Hepatitis C: From Discovery to Cure[1]

Nobel Lecture, December 7, 2020 by
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To begin, I want to thank the Nobel committee and organization for recognizing the hepatitis C story and allowing me and my esteemed colleagues and friends Harvey Alter and Michael Houghton to represent the many in this field who made this happen. I'm humbled and deeply honored to be awarded this prize but as you will learn shortly, this really came about through a series of chance events.

Now let's begin with some history and context on how we came to work on hepatitis C. Going back into the 17th and 19th centuries there were outbreaks of jaundice. This traces back even to Greek and Roman times of unexplained jaundice outbreaks. In the 1930s it was noticed that individuals vaccinated with an attenuated strain of yellow fever virus stabilized with human serum often came down with hepatitis or jaundice that was not characteristic of yellow fever. Then later, there was recognition of a link between hepatitis and people administered human serum. In the 1940s it was recognized that there were indeed two types of viral hepatitis – infectious, which was likely the form causing problems during

1. The references cited in this transcript are sparse and do not reflect the numerous seminal contributions from the many others who have contributed to the hepatitis C success story.

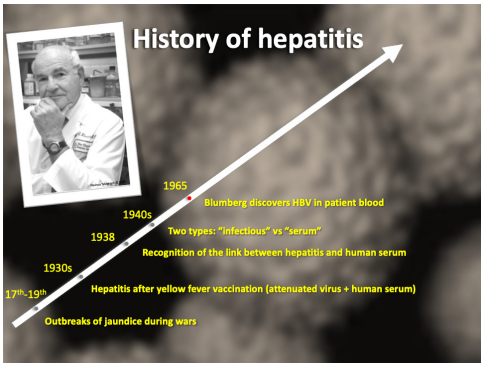


Figure 1. History of hepatitis. Events leading up to the breakthrough discovery by Baruch Bloomberg that the Australia antigen was the surface antigen of hepatitis B virus are depicted. These advances led to the development of a hepatitis B vaccine.

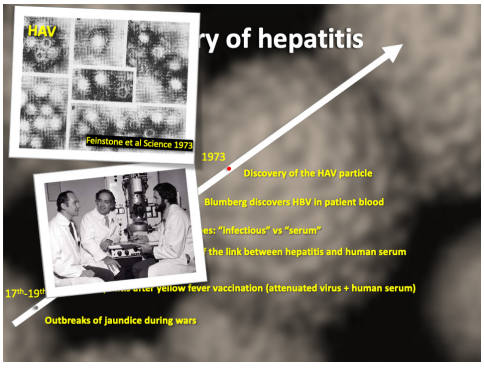


Figure 2. Discovery of the hepatitis A particle. Steve Feinstone, Al Kapikian, and Bob Purcell (lower photo) utilized immuno-electron microscopy to identify hepatitis A particles (upper photo) in 1973. These discoveries led to the development of assays to screen the blood supply for both hepatitis A and hepatitis B.

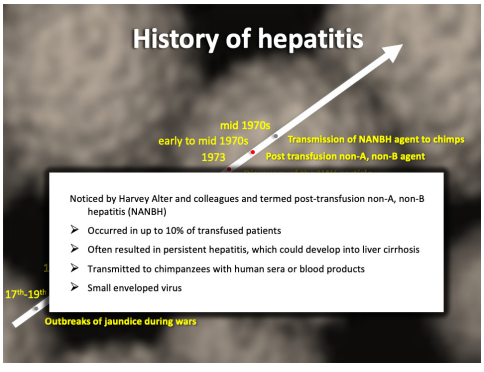


Figure 3. Post-transfusion hepatitis continues. Despite testing of the blood supply, Harvey Alter and colleagues noticed continued cases of hepatitis in up to 10% of transfused patients, which they termed non-A, non-B hepatitis (NANBH). The successful transmission of NANBH to chimpanzees led to the discovery that the agent was filterable, and efforts by many groups led to the conclusions that the agent was virus-like and probably enveloped given its sensitivity to organic solvents.

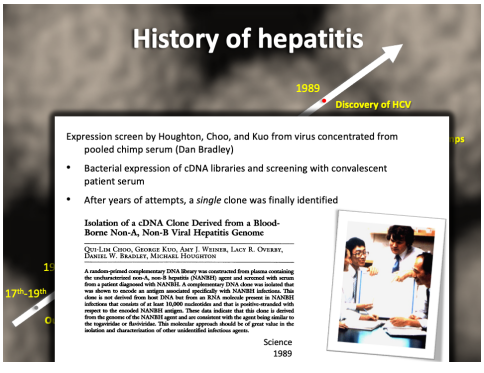
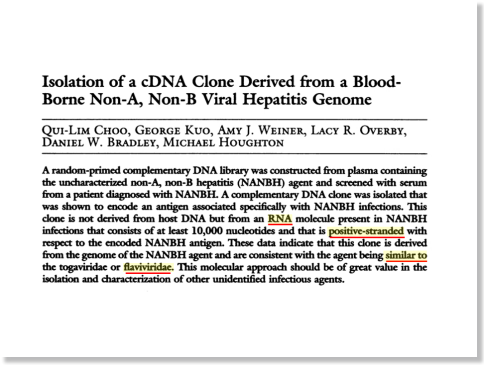


Figure 4. The discovery of hepatitis C virus. Using an expression screening approach, Michael Houghton and his team at Chiron Corporation and Dan Bradley at the Centers for Disease Control, used virus from infected chimpanzees and serum from a convalescent NANBH patient to isolate a cDNA clone.

wars, versus – serum, which I just mentioned. A major breakthrough came in 1965 when Baruch Bloomberg (Figure 1) discovered that the Australia antigen was actually the surface antigen of hepatitis B virus and this led to the development of a vaccine for hepatitis B and other advances. In 1973, Steve Feinstone, Al Kapikian, and Bob Purcell identified hepatitis A virus (1) using immuno-electron microscopy (Figure 2). With this breakthrough, it became possible to develop assays that could be used to screen blood products for both these viruses. However, that was not the whole story. Harvey Alter and others had noticed that there were still cases of post-transfusion hepatitis not associated with HAV and HBV, so they named this non-A, non-B hepatitis (2, 3). This occurred in about 10% of transfused patients and often resulted in persistent hepatitis that could lead to liver fibrosis, cirrhosis and was later associated with liver cancer (Figure 3). Several groups succeeded in transmitting this agent to our closest relative, the chimpanzee, but not to other non-human primates or more distant mammalian species. It was therefore pioneering chimpanzee studies that showed that the agent (or agents) was filterable, and virus-like, and probably enveloped given its inactivation by organic solvents. These findings spawned a great deal of effort among many groups to identify the responsible mystery virus. But that took almost 15 years from the time that post-transfusion non-A, non-B hepatitis was noticed until 1989. The transformative breakthrough came from Michael Houghton and his team at Chiron Corporation (Figure 4), collaborating with Harvey Alter at NIH and Daniel Bradley at the Centers for Disease Control. Using a cDNA expression screening approach, they identified a single short cDNA clone that was immunoreactive with serum from a convalescent non-A, non-B hepatitis patient. They extended this sequence to reveal a nearly 10 kb RNA viral sequence; serologic assays confirmed that this new virus, called HCV, was indeed the mysterious non-A, non-B agent (4). This was where we were in 1989. What caught my attention is highlighted in the abstract of the landmark Science paper (Figure 5). The HCV RNA genome was positive-sense with a size and organization reminiscent of the flaviviruses, which were also small, filterable, and inactivated by organic solvents.

At the time, we were intensively studying the prototype flavivirus, yellow fever virus. The lab phone rang and on the line was Stephen "Steve" Feinstone. As mentioned earlier, Steve co-discovered HAV. Still working with Robert "Bob" Purcell at the NIH, he had been a key player in several of the early chimpanzee studies with the non-A, non-B agent. He called me up out of the blue and said "Hey Charlie, how about making a yellow fever 17D – HCV chimera as a vaccine?" Steve was then at the FDA and interested in making a preventative HCV vaccine. We had been working with a tissue culture passaged, live-attenuated yellow fever virus strain,

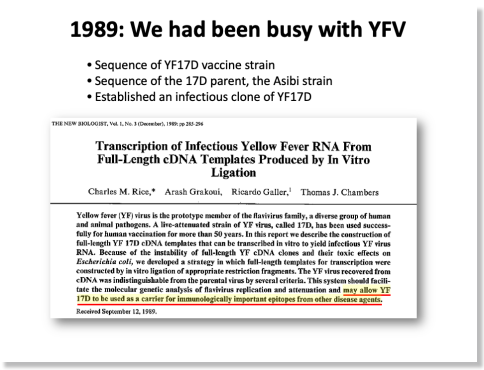


Isolation of a cDNA Clone Derived from a Blood-Borne Non-A, Non-B Viral Hepatitis Genome

QUI-LIM CHOO, GEORGE KUO, AMY J. WEINER, LACY R. OVERBY, DANIEL W. BRADLEY, MICHAEL HOUGHTON

A random-primed complementary DNA library was constructed from plasma containing the uncharacterized non-A, non-B hepatitis (NANBH) agent and screened with serum from a patient diagnosed with NANBH. A complementary DNA clone was isolated that was shown to encode an antigen associated specifically with NANBH infections. This clone is not derived from host DNA but from an RNA molecule present in NANBH infections that consists of at least 10,000 nucleotides and that is positive-stranded with respect to the encoded NANBH antigen. These data indicate that this clone is derived from the genome of the NANBH agent and are consistent with the agent being similar to the togaviridae or flaviviridae. This molecular approach should be of great value in the isolation and characterization of other unidentified infectious agents.

Figure 5. The NANBH agent is similar to the flaviviruses. The agent, named hepatitis C virus, was a positive-sense RNA virus with a size and genome organization similar to the flaviviruses, and also shared the characteristics of being small, filterable and inactivated by organic solvents.



1989: We had been busy with YFV

- Sequence of YF17D vaccine strain
- Sequence of the 17D parent, the Asibi strain
- Established an infectious clone of YF17D

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Transcription of Infectious Yellow Fever RNA From Full-Length cDNA Templates Produced by In Vitro Ligation

Charles M. Rice,* Arash Grakoui, Ricardo Galler,† Thomas J. Chambers

Yellow fever (YF) virus is the prototype member of the flavivirus family, a diverse group of human and animal pathogens. A live-attenuated strain of YF virus, called 17D, has been used successfully for human vaccination for more than 50 years. In this report we describe the construction of full-length YF 17D cDNA templates that can be transcribed in vitro to yield infectious YF virus RNA. Because of the instability of full-length YF cDNA clones and their toxic effects on *Escherichia coli*, we developed a strategy in which full-length templates for transcription were constructed by in vitro ligation of appropriate restriction fragments. The YF virus recovered from cDNA was indistinguishable from the parental virus by several criteria. This system should facilitate the molecular genetic analysis of flavivirus replication and attenuation and may allow YF 17D to be used as a carrier for immunologically important epitopes from other disease agents.

Received September 12, 1989.

Figure 6. Development of molecular tools for working with yellow fever virus. The discovery of HCV in 1989 coincided with work in my lab to sequence both the 17D vaccine strain and its virulent Asibi parent, and to develop an infectious clone system to generate wildtype and mutant derivatives for study. The underlined last sentence caught the attention of Steve Feinstone.

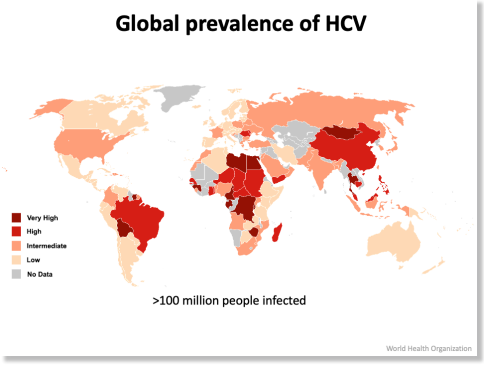
Yellow fever meets hepatitis C:

How a phone call from Steve Feinstone changed my life

Steve! Great idea! But what do we know about HCV?

Hey, Charlie! How about making YF17D/HCV chimeras as a vaccine?

Figure 7. A phone call from Steve Feinstone propels me to work on HCV. During discussions with Steve about his idea to use YFV to make an HCV vaccine it became clear that we knew little about the HCV genome and its expression.



Global prevalence of HCV

Very High
High
Intermediate
Low
No Data

>100 million people infected

World Health Organization

Figure 8. The global prevalence of HCV. At the time, HCV infection was present across the globe with estimates of more than 100 million chronically infected individuals.

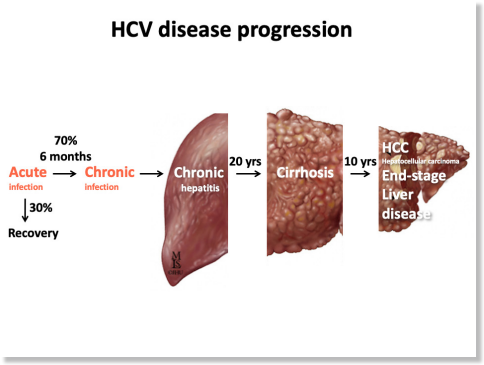


Figure 9. HCV disease progression. Acute HCV infection, usually asymptomatic, often progresses to chronic infection and chronic hepatitis. After many years, individuals can suffer from advanced liver fibrosis, cirrhosis, hepatocellular carcinoma, and end stage liver disease.

called 17D, that was developed by Max Theiler at the Rockefeller Foundation and reported in 1937. It has been used to vaccinate about half a billion people and is one of the most safe and effective human vaccines ever developed. Max was awarded the Nobel prize for this work in 1951. In 1985, we reported the 17D sequence and the sequence of the virulent parental virus, the Asibi strain, a few years later. After much work, we had established an infectious cDNA clone for 17D, which allowed manipulation of the viral genome as DNA to investigate the effects of specific mutations or other alterations on virus replication. This work was published (5) in *The New Biologist* – an aspiring new journal at the time, that went extinct shortly thereafter; the last sentence of the abstract (Figure 6) stated *"this may allow yellow fever 17D to be used as a carrier for immunologically important epitopes from other disease agents".* This is what caught Steve's attention (Figure 7). We got to talking about this but realized that we knew very little about the hepatitis C genome and how it was expressed. We did know by that time that HCV was a major problem, with more than 100 million people chronically infected (Figure 8). That became increasingly obvious when diagnostic tests were developed shortly after HCV's discovery. As Harvey and others had shown in the 1970s and 1980s, this was an infection that was usually asymptomatic in the acute phase, often went chronic, and chronic infection could lead to severe liver disease (Figure 9). Chronic HCV was a major public health problem. The immediate goals were clear: Clean up the blood supply, which was done quickly in the early 1990s; Try to educate high-risk groups – maybe develop a preventative vaccine; Identify those infected and improve treatment. The hope at that point, although this was a bit of a pipe dream, was to eradicate this exclusively human virus (Figure 10).

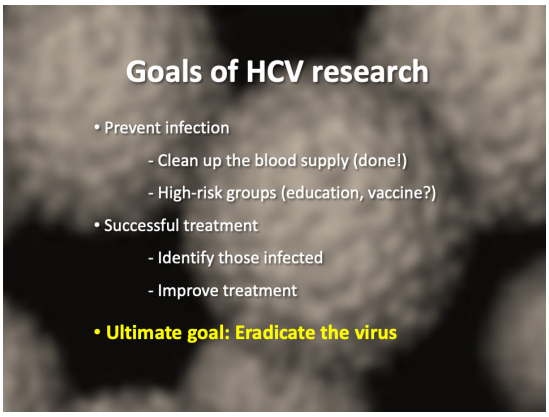


Figure 10. Goals of HCV research. At that time, the goals were clearcut. The blood supply was quickly cleaned up, but high-risk groups needed to be educated and potentially vaccinated, infected individuals needed to be identified, and treatment options needed improvement. The ultimate goal was to eradicate the virus.

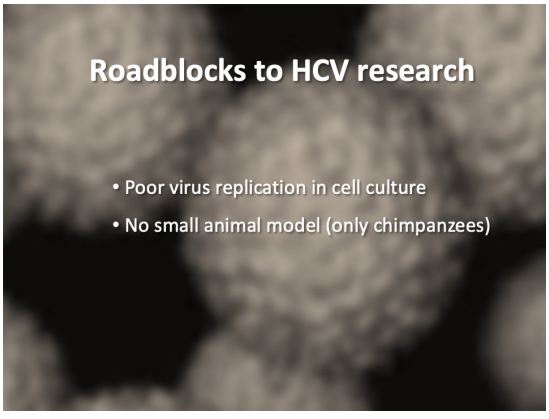


Figure 11. HCV research roadblocks. The lack of HCV replication in cell culture and in a small animal model limited researchers to working with clinical samples, chimpanzees, and information gleaned from the HCV sequence.

So, now what? There were some real challenges for hepatitis C (Figure 11). The virus had not been shown to replicate in any cell culture despite repeated attempts. There was no animal model other than chimpanzees, which were extremely important but available to only a few groups, and in very limited numbers. This left the field with clinical samples or chimpanzee passaged material, and what we could glean from the HCV genome sequence: A 9.6 kb RNA with a long open reading frame that could encode a polyprotein of over 3000 amino acids, flanked by short 5' and 3' non-coding sequences. An RNA structure near the 5' end of the genome was presumed (and later shown) to promote internal initiation of protein translation. But how was this polyprotein processed into the proteins the virus needed to replicate its RNA and produce progeny virus? Along with groups in Europe and Japan, Arash Grakoui who was a technician in the lab at that time took on the challenge. What he and others found were ten hepatitis C virus proteins including likely components of virus particles and the viral RNA replication machinery. Cleavages to produce the mature proteins were catalyzed by a combination of host and viral pro-

teases. This defined the HCV protein boundaries and allowed investigators to begin to study viral enzymatic activities that could be attractive drug targets. Examples included an NS3-4A complex, which is a serine protease/helicase whose structure was later determined, and NS5B, the RNA-dependent RNA polymerase of the virus (Figure 12, reviewed in ref. 6).

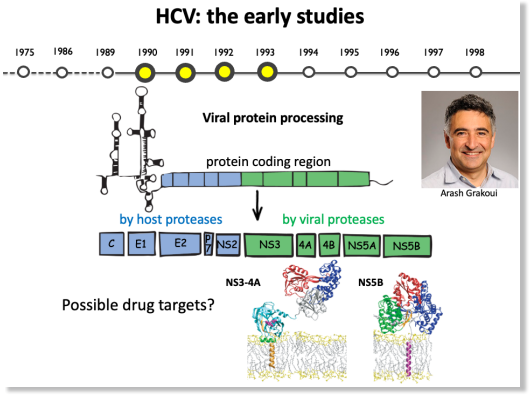


Figure 12. Early studies on HCV. Arash Grakoui in my lab, along with other investigators around the world, took on the task to study how the HCV polyprotein is processed. It was found that both host and viral proteases are required to yield 10 viral proteins, two of which had enzymatic activity that could be targeted for drug development. In subsequent years structures of the NS3-4A protease/helicase and the NS5B RNA-dependent RNA polymerase were determined by several groups to aid these efforts.

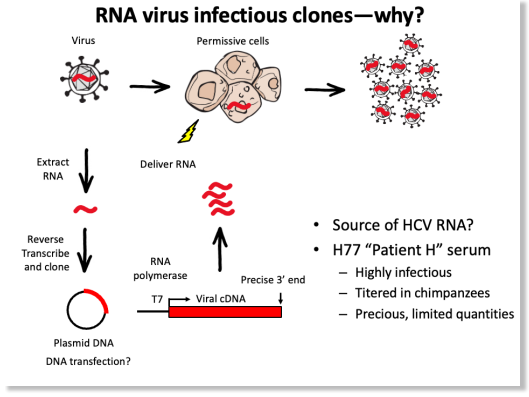


Figure 13. The utility of RNA virus infectious clones. In a normal virus infection (top) the virus particle efficiently delivers its genome RNA to the cell, and if permissive for infection, results in a burst of progeny viruses. For positive-strand RNA viruses like HCV, RNA isolated from the virus particle, if introduced into susceptible cells, can function as a messenger RNA and set off the entire virus life cycle (bottom). Generating a cDNA intermediate of the genome RNA allows virologists to use DNA manipulations to introduce desired changes. The resulting plasmid DNA construct can be transfected into cells, where if transcribed into a functional RNA, can launch the viral life cycle. Alternatively, the plasmid can be designed more precisely to use a phage-derived RNA polymerase to produce unlimited quantities of a synthetic mimic of the viral genome RNA. We utilized this latter approach using RNA from the highly infectious serum from "Patient H".

Advances were being made, but we still lacked the ability to test the importance of these findings for actual virus replication. The next obvious step was to make a cDNA clone of HCV that could be transcribed in vitro to produce synthetic HCV genome RNA that was infectious. If this succeeded, we would be able to produce unlimited quantities of infectious RNA and test different cell types and culture conditions in the hope of finding one that allowed HCV replication. Figure 13 displays the concept and possible routes to making functional cDNA clones. Success for this general approach was first reported in bacteria for the RNA phage QB (7) and a few years later for poliovirus (8). We had also succeeded in establishing such systems for the prototype alphavirus, Sindbis virus (9) and, as mentioned earlier, the vaccine strain of yellow fever virus (5).

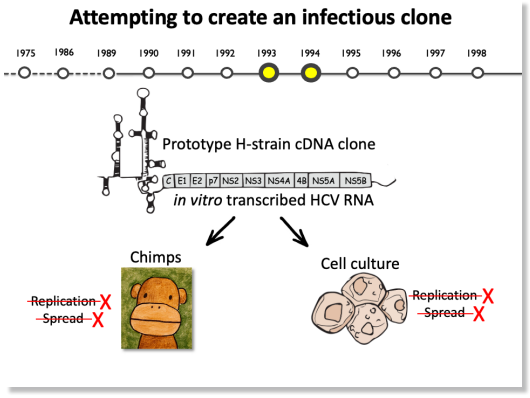


Figure 14. Launching HCV infection from an infectious clone. Using RNA from Harvey Alter's patient serum containing the genotype 1a "H77" strain of HCV, we generated a prototype HCV infectious clone from which we transcribed the RNA in vitro. However, no permissive cell lines had been established, and attempts to launch viral replication in cell culture failed. Working with Steve Feinstone, the RNA was injected into the liver of chimpanzees, but this also failed.

We began to try and accomplish this for hepatitis C (Figure 14). Our initial work had been carried out with the genotype 1a "H77" strain of HCV. This was obtained in 1977 by Harvey Alter at NIH from a post-transfusion patient, Mr. Hutchinson, in the acute, pre-seroconversion phase of infection (10). It had been titered in chimpanzees and was highly infectious, but it was precious and in limited quantities. The next dilemma was how to confirm that RNA transcripts from a cDNA clone were indeed infectious. The obvious and usual path was to simply transfect either a plasmid, or in vitro transcribed RNA, into cells shown to be permissive for virus replication. However, for HCV we didn't have a cell culture system that had been proven to work. Transfection of cells might not work, unless the block to infection was at virus entry, which would be bypassed. The only remaining possibility was the chimpanzee – by taking HCV genome RNA transcripts and injecting them directly into the liver and hoping that some intact RNA would successfully enter hepatocytes and initiate replication, which could then spread and amplify. This

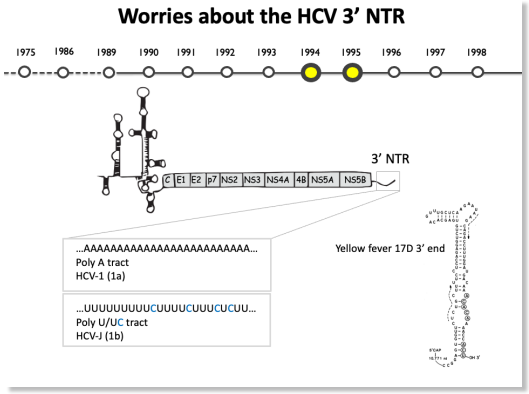


Figure 15. Conflicting reports on the sequence of the HCV 3'-non-translated region. The original 1989 report suggested a 3' terminal polyA tract, like normal cellular mRNAs, but sequences of other isolates reported later suggested a poly U/UC tract. However, it was known that distantly related yellow fever virus terminates with neither of these, but rather a stable stem loop RNA structure.

seemed like a crazy thing to try. However, Sue Emerson in Bob Purcell's lab, again at the NIH, had shown more than a decade earlier that you could use in vivo RNA transfection to launch hepatitis A virus infection in marmosets (11). It wasn't such a crazy idea – it had been shown to work, at least for one other virus. Steve Feinstone had access to chimpanzees and gave our prototype HCV-H full-length RNA a try. It failed. This was frustrating but we didn't give up.

We began to worry about the HCV genome sequence, might there be something missing? We focused on the 3' non-translated region given conflicting reports in the literature (Figure 15). The initial 1989 report suggested a 3' terminal polyA tract like normal cellular mRNAs, other, later isolate sequences, reported a polypyrimidine (poly U/UC) tract. We knew from our work on yellow fever that there was a different possibility. The yellow fever virus genome didn't terminate with either of these homopolymer tracts but rather a stable RNA stem loop structure. Alexander "Sasha" Kolykhalov, a remarkably talented molecular biologist from Novosibirsk, had immigrated to the US and joined my lab. Sasha took on this challenge and developed a fairly simple, at least on paper, 3' RACE protocol (Figure 16), which many of you reading this will think is just standard fare, but we were challenged by the fact that we had only tiny amounts of HCV RNA; every step had to be optimized in order to retrieve clones that might have arisen from the 3' end of HCV genome RNA. Sasha did all this, and what he found was quite a surprise (Figure 17). Following the stop codon of this long open reading frame there was a short region, quite variable among HCV genotypes, followed by an internal polyU/polyUC tract of variable length, and then a new sequence of about 98 bases (12), highly conserved among different HCV isolates (13). These kinds of terminal RNA elements in positive strand RNA virus genomes are absolutely required for replication. Simultaneously, the same sequence was discovered by Kunitada Shimotohno's lab in Japan (14).

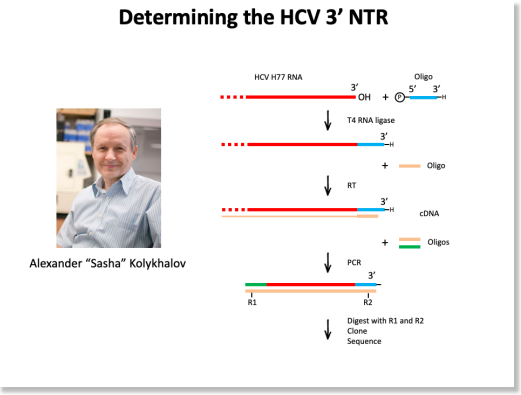


Figure 16. 3′ RACE determination of the HCV 3′NTR sequence. Using optimized conditions for each of the steps, Sasha Kolykhalov performed 3′ rapid amplification of cDNA ends (RACE) in order to determine the HCV 3′NTR sequence. Starting with HCV RNA from patient serum, T7 RNA ligase was used to attach an oligo at the 3′ end. A complementary DNA oligo was then used to prime cDNA synthesis using reverse transcriptase (RT). The polymerase chain reaction (PCR) and the same oligo in combination with an oligo complementary to known upstream HCV sequences were used to amplify the cDNA. The DNA was then digested with restriction enzymes, cloned, and sequenced.

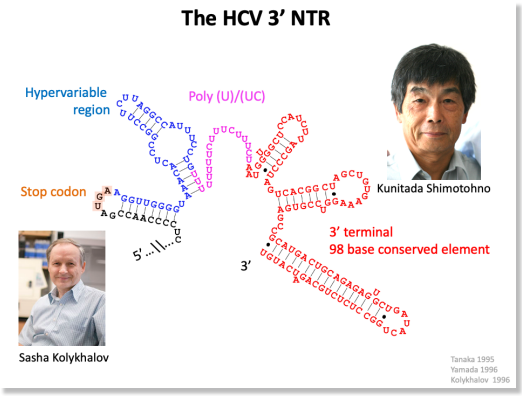


Figure 17. RNA structure of the HCV 3′NTR. Sequencing revealed that the HCV polyprotein stop codon is followed by a short sequence, highly variable between isolates, an internal polyU/polyUC tract of variable length, and a novel ~98 base pair sequence, highly conserved across different HCV isolates. This conserved sequence was simultaneously discovered in the laboratory of Kunitada Shimotohno.

The missing piece found, we modified our HCV-H clone to include the proper 3' non-coding region and repeated what we had tried before. No luck. We again failed to see any evidence for HCV replication in cell culture or in the chimpanzee model. After discovery of this new, highly conserved sequence element, likely to be essential, this was hugely disappointing.

We began to worry about everything. Perhaps this viral infection could not be initiated by injecting naked RNA into the liver of an animal. Could there be part of the genome still missing? Was HCV genome RNA not infectious? Could there be a virus-associated protein needed to initiate replication? What about RNA modifications deposited in an infected cell that would absent from synthetic HCV genome RNA made in a test tube? We knew that HCV was highly error prone, like all RNA viruses. There are

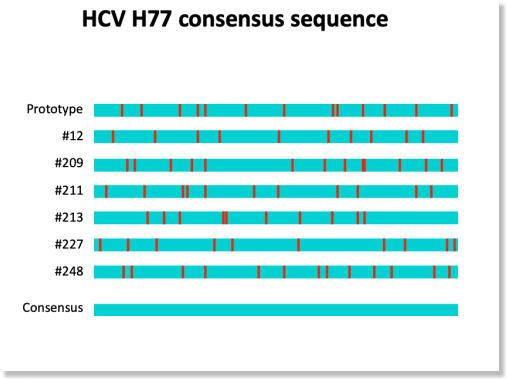


Figure 18. Determination of the HCV H77 consensus sequence. Sequences from 6 clones from a combinatorial library of more than 10^5 full length HCV cDNAs, and sequences from uncloned PCR products were all used to generate a consensus sequence. The prototype HCV clone and each of the 6 clones contained non consensus, potentially deleterious, sequence changes scattered across the genome, which likely explained our negative results.

an estimated trillion variants generated per day in a single chronically infected individual. That's a lot of variation; some of these mutations would be deleterious or lethal. In those days, the enzymes used for cDNA synthesis and cloning were also not as high fidelity as they are today. The one "worry" that we could address was to try and filter out possibly deleterious mutations.

Sasha began by constructing a combinatorial library with a complexity of $>10^5$ full-length HCV cDNAs using high-fidelity assembly reverse transcription–polymerase chain reaction (RT-PCR) followed by subcloning into a recipient plasmid vector containing the 5'-and 3'-terminal HCV consensus sequences. A flanking T7 promoter and an engineered specific restriction site allowed for production of runoff RNA transcripts; 233 of the clones were prescreened by restriction analysis, polyprotein processing, and production of the C-terminal NS5B protein (the HCV RNA-dependent RNA polymerase).

In yet another unsuccessful experiment, RNAs transcribed from 34 clones that passed these analyses were assayed for infectivity by direct intrahepatic injection in two HCV-naïve chimpanzees. As a negative control, a third animal received similar injections with transcripts from a clone containing a 20-amino acid in-frame deletion encompassing the NS5B polymerase active site. Serum samples were collected for 2 months after inoculation and analyzed for evidence of HCV replication. Neither experimental animal nor the negative control animal exhibited signs of productive infection. Of note was the absence of detectable circulating HCV RNA 2 days after inoculation. Missing terminal sequences, low RNA transfection efficiencies in vivo, and errors introduced during cDNA synthesis or PCR amplification might account for these negative results. Sasha sequenced six clones from the combinatorial library, as well as uncloned RT-PCR products, to determine a consensus sequence for the

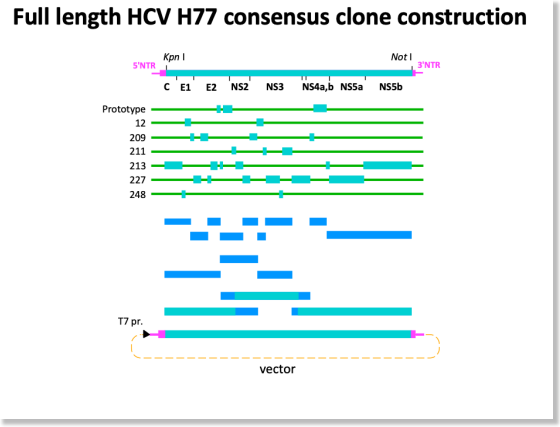


Figure 19. Assembly of an HCV H77 consensus clone. The prototype and 6 clones are shown schematically below the resultant consensus infectious clone. PCR and standard restriction enzymes were used to assemble the consensus clone. Regions from the clones used in the assembly are shown in teal rectangles. Blue rectangles below show intermediates generated for the assembly process. The teal and blue rectangles show the three final pieces that were assembled and cloned into the Kpn I and Not I sites of the prototype infectious clone to generate the consensus clone.

H77 isolate (Figure 18). From this analysis, it became clear that each of the six clones sequenced contained numerous non consensus sequence changes scattered throughout the genome, which could be deleterious and would explain the negative results. Sasha used this information to painstakingly direct the assembly of a full-length clone reflecting the H77 consensus sequence (Figure 19).

Aside from the sporadic changes corrected in this consensus clone, even greater sequence heterogeneity was noted in three regions of the HCV genome. During analysis of the extreme 5' terminus, we found a substantial number of clones that contained one or more bases in addition to the reported 5'-terminal sequence (5'-GCCAG . . . -3'). In the structural protein coding region, the N-terminus of the E2 virion envelope glycoprotein was highly variable, but a predominant sequence was identified. Near the 3' terminus, the poly(U/UC) tract was variable in length and composition. Because these differences might be functionally significant, variants of the consensus clone with additional bases at the 5' terminus and two different poly(U/UC) tracts were constructed (Figure 20). Silent nucleotide substitutions were engineered as markers so that virus recovered from transfected animals could be sequenced to identify which clones were infectious. From each of the 10 clones, full-length uncapped RNAs were transcribed from linearized template DNAs and again sent to Steve Feinstone at the FDA – Steve and his team did the chimpanzee work for the studies that I've mentioned and was involved in essentially all of our HCV work in the 1990s and beyond. This was probably going to be our final attempt.

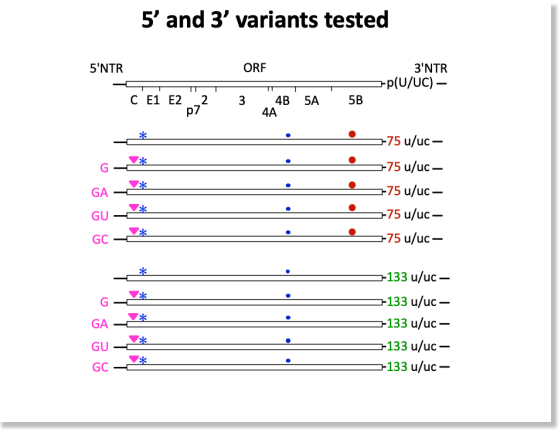


Figure 20. Variants constructed for testing. The HCV genome is shown schematically at the top. Variants constructed for testing are shown below. Pink indicates the 5′ terminal variants with extra bases, the asterisk represents the predominant hypervariable sequence of the E2 virion envelope glycoprotein N-terminus, and red and green show the two lengths of the poly (U/UC) tract. Red and blue circles indicate the location of silent mutations engineered to allow determination of which sequences were infectious.

Two chimpanzees were identified that were seronegative for all known hepatitis viruses, negative for HCV RNA by nested RT-PCR, and had normal baseline levels of liver enzymes. Animals were inoculated by direct intrahepatic injection at multiple sites (Figure 21). Serum samples and liver biopsies were taken before inoculation and at weekly intervals thereafter. For 3 months after inoculation, serum samples were assayed for liver enzymes, antibodies to HCV, and viremia, as assessed by quantifying circulating HCV RNA (15).

The results finally began trickling in and they were breathtaking (finally) (Figure 22).

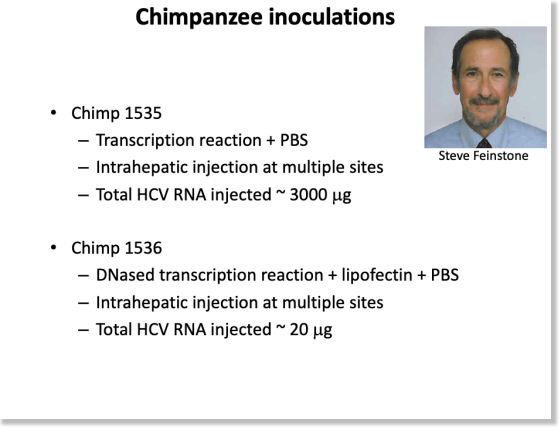


Figure 21. Inoculation of two chimpanzees with the 10 HCV clone variants. In collaboration with Steve Feinstone at the FDA, two chimps were inoculated with the pool of 10 HCV RNA variants. The conditions for each chimp are listed.

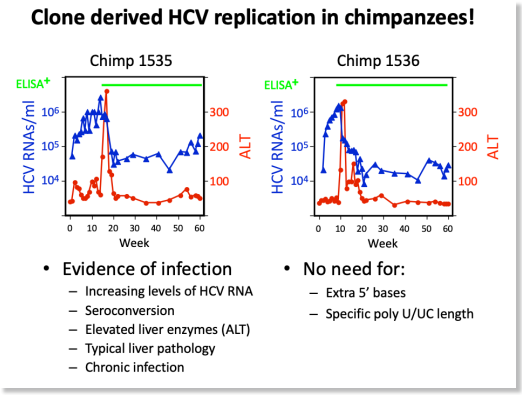


Figure 22. Clone-derived HCV replication in chimpanzees. Serum HCV RNA levels are shown in blue. ALT (red) and ELISA positivity for antibodies to HCV (green) are shown for each of the chimpanzees. Increasing HCV RNA, seroconversion, elevated liver enzymes, liver pathology and chronic infection all were evidence for HCV infection from the clones. Sequencing of the replicating variants determined that there was no need for the extra 5′ bases, nor a specific poly(U/UC) tract length.

Both chimpanzees had increased concentrations of serum alanine aminotransferase (ALT), which is a marker for liver damage; after a large spike, ALT gradually declined to the preinoculation baseline levels. HCV RNA titers were undetectable before inoculation, rapidly rose thereafter and peaked shortly before the ALT spike after which they rapidly declined. Histologic changes in liver biopsies were typical for hepatitis C in chimpanzees and were characterized predominantly by portal inflammation and focal necrosis. The severity of histologic lesions appeared to parallel the ALT elevations. These pathogenesis profiles were strikingly reminiscent of those obtained in chimpanzees inoculated with the infectious H77 material or other HCV-containing samples (16). Both animals eventually became chronically infected (17).

To prove that the HCV RNA signals detected were not due to residual template DNA from the transcription reactions, we assayed all the samples by PCR without reverse transcription. No products were obtained, which demonstrated that the signals detected were due to HCV RNA. If the HCV RNA detected in serum was from replicating virus, RNAs should be packaged in enveloped virions and resistant to degradation by ribonuclease (RNase), whereas residual transcript RNA should be RNase sensitive. Indeed, the HCV RNA in samples of chimpanzee serum was resistant to RNase digestion under conditions that completely degraded excess naked competitor RNA. Moreover, there was no correlation between the level of apparent viremia and the amount of inoculated RNA, which differed by ~150-fold between the two inoculated animals. Finally, in two previous animal experiments (a total of six animals), circulating HCV-specific nucleic acid was never detected, even as early as 2 days after inoculation. These experiments suggested that circulating RNA was

a result of authentic virus replication rather than release of inoculated nucleic acid. Restriction enzyme digestion and sequence analysis of recovered viral RNA revealed the presence of engineered markers, proving that these infections stemmed from the inoculated transcript RNAs.

Silent markers were analyzed to identify the 5'-terminal sequence(s) and the length(s) of poly(U/UC) tract required or preferred for initiating infection. Although it was not possible to draw firm quantitative conclusions about differences in specific infectivity, the results clearly demonstrated that the RNA transcripts without any additional 5' nucleotides were infectious (15). Transcripts with additional 5' nucleotides could also initiate infection, although our analysis did not allow us to distinguish among the various derivatives tested. Transcripts containing either the 75-base or the 133-base poly(U/UC) tracts were infectious, but the 133-base poly(U/UC) tract was preferred.

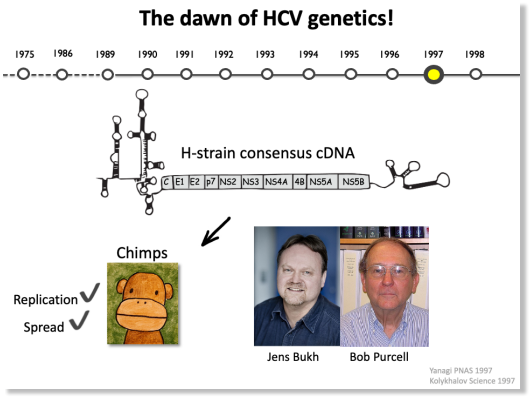


Figure 23. The dawn of HCV genetics. Nearly identical results were obtained by Jens Bukh and Bob Purcell. HCV genetics were now possible.

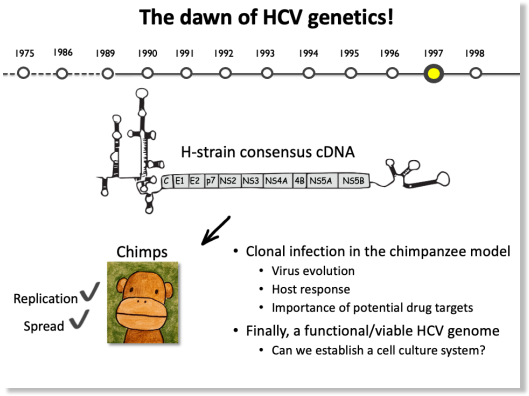


Figure 24. Potential uses of the HCV infectious clone. Given that the generated clones were infectious, clonal infection in the chimpanzee model would allow evaluation of virus evolution, host responses, and the importance of potential drug targets. Whether the functional viable HCV genome could be used to establish a cell culture system remained to be seen.

The demonstration that RNA transcribed from cloned HCV cDNA can initiate infection and cause hepatitis in transfected chimpanzees provided formal proof that HCV alone is the causative agent of this disease. Our experiments also defined the elements of a functional HCV genome RNA. Aside from a consensus genome sequence, no additional 5' sequences were required for infectivity and 3' poly(U/UC) tracts of variable length were tolerated. Very similar results were reported shortly thereafter by Jens Bukh and Bob Purcell (18), also using the HCV-H strain and the chimpanzee model (Figure 23). With this model, HCV infection could be launched from clonal derivatives to facilitate studies on virus evolution, pathogenesis, and host immune response relevant to understanding the factors that determine viral clearance versus chronic infection (Figure 24). Infectious HCV RNAs could be produced in unlimited quantities and used to identify and optimize cell-culture replication systems and to begin genetic analyses of HCV replication in vivo and in vitro. For example, the importance of potential drug targets could now be tested by mutagenesis of active site residues, which showed that each of the four HCV-encoded enzymes was absolutely required for infectivity (19).

With a functional, viable HCV genome sequence that we could make in unlimited quantities, could we use this to establish a cell culture replication system? One would hope so, but that didn't turn out to be the case. These RNAs, that were infectious in vivo in the most stringent model that you could imagine, – the chimpanzee model – were unable to replicate when transfected into a variety of different cell types. Similar results were being seen by other groups around the world, working with different HCV isolates including chimpanzee-validated infectious clones.

The next breakthrough came from Ralf Bartenschlager's lab (Figure 25). Ralf and Volker Lohmann engineered what is called a subgenomic replicon using a consensus sequence from a German genotype 1b isolate. This modified HCV RNA was engineered to express an antibiotic resistance gene product under the control of the HCV RNA replication machinery. The structural protein coding region was substituted with the neomycin resistance gene, followed by an internal ribosome entry site to drive translation of the downstream non-structural proteins believed to comprise the HCV RNA replication machinery. If such an RNA found itself in a cellular environment where it could replicate, not kill the cell, and express the neomycin resistance gene product, then those cells should become neomycin resistant and propagate in the presence of the antibiotic. Such an approach had the advantage of employing positive selection for even very rare events. After electroporation of this subgenomic replicon RNA into a human hepatoma cell line, Huh-7 cells, and selection with the neomycin analog G418, they were able to isolate drug resistant colonies that harbored continuously replicating HCV RNAs (20). This was a

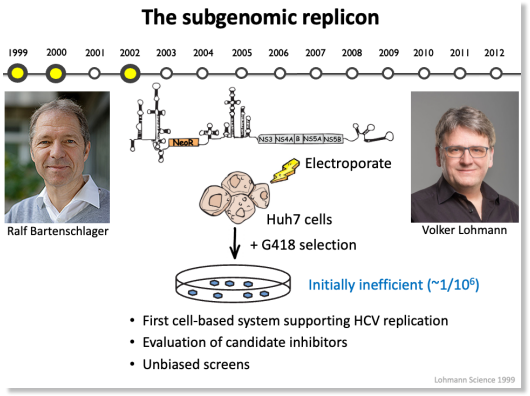


Figure 25. The HCV subgenomic replicon. Using a consensus sequence from an HCV genotype 1b isolate, Ralf Bartenschlager, Volker Lohmann and colleagues engineered a replicon that expressed the neomycin resistance gene in place of the structural protein coding region and under the control of the HCV replication machinery. Translation of the HCV proteins believed to be responsible for RNA replication was driven by an internal ribosome entry site from encephalomyocarditis virus. After delivery of the RNA into Huh-7 cells by electroporation, cells that survived and allowed replication of the RNA were selected by growth in the presence of a neomycin analog (G418), giving rise to colonies harboring the replicating HCV RNA. Although initially inefficient, this was the first cell-based system supporting HCV replication and opened the door for viral target-based and unbiased inhibitor screens.

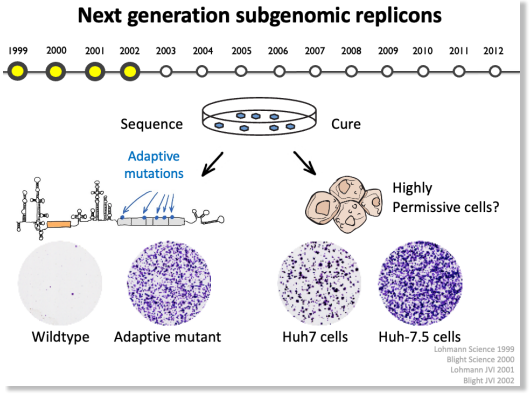


Figure 26. Next generation subgenomic HCV replicons. Sequencing of the replicon RNA within the surviving colonies revealed mutations, which when engineered back into the replicon clone resulted in markedly increased colony formation efficiency, as shown by the purple stain. On the other hand, curing of cells that were permissive for HCV replication by treatment with the antiviral cytokine, interferon, resulted in a cell line (Huh-7.5) more permissive for initiation of HCV replication.

major breakthrough – the first cell-based system supporting HCV RNA replication. This enabled the evaluation of inhibitors that were being developing against HCV enzymes using biochemical assays, and unbiased, cell-based screens to uncover new antiviral targets.

However, a remaining problem was the transduction efficiency of these replicons – only about one in a million transfected cells successfully initiated replication. Next generation replicons emerged from the efforts of several groups (21–23), including the Bartenschlager lab and ours (Figure 26). When we sequenced the HCV RNA present in these drug-resistant colonies, the recovered sequences were not the same as the input replicon

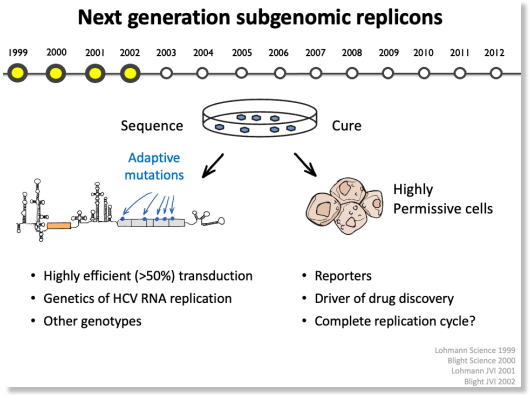


Figure 27. Applications of next generation subgenomic HCV replicons. With improved replicons and highly permissive cells, highly efficient transduction now allowed study of the genetics of HCV RNA replication, the evaluation of other genotypes, and generation of reporter replicons to allow facile drug discovery. But would adaptive mutations and permissive cells allow the complete HCV replication cycle?

RNA. Rather, they harbored mutations, which when re-engineered back into the parental replicon, enhanced the ability of these RNAs to initiate replication. Some single amino acid substitutions could increase the efficiency by more than 10,000-fold. You could cure replicon-containing cells with the antiviral cytokine interferon and identify hepatoma cells that were inherently more permissive for HCV replication. Replicons for other HCV genotypes and subtypes followed, as did replicons that expressed convenient reporter genes. Ten years after the initial discovery of HCV, we now had efficient HCV cell culture systems. It was finally possible to study HCV RNA replication in the laboratory. This was a major driver of drug discovery – compounds could now be tested and improved in a physiologically relevant context (Figure 27).

But were these cell culture adaptive mutations really the secret to getting a complete replication cycle? The subgenomic replicons lacked the HCV proteins important for assembling infectious virus particles, a basic capsid protein and two envelope glycoproteins. The obvious next step was to engineer replicon adaptive mutations into full length genomes that were otherwise unmodified. Upon delivery of these full-length RNAs to hepatoma cells, they replicated efficiently and expressed the expected structural and non-structural viral proteins. But no virus was produced, another roadblock was reached (Figure 28).

Enter Takaji Wakita, a hepatitis virus investigator in Japan. Takaji had been studying HCV isolates from different cases of post-transfusion hepatitis. One patient exhibited a rare case of acute fulminant liver disease. This HCV genotype 2a isolate was called Japanese fulminant hepatitis 1, or JFH-1. When the Wakita lab constructed and tested a subgenomic replicon of JFH-1, it was able to replicate with high efficiency without adaptive mutations. Puzzled by the lack of virus produced by full-length HCV genomes with cell culture adaptive mutations, many in the field were getting suspicious that adaptive mutations, selected for persistent HCV RNA

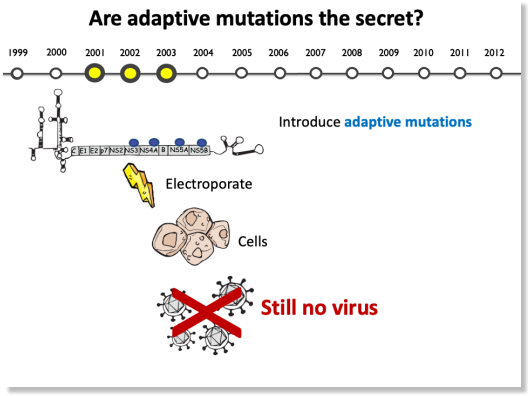


Figure 28. Testing adaptive mutations in the context of the complete HCV genome. Adaptive mutations (blue circles) were cloned into the infectious clone. After electroporation of HCV RNA into cells, the RNA was able to replicate, viral proteins were expressed, but no virus was produced.

replication in hepatoma cells, were somehow deleterious for downstream steps leading to the assembly of infectious virus. Three papers published in 2005 demonstrated that JFH-1 full length RNAs, or HCV chimeras substituting a structural region from other genotype 2a isolates, could produce HCV in cell culture (24-26). The released virus was infectious in naive hepatoma cells, transmissible to chimpanzees and liver-humanized mice, and virus recovered from these animals was infectious in cell culture (27, 28) (Figure 29).

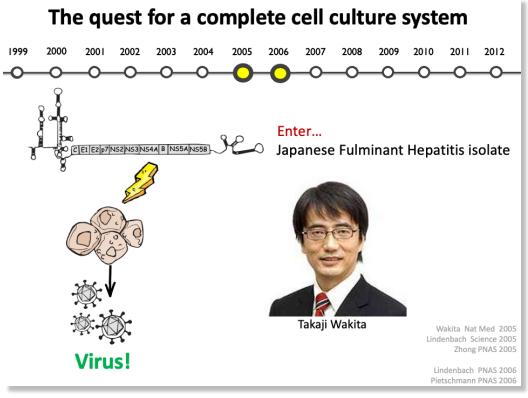


Figure 29. A unique Japanese HCV isolate. Takaji Wakita used HCV sequences from an unusual case of fulminate acute hepatitis to generate a genotype 2a based replicon that was able to replicate efficiently without adaptive mutations. This isolate, designated JFH-1, or chimeric genomes containing the structural region sequences from this or other genotype 2a isolates, resulted in full-length HCV RNA genomes that were able to replicate and produce infectious virus. The released virus was infectious in naïve cells, transmissible to chimpanzees and liver-humanized mice, and virus recovered from the animals was infectious in cell culture.

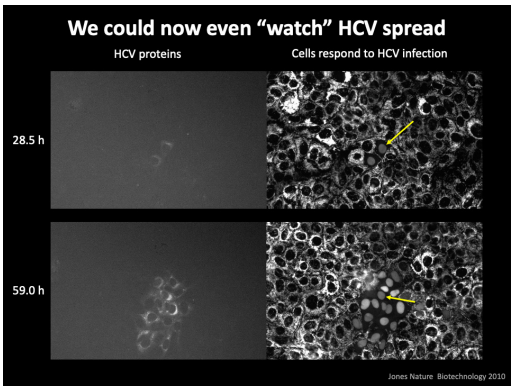


Figure 30. Watching HCV spread in cell culture. Two stills from a movie taken at 28.5 and 59 hours after infection show the expression of the viral protein (left) and the movement of a reporter protein domain that is released upon cleavage by the HCV protease and traverses to the cell nucleus. Arrows point to the same cell at the two different times.

We finally had a complete replication system for HCV – more than 15 years after its discovery! This ushered in a golden age in HCV research. We could study the complete virus life cycle, begin to look at virus – host interactions, the association of viral replication and assembly with membranes and lipid droplets in cells, study innate and adaptive immune responses, and develop neutralization assays useful for vaccine development. We found that HCV and related viruses were not that rare and commonly found in other species in nature. HCV became one of the best studied positive strand RNA viruses – surpassed only recently by SARS-CoV-2. Figure 30 shows you an example of how far we had come – we could devise systems where we could monitor hepatitis C virus spread in culture in real time (29). After this long struggle, not having a virus to work with in the laboratory, this was very gratifying.

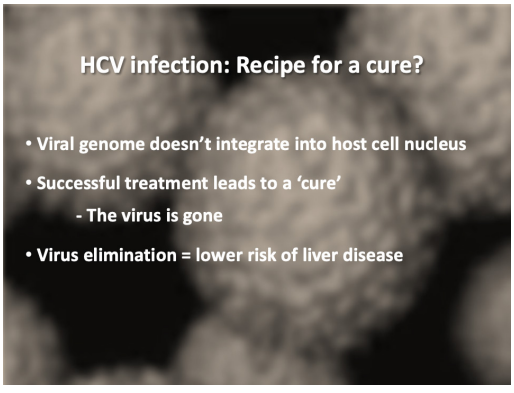


Figure 31. Can HCV be cured? The HCV features suggesting that a cure can be achieved are listed.

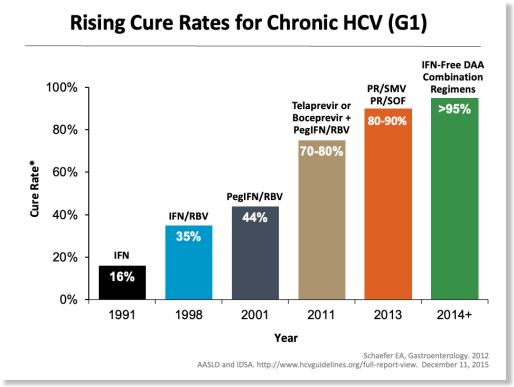


Figure 32. HCV treatment history. Treatments introduced in the indicated year and their cure rates are shown.

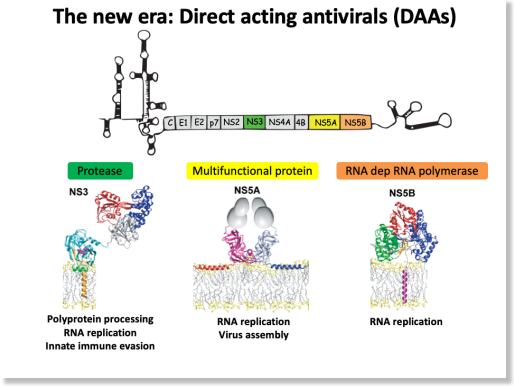


Figure 33. HCV direct acting antiviral targets. The location of the drug targets in the HCV genome is shown schematically at the top. Below, the viral protein functions and their experimentally determined structures are shown.

A few concluding comments about treatment. HCV is a virus that was ripe for coming up with a cure (Figure 31). We already knew this from the development and progression of interferon-based therapies for treating hepatitis C: The viral genome doesn't integrate, it's a cytoplasmic RNA virus, and successful treatment had been already shown to eliminate the virus. When the virus is gone there was accumulating evidence for lower risk of liver disease. To provide a brief history of HCV treatment (Figure 32), back in the non-A, non-B agent era, physicians began treating with systemic type I interferon with poor single digit sustained response rates. By 1991, with higher and longer interferon regimens, the efficacy of virus elimination was about 16%. That improved in subsequent years by combining interferon with ribavirin and making a pegylated, stabilized, version of type I interferon. In 2011, the first HCV serine protease inhibitors, telaprevir and boceprevir, were approved in combination with the current interferon-based regimens. Addition of these direct-acting antivirals (drugs that directly target viral functions) further improved efficacy, but also led to severe side effects. Finally, 2014 marked the end of interferon-based therapies. We had interferon-free, direct-acting, antiviral com-

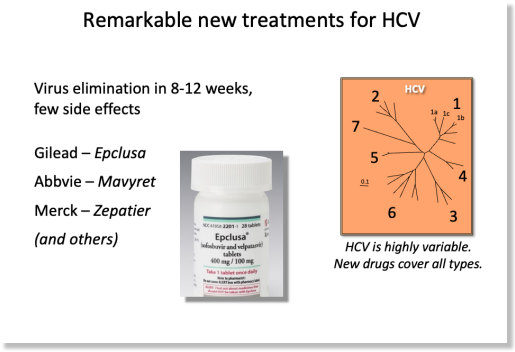


Figure 34. Effective treatments for genetically diverse HCV. Examples of effective drugs are shown on the left, while the genetic diversity of HCV strains is highlighted on the right.

binations with more than 95% cure rates. These potent inhibitors targeted several different essential viral functions (Figure 33); the NS3-4A serine protease, involved in polyprotein processing, RNA replication, as well as innate immune evasion, the NS5B RNA-dependent RNA polymerase, the engine of the HCV RNA replication machine, and NS5A, another non-structural protein involved in RNA replication and virus assembly. Different combinations of these inhibitors are now in widespread use and HCV can now be eliminated in two or three months. Remarkably, these combination therapies are broadly effective despite the HCV's immense global diversity (Figure 34).

It has been a remarkable journey (Figure 35) from a mystery virus in the 1970s and 1980s with a low cure rate, to the landmark discovery in 1989 – the virus identified, diagnostics in place, 2011 with the introduction of the first direct-acting antivirals, with a 75% cure rate, to 2014 and beyond – with highly effective regimens. But there are still challenges and the story is not finished (Figure 36). To get to 100% cure and eradication, which theoretically should be possible for HCV because it is an exclusively human virus, we need to identify those infected. Since this virus is often asymptomatic, there is a large segment of the world's population that is unaware of their infection. Once identified, getting patients into care and successfully treated can be a challenge. Affordable treatment is quite varied across the globe but lowering the price and making treatment universally available are important goals. With interferon-based therapies we were challenged by certain patient groups who were more difficult to treat, like HIV/HCV co-infected individuals. Fortunately, this seems to have disappeared in this new era of direct-acting antivirals. Virus resistance, always a potential problem, has not been a major issue with these next generation drug combinations. Finally, we do not have a preventative HCV vaccine, which many of us think is going to be needed to achieve global eradication. Hopefully the current efforts being put into developing COVID-19 vaccines will provide useful lessons for making a vaccine for hepatitis C.

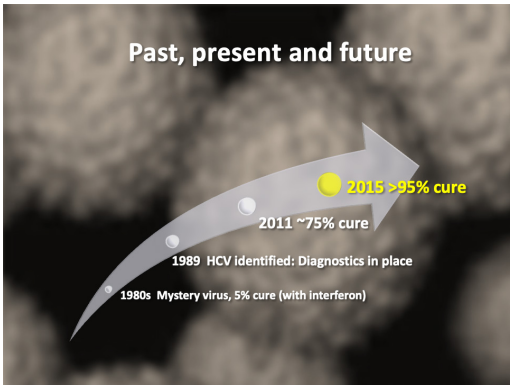


Figure 35. HCV cure trajectory. Key events and times in the development of an HCV cure are indicated.

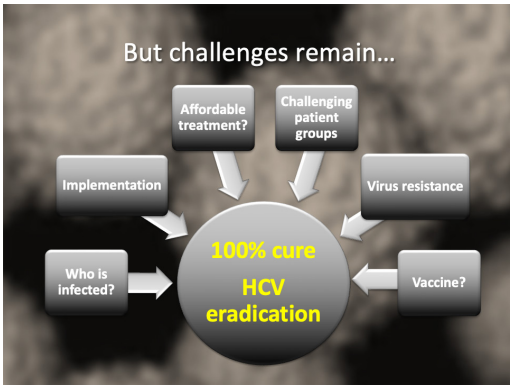


Figure 36. Remaining challenges on the path to HCV eradication.

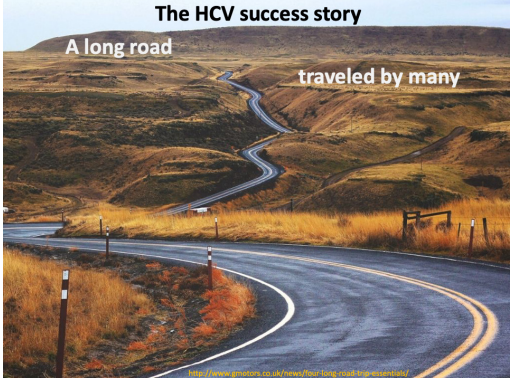


Figure 37. The HCV success story. Success required a long road, traveled by many.

I want to sum up with just a few thoughts. The HCV success story, at least from a molecular virology standpoint, has been a long road (Figure 37), but there have been many, many people involved – basic scientists, clinicians, people in biotech and pharma, people running these clinical trials, and the patients themselves. It has been a great team effort and wonderful to have been involved. I do want to thank my mentors and special friends (Figure 38): Dennis Barrett who got me interested in a

Figure 38. Mentors and special friends. A few of the many mentors and special friends who were instrumental in my career choices and the ultimate path it took are listed. Of course, I am indebted to my parents who were always supportive, and to Dennis Barrett who got me hooked on biology and gently guided me on the right path. I appreciate the incredible mentorship of Jim and Ellen Strauss, who allowed me the freedom to explore my scientific interests, and other friends and colleagues at Caltech, whose work inspired me. At Washington University, I was surrounded by amazing virologists, Sondra and Milt Schlesinger, Henry Huang, and John Majors, and developed an enduring friendship with Stan and Claire Birge, who generously shared their Wyoming cabin for much needed rest and relaxation. Jim Darnell was instrumental in convincing me to accept the offer to come to Rockefeller, and I have immensely enjoyed working with many colleagues in the city, including Ira Jacobson and Andrea Branch.

Figure 39. The many important support staff. Support staff members critical for our lab's work are shown in a Word Cloud.

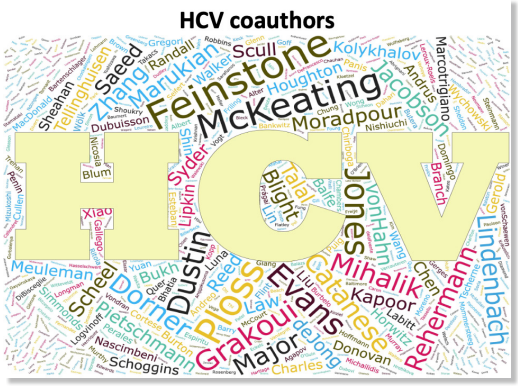


Figure 40. The many important collaborators. Names of co-authors who contributed to our HCV work are shown in a Word Cloud.

research career, and Jim and Ellen Strauss at Caltech, who were the ones that introduced me to the wonders of viruses and virology. In order to accomplish anything, we all depended on an incredible support staff (Figure 39) and a wonderful cast of collaborators (Figure 40). More broadly, I am profoundly grateful to all of my colleagues in this field for the many years of helpful input, encouragement, shared advances and failures, and friendship.

I'd also like to thank Peggy MacDonald, my partner in life and in science, and two of our canine support staff (Figure 41).

Finally, I would like to leave you with a quote that I love, written by Lewis Thomas from a book of essays that he published more than 30 years ago (30). He writes:

> In the course of scientific progress things tend to pop up. Most of the celebrated achievements had their origins in moments of surprise in the laboratory, unplanned for, unanticipated, unpredicted. No committee convened ... to survey the future prospects for biomedical research could possibly have guessed at the things that lay ahead. No branch of government could have laid out detailed plans for scientific needs of the future, beyond asserting ... that what the country needed was more fundamental knowledge about the human form and function, about the agents and influences responsible for disease, and, in a certain sense, about nature itself.
>
> Scientific research works, it is the only way to get at the underlying mechanisms of disease, and the only way to learn what to do about them.

So, with that I will end this story, and thank those of you who have managed to make it this far without dozing off. It's been a real pleasure for a basic scientist to be involved in this medical success story.

Figure 41. Other very important players. Peggy MacDonald and two of our former dogs, Wrangler and Sadie, are shown outside of the Rockefeller University Faculty-Student Club.

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