The Role of NS5A RNA Binding Activity in Hepatitis C Virus Replication

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Abstract

Approximately 2% of the world's population is infected by hepatitis C virus (HCV), for which there is no vaccine. HCV nonstructural protein 5A (NS5A), shown by the Cameron laboratory to specifically bind RNA, is a target of new drug development against the virus. We have made amino acid substitutions within regions of NS5A shown to be involved in RNA binding to better understand the function of this interaction. This project explores the impact of these substitutions using biochemical and cell culture-based assays to determine the role of NS5A RNA binding activity in HCV replication.

Introduction

Hepatitis C Virus (HCV) infects about 3% of the world's population and can lead to liver cirrhosis, liver cancer, and ultimately death. HCV is transmitted through the blood and, in some rare cases, can be passed to unborn children from infected mothers. There is no vaccine available against HCV, and current treatments combining the nucleoside analogues interferon and ribavirin are not only expensive, but are also effective in only 50% of cases[1]. These treatments were developed to inhibit virus activity using specific RNA sequences. However, viral RNA polymerase is prone to errors leading to multiple genetic forms of the same virus, making this treatment strategy ineffective in many cases[1]. A different approach to developing treatments is to target the HCV proteins needed for viral genome replication.

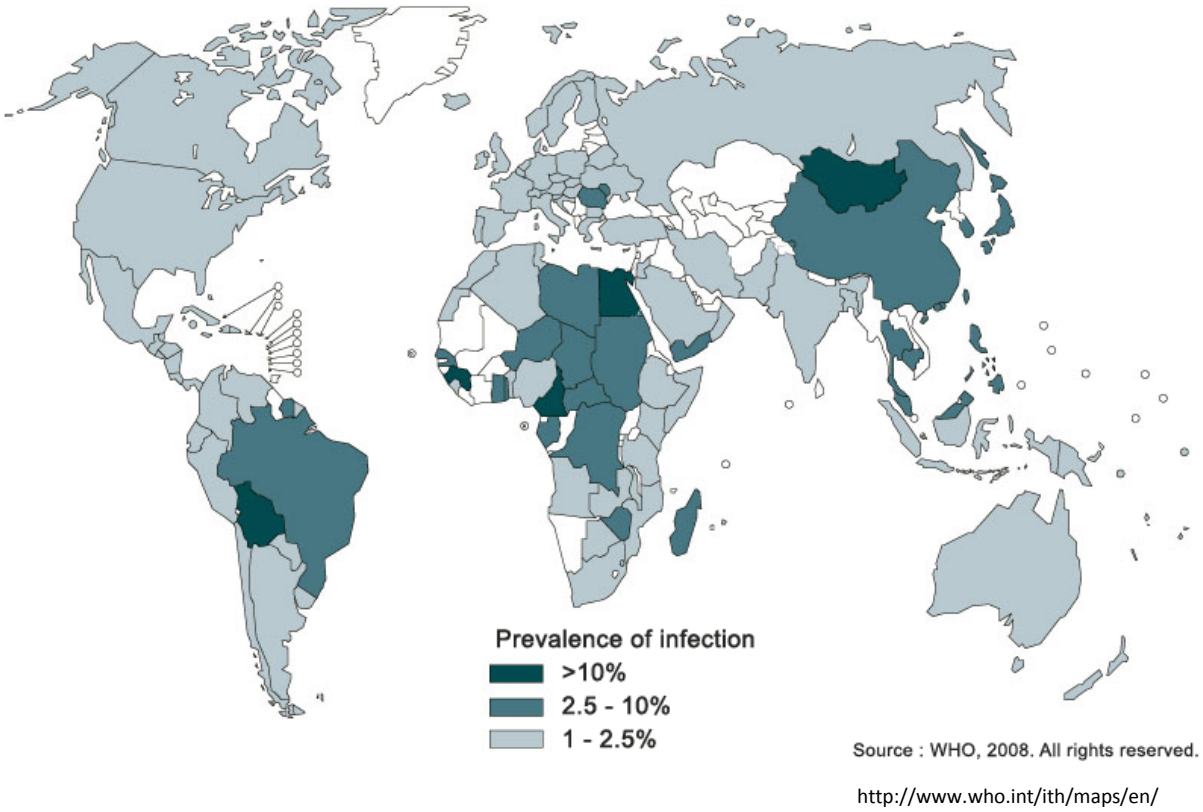

Figure 1. HCV is a global health concern. The map shows the prevalence of infection of HCV around the world.

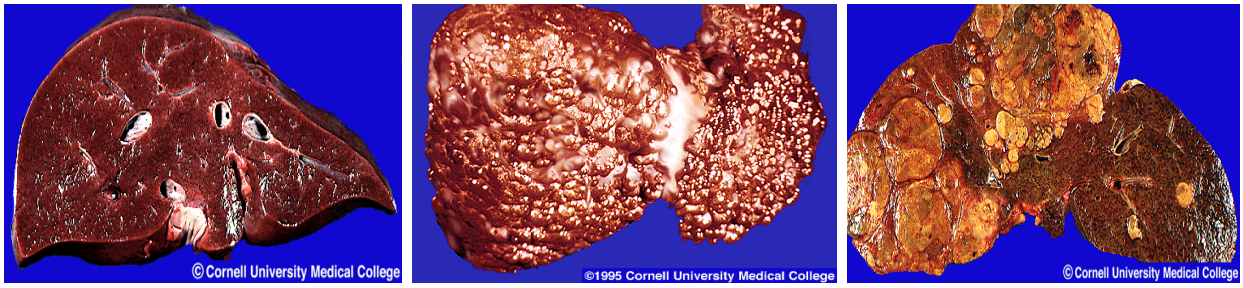

Figure 2. HCV affects the liver (left). HCV can cause liver cirrhosis (center) or liver cancer (right).

viruses contain RNA as their genetic material that can be translated directly into protein. In contrast, negative sense RNA viruses must first convert their negative sense RNA into positive sense RNA before making protein. HCV targets liver cells for infection. In order for HCV to replicate, it must bind to a cell's outer membrane and then enter the cell in a process known as endocytosis. Once inside the cell, the virus releases its RNA, which moves to ribosomes that are responsible for translation[2]. Once there, RNA can use the host cell's machinery to translate its RNA into protein. The new viral proteins either make up the structure of the virus or are used for other functions, such as aiding with genome replication[2]. Using its RNA as a template, the virus makes many copies of its own RNA, which is then packaged and assembled into new viruses using the newly formed proteins. These new viruses are transported out of the cell in order to spread to other cells and repeat the process[2]. In this way, viruses can spread throughout the body in a short period of time.

The HCV genome encodes many proteins: structural proteins make up the virus protein coat and nonstructural proteins are responsible for genome replication. The nonstructural (NS) proteins, NS2, NS3, NS4A, NS4B, NS5A, and NS5B, play various roles in the function of HCV. The protein examined in this study is NS5A. NS5A is a multifunctional protein separated into three domains and two linker regions. It is capable of interacting with cellular and viral factors, including viral RNA, and participates in cell signaling pathways that may promote HCV replication.

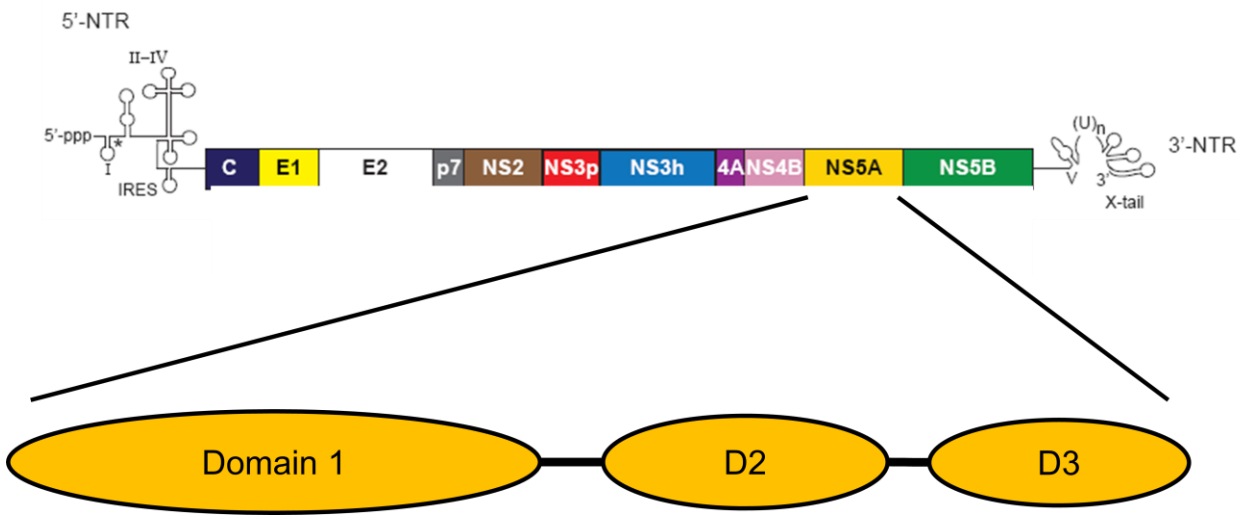

Figure 3. HCV encodes multiple proteins, structural and non-structural. Nonstructural protein 5A is the focus of this study.

protein that may alter protein structure and function. NS5A can exist in three forms: unphosphorylated, basally phosphorylated, and hyperphosphorylated. The phosphorylation of NS5A occurs at various sites within the protein, though one site in particular, the amino acid serine (S) at residue 2204, is an important site of phosphorylation. Phosphorylation at this site is necessary for NS5A to reach its hyperphosphorylated form. It has been shown that an adaptive mutation that changes the serine to an isoleucine (I) allows the virus to replicate in cells, though the mechanism behind this adaptation remains undefined.

Domain 1 of NS5A has been shown to be capable of binding RNA, in particular uracil (U) and by inference, guanine (G) nucleotides. Based on analysis of NS5A structure, it is known that NS5A can form a dimer, two NS5A molecules joined in a two-protein complex[3]. This conformation is optimal for RNA binding because this new structure creates a groove where RNA can be held while it interacts with NS5A[3].

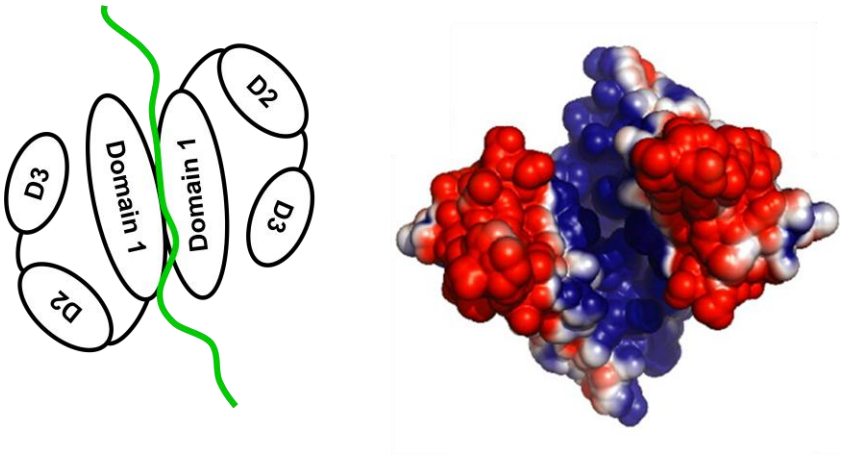

Figure 4. Domain 1 of NS5A forms a dimer which contains a groove sufficient for binding RNA.

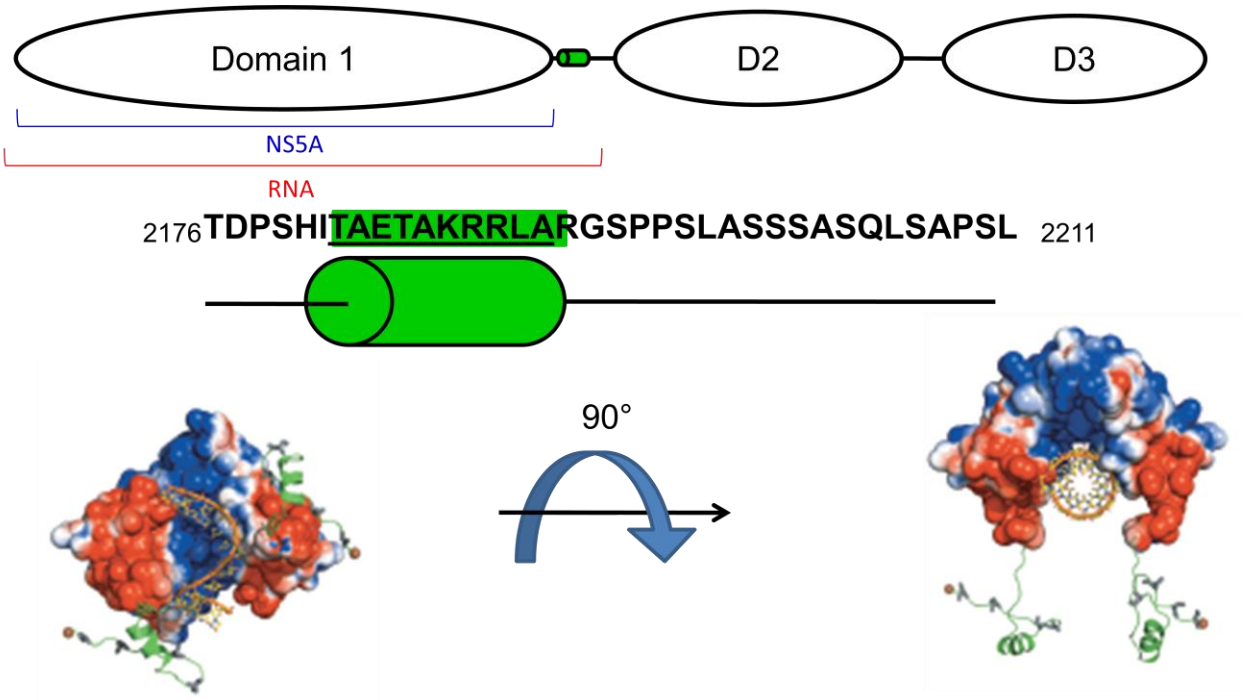

Figure 5. The helix within the linker region between domains 1 and 2 may play a role in NS5A binding activity.

plus. This small helix has been thought to be important for RNA binding because of a few positively charged amino acids: a lysine (K) and two arginine (R) residues at positions 2187, 2188, and 2189. Because domain 1 plus of NS5A is the optimal RNA binding domain, these residues could play a role in HCV genome replication[4].

Previous data analyzing RNA binding activity using fluorescent polarization was done with rU15 and rA15 and NS5A domain 1 plus. For rU15, the RNA binding activity was lower in the KRR-DDD protein when compared to wild type, as shown by the higher dissociation constant (K_d) value. For rA15 the K_d values between wild type and the mutant are similar (Figure 6). A difference in rU15 binding suggests that these residues in domain 1 plus play a role in specific binding of RNA to NS5A.

This project aims to address the importance of K2187, R2187, and R2189 in HCV genome replication through cell culture techniques.

	rU15		rA15	
	wt	K2187D, R2188D, R2189D	wt	K2187D, R2188D, R2189D
K_d (nM)	42.23 ± 5.5	96.06 ± 5.2	440.6 ± 31.8	412.7 ± 12.7

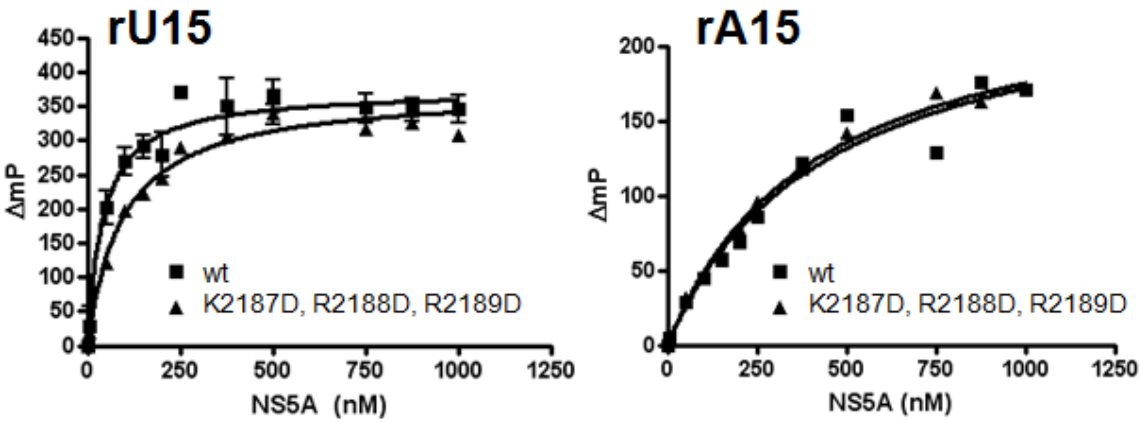

Figure 6. RNA binding activity was observed with NS5A domain 1 plus protein and rU15 and rA15.

Procedure

Cloning

Overlap extension PCR was used to engineer the desired mutation (KRR-DDD) into the replicon DNA. The first piece of DNA used the EcoR1 primer and the S2204I reverse primer with the triple mutant (KRR-DDD) as a template. The second PCR used S2204I forward and Sac 1 reverse primers with the SI/neo plasmid DNA as the template. The third PCR combined the first two in order to make the insert DNA.

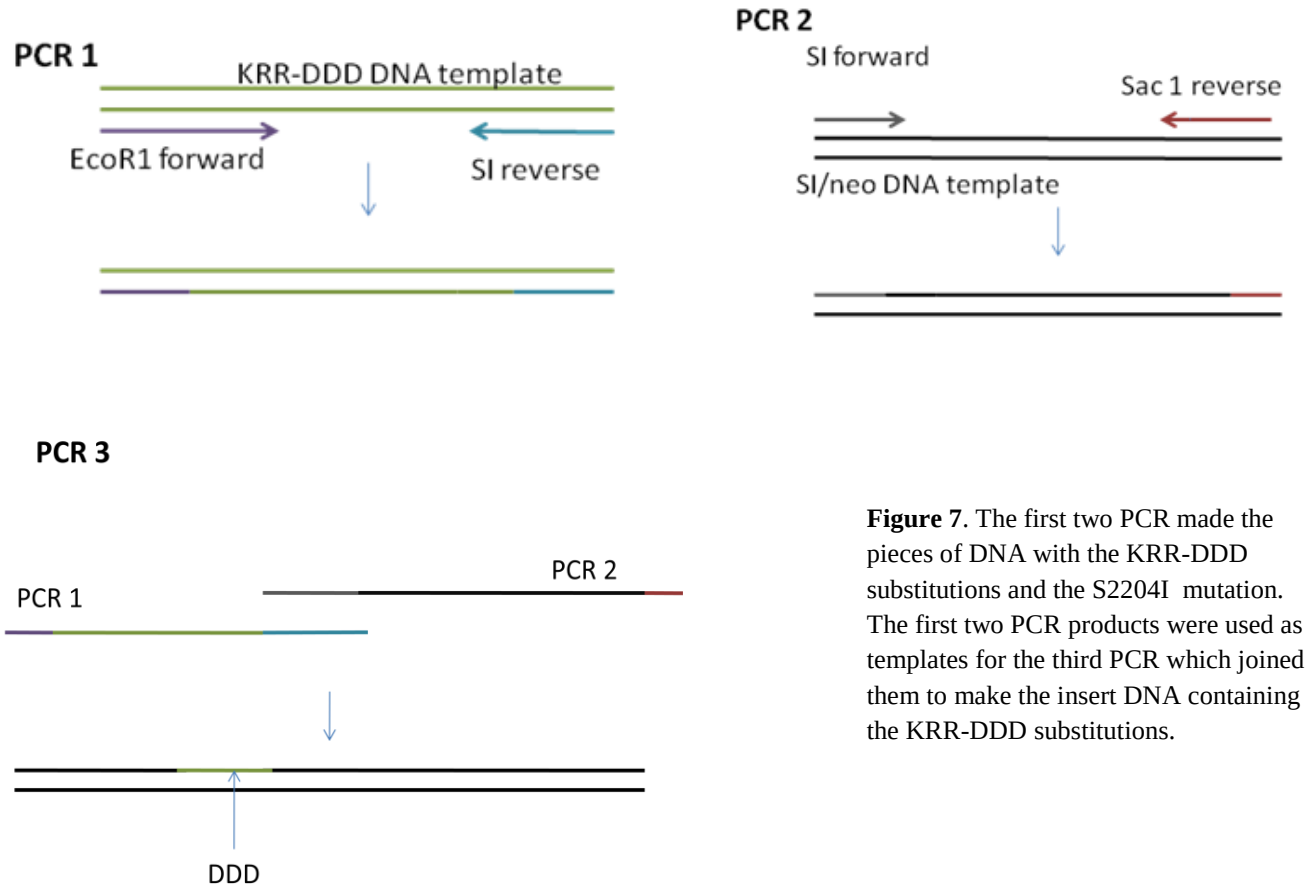

Figure 7. The first two PCR made the pieces of DNA with the KRR-DDD substitutions and the S2204I mutation. The first two PCR products were used as templates for the third PCR which joined them to make the insert DNA containing the KRR-DDD substitutions.

Once the insert was made, it was digested with Xho 1 and EcoR1 restriction enzymes so that it could be ligated into the replicon. This procedure involved incubation of the DNA at 37°C for 3 hours with the enzymes. After incubation, the QIAEX procedure was followed in order to purify the DNA from the reaction mixture.

The DNA vector was also digested with the same enzymes so that the ends of both pieces of DNA would ligate. A shrimp alkaline phosphatase (SAP) reaction prevented the DNA from reattaching to itself after being cut by restriction enzymes. The QIAEX procedure was followed to purify the DNA.

The vector and insert were incubated at 15°C with ligase. Two control groups, the vector with ligase but no insert DNA, and the vector with neither the ligase nor the insert DNA were used as comparisons for the ligation reaction.

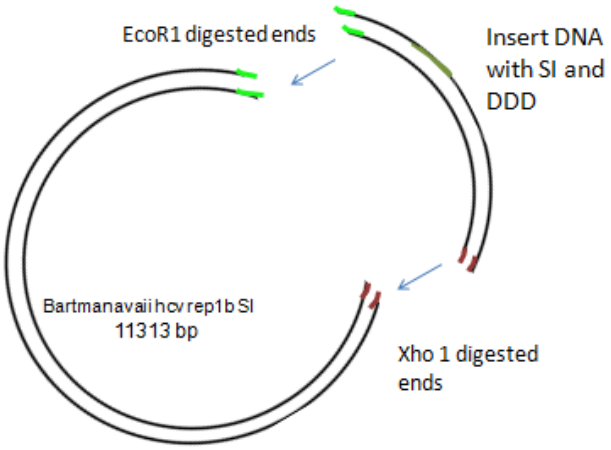

Figure 8. The ligation joined the insert DNA to the DNA vector.

The ligated DNA was expressed by transforming the DNA into MC1060 cells grown in the presence of tetracycline. The bacterial colonies containing the replicon with the ligated insert DNA were chosen and grown in 5 mL cultures at 30°C until they reached an optical density of 1. Then the DNA was extracted from these cells using the MINI prep procedure.

Because the engineered mutation removes a site on the DNA that is usually cut by the restriction enzyme Blp1, the DNA was screened to verify the presence of the mutation by double digesting with Blp1, which cuts within the HCV genome, along with Sca1, which cuts outside of the HCV genome. The reaction product was checked on an agarose gel.

The DNA was also sequenced in order to verify that the DNA coding for the KRR to DDD and the S2204I were present, and that all other parts of the sequence remained the same.

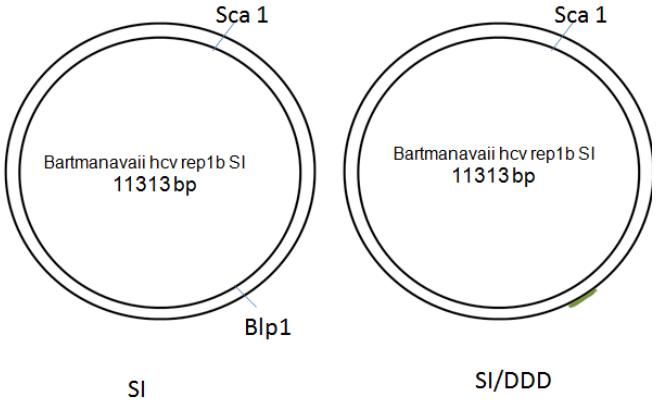

Figure 9. The DNA was screened with Sca1 and Blp1 to verify the correct sequence.

The DNA that contained the correct mutation was then further amplified by inoculating two 500mL cultures with the bacterial cells containing the correct DNA and growing to an optical density of 1. Then, MIDI prep procedures were followed in order to extract and purify the DNA from the bacteria.

Linearization and In Vitro Transcription
The plasmid DNA was linearized by incubating with a restriction enzyme, Sca1. The DNA was made into RNA with in vitro transcription using RNA polymerase.

Figure 10. The circular plasmid DNA was linearized and then translated into RNA.

Transfection
The RNA was used to transfect Huh 7.5 cells so that they could begin replicating the HCV RNA. Huh 7.5 cells were grown in DMEM growth medium containing 10% fetal bovine serum and 1% penicillin and streptomycin. The Huh 7.5 cells were incubated at 37°C in the presence of the RNA: wild type, SI, or SI/3D. A mock sample contained cells without any RNA.

Western Blot
After transfection, cells were analyzed with a Western blot by first running the samples on an 8% SDS-PAGE gel. The gel was run at 40mA for 35 minutes then placed in the Genie Blotter Apparatus and run at 24 volts for 45 minutes. The gel was blocked with 5% milk solution and washed with TBS-T. The gel was probed with Anti-NS5A as the primary antibody and goat anti-rabbit HRP Antibody as the secondary antibody. Once the antibodies had been removed, the gel was analyzed after being exposed to X-ray film in the dark room.

Colony Formation Assay
The transfected cells were plated at $0.75x10^6$, $0.5x10^6$, and $0.35x10^6$ cells per plate for mock, wild type, S2204I, and S2204I/3D in G418 medium. The G418 media was changed every 3 days. After 3 weeks, the cells were stained with crystal violet to visualize the colonies on the plates.

Results

Overlap extension PCR was used to engineer the KRR to DDD and the S2204I substitutions in DNA as PCR 1 and 2 and then the subsequent pieces of DNA were verified for the correct sizes using an agarose gel. The pieces of DNA from reactions 1 and 2 were shown to be the correct sizes.

The next PCR joined the two pieces of DNA that had been previously made. The two strands were used as templates to engineer a complete DNA product with the EcoR1 forward and Domain 2 reverse primers, providing the necessary digestion sites at the ends of the DNA. This reaction when completed and run on a gel, was shown to be successful as indicated by the appropriate band length of 580 base pairs.

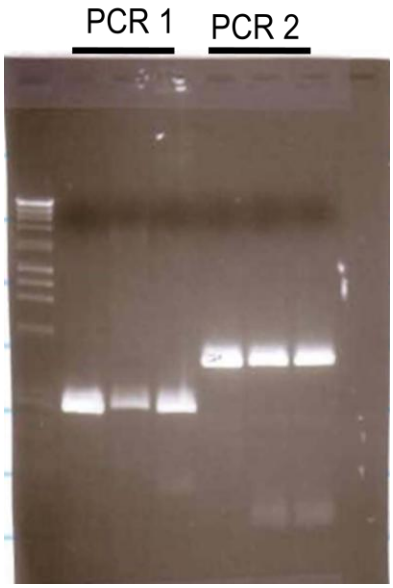

Figure 11.The first two PCR steps were run on an agarose gel to check for bands at the expected lengths.

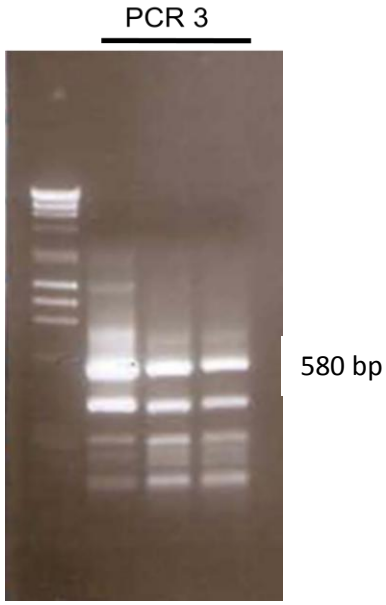

Figure 12. The third PCR was run on an agarose gel to check for the desired band length.

The DNA product was digested with EcoR1 and Xho1 restriction enzymes to prepare the ends of the DNA for ligation. The reaction joined the digested DNA product into a previously prepared DNA vector with EcoR1 and Xho1-digested ends. Successful ligation of the two pieces of DNA allowed creation of a template of the HCV genome with the desired mutations present. Ligations were checked on agarose gels for an upward shift in relation to the vector alone, which lacked the DNA insert.

Figure 13. The ligation reaction was checked on a gel to look for the change in the bands that occurred when the piece of DNA was inserted.

Verification of the ligation reaction allowed for a transformation of the vector into *E. coli* MC1060 cells. Purified MINI Prep DNA was sequenced to verify that the mutation was present. The DNA was screened to check for the presence of the desired DNA sequence by screening with the restriction enzymes Sca 1, which cut outside of the HCV genome, and Blp1, which cut within NS5A. The DNA with the KRR to DDD removed a site on the DNA that is usually cut by the enzyme Blp1, and so in order to analyze the DNA for the presence of the mutation, the DNA was incubated with Blp1. The DNA showed that the mutation was present because it remained intact when exposed to this enzyme.

Figure 14. The screening with the enzymes showed that the DNA contained the mutation when compared to the DNA containing only the S2204I substitution, which was cleaved in the presence of the enzyme.

Further verification of the correct sequence was done by sequencing the DNA. The electropherogram showed that all nucleotides were the same between the wild type and mutant protein, except for the S2204I and the KRR-DDD.

With the desired mutations present, MIDI preps were performed to gain greater amounts of purified DNA. The next step was to linearize this circular plasmid DNA so it could be made into RNA. Using the enzyme Sca1 the DNA was linearized and checked as shown by the gel indicating that the DNA was still the correct size.

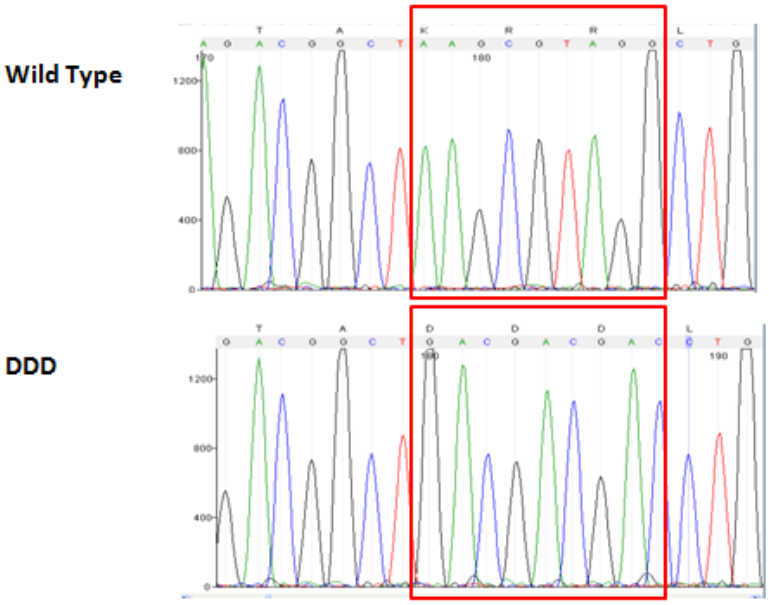

Figure 15.The DNA was sequenced to determine if the mutation was present.

Figure 16.The circular DNA was successfully linearized.

RNA was made from DNA using *in vitro* transcription for the wild type DNA, the DNA containing the S2204I substitution, and the DNA containing both the S2204I and the DDD substitutions. This RNA was run on an agarose gel.

Figure 17.The linearized DNA was made into RNA. The RNA was run on an agarose gel.

Once RNA was made, cells were transfected into Huh 7.5 cells that were grown over night and then analyzed with a Western blot in order to detect the presence of the NS5A protein. Transfected cells were observed for three weeks for the colony formation assay to observe viral genome replication in cells.

Discussion

The results show that the triple amino acid mutation was successfully engineered into the DNA. The DNA was transcribed into RNA and then used to transfect Huh 7.5 cells. These cells were examined for levels of NS5A using a Western Blot and for viral replication using a colony formation assay. These remaining steps are currently ongoing.

The first two PCR steps were completed using the EcoR1, Sac 1, and S2204I forward and reverse primers. Three reactions were made with varying concentrations of $MgSO_4$: 0μM, 1μM, and 2μM. After following normal PCR settings, the reactions were checked on an agarose gel (Figure 11.) The gels show that for PCR #1 and #2, all reactions were successful, so these reactions were purified.

After PCR purification, the two DNA samples were used as templates for PCR #3. This reaction was checked on an agarose gel (Figure 12). Combining the two DNA fragments from PCR #1 and #2 gave the product from PCR#3 an expected length of 580 bp.

Upon completion of the three PCR steps, a digestion reaction was set up using EcoR1 and Xho1 restriction enzymes and a ligation reaction was done and checked on a 1.5% agarose gel. When compared to the control vector, the new vector had a band shifted slightly upward which showed that it had taken in the insert DNA and gained the 580bp insert DNA (Figure 13).

The successful ligation allowed for the vector to be transformed into *E. coli* MC1060 cells. MINI prep was conducted in order to receive a greater amount of DNA. A screen was done to verify the presence of the DDD mutation since this mutation removes a Blp1 cut site that is found in wild type DNA. Screening with the Blp1 showed that the DDD was present because the plasmid DNA was not cut by the Blp1 enzyme (Figure 14). Sequencing the DNA showed that the DDD was the only change in DNA, and the rest of the sequence remained unchanged (Figure 15). Because the mutation was seen, MIDI prep procedures were followed in order to gain greater amounts of purified DNA.

The MIDI prep DNA was cut with Sca1 which made it linear (Figure 16). Using in vitro transcription, the DNA was successfully made into RNA (Figure 17) and used to transfect Huh 7.5 cells. Once the Western blot and colony formation assay are complete, final conclusions will be made.

Conclusion

The effect of NS5A on RNA binding activity was examined by changing three amino acids in domain 1 plus of NS5A: K2187D, K2188D, and K2189D. This substitution altered the RNA binding activity between rU15 and NS5A domain 1 plus, leading to the conclusion that these residues play a role in RNA binding activity.

The ongoing colony formation assay will further elucidate the role of this region in HCV genome replication. Future studies will continue to examine the role of NS5A in HCV function and allow for the development of better antivirals and eventually, a vaccine.

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