THE EFFECT OF THE HCV PROTEIN, NS5A, ON THE TRANSLOCATION OF THE TRANSCRIPTION FACTORS IN THE INTERFERON BETA RESPONSE.

By

Jordan Talley

Submitted in partial fulfillment of the requirements for Departmental Honors in the Department of Biology

Texas Christian University

Fort Worth, Texas

Monday, December 12th, 2016
THE EFFECT OF THE HCV PROTEIN, NS5A, ON THE TRANSLOCATION OF THE TRANSCRIPTION FACTORS IN THE INTERFERON BETA RESPONSE.

Project Approved:

Supervising Professor: Giridhar R. Akkaraju, Ph.D

Department of Biology

Matthew M. Chumchal, Ph.D

Department of Biology

Julie A. Fry, Ph.D

Department of Chemistry
ABSTRACT

Hepatitis C Virus infects hepatocytes in humans. Infection with the virus yields a range of symptoms from asymptomatic to liver disease or hepatocellular carcinoma. The virus is estimated to be infecting 3% of the world’s population and transmits via contaminated blood. There are current treatments for the virus, but they are not ideal because of their extreme side effects. Achieving a greater understanding of the virus and viral proteins can generate new ways to inhibit viral replication and progression of disease. To improve its reproductive success, HCV inhibits the immune system of the host. Specifically, HCV inhibits the antiviral response by decreasing the expression of IFN-β, a cytokine that activates the host defense against a pathogen. Three transcription factors, ATF-2, NFκB and IRF-3, are activated by viral infection, which stimulates the transcription factors to translocate to the nucleus to bind to the IFN-β promoter and turn on expression of IFN-β gene. An HCV protein, NS5A, is known to inhibit the expression of IFN-β, possibly by inhibiting transcription factor translocation. The effect of NS5A on the localization of each transcription factor pre- and post-viral infection was examined. NS5A inhibits translocation of one of the transcription factors, NFκB, but does not inhibit translocation of the other two, ATF-2 nor IRF-3. This supports the hypothesis that NS5A decreases the expression of IFN-β by inhibiting transcription factor, NFκB, from entering the nucleus and binding to the IFN-β promoter.
ACKNOWLEDGEMENTS

The completion of my Honor's Thesis would not have been possible without the guidance and assistance of many people. I would like to thank Dr. Akkaraju for the opportunity to work on his research team. I am grateful for his patience and challenges, which equally helped me learn. I would like to thank Dr. Fry and Dr. Chumchal for their time and guidance on improving my thesis. I could not have completed my thesis without my research team members; their encouragement and advice is much appreciated. It has been an honor to work with my team and I cannot wait to see the success of each of their futures. I am also grateful for the consistent support from my family and friends; I would not have finished without their constant reassurance in my ability. Thank you, TCU, for having faith in me as a student and for giving me the resources to grow and learn. Most importantly, I would like to thank God for his favor on my life and all He has provided me. I cannot wait to glorify His name in science. Through this process, I have learned to value curiosity and to trust my own knowledge; I look forward to taking all that I have learned to my future career.
Table of Contents

Introduction
  i. History
  ii. Infection
  iii. Prevention and Treatment
  iv. Virology
  v. Antiviral response
  vi. Interference of Anti-Viral Response by Virus
  vii. NS5A
  viii. Purpose and Research Questions

Materials and Methods
  i. Cell Culture
  ii. Cell Plating
  iii. Transfection
  iv. Infection
  v. Immunofluorescence

Results
  i. The effect of NS5A on virus-mediated translocation of ATF-2
  ii. Virus-mediated translocation of NFκB into the nucleus
  iii. The effect of NS5A on TNF-α-induced translocation of NFκB
  iv. The effect of NS5A and NS3/4A on virus-mediated translocation of NFκB
  v. The effect of NS5A and NS3/4A on virus-mediated translocation of IRF-3

Discussion

Conclusion
INTRODUCTION

Hepatitis C Virus History and Prevalence

The World Health Organization estimates that 3% of the world's population is currently infected with Hepatitis C virus (HCV). In 2007, HCV surpassed HIV as a leading cause of death in the United States. The population most commonly infected with HCV is the Baby Boomer generation, those born between 1946 and 1964 (5). Specifically, those most at risk are injection drug users, previous blood transfusion recipients, or infants born to infected mothers. Prior to the discovery of HCV, the correlation, but not causation, between blood transfusions, injection drug use and multiple sex partners with non-A, non-B hepatitis was made. In 1989, the infectious agent of a majority of non-A, non-B hepatitis was identified as the hepatitis C virus (10). Hepatitis refers to inflammation of the liver, thus HCV is the third viral infection identified that damages the liver, following hepatitis A virus and hepatitis B virus. The discovery of HCV led to an increase in preventative measures to stop the infection. In 1992, anti-HCV screening of donated blood began in order to stop the spread of infection. A drastic decrease in the population infected with HCV has occurred likely because of the use of clean needles and HIV preventative behavior (2). Screening for the virus has shown a global distribution of HCV infection, with a greater prevalence in East Asian and African countries (11,12).

Infection

Human hepatocytes are the host cells for HCV. In order for the virus to infect hepatocytes, it travels in the blood stream to the liver. Virus particles utilize blood circulation for transportation to hepatocytes, and therefore the virus spreads via blood contamination. This contamination ranges from blood transfusions to sexual encounters. Any form of blood-to-blood contact with HCV-infected blood is an opportunity to spread the virus. The most prominent opportunities for infection in the United States are blood transfusions before 1992, injection drug use, and unprotected sex. Health-related work also accounts for a small amount of transmission through accidental needle sticks of medical professionals after contamination with a patient's infected blood (1).

Acute hepatitis C is the first response of hepatitis C virus infection. A patient with acute hepatitis could be asymptomatic or demonstrate symptoms, such as malaise and jaundice. Typically,
it takes 3-12 weeks after exposure to HCV for acute hepatitis C symptoms to occur. The lack of symptoms post HCV infection causes the virus to infect many hosts before detection. Techniques used for the diagnosis of HCV currently include testing the serum for HCV RNA and alanine aminotransferase (ALT). ALT is a liver enzyme whose blood concentration elevates when the liver is damaged. HCV RNA levels can be recorded within the first two weeks after infection, while ALT levels will rise two weeks after infection. Testing via the HCV antibody commonly gives false negative results less than three months after infection, and therefore is not used to diagnose acute hepatitis C. (2) The asymptomatic characteristic of acute hepatitis C prevents diagnosis and treatment of HCV infection in many patients. This causes an infected patient to progress towards chronic hepatitis without any treatment.

In addition to acute hepatitis C infection, early stages of chronic hepatitis C infection can persist without diagnosis. Chronic HCV can remain asymptomatic until the patient develops end-stage liver disease or hepatocellular carcinoma. At this point, treatment options emphasize improving the health of the liver. Though commonly undiagnosed at an early stage, chronic hepatitis C infection occurs in 75-85% of patients infected with HCV; chronic infection begins if the patient is unable to overcome viral infection after six months of exposure. HCV infection progression varies among patients post chronic hepatitis C infection, possibly leading to cirrhosis of the liver, hepatocellular carcinoma or death. The development of HCV infection from acute infection to liver fibrosis is dependent on a wide range of factors. HCV infection is more likely to yield a chronic infection in patients infected at an age older than 25, without symptoms during acute infection and of the male gender. Factors that increase the progression of chronic HCV infection to fibrosis include alcohol consumption, age of infection and co-infection with Human Immunodeficiency Virus or Hepatitis B Virus (2).

**Prevention and Treatment**

The identification of the hepatitis C virus has lowered the infection rate of HCV over the past two decades. Currently, donors for blood transfusions, transplants, and clotting factors are screened for the presence of the virus. Instead of HCV infection predominantly spreading via blood and organ donations, HCV is more commonly spread through contaminated needles, intravenous drugs use,
unprotected sex or accidental needle sticks (3). An increase in awareness of the mode of transmission for the virus decreases the spread of infection. The late onset of symptoms caused by HCV, and the long persistence of the virus in an individual, allows for undetected transmission of the virus between people. Consistently being aware of possible HCV transmission and avoiding it by utilizing clean needles and condoms can prevent the spread of the virus. During treatment, a patient is not cured and is still considered infectious until he/she has sustained absence of HCV RNA for 24 weeks without treatment (14). Current treatment combines stimulating the antiviral response via interferon and abrogating virus production via inhibitors of HCV proteins. Depending on the progression of HCV infection, treatment also includes the prevention or decrease of liver damage.

Interferon was utilized as an initial form of treatment, even prior to the identification of HCV as the source of most non-A non-B hepatitis. This treatment increases the immune response towards a virus. (15). Increasing the amount of interferon within a patient stimulates the antiviral pathway to respond to the virus. Patients infected with HCV receive interferon treatment for a long duration, but the protein loses its effect over time. In order to increase the effect that IFN treatment has long term, pegylated interferon is currently used for treatment. The PEG attachment decreases the speed that IFN is broken down within the body (9).

Current treatment for HCV is a combination of antiviral drugs and PEG-IFN therapy; treatment is specific to the genotype of HCV causing the infection. The first widely used combination treatment for HCV was that of interferon and ribavirin. Ribavirin is a nucleoside analog, which inhibits the synthesis of viral RNA. Recently, direct acting antiviral drugs (DAAs) have been approved for treatment of HCV. These antivirals specifically target different proteins made by hepatitis C genome. For example, simeprevir targets an HCV protease, NS3/4A, and sofosbuvir targets the HCV polymerase, NS5B. Combined DAA treatments target different viral proteins by using two different inhibitors, such as an inhibitor that targets a viral protease and an inhibitor that targets a viral polymerase (5).

Although there are current treatments for hepatitis C virus infection, the side effects of these treatments increase the need for greater understanding of the virus life cycle in order to develop more efficient and less damaging treatment protocols. Interferon and antiviral treatment have
severe side effects such as anemia, fatigue, depression, and flu-like symptoms (6,8). Ribavirin can cause severe hemolytic anemia and simeprivir is known to cause photosensitivity (5). A more efficient drug can also decrease the duration of treatment, which currently ranges from 12 to 24 weeks (7). Along with side effects and long duration, current HCV treatments are very expensive.

**Virology**

Hepatitis C virus belongs to the *flaviviridae* family and *hepacivirus* genus. As characteristic of the *flaviviridae* family, HCV depends on humans and other mammals as the host. Specifically, HCV is only found to infect humans and chimpanzees. The virus is enveloped with a positive-sense RNA genome. The genome is composed of about 9.6 kilobases, but encompasses enough variability for 30 different genotypes of the virus. Similar to other viral genomes, the HCV genome is translated into a polypeptide that is cleaved into 10 functional proteins: three structural proteins and seven nonstructural proteins. The structural proteins (core, E1 and E2) are responsible for producing the nucleocapsid and envelope. The nonstructural proteins function in producing viral proteins and completing viral replication. NS5B is an RNA-dependent RNA polymerase, which replicates the RNA genome to produce viral progeny. NS3/4A is responsible for the processing of the polypeptide into the viral proteins and suppression of the host immune response (5).

Six different genotypes of the hepatitis C virus have been identified in patients. The molecular differences within a genotype are associated with geographic locations, response to treatment and impact of the virus. Genotype 1 is the most prominent globally. Genotypes 4 and 5 are typically diagnosed in lower-income countries. The treatment protocol is based on the genotype of HCV causing infection. For example, a protease inhibitor is not used for genotype 4 treatment but is used for genotype 1 (7).

**Antiviral Response**

The immune system has mechanisms to inhibit progression of viral infection within the body. Specifically, the innate immune response is composed of cells that kill virally-infected cells nonspecifically and the adaptive immune response generates cells against the antigen of a virus. Natural killer cells recognize changes on the surface of infected cells and release their granules onto
these cells inducing the cells to undergo apoptosis. Cytotoxic T cells of the adaptive immune response reach the site of infection days after NK cells arrive and also kill remaining infected cells.

Prior to the death of infected cells, an infected cell secretes interferons to activate the host response against a virus. Pathogens within a cell are recognized by a variety of receptors, known as pattern recognition receptors (PRR) that activate signaling pathways leading to downstream effects such as activation of antiviral gene expression. RIG-I is a PRR that recognizes tri-phosphorylated RNA characteristic of many RNA viral genomes. When a RIG-I-like receptor recognizes viral RNA, it binds to and induces the activation of a mitochondrial antiviral signaling protein, MAVS. The dimerization of MAVS leads to the activation of signaling proteins such as TBK, TAB and IKK and ultimately leads to the activation of proteins NFκB and IRF-3. IRF-3 and NFκB activation stimulates type-1 interferon production and NFκB activation stimulates secretion of inflammatory cytokines (Figure 1). Viral infection stimulates the transcription of IFN-β, which increases the infected cells host defenses and prepares neighboring cells for potential viral infection by turning on other interferon sensitive genes (ISG). Neighboring cells will thus be prepared to inhibit viral infection and replication, and NK cells will be stimulated to kill infected cells (13).

**Interference with the Anti-Viral Response by Virus**

In order to promote viral reproductive success, many viruses have evolved mechanisms to inhibit the antiviral response. These mechanisms inhibit aspects of the host immune system involved in the elimination of the virus. For example, human cytomegalovirus produces proteins that prevent the expression and function of MHC class I. The differentiation of the killer lymphocytes NK cells and

![Figure 1: Virus induced activation of the RIG-I pathway leading to the activation of IFN-β.](image-url)
killer T cells is dependent on the expression of this molecule. Without MHC class I, viral antigens will not be presented to killer T cells, preventing the targeted death of infected cells.

There are many viruses known to target the activation of interferon-β gene expression, within the antiviral response. Vaccinia virus prevents NFκB activation and blocks cytokines by producing inactivating binding proteins. The RIG-I protein recognizes dsRNA and activates IRF-3 and NFκB, and this promotes the transcription of IFN-β. Toscana virus produces a protein that interacts with RIG-I and promotes its degradation, preventing the interferon response. Influenza and polio also target upstream proteins that activate IFN-β expression (4). HCV is also known to inhibit the antiviral response through NS34/A and NS5A proteins. NS3/4A cleaves the MAVS protein, a protein that results in the activation of NFκB and IRF-3 activation (16).

**NS5A**

NS5A is one of the nonstructural proteins of HCV that is known to inhibit the antiviral response by decreasing the interferon response. Although not all functions of the protein have been determined, it does have a role in viral replication and interaction with host cell proteins. There are two forms of the NS5A protein dependent on phosphorylation. Basal phosphorylation produces the 56 kDa form while hyperphosphorylation produces the 58 kDa form. Although the purpose of the two forms are not explicitly elucidated, it is predicted that the two forms play a role in different phases of the viral lifecycle. NS5A interacts with many different cellular and viral proteins, including cellular transcription factors, cell cycle control proteins and HCV polymerase. NS5A is known to decrease the antiviral response by interfering with IFN. The exact mechanism is unknown, although interactions with signaling kinases and antiviral proteins associated with IFN are known (16).

**Purpose and Research Questions**

The purpose of this research was to evaluate the effect of HCV protein NS5A on the interferon response. NS5A is known to inhibit the interferon response in order to increase hepatitis C virus replication and production of virus particles. The transcription of interferon beta (IFN-β) is regulated by three transcription factors, ATF-2, NFκB and IRF-3. In order to determine the mechanism that NS5A uses to inhibit interferon, the effect of NS5A on each transcription factor was
evaluated. Transcription factors move into the nucleus, bind to the promoter region of DNA and turn on transcription of a gene. In this case, ATF-2, NFkB and IRF-3 move to the nucleus and bind to the promoter of the IFN-β gene, turning on the expression of the gene. In order to inhibit IFN-β expression, NS5A may prevent one of the transcription factors from translocating to the nucleus to turn on IFN-β expression. This series of experiments focuses on the effect of NS5A on the translocation of the transcription factors acting on IFN-β.

**MATERIALS AND METHODS**

**Cell Culture**

Four different cell lines were used for the experiments. HeLa (human cervical cancer cells), HeLa-NS5A (human cervical cancer cells expressing NS5A-10A protein), HEK293 (human embryonic kidney cells) and MCF-7 (breast cancer cells). Cells were maintained in a 25 cm² cell culture flask with 6 mL of complete DMEM (Sigma-Aldrich®) in a tissue culture incubator in an atmosphere of 5% CO₂ and 95% air. DMEM is composed of 500 mL of Dulbecco’s Modified Eagle Medium, 50 mL of 10% fetal bovine serum, 5 mL of 200 mM L-Glutamine, 5 mL of 100% Penicillin-Streptomycin and 5 mL of 100% Non-essential Amino Acid Solution. When cells were 80% confluent, they were split and 10% were transferred to a new flask. In order to split the cells, the medium was aspirated and cells were washed with 1 mL of 1x Phosphate Buffered Saline (PBS). PBS was aspirated and 1 mL of 0.05% Trypsin (Gibco®) was added to dissociate cells from the flask. The Trypsin and cell mixture was triturated and 0.1 mL of Trypsin with cells was added to a 25 cm² flask with 6 mL of medium. All cell culture protocol was carried out using sterile technique in a biosafety cabinet.

**Cell Plating**

Glass coverslips were prepared for cell plating by being soaked in 1M HCl for 6 hours at 50-60° Celsius. Then the coverslips were washed twice with double distilled water, once in 100% ethanol and allowed to air-dry. Before plating cells, the coverslips were added to a 9 cm² well, rinsed with 0.5 mL of 1x PBS, and left to dry.

![Figure 2: Experiment protocol.](image-url)
Approximately 10,000 cells were plated on each coverslip per experiment. After following the same procedure for splitting the cells, 3 mL of medium was added to the cells in 0.9 mL of Trypsin. Cells were counted with a hemocytometer to determine the amount of solution that contained 10,000 cells. This amount was combined with 0.5 mL of medium and placed on a coverslip within a well. Cells were allowed to settle on coverslips for 30 minutes in the tissue culture incubator, and then 2 mL of medium was added to the well. Plated cells were placed in tissue culture incubator for 24 hours before continuing the experiment.

For an experiment, cells were always plated on day one. The following days varied depending on the experiment. If transfection of NS3/4A via plasmid vectors were needed, that would occur 24 hours post plating and infection 48 hours post plating. If the experiment did not call for transfection, infection with Sendai Virus would occur 24 hours post plating. About 24 hours after infection, coverslips were stained for primary and secondary antibodies (Figure 2). All cell lines follow the same protocol during experiments.

**Transfection**

In order for cells to express the HCV protein NS3/4A, cells were transfected with a plasmid containing the NS3/4A gene. To transfect, a solution of DNA and Lyovec was made. Under the hood, 100 μL (per well) of Lyovec was added to 3 μg (per well) of DNA and the solution was mixed. After 20 minutes incubation at room temperature, 100 μL of the solution was added to each well dropwise all over the coverslips 24 hours after plating. The cells were placed back in the tissue culture incubator for 24 hours before the next step of the experiment.

**Infection with Sendai Virus**

Cells were plated 24 hours before the infection with a nonpathogenic RNA virus, *Sendai Virus* (SV). Depending on the experiment, 5 to 40 μL of SV (4000 HAU/mL) was added to a well containing a coverslip, cells and medium. The well was slightly rocked in order to evenly distribute the virus particles. Infected cells were placed back in the tissue culture incubator for a desired amount of time before immunofluorescent protocol.

**Immunofluorescence**

![Figure 3](Reference 17) Antibody binding used for immunofluorescence.
Immunofluorescence utilizes primary and secondary antibody binding. The primary antibody specifically targets the protein, while the secondary antibody targets the primary antibody. In my experiments, the primary antibody (Santa Cruz Biotechnology) either targets a transcription factor (ATF-2, NFκB, or IRF-3), a viral protein (NS5A or NS3/4A), or virus (SV). The secondary antibodies (Life Technologies) are conjugated with Alexa Fluor 488 or 546 fluorophores used to differentiate the location of an antibody under a confocal microscope (Figure 3). All transcription factors utilize the secondary antibodies with a 488nm excitation fluorophore. The viral proteins and virus bind to a secondary antibody with a 546nm fluorophore. The two different fluorophores (488nm or 546nm) used in each experiment identifies the subcellular location of two different proteins per experiment. Primary antibodies and secondary antibodies were diluted in 1x PBS by a 1/250 ratio and 1/500 ratio, respectively; 0.5 mL of PBS and antibody solution was added to coat each coverslip.

After cells were plated, transfected and/or infected, they were stained for the protein of interest. Medium was removed from the wells and coverslips were washed with 0.5 mL of PBS. PBS was removed from cells and 0.5 mL of 4% paraformaldehyde was added to the cells. After 30 minutes, the paraformaldehyde was removed and 0.5 mL of 10% Triton was added for 15 minutes. The triton was then removed, 0.5 mL of 10% calf serum in PBS was added to each coverslip and cells were allowed to soak for one hour. Calf serum was removed and 0.5 mL of the primary antibody solution was added for one hour. The primary antibodies were removed, and the coverslips were washed three times with 0.5 mL of 0.5% Tween 20 (Sigma-Aldrich®) in PBS. Then, the 0.5 mL of the secondary antibody solution was added to the cells for one hour. The secondary antibodies were removed and the coverslips underwent the Tween 20 wash again.

The coverslips were mounted with Fluoromount-G® (Southern BioTec) on glass slides after being washed with Tween 20. One small drop of mounting medium was added to a glass slide and the coverslips were lowered cell face down on top of the mounting medium slowly and at an angle to prevent the formation of air bubbles. The coverslips were then fixed to the glass slide with nail polish along all four edges. Prepared glass slides were stored in darkness to prevent photo bleaching of the antibodies. Glass slides were viewed under a Leica 500 Confocal microscope at an excitation
wavelength of 488nm and 546 nm. The locations of desired proteins were determined by the fluorescence of secondary antibodies within the cell.

RESULTS

The effect of NSSA on Sendai Virus-mediated translocation of ATF-2

ATF-2 is a transcription factor possibly inhibited by the NSSA protein of HCV in order to inhibit viral infection. To test the effects of NSSA on ATF-2 translocation, a control was established. HeLa cells, 293HEK cells, and MCF-7 cells were used to identify the best cell type for immunofluorescence experiments. HeLa cells were plated in two wells with 10,000 cells per well. After 24 hours, one of the coverslips was infected with 10 μL of Sendai virus. Then, all coverslips were stained for ATF-2 24 hours after infection (Figure 4). This experiment was repeated on HEK293 cells and MCF-7 cells. The HeLa cell line was selected for future experiments because the cells adhere well to the coverslips and have an easily identifiable morphology with clearly defined nucleus and cytoplasm. In contrast, the 293HEK and MCF-7 cell lines did not adhere well to the coverslips preventing adequate evaluation of nuclear versus cytoplasmic localization. These cells were also stained with an antibody against Sendai Virus (Figures 5 and 6). Interestingly, ATF-2 was localized in the nucleus pre- and post-infection. It appears that ATF-2 does not translocate to the nucleus after viral infection; it is constitutively present in the nucleus.

![Figure 4: Cells stained for ATF-2 (green). Red arrow represents the nucleus and blue arrow represents the cytoplasm.](image-url)
The effect of NS5A on ATF-2 translocation was tested using the HeLa-NS5A cell line, which expresses the viral protein NS5A. HeLa-NS5A cells were plated on coverslips at a density of 10,000 cells per well in two wells. 24 hours after plating, one well was infected with 20 μL of Sendai Virus. 24 hours post viral infection, the coverslips were stained with a primary antibody against ATF-2 and a secondary antibody against the primary antibody. Both groups of cells display nuclear localization of ATF-2 (Figure 7), which is consistent with our previous experiments. Therefore, NS5A does not have an effect on the location of ATF-2 in infected cells.
**SV-mediated translocation of NFκB into the nucleus**

NFκB is another transcription factor possibly targeted by NS5A. First, the optimal conditions for NFκB translocation were determined. The translocation of NFκB in response to virus was examined by varying the amount of virus infecting the cell (the multiplicity of infection, MOI), or the duration of viral infection. An experiment was performed to test the effect of the MOI used for infection on NFκB translocation. HeLa cells were plated in three wells with 10,000 cells per well. Cells were infected with either 0 µL, 10 µL or 20 µL of Sendai Virus (4000 HAU/mL). Cells were infected 24 hours after being plated, and then were stained for NFκB 24 hours post infection. Cells infected with virus show nuclear NFκB, and increasing the amount of virus did increase the amount of nuclear NFκB (Figure 8 and 9).
The next experiment tested the duration of virus infection on NFκB location. Cells were infected with 10 μL of virus 2 hours, 4 hours, 6 hours or 24 hours before they were fixed and stained for NFκB. A control group of uninfected cells were also stained for NFκB. The cells infected with virus 24 hours showed the highest amount of NFκB nuclear localization. NFκB does move from the cytoplasm to the nucleus after infection with Sendai virus (Figure 10).

The effect of NS5A on TNF-α induced translocation of NFκB

TNF-α is known to induce NFκB activity through a different pathway than a virus induces NFκB activity (Figure 14). In order to evaluate if NS5A causes global inhibition of NFκB, the effect of NS5A on TNF-α-induced NFκB translocation was tested. For the control, HeLa cells were plated 10,000 cells per well onto two wells. After 23 hours, one μL of TNF-α (10 ng/mL) was added to one well. One hour later, both wells were stained with antibodies for NFκB. This experiment shows that
TNF-\(\alpha\) induces NF\(\kappa\)B translocation into the nucleus (Figures 11a and 11b). In comparison to previous NF\(\kappa\)B translocation experiments, the response of NF\(\kappa\)B by TNF-\(\alpha\) is much stronger than the response to SV infection (Figure 8); the concentration of NF\(\kappa\)B in the nucleus is greater under TNF-\(\alpha\) conditions than viral infection. Two wells were plated with HeLa-NS5A cells, and the same experiment was performed. This experiment yielded similar results to the experiment performed on HeLa cells. TNF-\(\alpha\) induces nuclear translocation of NF\(\kappa\)B in HeLa cells and HeLa-NS5A cells (Figure 11c and 11d).

**Figure 11:** Cells stained for NF\(\kappa\)B (red in images A and B, green in image C and D).

The effects of NS5A and NS3/4A on SV-mediated translocation of NF\(\kappa\)B
HeLa-NS5A cells were utilized to test the effect of HCV protein NS5A on NFκB translocation after viral infection. HeLa cells and HeLa-NS5A cells were plated in four wells. After a 24-hour, one well of each cell type was infected with 20 microliters of Sendai virus. After 24 hours of infection, cells were stained with antibodies. The HeLa-NS5A cells were stained for NFκB and NS5A, with corresponding secondary antibodies to allow for fluorescence. HeLa cells and HeLa-NS5A cells without viral infection have cytoplasmic localization of NFκB. After viral infection, HeLa cells show nuclear localization of NFκB, while it remains in the cytoplasm in HeLa-NS5A cells (Figure 12).

![Figure 12: Cells stained for NFκB (red) and NS5A (green).](image)

![Figure 13: Cells stained for NFκB (red) and NS3/4A (green).](image)
NS3/4A is another HCV protein that has been shown to inhibit the activation of IFN-β gene expression. In order to analyze the effect that NS3/4A has on NFκB translocation, a similar experiment was performed on HeLa cells transfected with an expression vector for NS3/4A. Four wells of HeLa cells were plated 10,000 cells per well. After 24 hours, two wells of HeLa cells were transfected with a NS3/4A plasmid. After twenty-four hours post transfection, one well with HeLa cells and one well with HeLa-NS3/4A cells were infected with 20 μL of Sendai virus. After 24 hours, cells were stained with antibodies. Consistent with prior experiments, the control group, HeLa cells, showed NFκB translocation to the nucleus after SV infection. The HeLa cells expressing NS3/4A did not show this translocation. HeLa-NS3/4A showed NFκB localized to the cytoplasm in uninfected and infected cells (Figure 13).

The effects of NS5A and NS3/4a on SV-mediated translocation of IRF-3

IRF-3 is the third transcription factor required for interferon-beta expression that might be inhibited by NS5A. To test NS5A inhibition of IRF-3, another translocation experiment was performed on HeLa cells. The control group consisted of HeLa cells not expressing the proteins NS5A and NS3/4A. Twenty-four hours after infection with Sendai virus, cells were stained with antibodies for IRF-3. IRF-3 is found in the cytoplasm prior to viral infection and moves to the nucleus after viral infection in HeLa cells (Figure 14a and 14b).

The same experiment was repeated on two groups of HeLa cells, one expressing viral protein NS5A and the other expressing viral protein NS3/4A. A HeLa-NS5A cell line was used to analyze the effect of NS5A on the translocation of IRF-3. HeLa-NS5A cells were plated in two wells, after twenty-four hours one well was infected with SV and then all wells were stained with antibodies twenty-four hours after infection. The cells were stained with antibodies for IRF-3 and NS5A. This experiment supported the hypothesis that NS5A does not inhibit IRF-3 translocation. In HeLa-NS5A cells, IRF-3 localization was similar to HeLa cells. IRF-3 translocated to the nucleus only after viral infection (Figure 14c and 14d).

HeLa cells were transfected with an expression vector containing the NS3/4A gene to test the effect of NS3/4A on SV-mediated IRF-3 translocation. HeLa cells were transfected with NS3/4A, following which one well was infected with SV. All wells were stained with antibodies forty-eight
hours after transfection. The antibodies utilized in this experiment were for IRF-3 and NS3/4A. IRF-3 localization was cytoplasmic in non-infected cells and nuclear in infected cells. The amount of IRF-3 in the nucleus is substantially less in NS3/4A transfected HeLa cells than in non-transfected HeLa cells (Figure 14e and 14f).

DISCUSSION

In order to analyze the inhibition of IFN-β by NS5A, the influence of NS5A on three transcription factors were analyzed. In the experiments described above, ATF-2 consistently showed
nuclear localization regardless of whether the cell was infected with virus or not. Transcription factors typically translocate into the nucleus, bind to a specific promoter, and turn on the expression of the downstream gene. As a transcription factor of IFN-β, which is expressed in response to viral infection, ATF-2 would be expected to translocate to the nucleus under virus influence. The nuclear localization of ATF-2 was not altered by viral infection, nor by the presence of NS5A. ATF-2 also plays an important role in environmental stress, which the cells might have experienced during the plating process. Cell death in response to stress is regulated by ATF-2; the protein stimulates mitochondrial leakage leading to death. ATF-2 nuclear localization might prevent interaction with the mitochondria and prevent this mechanism.

During the ATF-2 translocation experiments, different cell types were used to identify the one that would work best for the subcellular localization experiments. Comparing the results between MCF-7, HEK293 and HeLa cells, it was determined that HeLa cells were the most efficient in fluorescent localization experiments and were used in all future experiments. HeLa cells adhered the best to the coverslips, and showed a clear morphological distinction between the nucleus of the cell compared to the cytoplasm.

After concluding that ATF-2 was constitutively localized to the nucleus, experiments were performed on NFκB. NFκB showed translocation in HeLa cells under the influence of Sendai Virus. Cells that were infected with 20 μL of SV for twenty-four hours showed consistent nuclear translocation, and therefore these conditions were used in the remainder of experiments. TNF-α also showed nuclear translocation in HeLa cells. HCV NS5A, via HeLa-NS5A cell line, inhibited virus-mediated NFκB translocation, but did not inhibit TNF-α induced NFκB translocation. NFκB is activated in two different pathways, one signaled by viral infection and the other signaled by cytokines binding to a receptor. One possible interpretation for the inability of NSSA to inhibit TNF-alpha induced translocation of NFκB is that NSSA targets the SV-mediated RIG-I signaling pathway of NFκB translocation and not the TNF-alpha.

**Figure 14:** TNF-α activates NFκB translocation.
induced signaling pathway (Figure 14). This data supports that the inhibition of NFκB by NS5A is upstream of IKK activating NFκB by degrading IKB. This part of the pathway occurs in the activation by TNF-α and virus infection. NS5A may inhibit NFκB upstream of IKK activation and future experiments on the effect of NS5A on the RIG-I/NκB pathway could result in insight to the mechanism of NS5A inhibition (Refer back to Figure 1). NS5A did not inhibit SV-mediated translocation of IRF-3, supporting the hypothesis that NS5A decreases production of IFN-β by inhibiting NFκB.

NS3/4A is a known inhibitor of IRF-3, and was shown to also inhibit NFκB. The NS3/4A experiments lacked consistency in inhibiting IRF-3 translocation. The experiments utilized an NS3/4A expression vector that transiently expresses the gene instead of a NS3/4A cell line, which constitutively expresses a gene. Unlike transfection and transient expression, maintaining a cell line in the presence of an antibiotic ensures that all cells are expressing the NS3/4A protein at a high level. Future analysis of the effect of NS3/4A on NFκB and IRF-3 translocation could utilize a cell line for more accurate results.

The experiments utilized 488 nm and 546 nm wavelength fluorophores to differentiate between different proteins within the cell. This limited each experiment to two antibodies, and thus the analysis of only two antigens. In one experiment, a cell could be infected with virus, expressing a virus protein and analyzed for the location of a transcription factor. Using three antibodies could identify location of the transcription factor, as well as ensure that the cell is infected with virus (by staining for viral antigens) and is expressing the virus protein (e.g., NS5A) improving the accuracy of the results.

The objectivity of the experiments would improve by analyzing the fluorescence through other techniques. Quantitatively analyzing the amount of cells with cytoplasmic localization of a protein compared to nuclear localization of the same protein could increase the credibility of analysis. This ratio could be compared between different variables. The fluorescence between the cytoplasm and the nucleus is not always easily differentiated; comparing a ratio between the fluorescence in the nucleus compared to fluorescence in the whole cell (n/(n+c)) would provide objective quantitative data.
This data supports our hypothesis that NS5A has an inhibitory effect on the transcription factor NFKB, and therefore future experiments should focus on the analysis of the interaction of NS5A and NFKB to specifically determine what component of the NFKB signaling pathway is specifically inhibited. NS5A 10A was the mutant form of the protein used in this research; a next step would interpret the effects of another NS5A mutant on NFKB translocation. With the NFKB activation pathway being well known, binding studies of NS5A with NFKB and upstream signaling proteins, such as IKK and IKBα, could be analyzed. This would explain exactly how NS5A inhibits NFKB translocation. NS5A is used as a drug target for patients infected with HCV; it is known that NS5A inhibits the antiviral response; targeting this protein could allow proper activation of this response. Another possible drug target could be to up-regulate NFKB in infected cells in order to combat the inhibitory effects of NS5A.

CONCLUSION

This series of experiments analyzed localization of the three transcription factors of IFN-β. The location of ATF-2, NFKB and IRF-3 within a cell was analyzed after virus infection. ATF-2 is constitutively found in the nucleus and did not respond to viral infection. This was consistent in all four cell-types used, which suggests that viral infection does not affect the location of ATF-2 within cells and NS5A has no effect on the location of ATF-2. To analyze NFKB virus mediated translocation, cells were treated 20 μL of Sendai virus for twenty-four hours. NFKB translocated to the nucleus after virus infection. HeLa-NS5A cells did not exhibit this translocation, supporting the hypothesis that HCV NS5A protein inhibits NFKB translocation to the nucleus. This could be the mechanism used by HCV to inhibit the expression of the IFN-β gene and consequently the antiviral response. TNF-alpha is another inducer of NFKB translocation. TNF-alpha-mediated translocation of NFKB was not inhibited by the NS5A protein. NFKB translocates to the nucleus in response to different pathways, one being virus mediated (to turn on transcription of IFN-β) and other being cytokine binding to a receptor. Cells transfected with NS3/4A did not show virus-mediated NFKB translocation, supporting the conclusion that NS3/4A does inhibit this pathway. SV-mediated translocation of IRF-3 in the presence of HCV proteins NS5A and NS3/4A was examined. The data reiterated that SV infection
stimulates IRF-3 translocation. In the experiments described above, NS5A did not inhibit the nuclear translocation of IRF-3, while NS3/4A did.
References


