HOST-PATHOGEN INTERACTIONS: USING VESICULAR STOMATITIS VIRUS TO DISCOVER THE RELATION BETWEEN UNTRANSLATED REGIONS OF MRNA AND HUMAN IMMUNITY

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ABSTRACT

Vesicular stomatitis virus (VSV) is a negative-sense RNA pathogen that infects livestock and humans with major implications in modern-day vaccines. Recently, VSV has been found to decrease the length of the human 3' untranslated regions (3'UTR) of messenger RNA in cells. Prior research suggests that 3'UTR length contributes to innate immune system regulation. Double-stranded RNA (dsRNA) is often used as a viral genome, so when long 3'UTRs form dsRNA, PRRs mistake "self" dsRNAs as foreign from viral pathogens, thus activating the immune response. This indicates that VSV has a selective advantage by shortening 3'UTR length to avoid detection and reduce host innate immunity. A more novel UL (ultra long) to ORF (open reading frame) ratio technique, derived from qPCR fold change, was normalized to a negative control to ascertain which gene (and associated protein) in VSV was responsible for this phenomenon. We found that the VSV glycoprotein (G protein) is most likely to be responsible. Compared to VSV's other protein-coding genes, only the qPCR fold change ratios for the *VSV-G* gene indicate 3'UTR shortening of both the PKR and SMC1A genes' mRNAs in THP-1 cells, being 0.349 and 0.254, respectively. Additionally, qPCR fold change values of IFN production with *VSV-G* demonstrate a decreased abundance of these innate immunity signalling molecules. Such data imply *VSV-G*'s immense role in host cellular immune dysregulation amidst viral infection, bringing into question the safety of recombinant VSV vaccines and calling for future studies that investigate the exact mechanisms utilized by the VSV-G protein for pseudotyping purposes.

INTRODUCTION

Belonging to the *Vesiculovirus* genus of the *Rhabdoviridae* family, VSV possesses an approximately 11kb single-stranded, negative-sense RNA genome that codes for five structural proteins (Figure 1): nucleoprotein (N), phosphoprotein (P), RNA-dependent RNA polymerase (L), matrix protein (M), and glycoprotein (G) (1, 2). Proteins N, P, and L form the ribonucleoprotein (RNP) complex required for the transcription process; protein M condenses the nucleocapsid and drives virion budding; and protein G studs the surface of the virion.

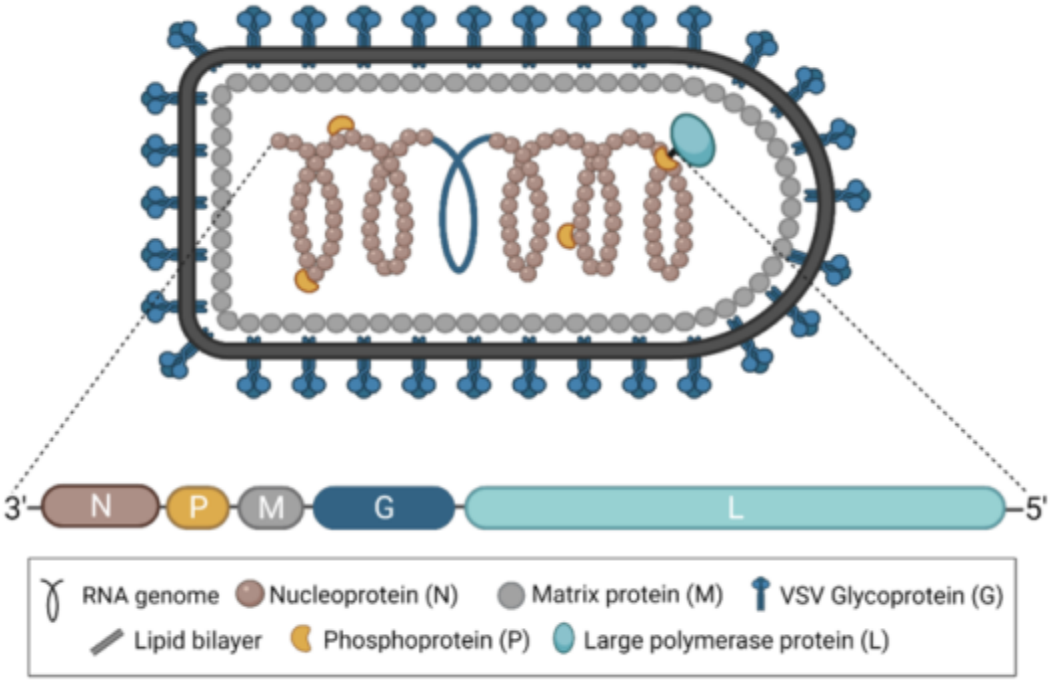


Figure 1. VSV cell and RNA genome (4). Vesicular stomatitis virus cell with a negative-sense RNA genome and five labeled proteins.

In human cells, the 3'UTR section of the mRNA sequence immediately follows the stop codon. The known functions of 3'UTR are the regulation of mRNA and protein-protein interactions (PPIs). Early findings of 3'UTRs revealed that divergent 3'UTR sequences correlate with differing functions for proteins and tissues, suggesting 3'UTRs play a crucial role in influencing the function of similar proteins. In addition, homology across 3'UTR gene sequences is highly conserved across organisms, indicating their important role in the regulation of mRNA stability. Fragments of 3'UTRs were also necessary for subcellular mRNA localization, regulating the spatial organization and aiding in early development and appropriate gene expression. When linked with RBPs, 3'UTRs aid in temporal protein production through regulation of translation to ensure gene expression at necessary developmental stages. Some protein-protein interactions also require one of the proteins to be bound with a 3'UTR, demonstrating the importance of 3'UTRs in a variety of protein functions. The length of 3'UTR is determined by 3'end cleavage and polyadenylation (6). Polyadenylation is a step in RNA processing involving the addition of the poly(A)-tail onto polyadenylation sites (PAS) within the 3' end of mRNA. Because a single gene can have multiple PASs, the differential usage of these PASs, or alternative polyadenylation (APA), will result in 3'UTR ends and mRNA strands of varying lengths. Depending on the expression levels of different polyadenylation factors, proximal or distal PAS usage—attaching the poly(A)-tail to the first PAS or to a PAS further downstream—will be favored. Proximal PAS usage shortens the mRNA strand, and distal PAS

usage lengthens it (Figure 2, 7). With the lengthening of such 3'UTR ends, the mRNA strands can fold over, forming hairpin loops or bulges. As one strand folds back to link with another, complementary sequences will pair. As hydrogen bonds between the paired bases stabilize this new structure, a double-stranded RNA molecule (dsRNA) is created (8). Since this nucleic acid structure is characteristic of many viral genomes, human immune systems recognize dsRNA as foreign microbial-associated molecular patterns (MAMPS), inducing a response in the immune system. Dorrity et al. (9) have hypothesized that 3'UTR double-stranding will trigger an antiviral response through the activation of pattern recognition receptors, or PRRs.

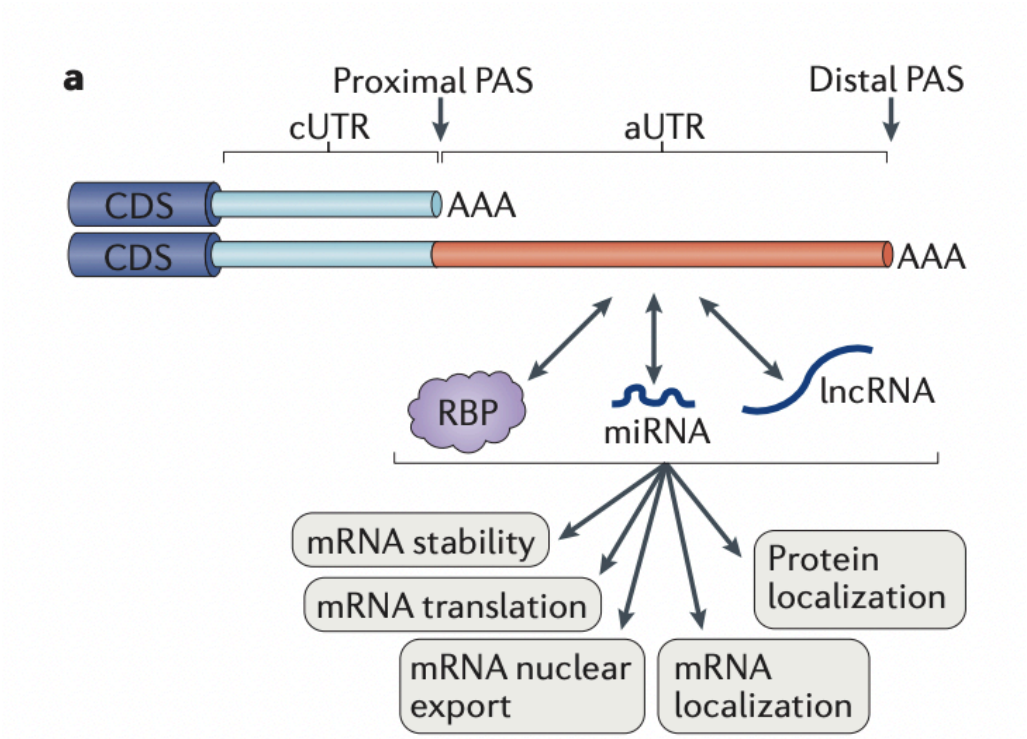


Figure 2. Alternative Polyadenylation (APA) Mechanism (*7*). Alternative Polyadenylation, binding partners, and functions of 3'UTR.

PRRs are a class of receptors within the innate immune system. Because dsRNA is commonly used as a viral genome, a number of PRRs recognize dsRNA as a ligand, which initiates signaling cascades that promote the production of type 1 interferons (IFNs) (10-12). IFNs are critical innate immunity signaling molecules, and their production allows for the expression of interferon-stimulated genes (ISGs) that have antiviral effects (13). Secreted as part of the initial cellular response to infection, type 1 IFNs regulate inflammation, promote the production of proteins that interfere with replication in infected cells, and enhance the activation and function of the adaptive immune response (14, 15). The effects of type 1 IFN on the pathology of an infection are highly context-dependent, and their ability to both limit and exacerbate inflammation can directly promote or hinder the body's immune response (14, 16).

The regulation of type 1 IFN production, therefore, plays a crucial role in the fate of viral infection.

Because longer 3'UTR regions on mRNA may become double-stranded, researchers have debated whether the choice between adding the poly-A tail to the distal or proximal PAS enhances the efficacy of an organism's immune response. Whereas the research of Jia et al., which experimented with VSV, suggests that longer 3'UTR regions increase the efficacy of the immune response, research by Bergant et al. found that lengthened 3'UTRs as a result of influenza A caused a decrease in interferon production (17). Jia et al. (5) found that VSV could shorten 3'UTRs, which could render the cell vulnerable to viruses such as VSV. However, an experiment conducted by Bergant et al. (17) increased the formation of dsRNA by increasing the length of the 3'UTR. They found that increased 3'UTR length led to decreased effectiveness in fighting the Influenza A virus (IAV). This discovery suggests that the relation between longer 3'UTRs and increased immune response may be more complicated than previously thought.

While Bergant et al. were able to identify the proteins responsible for 3'UTR lengthening in IAV, Jia et al. have yet to find the specific proteins that cause differential 3'UTR length, particularly in VSV. Thus, by identifying the specific gene responsible for the modification of 3'UTR length in VSV, this project seeks to further the understanding of possible mechanisms of 3'UTR immune pathway evasion by other viral pathogens. Moreover, with VSV being an effective vaccine platform (4), a thorough understanding of its impacts on human cells is crucial for safe clinical use.

Researching how VSV alters 3'UTR length sheds light on viruses with similar effects on 3'UTR length and their impact on the immune response. For instance, illness-causing viruses such as Herpes Simplex Virus 1 (HSV-1) and La Crosse Virus (LACV) have been shown to induce APA, altering 3'UTR length as a result (17). Shortened 3'UTRs in HSV-1 caused a decrease in cytokine production, preventing ISGs from being properly translated. As a result, inflammatory mediators are expressed at a lower rate. Similarly, LACV decreases the natural immune response by lengthening 3'UTRs to an extreme level (17). As further suppressive RNA-binding proteins (RBPs) are able to bind to the lengthened mRNA transcript, ISGs are silenced, failing to make an adequate amount of IFNs (18). In identifying the specific gene responsible for the modification of 3'UTR length in VSV, this project seeks to further the understanding of possible mechanisms of 3'UTR immune pathway evasion by other viral pathogens.

METHODOLOGY

Cell Preparation

For the purpose of this experiment, THP-1 cells were used. THP-1 is a monocyte-like cell line of a young male patient with acute myeloid leukemia. Due to its ability to differentiate into

macrophages, THP-1 could act against bacterial infection, which is why it was chosen when working with VSV. Additionally, the ability to be suspended in the cell medium, which maintains a uniform distribution and permits movement of cells around the medium, was beneficial for this study.

The THP-1 cells were grown and maintained in RPMI medium supplemented with the following: glutamine, non-essential amino acids to stimulate faster growth, fetal bovine serum (FBS), and a mixture of antibiotics (penicillin and streptomycin). The cells were then incubated at 37°C and a 5% CO2 level, mimicking the environment of a human body. Cell density was monitored, and cells were passaged as needed.

Counting and Plating

After the cells had grown to sufficient density, they were plated. To determine how many cells would be added to each well, the cells were first counted using a hemocytometer in order to determine the cell concentration in cells/mL. One million cells were added to each well.

Transfection

Lipoplexes, nonviral lipid carriers of genetic code, transported the desired VSV gene in plasmids, which are molecules of circular DNA. This process, referred to as transfection, transports each gene of interest into the plasma membrane of THP-1 cells.

All 5 VSV genes— *VSV-N*, *VSV-P*, *VSV-L*, *VSV-M*, and *VSV-G*— were transfected individually. *IAV-NS1*, a gene in the influenza A virus known to lengthen UTRs (16), was transfected as a positive control. The other two controls were negative: a mock and an unstimulated sample. The mock well was made to test if the act of using lipoplexes would affect 3'UTR length and immune response; thus, only the lipofectamine mix with no RNA was added. The unstimulated well was used as a baseline to see the 3'UTR length and immune response of a regular cell, so only Opti-MEM was added.

First, 5 µL of lipofectamine to 245 µL of Opti-MEM were mixed. The mock control well contained 250 µL of lipofectamine mix and 250 µL of Opti-MEM; the untransfected control well contained 500 µL of Opti-MEM. For the GOI and *IAV-NS1* wells, 250 µL of lipofectamine mix was combined with 250 µL of a plasmid and Opti-MEM combination, with the amount of plasmid solution used depending on the concentration (Table I). Each of the 500 µL solutions was then added to the THP-1 wells and incubated for roughly 48 hours.

Table I: The concentration of plasmid in the solution provided.

Gene of Interest	*VSV-N*	*VSV-P*	*VSV-M*	*VSV-L*	*VSV-G*	*IAV-NS1*
Concentration (ng/µL)	738.2	676.6	396.9	124.7	657.5	761.1

RNA Extraction

The THP-1 cells were extracted from each of the wells through centrifugation and were resuspended in 350 µL of Monarch StabiLyse lysis buffer in order to lyse the cells. Through a series of centrifugation and rinsing with Monarch Buffer WZ, Monarch Buffer BX, and a DNase mix composed of 10 µL DNase I and 70 µL of reaction buffer, the RNA was extracted and purified from DNA, salts, proteins, and nucleases that could have led to RNA degradation. Nuclease-free water was then used for the elution of the pure RNA from an S2A spin column into a microfuge, and a Nanodrop was used to measure the concentration of RNA extracted (Table II). The samples were set in a freezer at -20°C for 48 hours in order to preserve the RNA and prevent degradation.

Table II: The concentration of purified RNA extracted from each THP-1 well

Type of Well	RNA Concentrations (ng/µL)					
GOI in the RNA	*IAV-NSI*	*VSV-N*	*VSV-P*	*VSV-M*	*VSV-L*	*VSV-G*
Unstimulated	N/A	N/A	N/A	465.1	699.2	571.4
Mock	50.0	326.3	469.7	683.7	532.5	480.5
Gene - 2500ng	361.5	363.0	304.9	489.9	368.9	391.4
Gene - 5000ng	N/A	N/A	N/A	580.8	319.6	N/A

All samples displayed clean curves; there was a high peak for RNA concentration and low dips for salt and protein concentration, indicating no contamination.

cDNA Conversion and qPCR

To measure the RNA, a method called qPCR, or quantitative polymerase chain reaction, was employed. As PCR requires heat, RNA has to be converted to complementary DNA (cDNA), which is more stable than RNA and does not denature under the heat necessary for PCR. This was done through a process called reverse transcription. First, a predetermined amount of RNA was added to equalize all cDNA concentrations and combined with 2 µL of oligo dT primer, attaching the T chain to the poly-A tail of the RNA, providing a starting point for the reverse transcriptase enzyme to synthesize a cDNA of mRNA. The mixture was then filled to a total volume of 8 µL with nuclease-free water and denatured at 65°C for five minutes. After cooling the newly formed DNA on ice, 10 µL of ProtoScript II Reaction Mix and 2 µL of the PhotoScript II enzyme mix were added. In order for cDNA to be formed, the mixture was incubated at 42°C for 1 hour. The reverse transcriptase was then inactivated by heating the mixture to 80°C for 5 minutes and diluted with 80 µL of nuclease-free water.

For the qPCR, seven different PCR tubes were created for each sample, with each tube containing a different primer mix: 18S, IFNβ, PKR orf, PKR UL, SMC1A orf, SMC1A UL (Table III), and the complementary primer for each viral gene present in the sample. The IFNβ acted as a measure of immune response. The PKR orf and PKR UL were used to measure the ratio of long and short UTR; SMC1A orf and SMC1A UL had a similar purpose but to a different gene. Showcasing multiple genes helps clarify if the target is a specific gene or all UTRs. The viral gene primer was used to see if transfection was successful, and the 18S was used as a housekeeping gene (HKG) and a control group, acting as a reference point to ensure there is the same amount of genes present throughout the whole qPCR process. In the PCR tubes, 10 μL of the diluted cDNA was combined with 4 μL of nuclease-free water and 10 μL of its respective primer mix. Then, 20 μL of a master mix containing SYBR green, a fluorescent dye, was added to the tube. This SYBR master mix glows green when it binds to double-stranded DNA. By measuring the fluorescence, we could find the amount of cDNA present in each sample.

Table III: The Sequences of Primers used in qPCR (19)

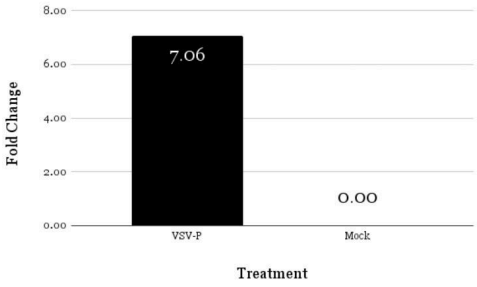
Primer (Forward)	Sequence	Primer (Reverse)	Sequence
18S F	AACCCGTTGAACCCCATT	18S R	CCATCCAATCGGTAGTAGCG
IFNβ F	GTCAGAGTGGAAATCCTAAG	IFNβ R	ACAGCATCTGCTGGTTGAAG
PRK orf F	TGGAAAGCGAACAAGGAGTAAG	PKR orf R	CCATCCCGTAGGTCTGTGAA
PKR UL F	AGAACACTGCACTGAAGATAGG	PKR UL R	GGATGGCCGTTTAGAGAGAAA
SMC1A orf F	CATCAAAGCTCGTAACTTCCTCG	SMC1A orf R	CCCCAGAACGACTAATCTCTTCA
SMC1A UL F	TGTTAGCTGGACTGGCATAAG	SMC1A UL R	ACATTCTGCCTCCCTGAAAG
VSV G F	TGCAAGGAAAGCATTGAACAA	VSV G R	GAGGAGTCACCTGGACAATCACT
VSV L F	TAAATGACGATGAGACTATGCAATC	VSV L R	ACACAAGTCACTCGTGACCATCT
VSV M F	TTTCCCTGCCATTCCGATGT TGGAGTTGACGAGATGGACAC	VSV M R	TTTCCCTGCCATTCCGATGT
VSV N F	CTTGGCTTATACAGAGTGGG	VSV N R	TTTGAACATGTGGAAGAACA
VSV P F	TAACCATGTTCATGCCTT	VSV P R	ATGGATAATCTCACAAAAGT
IAV NS1 F	GGAAGAAGCAGCACTCTTGG	IAV NS1 R	TTTCTGTTTGGGAATGAGCA/

Two qPCR plates were aligned, and the samples were plated consistently across them. The qPCR plates were heated to 95°C for 3 minutes, and then the cDNA samples went through 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 60°C for 1 minute. The samples were then analyzed through a standard melt curve in addition to fold change.

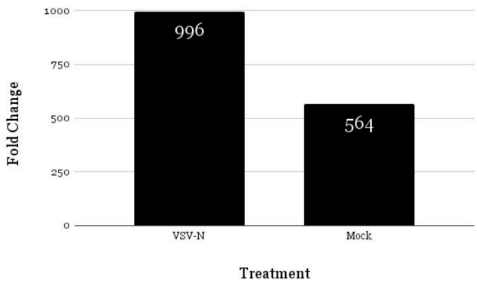
The qPCR provided C_q values for each sample and its corresponding target, which represents the cycle number at which the fluorescence signal crosses the threshold. Utilizing these values, the average and standard deviations were calculated for each target. Then, the housekeeping gene 18S was averaged in order to calculate the ΔC_q. The experimental samples were also compared to a known negative control, which was designated as unstimulated 2, to calculate a $\Delta\Delta C_q$. To ensure the sample was properly inserted into cells, the fold change value was analyzed, confirming all cells received the treatments. To determine whether the length of 3'UTR was altered, a ratio was calculated from the long UTR fold change and ORF fold change for each sample. The ratios and fold change values were compared and graphed to examine the effect of the target on specific sample 3'UTR lengths and interferons.

RESULTS

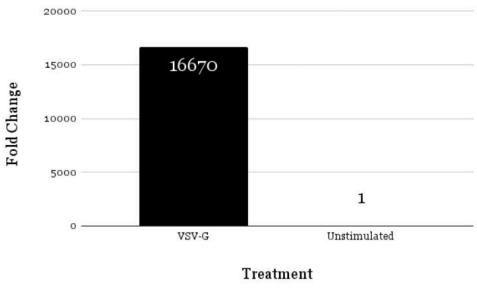
For all tested VSV genes, wells transfected with a gene showed higher expression than the mock and untransfected wells, our negative controls (Figure 3).



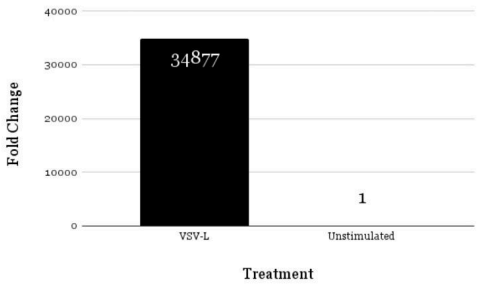
(a) *VSV-P* fold change versus Mock 1.



(b) *VSV-N* fold change versus Mock 2.



(c) *VSV-G* fold change versus Unstimulated 1.



(d) *VSV-L* fold change versus Unstimulated 2.

Figure 3. *VSV (P, N, G, L)* gene fold change versus negative controls. Shown above are the measured fold change values of VSV genes compared to negative controls.

The results of the qPCR display UTR length and interferon expression levels for samples and their corresponding targets. The ratio of ultra-long (UL) to the open reading frame (ORF) for PKR and SMC1A represented whether the 3'UTR lengthened or shortened in response to the addition of the plasmid. A greater UL to ORF ratio correlates to a lengthened UTR compared to the untransfected sample, whereas a lower ratio suggests shortening, since the UL primer targets a distal region that would be lost in shortened transcripts (Figure 4). For example, if the ratio of UL to ORF is normalized to 1 in the control, a normalized ratio of 0.3 in an experimental group would indicate a decrease in 3'UTR length. Thus, by comparing the ratios of UL to ORF caused by the various plasmids to the negative controls, the VSV protein responsible for shortened 3'UTR length can be ascertained.

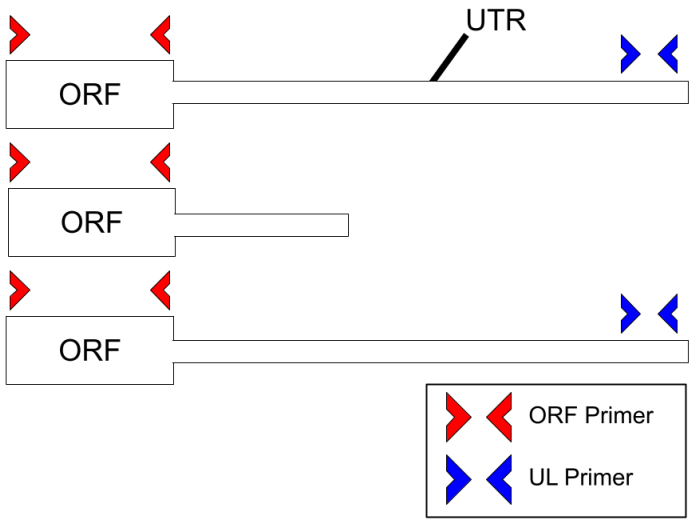


Figure 4: ORF compared to UL. Length of Open Reading Frame primer versus the length of the ultra-long primer.

Ratios of UL Fc/ORF Fc pertaining to PKR can be seen in Figure 5, where the unstimulated sample shows a ratio of 0.801, the mock sample shows a ratio of 1.338, the *VSV-G* and *VSV-L* display a ratio below 1, the ratios of *VSV-P, VSV-M*, and *VSV-N* are above 1, and the positive control *NS1* sample has a ratio of 4.141, enforcing Bergant's finding that NS1 lengthens UTRs (Figure 5) (17).

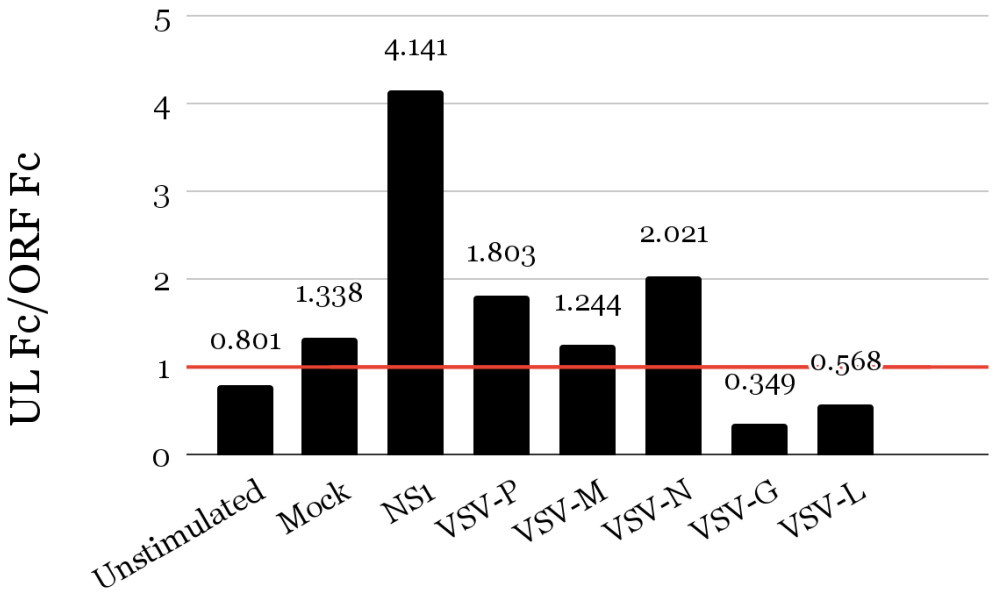


Figure 5. PKR UL and PKR ORF fold change. The ratio between protein kinase R ultra-long fold change and protein kinase R Open Reading Frame fold change.

Ratios of UL Fc to ORF Fc can be seen in Figure 6. Ratios of UL Fc/ORF Fc of 0.771 for the unstimulated sample, 7.546 for the mock sample, a ratio below 1 for *VSV-G*, a ratio above 1 for the *VSV-P*, *VSV-M*, *VSV-N*, and *VSV-L* samples, and a ratio of 11.353 for the *NS1* sample (Figure 6) were found.

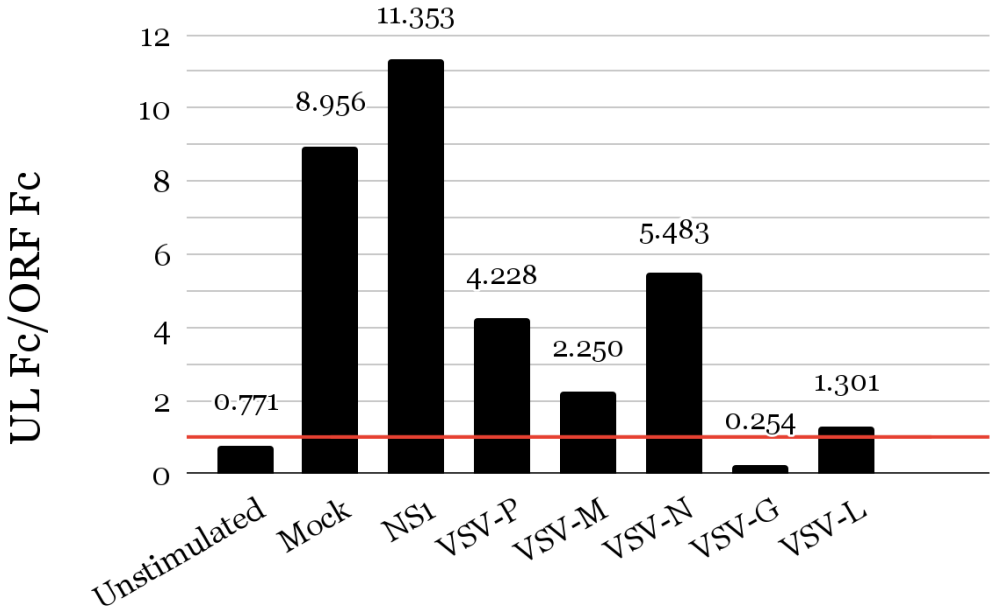


Figure 6. SMC1A UL and SMC1A ORF fold change. The ratio between SMC1A ultra long fold change and SMC1A Open Reading Frame fold change.

In order to measure immune activation, we ran qPCR for IFNβ. *NS1* and *VSV-G* show an IFNβ fold change below the unstimulated treatment, the negative control. *VSV-P*, *VSV-M*, *VSV-N*, and *VSV-L* transfection result in an IFN fold change above the unstimulated treatment (Figure 7).

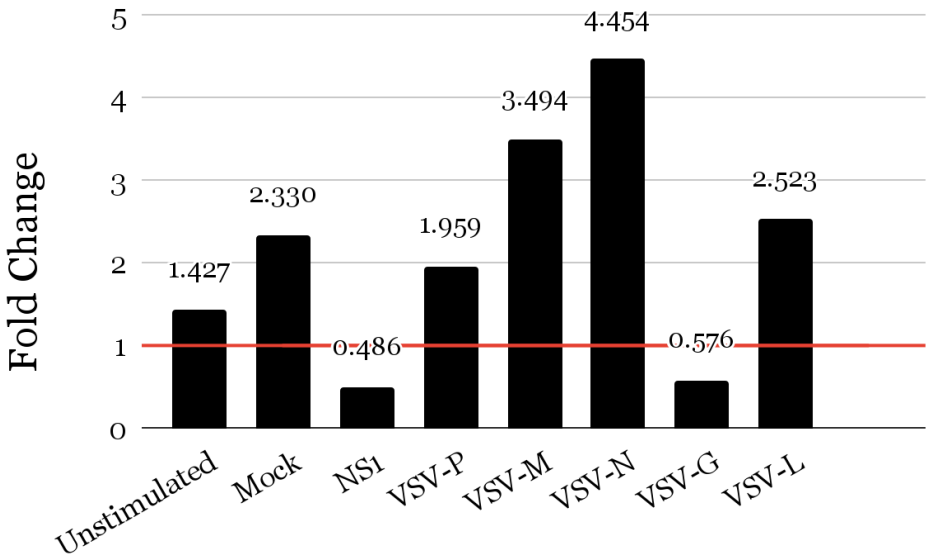


Figure 7. Fold change with IFN across eight treatments. The fold change ratios with interferon beta for unstimulated, mock, NS1, and all five VSV genes.

DISCUSSION

Considering VSV's role in modern vaccines, our study sought to build on the correlation between reduced immune response and altered 3'UTR length due to VSV. To determine which VSV proteins are responsible for this phenomenon, qPCR was utilized to evaluate the relative amount of mRNA sequences associated with 3'UTR length and immune response.

By analyzing the ratio of the UL fold change to the ORF fold change with the PKR and SMC1A primers, the specific treatment that resulted in 3'UTR shortening was deduced. From the 5 VSV proteins analyzed, the results indicate that protein G is most implicated in the shortening of the 3'UTR.

The NS1 gene, a positive control known to cause 3'UTR lengthening (17), performed as expected, with the fold change ratio of UL to ORF PKR genes being substantially greater than the ratios of the negative controls. This indicates that the NS1 gene played an important role in lengthening the UTR and was a successful positive control. When comparing the results of the NS1 gene to the VSV genes, none of the VSV genes extended UTR length to the same extent; however, when comparing the results of the negative control groups to the VSV genes, the differential effects of the VSV genes became more pronounced.

When only using the PKR primers to measure UTR length, the *VSV-G* and *VSV-L* proteins were the only 2 proteins of the 5 to result in 3'UTR shortening in comparison to the controls. The UL to ORF fold change ratios for these genes were below that of the unstimulated and mock (Figure 5), suggesting that these two proteins may contribute to 3'UTR shortening. However, upon combining the results of the PKR genes with those of the SMC1A genes, *VSV-G* was the only protein of the five that consistently resulted in shortened 3'UTR length. Though *VSV-L* didn't result in 3'UTR shortening for the SMC1A, it should still be further examined due to the effects it had on the PKR gene, for which it did cause shortening. Therefore, the primary genes that should be further investigated in the context of 3'UTR shortening are *VSV-G* and *VSV-L*, with an emphasis on the G gene.

In both PKR and SMC1A, cells with the *VSV-G* gene have a substantially shorter 3'UTR. As such, it is likely that the protein G alone causes the 3'UTR shortening that Jia et al. (5) have observed. However, in order to confirm that the uptake of the *VSV-G* gene occurred at all, the fold change of the *VSV-G* gene itself was compared between cells that received the *VSV-G* plasmid and those that did not (Figure 3). The difference in fold change was extremely notable, confirming that the *VSV-G* gene was well incorporated into the genomic sequences of the cells. This allows for greater certainty that the activity of the *VSV-G* protein is correlated with the shortening of 3'UTRs. Beyond this, the other three VSV genes of interest showed positive transfection results, further certifying our hypothesis that the *VSV-L, VSV-P,* and *VSV-N* proteins are not the cause of 3'UTR shortening, whereas *VSV-G* is.

Because dsRNA binds to PRRs that trigger the immune response, longer 3'UTRs that form more dsRNA will trigger a greater immune response and vice versa. Thus, qPCR measuring the relative amount of the IFNβ gene— which codes for the type 1 interferon beta, a protein essential in stimulating the immune response— was run in order to examine the magnitude of the immune signal. The results supported some past theories: a small increase in 3'UTR length is related to a greater immune response, and shortening is related to a weaker response. The fold change for the IFNβ of cells with the *VSV-N* protein, a protein that had a slightly increased 3'UTR length relative to the negative controls, was nearly double that of the mock and nearly quadruple that of the unstimulated group (Figure 7). With 3'UTR lengthening, the immune response increased. While these findings may indicate that *VSV-N* leads to extreme increases in 3'UTR length and therefore immune response, its UL to ORF ratio is still substantially smaller than NS1, a known 3'UTR lengthener. Still, further research should be conducted into *VSV-N*'s role in substantially increasing 3'UTR length, thus IFNβ levels, and the exact mechanisms undertaken by the protein to elevate host immune response. In contrast, VSV-G protein's role in shortening 3'UTR length is correlated with a lowered immune response.

The impacts of *NS1* and *VSV-G* activity on 3'UTR length, despite the similar effects of both genes on IFNβ expression, may be a source of confusion. *NS1* is already established as a cause of 3'UTR lengthening, while *VSV-G* is found to reduce the length (Figure 8). It may then seem counterintuitive that both genes lower IFNβ protein levels (Figure 9). However, *VSV-G* may use 3'UTR shortening to lower the immune response. In contrast, *NS1* decreases IFNβ expression unrelated to 3'UTR length; this gene inhibits IFN-I production by downregulating RIG-I expression, a PRR that is involved in responding to viral pathogens (20). *NS1* may also block the signaling cascade of the immune response by inhibiting NLRP3 activity, a primary sensor for cellular damage and infection (20). Overall, *NS1* has various mechanisms by which it slows the immune system. In addition, it is important to note once again that the mechanisms by which Influenza A and VSV invade cells are context-dependent. These genes are from two distinct viruses with two distinct structures and evolutionary backgrounds; thus, the genes may affect 3'UTR lengths in different ways.

During viral infection, transcription factors bind to a distal PAS, forming a longer mRNA and thus, a longer 3'UTR sequence. When the longer sequence folds over, PRRs recognize the dsRNA sequences as MAMPS, triggering the transcription of ISGs. These degrade viral RNA, eliminating the "foreign" molecules from the cell. However, when *VSV-G* causes 3'UTR shortening, the mRNA sequence is recognized less by PRRs, and the necessary amount of interferons is not produced. Thus, the alterations in 3'UTR length caused by *VSV-G* cause a decreased immune response, as supported by the results of the qPCR (9).

Interferons play a crucial role in innate immunity by signaling cells to act against viral replication; a large decrease in interferon expression would cause a decrease in immune response. Because a greater fold change is associated with a greater presence of that gene and therefore greater gene expression, cells with a low fold change of IFNβ would therefore have a weakened immune response. In the case of the *VSV-G* gene, the fold change was approximately 0.5, indicating that expression of interferons was reduced as a result of the gene's incorporation. This suggests that shortened 3'UTR length does, in fact, lower the immune response of the cells, which would support the findings of Dorrity et al. (9). The subsequent conclusion was that the *VSV-G* gene hinders innate immunity via UTR shortening. This finding could help develop theories on the reason why viruses alter 3'UTR length (9), as well as help expand on the functions of 3'UTRs. By understanding the mechanisms behind reductions in 3'UTR lengths, researchers could develop more sustainable practices in vaccination development.

The ratio of UL fold change to ORF fold change, as calculated from the results of the qPCR, shows that *VSV-G* was the gene of interest that caused 3'UTR shortening in organisms infected with VSV. As a result of the alterations in 3'UTR length, the IFNβ fold change saw a similar decrease, with a value of approximately 0.0003 – a major drop in comparison to the strong immune response of the negative controls: unstimulated and mock. This is because a

shorter 3'UTR hinders the mRNA from folding over and forming double RNA. As a result, PRRs can not recognize "foreign" RNA and initiate the immune response. Understanding this correlation between 3'UTR shortening and an inhibited immune response is of key importance as researchers continue to incorporate VSV in vaccines and deepen their understanding of the mechanisms behind human immune response.

Limitations

Despite compelling results implicating *VSV-G* in the shortening of 3'UTR length and therefore a weakened immune response, the lack of trials allows for the possibility of greater error. For *VSV-G*, there was only 1 biological trial, consisting of two tested genes, SMC1A and PKR. As a result, there was insufficient evidence to definitively claim that the *VSV-G* protein consistently shortens 3' UTR length and reduces immune response. This lack of certainty also applies to conclusions drawn about the other VSV genes, such as *VSV-L*, which we have suggested to also shorten certain 3'UTR lengths. Additionally, the VSV genes were only tested in THP-1 cells to show how VSV affects humans, but this cell line may not always be physiologically accurate, as hinted at by the fact that lipofectamine caused 3'UTR length to increase greatly, a result that had not been reported in previous studies. By only testing VSV in THP-1 cells, it is still unclear whether VSV-G reacts differently in non-human cell lines. Therefore, more research should be conducted on various cell lines, including human, other organisms, and live cells. By doing so, it could be confirmed whether the VSV-G causes 3'UTR shortening in only human monocyte cells, or if VSV-G also affects the 3'UTR and immune response in non-human organisms, which would suggest that a correlation between 3'UTR shortening and immune response has been evolutionarily conserved.

Additionally, lipofectamine may have impacted the immune response of the cells, as the mock showed an increased IFNβ compared to the unstimulated. As such, the effect of the VSV and NS1 genes on the immune response might be influenced by the heightened immune response due to the lipofectamine used for transfection, and more research should be done using transfection methods that do not include lipofectamine. Investigations should also be done on lipofectamine to uncover the nature of its effect on the immune response. Regardless, the idea that lipofectamine increases immune response underscores the claim that *VSV-G* substantially decreases immune response, as VSV-G displayed an IFNβ fold change of 0.576 (Figure 7), despite the increase in levels caused by mock.

Moreover, the possibility for complex interactions between multiple genes was not considered throughout the short duration of this experiment. Each gene was isolated and inserted individually, causing the protein to act alone on 3'UTR length and the immune response. However, gene and protein interactions are very common; it is therefore possible that one gene could act to regulate the expression of another, or that the proteins encoded by those genes combine to produce synergistic or antagonistic effects. Further research investigating the

combined effects of various genes would greatly improve our understanding of how the proteins of VSV might interact with one another in order to impair immune function.

Although the results have suggested that VSV-G shortens 3'UTR length and causes a decreased immune response, these results cannot be extrapolated to form a causal relationship between the two, as much is still unknown about the specific genomic sequence or mechanisms behind this phenomenon. Additional research could include looking into the exact amino acids in the VSV-G protein that result in 3'UTR shortening and lowered immune responses. Such investigation would then allow for a more definitive relationship between the two effects found by this paper to be better understood.

Following similar methodologies to previous researchers' findings regarding other viruses (17), future research should investigate the UTR-shortening mechanism behind *VSV-G*. Bergant et al. tested two parts of the NS1 protein individually: the RNA-binding domain and the effector domain. They noticed that the effector domain alone was able to cause 3'UTR lengthening. Similar to the methodology employed by Bergant et al. (17), we could section *VSV-G* by splitting the gene into its basic parts, placing sectioned sequences in individual cells, and repeating the reverse transcriptase qPCR methodology used in this study to determine which part of *VSV-G* causes the shortening of 3' UTRs. Furthermore, we can induce point mutations in the DNA sequence of the part of the VSV-G protein that works to shorten 3'UTRs to identify the specific mechanism by which the VSV-G protein shortens 3'UTRs.

Implications

Observing the impact of the VSV-G protein on 3'UTR length could lead to further research regarding the safety of recombinant VSV vaccines. Recombinant VSV has clinical value as a potent vaccine platform; its genome can be altered to deliver genes of other viruses that may cause detrimental effects to humans if injected directly. In this process of creating recombinant VSV, the G spike protein gene is often the target gene to be replaced. As briefly discussed in the introduction, the Ebola Virus vaccine VSV-EBOV is a VSV with its G gene replaced with the EBOV GP gene (21). In our research, *VSV-G* appeared to be associated with lowered immune response, supporting the safety of VSV-platform vaccines. Nevertheless, a future study could explore recombinant VSV's effects on human cells, determining whether or not a recombinant virus without the G gene also impacts 3'UTR length or immune responses.

In contrast to *VSV-G* being replaced for vaccination in humans, spike protein genes in other viruses are replaced with *VSV-G* for infecting a variety of organisms in research settings. Pseudotyping involves replacing the outer protein layer of a virus with that of a different virus (20). *VSV-G* is a common pseudotyping agent due to its ability to infiltrate a large range of organisms' cells. The human immunodeficiency virus (HIV), for example, can be recombined with the *VSV-G* spike protein gene, allowing the new recombinant virus to infect a more diverse

group of organisms (4). If further research confirms our findings of *VSV-G* shortening 3'UTR lengths, *VSV-G* may not be an ideal pseudotyping agent. Especially with viruses that impact the immune system, such as HIV, studying a recombinant version with VSV may result in unwanted, confounding immune effects on host cells. Therefore, the effect of viruses pseudotyped with VSV-G as an envelope protein on host cells must be studied. Furthermore, research needs to be conducted regarding the effect of VSV on the 3'UTR length of non-human cells, as they may yield different results.

This adaptability allows for effective infection of multiple cells. Notably, *VSV-G* was utilized due to the belief that it had no major impact on human cells (4). However, the implications of this study regarding the decreased immune response should be taken into consideration for future use of *VSV-G*, as its use could be causing negative side effects during vaccination. Especially with viruses that impact the immune system, such as HIV, studying a recombinant version with VSV-G protein may result in unwanted immune effects. Thus, examining the effects of the VSV-G protein as an envelope protein for pseudotyping is necessary for further understanding of the protein's properties.

The knowledge of the effect of VSV on 3'UTR length and the human immune system paves the path for further research into the manipulation of 3'UTRs in cell lines and animal models. For instance, various genetic therapies, such as CRISPR, can target specific sections of RNA, possibly altering the length of 3'UTRs (22). Researchers can technologically delete or modify PASs or their proximal regions to influence PAS usage and resulting 3'UTR length (23). Thus, a deeper understanding of the way viruses impact 3'UTRs can open up opportunities for intentional genetic alterations through emerging advancements.

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APPENDIX

Abbreviations

1. 3'UTR: 3' untranslated region
2. APA: alternative polyadenylation
3. cDNA: complementary DNA
4. DNA: deoxyribonucleic acid
5. dsRNA: double-stranded RNA
6. EBOV: Ebola virus
7. FBS: fetal bovine serum
8. Fc: fold change
9. GOI: gene of interest
10. HIV: human immunodeficiency virus
11. HKG: housekeeping gene
12. HSV-1: Herpes Simplex Virus
13. IAV: Influenza A Virus
14. IAV-NS1: non-structural protein-1
15. IFN: interferon
16. IFNβ: interferon beta
17. ISG: interferon-stimulated gene
18. LACV: La Crosse Virus
19. MAMPS: microbial associated molecular patterns
20. mRNA: messenger RNA
21. ORF: open reading frame
22. PAS: polyadenylation sites
23. PCR: polymerase chain reaction
24. PPI: protein-protein interaction
25. PRR: pattern recognition receptor
26. qPCR: quantitative polymerase chain reaction
27. RBP: RNA-binding protein
28. RNA: ribonucleic acid
29. RNP: ribonucleoprotein
30. UL: ultra long
31. VSV: vesicular stomatitis virus
32. VSV-EBOV: Vesicular stomatitis virus-based Ebola virus vaccine
33. VSV-G: glycoprotein
34. VSV-L: large RNA-dependent RNA polymerase protein
35. VSV-M: matrix protein
36. VSV-N: nucleoprotein
37. VSV-P: phosphoprotein