BRIDGING THE GAP:
THE ROLE OF HEPATITIS C VIRUS NONSTRUCTURAL PROTEIN 5A IN THE INHIBITION OF INTRACELLULAR ANTIVIRAL PATHWAYS

by

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To our heavenly Father all things, and all things through Him.

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

It is estimated that over two hundred million people worldwide are infected with hepatitis C virus (HCV), far more than HIV/AIDS, and that number grows by three to four million each year (5). It is a leading cause of cirrhosis, replacement of the liver tissue by fibrosis, or scar tissue, and is consequently the primary reason for liver transplantation in the United States (2, 5).

Infectivity and Treatment

First identified in the 1970's, it was originally called non-A non-B hepatitis until it was re-named 'hepatitis C' in 1989. It is an enveloped, positive single-stranded RNA virus, which means that upon entry into the cell it is capable of being directly translated into a protein. It is a member of the Flaviviridae family which gets its name from another member, yellow fever virus, where *flavus* is Latin for yellow, stemming from the condition known as jaundice. This yellow staining of the skin and deeper tissues is a symptom of both yellow fever virus and hepatitis C virus infection, which lead to liver dysfunction and subsequent increased levels of bilirubin, the yellow breakdown product of heme catabolism, in the blood. Additionally, HCV is the most common chronic blood-borne infection in the United States and can be transmitted in a number of ways including the sharing of drug paraphernalia, especially needles, improper sterilization of body piercing and tattoo equipment, sexual contact, blood transfusion, and organ transplantation. With reliable tests to screen for the virus, the last two modes of transmission have decreased in number. However, since these tests did not become available until the 1990s, those receiving a transfusion beforehand may have been exposed to the virus (1, 2, 3, 5).

The stages of hepatitis C virus infection are divided into acute and chronic. Acute refers to the first six months following infection and is generally asymptomatic, but any symptoms that do occur are generally nonspecific and mild. While infection clearance rates vary from patient to

patient, approximately eighty-five percent of patients will go on to develop chronic hepatitis C infection where the virus persists for more than six months. Liver inflammation due to inflammatory cells in the tissue results in death of the tissue (necrosis) and replacement by scar tissue (fibrosis). Cirrhosis, fibrosis of the liver, is a common cause for liver transplants. In addition, death of hepatocytes, cells in the liver's main tissue, leads to regeneration of the liver by activating the cell cycle in quiescent liver cells. This can increase the chances of errors during DNA replication. In addition, the liver processes many toxins present in the body including possible mutagens. Both of these factors increase the chance of cellular mutation, which can lead to various forms of cancer should this occur in the coding or regulatory regions of a cell cycle regulatory protein (2, 3, 5).

Currently, no vaccine is available for the virus. The treatment is a combination of IFN-α and ribavirin. IFN-α is a protein produced by leukocytes and is mainly involved in the innate immune response against viral infection. Ribavirin is a prodrug that when metabolized resembles purine RNA nucleotides, which interfere with RNA metabolism required for viral genome replication. However, the treatment lasts for extended periods leading to serious side effects, and because the virus is constantly mutating during viral genome replication, resistant strains are predominant. Due to all of these factors, treatment has a very low success rate where 50% of patients don't even respond to the therapy (1, 2, 5).

Hepatitis C Viral Genome

The viral genome consists of a 9.6 kilobase positive single-stranded RNA that codes for a polyprotein consisting of three structural and seven non-structural proteins. The structural proteins are responsible for the infectivity of the virus and encode the capsid and envelope glycoproteins, while the non-structural proteins are responsible for genomic replication and viral

pathogenesis. Two of the non-structural proteins are NS3 and NS5B. NS3 (in combination with its cofactor NS4A) acts as a protease and cleaves the polyprotein into its different segments. NS5B acts as an RNA-dependent RNA polymerase, an enzyme not found in a eukaryotic cell, and is required to make copies of the viral genome. Both are good targets for anti-viral drugs (2).

NS5A is another non-structural protein of HCV. It is known to inhibit RNA-activated protein kinase R (PKR) (3, 4) and block activation of the IFN-β promoter (8). IFN-β, like IFN-α, is a protein that is mainly involved in the innate immune response against viral infection. Unlike IFN-α, which is produced by leukocytes, IFN-β is produced in large amounts by fibroblasts (1). Fibroblasts are activated fibrocytes and play a vital role in wound healing. NS5A blocks activation of the IFN-β promoter, and it is believed that this is through its inhibition of PKR. Also of particular interest is the possible role of NS5A on the deregulation of cell growth and apoptosis and potential role in the development of hepatocellular carcinoma (HCC), or liver cancer.

When looking at NS5A, it's important to note the mutants. The purpose of proofreading during replication is to avoid mutations. HCV doesn't proofread during replication (true of any RNA virus), and constantly mutates. So, in a patient, as a virus replicates, it generates mutants which gives rise to multiple quasispecies or strains. These strains have different properties (1, 5). Two mutants that were generated in a cell culture model of viral replication were used. The only mutation found in these strains was in the NS5A gene, which accounts for the different phenotypes. With H27 [L2198S substitution], replication occurs at a lower rate in cells. With 10A [K2040 deletion], replication occurs at an increased rate. The 10A mutant was used because it is better able to suppress the antiviral response and allow the genome to replicate at a

higher level (14). Additionally, previous research has shown that wild-type (WT) NS5A behaves more like 10A than H27 (Molli Crenshaw and Giri Akkaraju, unpublished data).

Host Immune Response and Interference by NS5A

PKR is a protein in the immune response that protects against viral infections. It does so by being activated by double-stranded RNA which is not present in healthy eukaryotic cells but is a result of viral infection as many viral RNAs fold upon themselves to create dsRNA. Binding of dsRNA causes dimerization and autophosphorylation of PKR. Activated PKR inactivates eukaryotic translation initiation factor 2α (eIF2α), a cellular protein required by ribosomes to recruit the initiator tRNA to the 40S ribosomal subunit leading to the initiation of protein synthesis. The subsequent downregulation of translation will inhibit protein synthesis which could induce apoptosis. In addition to its contributions to the antiviral response, PKR has been shown to be involved in apoptosis and act as a tumor suppressor (3, 4).

The current research is examining the role of NS5A in relation to interaction with the host innate immune response. Unlike the adaptive, or acquired, immune response, the innate pathways are not pathogen specific nor provide immunity (memory) to the host from any future infection. Many components comprise the innate immune system including the activation of natural killer (NK) cells, which are nonspecific cytotoxic lymphocytes, the production of complement proteins, and the induction of apoptosis. An additional component of interest is the induced secretion of cytokines, including interferons and interleukins, which serve as protein cell immunomodulating agents (1, 2, 10).

The cytokine family contains over thirty members that respond to different stimuli from infection. They are produced over a very brief time span following induction due to the possible negative effects of a prolonged presence of some of the protein agents including chronic

inflammation. The cytokines target cell surface receptors and bind them with high affinity, which initiates a series of intracellular interactions that ultimately leads to altered gene expression. This in turn will lead to changes in the differentiation and proliferation of the target cells. Cytokines can be divided into groups based on their function and therapeutic targets. Given the focus on HCV, the group of interest consists of antiviral cytokines called interferons (1, 10).

Interferons (IFNs) function to induce resistance in cells to infection. This group of cytokines may be further divided into two subgroups, IFN I and IFN II. While both are involved in defense against bacteria, IFN I is more involved in viral defense than IFN II. Also, while IFN II only contains one member, IFN-γ, the IFN I family consists of multiple members, including two main cytokines, IFN-α and IFN-β. Induction of interferons can be via viral infection and subsequent dsRNA exposure or the fellow cytokine tumor necrosis factor-alpha (TNF-α), an inflammatory cytokine. While IFN-α and IFN-β are capable of being produced in almost any cell type, IFN-γ, following viral infection, is produced primarily by NK cells and T cells. In humans, thirteen genes encode for IFN-α while there is only one for IFN-β. Upstream of the IFN-β promoter is an enhancer region of the gene to which multiple transcription factors bind – nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), interferon regulatory factor-3 (IRF-3), IRF-7, and activating transcription factor-2 (ATF-2) forming a large transcription activation complex called an enhanceosome. In response to viral infection, the mitochondrial antiviral signaling protein (MAVS) is responsible for the activation of NFκB and IRF-3 (11). The complexity of the enhanceosome prevents accidental expression of the protein agent to avoid the unwanted damaging of healthy cells (1, 10). The transcription factors themselves are also tightly regulated. NFκB is bound and retained in the cytoplasm by a protein

called IκB. Upon stimulation (viral, TNF-α, etc.) IκB is phosphorylated by IκB kinase (IKK) and subsequently degraded, releasing NFκB for subsequent translocation into the nucleus (10). It has been hypothesized that PKR plays a role in activation of the promoter due to its activation of the NFκB pathway by activating the IKK complex (3). Additionally, NS5A, along with other HCV proteins, has been shown to inhibit activation of the IFN-β promoter (8). Specifically, NS3/4A inhibits the promoter's activation by cleaving MAVS (12).

Upon secretion, IFNs are capable of binding receptors on the same and/or a different cell (autocrine and paracrine signaling, respectively) to effect a response. Binding to the receptor induces the activation of its tyrosine kinase domain which induces the JAK-STAT pathway. IFN binds to the JAK-associated IFN receptor which results in the phosphorylation of STAT1 and STAT2. IFN-stimulated gene factor 3 (ISGF3) complex, which is comprised of transcription factors STAT1, STAT2, and IRF-9, translocates into the nucleus where it binds to specific sequences of DNA called interferon-stimulated response elements (ISREs). These are positioned within the promoters and induce transcription of genes called IFN-stimulated genes (ISGs). The varieties of proteins produced have a number of effects, including stimulating both the innate and adaptive immune response. Inducing fever (innate) can inhibit viral replication, and upregulating major histocompatibility complex (MHC) molecules leads to an increase in surveillance by cytotoxic T lymphocytes (CTLs) (adaptive). Additionally, interferon response proteins induce an antiviral state in cells by upregulating PKR and therefore its previously discussed pathway of shutting down the infected cell's ability to synthesize proteins (1, 10).

Also under current investigation is the possible role of NS5A on the deregulation of cell growth and apoptosis and potential role in the development of hepatocellular carcinoma (HCC), or liver cancer. Apoptosis is a process of programmed cell death governed by a family of proteins called caspases. These are proteolytic enzymes that activate the subsequent members of the cascade by cleaving them. They are divided into two types: initiator and effector. Initiator caspases, e.g. caspase 8 and 9, activate effector caspases by cleaving its pro form while effector caspases, e.g. caspase 3, cleave other cellular apoptotic proteins. Cell death may be induced by a number of factors, which may be divided into extrinsic and intrinsic signals as they originate from outside and from inside the cell, respectively. The intrinsic pathway can be activated by factors including stress, chemotherapy, DNA damage, and UV exposure and results in mitochondrial release of cytochrome c which activates the cascade through caspase 9. Extrinsic signals secreted from T cells (death ligands), e.g. Fas ligand and TNF-α, bind to their receptors and activate the cascade via caspase 8. The correlation between apoptosis and cancer is that inhibition of apoptosis increases the chances for a cell to accumulate detrimental mutations which can eventually result in cancer, especially if the mutations contribute to the deregulation of the cell cycle (1, 5).

Previous research has shown that NS5A interacts with the tumor suppressor protein p53 (6). p53 is capable of activating DNA repair, inducing cell cycle arrest, and initiating apoptosis. More than fifty percent of human tumors are found to contain a deletion or mutation in *TP53*, the gene that codes for p53. NS5A binds p53 and colocalizes it within the perinuclear space. This prevents p53 from transcriptionally transactivating the expression of proteins including those responsible for apoptosis, e.g. p21 through WAF1/CIP1. Thus, NS5A was found to inhibit p53-mediated apoptosis. Additionally, it has been shown that NS5A binds the protein, mammalian

target of rapamycin (mTOR) (9). This serine/threonine kinase helps regulate many cellular processes, especially proliferation and metabolism. By binding mTOR, NS5A promotes the cell's survival. Finally, in a previously unknown anti-apoptotic viral mechanism, NS5A prevents activation of a pro-apoptotic K+ channel in human HCC cells by inhibiting its phosphorylation (7).

MATERIALS AND METHODS

Cell culture

The cells lines used in this study include HeLa (a cervical cancer cell line), human embryonic kidney cell line (HEK 293), and HEK 293 cells expressing the hyperactivated mutant form of hepatitis C virus nonstructural protein 5A (HEK [293] NS5A-10A). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 2mM glutamine (Sigma), 0.1mM MEM non-essential amino acid solution (Sigma), 100μg/mL streptomycin (Sigma), and 100units/mL penicillin (Sigma). All cell lines were grown in a water-jacketed incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

DNA plasmid purification

Bacteria containing plasmids of interest were grown overnight in 30mL of LB medium. Using the Column-Pure Plasmid Miniprep Kit (Lambda Biotech, St. Louis, MO), following lysis

and DNA binding, the plasmid DNA was eluted into a 1.5mL microcentrifuge tube. Its concentration was then determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA).

Transfections

CalPhos protocol

0.5 to 3 hours before the transfection, the medium was replaced with the appropriate volume of fresh culture medium. Using the CalPhos Mammalian Transfection Kit (BD Biosciences Clontech, Mountain View, CA), the cell line was transformed with the desired plasmid(s) following the manufacturer's protocol.

LyoVec protocol

Following the protocol accompanying the LyoVec transfection reagent (InvivoGen, San Diego, CA), the cell lines were transfected with the desired plasmid(s). For each transfection preparation the following proportions were utilized:

Culture format	24-well plate	12-well plate	6-well plate	60 mm dish	100 mm dish
Medium per well/dish (mL)	0.5	1	2	4	8
LyoVec-DNA complex per well/dish (µL)	25	50	100	200	400
Cells per well/dish	$0.5x10^5$	$1x10^5$	$2x10^5$	$0.5x10^6$	$1x10^6$

Luciferase assay

The cells were prepared for the luciferase assay by first removing the medium, rinsing with PBS [2.7mM KCl, 1.4mM KH_2PO_4, 137mM NaCl, 4.3mM $Na_2HPO_4 \cdot 7H_20$], and adding 100µL of passive lysis buffer (Promega, Madison, WI) per well in a 24 well plate (or 250µL per well in a 12 well plate). The plate was placed on a rocker at room temperature for 15 minutes. To perform the assay, 20µL of the cell lysate was placed in a 1.5mL microcentrifuge tube, 50µL of

10X Luc Assay Reagent (LAR II) (Promega, Dual Luciferase assay kit) was added, and the light emitted was recorded using a Luminometer (Sirius). This detects the luminescence produced by the breakdown of substrate by firefly luciferase. To detect the luminescence produced by the breakdown of substrate by Renilla luciferase, 50μL of Stop and Glo reagent was added to the same tube and light emitted was again measured. To determine the relative luciferase activity (luciferase expression over background), Renilla luciferase activity in relative light units (RLU) was divided by the Luc RLU. The fold increase was found by dividing the relative luciferase activity of a single sample by the average of its control triplicate. The fold increases within a triplicate sample provided an average fold increase along with a standard deviation.

Protein analysis

Cells were rinsed with PBS and intracellular proteins were extracted using SDS lysis buffer [0.2mM tris (pH 7.5), 0.05M sodium chloride, 0.025M β-glycerophosphate, 1mM sodiumorthovanadate, 10% glycerol, 1% Triton X100, and 1mM DTT]. Extracts were placed on ice for 15 minutes then centrifuged at 14000 rpm at -4°C for 10 minutes. The supernatant was collected, mixed with SDS loading buffer, vortexed, and boiled at 100°C for 5 minutes. Total protein concentration was determined using Bradford assay (Sigma) according to the manufacturer's directions. Determination of protein expression was done by immunoblot analysis. Proteins were separated using 9% SDS page gel electrophoresis [1.5M Tris HCL (pH 8.8/6.8), Acryl:Bis (37:1), 10% ammonium persulfate, and TEMED]. After loading equal protein concentrations (determined using a Bradford assay), the gel was subjected to 100V for approximately 1.5 hours, following which it was transferred to a Polyvinylidene difluoride (PVDF, Millipore) membrane at 100V for one hour. Following transfer, the membrane was incubated for 1 hour in 5% BLOTTO solution where 25g of non-fat dry milk was dissolved in

500mL PBST [PBS, 0.1% Tween 20]. Bound protein was detected by probing the membrane with primary antibodies (in 5% BLOTTO) specific for the protein for 2 hours. The membrane was subjected to three, 10 minute washes in 5% BLOTTO. Following washing, the membrane was then exposed to alkaline phosphatase conjugated secondary antibody (in 5% BLOTTO), subjected to the same previously mentioned washing, rinsed with PBST, and then stained with NBT and BCIP (Promega) diluted in 10mL alkaline phosphate buffer [0.1M Tris (pH 9.5), 0.2M sodium chloride, and 5mM magnesium chloride]. The membrane was then scanned, and a densitometer was used to quantify the bands.

Immunofluorescence

Cells grown on a cover slip were rinsed with PBS and then fixed with ice-cold methanol. After completely removing the methanol via five, 1mL washes with PBS, the cells incubated in primary antibody solution [PBS, 3% bovine serum albumin (BSA) (Sigma) or 3% goat serum (GS)]. Following four, 0.5mL washes with PBS+3%BSA (or GS), the remainder of the experiment is carried out in the dark to prevent photo-bleaching of the fluorescent secondary antibody. After adding the secondary antibody solution [PBS, 3%BSA (or GS)], the plates are wrapped in tin foil, to block out the light, for the remainder of the incubation. Following washings with PBS, the cover slip was mounted onto a labeled microscope slide with a drop of Fluoromount-G (SouthernBiotech, Birmingham, AL). The slide was then fixed by coating the cover slip's edges with nail polish and allowing time to dry before storing at 4°C. Imaging was accomplished via a Zeiss fluorescence microscope with Axio Imager Z1 stand and AxioCam (Carl Zeiss, Thornwood, NY) and AxioVision Rel. 4.8, the company software provided. Pictures taken were at 40X oil immersion. In addition to natural light, the fluorescent lights used were AF488 for the 488nm antibody and Cy3 for the 546nm antibody.

Antibodies

	Immunoblots	
Antibody	*Company, Product Number*	*Dilution Used*
Primary		
Monoclonal mouse anti-FLAG M2	Sigma, F3165	1:1000
Polyclonal rabbit anti-IkB-a IgG	Santa Cruz Biotechnology, sc-371	1:1000
Polyclonal rabbit anti-Caspase-3 IgG	Santa Cruz Biotechnology, sc-7148	1:1000
Polyclonal rabbit anti-PKR IgG	Santa Cruz Biotechnology, sc-708	1:1000
Monoclonal mouse anti-a-Tubulin IgM	Santa Cruz Biotechnology, sc-8035	1:1000
Mouse monoclonal anti-Hep C NS5A IgG	Santa Cruz Biotechnology, sc-52417	1:1000
Secondary		
Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG	Promega, S372B	1:10000
AP-conjugated goat anti-rabbit IgG	Promega, S373B	1:10000
	Immunofluorescence	
Primary		
Monoclonal mouse anti-FLAG M2	Sigma, F3165	1:500
Rabbit polyclonal anti-IRF-3 IgG	Santa Cruz Biotechnology, sc-9082	1:100
Rabbit polyclonal anti-NFκB p65 IgG	Santa Cruz Biotechnology, sc-372	1:100
Secondary		
Alexa Fluor 546 goat anti-mouse IgG	Invitrogen, A11003	1:250
Alexa Fluor 488 goat anti-rabbit IgG	Invitrogen, A11008	1:250

RESULTS

NS5A inhibits activation of the IFN-β promoter. Previous research has shown that NS5A blocks the activation of the IFN-β promoter, thereby reducing the production of IFN-β (8). Knowing this, a stable HEK 293 cell line containing NS5A-10A was tested to confirm inhibition of the gene. After plating control HEK cells in triplicate, they were transiently transfected with a plasmid that encoded IκBΔN. When expressed, this protein inhibits the release of NFκB which inhibits activation of the IFN-β promoter. Along with some cells which were transiently

transfected with a plasmid that encoded the hepatitis C virus nonstructural protein 3/4A, a known inhibitor of IFN-β activity, these served as positive controls for inhibition of the promoter. All of the cells were transfected with a plasmid that encoded an IFN-β luciferase (IFN-β-Luc) reporter gene to measure IFN-β promoter activity. The cells were then stimulated with Sendai virus (SV) to induce activation of the promoter. A luciferase assay was performed where IFN-β promoter activity was measured (Fig. 1). Analysis of the results showed inhibition of promoter activation in cells that expressed NS5A by approximately 40%.

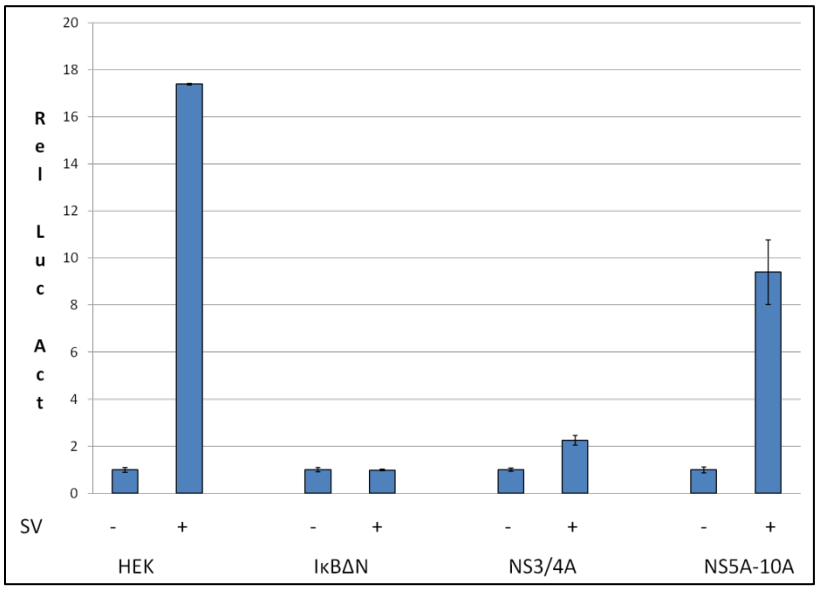


Fig. 1. **NS5A inhibits activation of the IFN-β promoter.** Luciferase assay where IFN-β promoter activation was measured following stimulation with SV.

D/NPKR inhibits activation of the IFN-β promoter to the same degree as NS5A. The next goal was to determine if PKR plays a role in the IFN-β activation pathway. Using the same method, both control and stable NS5A-10A cells were transfected with a plasmid that encoded dominant negative PKR (D/NPKR). D/NPKR is a PKR mutant [K296R substitution] that binds and inhibits WT PKR. The mutation results in a defective ATP-binding site. D/NPKR dimerizes

with WT PKR but is unable to autophosphorylate (13). Cells expressing D/NPKR showed promoter inhibition in a similar fashion to those expressing NS5A-10A, or 50% to 60% respectively (Fig. 2). However, when cells were expressing both proteins, IFN-β activity is restored to the near normal level as seen in the control cells expressing neither protein.

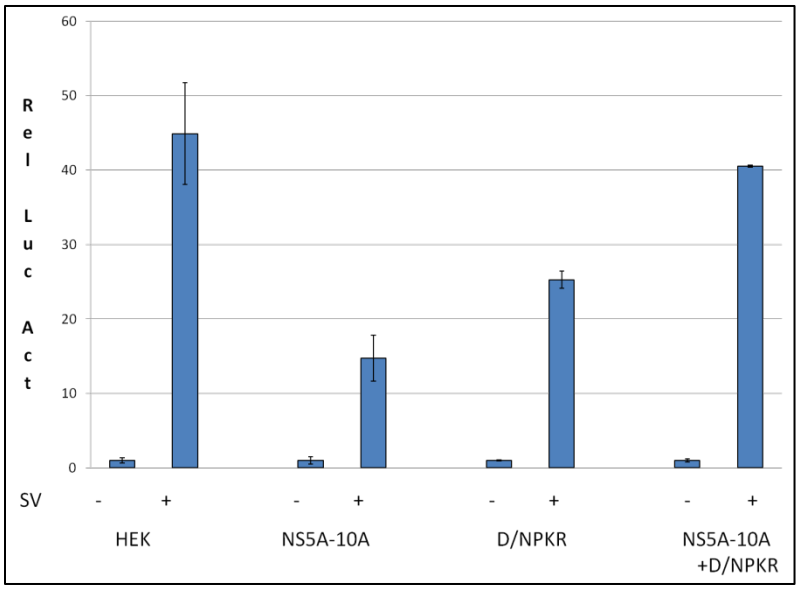


Fig. 2. **D/NPKR inhibits activation of the IFN-β promoter to the same degree as NS5A.** Luciferase assay where IFN-β promoter activation was measured following stimulation with SV.

NS5A inhibits activation of the PRDII promoter. The goal here was to establish if NS5A inhibits the promoter's activity at least in part by blocking the NFκB pathway. Activation of the complex IFN-β promoter requires the activation of three transcription factors (ATF-2, NFκB, and IRF-3) and their assembly into an enhanceosome (10). To help further elucidate the effect of NS5A effect on the IFN-β promoter via transcription factor NFκB, a luciferase assay using cells expressing a positive regulatory domain II (PRDII) luciferase (PRDII-Luc) reporter gene was used in addition to the IFN-β luciferase reporter. PRDII is the NFκB binding site on the

complex IFN-β promoter. In cells expressing NS5A there was a reduction in the activity of both promoters (Fig. 3) – approximately 60% with IFN-β and 85% with PRDII.

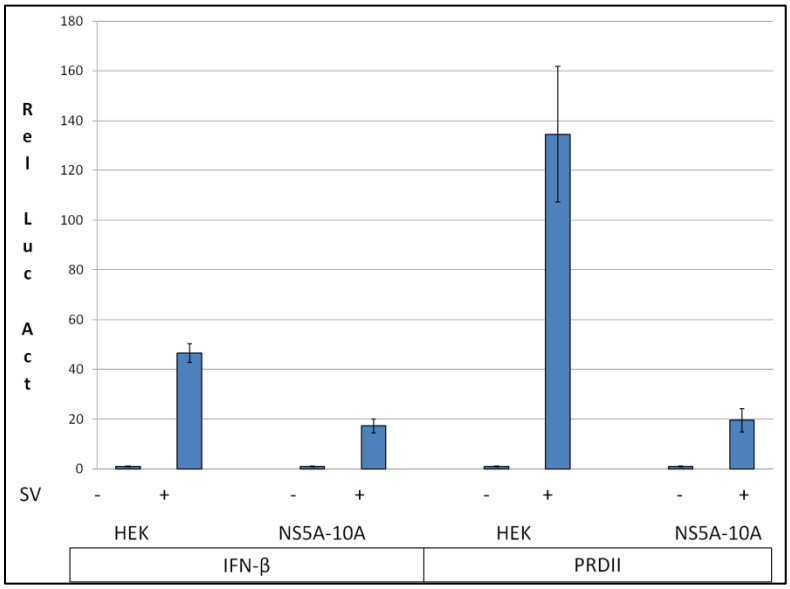


Fig. 3. **NS5A inhibits activation of the PRDII promoter.** Luciferase assay where IFN-β and PRDII promoter activation was measured following stimulation with SV.

NS3/4A is dependent on the MAVS pathway for inhibition of the PRDII promoter. In response to viral infection, the mitochondrial antiviral signaling protein (MAVS) is responsible for the activation of NFκB and interferon regulatory factor 3 (IRF-3), another transcription factor required for IFN-β activation (11). NS3/4A inhibits the promoter's activation by cleaving MAVS (12). While SV induces activation of IFN-β, tumor necrosis factor-alpha (TNF-α) only activates the NFκB pathway. The PRDII luciferase reporter gene was stimulated with either SV or TNF-α, however in cells expressing NS3/4A there was no inhibition of the PRDII promoter activity when stimulated by TNF-α (Fig. 4).

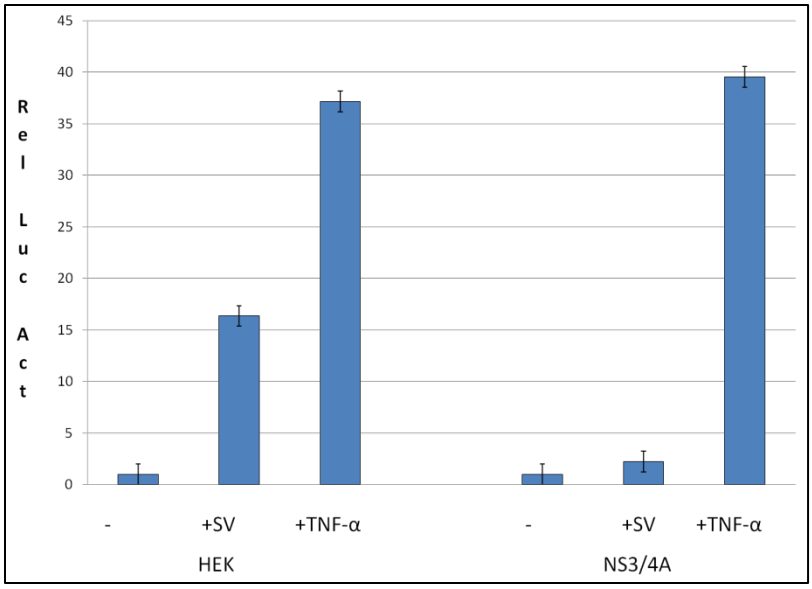


Fig. 4. **NS3/4A is dependent on the MAVS pathway for inhibition of the PRDII promoter.** Luciferase assay where PRDII promoter activation was measured following stimulation with SV or TNF-α.

NS5A appears to be partially blocking nuclear entry of transcription factor NFκB while having no effect on IRF-3. After receiving the appropriate stimulus, e.g. SV infection, both IRF-3 and NFκB enter the nucleus to serve as transcription factors for IFN-β (10). Blocking the nuclear entry of either inhibits the promoter's activation. To determine whether NS5A was blocking translocation of these transcription factors into the nucleus, both proteins were tracked using immunofluorescence in HEK 293 cells (Fig. 5). Nuclear translocation of these proteins upon stimulation with SV can be seen in the control cells expressing no viral proteins (Fig. 5a,d). Cells expressing NS3/4A were used as a positive control for inhibition of nuclear translocation (Fig. 5b,e). In cells expressing NS5A, nuclear entry of IRF-3 was not blocked (Fig. 5c). However, nuclear translocation of NFκB was partially blocked in cells expressing NS5A (Fig. 5f).

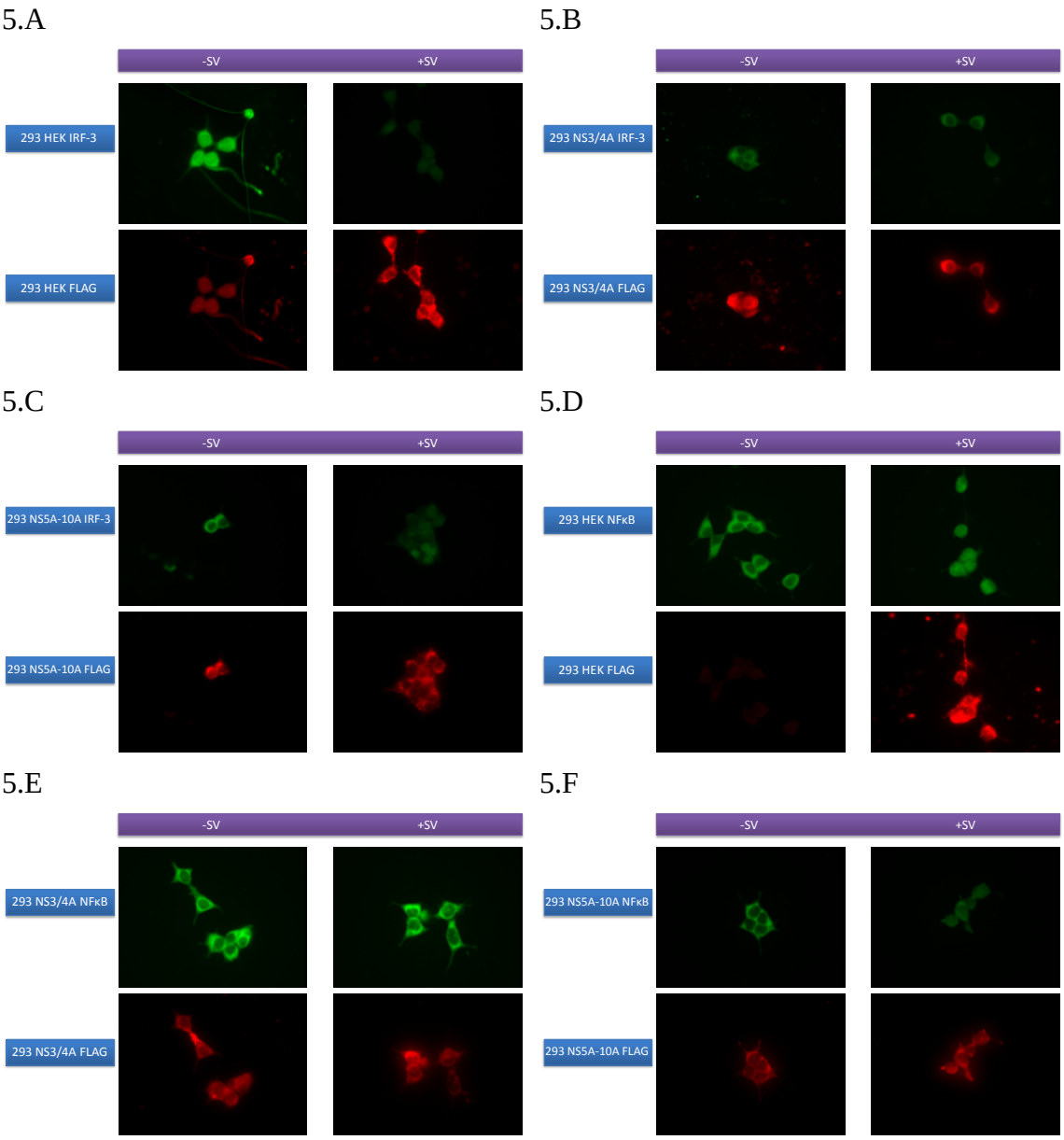


Fig. 5. **NS5A appears to be partially blocking nuclear entry of transcription factor NFκB while having no effect on IRF-3.** Immunofluorescence images where FLAG-tagged NS3/4A and NS5A (red) and either IRF-3 (A-C) or NFκB (D-F) (green) is tagged. Cells were either control (A,D), expressing NS3/4A (B,E), or expressing NS5A (C,F) and were stimulated with SV.

NS5A inhibits degradation of IκB. NFκB is bound and retained in the cytoplasm by a protein called IκB. Upon stimulation (viral, TNF-α, etc.) IκB is phosphorylated by IκB kinase (IKK) and subsequently degraded, releasing NFκB for subsequent translocation into the nucleus.

Following SV stimulation, IκB levels were compared in control cells versus cells expressing NS5A-10A (Fig. 6). If the expression of NS5A results in an increase in IκB levels, then this would confirm that the inhibition of the NFκB pathway by NS5A occurs by blocking the pathway somewhere prior to the degradation of IκB. Cells expressing NS5A showed approximately 40% reduction of IκB degradation within the first hour of stimulation.

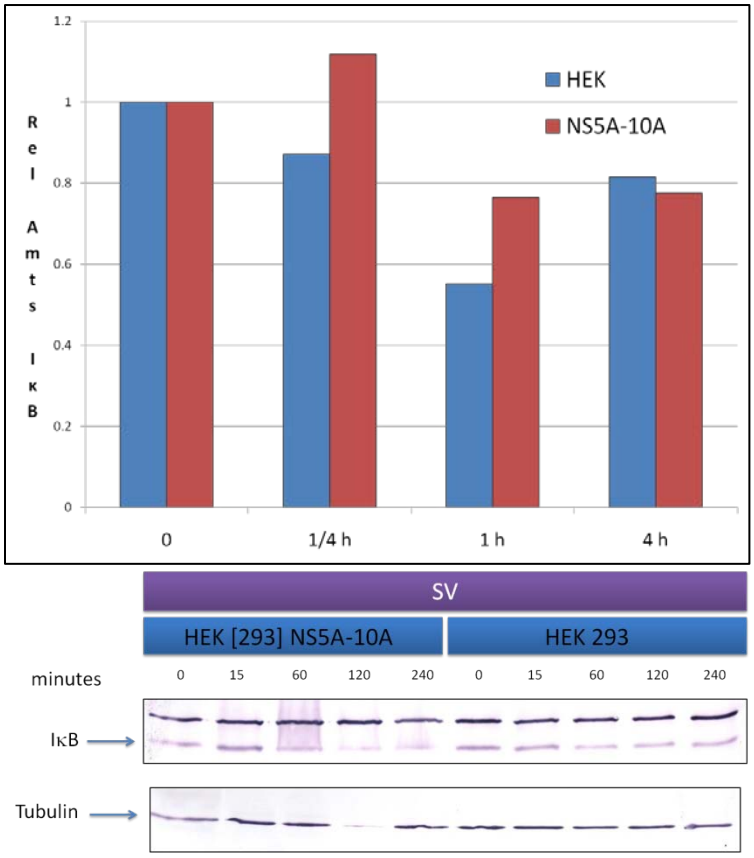


Fig. 6. **NS5A inhibits degradation of IκB.** Western blot analysis of IκB following a time course stimulation with SV using Tubulin as a normalizing control.

NS5A inhibits cleavage of procaspase 3. Previous research has shown that NS5A decreases cell sensitivity to etoposide-induced cell death (Molli Crenshaw and Giri Akkaraju, unpublished data). To determine if NS5A plays a role in inhibition of apoptosis, the cleavage of procaspase 3

following stimulation with etoposide was used in HeLa cells. Cells expressing NS5A showed a twofold reduction in procaspase 3 cleavage (Fig. 7). In addition to utilizing etoposide, the apoptotic drug staurosporine (STS) was also used as another apoptotic drug for additional data. In a dose-dependent (Fig. 8a) as well as a time course (Fig. 8b) treatment with STS, cells expressing NS5A showed a reduction in procaspase 3 cleavage, specifically twofold at the molarity and time of 4µM and 12 hours respectively.

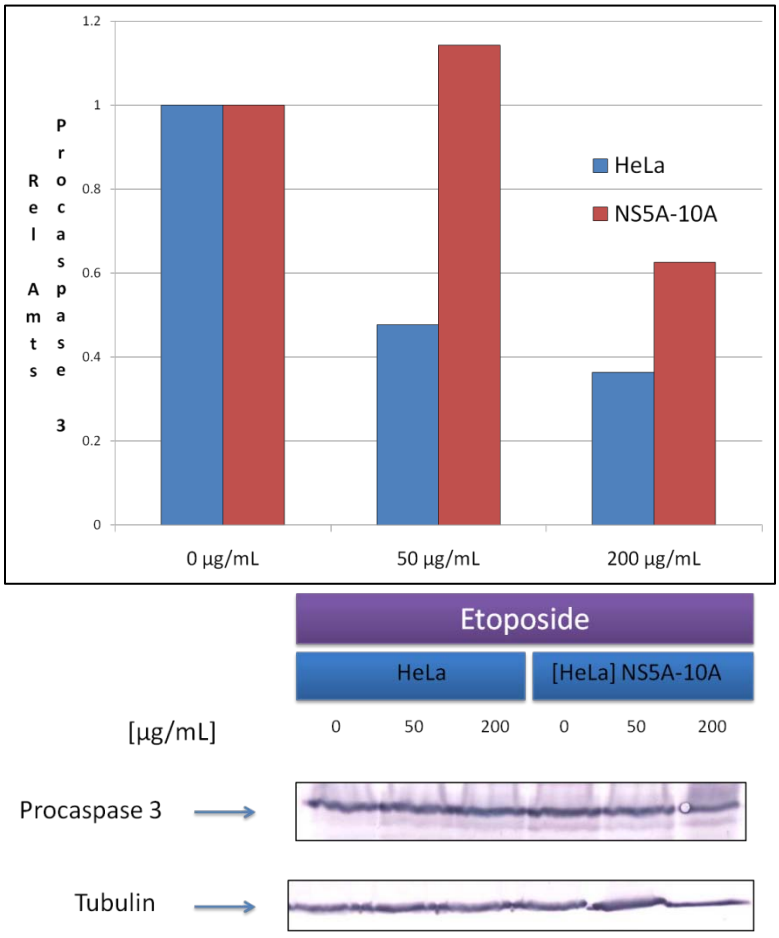
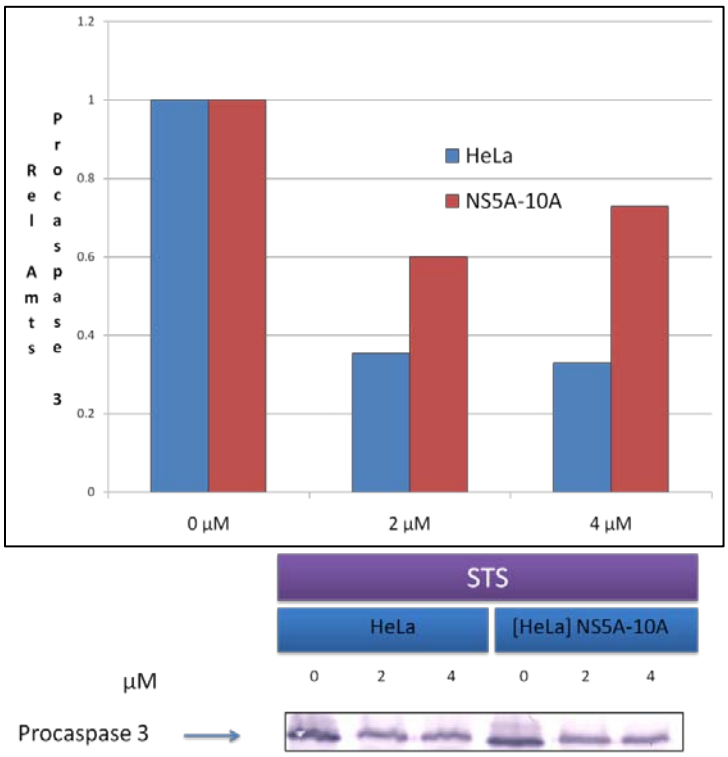


Fig. 7. **NS5A inhibits cleavage of procaspase 3.** Western blot analysis of procaspase 3 following a dose-dependent treatment with etoposide using Tubulin as a normalizing control.

8.A



8.B

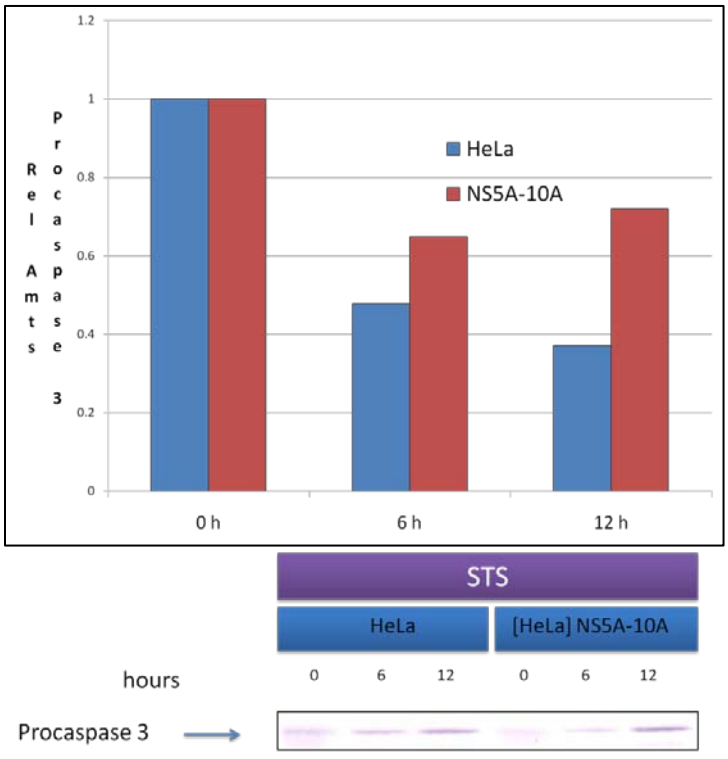


Fig. 8. **NS5A inhibits cleavage of procaspase 3.** Western blot analysis of procaspase 3 following a dose-dependent (A) or time course (B) treatment with STS using a nonspecific protein as a normalizing control.

DISCUSSION

The goal of treating HCV directs us to further elucidate the mechanisms behind its infectivity, persistence, and progression to HCC. Due to its involvement in viral replication, and inhibition of the host antiviral response and tumor suppression, the HCV protein NS5A is a key component in the natural history of the disease. The objective of this project was to understand the role played by NS5A in inhibiting two aspects of the host antiviral response – gene expression and apoptosis.

When cells are infected or stimulated, different pathways signal the formation of a multi-transcription factor enhanceosome that binds to an enhancer region upstream of the IFN-β promoter. It has been shown that NS5A downregulates the antiviral response through inhibiting activation of the IFN-β promoter by possibly blocking the activation of one or more of the transcription factors, ATF-2, NFκB, and IRF-3. We also show this with cells expressing NS5A-10A. We also investigated PKR, a protein involved in the antiviral response. D/NPKR was used to bind WT PKR and inhibit its activation. D/NPKR inhibited activation of the promoter to the same degree as NS5A-10A, providing evidence that PKR is involved in the IFN- β promoter activation pathway. When cells were expressing both D/NPKR and NS5A-10A, virus-mediated IFN-β promoter activation was restored to levels similar to the control cells expressing neither protein. This suggests that D/NPKR may be binding to NS5A and restoring PKR function in activating the promoter. The results provide evidence that NS5A inhibits activation of the IFN-β promoter by binding PKR.

To help further elucidate the effect of NS5A on the IFN-β promoter via the transcription factor NFκB, several methods were used. One technique compared PRDII promoter activation

with IFN-β promoter activation. PRDII is the NFκB binding site on the complex IFN-β promoter. The goal was to determine whether NS5A inhibits IFN-β promoter activation at least in part by blocking the NFκB pathway. The data showed inhibition of the PRDII promoter in cells expressing NS5A-10A. Activation of NFκB and another transcription factor that helps activate the IFN-β promoter, IRF-3, causes them to translocate into the nucleus where they induce gene expression. We next examined these translocations using immunofluorescence. In cells expressing NS5A-10A, nuclear entry of IRF-3 was not blocked, but nuclear translocation of NFκB was partially blocked which could downregulate the expression of IFN-β. Finally, Western blots comparing IκB levels were used to narrow down the location within the NFκB pathway where NS5A inhibits its activation. NFκB is bound and retained in the cytoplasm by a protein called IκB. Upon stimulation by virus, TNF-α, etc., IκB is phosphorylated by IκB kinase (IKK) and subsequently degraded, releasing NFκB for subsequent translocation into the nucleus. Following SV stimulation, IκB levels were compared in control cells versus cells expressing NS5A-10A. The expression of NS5A inhibits degradation of IκB which confirms that the inhibition of the NFκB pathway by NS5A occurs prior to this step in the pathway. Because PKR's activation of the pathway involves interacting with IKK which is also upstream of this step, this suggests a mechanism by which NS5A blocks activation of the IFN-β promoter through binding to PKR.

In addition to NS5A, another HCV nonstructural protein that inhibits activation of the IFN-β promoter is NS3/4A. In response to viral infection, NS3/4A inhibits the promoter's activation by cleaving MAVS which is part of the activation pathway. NS3/4A inhibits SV-mediated PRDII promoter activation but has no effect when stimulated by TNF-α. While SV

induces activation of the IFN-β promoter through RIG-I/MAVS, TNF-α only activates the NFκB pathway through a different mechanism. This shows NS3/4A blocks virally induced activation of the NFκB pathway but not cytokine, specifically TNF-α, induced activation.

In addition to understanding NS5A's role in inhibiting the antiviral response, this study also provides evidence that NS5A's possible role in the development of HCC stems from its ability to block apoptosis. In doing so, NS5A may be able to interfere with host cellular control of normal growth and proliferation. Previous research has shown that NS5A decreases cell sensitivity to etoposide-induced cell death (Molli Crenshaw and Giri Akkaraju, unpublished data). To determine if NS5A plays a role in inhibition of apoptosis, we examined the cleavage of procaspase 3 to effector caspase 3 (a component of the apoptotic pathway) following treatment with etoposide. Western blot analysis revealed that cells expressing NS5A-10A showed a decrease in the degradation of procaspase 3. In addition to utilizing etoposide, STS was also used as another apoptotic drug. Again, cells expressing NS5A-10A showed a reduction in procaspase 3 cleavage. This suggests NS5A is also blocking cell death by inhibiting activation of caspases. The data above suggests a role for NS5A in the etiology of HCC. For example, previous research has shown that NS5A inhibits p53-mediated apoptosis by binding to p53 and preventing its translocation into the nucleus. Given NS5A binds to PKR, a protein that in addition to its contributions to the antiviral response has been shown to be involved in apoptosis and act as a tumor suppressor, this might suggest a mechanism by which NS5A inhibits apoptosis.

In summary, we show that NS5A affects antiviral gene expression by inhibiting production of IFN-β at least in part through interfering with the NFκB signaling pathway. Additionally, NS5A inhibits degradation of procaspase 3, a protein in the caspase pathway for apoptosis, and therefore inhibits apoptosis, setting the stage for HCC. NS5A may do the above by inhibiting PKR, which is known to be involved in both the apoptosis and the regulation of gene expression.

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ABSTRACT

BRIDGING THE GAP:
THE ROLE OF HEPATITIS C VIRUS NONSTRUCTURAL PROTEIN 5A IN THE INHIBITION OF INTRACELLULAR ANTIVIRAL PATHWAYS

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It is estimated that approximately over two hundred million people worldwide are infected with hepatitis C virus (HCV), far more than HIV/AIDS, and that number grows by three to four million each year. It is a leading cause of cirrhosis, replacement of the liver tissue by fibrosis, or scar tissue, and is consequently the primary reason for liver transplantation in the United States. It is an enveloped, positive single stranded RNA virus and is the most common chronic blood-borne infection in the United States. The stages of hepatitis C virus infection are divided into acute and chronic. While infection clearance rates vary from patient to patient, approximately eighty-five percent of patients will go on to develop chronic hepatitis C infection. Liver inflammation results in death of the tissue (necrosis) and replacement by scar tissue (fibrosis). Death of hepatocytes leads to regeneration of the liver by activating the cell cycle in quiescent liver cells. This can increase the chances of errors during DNA replication. In addition, the liver processes many toxins present in the body including possible mutagens. Both of these factors increase the chance of cellular mutation, which can lead to various forms of cancer (including hepatocellular carcinomas) should this occur in the coding or regulatory regions of a cell cycle regulatory protein. Currently, no vaccine is available for the virus. The only treatment is a combination of IFN-α and ribavirin. However, the treatment lasts for extended periods, has serious side effects, and because the virus is constantly mutating during viral genome replication, resistant strains are predominant. Because of these combined factors, treatment has a very low success rate. The viral genome consists of three structural proteins and seven nonstructural ones. One of the nonstructural proteins, NS5A, has been attributed to the blocking of the cellular antiviral response including the apoptotic pathway. It is known to inhibit RNA-activated protein kinase R (PKR). The objective of this project was to understand the role played by NS5A in inhibiting two aspects of the host antiviral response – gene expression and apoptosis. In this study we show that NS5A affects gene expression by inhibiting production of the antiviral protein IFN-β. Additionally, NS5A inhibits degradation of procaspase 3, a protein in the caspase pathway for apoptosis, whose degradation is required for the pathway to continue. NS5A may do the above by inhibiting PKR, which is known to be involved in both apoptosis and the regulation of gene expression.