THE EFFECTS OF HEPATITIS C VIRUS NS5A PROTEIN ON ANTIVIRAL GENE EXPRESSION

by

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ABSTRACT

Hepatitis C virus (HCV) currently infects about 170 million people worldwide. HCV protein NS5A is a known inhibitor of the host innate immune response, specifically, the IFN-β production pathway. However, little is known about the overall structure of the NS5A protein and where NS5A interacts with this pathway. The goal of our study was to determine the effects of two different NS5A mutants, K2040 (10A) and L2198S (H27), on IFN-β promoter activity, as well as to determine which NS5A domain is particularly involved in blocking the IFN-β production pathway.

ACKNOWLEDGEMENTS

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INTRODUCTION

Natural History and Virology

Hepatitis C virus (HCV) currently infects around 170 million people worldwide and accounts for about 40% of chronic liver disease cases.[5] HCV, a member of the *Flaviviridae* family and the *Hepacivirus* genus, is an enveloped virus containing a 9.6-kb, positive-stranded RNA genome.[1] Infected patients typically experience a delay in presentation of symptoms, prolonging diagnosis and treatment. In the chronic stage, which occurs in about 75 to 85% of infected persons, [8] HCV presents with symptoms which may include fatigue, joint pain, nausea, vomiting, abdominal pain, fever, jaundice, and dark-colored urine.[6] Chronic infection, which occurs in about 75% of patients, can lead to liver disease, which can progress to liver cirrhosis, occurring in about 17% of all patients, or hepatocellular carcinoma, occurring in about 4% of all patients. HCV infection can be managed with medications, but often times a liver transplant is needed. There is currently no vaccine for HCV due to high variance among the different HCV genotypes.[5]

Transmission and Populations at Risk

HCV is primarily transmitted via blood-to-blood contact, which is commonly by way of blood transfusions, injection drug use and needle sharing, and sexual contact. HCV can be transmitted, although more rarely, from mother to child during childbirth.[8] HCV can infect anyone, however, there are a few key populations that have higher incidence rates. People born between 1945 and 1965, also known as the Baby Boomers, have the highest rates of death due to HCV infection in the U.S. Many of the Baby Boomers became infected from nonsterile medical equipment, a common occurrence in hospitals before sanitization protocols were mandated.

Other Boomers may have been infected from blood transfusions before 1992. Before 1992, there was no screening of blood donors for HCV.[8] Another population that is widely affected are adults under 40. Adults under 40 have the highest rates of HCV infections because of injection drug use, which is the most common way to contract HCV.[9] In fact, according to the CDC, 80% of new injection drug users contract HCV within the first six to twelve months. [9] Injection drug use is largely associated with poverty, which results in lack of access to treatment.[10] The population of injected drug-users has dramatically increased due to the opioid crisis.[9] In addition, people who inject drugs are prevalent in U.S. prisons, making prisons a frontrunner for populations with high incidence of HCV. Furthermore, HCV is endemic in Egypt and has the highest rate of HCV infection in the world at 14.7% (NCBI). These rates are even higher, at around 26%, in the Nile Delta and Upper Egypt. The HCV epidemic can be attributed to the use of tartaric emetic injections to treat schistosomiasis in Egypt from the 1950s-1980s. This protocol was perpetuated and encouraged by Egyptian health organizations, as well as the World Health Organization (WHO). Poor sanitation, infection control, and lack of sterilization in medical clinics is responsible for the continuation of HCV transmission today.[12]

Life Cycle and Gene Expression

The HCV genome has a high mutation rate due to rapid replication and inability to proofread.[5] HCV first enters the hepatocyte cytoplasm via low affinity receptors including a LDL receptor and glucosaminoglycans followed by binding to high affinity receptors SR-BI, CD81 Claudin-1 and occludin. Entry occurs via clathrin-mediated endocytosis which subsequently fuses with endosomes via acidification and interaction with the NPC1L1 receptor in the endosome. This releases the HCV genome into the cytoplasm where translation of viral proteins can then take place.[1]

HCV contains a single-stranded RNA genome of 9.6 kb which codes for ten proteins. These proteins include: core, E1, and E2, the structural proteins, and p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B, the nonstructural proteins. The HCV genome is translated in the cytoplasm via ribosome binding to an internal ribosome entry site (IRES) into a precursor polyprotein that is then cleaved by cellular proteases as well as NS3-NS4A, a viral protease, into ten individual proteins. This genome exists in seven different genotypes and several subtypes.[1] Genotypes 1a and 1b are estimated to account for about 60% of all HCV infections.[3] Genotypes 1, 2, and 3 are most common in the Americas, Europe, and Japan. Genotypes 3 and 6 are widespread in Southern Asia, while Genotype 4, 5, and 7 are most commonly observed in Africa.[1]

Innate Immune Response

When the viral genome is inserted into the cell, pattern recognition receptors (PRRs), such as Toll-Like receptors and retinoic acid-inducible gene I (RIG-I)-like receptors recognize the triphosphorylated characteristic of the foreign dsRNA. This recognition leads to RIG-I binding to and activating the mitochondrial antiviral signaling protein (MAVS) which subsequently leads to activation of transcription factors, specifically NF-κb and IRF3/7, which bind to and activate the type I IFN promoter. Type I IFNs are a type of cytokine necessary for inhibiting viral replication. When type I IFNs are secreted from the cell, they bind to type I and type II IFN receptors on surrounding cells. Once these receptors are activated, the Janus kinase (JAK)-signal transducer and activator of transcription factor (STAT) pathway is initiated. This pathway leads to production of IFN-stimulated genes (ISGs), which are involved in cellular antiviral defense.[4]

Viral Anti-Immune Mechanisms

Several proteins of HCV are known to inhibit host antiviral gene expression. Previous research suggests the viral protein NS2 inhibits apoptosis and cell growth by interfering with the cell cycle, which most likely helps with viral replication. Specifically, NS2 was shown to inhibit IFN-β production. HCV NS3 protein was found to block the activation of transcription factors, NF-κB and IRF3/7, both necessary proteins for production of IFN-β. NS3/4A, with 4A as a cofactor of NS3, was shown to cleave MAVS and block phosphorylation of IRF3/7, thus blocking activation of the IFN-β promoter. NS4A has been shown to significantly increase the efficiency of the protease activity of NS3. NS4B has been shown to interfere with the recognition of viral RNA by RIG-I, thus diminishing production of antiviral genes. Finally, viral protein NS5B is an RNA-dependent-RNA polymerase (RdRp), which is essential for viral genome replication. Cytoplasmic protein NS5A is also involved in viral replication, however, its exact function is not known. NS5A has been a major topic of interest in HCV research. Extensive research has shown that NS5A plays an important role in inhibiting antiviral gene expression through interference with multiple proteins involved in this pathway. One of the proteins is STAT1 of the JAK/STAT signaling pathway, which NS5A physically interacts with to prevent phosphorylation, thus diminishing activation of antiviral gene expression. NS5A has also been shown to inhibit the function of PKR and 2', 5'-oligoadenylate synthetase, two major antiviral gene products activated by type I IFNs. [(4)]

NS5A

NS5A protein has three domains. Domain I (a.a. 33-213), Domain II (a.a. 250-342), and Domain III (a.a. 356-466). Domain I, which contains a zinc-binding motif, is thought to specifically be involved in viral replication, however, the exact mechanism remains unknown. The functions of Domain II and Domain III are less well characterized.[4]

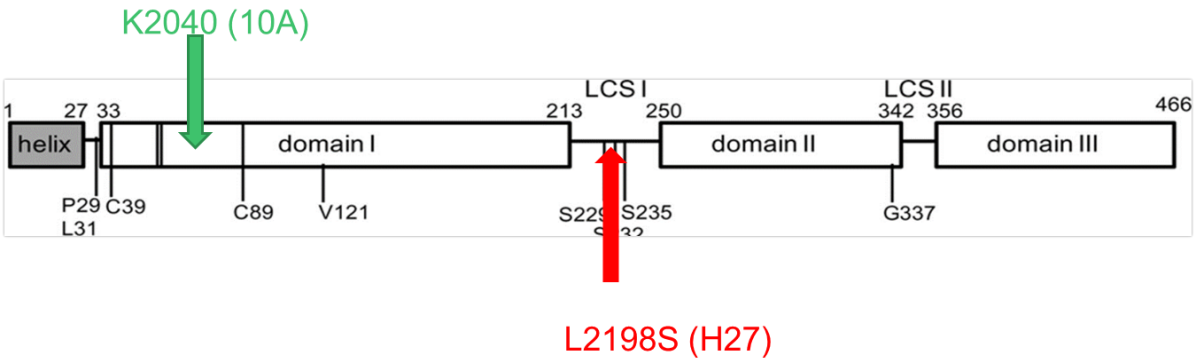


Figure 1 NS5A mutants.

Previous research identified two mutant form of HCV in a Huh7 replicon cell line. The mutants were found to replicate at differential rates. One mutant replicated faster than wild-type HCV, and one mutant replicated much slower. The genomes of the two different mutants were sequenced and it was found that the mutants had different mutations but in the same gene: NS5A. The mutant that replicated faster had a lysine deletion in Domain I, termed NS5A K2040 (10A), and the mutant that replicated slower had a missense mutation of a leucine to a serine in the LCSI region, termed NS5A L2198S (H27) (*Figure 1*).[13]

Treatment

HCV is highly curable; a sustained virologic response (SVR) can be achieved with the use of oral antiviral medications. SVR means there is no virus detected in the blood for an

extended period of time after treatment.[7] Commonly, treatment consists of Sofosbuvir, an NS5B inhibitor, Declatasvir, an NS5A inhibitor, or a combination of the two drugs.[6]

Prevention: Needle and Syringe Programs

There are many preventative measures that have been proposed to halt the spread of HCV. One effective measure is the implementation of needle and syringe exchange programs. These programs have been widely discussed by sociologists and public health workers. Needle and syringe programs benefit cities that are facing an injection drug use crisis by providing drug rehabilitation programs and reducing spread of HCV through vaccination, testing, access to healthcare professionals, and access to sterile needles as well as the safe disposal of used needles.[11] For interested states, all levels of state government would have to communicate with the CDC and prove that their jurisdiction is in the midst of an injection drug use crisis or at risk for one. If approved, these programs would be funded by the federal government. As of 2018, thirty-five states, eight counties, and one city had consulted with the CDC and were determined to have a need for needle syringe programs, but eighteen states still have not requested help from the federal government to implement these programs.[11]

It is crucial that these programs be integrated in all U.S. cities. These programs significantly decrease the spread of hepatitis C and other blood-borne diseases contracted from unclean needle usage, a growing problem in light of prisons and the opioid crisis. These programs help save health care dollars through prevention of infections, reduced hospital stays, and subsequent liver transplants. Moreover, these programs can help save lives.

HYPOTHESIS AND OBJECTIVES

This project aims to investigate the effects of different NS5A mutants on IFN-β promoter activity. We also aim to determine the functionality of the different domains in regard to the ability to inhibit the IFN-β gene expression pathway by creating truncations of the NS5A domains and test their effects on IFN-β promoter activity in isolation. We hypothesize that Domain I of HCV NS5A protein plays an important role in blocking activation of the IFN-β production pathway because the mutation in domain I enables the virus to replicate faster and subsequently greatly diminish the activity of the IFN-β promoter, thus attenuating the innate immune response.

METHODS AND MATERIALS

Cell Culture

For the promoter reporter assays, HEK 293 (human embryonic kidney) cells were used. The cells were grown in complete DMEM (200 μg/mL Penicillin/Streptomycin, 1mM Glutamine, 11 g/L inorganic salts, 4,500 mg/ L glucose, 945.4 mg/L Amino Acids) obtained from Sigma Aldrich. The cells were incubated in 5% CO_2 and 95% air, at 37°C. When the cells in the flask were almost 100% confluent, ~10% of the cells were transferred into a new flask following standard cell culture protocols.

Cell Plating

After splitting the cells into a new flask, the leftover cells were counted using a Brightline hemocytometer to determine the density of the cells. For the promoter reporter assays, cells were plated into a 12 or 24 well plate at a concentration of 100,000 or 50,000 cells per well

respectively. The cells were then kept in the incubator for 24 hours in order to allow the cells to reattach.

Cell Transfection

For the reporter assay, the cells were transfected with pIFN-β-Luc, p-EF-IRES-NS5A, and pRL-CMV-Luc. The IFN-β luciferase plasmid was added at a concentration of 100ng/well, RL-CMV at a concentration of 50ng/well, and the NS5A expression vectors at 400 ng/well. The vectors were premixed with Lyovec at a concentration of 25μl/well. The cells were incubated overnight.

Infection with Sendai Virus

In order for the IFN-β promoter to be activated, the cells were infected with Sendai virus (SV) (4,000HAU/mL) at a concentration of 5 μL/well, a lab-safe virus often used to model viral infection. Under the hood, of SV was added to each well. The cells were then put back in the incubator and left overnight.

Harvesting Cells

In the hood, the medium was aspirated from each well. Cells were washed with 1mL of PBS, and the PBS was then aspirated. 250μL 1X Passive Lysis Buffer (PLB) is added to each well to detach and lyse cells. Plate is rocked for 20 minutes. PLB is transferred from each well to a labeled tube.

Luciferase Assay

Luciferase activity was carried out using the Promega Dual Luciferase assay kit following the manufacturer's protocol. Light production was measured in a luminometer. 50 μL

of Luciferase Assay Reagent was added to each sample and put in the chamber and the light emission was measured. The sample was removed and 50 µL of Renilla substrate (50X) and STOP & GLO mixture was added to the sample. Sample was placed in the chamber again and Renilla activity was measured.

Primer Design

The NS5A gene sequence was obtained from GenBank. Base pairs were counted and domains of the gene were marked based on known size of the regions of NS5A.[13] Primers were designed to anneal to just outside of Domain I on both sides, and Domain II, on both sides. Four primers were designed in order to make three isolate truncations of NS5A: Domain I, Domain II and Domain I and II. A start codon is added to the start the coding sequence within the primer and restriction enzyme sites that correspond to the sites in the pEFIRES-p (Pst I and Not I) were added to each end of the primer:

Primers used:

Domain I:

5’ primer: 5’ GCG CTG CAG GCC GCC ATG TCC GGC TCG TGG CTA AGA GAT 3’

3’ primer: 5’ GCG GCG GCC GC TCA CTT AGC CGT CTC CGC CGT AAT 3’

Domain I and II:

5’: 5’ GCG CTG CAG GCC GCC ATG TCC GGC TCG TGG CTA AGA GAT 3’

3’: 5’ GCG GCG GCC GC TCA GTG TAC CAC TGG AGG GAC GTA 3’

Domain II:

5’: 5’ GCG CTG CAG GCC ATG GAC TCC CCG GAC GCT GAC CTC 3’

3’: 5’ GCG GCG GCC GC TCA GTG TAC CAC TGG AGG GAC GTA 3’

PCR with Q5 Reaction Mix

PCR was carried out using primers obtained from New England Biolabs and the Q5® Hot Start High-Fidelity DNA Polymerase following the manufacturer's protocol (New England Biolabs Inc.). We used the following PCR conditions for 30 cycles:

Phase of PCR Cycle	Domain I	Domain I and II	Domain II
DENATURATION	94°C 4 min 30 sec	94°C 4 min 30 sec	94°C 4 min 30 s
ANNEALING	67°C 30 sec	67°C 30 sec	60°C 30 sec
ELONGATION	72°C 6 min	72°C 6 min	72°C 11 min 30 sec

Gel Electrophoresis

PCR product was loaded onto a 12-well, 1% agarose gel. PCR product was mixed with 5 μL of loading buffer containing 1/10,000 dilution of GelRed®. 20 μL of sample was loaded into a well. A 10kb ladder was used. Electrophoresis machine was set to 200 Volts, 450 mA, and 300 Watts. The gel was ran for 30 minutes to an hour depending on the visible movement of the GelRed®.

Purification

Bands were cut out with a scalpel under a black light and purified using GeneClean® manufacturer's protocol (MP Biomedicals).

Digestion

After purification, the DNA was digested by Pst1 and Not1 (New England Biolabs) for two hours in order to create overhangs so that the isolated DNA could be inserted into the EFP plasmid.

Cloning

After digestion, the DNA was ligated into pEFIRES-p using T4 ligase and the manufacturer's protocol (New England Biolabs). Competent Ampicillin-resistant E. coli was transformed with the ligation reactions and the bacteria was plated on an LB/Ampicillin agarose dish.

RESULTS

Effects of NS5A Mutants on IFN-β Promoter Activity

As a part of the host innate immune response, IFN-β production is known to markedly increase after a viral particle enters a cell. We infected the 293 HEK cell line with Sendai virus as a positive control for the activation of the IFN-β promoter. Upon infection with Sendai virus, the cells exhibited a twenty-nine fold increase in luciferase activity of the IFN-β promoter (*Figure 2*).

HCV mutant NS5A-10A is a known inhibitor of the IFN-β promoter. We transfected cells with the IFN-β luciferase plasmid, the Renilla-CMV plasmid, and NS5A-10A and then incubated them overnight. We then infected the cells with Sendai virus and incubated them again over night. A luciferase assay was performed the next day. The results showed that NS5A-10A diminished IFN-β promoter activity by about 34% (*Figure 3*).

Next we wanted to test the effects of the NS5A-H27 mutant on IFN-β promoter activation. NS5A-H27 if known to increase IFN-β promoter activity. We carried out the same procedure as before but also transfected with NS5A-H27. Our results showed that NS5A-10A decreased IFN-β promoter activity by about 48% NS5A-H27 increased IFN-β promoter activity by about 19% (*Figure 4*).

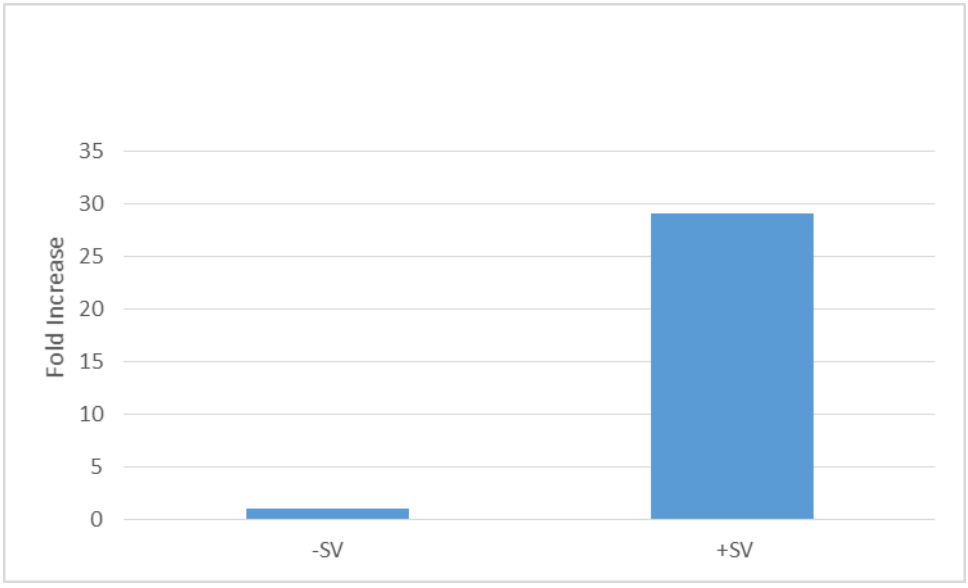


Figure 2 Luciferase activity of IFN-β promoter activity after infection with Sendai virus.

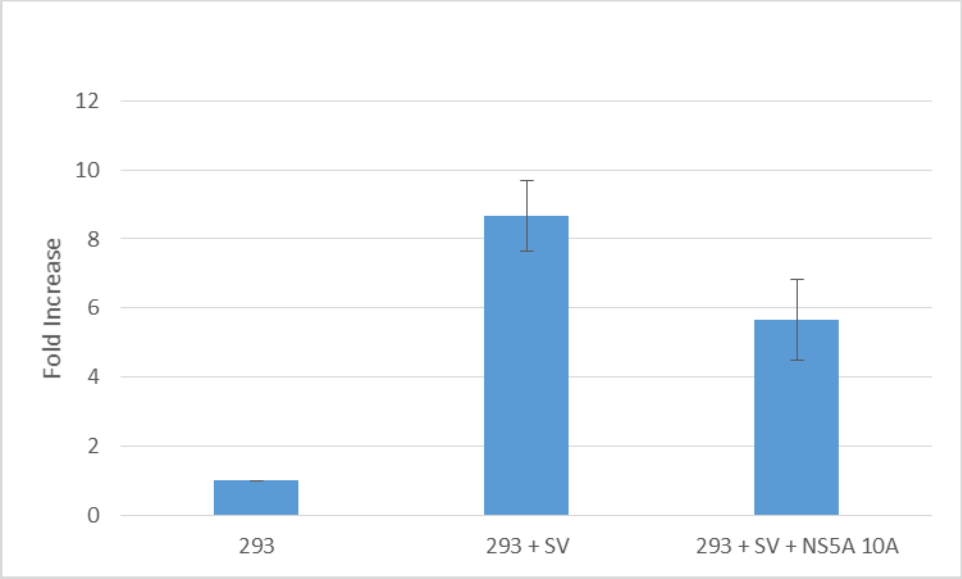


Figure 3 Luciferase Activity of IFN-β promoter after transfection with NS5A-10A.

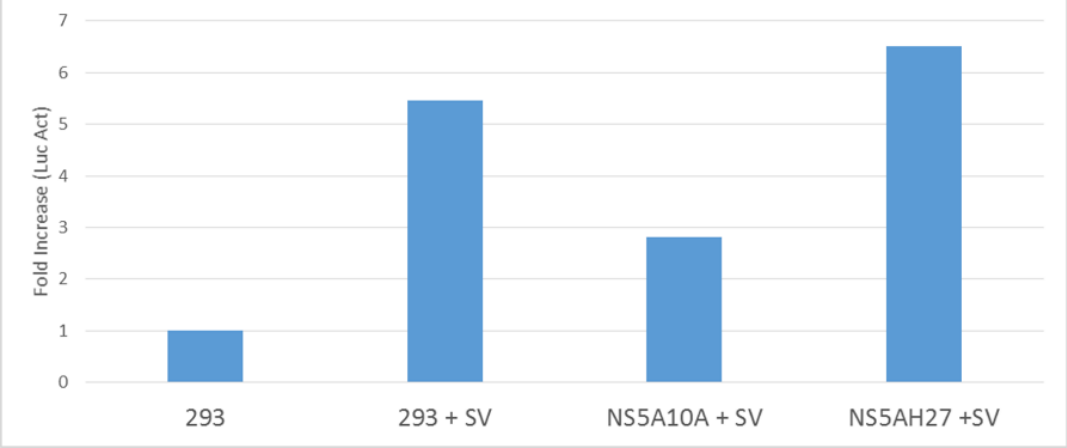


Figure 4 Luciferase Activity of the IFN-β promoter after transfection with NS5A-H27.

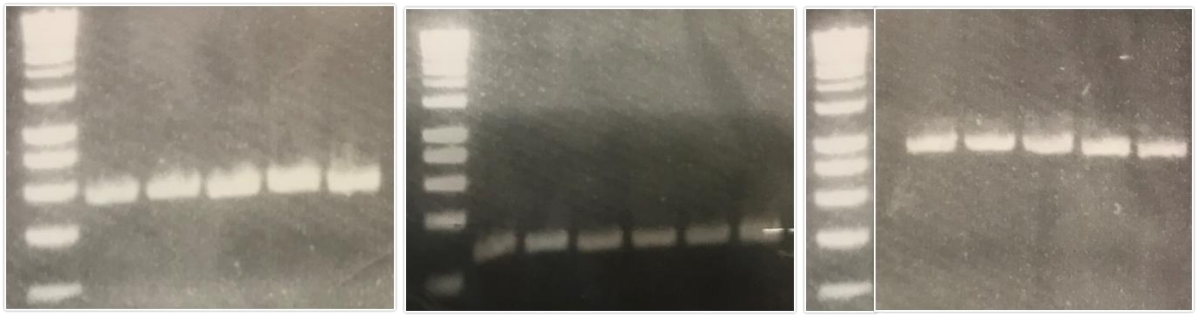
Isolation of Domains of NS5A and Effects on IFN-β Promoter Activity

Using the primers from NEB for PCR, we were able to successfully amplify Domain I, Domain II and Domain I & II. We expected Domain I to be 642 base pairs (bp), Domain II to be 303 bp, and Domain I & II together to be 945 bp. These are the results we observed when we ran the PCR product on a gel based on a 10kb ladder obtained from Denville Scientific (*Figure 5*).

We then purified the DNA from the gel and digested the product with Pst I and Not I. We

ran this product on a gel to purify the fragment and remove it from the restriction enzymes (*Figure 6*).

We then set up separate ligation reactions for DI, DII, and DI&II and ligated pEFIRES-p using T4 ligase and following the manufacturer's protocol. We then transformed competent Ampicillin-resistant E. coli with the ligation reactions, a positive control (full pEFIRES-p) and a negative control (ligase and digested pEFIRES-p). We then plated the bacteria and incubated overnight. If the ligation worked, we would expect to observe colonies on the positive control and our truncations ligated into pEFIRES-p, which would denote the presence of a full circular plasmid. A lawn of colonies was observed on the positive control, however, minimal colonies were seen on the truncation plates in comparison to the negative control plate, suggesting that the ligation was unsuccessful and the plasmid of interest containing the domain isolations was not present.



Domain I Domain II Domain I&II

Figure 5 PCR amplifications of DI, DII, and DI&II

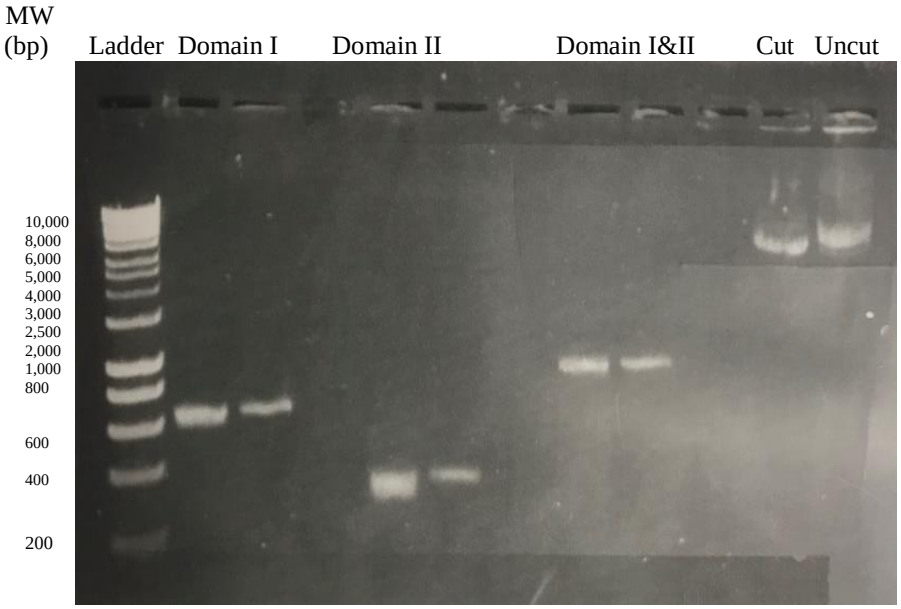


Figure 6 Domain I, Domain II, Domain I&II, cut and uncut pEFIRES-p

DISCUSSION

Overall, we found that the NS5A mutants were able to affect activation of the IFN-β promoter, consistent with previous research.[13] The NS5A-10A mutant was able to decrease IFN-β promoter activity by about 48%, allowing the virus to avoid the innate immune response. The NS5A-H27 was able to increase IFN-β promoter activity by about 19%, thus enhancing the cell's ability to fight viral infection. Due to the fact that NS5A-10A has a mutation in Domain I rendering it able to better attenuate cellular antiviral signaling, we hypothesized that Domain I may be particularly important for the ability of the NS5A protein to block the activation of the IFN-β production pathway. We aimed to isolate Domain I and observe its effects on IFN-β promoter activity via a luciferase assay and compare the activation to other domain isolations. We were successfully able to isolate the domains with the primers we designed and were able to cut the DNA with Pst I and Not I.

Future directions of this project include testing the effects of WT NS5A and HCV NS3/4A protein on IFN-β promoter activity. Also, we want to successfully ligate the NS5A domain isolations into pEFIRES-p, transfect cells with this plasmid, and then test the individual effects of the domains on the IFN-β activation pathway.

Additionally, Zika virus, another member of the *Flavivirus* family, is also known to inhibit the anti-viral response. Given the similarity of the two viruses, we hypothesize that it might express a protein that exerts a similar effect on IFN-β promoter activity.

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