THE EFFECTS OF HEPATITIS C VIRUS PROTEIN, NS5A,
ON TRANSCRIPTION FACTOR, NF-κB

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THE EFFECTS OF HEPATITIS C VIRUS PROTEIN, NS5A, ON TRANSCRIPTION FACTOR, NF-κB

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ABSTRACT

Hepatitis C Virus (HCV) infects liver cells in approximately 180 million people worldwide and will produce a chronic infection in roughly 85% of those affected. In order to develop more effective treatments for the disease, a more in-depth understanding of the mechanism the virus uses to block our immune response is needed. During a normal immune response, a signal transduction pathway turns on the Interferon-beta (IFN-β) promoter, which makes proteins to elicit an inflammatory response to clear the body of the infection. HCV, which has an RNA-based genome that codes for both structural and non-structural proteins, blocks this pathway by an unknown mechanism. NS5A, one of the nonstructural proteins made by HCV, is known to block the IFN-β promoter, but it is unclear which of the three transcription factors NS5A blocks—ATF-2, IRF-3, and NF-κB—or a combination of them. It is our hypothesis that HCV protein NS5A inhibits the activation of one or more of these transcription factors, allowing the virus to evade the immune system and establish a chronic infection.

To test this hypothesis, 293HEK cells were transfected with the promoters of interest—IFN-β and NF-κB—attached to a Luciferase reporter gene, which upon expression gives off light that can be measured using a luminometer. Next, the cells were co-transfected with NS5A and infected with Sendai virus (SV). Cells were harvested and the activity of the promoter was measured to determine if NS5A inhibits the activation of NF-κB. Our results suggest that NS5A inhibits the SV-induced activation of NF-κB but not the TNF-induced activation of NF-κB, possibly indicating that the step being inhibited by NS5A is unique to the virus-induced RIG-I signaling pathway. Identifying the exact step that NS5A inhibits could lead to the development of more effective antiviral drugs.
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INTRODUCTION

Hepatitis C Virus (HCV), a blood-borne virus that infects liver cells, can be transmitted through needle sharing, contaminated blood transfusions, sexual intercourse, or during childbirth. An estimated 180 million individuals are infected with HCV worldwide, but this number may be even higher due to the asymptomatic nature of the disease, making it a major public health threat (Horner & Gale, 2013). For a few fortunate individuals, the acute infection spontaneously clears by itself, but in approximately 85% of cases a chronic infection is established (Fig. 1). The silent progression of the disease causes approximately 30% of those infected to develop chronic liver failure (Hoofnagle, 1997).

The spectrum of the HCV infection is extremely variable—fewer than 20 percent of patients with chronic HCV infection exhibit symptoms, largely being fatigue and malaise. Approximately 20-30 percent of people chronically infected with HCV develop cirrhosis; at which time symptoms such as jaundice, gastrointestinal bleeding, and weakness typically appear. Following cirrhosis, the liver damage may progress to hepatocellular carcinoma or end-stage liver disease. At this stage, the only way to regain health is a liver transplant. Between 1987 and 1995, approximately 21 percent of adult liver transplantations in the United States could be attributed to HCV—this number has since risen to nearly 30 percent.

Figure 1. Progression of Hepatitis C Virus infection. More than 85% of acute infections result in chronic infections (Hoofnagle, 1997).
Unfortunately, almost all of these patients who undergo a liver transplantation develop a more mild, recurrent HCV infection (Mukherjee & Sorrell, 2008).

For many years, the primary treatment for HCV was a two-drug regimen including Interferon and Ribavirin administered for 12-72 weeks. Although this treatment works for some patients, it is not effective against all genotypes of HCV. Furthermore, long-term use of Interferon therapy is expensive and causes adverse effects such as chills and body aches (“A brighter”, 2013). Recently, antiviral drugs have been developed that target specific proteins in the HCV genome. Telaprevir targets the serine protease NS3/4A, while Declatasvir inhibits NS5A and prevents viral replication. Unfortunately, HCV mutates at a very high rate allowing it to develop resistance to these antiviral drugs over time (Soriano et al., 2009). A better understanding of how the virus replicates and evades the host immune response will allow for the development of more effective treatments and possibly a preventive vaccination (Sumpter et al., 2004).

**Hepatitis C Virus Life Cycle:**

As a member of the Flaviviridae family, HCV is an enveloped virus that has a 9.6kB positive-stranded, RNA-based genome. Once the virus enters a liver cell, it uses the host cellular machinery to transcribe and translate its genome. Upon transcription and translation, a long polyprotein is produced which is then cleaved by viral and cellular proteases to produce seven nonstructural and three structural proteins: Core, E1, and E2 (Fig. 2). The Core protein makes up the nucleocapsid, while E1 and E2 are glycoproteins that stick out from the envelope, assisting in viral entry and fusion. The seven nonstructural proteins play important roles in viral replication. Several of the
nonstructural proteins, such as NS2, NS3, and NS4A, have protease ability to cleave the polyprotein. NS5B serves as an RNA-dependent RNA polymerase during transcription, and NS5A is the regulator of replication (Chevaliez & Pawlotsky, 2006). As an RNA virus, these HCV proteins lack proofreading ability, resulting in numerous errors and the creation of new strains of the virus (Nakano et al., 2011). After the polyprotein is cleaved into the separate proteins, the genome is packaged into the nucleocapsid, and the mature virions leave the infected cell via exocytosis (Chevaliez & Pawlotsky, 2006).

Figure 2. Map of the HCV polyprotein showing the cleavage sites and functions of each protein. There are three structural proteins: Core, E1, and E2. The seven remaining nonstructural proteins function in regulating transcription and viral assembly (Scheel et al. 2013).

Innate Immune Response:

When a foreign pathogen enters the body, the innate immune response is activated and secretes type I interferons (IFN) to clear the body of infection. Interferon-Beta (IFN-β), a type I IFN, is produced as a result of a signal transduction pathway that is turned on after the recognition of a pathogen associated molecular pattern (PAMP). Cells of the innate immune system contain pattern recognition receptors (PRRs) to detect these PAMPs (Mogensen, 2009). RIG-I is an intracellular PRR that detects the presence of viral RNA such as HCV. After RIG-I detects viral RNA in the cell, it binds to and activates MAVS, which is
located on the mitochondria. At this point, MAVS stimulates IκB Kinase (IKK) to phosphorylate IκBα, tagging it for ubiquination and degradation by the proteasome. Once IκBα is degraded, NF-κB is free to move into the nucleus and bind to the IFN-β promoter (Seth et al., 2006). The IFN-β promoter is regulated at the transcriptional level and requires all three transcription factors—ATF-2, IRF3, and NF-κB—to move from the cytosol into the nucleus, bind to the promoter, and form an enhancesosome (Fig. 4). If even one of the transcription factors is not bound to the promoter, the gene will not be transcribed and the antiviral response will be suppressed (Kaukinen et al., 2006). HCV is able to establish a chronic infection in liver cells by suppressing the production of antiviral proteins, but the exact mechanism is unknown.

Figure 3. Intracellular viral RNA triggers the signal transduction pathway resulting in the nuclear translocation of NF-κB and IRF3 (Seth et al., 2006).

Figure 4. Interferon-β (IFN-β) promoter regulated by enhancesosome made of transcription factors: ATF-2, IRF3, and NF-κB. Once the transcription factors bind, RNA polymerase transcribes the IFN-β gene to produce antiviral proteins. The PRDII element of the IFN-β promoter is where NF-κB binds (Acheson, 2011).
**HCV Counter defenses:**

Recent studies have shown that HCV evades the host immune response by inhibiting the activation of IFN-β through several pathways. A few of the HCV non-structural proteins have well-characterized roles in blocking the antiviral response. In addition to its helicase and protease abilities, HCV nonstructural protein NS3/4A blocks the antiviral response by cleaving MAVS from the mitochondria (Fig. 3). This terminates the signal transduction pathway and prevents the nuclear localization of the transcription factors required to turn on the production of IFN-β (Kaukinen et al., 2006).

Research in our lab has shown that another HCV nonstructural protein, NS5A, inhibits the activation of IFN-β but the exact mechanism has not been elucidated. It has been hypothesized that a variation of NS5A with a lysine insertion at nucleotide position 2040 blocks the production of IFN-β by interfering with the nuclear localization of the transcription factors (Sumpter et al., 2004). Recent research in our lab showed that NS5A blocks the nuclear localization of NF-κB but not IRF-3 (Akkaraju, Unpublished). ATF-2 has not yet been analyzed. Through the use of Dual Luciferase Assays it is possible to quantify the activation of the individual transcription factors. It is our hypothesis that HCV protein NS5A K2040 inhibits the expression of IFN-β and helps the virus establish a chronic infection by blocking the activation of NF-κB.

**MATERIALS AND METHODS**

**Cell culture:**

293 Human Embryonic Kidney (HEK) cells were a kind gift from Dr. James Chen at the University of Texas Southwestern Medical Center Department of Molecular Biology.
The cell line HEK 293 NS5A K2040, made in the laboratory of Dr. Giridhar Akkaraju at Texas Christian University, are human embryonic kidney cells that constitutively produce the HCV protein, NS5A, with a lysine insertion at position 2040. 293HEK cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 1x nonessential amino acids, 2mM L-Glutamine, and penicillin/streptomycin (100 U/mL). The 293 NS5A K2040 cell line was cultured in the same medium and treated with Puromycin (0.1 μg/mL) to ensure only cells expressing NS5A K2040 would grow. Cells were cultured in 25 cm² tissue culture flasks at 37°C humidified air containing 5% CO₂ and passaged every three to four days.

**Cell Treatment:**

Sendai Virus (SV) is a negative-stranded, RNA virus that elicits a similar response as HCV but is much safer to use in the laboratory (Strähle et al., 2003). The Cantell Strain of SV was purchased from Charles River Laboratories (Wilmington, MA, USA). The titer was $6 \times 10^3$ hemagglutination units/mL (HAU/mL). Tumor Necrosis Factor-alpha is a cytokine that activates transcription factor, NF-κB, in response to a pathogen or an inflammatory stimulus. TNF-α was purchased from Sigma-Aldrich and had a final concentration of 10 μg/mL. Polyinosinic:polycytidylic acid [poly(I:C)] is double-stranded RNA that activates IFN-β promoter through the RIG-I-mediated antiviral pathway. The final concentration of poly(I:C) was 25mg/mL. IL-1β is a cytokine that activates NF-κB transcription factor during the inflammatory response. IL-1β was a kind gift from Dr. Gary Boehm in the Department of Psychology at Texas Christian University. The IL-1β was added to the cell culture medium at a final concentration of 2 μg/mL.
**Plasmids:**

Plasmid expression vectors were used to express the promoter reporter constructs and inhibitor proteins that were used in the study. The plasmids were prepared using the Zyppy plasmid midiprep protocol from Zymo Research. The following plasmids were a kind gift from Dr. James Chen at the University of Texas Southwestern Medical Center: 3x NF-κB-Luc, PRDII-Luc, NS5A K2040, NS3/4A, and IκBΔN. The 3x NF-κB-Luc plasmid contains three repeats of the binding domain for transcription factor, NF-κB preceding the luciferase reporter gene. The PRDII-Luc plasmid is composed of the PRDII element of the IFN-β promoter, which contains the binding site for NF-κB transcription factor, attached to the luciferase reporter gene. The NS5A K2040 plasmid contains the coding sequence for the HCV protein, NS5A, with a lysine insertion at nucleotide position 2040. Prior research has shown that strains of HCV with the NS5A K2040 mutation inhibit the antiviral pathway more than strains with different variations of the protein (Sumpter et al, 2004). The IκBΔN plasmid contains the gene for a dominant negative form of the NF-κB inhibitor IκBα. IκBΔN binds to NF-κB, preventing it from translocating to the nucleus. The IFN-β-Luc plasmid was made by Dr. Giridhar Akkaraju and consists of the promoter region of the human IFN-β gene linked to the reporter gene, luciferase. The plasmid RL-CMV-Luc containing the Renilla luciferase reporter gene was used as an internal control for transfection efficiency and was purchased from Promega (Madison, WI, USA).

**Dual-Luciferase Reporter Gene Assay:**

The host 293 HEK cells were plated in 24-well plates (100,000 cells per well) in Dulbecco’s Modified Eagle Medium 24 hours before transfection. To measure PRDII
activity, cells were transfected with 100ng/well PRDII firefly luciferase reporter and co-transfected with 50ng/well Renilla luciferase reporter (RL-CMV-Luc) for normalization using the LyoVec transfection reagent (Invivogen) and using the supplied protocol. Cells were treated with either Sendai virus or TNF-α 24 hours post-transfection. 24 hours after treatment, cells were rinsed once with 1x PBS (pH 7.4) and lysed with 1x Passive Lysis Buffer (100mL/well) as supplied in the Dual-Luciferase Reporter Assay kit (Promega). Then, the Firefly and Renilla luciferase activities were separately analyzed by measuring the luminescence using a luminometer. The data was then normalized to the corresponding control values.

**RESULTS**

*Activation of NF-κB promoter:*

As previously mentioned, the IFN-β promoter consists of three domains—ATF-2, IRF-3, and NF-κB binding domains—to which the respective transcription factors bind after activation by a pathogen-associated molecular pattern (PAMP). Sendai virus (SV) and poly(I:C) are known to activate the IFN-β promoter through this antiviral pathway. SV is a negative-stranded RNA virus that elicits a similar cellular response as HCV but is much safer to use in the laboratory. Poly(I:C) is double-stranded RNA that activates the IFN-β promoter through the RIG-I-mediated antiviral pathway. The NF-κB binding domain within the IFN-β promoter was isolated and attached to the Luciferase reporter gene (called 3x NF-κB-Luc), in order to measure the activation of only NF-κB. The NF-κB promoter is activated by SV, poly(I:C), and tumor necrosis factor-alpha (TNF-α), a cytokine involved in the inflammatory response. The goal of this experiment was to find an agent that would
reliably activate the NF-κB promoter, so the effects of HCV protein, NS5A, could later be tested. Since all three agents—SV, TNF-α, and poly (I:C)—are known to activate the NF-κB transcription factor, there should be increased activity of the 3x-NF-κB-Luc promoter following treatment with the agents.

293HEK cells were plated in a 24-well plate (100,000 cells/well). The IFN-β and NF-κB firefly luciferase reporter plasmids were transfected (100ng/well) into 293HEK cells using the LyoVec protocol, and an internal control RL-CMV luciferase reporter was co-transfected (50ng/well) for normalization. 24 hours post-transfection, the cells were either left untreated (-SV) or treated with the respective stimulatory agent—either 10μL/well SV (6000 HAU/mL), TNF-α (10ng/mL), or poly(I:C) (25µg/mL). The cells were harvested 24 hours later, and a Luciferase assay was performed. The SV-induced cells transfected with IFN-β experienced 16-fold increase in luciferase activity when compared to the untreated IFN-β control. In the cells transfected with NF-κB, the cells treated with poly(I:C) showed a 0.97-fold change in Luciferase activity when compared to control, TNF-α-induced cells showed a 1.18-fold increase in Luciferase activity, and the SV-induced cells showed a 8.6-fold increase in Luciferase activity when compared to the untreated NF-κB control (Fig. 5).
Activation of PRDII promoter:

The 3x-NF-κB Luciferase plasmid, which contains three tandem repeats of the binding domain for transcription factor NF-κB preceding the luciferase reporter gene, was used in repeated experiments, but consistent activation could not be achieved. Next, the PRDII-luciferase plasmid was tested to see if it would produce more consistent activation in response to Sendai Virus, TNF-α, or IL-1β. PRDII is an element of the IFN-β promoter that contains the NF-κB binding domain (Fig. 4). Therefore, the level of activation of the PRDII-Luc promoter should show the level of activation of the NF-κB transcription factor. Since PRDII is sub-domain of the IFN-β promoter, known activators of the antiviral pathway—SV, TNF-α, and IL-1β—would be expected to also activate the PRDII promoter.

293HEK cells were plated in a 24-well plate (100,000 cells/well). After 24 hours, the PRDII firefly luciferase reporter plasmid was transfected (100ng/well) into HEK293 cells following the LyoVec protocol, and an internal control RL-CMV luciferase reporter was co-
transfected (50ng/well) for normalization. 24 hours post-transfection, the cells were treated with the respective stimulatory agent—10μL/well SV (6000 HAU/mL), TNF-α (10ng/mL or 40ng/mL), IL-1β (10ng/mL or 20ng/mL)—or left untreated. The cells were harvested the 24 hours later, and a Luciferase assay was performed on the cell lysates. SV-induced cells showed 6-fold increase in Luciferase activity. The cells treated with 10μg/mL and 40μg/mL TNF-α showed 8.3-fold and 8.2-fold increase in luciferase activity, respectively. However, treatment with IL-1β showed negligible activation of PRDII-Luc (Fig. 6).

![Activation of PRDII promoter](image)

**Figure 6.** Luciferase assay showing the activity of PRDII promoter. The relative luciferase activity is shown as the ratio of (the results from the cells transfected by individual reporter)/ (the results from the cells transfected by the internal control in the same cell group). Error bars represent mean ± STDEV.

**Inhibition of PRDII by NS5A:**

Since the PRDII-Luc promoter showed consistent activation with both SV and TNF-α in repeated experiments, it was used to study the effects of HCV protein, NS5A, in future
experiments. Previously, it has been shown that the HCV protein NS5A inhibits the activation of the IFN-β promoter, but the exact mechanism by which it does so is unknown. By examining the activation of each transcription factor independently, it is possible to determine the exact manner through which NS5A acts. Prior research in our lab has shown that NS5A blocks the nuclear localization of NF-κB in the HeLa cell line, so NF-κB was the first transcription factor analyzed quantitatively using luciferase assays. If NS5A blocks the entry of NF-κB into the nucleus, cells transfected with NS5A and cells constitutively producing NS5A should inhibit the SV-induced activation of PRDII.

293HEK and 293HEK NS5A K2040 cells were plated in a 24-well plate (100,000 cells/well). 24 hours after plating, the PRDII firefly luciferase reporter plasmid was transfected (100ng/well) into 293HEK and 293HEK NS5A K2040 cells using the LyoVec protocol, and an internal control RL-CMV luciferase reporter was co-transfected (50ng/well) for normalization. Additionally, in one condition of 293HEK cells were transfected with 0.25μg/well NS5A. 24 hours post-transfection, the experimental conditions were infected with 10μL SV (6000 HAU/mL), and the controls were left untreated. The cells were harvested 24 hours later, and a Luciferase assay was performed. 293HEK cells infected with SV showed a 4.2-fold increase in Luciferase activity when compared to the untreated control. 293HEK cells that were co-transfected with 0.25μg/well NS5A and treated with SV showed a 1.6-fold increase in Luciferase activity. The cell line, 293HEK NS5A K2040, treated with SV showed a 2.6-fold increase in Luciferase activity when compared to the untreated control (Fig. 7).
Inhibition of PRDII by NS5A and IκBΔN:

After showing that NS5A inhibits the SV-induced activation of PRDII promoter by NS5A. The relative luciferase activity is shown as the ratio of (the results from the cells transfected by individual reporter)/ (the results from the cells transfected by the internal control in the same cell group). Error bars represent mean ± STDEV.

293HEK cells were plated in a 24-well plate (100,000 cells/well). 24 hours later, the PRDII firefly luciferase reporter plasmid was transfected (100ng/well) into 293HEK cells, and an internal control RL-CMV luciferase reporter was co-transfected (50ng/well) for
normalization. One condition was co-transfected with 0.5μg/well NS5A and a separate condition was co-transfected with 0.5μg/well IκBΔN. 24 hours post-transfection, the cells were infected with 10μL SV (6000 HAU/mL) or left untreated. The cells were harvested 24 hours, and a Luciferase assay was performed on the cell lysates. The SV-induced cells showed a 11.3-fold increase in Luciferase activity when compared to the untreated control. Cells co-transfected with 0.5μg/well IκBΔN and treated with SV showed a 7.5-fold increase in Luciferase activity, and the cells co-transfected with 0.5μg/well NS5A and treated with SV showed a 6.2-fold increase in Luciferase activity when compared to the untreated control (Fig. 8).

**Inhibition of PRDII Promoter**

![Inhibition of PRDII Promoter](image)

Figure 8. Luciferase assay showing the inhibition of the SV-induced activation of PRDII promoter by NS5A and IκBΔN. The relative luciferase activity is shown as the ratio of (the results from the cells transfected by individual reporter)/ (the results from the cells transfected by the internal control in the same cell group). Error bars represent mean ± STDEV.

**Inhibition of PRDII by NS3/4A and NS5A:**

After seeing convincing inhibition by NS5A in repeated experiments, the inhibitory activity of NS5A was compared to that of NS3/4A, another HCV protein that is known to
block the IFN-β pathway through a different mechanism than the one hypothesized for NS5A. Prior research has shown that NS3/4A inhibits the antiviral pathway by cleaving MAVS from the mitochondria, preventing the activation of both NF-κB and IRF-3 transcription factors (Li et al., 2005). In addition to comparing the effects of NS3/4A to NS5A, cells were transfected with a combination of NS5A and NS3/4A to see if the amount of inhibition in the condition with both proteins expressed in the same cells would be different than in conditions with either protein by itself. Since NS3/4A and NS5A are both thought to inhibit the activation of NF-κB, cells transfected with a combination of NS3/4A and NS5A should presumably show the most inhibition of the PRDII promoter, followed by cells only transfected with NS3/4A. Finally, cells with only NS5A would show the least inhibition of the three experimental conditions.

293HEK cells were plated in a 24-well plate (100,000 cells/well). 24 hours after plating, the PRDII firefly luciferase reporter plasmid was transfected (100ng/well) into HEK293 cells using the LyoVec protocol, and an internal control RL-CMV luciferase reporter was co-transfected (50ng/well) for normalization. In addition, one condition of cells was transfected with 0.5μg/well NS3/4A, one condition was transfected with 0.5μg/well NS5A K2040, and another condition was transfected with a combination of 0.5μg/well NS3/4A and 0.5μg/well NS5A K2040. 24 hours post-transfection, the cells were infected with 10μL SV (6000 HAU/mL) or left untreated. The cells were harvested 24 hours later, and a Luciferase assay was performed. The SV-induced cells showed a 10.4-fold increase in Luciferase activity when compared to the untreated control. Cells co-transfected with NS3/4A and treated with SV showed a 1.6-fold increase in Luciferase activity. Cells co-transfected with NS5A and treated with SV showed a 6-fold increase in
Luciferase activity, and the SV-induced cells co-transfected with both NS3/4A and NS5A showed a 3.3-fold increase in Luciferase activity when compared to the untreated control (Fig. 9).

![Inhibition of PRDII by NS3/4A and NS5A](image)

**Figure 9.** Luciferase assay showing the inhibition of the SV-induced activation of PRDII promoter by NS3/4A, NS5A and a combination of the two. The relative luciferase activity is shown as the ratio of (the results from the cells transfected by individual reporter)/ (the results from the cells transfected by the internal control in the same cell group). Error bars represent mean ± STDEV.

**Inhibition of PRDII by NS5A, Transfection versus Cell line:**

To further study the effects of NS5A on the transcription factor NF-kB, cells transfected with NS5A were compared to the cell line that constitutively produces NS5A K2040. In theory, cells constantly producing NS5A should have more NS5A in each cell than cells transfected with NS5A because transfection is not 100% efficient. Therefore, the average inhibition of the SV-induced action of PRDII should be lower for the 293HEK NS5A K2040 cell line than for 293HEK cells transfected with NS5A.

293HEK and 293HEK NS5A K2040 cells were plated in a 24-well plate (100,000 cells/well). After 24 hours, the PRDII firefly luciferase reporter plasmid was transfected
(100ng/well) into 293HEK and 293HEK NS5A K2040 cells following the LyoVec protocol, and an internal control RL-CMV luciferase reporter was co-transfected (50ng/well) for normalization. In addition, one condition of cells was co-transfected with 0.25 μg/well NS5A. 24 hours post-transfection, the cells were infected with 20μL SV (6000 HAU/mL) or left untreated. The cells were harvested the 24 hours later, and a Luciferase assay was performed on the cell lysates. 293HEK cells infected with SV showed a 9.8-fold increase in Luciferase activity when compared to the untreated 293HEK control. 293HEK cells co-transfected with NS5A and infected with SV showed a 2.2-fold increase in Luciferase activity. 293HEK NS5A K2040 cells infected with SV showed a 8.1-fold increase in Luciferase activity when compared to the untreated 293HEK control (Fig. 10).

**Inhibition of PRDII: Cell line Vs. Transfection**

![Bar chart showing inhibition of PRDII by NS5A](image)

Figure 10. Luciferase assay showing the inhibition of the SV-induced activation of PRDII by NS5A in transfected cells versus the 293HEK NS5A K2040 cell line. The relative luciferase activity is shown as the ratio of (the results from the cells transfected by individual reporter)/(the results from the cells transfected by the internal control in the same cell group). Error bars represent mean ± STDEV.
**Inhibition of TNF-induced activation of PRDII by NS5A:**

Since repeated experiments had shown substantial evidence that NS5A inhibits the SV-induced activation of PRDII, the focus of this research switched to studying the effects of NS5A on the TNF-induced activation of PRDII. As previously mentioned, TNF-α is a cytokine that is excreted from damaged cells and activates NF-κB during an inflammatory response. TNF-α activates NF-κB through a different pathway than that stimulated by an RNA virus. If TNF and viruses utilize different pathways to activate NF-κB, NS5A might block one or both of these pathways. If NS5A only blocks the activation of NF-κB following infection with SV but not treatment with TNF-α, the mechanism by which NS5A acts may be narrowed down to a step utilized only by the antiviral pathway. The goal of this experiment was to see if NS5A also inhibits the TNF-induced activation of PRDII. If this is true, cells co-transfected with NS5A should show a similar level of activity in PRDII-Luciferase activity as the cells co-transfected with known inhibitor, IκBΔN.

293HEK cells were plated in a 24-well plate (100,000 cells/well). After 24 hours, the PRDII firefly luciferase reporter plasmid was transfected (100ng/well) into 293HEK cells using the LyoVec protocol, and an internal control RL-CMV luciferase reporter was co-transfected (50ng/well) for normalization. In addition, six wells of cells were co-transfected with 0.25 μg/well NS5A and six wells of cells were co-transfected with 0.25 μg/well IκBΔN. 24 hours post-transfection, the cells were treated with 10μL TNF-α (10μg/mL) or left untreated. The cells were harvested 24 hours later, and a Luciferase assay was performed. 293HEK cells treated with TNF-α showed a 6.1-fold increase in Luciferase activity when compared to the untreated control. Cells co-transfected with IκBΔN and treated with TNF-α showed minimal Luciferase activity, but the cells co-
transfected with NS5A and treated with TNF-α showed a 16-fold increase in Luciferase activity when compared to the untreated control (Fig. 11).

**DISCUSSION**

Hepatitis C Virus is a major public health concern worldwide due to its ability to establish a chronic infection in liver cells sometimes without recognizable symptoms. Furthermore, HCV mutates quickly allowing it to develop resistance to the current treatment regimens and escape the host immune response more efficiently. Prior research has shown that a specific mutation in HCV nonstructural protein NS5A helps HCV suppress the production of IFN-β, thereby diminishing the host cell’s antiviral response. This mutation in HCV protein NS5A is a lysine insertion at position 2040 (NS5A K2040). IFN-β is a cytokine produced after RIG-I recognizes intracellular double-stranded RNA and triggers
the antiviral signal transduction pathway, ultimately ending in the nuclear localization of NF-κB and IRF3. The exact mechanisms that NS5A uses to inhibit the expression of IFN-β have not been elucidated, but recent studies in our lab suggest that NS5A is blocking the nuclear translocation of NF-κB (Akkaraju & Richards, unpublished). The focus of the research in this paper was to determine whether NS5A exerted its inhibitory effect on the IFN-β by influencing the activation of transcription factor, NF-κB.

The first step toward analyzing the effects of NS5A on transcription factor NF-κB was to find an agent that would reliably stimulate the promoters of interest. In the first experiment, known activators of the transcription factor NF-κB—SV, TNF-α, and poly(I:C)—were used, but only SV significantly activated the 3x NF-κB-Luciferase promoter. Although the genome of SV is not identical to HCV, the two viruses activate the antiviral response in a similar manner, so SV can be used in place of HCV (Sumpter et al., 2004; Strähle et al., 2003). Unfortunately, in later experiments, the 3x NF-κB-Luciferase promoter did not show SV-induced activation, so a different promoter, PRDII-Luciferase, was tested in Experiment 2.

In the second experiment, known activators of the antiviral pathway—SV, TNF-α, and IL-1β—were tested to see if consistent activation of the PRDII-Luciferase promoter could be achieved. Since PRDII is an element of the IFN-β promoter that contains the NF-κB binding domain, the activity of the PRDII-Luciferase promoter should show the activation of transcription factor NF-κB. In this experiment, both SV and TNF-α significantly activated the PRDII-Luciferase promoter, which meant both SV and TNF-α could be used in future experiments as a positive control. Neither concentration of IL-1β activated the PRDII-
Luciferase promoter probably because 293HEK cells do not have receptors that recognize IL-1β.

Once it was shown that SV and TNF-α could consistently activate the PRDII-Luciferase promoter, future experiments focused on attempting to show the effects of HCV nonstructural protein NS5A K2040 on the SV-induced activation of transcription factor NF-κB. In Figures 7 and 10, the effects of NS5A K2040 were studied in 293HEK cells co-transfected with NS5A K2040 and in the cell line, 293HEK NS5A K2040, which constitutively produces the HCV protein NS5A K2040. If NS5A K2040 is blocking the nuclear translocation of NF-κB as recent studies in our lab suggest, cells expressing NS5A K2040 should show less SV-induced activation of the PRDII-Luciferase promoter when compared to control. The results from Figures 7 and 10 support the hypothesis that NS5A K2040 is inhibiting the activation of NF-κB. Interestingly, in both experiments, 293HEK cells co-transfected with NS5A K2040 showed more inhibition of the SV-induced activation of PRDII-Luciferase promoter than the cell line 293HEK NS5A K2040. This suggests that the 293HEK cells expressed more NS5A K2040 than the cell line, which is the opposite of what should happen since transfection is not completely efficient.

In Experiment 4 (Fig. 8), the effects of NS5A K2040 were compared to a mutated form of the NF-κB inhibitor, IκBα. IκBΔN, a dominant negative form of IκBα, binds to NF-κB and prevents it from translocating to the nucleus to turn on IFN-β promoter. If NS5A K2040 is inhibiting the activation of NF-κB, cells co-transfected with NS5A K2040 should show similar levels of SV-induced activation of PRDII-Luciferase as cells co-transfected with the negative control, IκBΔN. Figure 8 illustrates that NS5A K2040 inhibited the SV-induced
activation of PRDII-Luciferase more than IκBΔN, thereby supporting the hypothesis that NS5A K2040 inhibits the activation of transcription factor, NF-κB.

In the fifth experiment, the effects of NS5A K2040 were compared to another HCV protein, NS3/4A, which is known to block the production of IFN-β by cleaving MAVS from the mitochondria and terminating the antiviral signal transduction pathway. If NS5A K2040 blocks the activation of transcription factor NF-κB, cells co-transfected with NS5A K2040 should show similar levels of SV-induced activation of PRDII-Luciferase as cells co-transfected with the known inhibitor, NS3/4A. The results from this experiment show that NS5A K2040 inhibited the SV-induced activation of PRDII-Luciferase, but to a lesser extent than NS3/4A. While the results do support the hypothesis that NS5A K2040 inhibits the activation of NF-κB, the results also suggest that NS5A K2040 might be less efficient at inhibiting the antiviral response than NS3/4A. In addition, cells co-transfected with both NS3/4A and NS5A K2040 showed an intermediate level of inhibition when compared to cells co-transfected with only NS3/4A or NS5A K2040. This might suggest that the proteins are interfering with each others function, but further research is needed to see if this pattern can be reproduced.

The seventh and final experiment attempted to show the effects of NS5A K2040 on the TNF-induced activation of transcription factor, NF-κB. As previously mentioned, TNF is a cytokine that activates the nuclear translocation of NF-κB during the inflammatory response, but it might work through a different pathway than the one mediated by RIG-I in the antiviral response. Since the data supports the hypothesis that NS5A K2040 inhibits the SV-induced activation of PRDII-Luciferase, if NS5A K2040 does not inhibit the TNF-induced activation of PRDII-Luciferase, then the pathway through which NS5A K2040 is working
may be narrowed down. In this experiment, NS5A K2040 was compared to the negative control IκBΔN. Figure 11 illustrates that while IκBΔN blocks the TNF-induced activation of PRDII-Luciferase, NS5A K2040 fails to do so. Therefore, NS5A K2040 might be blocking a step that is unique to the antiviral pathway and not shared with the TNF-induced pathway.

In conclusion, the research presented here supports the hypothesis that HCV protein NS5A K2040 is suppressing the production of IFN-β by inhibiting the activation of transcription factor, NF-κB. Furthermore, our data suggests that NS5A inhibits the activation of NF-κB following infection with SV but not treatment with TNF-α. Although the exact mechanism through which NS5A works remains unclear, the data suggests that NS5A is inhibiting a step further upstream in the signaling pathway that is unique to the antiviral response, but more research is needed to determine the exact step that NS5A is inhibiting. Moreover, NS5A could be blocking the activation of either ATF-2 or IRF3 in addition to NF-κB, so these other transcription factors should also be tested using Dual Luciferase Assays and Nuclear Localization studies.

Understanding the mechanism of action of NS5A in inhibiting the IFN-β promoter identifies it as a good target for the design of drugs against it. There are a few drugs that directly target NS5A including Daclatasvir and Ledipasvir, which are now being used in the treatment of chronic Hepatitis C infection. Since HCV is an RNA virus and mutates rapidly, it can acquire resistance to the antiviral drugs. Therefore, it is important to continue researching the Hepatitis C life cycle and how it evades the immune system to develop new effective antiviral drugs and win the battle against the worldwide public health concern, Hepatitis C Virus.
REFERENCES


of hepatitis C virus genotypes and subtypes based on the complete coding region.


