THE EFFECTS OF HEPATITIS C VIRUS PROTEIN NS5A ON THE INNATE IMMUNE RESPONSE

by

Coralee T. Toal

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THE EFFECTS OF HEPATITIS C VIRUS PROTEIN NS5A ON THE INNATE IMMUNE RESPONSE

Project Approved:

Supervising Professor: Giridhar Akkaraju, Ph.D.

Department of Biology

Michael Chumley, Ph.D.

Department of Biology

Jeff Coffer, Ph.D

Department of Chemistry

ABSTRACT

Hepatitis C Virus (HCV) infects liver cells, and is capable of setting up a chronic infection. HCV is a single-stranded, enveloped virus that has a genome that codes for 10 structural and non-structural proteins. Some of these proteins serve dual functions in the virus life cycle and in evading the host immune system. One of the antiviral pathways targeted by the HCV is the interferon-beta (IFN-b) production pathway of the innate immune system. Production of IFN-β alerts an infected cell and the surrounding cells to the presence of a pathogen and results in the production of antiviral proteins and the establishment of an antiviral state in the cell.

To be effective, transcription of IFN-β requires three transcription factors: IRF-3, NF-κB, and ATF-2. The focus of our research is to understand how an HCV non-structural protein NS5A, affects the activation of the IFN-β gene and the production of the interferon protein. Studies that a mutation in NS5A can inhibit activation of IFN-β gene. It is our hypothesis that HCV Non-structural protein 5A inhibits the expression of IFN-β by blocking nuclear localization of the transcription factors required to transcribe the Interferon-β gene.

To test this hypothesis, 293 HEK cells cells expressing NS5A were treated with Sendai virus. Following infection cells were fixed and stained with antibodies against IRF-3 and NS5A. Fluorescence microscopy was used to reveal the location of the different proteins post infection and to determine whether nuclear translocation was affected by NS5A.

ACKNOWLEDGEMENTS

I am able to submit this thesis thanks to several people and entities. First, I would like to thank Dr. Akkaraju, for giving me an opportunity to learn in the field of research, as well as for teaching and guiding me with support and advice along the way. I would like to thank Dr. Chumley and Dr. Coffer, for serving on my thesis committee and aiding my work with their intelligence and comments. The members of my lab have offered me friendship, collaboration and a helping hand throughout my research, and I am grateful for each of them.

My research has been supported with a grant from the Science and Engineering Research Center (SERC), and afforded me the opportunity to present my work at the Student Research Symposium. I want to thank SERC for their continued support of undergraduate research. I am grateful for TCU and the environment they create where undergraduate research is accessible and encouraged. Lastly, I would not be at this wonderful institution without the love and support of my parents. Thank you, Mom and Dad!

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INTRODUCTION

Hepatitis C Virus (HCV) is a member of the family Flaviviridae and infects liver cells. HCV is a blood borne virus and can be transmitted from mother to child at birth, via sexual partners, or through needle sharing. Before proper screening, HCV was transmitted via contaminated blood transfusions, but today that mode of transmission is very rare. Worldwide, over 170 million people are affected by HCV, which makes it a major public health threat (Horner et. al, 879.) When the virus infects its host in a small percentage of cases it can be resolved spontaneously, but in 70% of patients it establishes a chronic infection. Initial infection is relatively mild (jaundice or minor hepatitis) or completely asymptomatic, but HCV is able to evade the host immune response and establish a long-term infection (Thomas, 850). Long-term chronic infection causes continual destruction and regeneration of liver cells that leads to cirrhosis and an increased risk of cancer (Sumpter et. al, 11591). The continuing damage to the liver cells makes HCV one of the leading causes for liver transplants today (Foy et .al. 1145).

The primary treatment for HCV is Interferon, which activates the host immune system. HCV prevents the production of Interferon so treating with it supplements the host's immune system; however Interferon treatment brings with it all the side effects that are associated with illness like chills and aches. Currently there is no vaccine for HCV as it is a rapidly changing virus. The number one factor to predict how someone will respond to IFN treatment is the viral genotype of their infection (Horner et. al, 883). The virus has evolved many strains that are resistant to current treatments, thus a

better understanding of the viral etiology, how it performs its life cycle, would allow the possibility of more specific and effective treatment (Sumpter et al, 11592).

Hepatitis C virus life cycle:

HCV is an enveloped, single stranded, positive sense RNA virus meaning that once it enters a cell it can immediately the use host machinery to translate its genome. The HCV genome consists of one open reading frame that is 9.6 KB in length which, once it enters the cell, is translated into one large polypeptide chain and that is subsequently cleaved by viral and cellular proteases into 10 proteins, both structural and non-structural. The replication of the HCV genome relies on NS5B that acts as an RNA dependent RNA polymerase, and NS3/NS4A that serves as a protease/helicase (Sumpter et. al, 11591). HCV's viral replication machinery is error prone and this allows for a high replication rate and a genetically diverse range of quasispecies (Sumpter et. al, 11591). The HCV viral life cycle leads to cell destruction, thus causing a continuing cycle of cell regeneration. The increased level of DNA synthesis in chronic HCV infection combined with chronic inflammation is what is thought to lead to the increased risk of hepatocellular carcinoma due to increased opportunities for mutations to accumulate.

Innate immunity:

HCV is able to establish chronic infection by suppressing the host immune system. HCV specifically targets the Interferon-β (IFN-β) pathway of the innate immune response. Production of IFN-β alerts the surrounding cells to the presence of a pathogen and results in the production of antiviral proteins. The production of IFN- β is the result of a pathway that begins with the recognition of a foreign molecular pattern by the host cell. There are many molecular patterns that a cell can respond to collectively known as

Pathogen Associated Molecular Patterns (PAMPs). One example of a PAMP is double-stranded RNA (dsRNA) produced as a consequence of transcription by many viruses. When dsRNA enters a cell it is recognized by an intracellular pattern recognition receptor (PRR), RIG-I. When RIG-I binds to dsRNA it induces a conformational change that exposes its CARD domain (Kaukinen et. al). Exposure of the RIG-I CARD domain leads to the activation of mitochondrial-antiviral signaling protein (MAVS). MAVS also contains a CARD domain which, associates with RIG-I CARD domain and subsequently leads to activation of the IKK pathway (Kaukinen et. al). IKK is a kinase and its phosphorylation cascade ends in the activation and nuclear localization of the transcription factors NF-κB, IRF-3 and ATF-2, which enter the nucleus to turn on transcription of the IFN- β gene and begin the anti-viral response. Transcription of IFN-β requires all three of the above mentioned transcription factors and inhibition of even one of the three will render the gene un-inducible. (Kaukinen et. al.)

Viral countermeasures:

There are several pathways through which HCV inhibits the IFN-β pathway but not all are understood. Of the ten proteins of HCV three are structural in function, and the rest vary from metalloproteinases to an RNA-dependent RNA polymerase. One protein, NS3/4A, a protease and phosphorylation cofactor, is well characterized because of its ability to inhibit the antiviral response. NS3/4A attacks the host immune response by cleaving MAVS off the mitochondria and thereby inhibiting its function by blocking the activation of the signaling complex required to complete the pathway (Kaukinen et. Al).

MAVS is essential to the RIG-I-signaling pathway and the ultimate nuclear localization of NF-κB and IRF-3. Recent studies from our lab and outside sources have shown that certain mutants of HCV non-structural protein 5A (NS5A) (with a single amino acid deletion at position K2040 and titled NS5A-10A in this study) leads to decreased production of IFN-β (Sumpter et al, 11592)(Akkaraju, Unpublished). It is possible that NS5A-10A inhibits the nuclear localization of the transcription factors required for IFN-β production. Through the use of fluorescent antibodies it is possible to view the nuclear localization of these transcription factors following viral infection, and better understand the effect of NS5A on the host immune response. It is my hypothesis that HCV Non-structural protein 5A (NS5A) inhibits the expression of IFN-β by blocking nuclear localization of one or more of the transcription factors required to activate transcription of the Interferon-β gene, thus helping the virus persist in the infected individual.

MATERIALS AND METHODS

Cell Culture:

For initial experiments a line of human embryonic kidney cells, 293 HEK, was cultured. A second line used was 293 HEK NS5A, which are HEK cells that consistently express HCV protein NS5A. In later experiments HeLa cells, a line of cervical cancer cells, were cultured. A line of HeLa NS5A cells expressing the HCV protein were cultured as well. Cells were grown in nutrient rich Modified Eagle's Medium (MEM) (MediaTech) supplemented with fetal bovine serum, L-glutamine, penicillin, and streptomycin, which enhanced cell growth while preventing bacterial infection. Cells expressing NS5A were treated with 1 μL of puromycin to ensure only cells expressing

NS5A survive and grow. 293 HEK and HeLa cells were grown in an incubator at 37 degrees Celsius with an atmosphere of 5% CO_2.

Experimental Set Up:

For each experiment, 35mm wells were used. Cells were plated onto sterile coverslips at the base of each well that would be used later to transfer cells to microscopy slides. Depending on the experiment and cell type, 10,000-20,000 cells were plated on each coverslip. Initially, the cells are added to the coverslip in 0.5 mL of medium to allow proper attachment. The cells are incubated for 20 minutes at this volume. After that time, 1.5 mL of medium is added to the well to allow a proper volume for growth. Cells are allowed to incubate for 24 hours before the next stage of an experiment.

IKBΔN Transfection:

For experiments using IKBΔN as a positive control for a known inhibitor of NFκB nuclear localization following viral infection, HeLa cells were transfected with IKBΔN plasmid. Transfection occurred 24 hours after incubation using Lyovec, a transfection agent, and for each well of cells 100 μL of Lyovec was mixed with 1μg of IKBΔN plasmid. The Lyovec-plasmid mixture was tapped gently and then allowed to incubate at room temperature for 20 minutes. After incubation, the mixture was added to each well, distributed by gentle rocking, and then allowed to incubate for 48 hours at 37 degrees Celsius.

Cell Treatment:

Cells were infected with Sendai Virus (SV, Charles River Labs) to elicit the antiviral response. Sendai Virus is a commonly used research tool as it is safe to humans

but elicits a response very similar to that of HCV. Depending on the cell line and transcription factor being analyzed, different incubation times were used. In 293 cells analyzing intracellular movement of transcription factor IRF-3, cells were treated with 80 μL of SV and allowed to incubate for 16 hours at 37 degrees Celsius. In HeLa cells analyzing intracellular movement of transcription factor NFκB, cells were treated with 80 μL of SV and allowed to incubate for 4 hours at 37 degrees Celsius.

Preparation of Cells for Immunofluorescence:

Following appropriate incubation interval with SV, slides were washed with 0.5 mL of PBS. Then, cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature. Next, cell layers were permeabilized using a solution of 0.5 mL of PBS/0.2% Triton X-100 for 15 minutes. Next, cells were incubated for 1 hour at room temperature in 0.5 mL of a solution of PBS/10% normal goat serum. Cells were washed in PBS following incubation. Then, cells were incubated for 1 hour at room temperature in a 1/500 dilution of the primary antibody in PBS, which was IRF-3 Rabbit polyclonal IgG when tagging IRF-3, NFκB Rabbit polyclonal IgG when tagging NFκB, and Hep C NS5A Mouse monoclonal IgG when tagging NS5A. To ensure removal of all excess primary antibodies, the cells were washed three times with PBS/0.5% Tween-20. The remaining steps were completed in the dark to prevent photo-bleaching of the fluorescent secondary antibody. Then, cells were incubated for 1 hour, at room temperature in a 1/1000 dilution of the secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (H+L) or Alexa Fluor 546 goat anti mouse IgG. Following incubation, cells were washed three times with PBS before being dried and mounted to slides. Tweezers

were used to transfer coverslips to slides, and cover slips were mounted using a drop of Fluoromount-G (Southern Biotech). Coverslips were sealed using nail polish.

Visualization of labeled cells:

Prepared slides were analyzed under an Axiovert 200 Fluorescent Microscope (Zeiss). Fluorescent photos were obtained using an Axiocam MRm (Zeiss) attachment and observed on a computer using the AxioVision 4.6.1.0 program (Zeiss). Fluorescent images were compared to light images of the structure of the cell. The light images helped confirm the location of the cell nucleus. Cell images for experiments analyzing the intracellular movement of IRF-3 were taken at 200X magnification and 1000x magnification using lens oil. Cell images analyzing the nuclear localization of NF-κB were taken at 400X magnification.

RESULTS

Intracellular localization of transcription factor IRF-3:

As mentioned above, HCV protein NS5A has been shown to inhibit the innate immune response by blocking virus infection-induced activation of the IFN-β promoter. To study the intracellular movement of transcription factor IRF-3 following viral infection 293HEK cells were infected with Sendai virus for 16 hours to elicit activation of the host antiviral state. As seen in Figure 2, 293HEK cells expressing HCV protein NS5A show nuclear localization of transcription factor IRF-3 following viral infection, suggesting that NS5A does not inhibit nuclear localization of IRF-3.

In this experiment we fluorescently labeled both IRF-3 and NS5A to observe their intracellular location and potential interaction following viral infection. Figures 3 and 4 show the intracellular location of both IRF-3 and NS5A following viral infection.

In Figure 3, IRF-3 appears to localize to the nucleus following SV infection. In Figure 4, NS5A appears to remain in the cytoplasm following SV infection. In addition to confirming the expression of NS5A in these cells it also suggests that NS5A does not physically interact during viral infection.

Time-course for nuclear localization of NFκB following viral infection:

Our previous experiments indicated that NS5A was not inhibiting the activity of IRF-3, so the ability of NS5A to inhibit the activity of another IFN-β transcription factor, NF-κB, was explored next**.** In experiments attempting to analyze the intracellular movement of transcription factor NF-κB, nuclear localization of NF-κB was carried out in HeLa cells which are a better model to study NF-κB translocation. However, in HeLa cells, nuclear localization of NF-κB was not observed after 16-hour incubation with SV (the time point used to study IRF-3 translocation). To explore the possibility that nuclear localization of NF-κB might be best observed at a different infection interval, a time course was conducted. HeLa cells were infected with SV at interval of: 30min, 1 hour, 2 hours, 4 hours, 8 hours, and 12 hours. The cells were also treated with TNF-α, a cytokine that is known to activate NF-κB, for comparison. TNF-α is involved in many cellular pathways from cell proliferation to apoptosis. TNF-α binds to its receptor, TNF-RI, and can lead to the recruitment of receptor associated factors that cause ubiquitin-mediated degradation of IκB, which binds to NF-κB preventing its nuclear localization (Machuca et al). Although both the TNF-α pathway and virally activated RIG-I pathway lead to the degradation of IκB and the exposure of the nuclear localization signal on NF-κB, they begin with different factors and signal receptors. NF-κB was observed to localize to the nucleus after 30-minute incubation with TNF-α, showing that we were

able to visualize nuclear localization of NF-κB; however since this does not activate the same pathways as viral infection, identifying the specific time needed for infection-induced translocation of NF-κB was important.

Figure 5 shows various examples of cells from the time course. It appears that at 1 hour and 12 hours of SV incubation, no nuclear localization is observed. However, at 4 hours of SV incubation it appears that several cells (25%) show NFκB localizing to the nucleus. Figure 6 shows a graph that quantifies the percent nuclear localization of NFκB in HeLa cells at various intervals of SV incubation. One set of cells was treated with TNF-α at 30 min incubation for comparison. The graph shows that at 4 hours of SV incubation a significant percent (36.08%) of HeLa cells show NFκB localizing to the nucleus. In experiments that followed HeLa cells were infected with SV for 4 hours.

Intracellular localization of transcription factor NFκB:

Since treatment of HeLa cells with SV does not cause nuclear localization of transcription factor NF-κB, in 100% of the cells, results were analyzed in terms of percent nuclear localization of NFκB. To calculate percent nuclear localization in HeLa cells, three different images were taken at 400x magnification. From these pictures the total number of cells was counted, and then the total number of cells exhibiting nuclear localization was counted. Dividing the total cells exhibiting nuclear localization by the total number of cells give percent nuclear localization. To be able to have a better control for nuclear localization of NF-κB in HeLa cells, a dominant negative inhibitor of NF-κB was used. IKBΔN, which is a mutated version of IκB that has two IKK phosphorylation sites deleted and therefore is degradation-resistant, is a known dominant-negative inhibitor of NF-κB nuclear localization (Wilson, 28). In Figure 7, it

appears that several HeLa cells show nuclear localization of NF-κB, whereas HeLa + IKBΔN and HeLa NS5A cells show NF-κB remaining in the cytoplasm. Figure 8 confirms this by quantifying the amount of NF-κB nuclear localization in each cell type, suggesting that NS5A causes similar levels of nuclear localization as the dominant-negative inhibitor of NF-κB nuclear localization, IKBΔN. Conversely, Figure 9 shows results from a duplicated experiment where HeLa cells expressing NS5A do appear to inhibit nuclear localization of NF-κB.

DISCUSSION

Hepatitis C virus has widespread and detrimental effects worldwide due to its ability to establish chronic infection. HCV manages to evade the host immune system long term while causing continual cell destruction. It can replicate quickly thereby incurring mutations that can render it resistant to current treatments and enhance its ability to evade the host immune system. A mutation in HCV non-structural protein 5A (NS5A) has been shown to diminish production of IFN-β, which is an antiviral cytokine produced by the host immune response. IFN-β is the product of the RIG-I pathway that is activated by the presence of viral dsRNA within the cell and leads to the nuclear localization of three transcription factors essential for IFN-β gene expression. The mechanisms by which NS5A diminishes the innate immune response have yet to be characterized. The focus of this research is to explore the possibility that NS5A inhibits the nuclear localization of one or more of these three transcription factors as a mechanism to inhibit host innate immunity.

Although the cells used in the experiments were not infected with actual Hepatitis C Virus, the use of cells that express the protein being studied along with

infection by Sendai virus allows us to study the effect of HCV proteins on the cellular response to viral infection. Experiments analyzing the intracellular movement of IRF-3 following viral infection do not support the hypothesis that NS5A inhibits IRF-3 from localizing to the nucleus. Figures 2 and 3 show IRF-3 entering the nucleus uninhibited by the NS5A being expressed by the cell. Figure 4 confirms that NS5A was being expressed in each cell, and further confirms that NS5A is not inhibiting the nuclear localization of IRF-3 following viral infection.

Exploring the relationship between NS5A and NFκB presented challenges and the SV infection interval that was used to activate IRF-3 was not effective for analyzing the intracellular movement of NFκB. Utilizing a time-course of various infection intervals we discovered that the appropriate incubation time for treatment with SV in order to observe nuclear localization of NFκB in HeLa cells was 4 hours. Although a 4-hour incubation with SV shows the highest level of NFκB nuclear localization, it is not uniform, meaning that only a percentage of nuclear localization can be compared. Figures 8 and 9 show the levels of NFκB nuclear localization in cells expressing NS5A in two separate experiments and, as can be seen, the results are contradictory. Due to the variable nature of these results, repeated experiments are needed to confirm whether NS5A inhibits the nuclear localization of NFκB following viral infection.

The third transcription factor required for Interferon expression, ATF-2, was not explored in these experiments but NS5A may inhibit the activation of the IFN-beta gene by inhibiting the nuclear localization of this factor as well. Alternatively, the mechanism by which NS5A inhibits the production of IFN-β may be at a different point in the antiviral pathway other than the nuclear localization of transcription factors, such as

inhibiting the exposure of the CARD domain after activation of RIG-I or preventing the activation of MAVS. Thus, although these results do not confirm the mechanism by which NS5A is affecting the host immune response, they do eliminate an option and thus focus the efforts of future research.

APPENDIX A: FIGURES

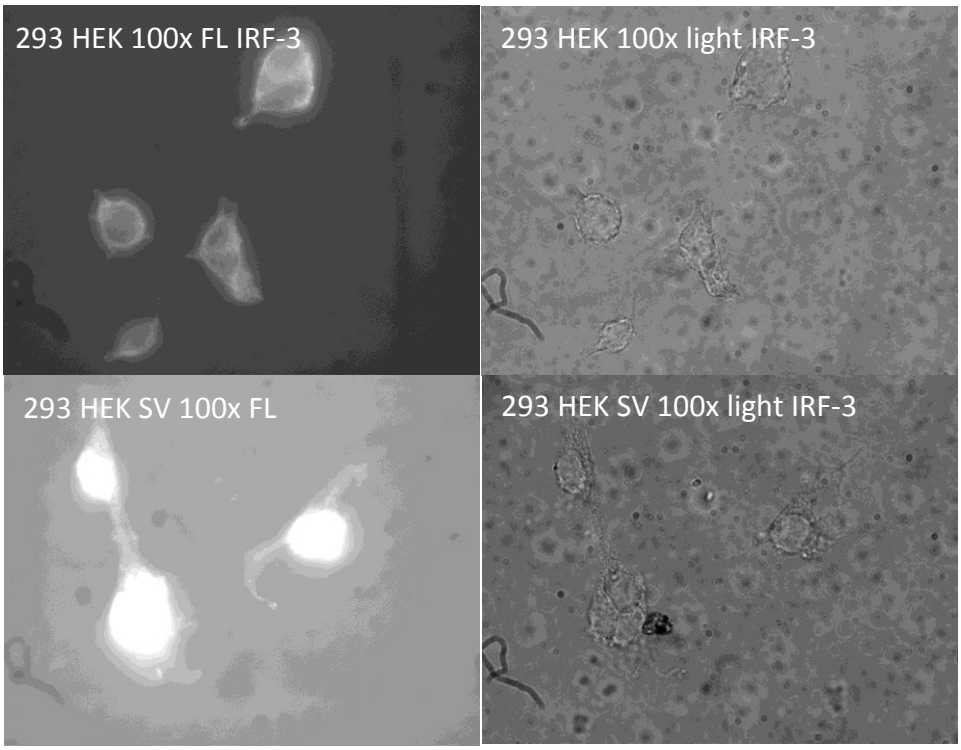

Figure 1: Nuclear localization of IRF-3 in SV-infected 293 HEK cells. immuno-fluorescent molecules attached to transcription factor IRF-3 are excited with 488nm light. In these images IRF-3 appears to move into the nucleus following viral infection in 293 HEK cells. 100x magnification. 10-11-12

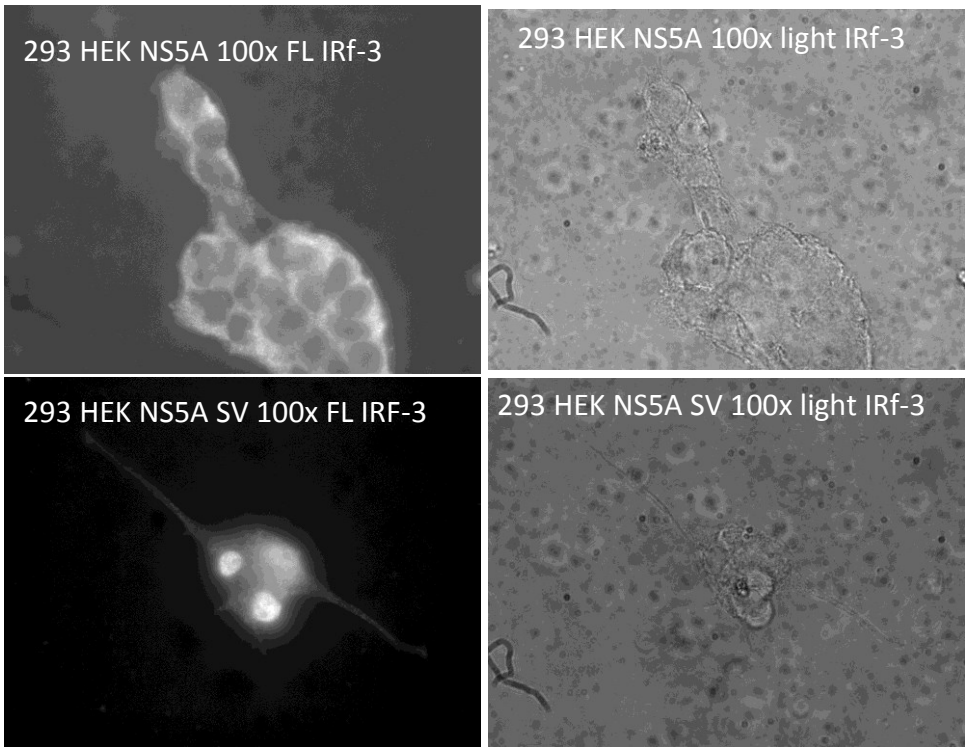

Figure 2: Nuclear localization of IRF-3 in SV-infected 293 HEK cells expressing NS5A. immuno-fluorescent molecules attached to transcription factor IRF-3, excited with 488nm light. In these images IRF-3 appears to move into the nucleus following viral infection in 293 HEK NS5A cells, suggesting that the presence of NS5A does not inhibit the nuclear localization of IRF-3 following viral infection. 100x magnification. 10-11-12.

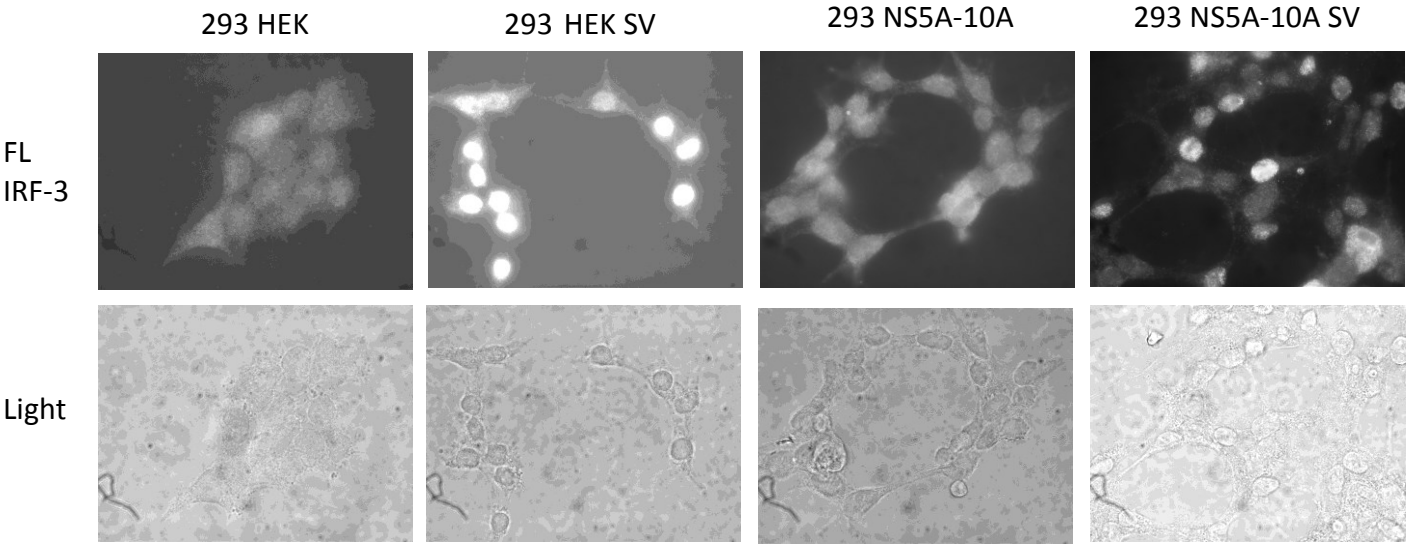

Figure 3: Intracellular localization of IRF-3 in 293 HEK cells and 293 HEK cells expressing NS5A. Immuno-fluorescent antibodies excited with 488nm light attach to transcription factor IRF-3. It appears that IRF-3 is able to localize to the nucleus following viral infection in cells expressing HCV protein NS5A. 100x magnification.

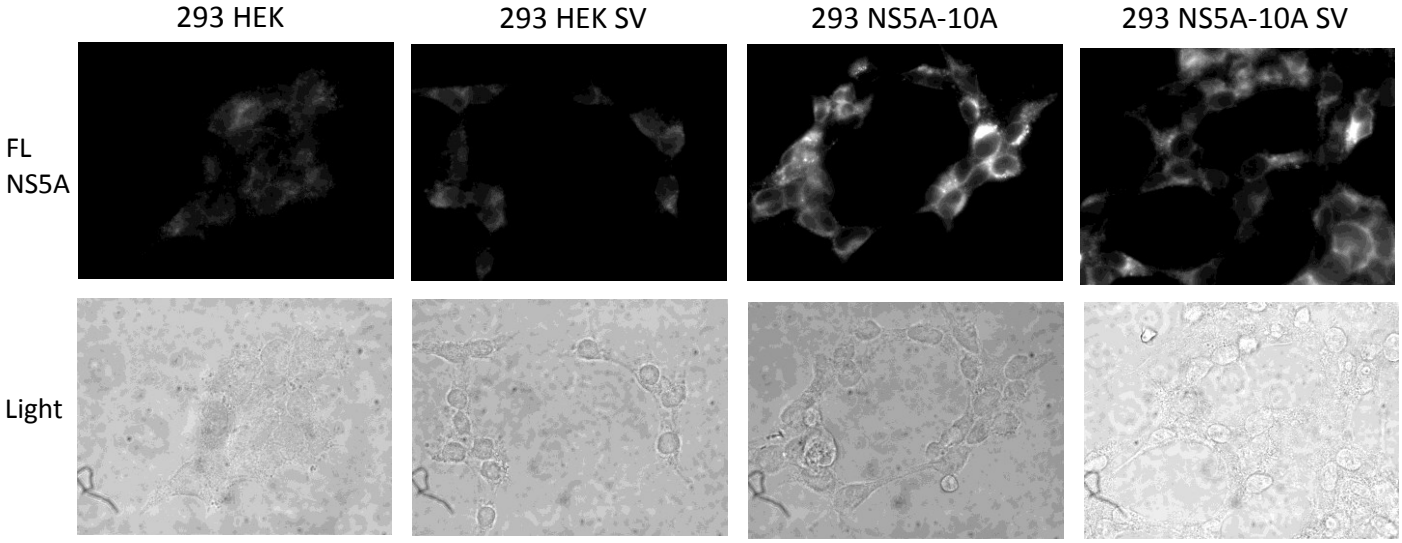

Figure 4: Intracellular localization of NS5A in 293 HEK cells expressing NS5A. immune-fluorescent antibodies excited with 546nm light attach to HCV protein NS5A. It appears that NS5A remains in the cytoplasm following viral infection. 100x magnification.

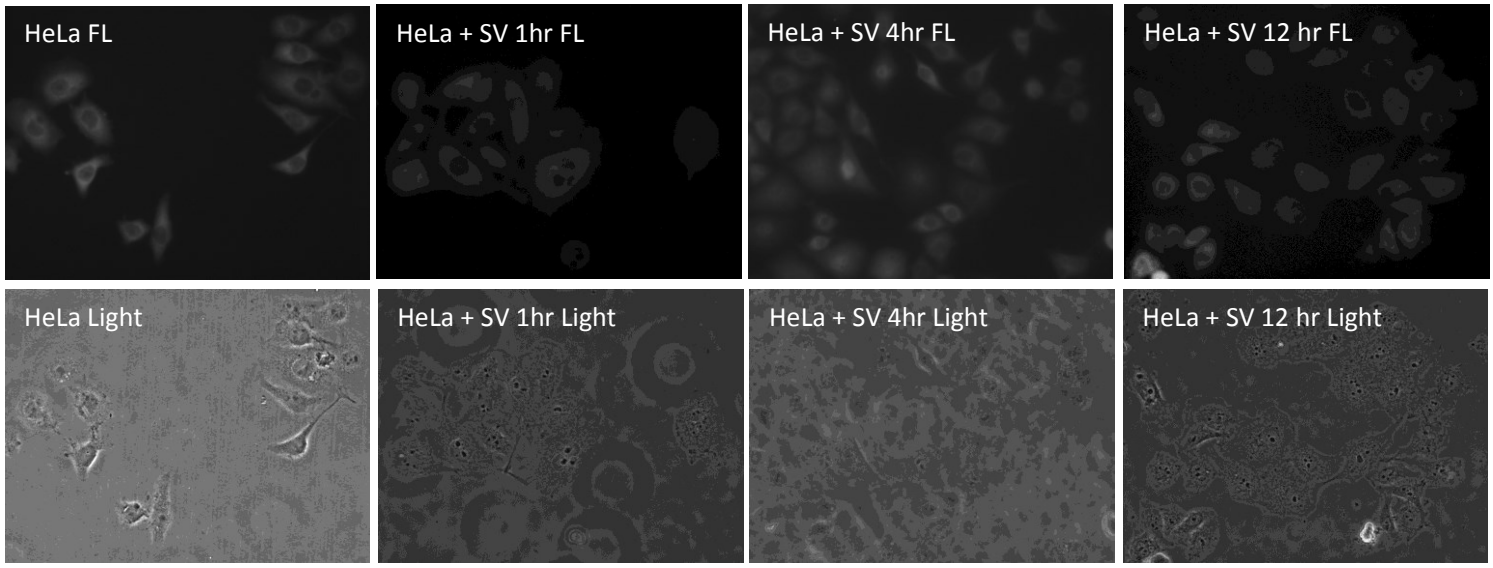

Figure 5: Nuclear localization of NF-κB in SV-infected HeLa cells at varying incubation times. Immuno-fluorescent antibodies excited at 488nm light attached to transcription factor NF-κB. Highest degree of nuclear localization of NF-κB in SV-infected HeLa cells seen at 4 hours. 40x magnification.

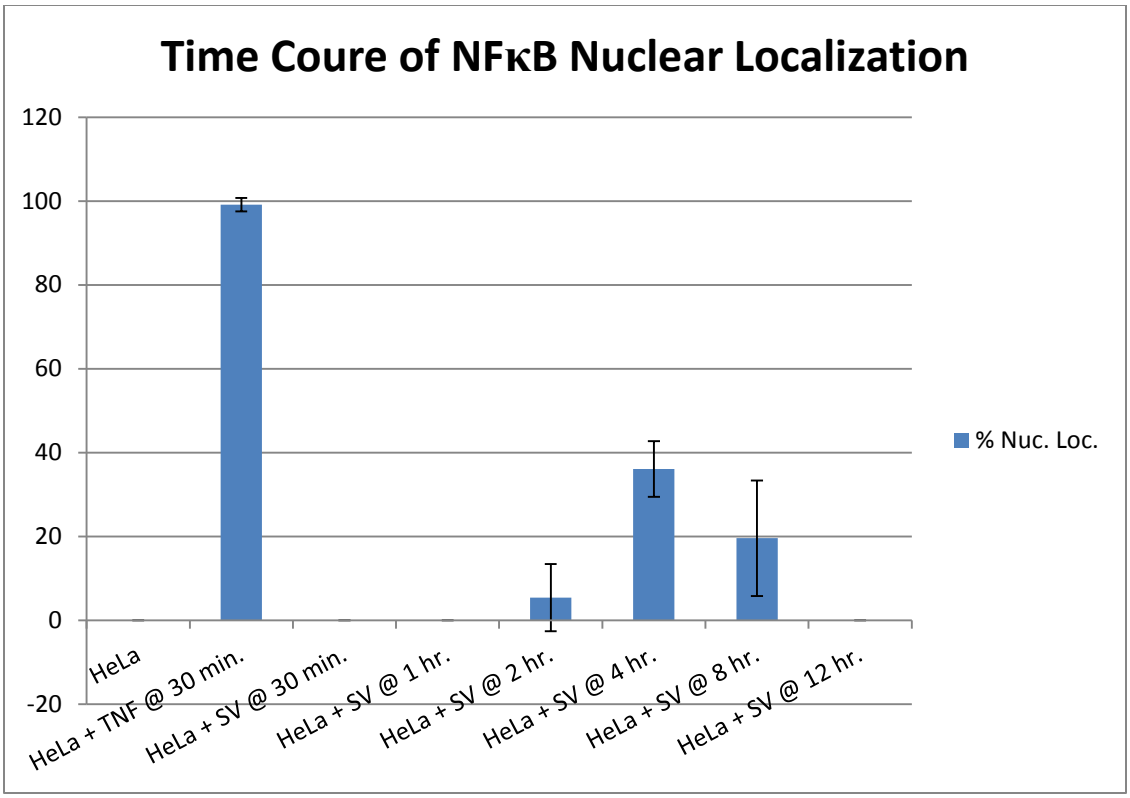

Figure 6: Percent nuclear localization of NF-κB in SV-infected HeLa cells at varying incubation times. It appears that for SV infection, a 4 hr infection interval elicits the greatest amount of nuclear localization of NF-κB.

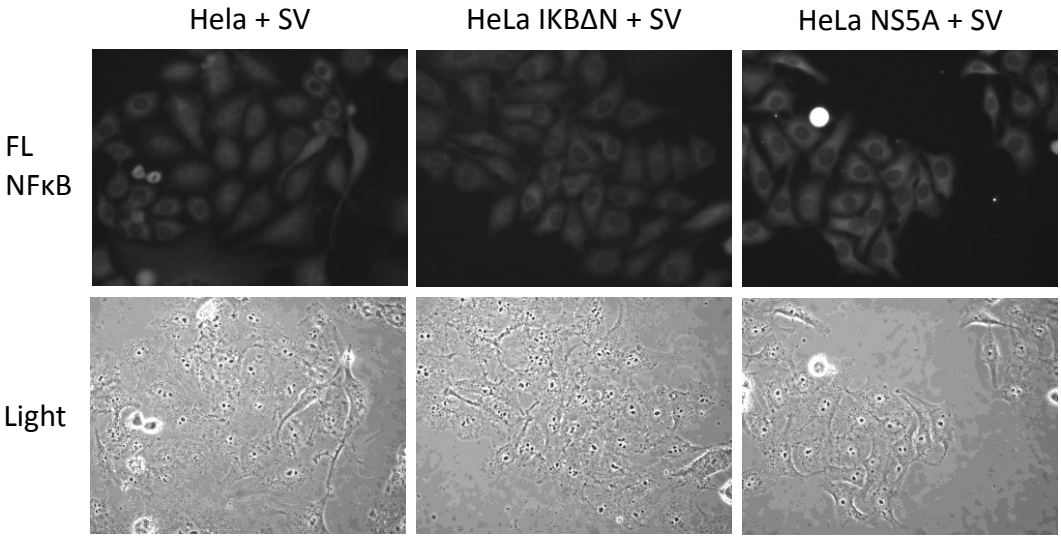

Figure 7: Intracellular localization of NF-κB in HeLa cells, HeLa cells expressing IKBΔN, and HeLa cells expressing NS5A. Immuno-fluorescent antibodies excited with 488nm light attach to NF-κB. It appears that NF-κB is able to localize to the nucleus in several HeLa cells, however in the presence of IKBΔN and NS5A there is less nuclear localization. 40x magnification.

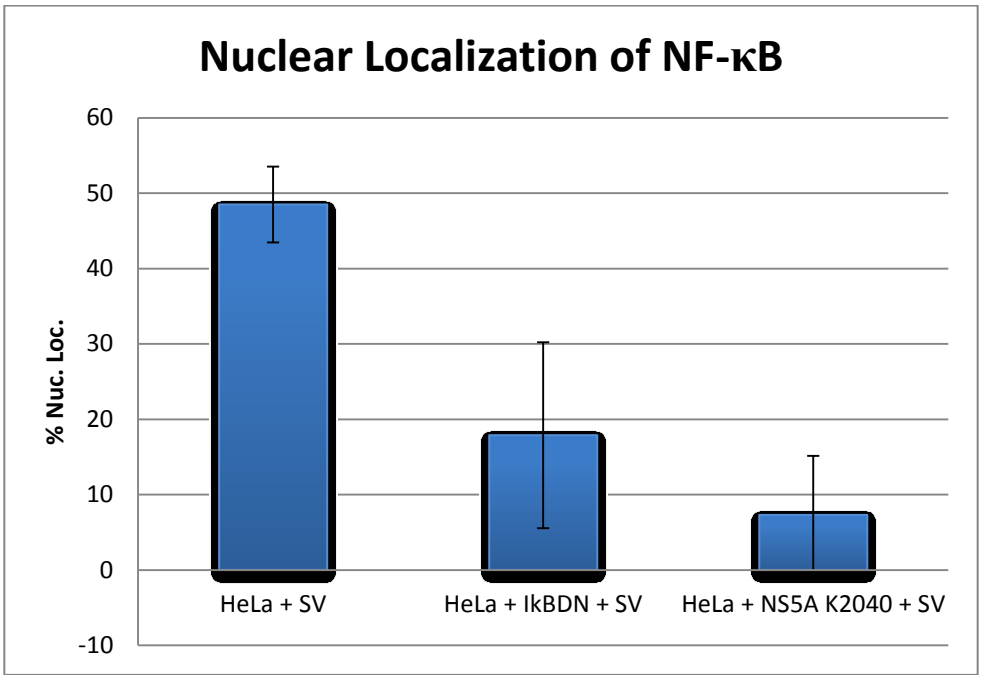

Figure 8: Percent nuclear localization of NF-κB in SV-infected: HeLa cells, HeLa cells expressing IκBΔN, and HeLa cells expressing NS5A. HeLa NS5A cells show a low level of nuclear localization.

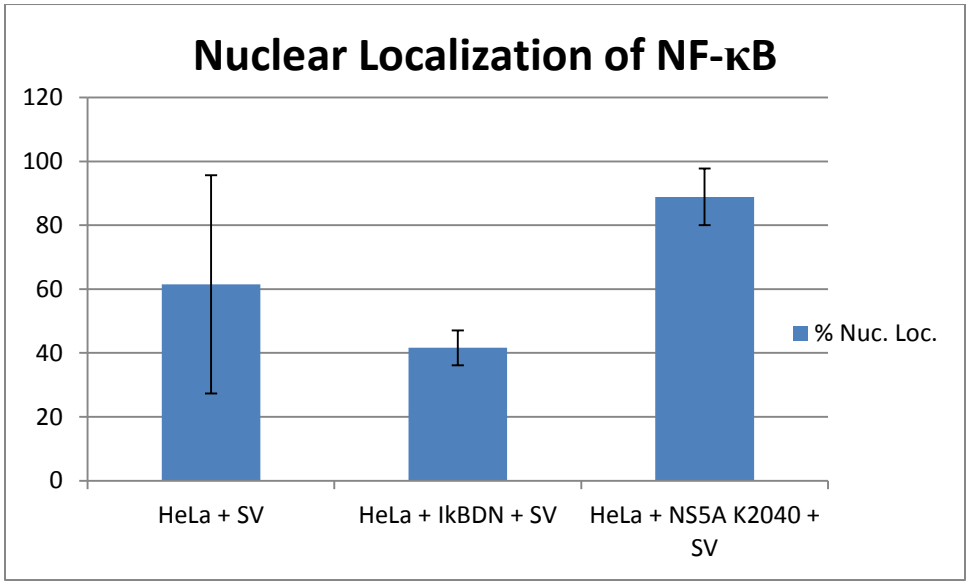

Figure 9: Percent nuclear localization of NF-κB in SV-infected: HeLa cells, HeLa cells expressing IκBΔN, and HeLa cells expressing NS5A. SV-infected HeLa cells expressing NS5A show a high level of nuclear localization.

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