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# Analyzing the Effects of Site-Directed Mutagenesis on the Structure of Coxsackievirus B3 Genomic RNA

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# Analyzing the Effects of Site-Directed Mutagenesis on the Structure of Coxsackievirus B3 Genomic RNA

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*ABSTRACT OF THESIS:*

Coxsackievirus B3 (CVB3) is known to cause myocarditis and pancreatitis in humans. The virus has a single stranded RNA genome that codes for 11 different proteins. CVB3 is found to have two serotypes: 28 (virulent and disease causing) and GA (avirulent and not disease causing). Like other members of the Picornaviridae family, CVB3 utilizes the genomic 5' untranslated region (5' UTR) to initiate viral replication mechanisms through interactions with host protein factors. Structural variations of the CVB3 5' UTR are hypothesized to influence the success of such interactions and consequentially determine viral virulence. The aim of this project was to investigate the effects of site-directed mutagenesis on the structure of the 5' UTR of the virulent CVB3/28 strain. Our lab has previously constructed a mutant strain with two C-to-A mutations in positions 122 and 124 in the genome of the naturally occurring 28 strain. As a result, the mutant strain contains a poly-A region from position 121 to 125. We utilized chemical probing to analyze the structure of the mutant 5' UTR. The modification data was analyzed via the software ShapeFinder and the resulting normalized modification values were used to generate a structural model of the mutant 5' UTR using the RNAstructure algorithm.

**Acknowledgements**

I would like to express my thanks to Dr. William Tapprich for allowing me the opportunity to work in his lab. Over the past year, I have learned a great deal from him about the virology and biochemistry behind our work, as well as how to think critically and scientifically. I greatly appreciate all the time and energy he has invested into my work and this thesis.

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Finally, I would like to thank my other fellow researchers at the University of Nebraska Omaha. They challenged and motivated me each and every day in lab, creating an atmosphere that allowed me to grow academically and personally. Their support, contributions, and friendship made this work much more rewarding.

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## A. Introduction

### 1. Purpose

The purpose of this study is to analyze the effects of site-directed mutagenesis on the secondary and tertiary structure of RNA genome from the virulent strain of Coxsackievirus B3 (CVB3) through chemical modification and primer extension. Since structure dictates function, the data generated will provide us with preliminary evidence of how a mutated strain's structure differs from other nonmutated strains. This information will help us understand the nature of viral virulence and assist the effort to design antiviral strategies against CVB3 and other viruses.

## B. Background

### 1. Coxsackievirus B3

Coxsackievirus B3 (CVB3) is an RNA virus and a member of the *Picornaviridae* family. Two CVB3 subtypes exist: a virulent subtype (28) and an avirulent subtype (GA). The virulent strain is known to cause myocarditis and pancreatitis, and is thought to play a role in type I diabetes (1). The avirulent type is not known to cause any diseases. The CVB3 viral genome is a single-stranded positive-sense RNA composed of approximately 7,400 nucleotides (1). The genome is organized into four structural regions: a 742 nucleotide 5' untranslated region (5'UTR), a single open reading frame, a 98 nucleotide 3'UTR, and a poly-A tail (1). The 5'UTR region is shown to determine virulence due to its role in recognizing cellular translation factors, ribosomes and other cellular proteins vital to the initiation of the viral replication (1). The 5'UTR region contains seven highly structured domains made up of two distinct functional regions. The first functional region comprises a 5' terminal cloverleaf which represents Domain I. The second functional region comprises Domains II-VII which are collectively known as the internal ribosomal entry site (IRES) (2).

Multiple studies have found that mutations within the 5'UTR significantly reduce virulence of the molecule (1). To further explore this concept, we focused on a previously constructed mutant strain with two C-to-A mutations in positions 122 and 124 of a naturally occurring 28 strain. As a result, the mutant strain contains a poly-A region from positions 121 to 125 in Domain II (Figure 1).

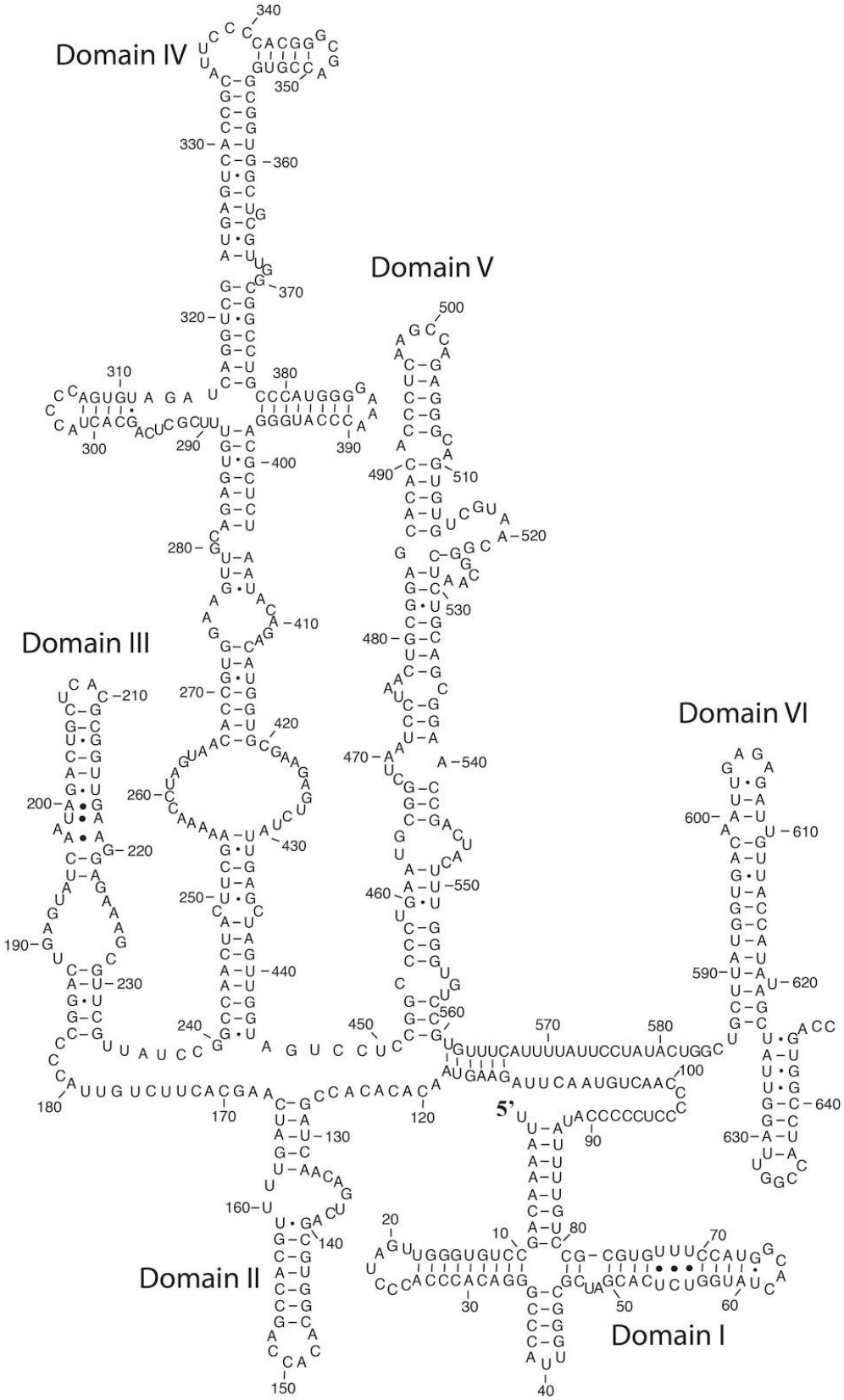


**Figure 1. 5' UTR structure of the virulent CVB3/28.** The genome was mutated at positions 122 and 124 from C to A to create a poly-A region from position 121-125.

## C. Materials and Methods

### 1. Production of DNA Fragment

*E. coli* Sure cells containing a plasmid vector with the full length CVB3 genome were generously provided by Dr. Nora Chapman from the University of Nebraska Medical Center (UNMC). The transformed cells where twice grown for 24 hours at 37°C on luria broth (LB) plates with ampicillin to generate isolated colonies. Three colonies of similar size and morphology were selected from the second plate with a toothpick and suspended in 100 μL of liquid LB. This mixture of colony/LB culture was then added to an overnight culture of 25 mL of liquid LB and 200 μL of ampicillin (25 mg/mL). The overnight culture was placed in a shaker bath at 37°C for 16 hours to allow for amplified growth of the plasmid-containing cells. To prepare the plasmids for extraction, the overnight culture was separated into 5 mL aliquots and centrifuged at 6,000 rpm for 10 min at 4°C. The resulting white pellet was used to isolate and purify the plasmid DNA via the Qiagen MiniPrep DNA Extraction Kit protocol. The subsequent DNA solution was stored at -80°C. Electrophoresis of the DNA products on a 1% agarose gel confirmed successful isolation of the plasmid DNA (Figure 2).

### 2. Restriction Digestion

A restriction digest was assembled by mixing 10 μg of DNA, 2 μL of 10X Reaction Buffer, and 2 μL of FastDigest *Ecl*136II. RNase-free water was added to the mixture to obtain a total reaction of 218 μL. The samples were incubated for 4.5 hours at 37°C. The newly cut DNA was purified via a phenol/phenol-chloroform extraction. The extraction procedure involved the following: 200 μL of phenol was added to the restriction digest sample, vortexed for 30 seconds, and centrifuged at 13,300 rpm for two minutes at room temperature. Two distinct layers formed and the upper aqueous layer was transferred to a new 1.5 mL Eppendorf tube. 200 μL of phenol-

chloroform was added to the new tube, vortexed for 30 seconds, and centrifuged at 13,300 rpm for two minutes at room temperature. Again, two distinct layers formed and the upper aqueous later was transferred to another new 1.5 mL Eppendorf tube. 20 μL of 3M sodium acetate (NaOAc) pH 5.2 and 600 μL of 95% ethanol (EtOH) was added to the Eppendorf tube and mixed by inverting. The sample precipitated overnight at -20°C.

The following morning, the overnight samples were centrifuged at 13,300 rpm for 20 minutes at 4°C. The EtOH was poured off and blotted dry before adding 400 μL of cold 70% EtOH and again centrifuging for five minutes at 13,300 rpm at 4°C. This was repeated twice to ensure successful precipitation of plasmid DNA. Following the two ethanol wash procedures, the samples were dried in the Speed-Vac for two minutes. The pellet was resuspended in 21 μL of TE (10mM Tris, 1 mM EDTA) pH 7.5. Electrophoresis on a 1% agarose gel confirmed a successful restriction digest (Figure 3).

**3. *In vitro* Transcription of 5' UTR RNA**

An 80 μL transcription reaction was set up using the 5X MEGAscript T7 kit (Invitrogen by Thermo Fisher Scientific) according to the manufacture's protocol. Briefly, the protocol required mixing 4 μg of DNA, 8 μL ATP, 8 μL CTP, 8 μL GTP, 8 μL UTP, 8 μL 10X Reaction Buffer, 8 μL T7 RNA Polymerase Enzyme solution, and RNase-free water to a total volume of 80 μL. The reaction was incubated at 37°C for six hours. Afterwards, 4 μL of DNAse were added to remove the DNA template, and the sample was incubated at 37°C for an additional 30 minutes. Following incubation, the transcription reaction was halted by adding 460 μL of RNase free water and 60 μL Acetate Stop Solution and mixing thoroughly. The resulting 5' UTR RNA was purified via a phenol/chloroform extraction similar to the phenol/phenol-chloroform

extraction previously discussed, but using 600 μL instead of 200 μL. Following extraction, 600 μL of isopropanol was added, mixed by inverting and precipitated overnight at -20°C.

The following morning, the sample was centrifuged at 13,300 rpm for 20 minutes at 4°C, resulting in a visible white pellet suspended in isopropanol. The alcohol was poured off, blotted dry, and 400 μL of 70% EtOH was added before the sample was centrifuged at 13,300 rpm for five minutes at 4°C. This ethanol wash protocol was repeated twice. The final sample was dried for two minutes in the Speed-Vac and resuspended in 21 μL of TE pH 7.5. Electrophoresis on a 1.5% agarose gel confirmed successful transcription of the 5' UTR (Figure 4).

**4. RNA cleanup via MEGAclear Kit**

Following successful *in vitro* transcription, the RNA was purified using a MEGAclear kit (Invitrogen by Thermo Fisher Scientific). The RNA sample was brought to 100 μL with Elution solution and mixed gently, but thoroughly. 350 μL of Binding Solution and 250 μL of 100% ethanol were added to the sample, with gentle mixing after both additions. After placing a Filter Cartridge in a Collection and Elution Tube, the RNA mixture was transferred to the Filter Cartridge. To ensure transfer of the entire sample, the pipet was set to slightly over 700 μL (100 μL + 350 μL + 250 μL). The tube was centrifuged at 13,300 rpm for one minute at 4°C, allowing the solution to flow through to the Collection and Elution Tube, trapping the RNA in the filter. The flowthrough was discarded, but the filter was returned to the same tube. Then, 500 μL of Wash solution was added to the center of the Filter Cartridge and centrifuged at 13,300 rpm for another one minute at 4°C. The flowthrough was discarded, and the same step was repeated. After the second wash, the sample was centrifuged for an additional minute on the same settings to remove any residual traces of Wash Solution that may still be present. The Filter Cartridge was transferred to a new, clean Collection and Elution Tube. To elute the RNA from

the filter, 50 μL of Elution Solution was pipetted onto the center of the Filter Cartridge and incubated at 65°C in the thermobath for 10 minutes. Following incubation, the tube was centrifuged at room temperature for one minute at 13,300 rpm to allow the RNA solution to fall through.

**5. Chemical Modification of RNA Using Dimethyl Sulfate**

The purified RNA was chemically modified using Dimethyl Sulfate (DMS). Two tubes were prepared, each with 2.385 μg of purified RNA, 50 μL of 1X DMS buffer and RNase-free water to bring the total volume to 100 μL. One tube was labeled with a (+) to represent the modified tube, and the other tube was labeled with a (-) to represent the control tube. To ensure that the RNA molecules were in their natural 3D structure before modification occurred, the RNA was denatured at 80°C for two minutes and then slowly cooled to 40°C to renature. Once cooled, the samples were placed on ice and a 0.4% DMS reagent was prepared by adding 2 μL of liquid DMS to 98 μL of 95% EtOH.

The modification process was started by adding 2 μL of 1X DMS buffer to the control tube and 2 μL of the prepared 0.4% DMS solution to the modification tube. Both tubes were incubated at 37°C for 10 minutes in the hood. To stop the modification reaction, 25 μL of DMS stop buffer was added. To precipitate out the RNA, 5 μL of 50 mM EDTA pH 8.0, 1.25 μL of 20 μg/μL glycogen, 12.5 μL of 3M NaCl, and 274 μL of 100% EtOH were added to both tubes and mixed by inverting. The glycogen acts as a co-precipitant, making it easier for the RNA to precipitate out. Therefore, the RNA incubated for only 20 minutes at -80°C rather than all night. Following the 20-minute incubation, the samples were centrifuged at 13,300 rpm for 20 minutes at 4°C and the supernatant was removed. To purify the modified RNA, an ethanol wash was twice performed by adding 150 μL of 70% ethanol, centrifuging it for 5 minutes at the same

conditions, and pouring off the EtOH. The samples were dried for two minutes in the Speed-Vac and the resulting pellet was resuspended in 90 μL of 0.5X TE pH 8.0.

**6. Primer Annealing and Extension**

First, the post-clean-up unmodified RNA (section C4) was diluted to 0.02641 μg/μL with 0.5X TE pH 8.0 for a total volume of 60 μL. Then, eight PCR tubes were prepared with 9 μL aliquots of RNA: Tube 1 with 9 μL of the modified RNA, Tube 2 with 9 μL of the control RNA, and Tubes 3-8 with 9 μL of the diluted unmodified RNA. All eight tubes were incubated in the thermocycler for three minutes at 95°C using the "*Unfold*" protocol to denature and unfold the RNA. Upon completion, the tubes were placed on ice, and the lights in the lab were turned off before taking out the primers. This was to minimize the fluorescent-tagged primers' exposure to light. 2 μL of 1 μM oligo primer 5'-tagged with the VIC fluorophore was added to tubes 1-4. 2 μL of 1 μM oligo primer 5'-tagged with the FAM fluorophore was added to tubes 5-8. To anneal the primers to the RNA strand, the tubes were incubated in the thermocycler for five minutes at 65°C and then for 10 minutes at 35°C using the "*SHAPE Anneal"* protocol. Upon completion, the tubes were placed on ice. 8 μL of the Extension mix (12 μL of 5X First-strand buffer, 3 μL 0.1M DTT, 3 μL 10 mM dNTP mix, 6 μL RNase-free water) was added to tubes 1 and 2. 8 μL of the C termination mix (8 μL of 5X First-strand buffer, 2 μL 0.1M DTT, 2 μL 10 mM dNTP mix, 2 μL 10 mM ddCTP, 2 μL RNase-free water) was added to tube 3. 8 μL of the A termination mix (24 μL of 5X First-strand buffer, 6 μL 0.1M DTT, 6 μL 10 mM dNTP mix, 6 μL 10 mM ddATP, 6 μL RNase-free water) was added to tubes 4-8. 1 μL of SuperScript III Reverse Transcriptase (200 U/μL) was added to each tube, mixed well and incubated in the dry bath for 15 minutes at 52°C to allow the reverse transcription of the cDNA.

After obtaining the cDNA, 2.5 μL of 1M NaOH was added to each tube and incubated in the thermocycler for 15 minutes at 98°C using the "*SHAPE NaOH*" protocol. During this time, the RNA template was deleted as NaOH deprotonates the 2'OH of RNA causing it to self-hydrolyze or "commit suicide." To neutralize the NaOH and bring the samples up to volume, 2.5 μL of 1 M HCl and 25 μL of RNase- free water was added to each tube. Tubes 1 and 5, 2 and 6, 3 and 7, and 4 and 8 were combined and transferred into Eppendorf tubes. 1 μg/mL of glycogen, 10 μL 3M sodium acetate pH 5.2, and 300 μL of 100% ethanol were added to each of the four Eppendorf tubes and incubated at -80°C for 20 minutes. Following incubation, the tubes were centrifuged at 13,300 rpm for 20 minutes at 4°C. The supernatant was poured off. Each sample was then washed with 1 mL of 75% EtOH and centrifuged for 5 minutes on the same settings before pouring the supernatant off. This was repeated to ensure optimal purity. The tubes were then dried in the Speed-Vac for two minutes and the resulting pellets were resuspended in 10 μL of HiDi formamide. The four samples were stored in a dark -80°C freezer before being transported in a sealed ice bucket to UNMC the following day.

## D. Results

### 1. DNA Extraction

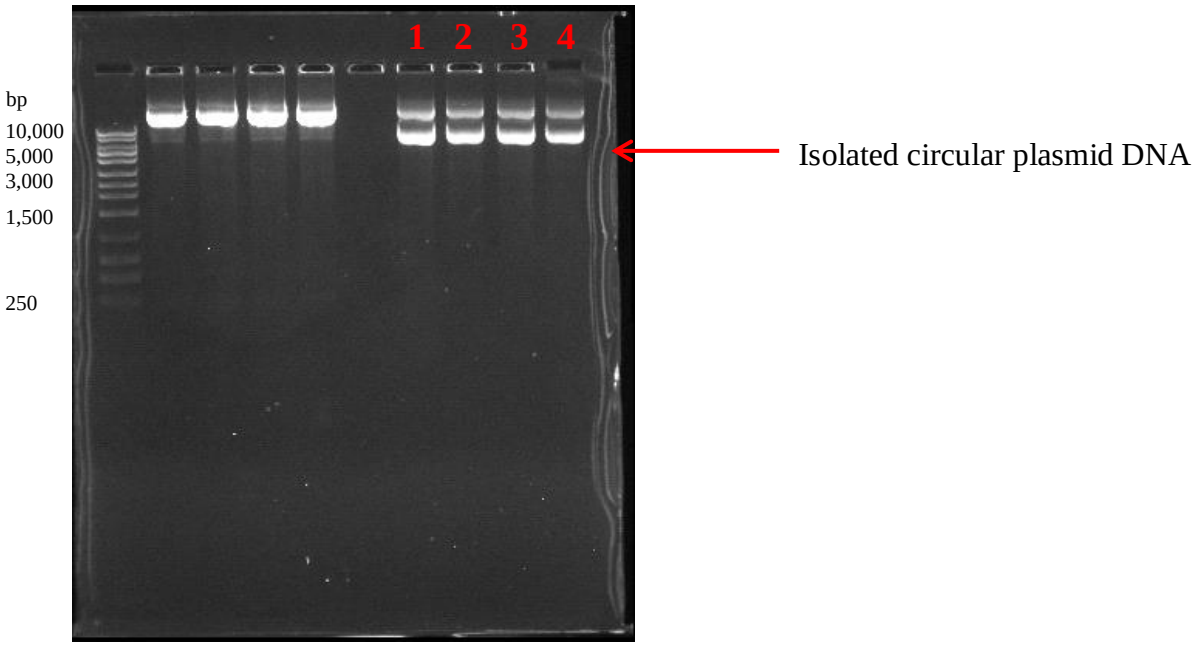


**Figure 2**. **Agarose gel electrophoresis of the isolated plasmid.** Plasmid DNA samples (lanes 1-4) were analyzed using a 1% agarose gel electrophoresed at 70 volts for 60 minutes. This confirms successful extraction of the plasmid DNA from *E. coli* Sure cells.

### 2. Restriction Digestion of DNA

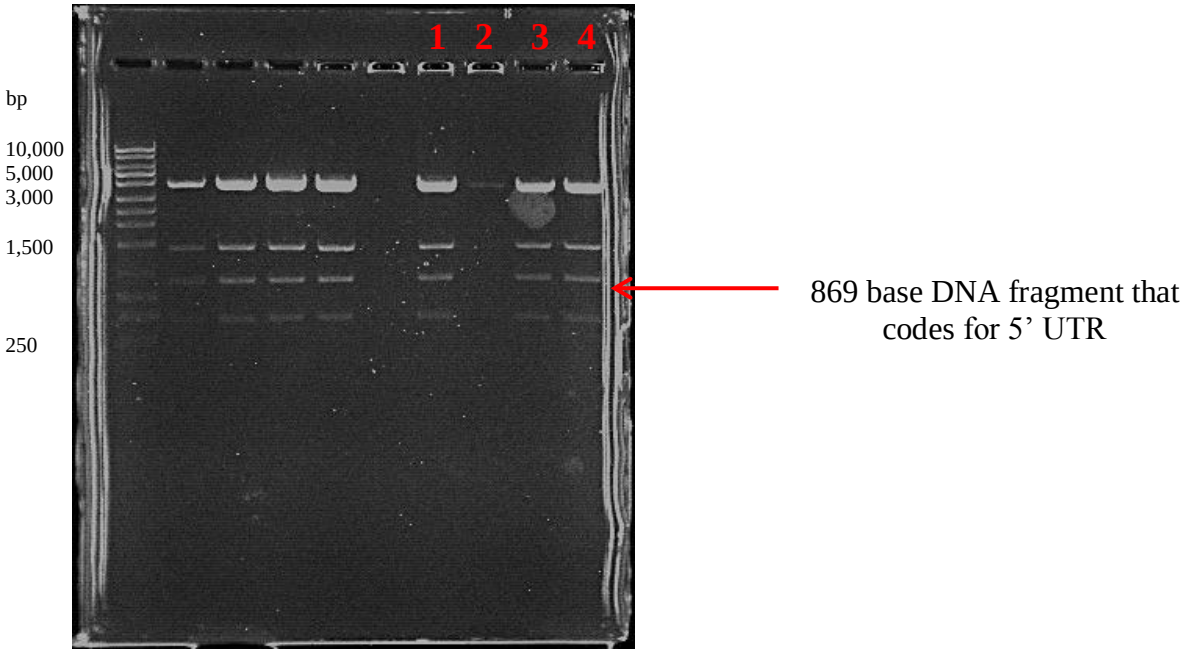


**Figure 3. Agarose gel electrophoresis of digested plasmid.** Plasmid DNA samples digested with *Ecl136II* were loaded into lanes 1-4. The 1% agarose gel was ran at 70 volts for 60 minutes. Lanes one, three and four show properly cut DNA. Digestion of the sample in lane 2 is shown to be unsuccessful.

### 3. *In vitro* Transcription of 5' UTR

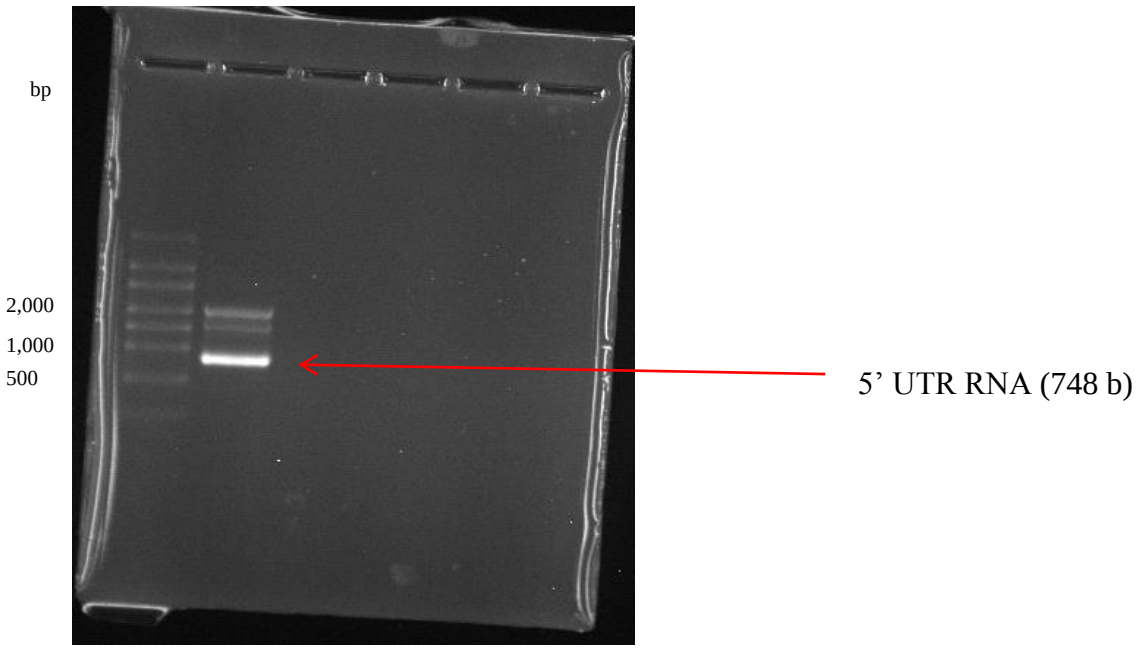


**Figure 4. Agarose gel electrophoresis of the 5'UTR RNA.** The *in vitro* transcribed 5'UTR sample was analyzed via a 1.5% agarose gel ran at 70 volts for 90 minutes.

### 4. Electropherogram via ShapeFinder

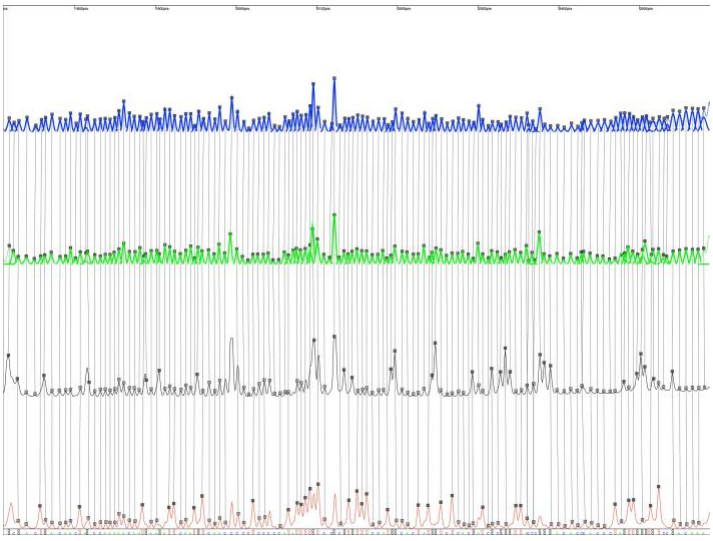

**Figure 5. Processed Electropherogram of the chemically modified 5'UTR.** The raw, capillary electrophoresis files, generated by UNMC, were processed with ShapeFinder. The first trace (blue) is the experimental sample with modifications. The second trace (green) is the control sample with no modifications. The third (grey) and fourth (red) tracks are the sequencing tracks.

### 5. Proposed RNA Structure via RNAstructure Algorithm

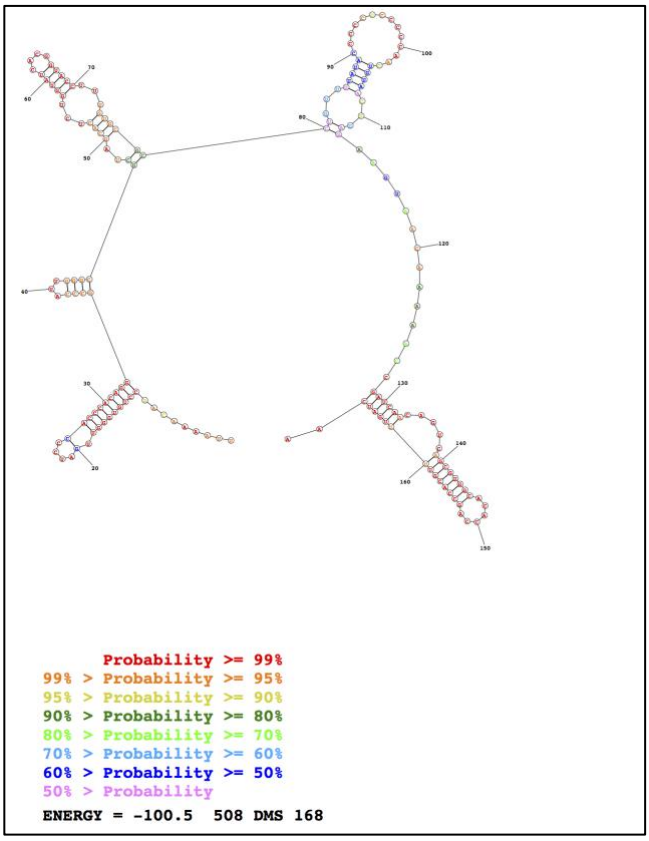


**Figure 6. Generated Structure for Domains I and II of the mutant 5'UTR.** After generating reactivity values from the electropherogram, the RNA structure of the mutant 28 strain was generated using the RNAstructure algorithm.

## E. Discussion

### 1. Chemical Modifications and Proposed Model of 5' UTR

RNA is capable of folding itself into specific conformations that are critical to its function and effectiveness. Chemical probing determines the secondary structure of RNA by utilizing reagents that selectively target and methylate nucleotides in flexible regions of the RNA (3, 4). Multiple factors affect the flexibility of a region, including base pairing and solvent exposure. Mutations in the nucleotide sequence will produce these reagent-accessible sites due to reverse transcription stops (3). Base-specific modification with DMS selectively modifies

Adenosine and Cytosine nucleotides in these flexible, solvent-exposed areas by methylating the nitrogenous bases (4).

Based on the agarose gels, 5' UTR RNA of the mutant strain was successfully isolated with concentrations suitable for chemical modification and primer extension. The preliminary structures generated via RNAstructure suggest of possible structural differences between the 5'UTRs of the mutant and the wild type 28 strains.

**2. Limitations**

Due to time constraints, only one set of chemical modification experiments was completed. More replicates are needed to confirm or reject the preliminary findings of the study. Additionally, the obtained electropherogram suggests of only low levels of chemical modification through the molecule, which does not let us accurately predict the details pertaining to the folding patterns of the 5' UTR.

**3. Future Directions**

After concluding this work, one future direction would be conducting additional chemical probing utilizing different modifying agents such as N-methylisatoic anhydride (NMIA) and N-Cyclohexyl-N'-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMCT). The data obtained, in addition to the modification patterns suggested by DMS modification, would allow us to define the structure of the mutant CVB3 strain with greater accuracy and confidence. Such analysis would contribute to the knowledge of how site-directed mutagenesis within the 5'UTR can affect the virulence.

## F. References

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