VIRUS VERSUS BODY:
THE EFFECT OF THE HEPATITIS C VIRUS NS5A PROTEIN
ON THE ANTIVIRAL RESPONSE AND CANCER DEVELOPMENT

by

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Introduction

In the 1970s, the National Institute of Health began to detect a non-A, non-B form of Hepatitis in many blood transfusion cases. However, it was not until 1988 that Dr. Michael Houghton and colleagues were able to identify the genome of the virus, at which time they named it Hepatitis C Virus (HCV) (1). Since then, the virus has been identified as the cause of liver disease and death in an estimated 200 million people world-wide (2). As this number continues to climb there is still no vaccine available, and the current treatments are both toxic and ineffective in more than 50% of patients. Although the virus has been studied extensively since its discovery, there remains a great deal of mystery surrounding the mechanisms which allow the virus to persistently infect a cell.

Infectivity and Treatment

Hepatitis C Virus is transmitted percutaneously, and prior to the development of diagnostic tests, it was transmitted through blood transfusions and organ transplants. Infection with HCV is initially asymptomatic, so many individuals are unaware of infection until the virus has begun to inflict considerable damage on the body, mainly on the tissues of the liver. Approximately 70% of acute HCV infections become chronic leading to liver disease and cirrhosis, which can result in the development of hepatocellular carcinoma or liver cancer in roughly 5% of these individuals (1). The virus has had a considerable impact on injection drug users and their sexual partners, along with prison inmates, 20-40% of which are infected and individuals with HIV, 25% of which are co-infected with HCV. (1)

Current treatment is a combination therapy of recombinant interferon-α (IFN-α) and ribavirin. IFN-α is a cytokine derivative which functions both as an antiviral and an immunomodulator, while ribavirin is an inhibitor of viral replication. Ribavirin is a nucleoside analogue of guanosine and is incorporated into viral RNA by an RNA polymerase during RNA replication. The insertion of Ribavirin blocks further addition of nucleosides by RNA polymerase, thereby inhibiting viral RNA replication (58). The therapy causes substantial toxicity in most patients, including symptoms of fatigue, depression, nausea, myalgia, and lowered red blood cell count (3). This combination therapy has been successful in an average of 47% HCV-infected patients, but is ineffective in most. Resistance to HCV therapy is most likely related to the existence of 6 viral genotypes, or quasispecies, numbered 1-6. These quasispecies arise as a result of faulty genome replication (1), and could have the ability to resist antiviral activities of the host cell induced by IFN-α and/or resist binding to ribavirin (59). The most resistant of the six strains is HCV 1, and according to the Center for Disease Control, even if treatment for this strain is begun during the acute, asymptomatic stage, only 50% of patients will be cured of the virus infection (4).

In order to develop more specific and effective remedies, scientists have attempted to understand the effects of the virus on the host cell immune response. Ideally, understanding the mechanisms of the anti-viral response to HCV *in vivo* would allow researchers to develop more effective therapies and a possible vaccine, a task made difficult for two reasons. First of all, the virus is only infective in humans and chimpanzees, so it has been difficult to find small animal models which would accurately display viral effects on the body (5). Second, until recently scientists have struggled to produce culture systems which can grow HCV in amounts large enough for testing. To combat this, self-replicating stretches of HCV RNA called replicons were developed. By replacing certain structural genes in the RNA with a drug-inducible gene sensitive to neomycin sulfate, cells transfected with the replicon could be selected for under presence of the drug. This method yielded enough viral RNA to be detected through testing and lead to development of cell culture-adaptive mutations producing more than 20 different cell lines. Comparison between RNAs of these replicons has led to a deeper knowledge of the roles of the individual viral genes (63). Finally, in 2001, scientists were able to develop a culture system by cloning the entire viral genome of a patient who developed fulminant hepatitis and then cleared the virus. The genome, referred to as "Wakita's Isolate", is capable of high reproductive output, but only represents the genome of one of the six forms of HCV (60).

Hepatitis C Virus Life Cycle

Despite the vast amount that is unknown about mechanism of HCV infection, it is important to reflect on what has been discovered about the virus. Hepatitis C virus is a small, enveloped virus, and is the only known member of the *hepacivirus* genus in the family *Flaviviridae*. Upon attachment and entry into a host cell, the virus uncoats releases a positive-sense, single-stranded RNA (ssRNA) genome, approximately 9.6 kb in length. The 5' end of the genome contains an Internal Ribosome Entry Site (IRES) which binds to cellular ribosomes and is translated into a large polyprotein. Viral and cellular proteases then cleave the polyprotein into ten individual proteins which are classified as either structural or non-structural in function. The structural proteins include a Core protein (C), two glycoproteins referred to as E1 and E2, and the p7 protein. The non-structural (NS) proteins include NS2, NS3, NS4A, NS4B, NS5A, and NS5B (6).

These newly cleaved proteins are then involved in the replication of the original positive-sense genome. Due to the single-stranded nature of the genome, an RNA-dependent RNA polymerase (RdRP) produced by the virus is forced to produce a negative-sense RNA intermediate template to be used for further protein translation and production of more positive-sense genomes. This RdRP replicates the genome rapidly, enabling the production of up to 10^{12} viruses per day in infected individuals, which is 100-fold greater than rates reported for HIV. The polymerase lacks a proofreading mechanism, however, and therefore has a high error-rate which is partially responsible for generating the six HCV genomes in existence today (1). The viral life cycle is completed as the positive-sense genomes produced by the RdRP are packaged into newly assembled nucleocapsids, which then bud into the cellular ER, and exit the cell by hijacking its secretory pathways. These extracellular, enveloped virions are then free to infect neighboring cells and continue the HCV life cycle (1).

The complexity of these events is highly dependent on the activities of the ten viral proteins produced upon the virus's entry into the cell. Research conducted on these proteins has further revealed their roles regarding both the viral life cycle and host cell interactions including anti-viral signal suppression. The core protein (C) is a dimeric protein which forms the nucleocapsid coat of the virus particle. This nucleocapsid forms in the cytoplasm of the host cell, and encapsulates the ssRNA replicate before exiting the cell as viral progeny. Certain regions of the protein have high affinity for lipid molecules, facilitating its attachment to the ER in order to form the viral envelope (16). E1 and E2 are glycosylated type-I transmembrane proteins that form a heterodimer in order to mediate host-cell recognition and entry. In addition to this, they are important for certain steps of HCV replication, viral particle assembly, and are targets of the host anti-viral response (17). The p7 structural protein is comprised of two transmembrane helices which embed in the surface of the endoplasmic reticulum. P7 functions as an ion channel and, while it is not involved in virus entry into a host cell, research shows that it is crucial for the assembly and release of infection virions from infected cells (18). HCV non-structural protein 2 (NS2) contains a cysteine protease domain responsible for its self-cleavage from NS3, but its other functions are not well understood. It can interfere with apoptotic mechanisms, and is essential for the phosphorylation of NS5A. NS2 is also capable of activating transcription of the inflammatory chemokine interleukin-8 (IL-8) through NF-κB, which indicates that it may be able to stimulate transcription of other genes downstream from the NF-κB promoter (19). The NS3 protein is a serine protease responsible for cleavage at the NS3/4A, NS4B/5A and NS5A/5B junctions. NS3 is dependent on NS4A for optimum protease activity, and has become a target of pharmaceutical research due to its role in viral replication (20). NS4A, in addition to its role as a cofactor for the NS3 protease, has also been shown to inhibit protein synthesis within the cell through interactions with the eukaryotic elongation factor 1A (eIF4E). This indicates that NS4A may play a role in down-regulating the translation of anti-viral proteins within the cell, allowing HCV to establish a chronic infection (21). NS5B functions as the RNA-dependent RNA polymerase (RdRP), and is responsible for replication of the viral genome. Unlike DNA polymerase, NS5B does not require a promoter region in the RNA, but binds to the 3' untranslated region (3' UTR) of the viral genome. The NS3, NS4A and NS5A proteins bind to NS5B to form a replication initiation complex, but the details of each protein's role in this complex are still unknown (22).

<u>The NS5A Protein</u>

Among all the viral proteins, NS5A has been studied for its apparent ability to regulate viral replication within the host cell, inhibit the cellular immune response, and to block tumor-suppression capabilities of the cell. NS5A is a large phosphoprotein (56-58kDa) composed of an NH_2-terminal amphipathic α-helix which anchors it to intracellular membranes, and three cytoplasmic domains containing sites of interaction many cellular proteins. NS5A is serine-phosphorylated and exists in both basally phosphorylated and hyperphosphorylated isoforms, although the roles of these different states is unclear (22).

Once phosphorylated, NS5A becomes an essential part of the HCV-replicon complex. As it localizes near the surface of the nucleus, its C-terminal region binds to c-Raf (a MAP kinase) and then to NS5B (the RdRP) to catalyze replication. Assays involving caspase-dependent cleavage of NS5A causes truncated fragments of the HCV protein to translocate into the nucleus, still attached to c-Raf, preventing its ability to bind to NS5B thus restraining replication (23). NS5A has also been revealed as a key factor for the assembly and release of infectious HCV virions. Cellular ER and lipid droplets are the assumed sites of viral assembly, and in patients infected with HCV the droplets are dotted with both Core and NS5A proteins. Deletions in domain III of NS5A coincide with a decrease in virion formation, along with the presence of only Core proteins on ER and lipid membranes (24).

Evidence for the role of NS5A in HCV RNA replication also comes from studies comparing the two mutants of the NS5A protein used in research today. These mutants are referred to NS5A 10A and H27 after the replicon quasispecies from which they were obtained. As mentioned before, these subgenomic replicon systems were developed for use under drug induction, and eventually accrued mutations during their replication in cell culture systems. Upon analysis of two replicons, the only differences found in the genomes were the mutations within the NS5A gene sequence. The replicon named H27, encodes a leucine substitution at amino acid 2198 in place of a serine (L2198S), and exhibits in basal levels of replication in cells. Replicon 10A encodes a lysine insertion at amino acid position 2040 (K2040) and produces an increased replication rate in infected cells(64). This indicates that alterations in only the NS5A gene are enough to cause changes in replication capabilities of the virus.

<u>Mechanisms of The Host Cell Antiviral Response</u>

Hepatitis C Virus is a non-cytopathic virus, which means it does not kill the cell, but rather manipulates the host cell immune response while it replicates, packages and releases its viral progeny from the cell. While HCV has several strategies for potential evasion of antiviral treatment and the host immune system, the NS5A protein has been demonstrated as a key player in HCV’s defense against clearance by the host (25). Under normal conditions, the presence of a virus will activate cellular pathways resulting in the antiviral response. The antiviral response is a multifaceted reaction to viral infection in which the infected cell transcribes proteins responsible for inhibiting viral replication, initiating apoptosis and stimulating the antiviral response in neighboring cells (61). This response typically begins on the outer surface of the cell as toll-like receptor proteins (TLRs), namely TLR3 in the presence of viral RNA, induce the phosphorylation of the cytoplasmic interferon regulatory factor (IRF)-3. This protein dimerizes, and translocates to the nucleus where it interacts with other transcription elements such as NF-κB in order to induce transcription of interferon (IFN)-β. NF-κB is also known for its role in regulating the gene expression of chemokines and inflammatory cytokines. These IFN-β proteins are released from the cell and bind to a class of tyrosine kinases called Janus kinases (Jaks). Jaks phosphorylate and activate signal transducers and activators of transcription (STATs) which associate with IRF-9 and migrate to the nucleus where they stimulate transcription of IFN-stimulated genes (ISGs) such as IRF-7 and RIG-I(6). Retinoic acid inducible gene (RIG)-I is an RNA helicase which initiates the antiviral response by binding to single or double-stranded viral RNA. Once bound to RNA, RIG-I activates the IPS-1/MAVS/Cardif/VISA protein on the surface of the mitochondria, thus activating a cascade to induce transcription of IFN-1 (62).

Another protein translated following activation of the Jak/Stat pathway is double-stranded RNA (dsRNA)-activated protein kinase, also referred to as PKR (protein kinase RNA-dependent). PKR is activated by binding to double-stranded RNA, the TLR (toll-like receptor) pathway (50), growth hormones and cytokines such as PDGF, TNF and

IL-1 (51), and cellular stresses such as toxins and H_20_2 (52). Activation induces dimerization and autophosphorylation of PKR, which regulates its binding with the α subunit of eukaryotic protein synthesis initiation factor 2 (eIF-2), impairing its activity and inhibiting mRNA translation (53). This primarily limits the ability of viruses to be replicated using the host cell translation machinery. Due to its nature as a primary defense against viral infections, many viruses have developed ways to counteract the functions of PKR until the viral life cycle has completed (53). Examples of this are the v-IRF-2 protein from Kaposi's sarcoma herpesvirus (KSHV) which prevents autophosphorylation of PKR (56), and the EBER-1 protein from EBV which binds as a competitive inhibitor to the ds-RNA binding site of PKR, preventing its recognition of viral RNA (57).

NS5A Represses the Antiviral Response

NS5A's ability to circumvent aspects of this antiviral response has made it the target of numerous studies, leading researchers to a deeper understanding of the role played by NS5A in the pathology associated with Hepatitis C virus. For example, recent research has demonstrated that NS5A may interact with the myeloid differentiation factor 88 (MyD88) in order to interfere with the production of HCV-specific T-cells during chronic infection with HCV. MyD88 is an adaptor protein essential for the activation of certain proteins in the TLR signaling cascade, namely ISGs. One such ISG, the interferon regulatory factor (IRF) 3, is phosphorylated and activated following MyD88's activation in the pathway, and subsequently mediates transcription of the cytokine IFN-1. Under normal circumstances, IFN-1 induces maturation of immune cells known as dendritic cells, which are then primarily responsible for the maturation of T-cells. However, under the influence of HCV, NS5A can bind to, and inhibit MyD88, thus reducing levels of IRF-3, IFN-1, mature dendritic cells and HCV-specific T-cells (26). This hinders the ability of the immune system to recognize HCV antigens, reducing the ability of the body to clear the virus following chronic infection.

Hepatitis C Virus Causes Hepatocellular Carcinoma

In addition to its ability to regulate the host cell antiviral response, HCV has also been identified as a leading cause of hepatocellular carcinoma (HCC) or liver cancer in the US. HCC is the fifth most common type of cancer, and the third leading cause of cancer-related deaths world-wide (27). The rate of incidence has increased approximately 80% in the last twenty years, and though once more prevalent in individuals 65 years and older, has lately shifted its prevalence toward younger patients. HCC usually develops in people with chronic liver inflammation induced by hereditary haemochromatosis, autoimmune liver diseases, alcoholism and viral infections (primarily Hepatitis B and C) (28). Current theory concerning this connection is that liver inflammation in these situations results in cell death, which then stimulates hepatic regeneration, increasing the risk of cellular mutations which could promote cell transformation and tumorogenesis (29). However, there is a rare occurrence of HCC in patients with autoimmune hepatitis, a disease which induces severe liver inflammation and persists indefinitely. For this reason, scientists have begun to evaluate the ability of viral proteins from HCV and HBV to directly induce HCC development without the necessity of inflammation (30). The resulting discoveries have added HCV to the long list of viruses, which, through their many protein products, can deregulate cellular mechanisms involved in the host cell cycle.

The Host Cell Cycle

The cell cycle is divided into stages which alternate cell growth and development, with genome replication and mitotic division. The growth phases are denoted as G1 and G2, the replication phase is denoted as S, and the period spent in mitosis is represented by the letter M. The life cycle runs in the order of G1, S, G2 and finally M. The timing of each of these phases is of considerable importance, and is tightly regulated by a number of cellular factors which are divided into two main categories. The first group consists of cell cycle promoters such as intracellular cyclin-dependent kinases (CDKs), and extracellular factors known as mitogens, which include platelet derived growth factor (PDGF) and epidermal growth factor (EGF) (8). Upon damage to host cell DNA which can be caused by chemical mutagens, UV radiation, and infection, CDK inhibitors are produced in order to stop progression of the cell cycle, allowing for DNA repair. If repair is unsuccessful, the cell is signaled to undergo programmed cell death through apoptosis.

Mechanisms of Apoptosis

Apoptosis is triggered by activation of a cascade of proteases called caspases. This proteolytic event causes the cell to shrink as its DNA is fragmented, and its surface proteins are altered to attract macrophages of the immune system, which then phagocytize and eliminate the cell. This form of cell death is far more neat than cell necrosis, the process in which cells burst, and release their contents into the environment causing an often harmful inflammatory response. Apoptosis can be induced by a wide variety cellular pathways, involving cell surface death receptors and intracellular proteins which are often referred to as tumor suppressors (8).

Fas (CD95) and TNFR are two examples of surface receptors which can activate the caspase cascade. The TNFR pathway is activated as this surface receptor binds to the extracellular factor TNF-α, and induces formation of a complex involving the TRADD, TRAF2 and FADD proteins, which then induce the cleavage of caspase-8. In contrast to this pathway, upon activation of the Fas receptor, it immediately binds to FADD in order to initiate cleavage of caspase-8 (32). The double-stranded DNA Hepatitis B virus, produces an HBX protein to prevent the cleavage of caspase-8 in the Fas pathway. Using cell lines expressing HBX, Diao and associates were able to successfully transform liver cells, mimicking the ability of HBV to deregulate this tumor suppressor (38). Another protective measure found in cells is the ability of mitochondria to activate apoptosis by releasing the factor cytochrome C, which activates APAF-1, which then cleaves caspase-9 (33). The Bcl-2-associated X (BAX) protein is believed to be able to produce a pore in the mitochondrial membrane, allowing the release of cytochrome C. Aubert and associates were able to demonstrate the ability of Herpes simplex virus to target and inhibit BAX, preventing cytochrome c-induced apoptosis in infected human epithelial cells, thus allowing possible transformation (39). A third way a cell can launch apoptosis is through expression of tumor suppressor proteins including p53, p21, pRb, and PKR. One of the most well-known of these is p53, and is mutated in about 60% of human cancers (34). p53 is a nuclear protein, and induces apoptosis in response to stress-associated signals including hypoxia and cellular starvation. It is normally bound and degraded by the MDM2 (murine double minute 2) protein, but is unbound when the protein ARF suppresses MDM2 (28). Once free of MDM2, p53 initiates a pathway which eventually activates expression of the p21/waf1 gene. p21 is a CDK inhibitor which prevents phosphorylation of CDK substrates, and blocks cell cycle progression (35). Aside from its role in cancer formation strictly due to mutagenesis, or its mutation, cell transformation can occur when p53 is inhibited by the protein products of certain viruses. One of the leading causes of adult T-cell leukemia is the human T-cell lymphotrophic virus type I (HTLV-1). The HTLV-1 TAX protein inhibits p53 activation through phosphorylation, thus preventing it from activating p21 and inhibiting cell cycle progression (41). Recently, Yoon and colleagues were able to demonstrate that p53 is also able to induce PKR expression (37). PKR, in addition to its role in the antiviral response, appears to play a role as a tumor suppressor by augmenting the apoptotic activity of p53 in a downstream fashion. The last of these tumor suppressors is pRb, a member of the retinoblastoma (Rb) family of genes. In addition to pRb, the Rb family is comprised of the p107 and p130 proteins. In response to DNA damage, these proteins bind to, and inhibit the E2F transcription factors which would otherwise initiate the transcription of S phase genes. Deactivation of these Rb proteins can result in S phase entry, and promote cell transformation (36). Human papillomavirus (HPV) has been shown to produce E6 and E7 proteins which bind to, and inhibit pRb, p107 and p130. This is theorized to be one of the primary mechanisms by which the virus is able to produce high-risk lesions that can progress to invasive squamous cell carcinomas (40).

Mechanisms of Growth and Proliferation

In the absence of tumor-suppressors and apoptosis-promoting factors such as these, the cell cycle is controlled by factors which promote growth and proliferation. In most cases, these factors are beneficial, but their deregulation can lead to uncontrolled growth resulting in cell transformation. Deregulation can be the result of viral infection, or it can be caused by mutations in these positive control genes resulting in what are known as oncogenes. Elements involved in cell cycle progression can be divided into three groups, mitogens, growth factors and survival factors (8).

Mitogens are extracellular factors such as the previously mentioned PDGF and EGF, which stimulate cell division, and inhibit the effect of tumor suppression. They often react with RTKs (receptor tyrosine kinases) on the surface of the cell, and activate Ras proteins which then initiate the Ras-MEK-ERK kinase cascade. This results in the phosphorylation and stabilization of a nuclear transcription factor c-Myc, which both represses CDK inhibitors, and promotes expression of cyclins involved in cell division (34). C-myc is the primary target of the EBNA-2 protein produced by the Epstein-Barr virus, an infective agent of primary B cells. By inducing the oncogenic behavior of c-myc, EBV is able to transform these B cells, resulting in both Burkitt's lymphoma and Hodgkin's lymphoma (42). The second group of these cell cycle factors are growth factors, many of which interact with PI(3)K (phosphatidylinositol-3-OH kinase), which then phosphorylate and activate the protein kinase Akt. Akt is able to prevent apoptosis by phosphorylating and inhibiting the GSK3-β kinase, preventing this kinase from phosphorylating and inactivating c-myc (34). The influenza A virus protein NS1 promotes indirect oncogenic activity of c-myc by activating the PI(3)K pathway, in order to facilitate more efficient viral replication (44). The final set of cell cycle promoters are survival factors, which include the anti-apoptotic proteins Bcl-2 and Bcl-x. These proteins function principally through stabilization of the mitochondria, preventing release of cytochrome c which would activate the apoptotic pathway (45). They are also a product of the Akt pathway, by way of the NFκB family, a group of transcription factors who regulate a wide array of genes involved in cell immunity, growth and apoptosis (43). NFκB dimers are inactive in the cytoplasm while they are bound to members of the IκB family. However, the Akt pathway activates IKKβ (IκB kinase β) which then phosphorylates IκB, marking it for proteolysis, allowing NFκB to translocate into the nucleus and initiate transcription of multiple genes including the anti-apoptotic Bcl-2 proteins. The highly oncogenic avian reticuloendotheliosis virus strain T (Rev-T) expresses the v-rel gene, a variant of the c-rel oncogene expressed in many human breast cancers. When this viral protein was expressed in transgenic mice, it amplified the expression of NFκB, resulting in oncogenic activity of Bcl-2 and other proteins, resulting in the formation of mouse mammary tumors (46).

Mechanisms by which Hepatitis C Virus causes Hepatocellular Carcinoma

One of the goals of my research is to gain understanding of the mechanism by which HCV deregulates cell cycle control to induce HCC. Research in this area has revealed that the HCV Core protein plays a very important role in HCC development. As liver cells undergo transformation associated with HCC, they undergo a morphological alteration referred to as epithelial to mesenchymal transition (EMT). Although the term is typically associated with embryologic development, it is also used to describe the genesis of fibroblasts promoting the development of liver carcinomas (47). Transforming growth factor-β (TGF- β) phosphorylates and activates gene regulatory proteins known as Smads, which initiate transcription of TGF-β target genes resulting in either apoptotic or anti-apoptotic activities depending on the gene. HCV Core protein is known to bind to Smad3, and coincide with upregulation of anti-apoptotic activity and HCC development. Therefore, the data suggests that the Core protein manipulates the Smad3 protein in such a way that it is insensitive to the apoptotic effects of TGF-β, but still sensitive to its anti-apoptotic properties (47).

Despite the important effect the Core protein seems to have in liver cell transformation, a lot of research is unveiling NS5A as a major player in HCV associated HCC. In studies conducted with transgenic mice harboring the NS5A gene over a period of 6 months, harvested hepatocytes revealed high levels of NFκB in addition to other anti-apoptotic factors, implying that the presence of NS5A is correlated with decreased cell-directed apoptosis. Although few mice presented HCC, most developed a condition known as steatosis, in which lipid droplets associated with liver cirrhosis accumulate in the hepatocytes (48). NS5A has also been shown to interact with p53, and to down-regulate the apoptotic activity of p21 (35,49). This is compounded by its ability to bind to Protein Kinase R (PKR), which regulates the antiviral response by phosphorylating and inhibiting the alpha subunit of eukaryotic initiation factor 2 (eIF-2α), preventing translation of both cellular and viral mRNA. In addition to this, it acts as a tumor suppressor by controlling the apoptotic activities in the cell that are regulated by such factors as NFκB (10).

MATERIALS AND METHODS

Tissue Culture: Adherent 293 IFN-β cells and MEF WT cells from the lab of James Z. Chen (University of Texas at Southwestern Medical Center) and HEK 293 purchased from ATCC (Manassas, VA) were grown in monolayer on Corning tissue culture dishes. All cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (MediaTech, Herndon, VA) and 5% CO_2 enriched air at 37°C in a tissue culture incubator (NuAire, Plymouth, MN). Added to the medium were: 10% fetal bovine serum (FBS), 1% glutamine, 1% non-essential amino acids, and 1% penicillin/streptomycin antibiotics (all from Sigma Aldrich).

Luciferase Assay: HEK 293 IFN-β cells were plated in a 24-well plate at $1x10^5$ cells and 500 µL DMEM complete medium per well. The following day, Lyovec Reagent was used to transfect specific wells with 0.5µg of either NS5A 10A, NS5A H27, NS5A WT or NS3/4A plasmids. Twenty four hours post-transfection, selected wells were infected with 4µl Sendai Virus. All cells were lysed the following day using Passive Lysis Buffer (Promega). Luciferase Assay Reagent, followed by Stop and Glo Buffer (Promega) were added to the samples as per the manufacturer's protocol. The samples were then read by a Luminometer (Berthold Detection Systems) to measure Luciferase activity.

Cell Death Assay: On day one, cells were trypsinized and plated in a 96-well plate at 5000 cells per well. 24 h later, the cells were treated with increasing concentrations of the apoptosis-inducing drug etoposide (Sigma-Aldrich) to elicit a response similar to that seen in apoptosis. After approximately 20 h, the cells were stained with the vital stain MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], which is reduced to a purple derivative by mitochondrial dehydrogenases contained in living cells, providing a color contrast between living and dead cells. The precipitated derivative is solubilized in DMSO for 5 min at room temperature (RT), and the cell vitality as indicated by MTT was determined by measuring absorbance with a spectrophotometer at 540nm. In the case of experiments involving transient expression of protein, 293 HEK cells were plated in a 60mm dish at a density of 100,000 cells, and transfected 24 hours later with 1ug of the indicated plasmid. 24 hours after transfection, the cells were trypsinized and plated in a 96-well tray.

Western Blot:

Preparation of SDS Page Gel: A 9% SDS Page gel was prepared (4X Tris buffer pH 8.8, 30%acrylamide/0.8% bisacrylamide, 10% (w/v) ammonium persulfate, 0.01% TEMED). Gels were run in SDS Running Buffer (0.125M Tris Base, 0.96M Glycine, 0.5 w/v SDS) at 100v for 1 hour and 20 minutes, or until the bromophenol blue band reached the bottom of the gel. Proteins were transferred to Polyvinylidene difluoride (PVDF, Millipore) membranes in transfer buffer (0.04M Tris Base, 0.29M Glycine, 20% Methanol) at 100v for 1 hour.

Immunoblotting: Equal amounts of Sample (normalized for protein concentration) and pre-stained SDS broad range ladder (Biorad) were loaded, and the gel was run. Membranes were washed in PBST (2.7mM KCl, 1.4mM KH_2PO_4, 137mM

NaCl, 4.3mM Na_2HPO_4 $7H_20$, 0.1% Tween 20). Once the transfer was completed, the membrane was incubated for 1 hour in 2% BLOTTO solution (10g Non-fat dry milk, 500mL PBST) and primary antibody was prepared (1:2000 in 2% BLOTTO). After one hour, the membrane was placed in a sealed tub with the primary antibody solution for 30 minutes and then incubated at 4®C overnight. The membrane was subjected to three, 10 minute washes in 2% BLOTTO. Secondary antibody was prepared (1:10,000 in 2% BLOTTO) and added to the membrane for one hour. The membrane was again subjected to three, 10-minute washes in 2% BLOTTO, and given a final wash in 1X PBS. Alkaline Phosphatase staining reagents, NBT and BCIP (Promega) were added, as per manufacturer's instructions, to Alkaline Phosphate Buffer (100mM Tris pH 9.5, 5mM $MgCl_2$, 100mM NaCl) for membrane staining.

Immunoprecipitation: Anti-flag M2 affinity gel (Sigma-Aldrich) was thoroughly suspended in its vial before being transferred (at 30uL per sample) to a 1.5-mL microcentrifuge tube. The tube was centrifuged at 10,000rpm for 1 minute and the supernatant removed. The resin was washed twice in TBS (0.15M NaCl, 0.05M Tris HCl pH 7.4), where on the final wash, it was equally divided among the samples. Harvested cell pellets were resuspended in hypotonic lysis buffer (10mM Tris HCl, pH 7.5, 10mM KCl, 0.5mM EGTA, 1.5mM $MgCl_2$), incubated at 4°C for 15 minutes, and then centrifuged at 14,000rpm for 10 minutes at 4°C. The cell lysates were transferred to fresh microcentrifuge tubes, and spun again at 14,000rpm for 30 minutes at 4°C. This supernatant was drawn through at 0.45-μm filter into the tubes containing 30uL washed Anti-Flag M2 affinity gel. The suspension was rocked at 4°C for 2 hours, then centrifuged for 1 minute at 10,000 rpm. The resin was washed 3 times with TBS/0.5% NP-40 (0.15M NaCl, 0.5M Tris HCl pH 7.4, 0.5% NP-40), with 5 minutes of rocking at 4°C in between each wash. The centrifuged resin was then incubated twice with 250mg/mL Flag peptide at 4°C for 30 minutes and the eluate saved. Twenty microliters sample buffer was added to each sample and tapped to mix. Samples were boiled at 95°C for 5 minutes and then centrifuged at 8,000rpm for 30 seconds. The supernatant, now ready to be loaded onto a gel, was transferred to a fresh tube.

RESULTS

<u>NS5A WT is able to repress activation of the IFN-β promoter in a manner similar to the NS5A 10A mutant.</u> Past research has demonstrated NS5A's ability to reduce activation of the IFN-β promoter, thereby reducing the production of IFN-β. This research, however, has largely been conducted using the NS5A 10A and H27 mutants, and not WT NS5A. We recently created a wild type version of NS5A by site-directed mutagenesis of the H27 mutant, work carried out by TCU junior Akshaya Maller. She used site-directed mutagenesis to back-mutate the NS5A H27 leucine at residue 2198 to the original serine. A luciferase assay using cells expressing an IFN-β luciferase reporter gene was used to compare IFN-β promoter activity in cells not expressing NS5A with those expressing NS5A 10A, NS5A H27 and NS5A WT (Fig. 1a). The assay was followed by Western blot analysis to ensure successful transfection of the NS5A plasmids (Fig. 1b). Data analysis revealed repressed promoter activity in cells expressing NS5A as compared to control cells. Upon comparison of promoter activation following viral infection in the

presence of the three variants of NS5A, it was evident that the highest level of IFN-β promoter repression was seen upon expression of NS5A 10A in 293 cells when compared to cells expressing NS5A H27. It also appears that cells expressing NS5A WT behave more similar to those expressing NS5A 10A than NS5A H27.

1.A

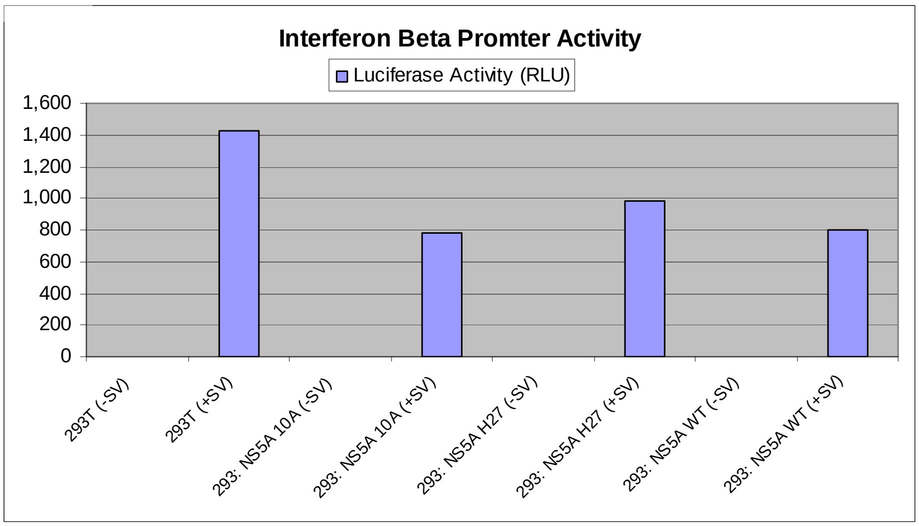
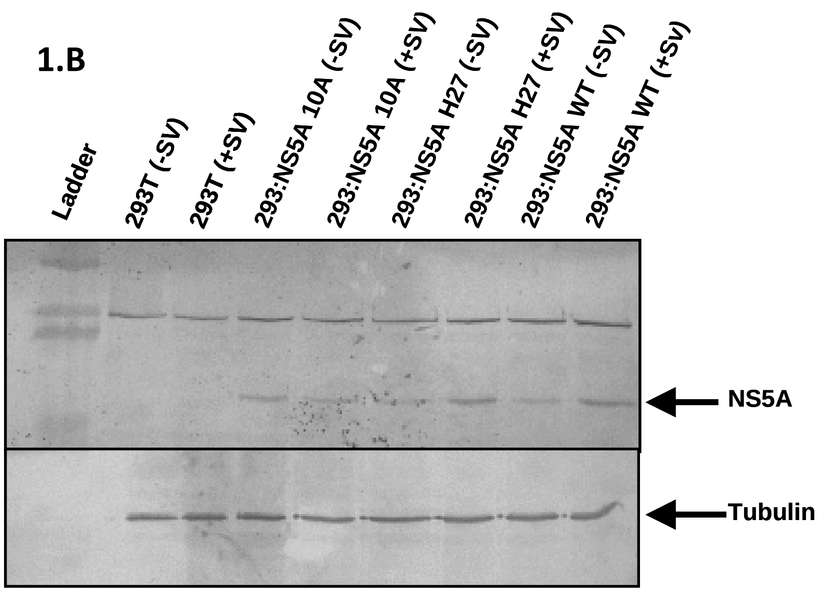


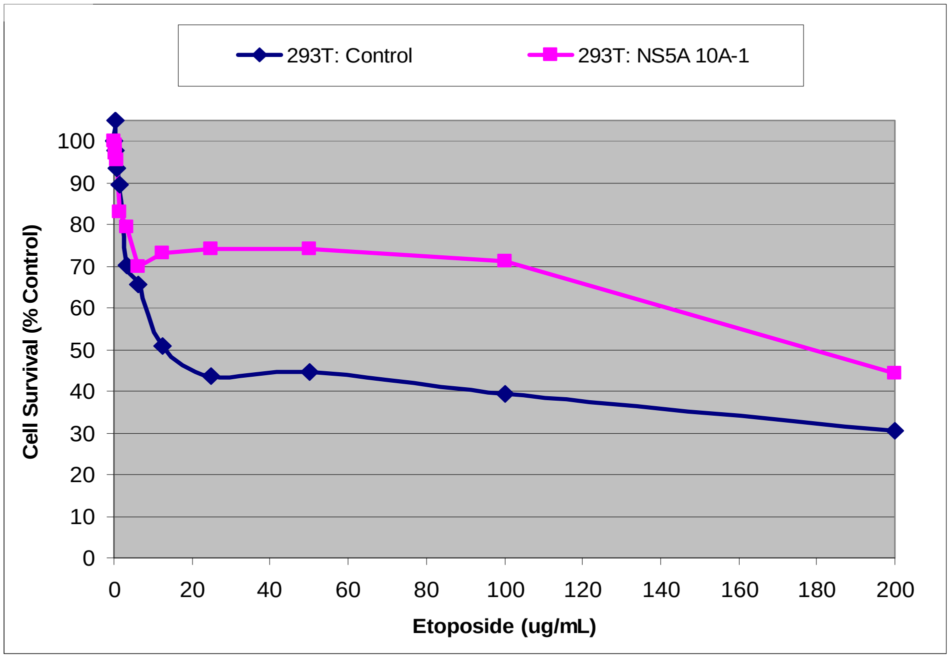
FIG 1. **NS5A WT is able to repress activation of the IFN-β promoter in a manner similar to the NS5A 10A mutant.** *A,* Luciferase assay (top) in which IFN-β promoter activation was measured following infection with Sendai Virus. *B,* Western Blot analysis (bottom) using anti-NS5A and anti-Tubulin.

1.B



NS5A decreases cell sensitivity to etoposide-induced cell death. Previous research has shown that NS5A interacts with a number of cellular proteins that are involved in tumor suppression. This indicates that the protein may be a significant factor in the interference of apoptosis leading to cell transformation, which can result in the development of hepatocellular carcinoma. To determine whether NS5A can disrupt a cell's tumor suppression capabilities, and thus block cell death, a cell death assay was used to assess the effect of the apoptosis-inducing drug etoposide on a HEK 293 cell line stably expressing the NS5A 10A plasmid (293:NS5A 10A, created by Shilpi Subramanian). As a control, I also treated non-transfected HEK 293 cells with the same concentrations of drug. Following staining with MTT, absorbance of the cell lysates was measured at 540nm. This was followed with Western blot analysis to confirm expression of NS5A in the 293:NS5A 10A cell line (Fig. 2b) Analysis of the data revealed that the LD50 (median lethal dose) of etoposide required for 293:NS5A 10A cells was much higher than that required for the 293 control cells (Fig. 2a), indicating that the 293:NS5A 10A-1 cells exhibited decreased sensitivity to etoposide-induced cell death as compared to the HEK 293 control cells.

2.A



2.B

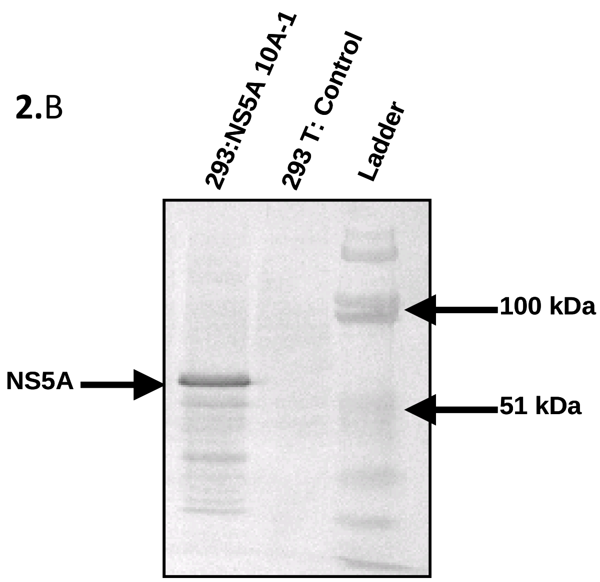
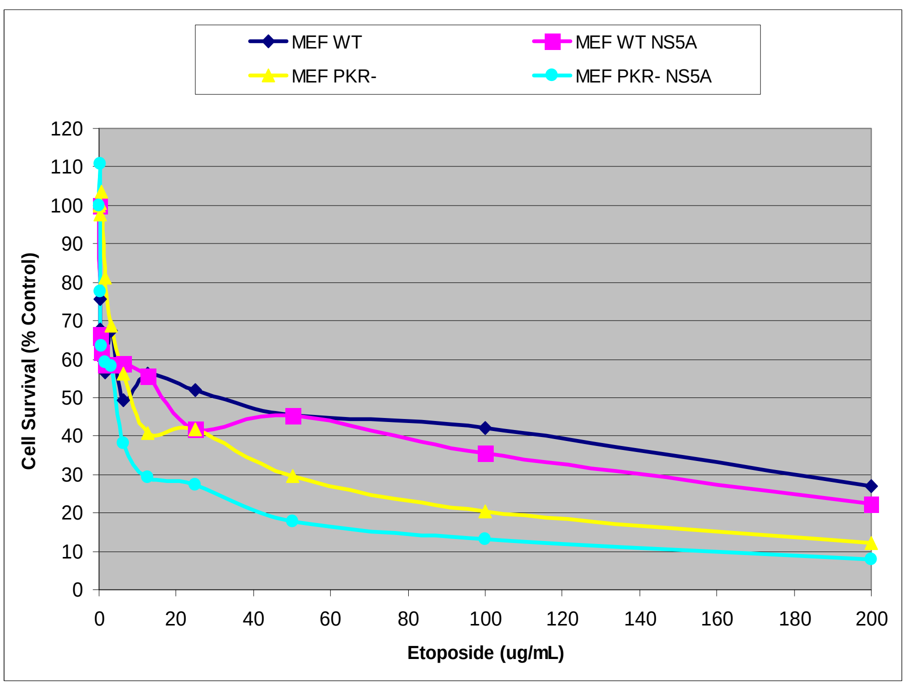


FIG 2. **NS5A decreases cell sensitivity to etoposide-induced cell death.** *A,* Cell death assay using the drug etoposide was carried out with HEK 293 control cells and cells expressing NS5A. Cell vitality was measured using a spectrophotometer following staining with MTT. The LD50 is approximately 12.5 µg/mL etoposide in control cells and 180 µg/mL in cells expressing NS5A. *B,* Western blot analysis using anti-NS5A antibody. NS5A is approximately 58 kDa.

<u>Cells expressing both PKR and NS5A are less sensitive to etoposide-induced cell death than cells not expressing PKR.</u> Previous research has shown that NS5A binds directly to PKR in infected cells, and suggests that NS5A may be inhibiting PKR's ability to induce apoptosis. If this is correct, a PKR $^{-}/^{-}$ cell line should exhibit the same characteristics as a cell line stably expressing both PKR and NS5A. To further explore the role played by the

interaction between NS5A and PKR in decreasing sensitivity to etoposide, wild type Mouse embryonic fibroblasts (MEF) and MEF PKR $^{-}/^{-}$ cells were transfected with 1μg pCDNA3: NS5A 10A, and treated with etoposide in a cell death assay. The assay also included controls of non-transfected MEF WT and MEF PKR $^{-}/^{-}$ cell lines. Following 18-20 hours of treatment with etoposide, cells were stained with MTT, and absorbance was measured. Analysis of data revealed that LD50 for MEF WT and MEF:NS5A 10A cells was higher than that of MEF PKR $^{-}/^{-}$ cells and MEF PKR $^{-}/^{-}$:NS5A 10A cells (Fig. 3). This indicates that PKR $^{-}/^{-}$ cells do not exhibit the same decrease in etoposide sensitivity as do cells producing HCV NS5A.

3.A



3.B

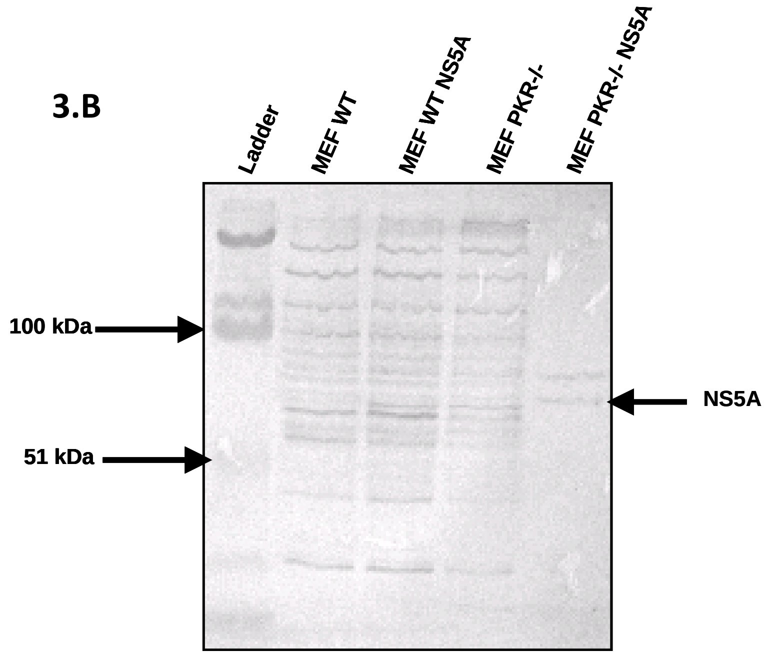
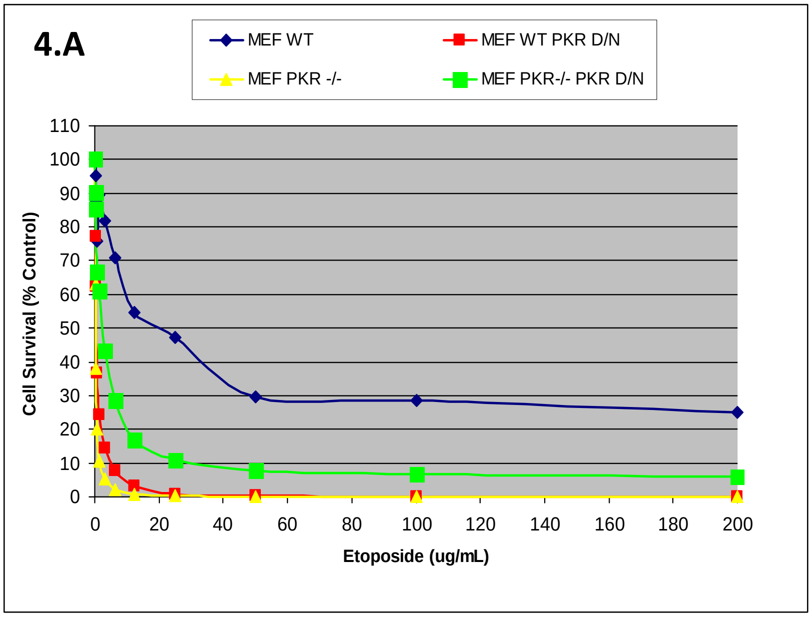


FIG 3. **Cells expressing both PKR and NS5A are less sensitive to etoposide-induced cell death than cells not expressing PKR.** *A,* Cell death assay using the drug etoposide was carried out with MEF WT and MEF PKR^{-}/$^{-}$ cells expressing NS5A. Cell vitality was measured using a spectrophotometer following staining with MTT. The LD50 is approximately 10 µg/mL etoposide in cells expressing both NS5A and PKR and 22 µg/mL in cells expressing both PKR and NS5A. *B,* Western blot analysis using anti-NS5A antibody. NS5A is approximately 58 kDa.

<u>Cells expressing PKR dominant-negative protein do not exhibit the same decrease in sensitivity as cells expressing NS5A.</u> To determine whether NS5A's binding to PKR is required to decrease sensitivity to etoposide, I transfected MEF WT and MEF PKR $^{-}$/$^{-}$ cells with 1µg PKR Δ/N plasmid. In cells expressing PKR Δ/N, PKR Δ/N will bind to WT PKR to inhibit its autophosphorylation and activation within the cell. Effectively, this would block the ability of PKR to carry out its function forcing the cell to behave like a PKR knockout cell line. I then ran a cell death assay with MEF WT and MEF PKR $^{-}$/$^{-}$ cells as controls. Analysis revealed that MEF WT PKR Δ/N cells exhibit a similar LD50 to MEF PKR $^{-}$/$^{-}$ cells, indicating that cells no longer expressing PKR behave like cells expressing an inhibited form of PKR (Fig. 4). This indicates that cells expressing WT PKR which is

bound and inhibited by PKR Δ/N do not show the same decrease in sensitivity to cell death as cells expressing NS5A.



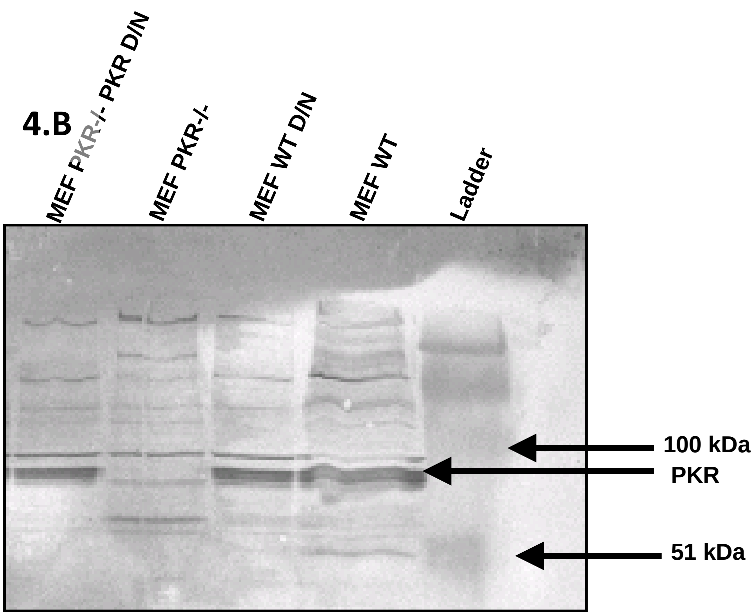
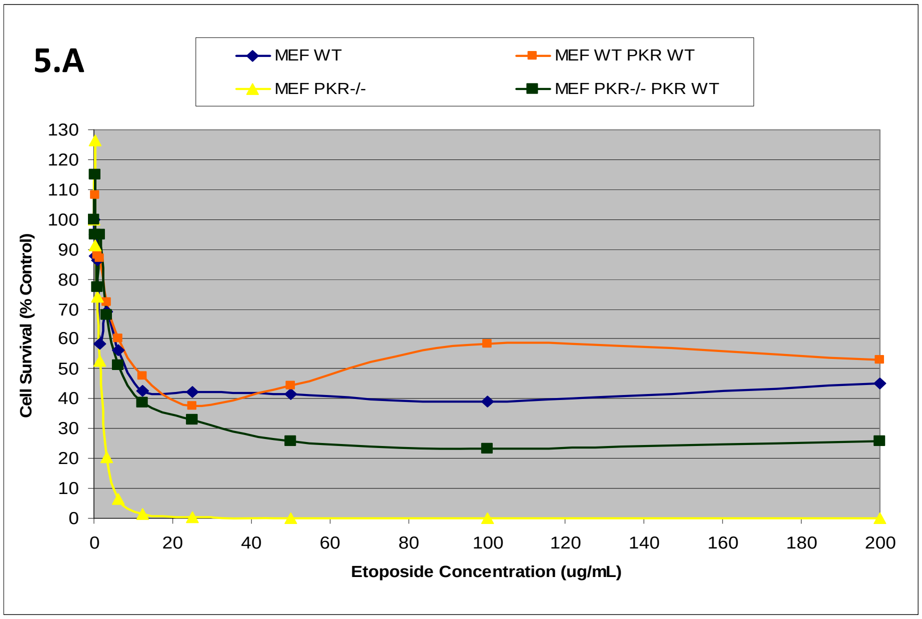


FIG 4. **Cells expressing PKR dominant-negative protein do not exhibit the same decrease in sensitivity as cells expressing NS5A.** *A,* Cell death assay using the drug etoposide was carried out with MEF WT and MEF PKR $^{-}/^{-}$ cells. PKR D/N expression is conveyed by transfection with PKR D/N. Cell vitality was measured using a spectrophotometer following staining with MTT. The LD50 is approximately 22 μg/mL etoposide in cells expressing PKR and 1-5 μg/mL in cells without active PKR or without PKR expression. *B,* Western blot analysis using anti-PKR antibody. PKR is approximately 63-69 kDa.

PKR function can be restored in PKR $^{-}/^{-}$ cells. To ensure that PKR expression in MEF PKR $^{-}/^{-}$ cells could be rescued, I transfected MEF PKR $^{-}/^{-}$ cells with 1μg PKR WT plasmid, tested with a cell death assay. LD50 was similar between MEF WT cells and MEF PKR $^{-}/^{-}$ cells expressing PKR WT, indicating that PKR function had been restored in MEF PKR $^{-}/^{-}$ cells (Fig. 5).



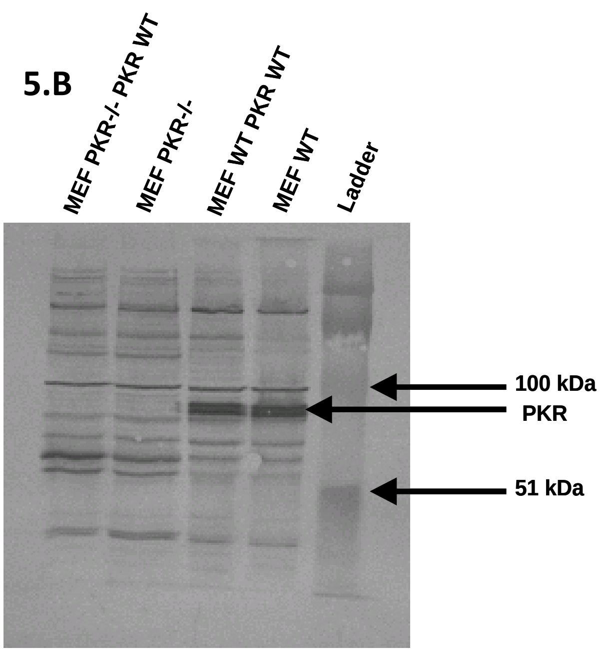


FIG 5. **PKR function can be restored in PKR $^{-}/^{-}$ cells.** *A,* Cell death assay using the drug etoposide was carried out with MEF WT and MEF PKR $^{-}/^{-}$ cells. PKR expression is conveyed by transfection with PKR WT. Cell vitality was measured using a spectrophotometer following staining with MTT. The LD50 is approximately 2 µg/mL etoposide in cells not expressing PKR and 5-10 µg/mL in cells expressing PKR. *B,* Western blot analysis using anti-PKR antibody. PKR is approximately 63-69 kDa.

Summary of the cell death assays. These assays have demonstrated first, that cells expressing NS5A are more resistant to cell death than control cells. The assays have also demonstrated that cells expressing NS5A are more resistant to cell death than cells which either lack PKR expression, or whose PKR activity is inhibited through protein-protein interaction within the cell. I am therefore able to conclude that NS5A's ability to block cell death does not require its ability to bind and inhibit PKR.

NS5A binds to the tumor suppressor protein p53. In order to determine the mechanism by which NS5A might regulate cell death other than its binding to PKR, it is important to identify proteins that NS5A interacts with in the cell. To do this we used co-immunoprecipitation (co-IP) to detect proteins that NS5A might bind to in addition to PKR. Using immunoprecipitation (IP), NS5A was pulled down from HEK 293:NS5A 10A cell lysates using M2 antibody-conjugated sepharose beads, and then eluted from the beads for further analysis by SDS PAGE and Western blot. A Western blot using anti-M2 revealed the presence of NS5A 10A in both the cell lysate and the eluate indicating that NS5A had been successfully pulled down(Fig.6a). Whole cell lysates made from 293 HEK cells, the 293:NS5A 10A cell line, along with the IP eluate were then separated on an SDS-PAGE gel, and stained using Coomassie brilliant blue in order to visualize bands corresponding to the presence of NS5A as well as any other protein that might be co-immunoprecipitated with it. The stain did not clearly reveal the presence of proteins other than NS5A in the IP eluate (Fig. 6b). Western blot analysis using anti-PKR (Fig. 6c) and anti-p53 antibodies (Fig. 6d) was preformed on these same samples, revealing the presence of both PKR and p53 in the NS5A IP eluate.

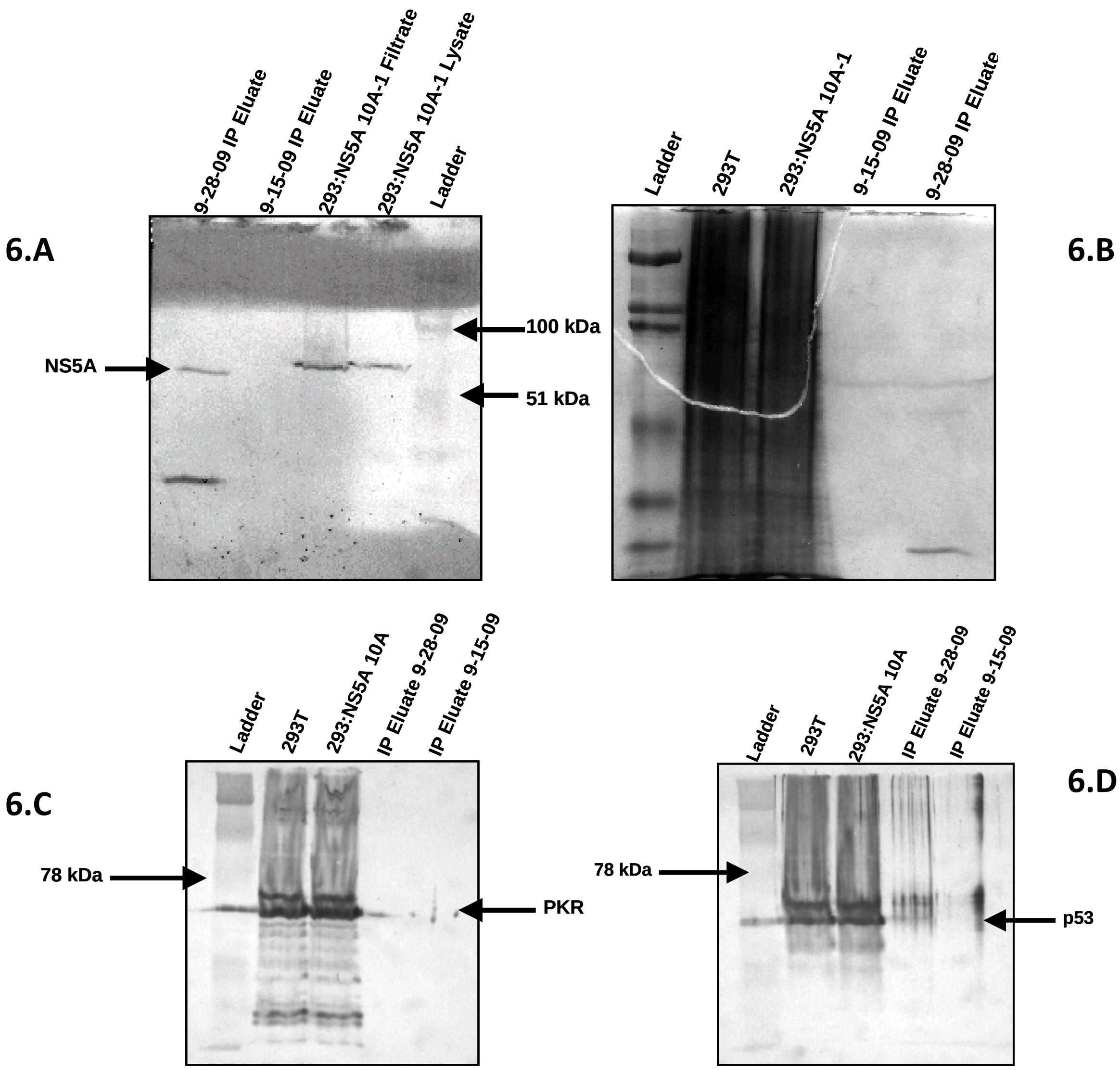


FIG 6. **NS5A binds to the tumor suppressor protein p53.** *A,* Immunoprecipitation of NS5A was followed by western blot analysis with anti-M2 antibody for NS5A. NS5A is approximately 58 kDa. *B,* IP eluate run on SDS-PAGE gel and stained with coommassie brilliant blue to identify all proteins in IP eluate. *C,* Western blot analysis of IP eluate with anti-PKR antibody. PKR is approximately 63-69 kDa. *D,* Western blot analysis of IP eluate with anti-p53 antibody. p53 is approximately 53-55 kDa.

DISCUSSION

The HCV protein NS5A has gained increasing recognition as an important player in HCV's infectivity, persistence, and its role in HCC. This is due to its apparent ability to co-regulate viral replication and virion egression, in addition to its capacity to regulate the functions of proteins involved in both the host cell antiviral response and tumor suppression. The goal of my research was to compare the behavior of NS5A observed *in vivo* during HCV infection with that of NS5A expressed in the replicon systems so widely used in research today. The replicon systems 10A and H27 are unique because they exhibit dissimilar replication rates when expressed in cell culture. The 10A replicon replicates at much higher rates than H27. Genome analysis shows that the only existing difference is located within the NS5A gene, suggesting that this protein is solely responsible for the difference in replication rates presumably due to its ability to affect cell cycle regulation and perhaps other cellular mechanisms. In my research I investigated the role of the two mutants, NS5A:10A and NS5A:H27, in the host cell's antiviral response and tumor suppression capabilities.

Upon viral infection, a transcription factor enhanceosome complex comprised of NFκB, ATF-2 and IRF-3, initiates expression of the antiviral gene IFN-β. Recent research has verified the ability of NS5A to suppress activation of this promoter, possibly by interfering with the activation of one or more of these transcription factors, thereby downregulating the antiviral response (65). Luciferase assays which use a reporter gene to measure the activation of the IFN-β promoter, have revealed that the NS5A 10A mutant is a stronger suppressor than NS5A H27 (G. Akkaraju, unpublished data). It is currently unknown how this compares with the ability of WT NS5A to suppress the promoter. WT NS5A was produced through site-directed mutagenesis of NS5A H27 and using this, I was able to compare activation of the IFN-β promoter in cells expressing WT NS5A, with cells expressing the 10A or the H27 mutants. These assays revealed that, in comparison to the 293 control cells, NS5A 10A inhibited promoter activation by 45% while NS5A H27 inhibted 31%. This confirms that NS5A 10A is a more effective suppressor than NS5A H27.

The luciferase assay also revealed that WT NS5A behaved more similarly to NS5A 10A than to NS5A H27 by inhibiting 44% of control cell promoter activity. However, luciferase assay results from previous research have revealed a more significant difference in suppression between NS5A 10A and NS5A H27 (66% and 1% respectively) than I was able to replicate in my experiments. This suggests that the relatively small difference I observed in IFNβ promoter suppression between cells expressing WT and 10A may not be reflective of the actual level of inhibition, and further testing would be required to verify results. My assay involved transfection of only 0.5 μg of NS5A DNA into the 293 IFN-β luciferase cell line, and increasing the amount could result in greater NS5A expression, producing amounts of NS5A similar to what is seen *in vivo* during HCV infection. An augmented level of expression might then result in a more accurate representation of NS5A expression, resulting in a more clear distinction in the suppression of the IFN-β promoter by cells expressing NS5A 10A and H27 mutants. Under these circumstances, I could then more clearly define the properties of WT NS5A.

To summarize results from my luciferase assays, I have been able to confirm that expression of NS5A has the ability to reduce activation of the IFN-β promoter, thus providing further evidence of NS5A's capacity to downregulate the host cell antiviral response. This data has confirmed that the NS5A 10A mutant is a stronger suppressor of the IFN-β promoter than the NS5A H27 mutant, although further testing might reveal greater variation between behavior of these two mutants. In addition, this data has revealed that NS5A WT expression inhibits the IFN-β promoter in a manner more similar to that seen in cells expressing NS5A 10A, than H27. This indicates that the NS5A 10A mutant behaves more like the NS5A expressed *in vivo* during natural HCV infection.

In addition to confirming the role of NS5A in mitigating the antiviral response, I was able to demonstrate NS5A's possible role in HCC development through its ability to block cell death in 293 HEK cells. This property, if shown to be true, would suggest that NS5A is able to deregulate cellular control of normal growth and division cycles in host cells presumably by repressing mechanisms involved tumor suppression. A loss of the ability to activate tumor suppressors such as pRb, p53 and p21, accompanied by secondary mutations in cell cycle control pathways could predispose the cell to undergo transformation leading to malignancies associated with HCC.

Results from cell death assays revealed that cells expressing NS5A required an LD50 of etoposide that was 15-fold greater than that required by control 293 cells. Since this signifies that NS5A expression is able to block cell death in 293 cells, my next step was to understand the mechanism by which NS5A might be blocking cell death in host cells. Previous research has revealed NS5A's ability to bind to and inhibit the antiviral protein PKR, which, in addition to its role in the antiviral response, has recently been labeled as a tumor suppressor protein due to the fact that several cancers types have been shown to have inactivating mutations in this gene. Therefore, it has been suggested that NS5A might block cell death by binding to and inhibiting PKR. However, cell death assays conducted on both cells lacking PKR gene expression and cells whose PKR proteins were inactivated revealed that inhibiting PKR does not reduce the sensitivity of the cell to death induced by etoposide. These results suggests that NS5A's ability to block cell death does not require its ability to inhibit PKR function, indicating that NS5A must mediate cell death through interactions with one or more other proteins besides PKR. This finding, if confirmed, would uncover a novel function of NS5A in regulating cell death.

If NS5A indeed acts independent of PKR to inhibit cell death, then the next task is to identify what other protein or proteins it interacts with to do so. To do this, we used an immunoprecipitation technique in which we first pulled down NS5A, and then tried to identify proteins which might have co-immunoprecipitated with NS5A. We assume that the function of any protein pulled down with NS5A would be modulated by its interactions with NS5A. To determine which proteins NS5A might interact with in order to block cell death, co-IP was performed on lysate from the 293:NS5A 10A cell line. After successfully pulling down NS5A protein with immunoprecipitation followed by Western blot analysis, I used Coomassie brilliant blue to determine if any other proteins might have been eluted during IP due to their interaction with NS5A. Although the stain did not reveal the presence of proteins besides NS5A, it would be important to follow up the next IP with a silver stain, which is more sensitive than Coomassie brilliant blue, and can detect proteins at much lower concentrations. Co-IP followed by immunoblotting revealed that the IP eluate containing NS5A also contained PKR and p53. While it has already been confirmed by previous research that NS5A binds to both of these proteins, this tells us that the technique is behaving as expected, and is pulling down cellular proteins interacting with NS5A. Further research should include co-IP with antibodies specific for other proteins involved in tumor suppression such as pRb (which regulates the cell cycle) and proteins of the Bcl-2 family (which regulate apoptosis).

In summary, my results confirm that NS5A has the ability to suppress activation of the IFN-β promoter, and reveal that WT NS5A may behave in a manner similar to the NS5A 10A mutant. This comparison will hopefully stimulate a series of tests to further analyze the similarities of WT NS5A to other NS5A mutants, revealing a deeper understanding of its role in areas such as cell replication rate and possible cell transformation capabilities. In addition to this, efforts to create a cell line constitutively expressing WT NS5A would increase the efficiency of these experiments, and produce more consistent results between experiments. My research has also demonstrated that NS5A does, in fact, block cell death, but that the search for its cellular targets in this event must continue. Cell death assays involving cell lines which lack the expression of tumor suppressor proteins such as p21 and pRb would be an important place to start, and assays involving a cell line expressing WT NS5A and NS5A:H27 would reveal other important data about the nature of the NS5A protein and the mechanism by which Hepatitis C virus infection can lead to different outcomes in different patients.

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VITA

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ABSTRACT

VIRUS VERSUS BODY: THE EFFECT OF THE HEPATITIS C VIRUS NS5A PROTEIN ON THE ANTIVIRAL RESPONSE AND CANCER DEVELOPMENT

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The Hepatitis C Virus (HCV) non-structural 5A protein (NS5A) has been studied for its ability to increase viral replication rates by repressing the host cell antiviral response and deregulating the host cell-cycle. Recent research conducted with NS5A mutants derived from the HCV replicon system demonstrated NS5A's capacity to repress activation of the IFN-β promoter. Using luciferase assays, I compared the suppression capabilities of the NS5A mutants 10A and H27 with WT NS5A, revealing that WT NS5A suppresses the IFN-β promoter more similar to the NS5A 10A mutant than the H27 mutant. I then used cell death assays to demonstrate NS5A's ability to block etoposide-induced cell death. To identify the mechanism by which NS5A is blocking cell death, I used cell death assays comparing cells expressing NS5A with cells lacking expression of the tumor-suppressor protein PKR, revealing that NS5A is must block cell death through interactions with a protein(s) other than PKR.