck 2001):

- Localization and membrane anchoring—During translation and cleavage, E1 and E2 enter the lumen, or interior, of the endoplasmic reticulum. Both envelope proteins subsequently remain anchored to the ER membrane.
- Glycosylation—Oligosaccharide chains, or sugar molecules, are added to up to 6 sites on E1 and 11 sites on E2 inside the ER.
- Heterodimerization—Following glycosylation, E1 and E2 can be folded into their proper three-dimensional forms and joined into heterodimers, with the help of cellular "chaperone" proteins that assist in protein folding.

These steps are crucial for viral infectivity. Without heterodimerization of E1 and E2, new HCV virions cannot attach to and infect other cells (see Stage 1: Host cell attachment, entry, and uncoating). Similarly, without glycosylation, E1 and E2 cannot be properly folded into heterodimers. Indeed, clumps of improperly folded E1 and E2 proteins (called misfolded protein aggregates) have been found in the lumen of the ER, suggesting that proper folding does not always occur automatically (Choukhi 1999; Deleersnyder 1997; Dubuisson 1994; Dubuisson 1996; Duvet 1998; Grakoui 1993a; Michalak 1997). In theory, the accumulation of misfolded

* All proteases are classified into one of five categories, depending on their mechanism of action and the type of amino acids involved in catalyzing cleavage: aspartyl, cysteine, serine, threonine, and zinc proteases (also called metalloproteases). A diverse range of proteases falls within each category; human genes encode hundreds of different serine proteases. This classification encompasses viral as well as cellular proteases; HIV, for example, encodes an aspartyl protease that has been successfully targeted by HIV protease inhibitors.

E1-E2 protein aggregates may trigger an ER stress response, or unfolded protein response. These cellular defenses could potentially shut down translation initiation and conceivably lead to cell death, as has been seen *in vitro* with the related flaviviruses bovine viral diarrhea virus (BVDV) and Japanese encephalitis virus (JEV) (Jordan 2002; H. L. Su 2002). While some research supports a role for ER stress in HCV pathogenesis, available evidence is ambiguous, and other viral proteins, such as NS5A, may be involved (Gong 2001; Pavio 2003a; Tardif 2002; Tardif 2003; Tardif 2004; Waris 2003). Advances in mouse models of ER stress may clarify these questions (Iwawaki 2004).

Other viral proteins are also modified following cleavage. For example, the HCV protein NS5A undergoes a chemical modification called hyperphosphorylation that adds multiple phosphate groups to the protein (Reyes 2002). The role of NS5A hyperphosphorylation in viral replication is unclear, though it may be necessary for the assembly of the viral replication complex (see Stage 4: Replication of HCV genome) or for NS5A's role in pathogenesis (see Chapter IX, Immune Response, Persistence, and Pathogenesis). A set of cellular enzymes called protein kinases catalyze phosphorylation; over 500 kinases have been identified in the human genome (Manning 2002). Screening of yeast kinases has provided clues into which human kinases may be responsible for NS5A hyperphosphorylation, and NS5A phosphorylation by the human kinase c-Raf1 (an NS5A binding partner) has been demonstrated *in vitro* (Buerckstuemmer 2004; Coito 2004). Other HCV nonstructural proteins such as NS3, NS4A, and NS4B probably mediate the hyperphosphorylation of NS5A (Kaneko 1994; Koch 1999; Neddermann 1999).

Directions for future research on HCV cleavage and processing

While several facets of viral protein cleavage and post-translational modifications require further investigation, many fundamentals of polyprotein processing are now well established. In particular, a significant body of research has examined the function and structure of the NS3 serine protease and its NS4A cofactor. The NS3 serine protease—arguably the most extensively researched HCV viral enzyme—makes an attractive target for drug development. The success of HIV protease inhibitors no doubt contributes to the prominence of the NS3 serine protease in HCV research, despite little structural similarity between the proteases (Bartenschlager 1999; Bianchi 2002; Lahm 2002; Narjes 2003). Moreover, several drug companies have directed significant resources to the study of NS3, increasing our understanding of its molecular biology and their ability to develop NS3 protease inhibitors*. Indeed, the productive convergence of industry and academic research activities around the NS3 serine protease is unparalleled in the field of HCV, distantly echoing the public-private collaboration leading to the discovery of HCV. This impressive body of research provides hope that many lingering mysteries surrounding HCV could finally be resolved if similar resources and talents are brought to bear upon other aspects of viral replication.

*Drug companies include Agouron (now Pfizer), Boehringer Ingelheim, Bristol-Myers Squibb, Hoffmann-LaRoche, Merck (working in part through the Italian Istituto di Ricerche di Biologia Molecolare P. Angeletti), Schering-Plough, Vertex, and Wyeth.

Summary of HCV cleavage and processing

- The HCV polyprotein contains ten viral proteins—four structural proteins and six nonstructural proteins.
- Polyprotein processing occurs at the ER during and after translation.
- The structural proteins, core, E1, E2, and p7, are cleaved by cellular signal peptidases.
- The NS2/NS3 juncture is cleaved by the NS2-NS3 protease, thought to be a zinc protease but also sharing properties with cysteine proteases.
- The remaining nonstructural proteins are cleaved by the NS3 serine protease, using NS4A as a cofactor.
- The envelope proteins E1 and E2 undergo further post-translational modifications in the ER lumen, where they are glycosylated and folded with the help of chaperone proteins into their proper heterodimeric form.
- Other post-translational modifications, including the hyperphosphorylation of NS5A, also occur following cleavage.

Stage 4: Replication of the HCV Genome

Replication of the HCV genome, pivotal event in the viral life cycle, follows translation and viral protein processing. Each new virion requires its own viral genome.

These new HCV genomes are RNA strands that have been copied, or transcribed, from the original HCV RNA; however, the original RNA cannot simply be duplicated; it must first go through an intermediary stage. Replication, therefore, has two major steps: 1) the original viral genome is used as a template for the synthesis of the negative-sense strand of viral RNA, and 2) the negative-sense RNA then serves as the template for the production of new, genomic, positive-sense HCV RNA. A type of enzyme called a polymerase carries out strand synthesis; another enzyme, called a helicase, keeps the RNA strands separated during synthesis. Both polymerase and helicase are viral enzymes but other viral and cellular proteins are also involved in the replication process. Indeed, replication involves virtually all of the nonstructural proteins, which assemble to form the viral replication complex, the site where RNA strand synthesis occurs. Genome replication, like HCV translation, is a tightly orchestrated process regulated by viral and host factors.

The study of the replication of the HCV genome has perhaps benefited the most from the development of HCV replicon systems. As with prior stages in HCV replication, the synthesis of new genomic RNA presents several opportunities for therapeutic intervention. In particular, the viral polymerase and helicase enzymes make attractive targets for drug development.

Research on HCV RNA replication has explored three major areas:

- How does HCV replicate its genome?
- Which viral and cellular proteins are involved in replication?
- Where does replication occur?

Synthesis of HCV RNA

HCV polymerase enzyme, located within the HCV NS5B protein, synthesizes new RNA strands. This enzyme is called the RNA-dependent RNA polymerase (RdRp; reviewed in Lesburg 2000; Lohmann 2000). The RdRp operates through mechanisms similar to those used by cellular polymerase enzymes in the production of RNA. RNA is composed of nucleotides, molecules supplied by cells and containing one of four possible chemical bases—adenine, cytosine, guanine, and uracil. Each base has a complementary base to which it binds. Adenine binds to uracil, forming a base pair, and cytosine base pairs with guanine.

The NS5B RdRp "reads" the nucleotides on the genomic, positive-sense strand of HCV RNA and begins to synthesize a complementary negative-sense RNA chain, matching each original nucleotide with its complementary base. If the polymerase reads an A, it adds a complementary U to the new negative strand; when it reads a C on the original strand, it adds a G on the growing RNA chain, and so on. The NS5B RdRp reads the original RNA strand from the 3′ end to the 5′ end, reversing the direction that the ribosome uses for translation. In this way, the RdRp synthesizes a new RNA strand that is fully complementary, but not identical, to the original genomic RNA. The negative-sense RNA strand is therefore a molecular mirror image of the viral RNA.

Because the positive-sense and negative-sense strands are complementary, each can serve as a template for synthesizing the other. After the RdRp synthesizes negative-sense RNA, it repeats the process, this time using the negative-sense strand as a template for synthesizing a new positive-sense strand. This new positive-sense HCV RNA will form the genome of a new hepatitis C virion. The HCV RdRp has no proofreading mechanism to correct errors during strand synthesis, so mistakes made by the HCV RdRp get incorporated into new HCV RNA as mutations (Behrens 1996). This propensity for error during genomic replication results in a quasispecies population of closely related but genetically distinct HCV variants.

Complementary RNA strands can bind to each other, forming double-stranded RNA. Double-stranded RNA (dsRNA) rarely appears in cells. Cells tend to interpret the presence of dsRNA as a sign of viral infection, triggering a set of cellular defenses (including interferon) that aim to block viral replication and destroy viral RNA (see also the discussion of interferon mechanisms of action in Chapter X, The Future of HCV Therapy). Furthermore, the HCV polymerase cannot "read" RNA strands bound into dsRNA form; strand synthesis requires a template of single-stranded RNA. Therefore, HCV must keep positive-sense and negative-sense RNA strands separated in order to avoid cellular defenses and maintain the replication process. The helicase enzyme, contained within the HCV NS3 protein, accomplishes this task by unwinding or separating RNA strands during genome replication. The helicase acts as a wedge, binding to an RNA strand and moving from the 3′ end to the 5′ end, disrupting dsRNA formation, and possibly dislodging cellular RNA-binding proteins that would otherwise interfere with HCV polymerase activity. The HCV helicase operates with another NS3 enzyme, called NTPase (nucleotide triphosphatase), which catalyzes chemical reactions that support the movements and RNA binding of the helicase (J. L. Kim 1998; Levin 2003; Paolini 2000). Multiple HCV helicase enzymes may be required for efficient RNA strand synthesis (Levin 2004).

Strand synthesis occurs within a viral replication complex (or replicase) formed by the HCV proteins NS3, NS4A, NS4B, NS5A, and NS5B (and possibly NS2) on the membrane of the endoplasmic reticulum (Dimitrova 2003; El-Hage 2003; Fipaldini 1999; Moradpour 1998; Mottola 2002; Pietschmann 2001; Tardif 2002; Waris 2004). This replication complex sequesters the process of strand synthesis, protecting viral RNA strands from host cell defenses and degradation. The replicase probably also provides the scaffolding for replication and helps to retain the negative strand for further rounds of synthesis. The viral replication complex has not been well characterized and likely involves cellular and viral proteins. One candidate cellular protein, alpha-actinin, interacts with HCV NS5B and appears to be required for HCV replication (S. Lan 2003). NS5B and NS5A can each bind to the cellular protein hVAP-33 (human vesicle–associated membrane protein–associated protein of 33 kDa), which facilitates the formation of the viral replication complex (Tu 1999). Interference with hVAP-33 expression dramatically reduces intracellular HCV RNA and viral protein levels *in vitro* (L. Gao 2004).

The viral protein structure formed on the endoplasmic reticulum during replication has been described as a "membranous web" (Egger 2002; Gosert 2003; Miyanari 2003). The HCV protein NS4B appears to play a major role in forming these membranous webs, presumed to function as the viral replication complex. NS4B also possesses a domain capable of binding nucleotides, and replicon studies show that mutations to that domain impair or abolish viral replication (Einav 2003a). However, other researchers have suggested that viral replication may actually occur not on the ER membrane, but on lipid rafts (cholesterol-rich bits of membrane) (Shi 2003).

HCV RNA strand synthesis is regulated by a variety of viral and cellular factors. Evidence from chimpanzees suggests that some mutations to HCV NS5B may increase the rate of viral RNA strand synthesis (Lou 2003). Conversely, some highly conserved elements in HCV RNA, common across genotypes and individual viral isolates, may also regulate viral replication. The NS5B coding sequence includes a conserved RNA structural element that folds into a stem loop and functions as a *cis*-acting replication element (CRE) (S. You 2004). *Cis*-acting replication elements function as conserved structures embedded in viral RNA that can mediate translation, RNA strand synthesis, or other aspects of the viral replication cycle. CREs have also been found in the HCV 5′ UTR, 3′ UTR, and core protein coding sequence; key RNA structures in the 3′ UTR are required for HCV strand synthesis (Friebe 2002; Penin 2004; Schuster 2002; Yi 2003a; Yi 2003b). The NS5B CRE is also essential for replication, presumably by interacting with one or more viral and/or cellular proteins (S. You 2004).

Interactions between NS5A and NS5B appear particularly important for effective HCV replication (Shimakami 2004). Viral proteins (including NS3, NS4B, and NS5A) may directly or indirectly contribute to the efficiency of HCV genome replication, inhibiting or stimulating HCV RdRp activity (Piccininni 2002; Shirota 2002). This may partly account for differing rates of synthesis for positive-sense and negative-sense RNA strands. Generally the amount of positive-sense HCV RNA exceeds that of negative-sense RNA, suggesting a regulatory mechanism at work (M. Chang 2003; Komurian-Pradel 2004).

Some cellular proteins may also regulate viral RNA strand synthesis, though their roles are less clear. Morphine can increase HCV RNA replication *in vitro*, possibly by stimulating intracellular signaling pathways rather than via direct interaction with viral proteins (Y. Li 2003). Evidence

suggests that NS5B interacts *in vitro* with nucleolin, an RNA-binding molecule, and hPLIC1 (human homolog 1 of protein linking intergrin-associated protein and cytoskeleton), a cellular protein that reduce replication by targeting NS5B for degradation via ubiquitination (L. Gao 2003; Hirano 2003). NS5A interacts with the cellular protein amphiphysin II, though the function of this interaction is unclear (Zech 2003). In vitro models suggest that the NS5A protein and the viral replication process itself trigger the ER stress response, with possible consequences for HCV RNA strand synthesis (Gong 2001; Tardif 2002; Waris 2002).

Cellular proteins such as PTB and hnRNP C that bind to the 3′ UTR, where negative-sense strand synthesis initiates, may modulate the efficiency of HCV genome replication (Ito 1997; Luo 1999). Further research may elucidate the role of these factors and identify additional cellular proteins that can regulate RdRp activity. Inhibition of geranylgeranylation (the transfer of chemical groups involved in protein prenylation, which directs cellular proteins to cell membranes) disrupts the HCV replication complex, suggesting a role for an unidentified geranylgeranylated host protein in viral replication and a possible avenue for therapeutic inhibition (J. Ye 2003). Cellular factors almost certainly influence HCV replication, given that viral replication may be most active during cell growth and division, as with protein synthesis (M. Honda 2000; Pietschmann 2001; Scholle 2004). The availability of pools of nucleotides for incorporation in new HCV RNA strands, and perhaps cytosine and uracil in particular, would also determine the rate of replication and may provide a strategy for inhibition (Stuyver 2003b).

Summary of HCV genome replication

- HCV genome replication entails synthesis of a negative-sense RNA strand complementary to the original viral RNA, which then serves as a template for synthesis of positive-sense, genomic viral RNA.
- Negative-sense and positive-sense strand synthesis is performed by the HCV NS5B protein, an RNA-dependent RNA polymerase, which assembles a chain of nucleotides complementary to the original strand.
- During RNA replication, the NS3 helicase/NTPase, with NS4A acting as a cofactor, moves along the template RNA strand to keep the original and newly synthesized RNA strands separate.
- RNA replication begins with the assembly of a viral replication complex consisting of all HCV nonstructural proteins and possibly one or more as yet unidentified cellular proteins.
- The viral replication complex is associated with the ER membrane, at least initially, though viral replication may occur on a lipid raft detached from the ER in association with the replication complex.
- The highest levels of viral genome replication are observed during cell growth and division, suggesting the importance of cellular factors in modulating replication efficiency.

Stage 5: Assembly of new virions and release from host cell

New HCV virions consist of three main components: single-stranded genomic HCV RNA, a capsid shell, and the viral envelope. HCV assembles new virions from components produced through translation (the core and envelope proteins) and strand synthesis (genomic HCV RNA). The genomic RNA is packaged within the capsid, which is formed from core proteins. The combination of HCV RNA with the capsid shell is known as the nucleocapsid, which is then enveloped within a section of cellular membrane, presumably derived from the endoplasmic reticulum. This envelope incorporates the viral envelope proteins, completing new virion assembly. These new viruses are then transported to the plasma membrane and released to infect new cells. It is unclear whether viral release results in the death of the host cell (see Chapter IX, Immune Response, Persistence, and Pathogenesis). The dynamics of viral assembly and release are not well understood, largely due to the limitations of experimental models; however, these late events in the viral replication cycle are crucial for maintaining HCV infection, and may also offer additional targets for antiviral intervention.

Research on the late stages of HCV replication poses the following questions:

- How are new HCV virions assembled?
- Where does assembly occur?
- How are new virions released from the cell?

Viral assembly can be roughly divided into two phases: the formation of the nucleocapsid and the formation of the viral envelope. The capsid is composed of at least 420 core protein subunits assembled into an icosahedral form (Kunkel 2001; Matsumoto 1996). Interactions between core proteins and the 5′ UTR of HCV RNA trigger the assembly of the capsid, and enclose genomic RNA within the capsid shell (Fan 1999; Kunkel 2001; Kunkel 2002; Santolini 1994; Shimoike 1999; Tanaka 2000). How this process is regulated remains an open question, though cellular factors may be involved. In particular, the cellular enzyme tissue transglutaminase promotes the formation of core protein dimers (two core proteins joined together), impairing the ability of the core protein to bind to RNA and thus possibly impeding nucleocapsid formation (W. Lu 2001).

How the HCV nucleocapsid acquires its envelope is not yet clear. The assembly of other members of Flaviviridae viruses is generally thought to involve a process called "budding" into vesicles, wherein the viral capsid encircles itself in a cellular membrane, which becomes the viral envelope (Garoff 1998; Lindenbach 2001). This membrane is studded with viral envelope proteins (E1 and E2, in the case of HCV). E1-E2 heterodimers are largely retained within the ER prior to virion assembly, providing indirect evidence that budding of new HCV virions likely occurs at the ER membrane, though functional E1-E2 heterodimers have also been detected on cell surface membranes in some *in vitro* systems (Charloteaux 2002; Cocquerel 1998; Cocquerel 1999; Drummer 2003; Dumonceaux 2003; Duvet 1998). The HCV core protein appears to play a key role in directing the budding process (Blanchard 2003b).

Not all viral particles are released in enveloped form. Nonenveloped viral particles that appear to consist solely of a nucleocapsid shell, presumably surrounding HCV RNA, have been found in the blood of people chronically infected with HCV (Maillard 2001). The role of these particles in viral replication and pathogenesis is unclear; however, HCV nucleocapsids can bind to certain types of

antibodies (IgG, or immunoglobulin G), which may interfere with immune responses or otherwise contribute to HCV pathogenesis (Maillard 2004; Sansonno 2003; see also Chapter IX, Immune Response, Persistence, and Pathogenesis).

The actual release from the cell of new hepatitis C virions has not been observed, so speculation on this process again relies on analogy to other members of the Flaviviridae family. In this model, newly enveloped virions would be transported in vesicles to the plasma cell membrane. When the vesicles fuse to the plasma membrane, new virions would be released from the cell in a process called exocytosis, the reverse of endocytosis (Lindenbach 2001; Serafino 2003). This form of release follows the cell's own secretory pathway (the route taken by proteins secreted by the cell) moving from the ER to the Golgi apparatus to the plasma membrane.

Alternately, viral release may involve the HCV p7 protein. Recent research has shown that p7 can form pores or protein-lined tunnels on the plasma cell membrane called ion channels, which allow ions such as calcium, sodium, and potassium to enter cells (Carrère-Kremer 2002; Griffin 2003; Pavlović 2003). These p7 ion channels may effectively remder the cell's plasma membrane permeable to substances that would otherwise be blocked. Increased permeability of the cell membrane might also facilitate the release of hepatitis C virions. This hypothesis remains speculative, but gains credibility from the observation that HIV produces a similar protein, Vpu, which forms ion channels that enhance the release of new HIV virions (C. Ma 2002). Despite uncertainty about its function, p7 appears essential to viral replication. Chimpanzee studies demonstrate that viral HCV RNA transcripts without p7 or with p7 mutations are not infectious, and that p7 function may be genotype-specific (Sakai 2003). The p7 protein may present a viable target for anti-HCV drug development Pavlović 2003).

HCV assembly and release are presumably not automatic or passive events, and the viral and cellular factors that mobilize and direct these factors require elucidation. Evidence from several other viruses, including HIV, indicates that cellular proteins can play a significant role in viral assembly and release (Erturk 2003; Freed 2002). The existence and nature of such cellular factors has not yet been determined for HCV (Pietschmann 2002). Despite progress in HCV replicons and surrogate viral replication models, the assembly and release of infectious virions or HCV-like particles following replication have not been observed in cell culture systems (Molenkamp 2003; Pietschmann 2002). Analogies to the maturation and budding processes seen in other viruses may aid in forming hypotheses, but a definitive model for HCV assembly and release will require further research. Better experimental models for HCV assembly and release are badly needed.

Summary of HCV assembly and release

- The capsid self-assembles from multiple subunits of the core protein.
- Capsid formation requires interactions between the core protein and the HCV 5′ UTR.
- The viral envelope is formed from cellular membranes by a process called budding.
- HCV is thought to acquire its envelope through budding into the lumen of the endoplasmic reticulum.
- In this model, the assembled virion would be released through the secretory pathway.
- The viral protein p7 may also be involved in viral release by forming ion channels, potentially increasing the permeability of the cell's plasma membrane.

Research Recommendations

Support and intensify research into the molecular biology of HCV.

The initial identification of hepatitis C virus (Q. L. Choo 1989) ushered in a highly productive era in virology research, as scientists began investigating the genetic structure of the virus and the role of viral proteins in HCV replication. Despite enormous advances, numerous challenges remain. Key aspects of the HCV replication cycle are not fully understood, and further work on cell culture systems and animal models is an urgent priority. Continued elucidation of the molecular biology of HCV will be critical in understanding viral pathogenesis and developing new therapies.

Efforts must be made to increase the utility of the mouse model and enhance the efficiency and reproducibility of *in vitro* cell culture systems. The chimeric mouse model incorporating human hepatocytes shows promise, though further work is necessary to better mimic human immune responses (Brass 2002; Pietschmann 2003). The National Institute of Allergy and Infectious Diseases' Division of Microbiology and Infectious Diseases' (DMID) currently supports small-animal model HCV research. Increased NIAID funding and additional resources from other funders of biomedical research would help to accelerate this work. Further exploration of the potential for chimeric GB virus B in tamarins and marmosets as a surrogate model for HCV infection should also be supported (Beames 2000; Beames 2001; Bright 2004; Bukh 1999; De Tomassi 2002; Sbardellati 2001).

Finally, recent advances in HCV replicon systems offer hope for the eventual development of a viable cell culture model for HCV (Bartenschlager 2002; Pietschmann 2003). Further refinements in replicon models will require sustained support from public and private funders. Prominent examples of such support include the recent five-year, unrestricted grant from Bristol-Myers Squibb to Ralf Bartenschlager's laboratory at the University of Heidelberg in Germany, and the support from government grants and the Greenberg Medical Research Institute for the work of Charles Rice and colleagues at Rockefeller University's Center for the Study of Hepatitis C. The National Institutes of Health and other governmental and private funders should coordinate and extend their efforts to ensure that the work of Bartenschlager, Rice, and other groups focusing on replicons continues. DMID must also update its "Framework for Progress on Hepatitis C" (NIAID 1997) and receive additional funding to increase its commitment to intramural and extramural basic research on HCV infection.

List of Terms Used in this Chapter

3′ UTR (3-prime untranslated region): non-coding region of HCV RNA; site of initiation of negative-sense strand synthesis.
5′ UTR (5-prime untranslated region): non-coding region of HCV RNA; contains the internal ribosomal entry site (IRES); site of initiation of translation.
Adenine: a nucleoside base; one of the four building blocks of RNA.
Amino acids: the building blocks of proteins; the sequence and composition of amino acids determines a protein's structure and function.
ARFP (alternate reading frame protein): an HCV protein encoded within an alternate reading frame of the core protein coding sequence; also called F protein or frameshift protein.
Cap-dependent translation: the method of translation (protein synthesis) predominantly used for cellular proteins.
Cap-independent translation: translation (protein synthesis) via an internal ribosomal entry site (IRES); the method used by HCV.
Capsid: the shell surrounding the HCV genome, formed by the HCV core protein.
CD81: a cell surface receptor believed to be involved with HCV entry into cells.
Cell culture: growing cells in a laboratory; an *in vitro* research tool.
Cell line: a group of cells maintained in cell culture that continue to survive and divide; an *in vitro* research tool.
Chimeric virus: an engineered hybrid of two different viruses (e.g., the core particles of a retrovirus bearing HCV envelope proteins on the surface).
***Cis*-acting replication element (CRE):** conserved structures embedded in viral RNA that can mediate translation, RNA strand synthesis, or other aspects of the viral replication cycle.
Cloning vectors: DNA molecules (i.e., plasmids) or viruses that smuggle HCV genetic material inside a cell to synthesize viral proteins.
Core: an HCV protein; forms the capsid.
Cryopreservation: freezing (e.g., of cells or tissue for later study).
C-type lectins: carbohydrate-binding molecules; includes DC-SIGN and L-SIGN.
Cytokine: secreted proteins that function as chemical messengers between cells by binding to cell surface receptors.
Cytoplasm: the main area of the cell inside its membrane but outside of its nucleus.
Cytosine: a nucleoside base; one of the four building blocks of RNA.
DC-SIGN (dendritic cell–specific intercellular adhesion molecule 3-grabbing non-integrin): a cell surface receptor; possibly involved with HCV entry into cells.
E1: one of two HCV envelope proteins.
E2: one of two HCV envelope proteins.
Endoplasmic reticulum (ER): a membrane within cells; the site of HCV translation (protein synthesis) and strand synthesis.
Envelope: the outer layer of HCV; a membrane containing HCV envelope proteins E1 and E2 that surrounds the nucleocapsid.

Eukaryotic initiation factors: cellular proteins involved in translation (protein synthesis).
Extrahepatic: cells or tissue outside the liver.
F protein (frameshift protein): an HCV protein encoded within an alternate reading frame of the core protein coding sequence; also called the alternate reading frame protein (ARFP).
Flaviviridae: the family of viruses that HCV belongs to.
Flavivirus: a genus within the Flaviviridae family; closely related to HCV.
Frameshift: the translation of 2 or more proteins from overlapping sections of RNA. HCV contains at least one frameshift protein (the F protein or ARFP).
Geranylgeranylation: the transfer of chemical groups involved in protein prenylation.
GAPDH (glyceraldehyde-3-phosphate dehydrogenase): a cellular protein that binds to the HCV 3′ UTR.
Genome: the total genetic information of an organism; the HCV genome is a single strand of positive-sense RNA.
Glycosaminoglycans: a group of polysaccharides (e.g., heparin and heparan sulfate) found on cell surfaces and possibly involved in HCV attachment to target cells.
Glycosylation: a chemical modification that adds sugar molecules to proteins. HCV envelope proteins E1 and E2 undergo glycosylation in the endoplasmic reticulum.
Guanine: a nucleoside base; one of the four building blocks of RNA.
Helicase: an HCV enzyme, contained within NS3, which unwinds and separates RNA strands during strand synthesis.
Hepacivirus: the genus of viruses that HCV belongs to.
Hepadnaviridae: the family of viruses that hepatitis B belongs to.
Heparan sulfate: a glycosaminoglycan found on cell surfaces and possibly involved in HCV attachment to target cells.
Heparin: a glycosaminoglycan found on cell surfaces and possibly involved in HCV attachment to target cells.
Hepatocyte: a liver cell; the main cell type that HCV infects and uses for replication.
Hepatoma cell line: a cell line derived from cancerous hepatocytes (liver cells); an *in vitro* research tool.
Hepatotropic: targeting the liver; HCV is a hepatotropic virus (as are hepatitis A and hepatitis B).
Heterodimer: complexes formed by two different proteins; the HCV envelope proteins E1 and E2 join to form heterodimers.
hnRNP (heterogeneous nuclear ribonucleoprotein): a type of cellular protein. HnRNP L binds to the HCV internal ribosomal entry site (IRES). HnRNP C binds to the HCV 3′ UTR.
Host cell: a cell infected by a virus.
hPLIC1 (human homolog 1 of protein linking integrin-associated protein and cytoskeleton): a cellular protein that reduces replication by targeting HCV NS5B for degradation via ubiquitination.
Hyperphosphorylation: a type of chemical modification performed by cellular enzymes; specifically, the addition of multiple phosphate groups to a molecule. HCV NS5A undergoes hyperphosphorylation.

In vitro: research performed outside of a living organism (e.g., not in humans or animals); test tube or laboratory research.
In vivo: research performed inside of a living organism (e.g., humans and animals).
Ion channels: a gateway, composed of one or more proteins, that allows ions (charged atoms or molecules such as calcium) to pass through cell membranes.
IRES (internal ribosomal entry site): a structure with the HCV RNA 5′ UTR that binds directly to the ribosome to initiate translation.
La antigen: a cellular protein that binds to the HCV internal ribosomal entry site (IRES) and increases the efficiency of translation (protein synthesis). The La antigen also binds to the HCV 3′ UTR.
LDLR (low-density lipoprotein receptor): a cell surface receptor; possibly involved with HCV entry into cells.
Ligand: molecules that bind to a particular receptor.
LSECs (liver sinusoidal endothelial cells): endothelial cells that line blood vessels in the liver and separate blood from surrounding organ tissue; express L-SIGN receptors.
L-SIGN (liver/lymph node–specific intercellular adhesion molecule-3-grabbing integrin): a cell surface receptor; possibly involved with HCV entry into cells.
Membraneous web: the structure formed by HCV replication complex.
Molecular cloning: duplication of genetic material (e.g., RNA) through PCR techniques.
mRNA (messenger RNA): positive-sense RNA that encode proteins; the HCV genome functions as mRNA.
Negative-sense RNA: the mirror image of positive-sense RNA; serves as a template for synthesizing positive-sense RNA; cannot function as messenger RNA (mRNA).
NS2 (non-structural protein 2): an HCV protein; part of the NS2-NS3 protease.
NS3 (non-structural protein 3): an HCV protein; contains part of the NS2-NS3 protease; the NS3 serine protease; and the NS3 helicase/NTPase
NS4A (non-structural protein 4A): an HCV protein; a co-factor for the NS3 serine protease and part of the HCV replication complex.
NS4B (non-structural protein 4B): an HCV protein; part of the HCV replication complex.
NS5A (non-structural protein 5A): an HCV protein; part of the HCV replication complex.
NS5B (non-structural protein 5B): an HCV protein; contains the RNA-dependent RNA polymerase (RdRp).
NTPase (nucleotide triphosphatase): an HCV enzyme, contained within NS3, which catalyzes chemical reactions that support the movements and RNA binding of the HCV NS3 helicase.
Nucleocapsid: the HCV genome, surround by the capsid shell.
Nucleolin: an RNA-binding molecule that interacts with HCV NS5B.
Nucleoside: a base (adenine, cytosine, guanine, or uracil) attached to a sugar molecule.
Nucleotide: the phosphorylated form of a nucleoside; the building block of RNA.
Open reading frame: the section of HCV RNA that encodes viral proteins; does not include the 5′ UTR and the 3′ UTR.
p7: an HCV protein of unknown function; may create ion channels.

PBMCs (peripheral blood mononuclear cells): white blood cells, including monocytes and lympocytes (e.g., T cells); HCV can infect PBMCs, and may replicate within them.
PCR (polymerase chain reaction; also RT-PCR for reverse transcriptase polymerase chain reaction): a technique to amplify and clone genetic material (DNA and RNA); PCR technology enabled the original identification of HCV.
Permissive cells: cells susceptible to HCV infection and capable of supporting HCV replication (e.g., hepatocytes).
Pestivirus: a genus within the Flaviviridae family; closely related to HCV.
Phosphorylation: a type of chemical modification performed by cellular enzymes; specifically, the addition of a phosphate group to a molecule.
Phylogenetic: referring to HCV's evolutionary history; i.e., HCV's "family tree."
Picornaviridae: the family of viruses that hepatitis A belongs to.
PKR (Double-stranded RNA-dependent protein kinase R): a cellular defense against viral infection that shuts down protein synthesis.
PCBP (polycytosine-binding protein): a type of cellular protein. PCBP 1 and 2 bind to the HCV internal ribosomal entry site (IRES).
Polymerase: an enzyme that synthesizes new DNA or RNA strands. HCV NS5B contains a polymerase enzyme, the RNA-dependent RNA polymerase, which synthesizes new HCV RNA.
Polyprotein: a long protein chain; HCV RNA is translated into a polyprotein, which is then cleaved (split or separated) into individual HCV proteins by viral and cellular protease enzymes.
Polysaccharides: carbohydrate molecules composed of sugars; glycosaminoglycans are a type of polysaccharide.
Positive-sense RNA: RNA strands that can function as messenger RNA (mRNA) and be translated into proteins; the HCV genome is a single positive-sense RNA molecule.
Prenylation: a type of chemical modification that directs cellular proteins to cell membranes.
Protease: an enzyme that breaks down proteins. HCV contains two viral protease enzymes: the NS2-NS3 protease, and the NS3 serine protease.
Protein: large molecules composed of amino acids. The genetic template or blueprint for proteins is stored (encoded) in RNA and DNA. HCV RNA encodes at least ten viral proteins. HCV structural proteins (core, E1, E2) become components of new virions; HCV non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) play a variety of roles in viral replication.
Protein expression: the production of individual HCV proteins; an *in vitro* research tool.
Pseudotyped virus: an engineered hybrid of two different viruses (e.g., the core particles of a retrovirus bearing HCV envelope proteins on the surface).
PTB (polypyrimidine tract binding protein): a cellular protein that binds to the HCV 3' UTR.
Quasispecies: a dynamic population of closely related but distinct genetic sequences; the population of HCV within an individual, including strains containing mutations.
Receptors: molecules on cell surfaces that other molecules bind to, for signaling and cell entry. HCV uses one or more receptors to enter cells.

Receptor-mediated endocytosis: the mechanism through which HCV is believed to enter cells; HCV envelope proteins would bind to one or more cell surface receptors, triggering the cell to internalize the virus, encapsulating it into a vesicle.
Replicase: the HCV replication complex, the structure within which HCV RNA strand synthesis occurs. Composed of non-structural (and probably cellular) proteins.
Replication: the process of creating new HCV virions; occurs within cells (primarily hepatocytes).
Replication complex: the structure within which HCV RNA strand synthesis occurs. Composed of non-structural (and probably cellular) proteins. Also called the replicase or membraneous web.
Replicon: genetic material capable of autonomous replication and protein synthesis; HCV replicons are the main *in vitro* model for research on viral replication.
Retrovirus: a type of RNA virus; HIV (but not hepatitis C) is a retrovirus.
Ribosome: cellular machinery responsible for translation (protein synthesis); the ribosome "reads" mRNA.
Ribosomal protein L22: a component of the ribosome that binds to the HCV 3′ UTR.
RNA: a molecule (composed of nucleotides) containing genetic information that can be translated into proteins; the HCV genome is a single strand of positive-sense RNA.
RNA-dependent RNA polymerase (RdRp): the HCV polymerase enzyme, contained within HCV NS5B and responsible for synthesizing new positive-sense and negative-sense HCV RNA.
Serial passage: taking sera (the clear fluid portion of blood) containing HCV and r epeatedly passing it through (i.e., infecting) animals, from one animal to the next; this can increase the virulence (infectiousness) of the virus.
Signal peptidases: a type of cellular protease enzymes; signal peptidases cleave (split or separate) part of the HCV polyprotein.
SR-BI (human scavenger receptor class B type I): a cell surface receptor; possibly involved with HCV entry into cells.
Strand synthesis: the creation of new RNA strands by a polymerase enzyme; HCV uses the NS5B RNA-dependent RNA polymerase to synthesize new negative-sense HCV RNA from a positive-sense HCV RNA strand, and new positive-sense HCV RNA from a negative-sense HCV RNA strand. Strand synthesis is vital to HCV replication.
Subgenomic: containing only part of the genome; subgenomic HCV replicons typically contain only the RNA sequences that encode the HCV non-structural proteins.
Taxonomies: systems of classification.
Tissue culture: organ tissue (e.g., from the liver) grown and maintained in a laboratory; an *in vitro* research tool.
Translation: protein synthesis; the ribosome translates HCV RNA into viral proteins. Translation is vital to HCV replication.
Ubiquitination: a cellular process that targets proteins for destruction.
Uracil: a nucleoside base; one of the four building blocks of RNA.
UTR (untranslated region): regions of HCV RNA that do not encode viral proteins (the 5′ UTR and the 3′ UTR).

Vesicle: a small pocket of fluid within cells surrounded by a thin membrane.
Viral heterogeneity: genetic diversity among viral isolates.
Viral isolates: an individual strain of virus.
Virion: an individual virus particle.