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Selection and Characterization of HCV Replicon Cells That Are Resistant to Cyclosporine A and Temperature Shift In Vitro

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SELECTION AND CHARACTERIZATION OF HCV REPLICON CELLS THAT ARE RESISTANT TO CYCLOSPORINE A AND TEMPERATURE SHIFT *IN VITRO*

By

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For Jimmy and Dane

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ABSTRACT

Rationale: The hepatitis C virus (HCV) is a serious health threat globally. Current therapies are not tolerated well, have a low response rate, and there is no available vaccine. New viral targets for drugs are urgently needed. The aim of this study was to characterize resistance to chemical and non-chemical treatments of the HCV replicon and determine a viral target for new treatment options.

Methods: Replicon cells were treated with both Cyclosporine A (CsA) and temperature shift treatment (39°C). Resistant replicon cells were attained by double treatment with selection antibiotics and anti-viral treatments, in concert with live cell sorting techniques. Resistant cell lines were analyzed and RNA was extracted. This RNA was electroporated into naïve or cured cells, creating new cell lines. These new cell lines were then tested for resistance. Resistant replicon RNA was also sequenced and compared to non-resistant strains.

Results: After cell lines had been attained with high levels of resistance, and RNA was electroporated into naïve or cured replicon cells, these new cell lines also showed resistance. This indicated that the viral RNA was the source of the treatment resistance. There were unique mutations at the amino acid level in both CsA and temperature shift resistant cell lines.

Conclusion: These unique mutations in both the CsA and temperature shift resistant replicon genomes indicate changes in the NS5B and NS3 proteins, respectively. Further work on the protein structures with the amino acid substitutions and their interactions could lead to new targets for therapies in patient

CHAPTER 1

INTRODUCTION

The hepatitis C virus

Hepatitis C virus (HCV) infects 170 million people globally and is a chief cause of cirrhosis, hepatocellular carcinoma, and liver transplantation. The Center for Disease Control estimates that nearly 20,000 new cases of HCV infection emerged in the U.S. in 2006, and 8-10,000 deaths occur annually due to chronic liver disease in this country alone (1). Current therapy options are limited and not well tolerated. The most commonly used strategy is the combination of interferon-α (IFN-α) and ribavirin, which has been the treatment of choice since 1998 and achieves results in only about 40-50% of patients (2). Due to the worldwide prevalence of HCV and the lack of a vaccine or reliable treatment options, new strategies for therapy are urgently needed.

By the middle of the 20th century, viral hepatitis had been recognized in the scientific community as having two distinct causes and two different modes of transmission. "Infectious hepatitis," later to be renamed hepatitis A virus (HAV), and "homologous serum hepatitis," to be later named hepatitis B virus (HBV), would have their causative agents identified in the mid-1970s (3). It became clear at about this time that there was a distinct and entirely separate agent also responsible for hepatitis infection that was neither of these viruses, and this was termed "non-A, non-B hepatitis," (NANB) (4).

NANB was investigated using a chimpanzee model system, as no reliable cell culture system had been established. The causative agent of NANB harvested from affected individuals was able to infect chimpanzees and begin chronic infection, as well as pass through filters of 50-80 nm in size and was demonstrated to be susceptible to lipid solvents – these data suggested that the NANB causative agent is both small and enveloped (5).

As molecular biology techniques evolved in the 1980s, so did knowledge of NANB. Michael Houghton, along with other scientists at the CDC and Chiron, was able to extract nucleic acids from the plasma of NANB infected chimpanzees. With this material, they created a cDNA library and screened these clones with serum antibodies from NANB infected patients. There was one clone that expressed an antigen to the patient antibodies, and this breakthrough led to the disease of non-A non-B hepatitis to be recognized as the hepatitis C virus (6, 7).

The hepatitis C virus, classified in the family *Flaviviridae* and genus hepacivirus, is an enveloped, plus-stranded RNA virus with a genome length of 9600 nucleotides. The HCV genome consists of a 5' non-translated region that contains an internal ribosomal entry site (IRES), one open reading frame that codes for a polyprotein, and a 3' non-translated region. The genome of HCV is utilized as messenger RNA once inside the cell, and translation of the open reading frame begins under the direction of the IRES to produce a polyprotein that consists of approximately 3,000 residues (8). The polyprotein is cleaved both co- and post-translationally by both viral and cellular proteases, which results in ten different viral proteins having structural and non-structural purposes (8). The non-structural proteins (NS3, NS4A, NS4B, NS5A, and NS5B) gather as a replication complex that disrupts cytoplasmic membranes, transforming them into scaffolding known as a membranous web on which negative strand RNA is produced (9). It is believed that the complete assembly of the virus occurs within or about this membranous web construction, as structural proteins join together to package progeny RNA and escape from the cell, to complete the cycle of viral propagation.

HCV targets hepatocytes, and there have been several proposed cell receptors for the virus, such as CD81, the LDL receptor, scavenger receptor class B type I, claudin-1, and occludin (10, 11, 12, 13, 44). The structure of HCV has not yet been visualized, however, based on filtration studies, particles have been shown to be between 40-70 nm in diameter (14) and based on related viruses, it is believed that envelope proteins E1 and E2 along with the core protein make up the virion.

HCV Replicon System for *in vitro* Replication

Since the 1989 breakthrough that identified HCV, several molecular biology advances have helped to elucidate the virus to researchers. In 1995, the 3' end of the genome was finally identified, which meant that the entire length of the HCV genome was known to researchers (15). HCV only infects humans and chimpanzees. The lack of a cell culture system in which to study the virus was a major setback for years. Then in 1999, Lohmann, et.al. reported a subgenomic replicon system for HCV that would allow *in vitro* replication and would prove to be an important breakthrough in the field (16). In 2000, another group – Blight, et al, identified a cluster of mutations in the non-structural 5A (NS5A) protein of HCV which allowed the virus to evade host immune responses. These mutations were engineered into a subgenomic RNA which contained the non-structural genes of HCV, along with a neomycin resistance gene, and robust replication in human hepatocytes in cell culture was achieved (17).

In 2004, Moradpour, et al, developed an HCV replicon system - based on the Blight replicon - with a green fluorescent protein (GFP) reporter inserted into the highly variable NS5A protein (18). This system allowed for convenient visualization of the replicon expression through fluorescent microscopy or flow cytometry. Our lab obtained this replicon, and through several rounds of cell sorting, generated a cell line with a more robust GFP signal. This cell line (GFP-Sort #4 cells, or GS4) is the replicon model system utilized for this project (19).

Hepatitis C virus *in vivo* and *in vitro* exists in a quasispecies nature, that is, its genetic diversity is very high. In addition to the cell culture-adaptation mutations found in the HCV replicon, the spontaneous mutation rate is estimated to be approximately 3.0×10^{-3} base substitutions/site/year (17,20,16). This is probably due to the error-prone RNA-dependent RNA polymerase and high turnover rate of HCV RNA. This high rate of mutation leads to drug resistant HCV species that emerge both *in vivo* and *in vitro.* Several mutations have been identified within the HCV genome which confer resistance to a variety of compounds, such as enzyme inhibitors, IFN-α, or ribavirin (21-32).

Cyclosporine A and Temperature Shift Treatments

Cyclosporine A (CsA) is a compound that is used primarily for immunosuppressive therapy. It was originally discovered by researchers at Novartis when obtained from a Norwegian soil sample, and is produced by *Hypocladium inflatum gams,* a fungus (45). Although mainly used for post-transplantation immune system suppression, it has recently been shown to suppress HCV both in patients and *in vitro* (33-37). Its mechanism of action is not entirely clear, but evidence indicates that CsA binds with cyclophilins. Cyclophilins are cellular proteins which have peptidyl prolyl isomerase and protein folding activities (46). There is evidence that CsA binds to cyclophilins and blocks their interaction with the HCV polymerase NS5B, thereby inhibiting viral replication (35,37,38).

Increasing the incubation temperature of various viral host cells has been shown to have effects on viral replication (39-42). It has been reported that shifting replicon cells to 39°C inhibits replication (49). By shifting our replicon cells from an incubation temperature of 37°C to 39°C, we also observed a dramatic decrease in replication.

In order to further investigate each of these replication-decreasing phenomena, we set out in this study to obtain replicon cell lines which were resistant to either CsA or temperature shift treatment. Screening replicon cells for resistance using both G418 (a selection antibiotic) and anti-viral compounds is a common technique. We utilized this technique, coupled with flow-cytometry, in order to obtain replicon cell lines with high levels of resistance to the compound CsA and a temperature shift to 39°C.

Once resistant replicon cell lines are isolated, and the source of that resistance is identified (viral replicon or host cell), the aim is to identify the replication knock-down mechanism. By determining a specific pathway by which a treatment inhibits HCV replication, one can identify targets for drug treatments, with a long-term goal of a treatment that can be applied to patients for therapy.

CHAPTER 2

MATERIALS AND METHODS

Cell culture

Replicon cells were maintained using Dulbecco's Modified Eagle Medium (Mediatech, Herndon, VA), supplemented with 10% fetal bovine serum, 1X non-essential amino acids and penicillin/streptomycin, and 500 μg/ml G418 (Mediatech, Herndon, VA). Huh-7 cells (liver cancer cells, not infected with HCV) were maintained in the same medium without the G418. Cells were harvested using 0.05% Trypsin EDTA (Mediatech, Herndon, VA), and grown in flasks that were treated for adherent cells (Nunc, Rochester, NY). During periods of temperature shift treatment, or chemical treatments, the G418 was omitted from the medium. All cells were maintained in cell culture incubators with 5% injected CO_2.

Flow Cytometry

The replicon cells used in this study which contain a GFP fusion protein were either fixed and analyzed using a BD FACSCanto Cytometer (BD Biosciences, Franklin Lakes, NJ) or sorted live and collected for further culturing using a BD FACSAria Cytometer. The software used to collect and analyze the data for both cytometers was BD FACSDiva. Cells which were harvested and fixed were collected and placed in a Fix Buffer solution (2% paraformaldehyde solution, in 1X Phosphate Buffered Saline [PBS]), passed through a sterile 50μM filter (PGC Scientifics, Frederick, MD), and subjected to analysis using a 510/23 band pass filter. Cells which were harvested for live-cell sorting were placed in a Sorting Buffer solution (4% fetal bovine serum in 1X Phosphate Buffered Saline), passed through a sterile 50μM filter, kept on ice, and subjected to analysis using a 510/23 band pass filter. After these cells were sorted, they were maintained in DMEM supplemented with 20% fetal bovine serum for 48 hours, and then switched to normal maintenance

media containing 10% fetal bovine serum. Huh-7 cells which do not contain the HCV replicon were used to set a negative GFP parameter prior to analysis of all replicon cells.

Selection and Cell Sorting of Cyclosporine A Resistant Cells

Replicon cells were seeded at a density of 5×10^6 cells in a 10 cm dish, and maintained in DMEM without G418. CsA (Alexis, San Diego, CA) was then added directly to the medium at a concentration of 1 μg/ml, and cells were maintained under these conditions for a period of 3 days. After 3 days, the media was aspirated, cells washed with 1X PBS, and growth media containing 500μg/ml G418 and CsA at a concentration of 1 μg/ml was added to cells. These conditions were maintained for a period of 3 weeks, with media changed once per week, and CsA added with each media change. After cell colonies appear, they are trypsinized and collected, pooled together and maintained in media supplemented with 500μg/ml G418. These cells are then treated with 2 μg/ml CsA for 3 days, as is a sample of replicon cells that have not been screened for resistance. These cells are subjected to fluorescence activated cell sorting (FACS) to determine the number of GFP positive cells in the population. The top 5-10% of GFP positive cells are then collected from the resistant population as previously described. For subsequent populations having a higher level of CsA resistance, the entire process is repeated again using a higher dosage of CsA.

Selection of Temperature Shift Resistant Cells

Replicon cells were seeded at a density of 5×10^6 cells in a 10 cm dish, and maintained in DMEM without G418. Cells were then placed in separate, identical cell culture incubators, with temperatures set at 37°C and 39°C. After a period of 3 days, media was aspirated, cells washed with 1X PBS, and media was replaced with DMEM supplemented with 500μg/ml G418. These conditions were maintained for a period of 3 weeks, with media changed once per week. After cell colonies appear, they were trypsinized and collected, pooled together and maintained in media supplemented with 500μg/ml G418. Separate samples of these cells were then subjected to 37°C and 39°C for 96 hours,

alongside replicon cells that have not been screened for resistance. These cells were subjected to FACS to determine the number of GFP positive cells in the population. The top 5-10% of GFP positive cells was then collected from the resistant population as previously described. For subsequent populations having a higher level of temperature shift resistance, the entire process was repeated.

Western Blotting

Cells were harvested and directly lysed in IP buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM phenylmethylsufonyl fluoride [PMSF], 0.5% NP-40), mixed with reducing SDS loading buffer (1M Tris-HCl pH 6.8, 1M DTT, 4% SDS, 0.2% Bromophenol Blue, 20% Glycerol), boiled at 100°C for 5 minutes, and loaded onto SDS-polyacrylamide gels. Gels were run using the Bio-Rad mini PROTEAN gel electrophoresis system (Bio-Rad, Hercules, CA). The gels were then transferred to a polyvinylidene fluoride membrane (PVDF) (Millipore, Billerica, MA), also using the PROTEAN system. The PVDF membrane was then blocked using 5% non-fat cow milk in 1X Phosphate Buffered Saline + Tween (PBST), and subjected to immunostaining using antibodies which were diluted in the same buffer used for blocking. The anti-NS5A monoclonal antibody was purchased from Virogen, (Boston, MA), and anti-Actin was purchased from Sigma-Aldrich (St. Louis, MO). The secondary antibodies used were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), which were conjugated with horseradish peroxidase (HRP). The horseradish peroxidase was detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA), the membrane was exposed to X-ray film (Kodak, Rochester, NY), and developed using a mini-medical series X-ray developer (AFP Imaging, Elmsford, NY).

RNA Extraction and q-RT-PCR

Total cellular RNA was extracted using the QIAGEN RNAeasy Plus kit (Qiagen, Valencia, CA), according to its protocol. The RNA was then analyzed using the

NanoDrop spectrophotometer and software (Thermo, Wilmington, DE) for concentration and purity. Real time quantitative reverse transcriptase polymerase chain reaction (q-RT-PCR) was performed with Applied Biosciences SYBR Green PCR kit (Applied Biosciences, Foster City, CA) according to its protocol. Primers which were designed and purchased from IDT DNA (Coralville, IA) were utilized to amplify the HCV internal ribosome entry site (IRES). The sequences of the primers used are noted in Table 1.

Table 1. Primers used for q-RT-PCR

Primers used for amplification of HCV IRES region
Forward Primer
GTC TGC GGA ACC GGT GAG
Reverse Primer
CGG GTT GAT CCA AGA AAG GAC

RT-PCR of Resistant Genome

RNA was extracted from temperature shift resistant cell lines, as was previously described. Next, RT-PCR was carried out to synthesize cDNA using random hexamers as primers, and the Superscript III RT kit (Invitrogen, Carlsbad, CA), according to its protocol. After cDNA synthesis, PCR was utilized to amplify specific gene fragments of the genome. The PCR was carried out using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA). The gene-specific primers used were designed by Tellinghuisen, et. al., (47) and purchased from IDT DNA (Coralville, IA). The primers are described in detail in Table 2. The resulting PCR products were next subjected to sequencing.

Table 2. Primers used for genome sequencing

Forward Primer	Nucleotide Sequence	Nucleotide Position
1	CTCTCCTCAAGCGTATTCAAC	1624-1644
3	TTCCAGGTGGCCCATCTACAC	2392-2412
5	GGCCTTGATGTATCCGTCATA	2983-3003
7	GACAACAGGCAGCGTGGTCAT	3750-3770
9	GGATGAACCGGCTGATAGCGT	4469-4489
11	GTCGGGCTCAATCAATACCTG	5170-5190
13	GCTAGTGAGGACGTCGTCTGC	5959-5979
14	ACAGGCCATAAGGTCGCTCAC	6732-6752
16	CTCCATGGCCTTAGCGCATTT	7378-7398
Reverse Primer	**Nucleotide Sequence**	**Nucleotide Position**
2	GTGGCGGCGACGGACGGGTTC	2487-2507
4	GGTAAAGCCCGTCATTAGAGC	3040-3060
6	GGGAGGTGTGAGGCGCACTCT	3852-3872
8	CGTGCTGCAGCGTCGCTCTCA	4533-4553
10	TTAGCCGTCTCCGCCGTAATG	5265-5285
12	TCTGCCCTTTAGAATTAGTCA	6779-6799
15	ACTGGGACGCAGCCGGGATTG	7615-7595
17	AAAAACAGGATGGCCTATTGG	7781-7801

Sequencing of Resistant Genome

PCR products were subjected to sequencing using the ABI 3130xl Genetic Analyzer (Applied Biosciences, Foster City, CA). Table 3 illustrates which primer sets were used for each fragment's amplification and sequencing. Sequences were visualized using Chromas Lite version 2.13 software, and aligned using DNA Strider™ version 1.3 software.

Table 3. Primer sets used for amplification and sequencing of replicon regions

Region of Replicon	Primer Set	Size (bp)	Position
EMCV IRES - NS3	1,2	883	1624-2507
NS3	3,4	668	2392-2412
NS3-NS4A	5,6	889	2983-3872
NS4A-NS4B	7,8	803	3750-4553
NS4B-NS5A	9,10	816	4469-5285
NS5A-NS5B	11,12	1629	5170-6799
NS5A-NS5B	12,13	800	6779-5979
NS5B	14,15	883	6732-7595
NS5B-3' UTR	16,17	423	7378-7801

Site-Directed Mutagenesis of the HCV NS3 Gene

In order to introduce a single site mutation into the Con1 replicon background, the Con1 plasmid and pUC19 (New England Biolabs, Ipswich, MA) were digested with *EcoR*I and *Xba*I (Invitrogen, Carlsbad, CA) for 1 hour at 37°C. The fragment of Con1 which contained the HCV NS3 replicon gene was then ligated into pUC19 using T4 ligase (Invitrogen, Carlsbad, CA), incubated at room temperature for 1 hour. For mutagenesis, the Stratagene QuikChange® Kit (Stratagene, La Jolla, CA) was used according to the manufacturer's protocol. Custom primers were designed and purchased (IDT DNA, Coralville, IA) in order to introduce the single nucleotide mutation, as described in Table 4.

Table 4. Custom primers utilized for site-directed mutagenesis.

Original Region in Resistant Replicon NS3 Sequence
CGT CGA GTC TAT GGG AAC CAC TAT GCG GTC C
Forward Primer
CGT CGA GTC TAT GGG AAC CAC TAT GCG GTC C
Reverse Primer
GCA GCT CAG ATA CCC TTG GTG ATA CGC CAG G
Nucleotides in red indicate the site of mutagenesis.

In Vitro Transcription of Mutant Replicon RNA

The mutated plasmid (NS3 E→G mutation ligated into Rep 1b plasmid background) was linearized with *Sca*I (Invitrogen, Carlsbad, CA) and incubated for 2 hours at 37°C. After running the plasmid on a DNA gel to ensure that digestion occurred correctly, the plasmid was purified with phenol, saturated, pH 6.6/7.9 (Fisher scientific, Suwanee, GA) and chloroform/isoamyl alcohol, 24:1 (Acros Organics, NJ), and precipitated with isopropyl alcohol. The DNA was suspended in nuclease-free water. Then 1µg of the resulting DNA was used in the MEGAScript T7 Kit (Ambion, Austin, TX). After following the manufacturer's protocol, the resulting RNA was purified with phenol, saturated, pH 4.3, and chloroform/isoamyl alcohol, 24:1, and precipitated with isopropyl alcohol. The resulting RNA was suspended in nuclease-free water, and run on a RNA gel to determine integrity, and analyzed using the Nanodrop software to determine concentration.

Northern Blotting

Total cellular RNA was extracted using the QIAGEN RNAeasy Plus kit (Qiagen, Valencia, CA), according to its protocol and analyzed using the NanoDrop spectrophotometer, as was previously described. Equal amounts of RNA (10 μg) from each sample was loaded onto formaldehyde-containing agarose gels and subjected to electrophoresis. RNA was then transferred onto a nitrocellulose membrane, and baked in a vacuum oven at 80°C for 2 hours. The probe used was a fragment of cDNA that corresponds to the RNA of HCV genotype 1B, nucleotides 8024 to 9563. The probe was radioactively labeled, hybridized, and bands were detected using a Storm 860 phosphoimager (Amersham/GE Biosciences, Piscataway, NJ). The bands were quantified using Quantity One software on a Bio-Rad ChemiDoc gel documentation system (Bio-Rad, Hercules, CA).

Creation of Interferon-α Cured Cell Lines

To make cured cell lines, GS5, RS2, or temperature shift resistant cells were treated with 100 units/ml IFN-α for a period of 4 weeks. Next, a sample of cells were treated with 500μg/ml G418 for a period of 3 weeks to ensure that cells did not contain replicons, and that no G418-resistant colonies would develop.

Electroporations

To perform electroporations, naïve liver cells were harvested, DMEM was removed and replaced with OPTI-MEM (Invitrogen, Carlsbad, CA). Cells were counted, and 4×10^6 cells were placed in a sterile 4mm cuvette. Next, 10 μg of total cellular RNA dissolved in RNAse-free water was added to the cuvette which was loaded into the Bio-Rad Gene Pulser Xcell (Bio-Rad, Hercules, CA). The parameters used were 270 V, 950 μF, and ∞ Ω. After electroporation, the cells were seeded onto 10cm dishes and given 20% fetal bovine serum-supplemented media to aid in recovery for 24 hours. Media was then

aspirated, cells washed with 1X PBS, and cells were then given 500µg/ml G418 selection media with 10% fetal bovine serum. Media was changed every 3 days to remove dead cells as colony formation occurred. After maintaining cells for a period of approximately one month, surviving cells had formed colonies and were harvested for expansion.

Compounds

Cyclosporine A was purchased from the Alexis Corporation, and dissolved in 100% ethanol as per manufacturer instructions. Interferon-α was purchased from Sigma-Aldrich and was dissolved in DMSO as per manufacturer recommendations. 2'-C-methyl-adenosine (2CMA) was provided by Steve Carroll.

CHAPTER 3

RESULTS

SELECTION AND CHARACTERIZATION OF CYCLOSPORINE A RESISTANT HCV REPLICON CELLS

This project utilized the genotype 1B HCV replicon system with a green fluorescent protein (GFP) reporter insert as illustrated in Fig. 1. The replicon was contained in immortalized human hepatocytes known as GFP-Sort#4 (GS4 cells).

Figure 1. Representation of HCV replicon with GFP reporter insert used in this project. The replicon includes a Neomycin resistance gene as well as the non-structural proteins of the virus.

When GS4 cells were treated with 1 µg/ml CsA, both the GFP signal and HCV RNA was inhibited by more than 90% (19). In order to obtain cells that were resistant to CsA, cells were treated with a double selection of CsA and G418 (see Materials and Methods). These resistant cell colonies were designated "CsA resistant" (CsA-R), pooled together, and analyzed by FACS to determine their level of CsA resistance. A portion of this cell population was then treated with 2 µg/ml CsA for a period of 3 days, and then cells were sorted and the portion of the population having high GFP levels (approximately 5-10% of the total population) were collected and expanded. This cell line was designated "CsA resistant Sort 1" (RS1), and the selection process was repeated using 4 µg/ml CsA, resulting in "CsA resistant Sort 2" cells (RS2). This process of selection is illustrated in Figure 2.

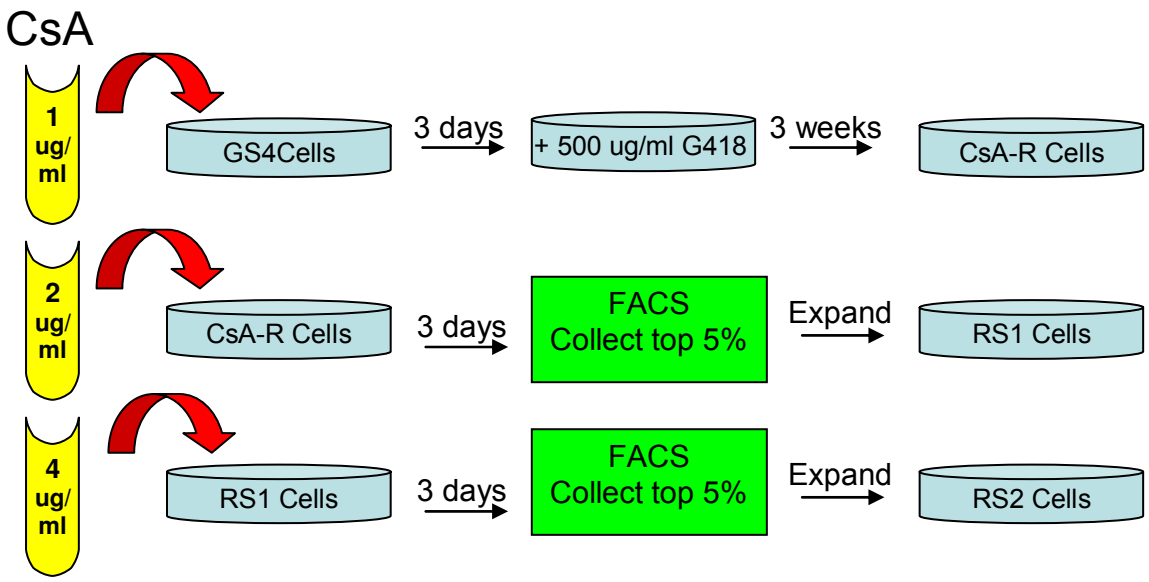


Figure 2. Schematic of selection process which was used to isolate CsA resistant cells. Cells were initially treated with both CsA and antibiotic for 3 weeks to form resistant colonies. Subsequent rounds of selection were achieved using higher levels of CsA and FACS.

In order to determine if the CsA resistance was specific to these cell lines, or if the replicon was resistant to a broad range of chemical treatments, each cell line was treated with 2 additional chemical inhibitors of HCV replication, IFN-α, and 2'-C-methyl-adenosine (2CMA). GS5 cells (GFP Sort #5) were used as a control. Samples of each of the representative cell lines were treated with either CsA, IFN-α, or 2CMA at different concentrations. The conditions were maintained for 96 hours before the cells were fixed and analyzed by FACS. While NS5A-GFP expression was reduced to a level of less than 10% in the GS5 cell line at 0.5 µg/ ml of CsA, all of the CsA resistant cell lines maintained a level of approximately 80% GFP expression. Treatments with IFN-α and 2CMA resulted in a knockdown of GFP expression in all cell lines without discrimination, indicating a specific resistance to CsA, as illustrated in Figure 3A-C.

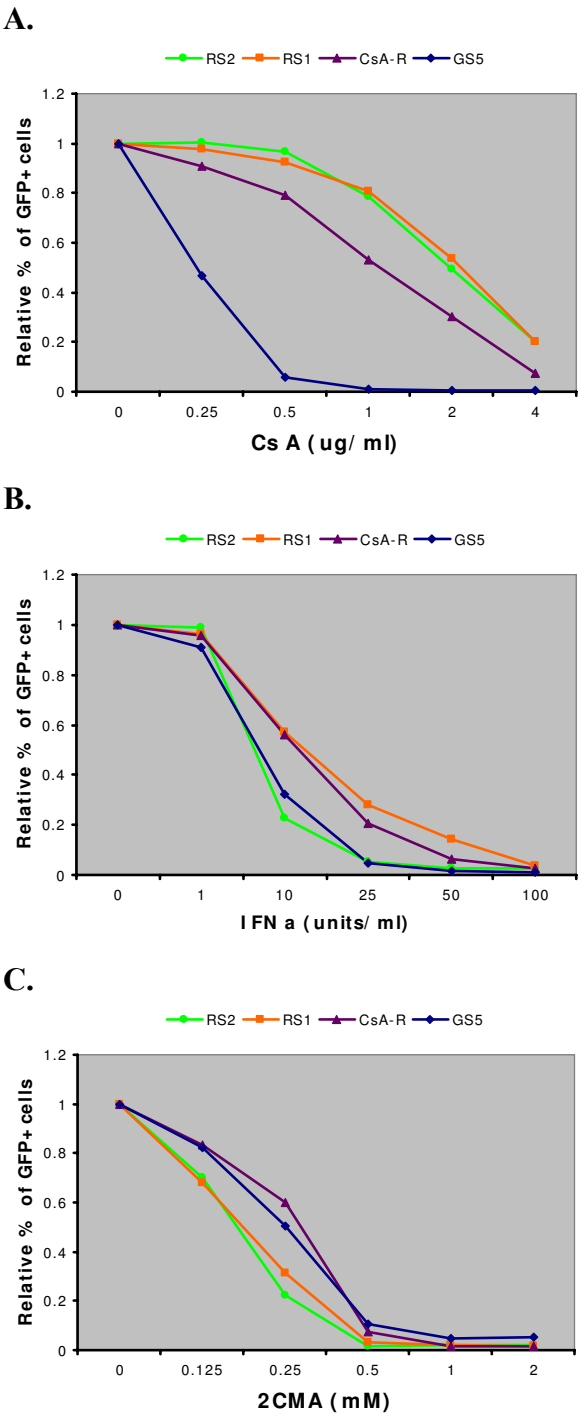


Figure 3. Resistance profiles of CsA resistant cell lines versus GS5. Percentage of GFP positive cells is normalized to the untreated sample, set at 100%. (A) Profiles of CsA resistant cells treated with CsA at increasing concentrations. GS5 cells serve as a negative control. (B) Profiles of CsA resistant cells and GS5 treated with IFN-α.

Inhibition of NS5A-GFP is similar in all cell lines. (C) Profiles of CsA resistant cells and GS5 treated with 2CMA. Inhibition of NS5A-GFP is similar in all cell lines. Next, in order to further establish that the observed CsA resistance was not the result of enhanced replicative ability, but rather a unique ability of the resistant replicon, the RS2 cells were subjected to long term treatment with CsA. Samples of identically treated GS5 and RS2 cells were analyzed by FACS to determine the level of NS5A-GFP expression. The results indicated that RS2 cells maintained high levels of expression for 10 days, as illustrated in Figure 4.

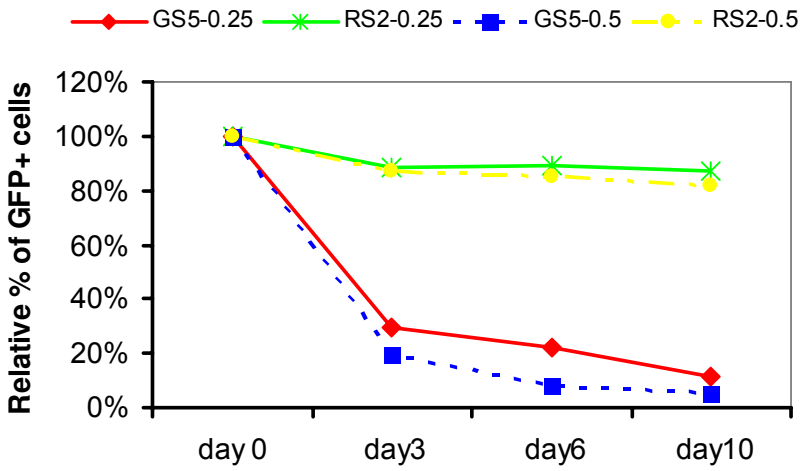
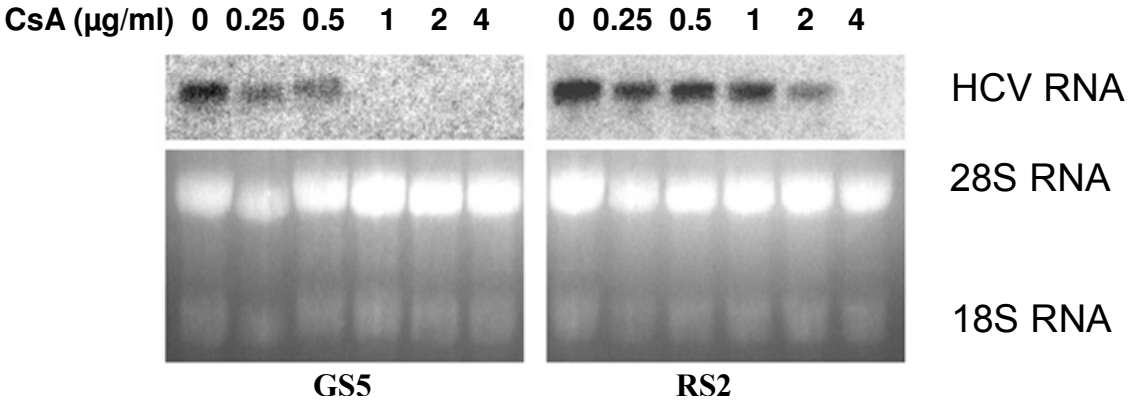


Figure 4. Long-term CsA treatment does not inhibit RS2's NS5A-GFP expression levels. Samples of GS5 and RS2 cells were treated with either 0.25 µg/ml or 0.5 µg/ml CsA, and were collected and analyzed by FACS at different time points.

It has been shown that CsA suppresses replication at the viral RNA level in replicon cells (36,48). In order to determine if CsA resistance was occurring at the RNA level, total cellular RNA was extracted from both GS5 and RS2 cells which were treated with CsA for 48 hours, and Northern blotting was performed (Figure 5A). The radioactive probe used was designed to correspond to the NS5B gene in HCV. The results indicated that while GS5 replicon RNA was not detected at a level of 1µg/ml CsA, RS2 replicon RNA was detectable until a level of 4µg/ml was applied. The band intensity from the resulting Northern was quantified (figure 5B).

A.



B.

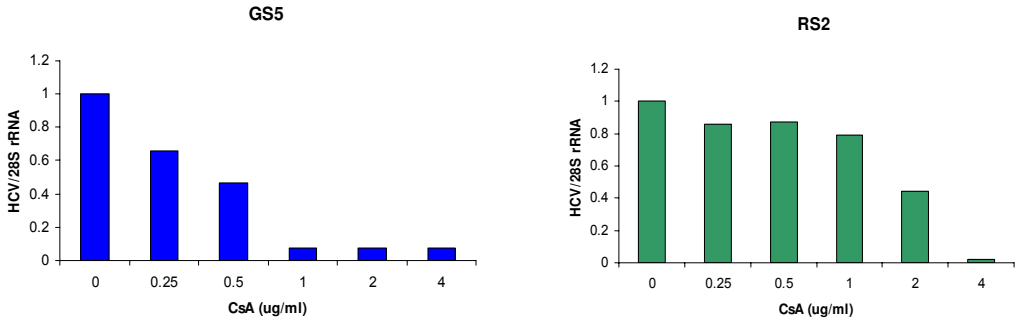
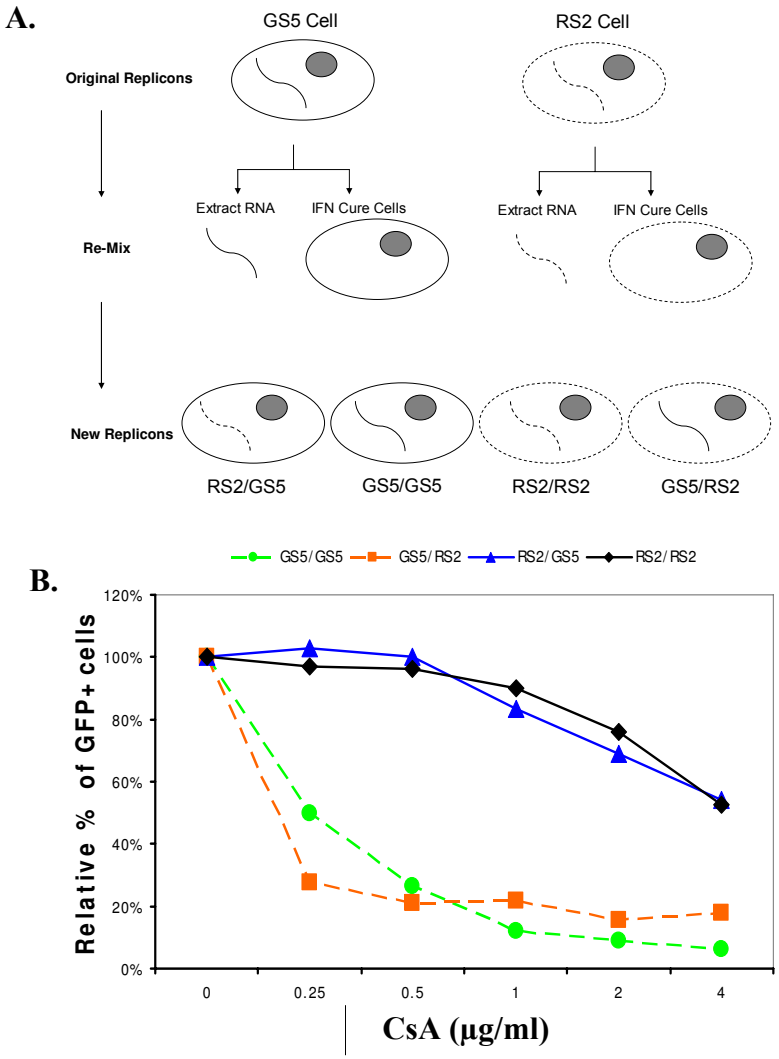


Figure 5. CsA resistance at the RNA level. (A) Northern blot results. Cells were treated with CsA for 48 hours before total cellular RNA was collected. RNA was subjected to Northern blotting with a radioactively labeled probe. The 28S and 18S RNA shown is from the electrophoresis gel before blotting was carried out and serves as a loading control. (B) Bands from (A) were quantified using the Bio-Rad ChemiDoc system, and the ratio of HCV/28S RNA was plotted.

To establish that the replicon RNA and not its host cell confers CsA resistance, the total cellular RNA was extracted from GS5 and RS2 cells and re-mixed with IFN-α cured replicon cells via electroporation. The neomycin resistance gene contained within the replicon will allow this RNA to persist while cells are under G418 treatment. The IFN-α cured cells were created by treating replicon cells with 100 units/ml of IFN-α for a period of one month, at which point the cells would die when treated with G418 (see Materials and Methods). A schematic of the re-mixing is illustrated in Figure 6A. After the new

cell lines were created, the cells were analyzed by FACS to determine relative levels of CsA resistance. The cells that had received RS2 RNA showed resistance to CsA, while those containing GS5 RNA did not (Figure 6B). These results were also confirmed by performing a western blot on all 4 cell lines and probing for NS5A (Figure 6C).



C.

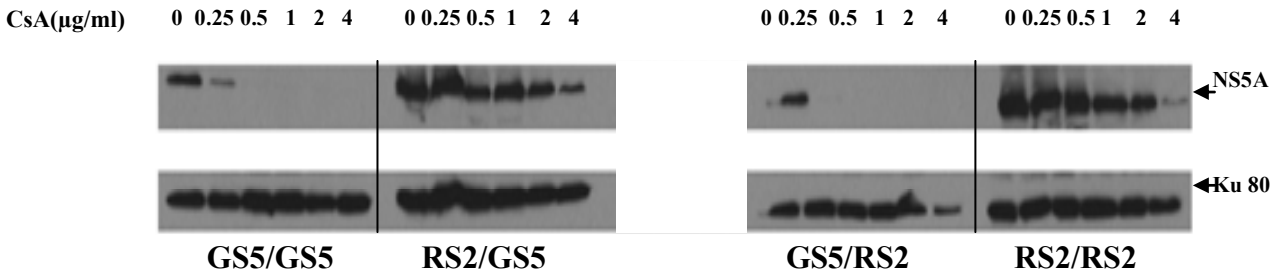


Figure 6. CsA resistance is conferred upon cells by RS2 RNA. (A) Re-mix scheme used to separate total cellular RNA from host cells. (B) FACS data shows that only cells containing RS2 RNA show resistance to CsA. RS2 cells containing GS5 RNA showed sensitivity to CsA comparable to original GS5 cells. (C) Western blot confirms the FACS data shown in (B). Anti Ku-80 serves as a loading control.

SELECTION AND CHARACTERIZATION OF TEMPERATURE SHIFT RESISTANT HCV REPLICON CELLS

We observed first through fluorescent microscopy that a shift from normal 37°C conditions to 39°C for a period of 96 hours produced a significant drop in the level of NS5A-GFP expression (figure 7).

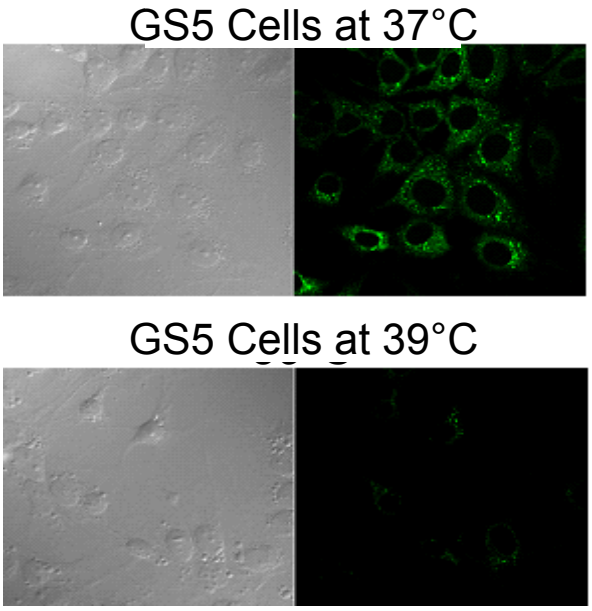


Figure 7. Fluorescent microscopy images of GS5 cells cultured for 96 hours under 37°C versus 39°C conditions.

After observing this phenomenon, we cultured a population of GS5 replicon cells that could maintain replicative ability under 39°C conditions. In order to do this, GS5 cells were cultured at 39°C under the selection of G418-supplemented media. Under these conditions, cells that lost the ability for HCV replication would die due to the pressure of G418, which would eliminate any cell not containing the neomycin resistance gene contained within the replicon. After approximately one month, the remaining living cells were collected. A schematic of the selection process is illustrated in figure 8. The new cell line, named New 39R, was subjected to 39°C treatment alongside original GS5 cells and analyzed by FACS to determine what level of resistance had been obtained. FACS analysis determined that New 39R cells showed an approximately 60% higher replication rate than GS5 cells when treated at 39°C for 4 days (Fig. 9).

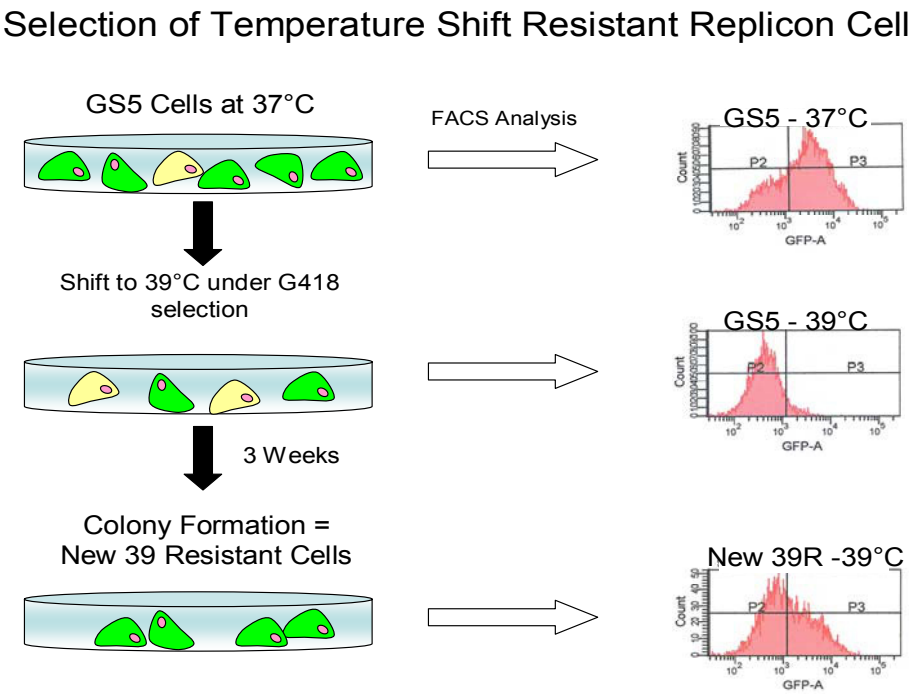


Figure 8. Schematic representation of selection process utilized to obtain replicon-containing cells that are resistant to temperature shift conditions. Whole cell populations were analyzed via flow cytometry on a BD Canto cytometer using BDFACS Diva software.

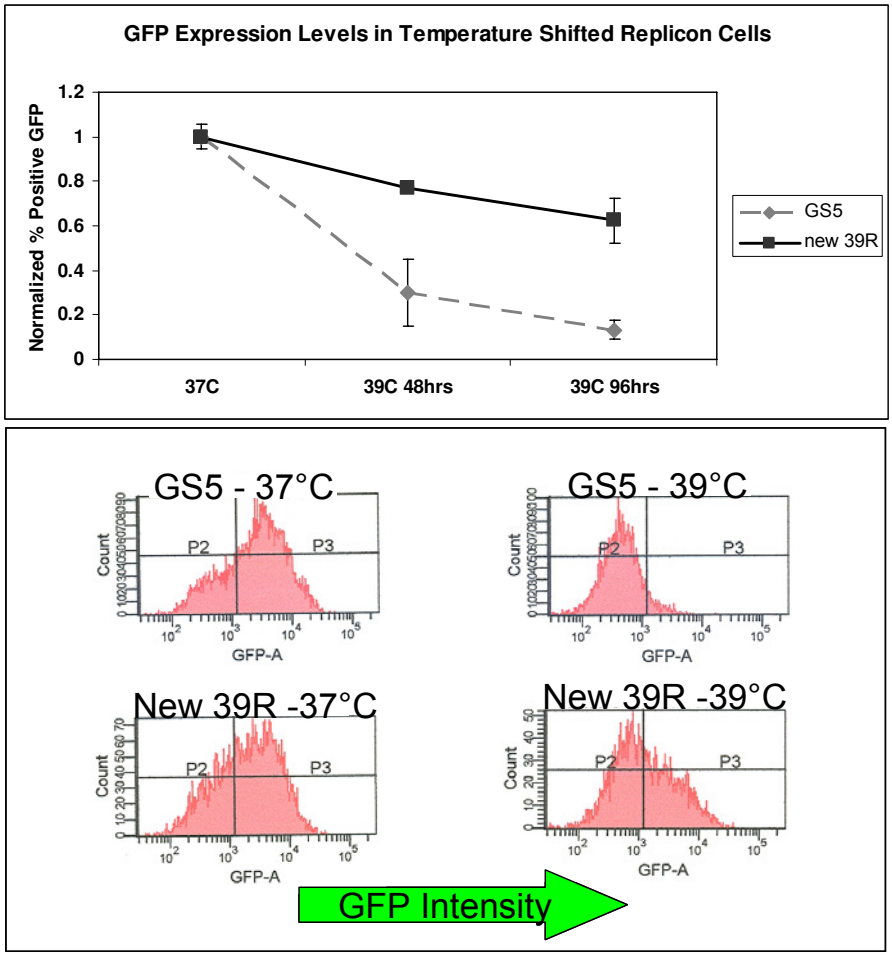


Figure 9. New39R cells show higher levels of replication under temperature shifted conditions than GS5 cells. **Top Panel:** GS5 and New 39R cells were collected in duplicate at 48 and 96 hour time points and analyzed by FACS. **Bottom Panel:** Representative FACS profiles of the two cell lines untreated and treated for 96 hours.

To demonstrate that this phenomenon is not strictly tied to GFP expression, we collected total cell lysates after treatment at 39°C that were subjected to immuno-blotting using an antibody against the NS5A protein contained in the replicon. The western blot showed a knockdown of NS5A in GS5 cells that was not mirrored in New 39R cells (Fig. 10, top panel).

To determine the extent of replication knockdown at the RNA level, total cellular RNA was collected after 39°C treatment and subjected to quantitative RT-PCR, with primers

directed at the HCV IRES region of the replicon to show the level of replication occurring at each time point. Primers directed at the cellular gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an enzyme involved in glycolysis and other cellular functions, were used as an internal control (Fig. 10, bottom panel). This data illustrated an approximate 10-fold difference in replication between GS5 and New 39R cells at the RNA level after 3 days of 39°C treatment.

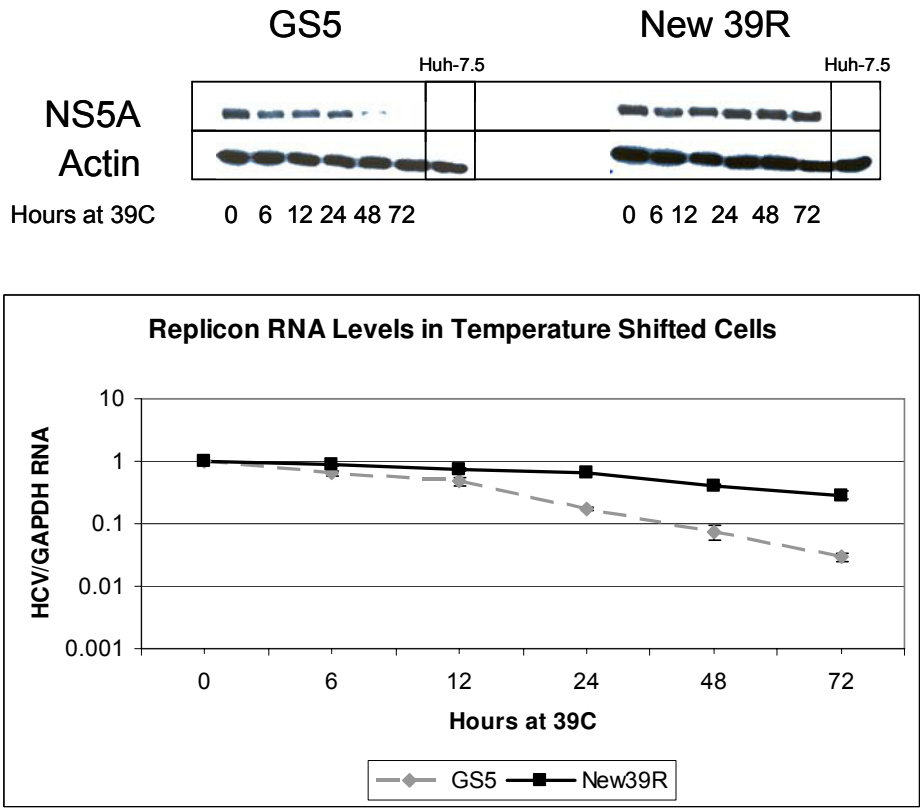


Figure 10. Short term 39°C treatment of GS5 versus New39R cells shows resistance at the protein and RNA levels. **Top panel:** Western blotting of total cell lysates collected after 39°C treatment and probed with anti-NS5A antibody. Huh-7.5 cell lysates (naïve liver cells) serve as a negative control. Anti-actin serves as a loading control. **Bottom panel:** Total cellular RNA samples collected after 39°C treatment were subjected to quantitative RT-PCR using primers directed at the HCV IRES region and GAPDH as an internal control.

To establish that the resistance of New 39R cells to 39°C treatment is specific, we treated the cells (alongside GS5 cells) with two common chemical HCV inhibitors – CsA and IFN-α - at identical time points. The cellular RNA was collected and subjected to quantitative RT-PCR as described previously. The results showed a similar knockdown pattern between GS5 and New 39R cells, indicating that the resistance to 39°C is specific, and not the result of a general increase in replicative ability (Fig. 11).

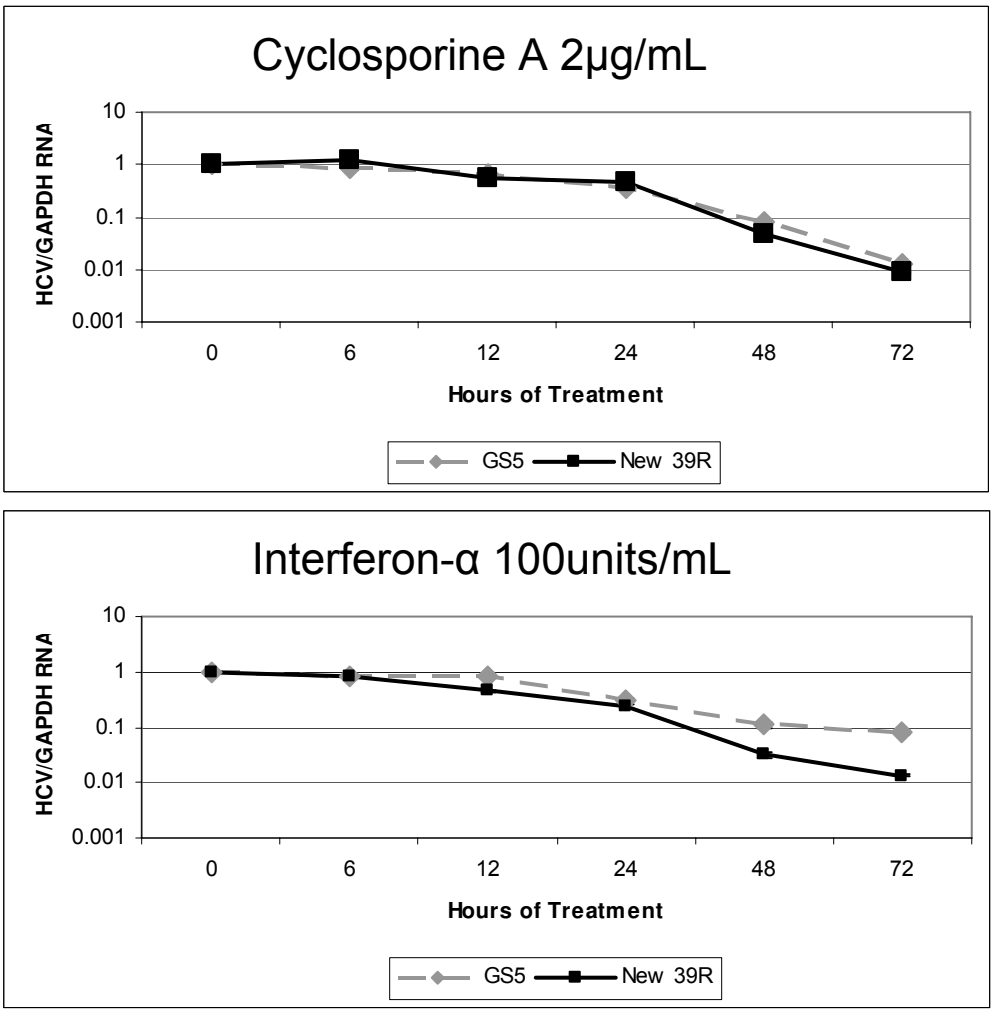


Figure 11. New 39R cells do not show resistance to common chemical HCV inhibitors, and GS5 and New 39R cells treated with either Cyclosporine A or Interferon-α show similar levels of HCV inhibition. Total cellular RNA was collected at the indicated time points and subjected to quantitative real time RT-PCR using primers directed at HCV IRES and GAPDH as an internal control.

We then set out to determine the length of time that the resistant cells would maintain robust HCV replication under 39°C conditions. To do this, GS5 and New 39R cells were seeded identically and maintained at 37°C and 39°C. Samples were harvested every 2 days and subjected to FACS analysis to determine their level of replication (Fig. 12). The GS5 cells showed a dramatic drop in HCV replication by day 4, while New 39R cells' replication tapered off slowly and maintained replication levels above 40% until day 14.

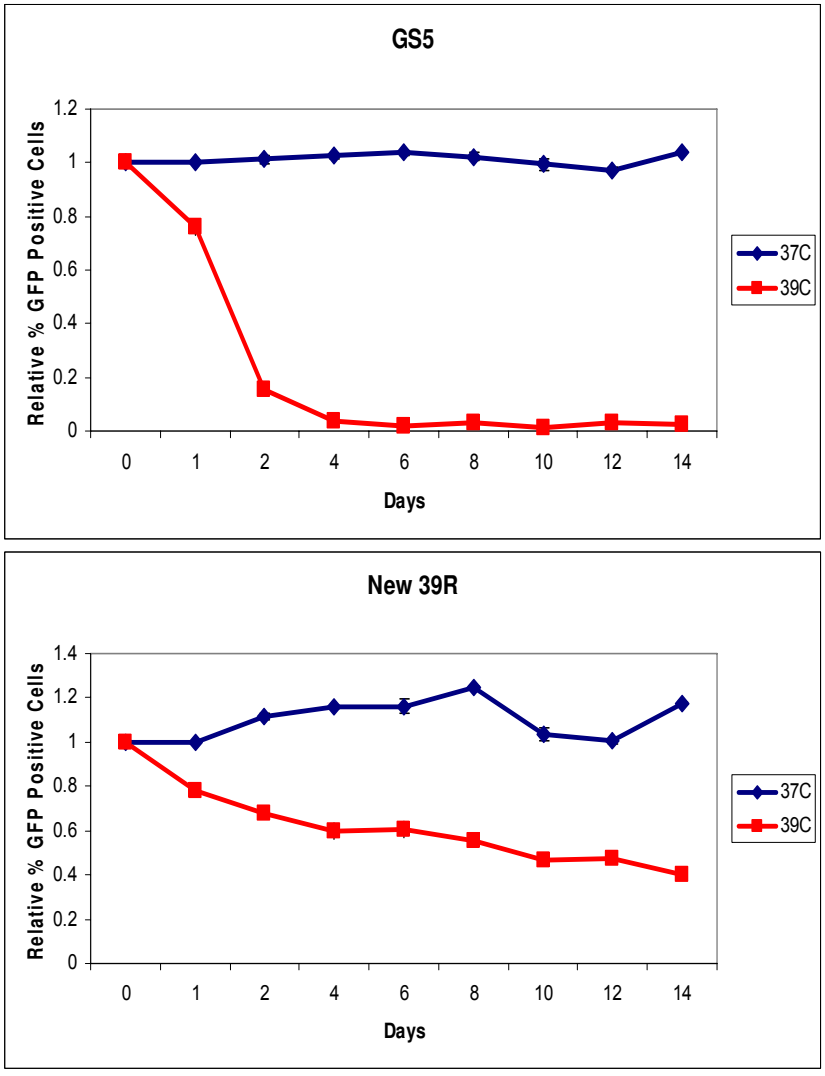


Figure 12. Long term treatment at 39°C shows that New 39R cells maintain resistance for 2 weeks. Cells were maintained at both 37°C and 39°C and duplicate samples were harvested every 2 days and analyzed by FACS.

The selection process to obtain temperature resistant cells was conducted two separate times, to create two independently selected cell lines – Temperature shift resistant cells (TSR) in addition to the New 39R cell line. TSR cells were also live-cell sorted an additional time to obtain the HeS3 cell line. TSR and HeS3 cells demonstrated a FACS profile that was similar to New 39R cells when temperature shifted.

To determine if resistance is indeed conferred upon cells by replicon RNA and not cellular adaptations to temperature shift treatment, we extracted total cellular RNA from both GS5 and TSR cells and introduced them into IFN-α cured cells (Fig. 13). These cells were selected with G418 media for approximately one month until surviving cells formed colonies. These cells were collected for further analysis.

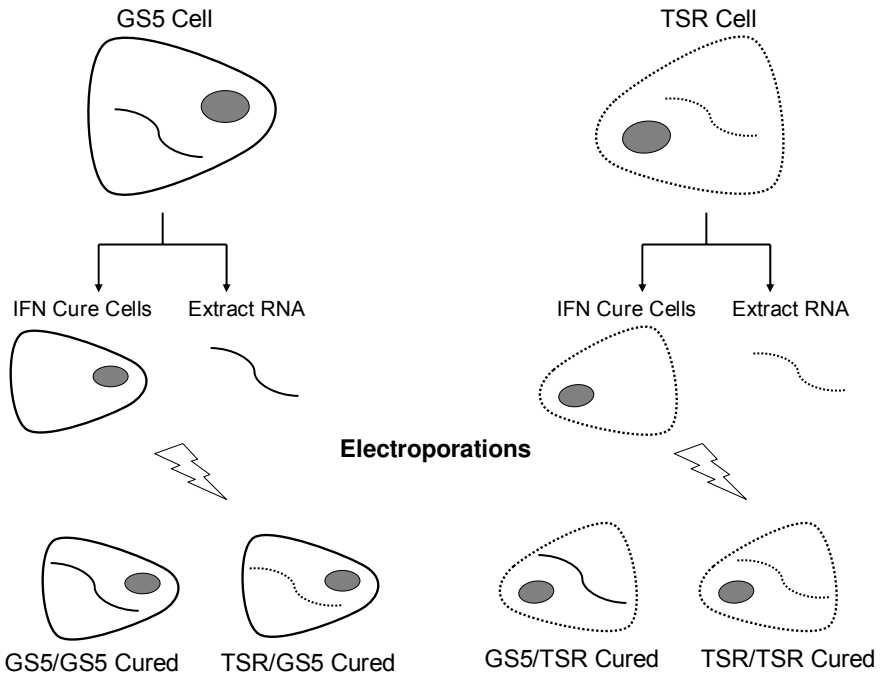


Figure 13. Schematic representation of electroporations performed. Total cellular RNA was extracted using QIAGEN RNAeasy Plus kit, and was electroporated into IFN-α cured cells as was previously described. Cells were allowed to recover for 24 hours and

then were given G418 selection media. Cell colonies formed and were harvested 4 weeks later.

The resulting cell lines were temperature shift treated for 96 hours, cell lysates were collected, and western blots performed. Figure 14 details the results of two independent treatments and western blots of these re-mixed cell lines. The results show that cells containing the temperature shift resistant RNA (TSR/GS5C) maintain resistance under 39°C conditions, while the replicon cells containing GS5 cell RNA (GS5 and GS5/TSRC) do not.

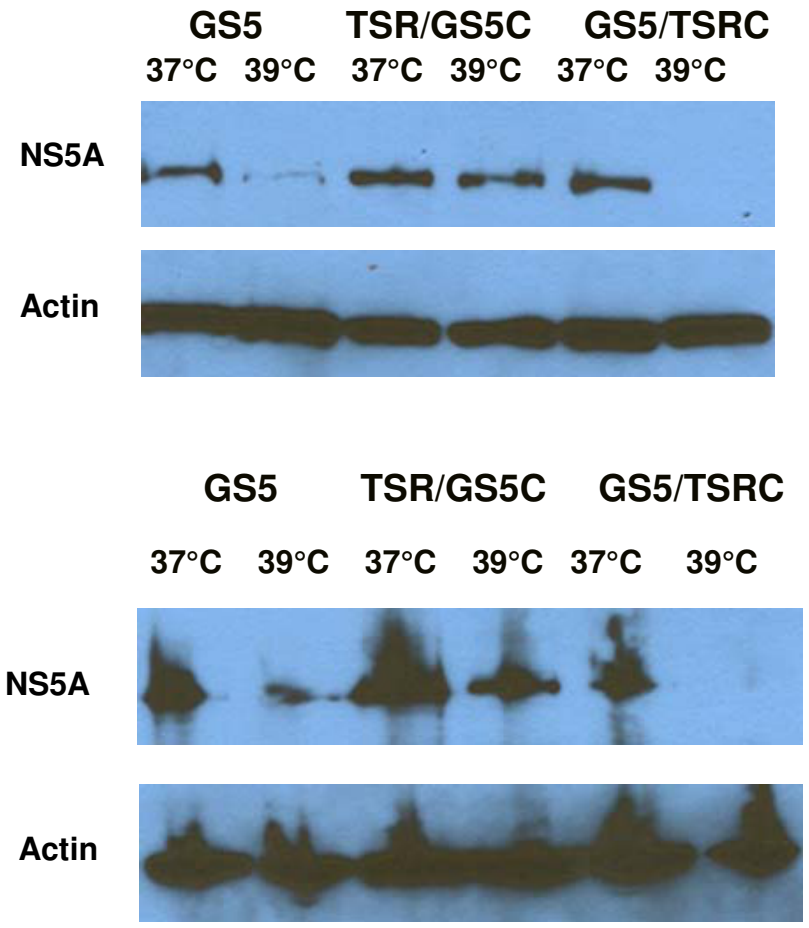


Figure 14. Western blot results of two independent 96 hour temperature shift assays of re-mixed cell lines. Anti-NS5A was used to show replicon presence, and anti-actin was used as a loading control.

After establishing that replicon RNA was responsible for the observed temperature shift resistance, the genome of the resitant replicons were analyzed in order to identify any mutations that may be contributing to the resistant phenotype. Total cellular RNA was collected from all temperature shift resistant cell lines, as well as non-resistant replicon cell lines (Con1, GS0, and GS5). RT-PCR was performed to obtain cDNA which was then subjected to sequencing (see Materials and Methods). Each replicon's amino acid sequence was analyzed and all were aligned. A position of interest developed, within the NS3 gene, which codes for the viral protease. Amino acid 178 showed a glutamic acid (E) in all replicon cells that were not temperature shift resistant, and a glycine (G) in all cell lines that were temperature shift resistant. A listing of cell lines and amino acids found at position 178 is illustrated in table 5.

Table 5. Comparison of amino acids found in position 178 within the NS3 gene of different replicon cell lines.

Replicon Cell Line	Amino Acid at Position 178
Con 1	E
GS0	E
GS5	E
TSR	G
HeS3	G
New 39R	G

In order to determine the effect of this single mutation on the replicon, mutagenesis was performed (see Materials and Methods) on the Rep 1B plasmid, which is the genotype 1B replicon that has not been sorted or gained resistance through treatments. By engineering this mutation into the Rep 1B background and electroporating the RNA into naïve liver cells, the effect of the mutation when treated with temperature shifting will be determined by future researchers.

During the course of this thesis work, a cell culture system was developed which would allow for HCV infection. This virus is of a different genotype (2A) than the replicon

used here, genotype 1B. Because of this valuable tool, we decided to investigate if temperature shifting would affect the infection or replication processes (or neither or both) of the genotype 2A virus. In order to separate the two, cells were infected with the 2A virus at either 37°C or 39°C and then incubated for a period of 10 days. RNA samples were collected at day 7 and day 10 and analyzed via quantitative RT-PCR. The results are illustrated in figure 15.

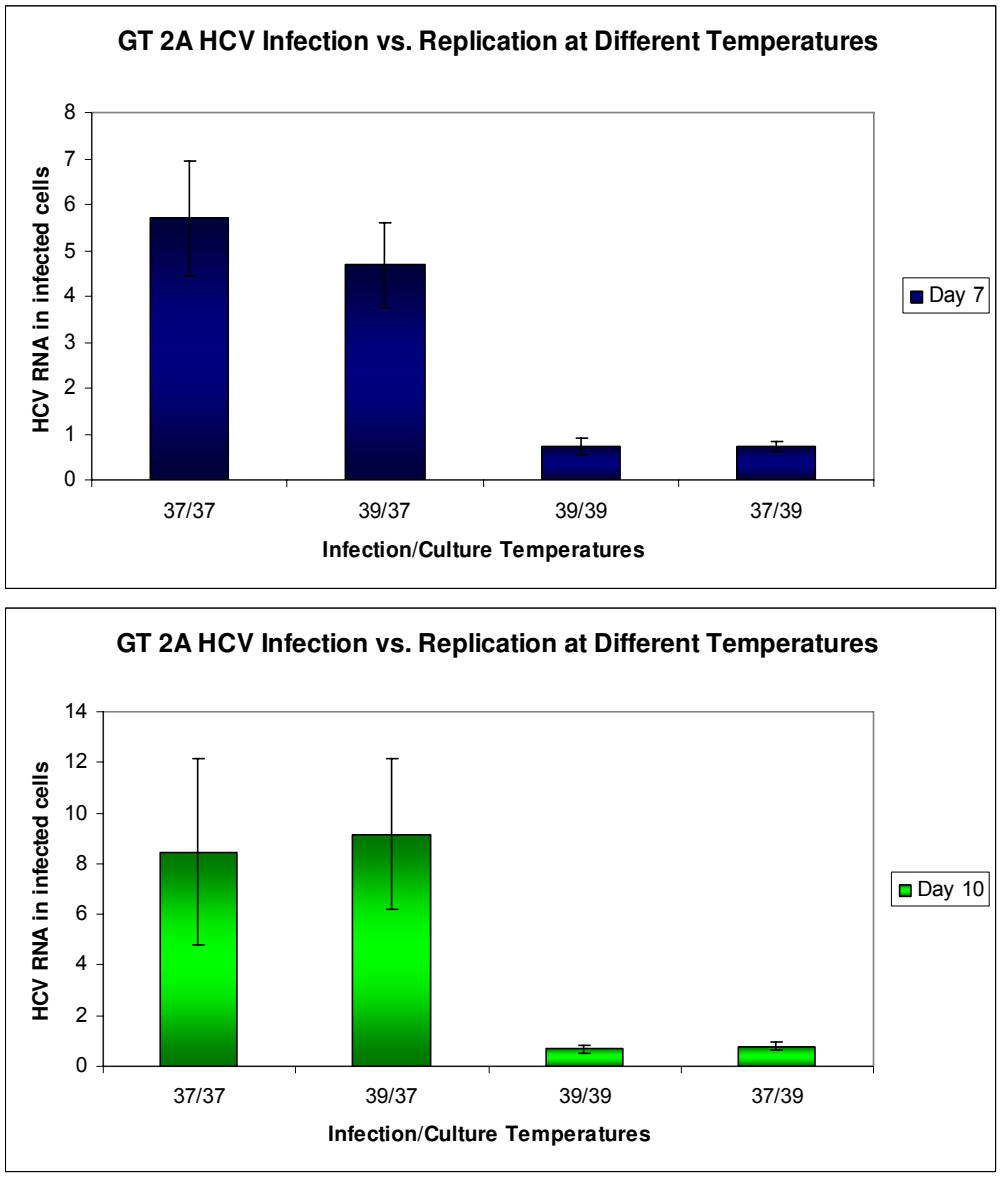


Figure 15. Infection and replication of genotype 2A HCV at different culture temperatures. RNA was collected from infected cells and analyzed via q-RT-PCR. **Top Panel:** Samples collected 7 days after infection. **Bottom Panel:** Samples collected 10 days after infection.

CHAPTER 4

DISCUSSION

In this study, we developed HCV replicon cell lines that were resistant to two treatments – CsA and temperature shifting. Different sources of resistance have been identified for other treatments of HCV. For treatments that act directly on the virus, such as protease and polymerase inhibitors, resistance may arise that can be attributed to the virus itself (21-27). Conversely, the host cell can also confer drug resistance, such as with IFN and ribavirin treatments (28-32). To further characterize the resistance that we attained to both CsA and temperature shift treatment, we needed to determine the source of our resistance.

In addition to confirming that resistance existed at the protein and RNA level, RNA was separated from host cells and electroporated into naïve liver cells in order to determine the host cell's impact on the observed resistance. In both the case of CsA and temperature resistance, naïve cells harboring resistant replicon RNA maintained resistance to treatment, while cured resistant cells harboring naïve cell RNA did not. This demonstrated that the resistance was conferred upon cells by the viral replicon RNA. Having identified resistance that is tied to the viral replicon, we then took a closer look at the sequence of the resistant replicon's RNA.

The work presented in this thesis on CsA resistance was carried forward by a fellow graduate student who went on to identify a single amino acid change in the RS2 replicon's genome. At position 432, which is located in the viral RNA polymerase NS5B, in RS2 cells (and a single cell clone isolated from the RS1 population, RS1-2) the isoleucine (I) was substituted with a valine (V). This mutation was engineered into a wild-type HCV replicon background (this replicon also did not contain the GFP gene to exclude any possible interference). When the replicon cells containing the I432V mutation were subjected to treatment with CsA and NS5A was probed in a western blot, the resistance was maintained up to a level of 0.375 μg/ml, while the wild-type replicon could only withstand a level of 0.25 μg/ml. I432V cells did not restore the full level of

resistance as identified in RS2 cells. The RS2 cells showed 10 times the resistance as GS5 cells, and I432V cells demonstrated about 2 times the resistance as Rep 1B cells. We believe that this is due to the multiple rounds of sorting GS5 cells have undergone in comparison to Rep 1B cells. Many mutations in the GS5 and RS2 cells were identified that were not found in the Rep 1B background. These mutations, while not contributing to CsA resistance, could improve the replicon's replicative abilities. In fact, the GS5 and RS2 cells' RNA showed a level of replication that was 50 times higher than the Rep 1B or original GFP replicon cells' RNA.

The mechanism of action of CsA on HCV has been recently elucidated. It is known that CsA binds to the cellular proteins cyclophilins, and evidence supports the idea that cyclophlins act as a cofactor for NS5B, the viral polymerase (35, 37,38). It has been demonstrated by subsequent study in our lab that one type of cyclophilin – cyclophilin A – is an essential cofactor for HCV replication *in vitro* (43). When CsA is introduced into the cell, a competitive environment arises between the HCV and CsA for binding to the cellular protein cyclophilin A. The replicon's proteins need the interaction with cyclophilin A, in order to be folded properly. It has been demonstrated that the RS2 cells show a reduced dependency on cyclophilin A, due to either a higher affinity for cyclophilin A, or conformational changes to NS5B which requires less interaction with it (43).

The replicon sequences of temperature shift resistant cells (New 39R, HeS3 and TSR cells) were analyzed, and a mutation common to all three, which was not found in our non-resistant replicons, was a glutamic acid (E) to glycine (G) change in position 178 of the replicon. This position is located within the NS3 protein. NS3 is a multifunctional protein, possessing both a serine protease domain and a helicase domain. The serine protease domain is located in the first one third of the protein, and houses our E178G mutation. NS3's viral protease activity is essential for viral replication, cleaving the polyprotein at the NS3/4A, 4A/4B, 4B/5A and 5A/5B junctions. In order to determine if the E178G change was alone capable of conferring temperature shift resistance to the genotype 1B replicon, the mutation was engineered into a 1B HCV wild-type

background, just as the CsA resistant I432V mutation was. The newly mutated RNA was electroporated into naïve cells, which at the time of this writing, have not formed colonies. Future plans for this project include temperature shifting this new cell line against both naïve cells and temperature shift resistant cells and determining if this one mutation is indeed responsible for resistance. When the sequence containing the E178G mutation was searched in the Los Alamos National Laboratory HCV database (http://hcv.lanl.gov/content/hcv-db/index), only 6 sequences were identified as having the same glycine residue in position 178, out of 69,165 total sequences in the database. This indicates that this particular mutation is extremely rare in naturally occurring HCV sequences.

The assay of temperature shifting HCV replicons has not been widely published. Because of this, we wanted to include an additional assay, which would separate the processes of entry and replication. This was made possible by using the infectious genotype 2A strain of HCV. When naïve cells were infected at 37°C and cultured at 37°C, the level of HCV RNA analyzed at both day 7 and day 10 post-infection were high. Levels were similar to cells that were infected at 39°C and switched to 37°C for replication. The HCV RNA levels dropped approximately 6-fold, however, in cells that were cultured at 39°C, regardless of which temperature setting they were infected at. These findings indicate that replication of both HCV genotypes 1B and 2A show sensitivity to a 2°C increase in temperature.

In addition to treating our temperature shift resistant cells with CsA (figure 11), we also treated our CsA resistant cells with temperature shifting. The CsA resistant cells did not show any resistance to temperature shift treatment (data not shown). This data further illustrates that the resistance to temperature shifting and CsA is specific.

The aim of identifying and characterizing drug resistant mutants is ultimately to determine a new mechanism of action that a treatment enacts on a virus. For this study, the CsA resistant mutants have had extensive research carried out with valuable results.

The development of the RS2 cell line led to novel findings by fellow researchers on the role of cyclophilin A in the HCV replication cycle.

The temperature shift resistance study is young in comparison to the CsA resistance study. The goal is that temperature shift resistant mutants will yield as valuable insight into this virus as the CsA resistant mutants have. Future work in the temperature shift resistant mutant study will entail testing cell lines with the E178G mutation in the Rep 1B background for temperature shift resistance. If these cells exhibit resistance, then the mutation will be studied in detail, for structural and functional impacts on viral replication. Because the NS3 protein has been a tempting target for drug design, the prospect of a single site mutation within the protease conferring resistance to treatment is an exciting one. This work will be carried out by future researchers, with an ultimate goal of determining a mechanism of action of temperature shift treatment.

APPENDIX

Table 6. Comparison of amino acids found in different replicon cell lines

		Con 1	GS0	GS5	39R.1S2	New 39R	HeS3	RS1-2
HCV Protein	**Amino Acid Position**							
NS3	81	Q	Q	Q	H	Q	Q	Q
	117	V	V	V	L	V	V	V
	177	E	E	E	G	G	G	G
	281	S	S	S	S	F	S	S
	310	Q	Q	Q	E	Q	E	Q
	380	A	A	A	V	A	V	A
	512	V	V	V	V	A	A	V
	584	K	E	K	E	K	E	K
	676	L	L	L	L	F	L	L
NS4B	687	A	A	A	S	A	A	A
	695	Q	R	Q	R	R	R	R
	812	A	A	A	A	V	A	A
	905	S	S	S	P	S	S	S
NS5A	1162	K	K	K	R	K	K	K
	1178	A	A	A	T	A	T	A
	1181	L	L	P	L	P	L	P
	1187	K	K	K	K	E	K	K
	1193	R	R	R	R	H	R	R
	1194	H	H	N	N	N	N	N
	1227	I	I	S	V	I	V	S
	1262	I	I	I	I	M	I	I
	1304	R	R	E	E	E	E	E
	1316	S	S	P	P	S	P	P
NS5B	1826	I		I	V	I	V	V
	1977	V		V	E	V	V	V
	1979	I		I	T	I	T	I
	1985	R						

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Publications

- *"Cyclophilin A Is an Essential Cofactor for Hepatitis C Virus Infection and the Principal Mediator of Cyclosporine Resistance in Vitro"*, Feng Yang, Jason M. Robotham, Heather B. Nelson, Andre Irsigler, Rachael Kenworthy, and Hengli Tang, Journal of Virology, June 2008, pp. 5269 - 5278, Vol. 82, No. 11.

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