THE EFFECT OF ZIKA VIRUS PROTEIN NS5 ON THE HOST INNATE IMMUNE RESPONSE

by

Jacey Elliott

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HOST INNATE IMMUNE RESPONSE

Project Approved:

Giridhar Akkaraju, Ph.D.
Department of Biology

Matthew Hale, Ph.D.
Department of Biology

Ryan Schmitz, Ph.D.
Department of Spanish and Hispanic Studies

ABSTRACT

Zika virus (ZIKV) is single stranded RNA virus that has raised global health concerns in recent years, causing diseases such as Guillain-Barré Syndrome, adult meningoencephalitis, and fetal microencephaly. ZIKV is known to interfere with the host innate immune system, the first line of defense against viral pathogens. ZIKV inhibits the production of Interferon-β (IFN-β), an essential cell signaling molecule for the immune system, by interfering with proteins along the signaling pathway leading to the activation of interferon gene expression. The goal of this research is to identify the role of ZIKV nonstructural protein NS5 in antagonizing the innate immune response. We hypothesize that ZIKV NS5 binds to a protein within the antiviral signaling pathway, thus inhibiting the pathway and decreasing IFN-β production. This research focuses on the impact of NS5 on cytokine production as well as the mechanisms by which the viral protein is able to achieve this result.

INTRODUCTION

Zika virus (ZIKV) was first identified in a rhesus monkey located in the Zika Forest of Uganda in 1947. The first documented human infections later occurred in 1952 in Uganda and Tanzania [(5)]. Historically, Zika virus was isolated to the Asian and African continents [(2)]. Between the 1960s and 1980s, 14 sporadic cases were documented throughout these regions which were found to have no serious consequences [(5)]. An outbreak in 2007 in the Yap Islands of the Pacific Ocean affected approximately 75% of the population [(2,17)]. The virus continued to move across the Pacific Ocean until appearing in the Americas in 2014, creating a medical

emergency as much of the population was affected [17]. In the 2015 outbreak in Brazil, infections totaled over 1.5 million and spread to 29 other countries within the American continents [11]. In 2016, following the recent association of ZIKV infection with clusters of microcephaly and neurological diseases, ZIKV was declared a Public Health Emergency of International Concern by the World Health Organization [19].

The virus is transmitted primarily via infected female Aedes mosquitoes. ZIKV establishes an active replication cycle within the salivary glands of the mosquitoes, and this infected saliva is injected into the skin of a human when a blood meal is taken [7]. ZIKV can also be transmitted sexually, vertically (from mother to fetus), and through bodily fluids such as breast milk and blood transfusions [5].

Over 80% of individuals infected with Zika virus are asymptomatic, while 20% of patients exhibit mild fever, rash, and joint pain for a period of one week [2]. While most symptoms are mild, Zika virus has been found to cause numerous neurological complications such as Guillain-Barré Syndrome, adult meningoencephalitis, and fetal microencephaly [17]. Guillain-Barré Syndrome is a disease in which the body's immune system recruits an attack on the nerves, eventually leading to paralysis [8]. Meningoencephalitis is inflammation and infection of the brain and meninges, the three membranes that surround the brain and spinal cord [6]. Fetal microencephaly is a condition in which the brain does not develop normally in the fetus, resulting in a smaller than normal head [3]. Previous studies suggest that the presence of ZIKV specific IgM immunoglobulins in infants are one of the main factors for the development of microencephaly [11]. Furthermore, it is suggested that the protein phospho-TANK binding kinase 1 (TBK1), necessary for neural cell division in fetuses, is diverted away from the fetus in infected mothers contributing to the development of microencephaly [11]. ZIKV has been shown

to cause infertility in male mice [17]. There is currently no vaccine or specific antiviral drug treatment for ZIKV. The current recommended treatment includes fever reducers such as acetaminophen and repurposed nucleoside inhibitors such as Sofosbuvir [11].

ZIKV is a single-stranded positive sense RNA (+ssRNA) arbovirus that belongs to the family Flaviviridae [5], along with other viruses such as Dengue, Yellow fever, and West Nile. The viral genome is approximately 11kb in length, encoding 3 structural and 7 nonstructural proteins [5]. These proteins include structural proteins for the capsid (C), pre membrane (PrM) and envelope (E), and nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [11]. The capsid protein forms a nucleocapsid for the budding viral particles containing the RNA genome. The PrM protein exists underneath the layer of E protein, which functions in the binding and membrane fusion process to allow viral particle entry into host cells. NS1 functions in viral replication along with NS2A; NS2A also controls the interferon response and the release of viral particles from host cells. NS2B functions in viral replication and, along with NS3, comprises a serine protease for polyprotein cleavage. NS3 functions as a helicase/NTPase and protease. NS4A contains determinant factors for viral pathogenesis. NS4B contains components of the viral replication complex. Lastly, NS5 comprises an RNA-dependent RNA polymerase and methyltransferase [11].

NS5 is the largest protein encoded by the genome at 2,712 bases in length [5]. NS5 is a polymerase that increases the efficiency of RNA replication, using host machinery, within cells infected with Zika virus [5]. Previous studies have shown that NS5 has specific functions in interrupting the innate immune response within the infected host [15].

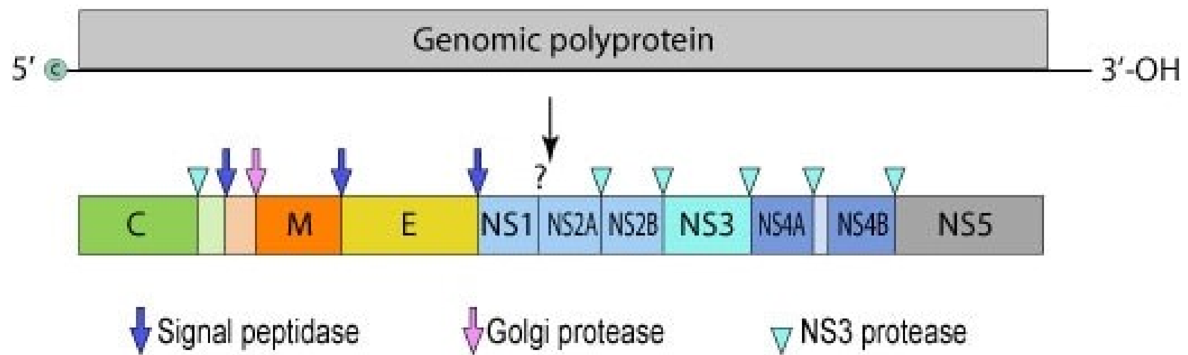

Figure 1: ZIKV genome. [1]

The innate immune response is the host's first line of defense against pathogens and is essential in the control of flavivirus reproduction within the host [2]. This response includes the production of cytokines IFN-α, IFN-β, and IFN-γ in response to the recognition of pathogen-associated molecular patterns (PAMPs) found on the surface of the pathogen [15]. Previous studies have shown that IFN type I (IFN-α and IFN-β) and type II (IFN-γ) signaling is inhibited by ZIKV NS5 [5].

When the +ssRNA enters a cell, the pattern associated molecular patterns (PAMPs) on the RNA are detected by intracellular proteins known as pattern recognition receptors (PRRs). RIG-I is a PRR that recognizes RNA. When RIG-I becomes activated upon binding RNA, the activated protein will initiate a response pathway that will eventually result in the expression of IFN-β. NF-KB and IRF-3 are both transcription factors which, when activated, move into the nucleus and bind to the promoter of the IFN-β gene, initiating transcription of IFN-β. The IFN-β produced will then leave the cell and bind to interferon receptors in both autocrine and paracrine fashion, recruiting an antiviral response against the pathogen [15].

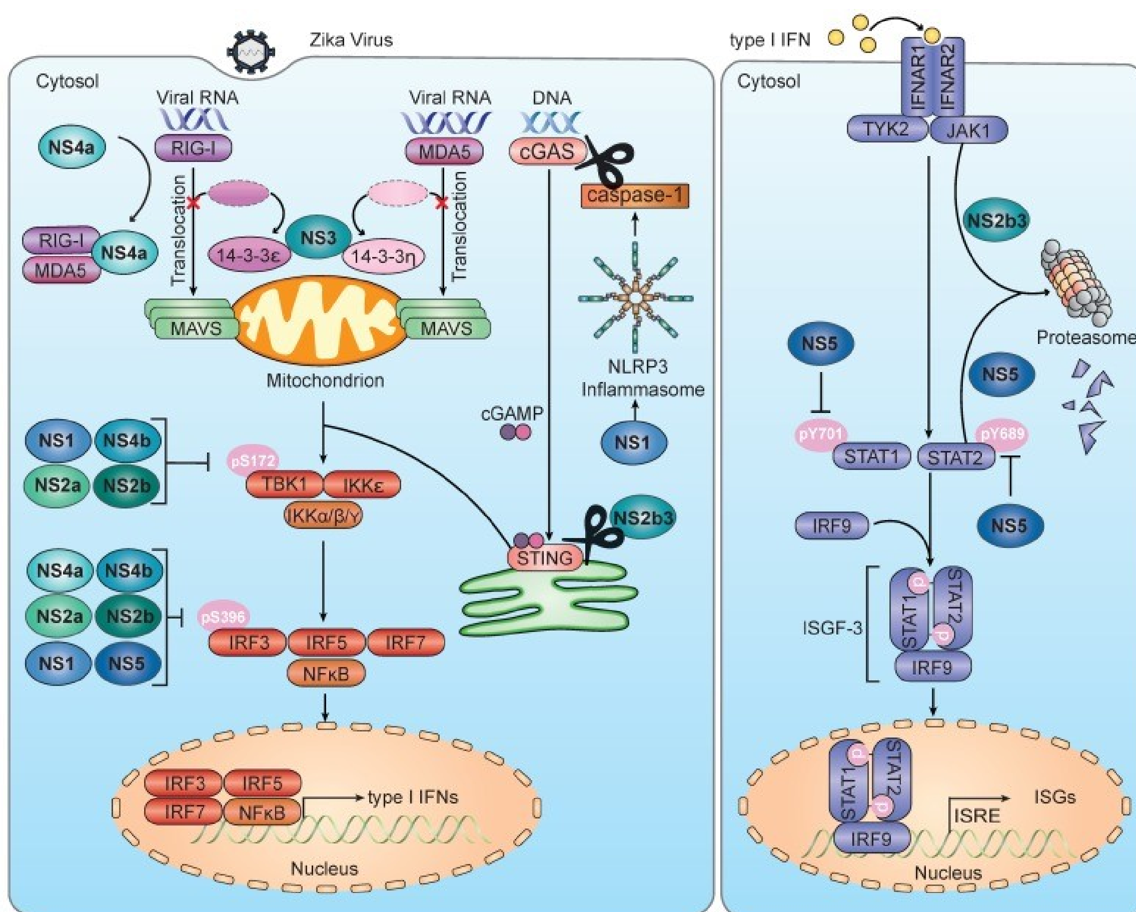

Figure 2: Antiviral immune response. [15]

In a successful viral life cycle, the virus particle gains entry into a cell where it will then release its +ssRNA genome into the cytoplasm of the cell. Host machinery is used to translate the polyprotein, which is then cleaved by viral proteases encoded by the ZIKV NS3 gene. Other viral proteins, such as NS5, interact with the RIG-I signaling pathway to inhibit the production of interferons. Without a recruited antiviral response, the virus is allowed to continue replication and release of new viral particles from the cell, furthering the infection within the host [15].

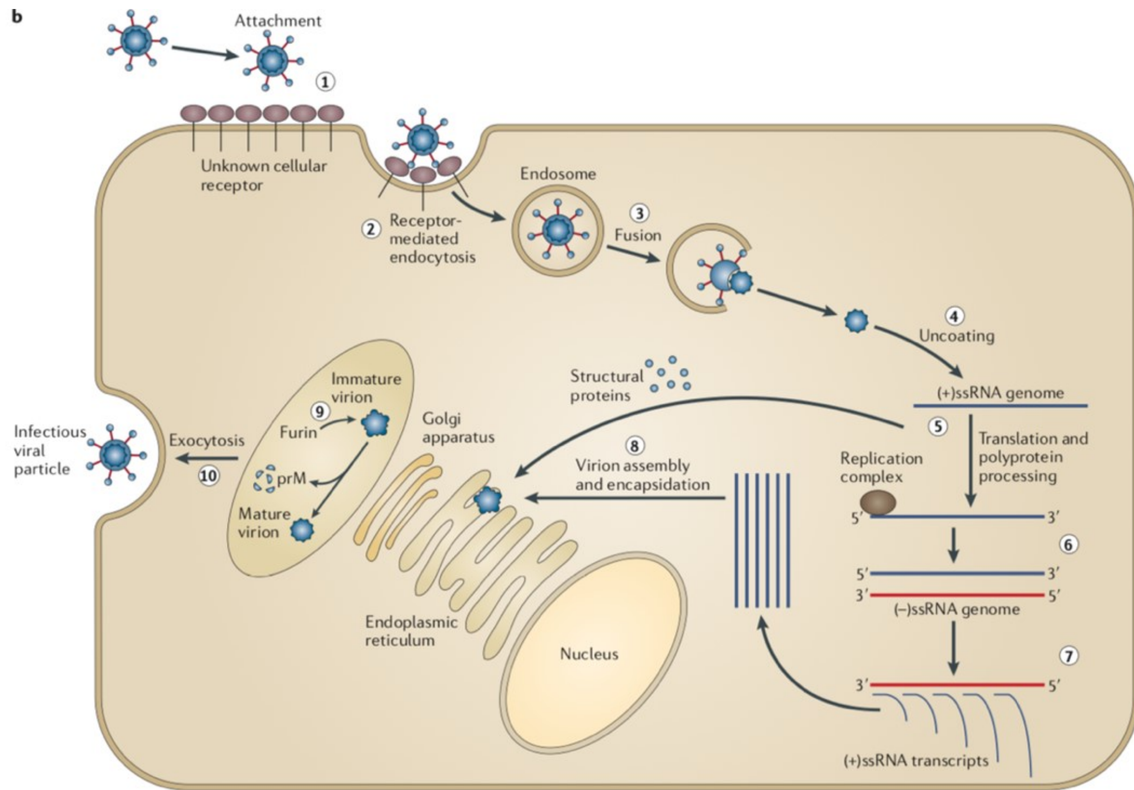

Figure 3: Life Cycle of an RNA virus. [16]

The goal of this research is to understand the mechanism of action of ZIKV nonstructural protein NS5, particularly within its role of antagonizing the innate immune response. Once the role of the NS5 protein is further understood, compounds that inhibit the function of NS5 can be identified and tested, thus allowing a successful antiviral immune response to be induced. This compound could have potential as a drug for the treatment of ZIKV.

METHODS

Transformation of E. Coli DH5α with ZIKV genome

The genome of ZIKV was used to transform chemically competent DH5α *Escherichia coli (E. coli)* bacteria using the heat shock method. The transformed bacteria were plated on LB/Amp^+ agar plates and allowed to incubate overnight. Colonies were selected from the plates and expanded using a mini-preparation containing ampicillin and LB. 100μL of these cultures were used to prepare a midi-preparation. The DNA from the midi-preparation was purified using QIAGEN buffers and elution through a gravity flow filter. To ensure the genome was successfully transformed into the bacteria, the purified DNA was run on an Agarose gel and analyzed against 10kb HyLadder. The size of the genome shown on the gel was approximately 11kb, the same size as the ZIKV genome [11]. The DNA was extracted from the Agarose gel and purified using GeneClean (MP Biomedicals) following the manufacturer's protocol.

Polymerase Chain Reaction for NS5 gene

RNA primers were synthesized for the ZIKV NS5 gene (idtdna.com).
The sequence of the 3' primer was as follows:
5'GCGGCGGCCGCTAGCAACACTCCGGGTGTGGACCCTTCCTCA3'.
The sequence of the 5' primer was as follows:
5'GCGGAATTCGCCACCATGGGAGGTGGGACGGGAGAGACTC3'.
Using polymerase chain reaction (PCR) and these specific 5' and 3' primers, the NS5 gene was selectively amplified. The PCR cycle included 30 seconds at 94°C, 30 seconds at 55°C, and 7 minutes 55 seconds at 72°C for 35 cycles. To confirm that the gene was successfully bound by

the primers, the PCR product was run on 1% Agarose gel. The PCR product had a size near 2,700bp, the approximate size of the NS5 gene. This PCR product was extracted from the gel and purified using GeneClean following the manufacturer's protocol.

Cutting NS5 and EFIRES-p with restriction enzymes

EFIRES-p was selected as an expression vector in which to insert the NS5 gene. EFIRES-p has a genome measuring approximately 5,700bp. NotI and EcoRI were identified as viable restriction enzyme locations to use for insertion of the NS5 gene into the plasmid. The purified PCR product and EFIRES-p were both cut in a 37°C warm bath for 2 hours with NotI and EcoRI restriction enzymes (New England Biolabs). The cut products were run on an Agarose gel next to uncut plasmid and insert to ensure that the plasmid and insert were both cut by the restriction enzymes. The cut plasmid and insert were extracted from the Agarose gel and purified using GeneClean following the manufacturer's protocol.

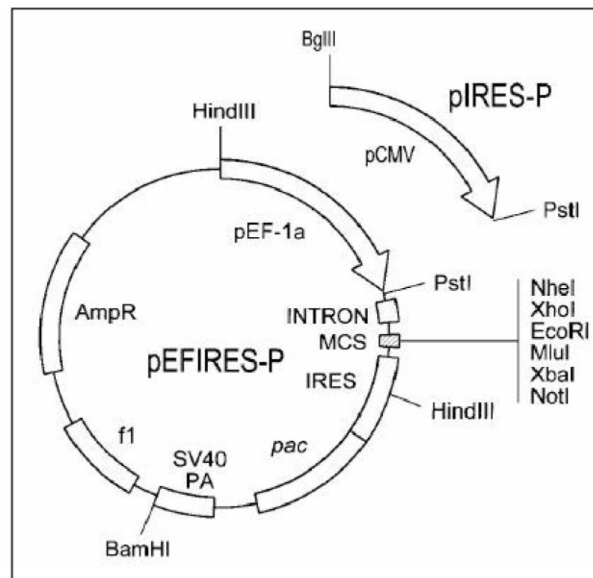

Figure 4: EFIRES-p expression vector with restriction enzyme sites. [13]

Ligation and transformation

The cut plasmid and insert were ligated together on ice for 2 hours, then used to transform chemically competent DH5α *E. coli* bacteria using the heat shock method. The bacteria were plated on LB/Amp^+ agar and allowed to incubate overnight. Colonies were selected from these plates and used to prepare mini-preparations containing LB and ampicillin. After shaking overnight in a 37°C incubator, the bacteria from these preparations were lysed and the DNA was purified using TEGL (25mM Tris-HCl pH 8, 50mM Glucose, 10mM EDTA, 4mg/mL Lysozyme), NaOH (0.2M NaOH, 1% SDS), and 7.5M ammonium acetate.

Cutting and analyzing ligated expression vector

The purified DNA was then cut again using EcoRI and NotI restriction enzymes for 2.5 hours in a 37°C water bath to examine whether the insert was successfully taken up by the plasmid, as shown by a successful extraction back out of the plasmid. The cut DNA was run on an Agarose gel and analyzed for bands resembling the extracted NS5 insert and EFIRES-p. The resulting bands appeared near 2,700bp and 5,700bp, corresponding to the sizes of NS5 and EFIRES-p, respectively. A band also appeared near 8,500bp, the size of ligated plasmid and insert together that remained uncut by the restriction enzymes.

RESULTS

The goal of this project is to create a functional eukaryotic expression vector containing the ZIKV NS5 gene that can be used to transfect human cells, allowing NS5 to be expressed in a human cell line. The ZIKV NS5 protein is hypothesized to inhibit the innate immune response pathway, inhibiting the ability of the cell to recruit a successful antiviral response. If this hypothesis is supported, cells expressing NS5 should show a decreased expression of IFN-β when infected with virus in comparison to cells not expressing NS5. Once the expression vector is successfully transfected into human cells, the baseline expression of IFN-β in the presence of NS5 will be measured using an IFN-β-luciferase promoter reporter construct and compared to cells not expressing NS5. The cells will then be infected with Sendai virus to stimulate an antiviral response in the cell. The level of expression of IFN-β in the presence of NS5 upon infection with virus will then be measured. This expression level will be compared to infected cells that do not express NS5 to determine the degree to which NS5 inhibits the antiviral response.

Transformation of DH5α with ZIKV Genome

After transformation of DH5α with the ZIKV genome, DNA was extracted from the bacteria, purified, and run on an Agarose gel next to HyLadder 10kb. This was performed to analyze the size of the transformed genome. The size of the genome shown on the gel was approximately 11kb, the same size as the ZIKV genome [11]. This suggests that the ZIKV genome was successfully transformed into the bacteria.

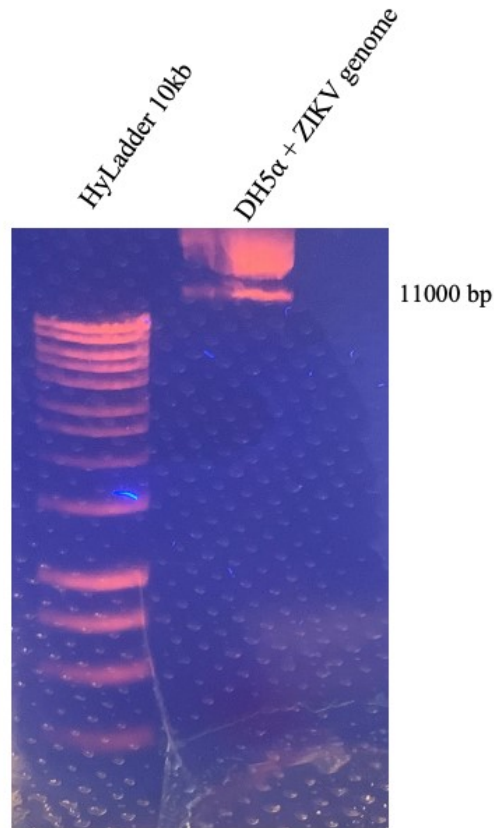

Figure 5: Agarose gel containing DNA from purified DH5α following transformation with ZIKV genome.

Polymerase Chain Reaction (PCR)

PCR was performed to selectively isolate and amplify the NS5 gene from the ZIKV genome. The amplified gene was to be cloned into an expression vector to allow the NS5 gene to be expressed in eukaryotic cells. The PCR product, obtained from using 3' and 5' primers for the NS5 protein, was run on a gel next to HyLadder 10kb to analyze the size of the gene product. The size of the amplified gene was shown to be near 2,700bp, which is the approximate size of the NS5 gene. This result suggests that the NS5 gene was successfully amplified using PCR.

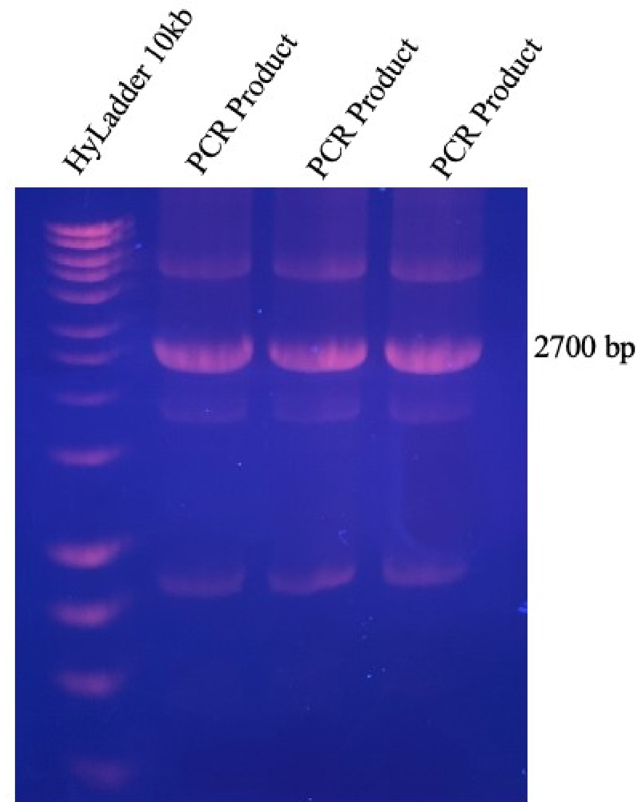

Figure 6: Agarose gel containing PCR product after amplification with NS5 primers.

Cutting Ligation Product

The amplified NS5 gene from PCR and the EFIRES-p expression vector were both cut with restriction enzymes to create complementary sticky ends. These complementary ends allowed the NS5 gene to be inserted into EFIRES plasmid to create a functional eukaryotic expression vector. After the NS5 insert and plasmid were ligated together and transfected into DH5α, the DNA from the bacteria was extracted and purified. This DNA was again cut with the same restriction enzymes, EcoRI and NotI, to determine if the NS5 insert could be cleanly extracted back out of the ligation product. After cutting the ligation product again with NotI and EcoRI restriction enzymes, the cut and uncut samples were run on an Agarose gel next to HyLadder

10kb in order to analyze the size of each component present in the ligation product. The uncut samples were run as a control to ensure that the cut products were successfully cut by the restriction enzymes. The cut samples produced bands at 2,700bp and 5,700bp. These bands suggest the presence of NS5 and EFIRES-p, respectively. The resultant band near 8,500bp suggests the presence of ligated plasmid and insert that remained uncut by the restriction enzymes. These results suggest that the NS5 insert was successfully taken up by the EFIRES-p expression vector.

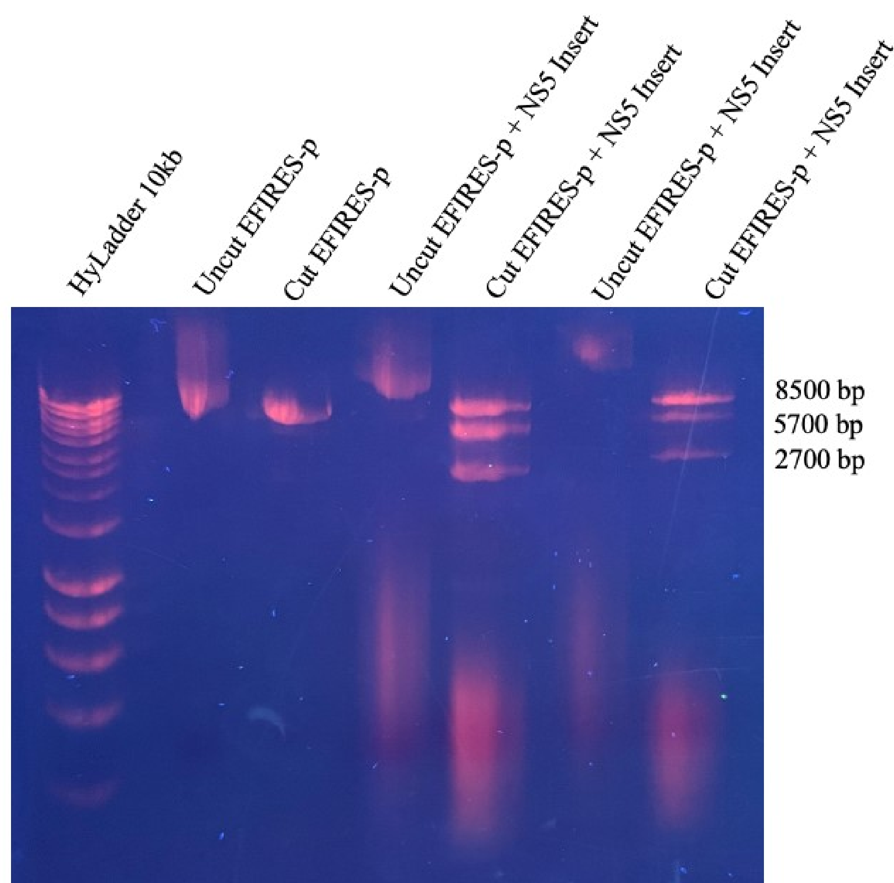

Figure 7: Agarose gel containing purified ligation product following cutting with NotI and EcoRI restriction enzymes.

DISCUSSION

These results suggest that the NS5 gene of ZIKV was successfully inserted into the expression vector EFIRES-p. This work will allow for further investigation of the proposed hypothesis that ZIKV NS5 binds to a protein within the immune response signaling pathway, thus inhibiting the pathway and decreasing IFN-β production. Future steps will involve transfecting HEK293 cells with this expression vector, allowing the NS5 gene to be expressed in a human cell line. These transfected cells will be infected with Sendai virus to analyze how the cells recruit an immune response in the presence of viral infection. If the proposed hypothesis is supported, the presence of the NS5 gene in these cells should inhibit the ability of the cell to activate the signaling pathway leading to the expression of IFN-β. Various drugs and compounds can be tested on these infected cells to determine how the immune response is altered by these compounds. The future goal of this project is to identify compounds that can block ZIKV NS5, allowing the full antiviral immune response to be recruited in response to viral infection. Ultimately, any identified compounds could be a potential drug for ZIKV treatment. An anti-NS5 drug would allow for an infected cell to increase production of IFN-β, stimulating surrounding immune cells to carry out an antiviral response. This response would include activating phagocytic cells to kill virally infected cells, halting the viral reproduction cycle within the host and terminating viral infection. This recruited immune response could prevent the development of acute infection and long-term neurological complications associated with ZIKV.

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