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Analyzing Coxsackievirus B3 Genomic RNA by Site Directed Mutagenesis and SHAPE Analysis

Sara Smith
University of Nebraska at Omaha, sarajsmith@unomaha.edu

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Sara Smith

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William Tapprich, PhD

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PROGRAM: UNIVERSITY HONORS PROGRAM

DIRECTOR: LUCY MORRISON

ADDRESS: KH 208

UNIVERSITY OF NEBRASKA AT OMAHA

OMAHA, NE 68182

TELEPHONE: (402) 554-2696

UNIVERSITY: YES

STATE: YES

PROGRAM SIZE: 450+

THESIS: REQUIRED THESIS

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ADVISOR: WILLIAM TAPPRICH

wtapprich@unomaha.edu

Abstract

Virulent Coxsackievirus B3 (CVB3) is linked to myocarditis, pancreatitis, and type I diabetes. Avirulent CVB3 doesn't cause disease. CVB3 possesses a positive, single-stranded ribonucleic acid (RNA) genome containing 7,400 nucleotides, organized into four sections: a 5' untranslated region (5' UTR), a single open reading frame, a 3' untranslated region, and a poly-A tail. The 5' UTR is 743 nucleotides divided into 7 domains. Mutations within the 5' UTR can alter RNA structure, and thus alter virulence. Nucleotide associations responsible for higher level RNA folding patterns and structure haven't been defined. We are investigating the 5' UTR by selective 2' hydroxyl acylation analyzed by primer extension (SHAPE) to learn about the flexibility of the RNA backbone, thus determining the relationship between RNA structure and virulence. New data generated through SHAPE will be compared to known models of CVB3. This will provide quantitative data regarding flexibility in the RNA structure. Previously, the virulent and avirulent strains were characterized by base-specific mutagenesis and the structure qualitatively analyzed. My work will determine the nucleotide associations responsible for virulence.

Acknowledgments

I would like to thank Dr. Tapprich for allowing me to work in his lab. Since August of 2016, Dr. Tapprich has enabled me to exponentially improve my technical skills, while also building my scientific knowledge.

I would also like to thank Jaime Luhr, MS. Without her presence in lab, I would have never completed an experiment. She is a very personable and knowledgeable. I could not have completed this research without her help.

Lastly, I would like to extend a thank you to all my peers/coworkers/friends I have collaborated with in my time at the University of Nebraska at Omaha and the University its self. If it wasn't for UNO, I would have never met any of these amazing individuals, who over the past few years have pushed me to improve personally and academically.

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A. Introduction

1. Purpose

The goal of this work was to execute the selective 2’ hydroxyl acylation analyzed by primer extension (SHAPE) protocol, thus generating quantitative data surrounding the secondary and tertiary structures of virulent and avirulent strains of coxsackievirus B3. Gaining new information about how the genomic RNA structures differ between the virulent and avirulent will help us comprehend how structure implicates virulence phenotype. The newly acquired data will be compared to data previously generated using chemical modifications to improve the models of CVB3 currently in use.

B. Background

1. Viruses

A virus is an infective agent only capable of life inside a host cell. Viruses consist of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) enclosed in a protein capsid. The survival and completion of the virus’ life cycle depends upon entering a permissible host cell, and manipuLating the host’s cell machinery, forcing the host to produce viral progeny. Picornaviridae, a family of viruses, includes Poliovirus, Enterovirus, Coxsackievirus, and many more. Characteristics of viruses in this family are RNA dependent RNA Polymerase, Vpg attached to the 5’ end, single open reading fame, and completion of the polyprotein prior to processing (1).

Coxsackievirus B3 (CVB3) is a pathogenic Enterovirus belonging to the *Picornaviridae* family. CVB3 is closely related to Poliovirus. CVB3 is a dominate etiological factor in the development of myocarditis (inflammation of the heart muscle); (2), pancreatitis (inflammation of the

pancreas); (3), and type I diabetes (4). CVB3 has been implicated in 25-45% of acute myocarditis and dilated cardiomyopathy cases involving infants and young adults (5).

2. Coxsackievurus B3 on a Molecular Level

The CVB3 genome contains 7,400 nucleotides organized into four structured regions: a 5' non-translated region (5'UTR), a single open reading frame, a 3' non-translated region (3'UTR), and a poly A tail (Figure 1). The 5'UTR plays an essential role in determining virulence. Slight mutations to nucleotides in the 5'UTR have been shown to have profound effects on virulence. The 5'UTR is 743 base pairs organized into seven domains. The 5'UTR aids in translation by recognizing ribosomes, proteins, and additional initiation factors. The domains covered in this work are domains I and II. The cloverleaf structure, also known as domain I is critical for replication and genome stability (6). Domain II is highly conserved and a determinant for virulence (3).

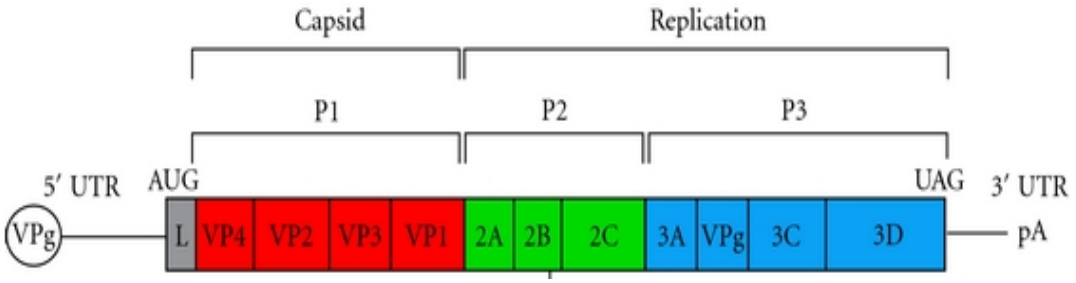

Figure 1. Overview of the entire CVB3 genome. Image courtesy of CVB3 genome overview. Image from https://upload.wikimedia.org/wikipedia/commons/e/e3/English-_Picornavirus_genome_diagram.png.

This research was conducted on two, naturally occurring strains of CVB3. The virulent CVB3/28 has the potential to cause the symptoms listed earlier. The avirulent CVB3/28 does not cause symptoms. Nucleotide sequences as well as secondary nucleotide interactions can be seen in

Figures 2 and 3. Work was also conducted on a mutant strain with mutations at 122 and 124, C to A.

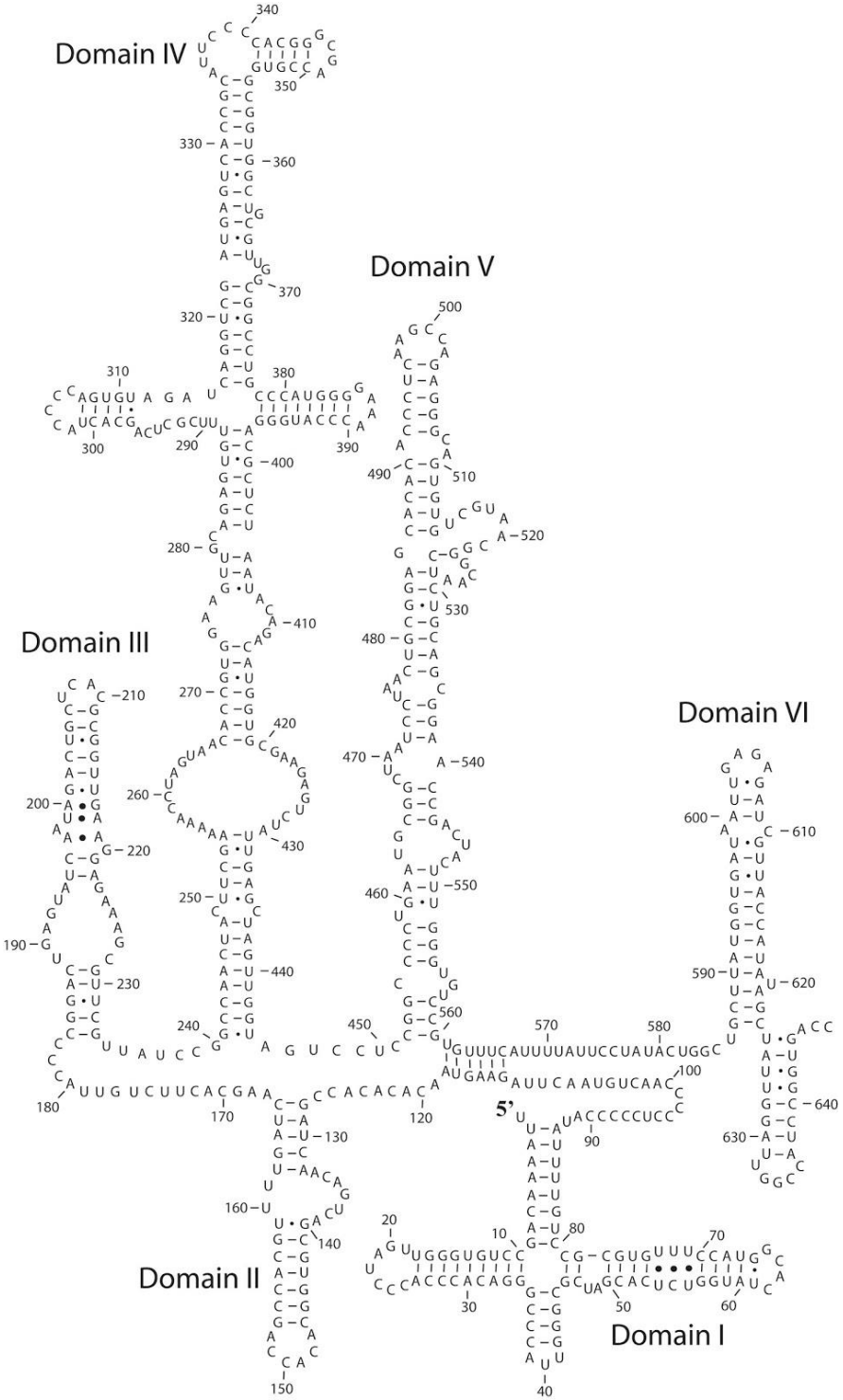

Figure 2. 5’ UTR structure of virulent CVB3/28. Model provided by Bailey and Tapprich, 2006.

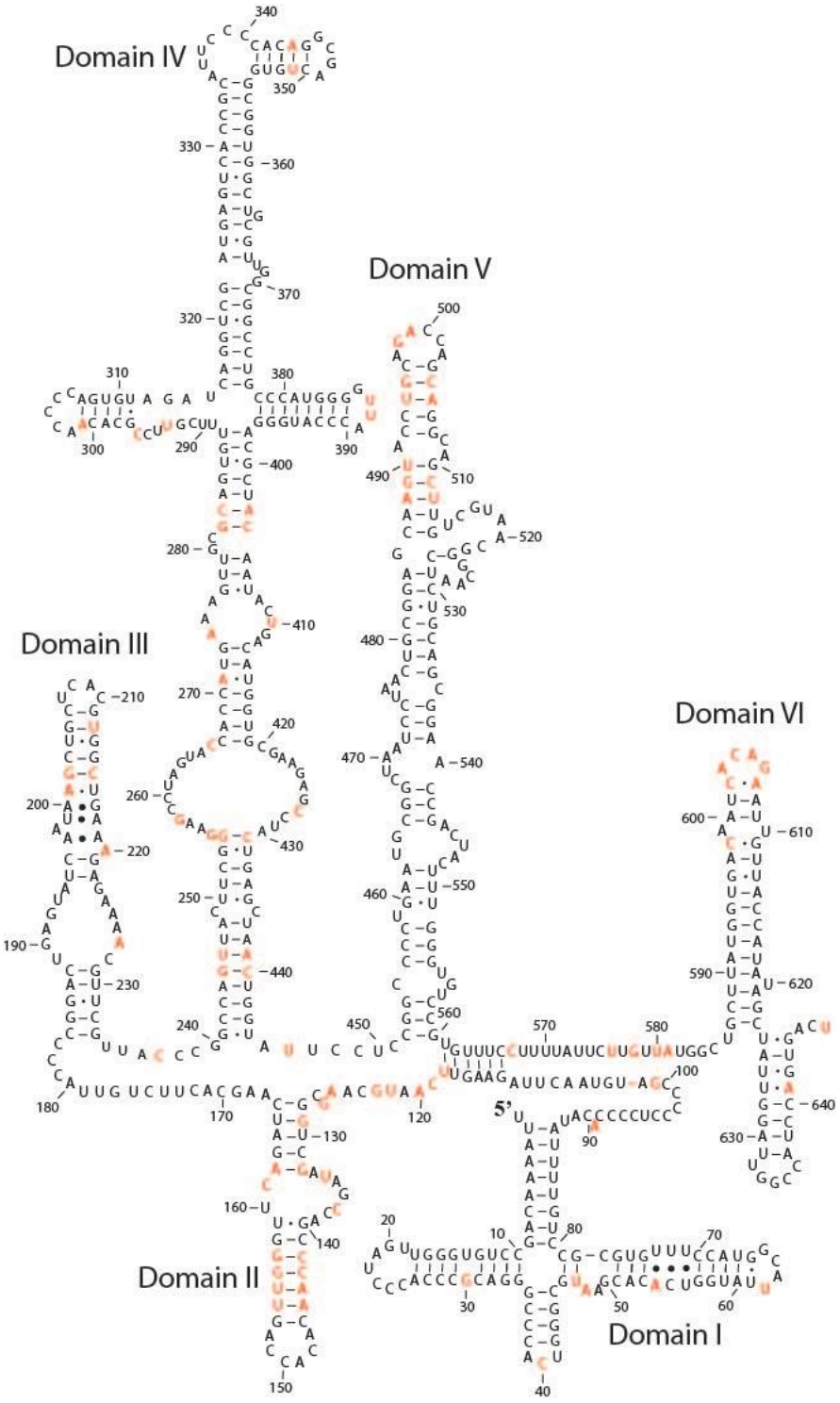

Figure 3. 5' UTR of avirulent CVB3/GA. Nucleotides shown in red indicate variance from CVB3/28. Model provided by Bailey and Tapprich, 2006.

C. Materials and Methods

1. DNA Extraction

E. coli Sure cells were transformed with a plasmid containing the entire CVB3 genome and genes for ampicillin resistance. Transformed cells were twice selected for optimal size and growth by overnight growth at 37°C on luria broth (LB) plates with ampicillin. One colony was removed and placed in 25 mL of liquid LB with 200 ug/mL ampicillin. Cells were grown for 14-18 hours at 37°C. The overnight culture was centrifuged at 6,000 rpm for 10 minutes at 4°C. The resultant pellet was used to harvest plasmid DNA via Quiagen Miniprep DNA Extraction protocol. Electrophoresis on a 1% agarose gel verified isolation of the correct product. See figure 4.

2. Restriction Digest

Isolated plasmid DNA was linearized using Fast Digest *BssHII* for CVB3/GA and FastDigest *Ecl136II* for CVB3/28 according to manufacturer's protocol (Thermo Fisher). CVB3/GA digest reactions contained 10 ug of DNA, 20 uL of 10X fast digest buffer, 16 uL of dNTP, 2 uL of Klenow (10 U/uL), 5 uL of *BssHII* enzyme, and RNase free water to 200 uL. CVB3/28 digest reactions contained 10 ug of DNA, 20 uL of 10X fast digest buffer, 2 uL of Ecl136II, and RNase free water to 200 uL. Reactions were incubated for 4 hours at 37°C. Reactions were purified by phenol and phenol/chloroform extraction. DNA was precipitated by the addition of 20 uL of 3M sodium acetate (NaOAc) pH 5.2 and 600 uL of 95% ethanol and stored overnight at -20°C. The following day, DNA was centrifuged at 13.3g for 20 minutes at 4°C. The pellet was washed twice with 70% ethanol followed by a 5 minute spin at top speed. The pellet was dried in a speed vacuum for 5 minutes. DNA was resuspended in 22 uL of TE (10 mM Tris, 1 mM EDTA) pH

7.6. A 4 uL sample was used for conformation via electrophoresis on a 1% agarose gel. See figure 5.

3. *In vitro* Transcription of 5' UTR

The 5' UTR is generated using linearized DNA fragments and a T7 RNA polymerase (MEGAscript kit, Ambion). Transcription reactions are 80 uL reactions consisting of 32 uL of dNTPs, 8 uL of T7 10X reaction buffer, 8 uL of T7 enzyme, 4 ug of DNA, and RNase free water to volume. Transcription reactions were incubated at 37°C for 6 hours. 4 uL of DNase 1 was added and the reaction was incubated for 30 minutes at 37°C. Transcription was stopped by the addition of 400 uL of RNase free water and 60 uL of ammonium acetate stop solution. Reactions were purified via phenol/chloroform and chloroform extraction. RNA was precipitated overnight in 600 uL of isopropanol at -20°C. RNA was pelleted by centrifugation at top speed for 20 minutes at 4°C. The pellet was washed twice with 70% ethanol followed by a 5 minute spin. RNA was dried in a speed vacuum for 5 minutes and resuspended in 80 uL of TE ph 7.6. See figure 6.

4. RNA Clean Up via MEGAclear Kit

RNA generated through in vitro transcription was cleaned using the MEGAclear Kit (Ambion). The sample was brought to 100 uL with elution solution, followed by 350 uL of binding solution and 250 uL of 100% ethanol and mixing. RNA mixture was applied to a filter cartridge and collection tube, then centrifuged for 1 minute at top speed. Discarded flow through. Washed filter twice with 500 uL of wash solution followed by a 1 minute spin. RNA was collected by addition of 50 uL of elution solution and incubated at 65°C for 10 minutes, then centrifuged for 1 minute.

5. SHAPE

5.1. SHAPE RNA Modification

RNA cleaned using the MEGAclear kit was diluted to 0.03975 ug/uL using 0.5X TE pH 8.0. RNA was separated into two, 60 uL reactions. Both tubes were incubated at 95°C for 3 minutes and moved to ice. While on ice, 30 uL of 3.3X RNA folding buffer (333 mM HEPES pH 8.0, 20 mM $MgCl_2$, 333 mM NaCl) was added. Tubes were incubated at 37°C for 20 minutes. 10 uL of dimethyl sulfoxide (DMSO) was added to the control tube. 10 uL of 32.5 mM N-methylisatoic anhydride (NMIA) in DMSO was added to the modification tube. Tubes were incubated at 37°C for 45 minutes. RNA was precipitated by adding 4 uL of 50 mM EDTA pH 8.0, 1 uL of 20 ug/uL glycogen, 10 uL of 3 M NaCl, and 300 uL of 100% ethanol, and incubating at -80°C for 20 minutes. Reactions were then centrifuged at top speed for 20 minutes at 4°C. Pellets were washed twice with 150 uL of 70% ethanol and centrifuged for 5 minutes. Pellets were dried in the speed vacuum for 2 minutes. RNA was resuspended in 90 uL of 0.5X TE pH 8.0.

5.2. Primer Annealing and Extension

Unmodified RNA was diluted to 0.02641 ug/uL with 0.5X TE pH 8.0. Unmodified and modified RNA was distributed into PCR tubes in 9 uL aliquots. Tubes were heated to 95°C for 3 minutes using the thermocycler. 2 uL of 1 uM fluorescently labeled primers were annealed to RNA by incubating at 65°C for 5 minutes, then 35°C for 10 minutes. 8 uL of extention mix and termination mixes were added to the appropriate PCR tube. RNA was reverse transcribed to DNA using SuperScript III RT (Thermo Fisher) and incubated at 52°C for 15 minutes. RNA template was removed by addition of 2.5 uL of 1 M NaOH, followed by a 15 minute incubation at 98°C, and 2.5 uL of 1 M HCl and 35 uL of RNase free water. Samples were transferred to 1.7

mL tubes. To precipitate DNA, 1 uL of 20 ug/ml glycogen, 10 uL of 3 M NaOAc pH 5.2, and 300 uL of 100% were added, and then incubated at -80°C for 20 minutes. Samples were centrifuged at top speed for 20 minutes and washed twice with 1 mL of 75% ethanol. Samples were dried for 2 minutes using the speed vacuum and resuspended in 10 uL of HiDi formamide. Samples were stored in the dark at -80°C until taken to University of Nebraska Medical Center (UNMC) for analysis.

5.3. SHAPE Data Analysis and Interpretation

Data received from UNMC was in the form of an electropherogram. Electropherograms present the fluorescence intensity for each sample as it passed through capillary detectors plotted against time. Each plot consisted of 4 traces corresponding to the 4 channels and their contents. Additionally, each trace is reflective of the unique cDNA contents. Electropherogram results are exported to ShapeFinder.

In ShapeFinder the peaks of the traces are integrated, aligned, and normalized. A reactivity profile is generated for the sample of interest. This profile is exported to RNAstructure software. Using RNAstructure, the normalized profile is fed into the RNA secondary structure algorithm. The output of RNAstructure is a figure with the lowest calculated energy for its secondary structure. See figures 9 and 10.

6. Chemical Modification of RNA

6.1. N-Cyclohexyl-N'-(2-morpholinoethyl)carbodiimide metho-p-toluensuLfonate (CMCT)

15 ug of RNA was renatured in 50 uL of 1X CMCT buffer (160 mM K-Borate pH 8.0, 40 mM $MgCl_2$, 200 mM NH_4Cl, 3 mM DTT) by incubation at 80°C for 2 minutes, then slow cooled to

40°C. RNA was modified by addition of 50 uL of 42 mg/mL CMCT in 1X CMCT buffer and incubated at 37°C for 10 minutes. Reaction was stopped by addition of 10 uL of 3M NaOAc pH 7.0 and 300 uL of 95% ethanol. Reaction was stored at -20°C overnight. RNA was pelleted by centrifugation at top speed for 20 minutes at 4°C. Pellet was twice washed with 400 uL of 70% ethanol followed by a 5 minute spin. Pellet was resuspended in 20 uL of TE pH 7.6.

6.2. Dimethyl suLfide (DMS)

15 ug of RNA was placed into 100 uL of 1X DMS buffer (160 mM K-Cacodylate pH 7.2, 40 mM MgCl2, 200 mM NH4Cl, 3 mM DTT) and renatured by heating to 80°C for 2 minutes. DMS was diluted to 1:10 in 95% ethanol and 2 uL were added to the RNA. Reaction incubated at 37°C for 10 minutes. Reaction was stopped by addition of 25 uL of DMS Stop Buffer (1 M Tris-HCl pH 7.5, 1 M β-mercaptoethanol, 0.1 M EDTA). RNA was precipitated by addition of 15 uL of 3 M NaOac pH 7.0 and 450 uL of 95% ethanol, and stored overnight at -20°C. RNA was pelleted by centrifugation at top speed for 20 minutes, followed by 2 washes with 70% ethanol. Pellet was dried in the speed vacuum for 5 minutes. RNA was resuspended in 20 uL of TE pH 7.6.

6.3. Kethoxal

15 ug of DNA was placed into 100 uL of 1X Kethoxal buffer (160 mM K-Cacodylate pH 7.2, 40 mM $MgCl_2$, 200 mM NH_4Cl, 3 mM DTT) and renatured by heating to 80°C for 2 minutes and slow cooled to 40°C. Kethoxal was diluted to 1:10 in 1X Kethoxal buffer, then 5 uL was added to the RNA. RNA was incubated at 37°C for 10 minutes. Reaction was stopped by adding 50 uL of Kethoxal Stop buffer (1 M Tris-HCl pH 7.5, 1 M β-mercaptoethanol, 0.1 M EDTA). RNA was precipitated by adding 15 uL of 3 M NaOAc pH 7.0 and 450 uL of 95% ethanol and stored at -20°C overnight. RNA was pelleted by centrifugation at top speed for 20 minutes followed by

two washes with 70% ethanol and 5 minute spins at 4°C. Pellet was dried in the speed vacuum for 5 minutes. Pellet was resuspended in 20 uL of K-Borate pH 7.0.

6.4. Primer Annealing and Extension

Oligonucleotide primers were labeled with ^{32}P by the following reaction: 5 uL of primer, 2 uL of 10X T4 polynucleotide kinase buffer (Invitrogen), 1 uL of T4 polynucleotide kinase (10 units/ uL; Invitrogen), 10 uL of Υ-^{32}P ATP (6000 Ci/mmol), and 2 uL RNase free water. The reaction was incubated in a thermocycler for 40 minutes at 37°C, then 20 minutes at 65°C, and held at 4°C. The final reaction was diluted with 30 uL of TE pH 7.6 to make the final concentration 2 pmol/uL. Primer was stored at -80°C.

Radioactive labeled primers were annealed to modified or unmodified RNA using the following 20 uL reaction: 2 ug of RNA, 4 uL of 5X annealing buffer, 2 uL of labeled oligonucleotide, and RNase free water to volume. The reaction was heated to 80°C for 2 minutes and slow cooled to 40°C.

Primer extension reactions were performed by adding 4 uL of the annealing reaction, 3 uL of extension mix (100 mM Tris-HCl pH 8.3, 80 mM KCl, 12 mM MgCl2, 4 mM each of dATP, dGTP, dTTP), and 2 uL of reverse transcriptase. Reactions incubated for 25 minutes at 45°C. Reactions were stopped with 2 uL of Stop solution (0.025% bromophenol blue, 0.025% xylene cyanol, 90% formamide, 1 mM EDTA pH 8.0). Primer extension reactions were stored on ice. Immediately before loading onto gel, samples were heated to 90°C for 2 minutes.

6.5. Urea Gel

Chemical modification results were visualized using a 12% urea gel electrophoresis. Gels were made by combining the following over low heat: 33.6 g of urea, 21 mL of 40% 19:1 acrylamide,

7 mL of 10X TBE (890 mM Tris base, 890 mM boric acid and 0.5 mM EDTA pH 8), and water to 70 mL. The mixture was polymerized by adding 100 uL of TEMED and 250 uL of 10% ammonium persulfate. The mixture was then injected between 2 glass plates and allowed to set overnight. Gels were pre-run for 60 minutes at 60W. Samples were run for 2-8 hours depending on desired separation.

After the gel was done electrophoresis, the gel was transferred to filter paper, vacuum dried for 1 hour, and placed on an imaging screen overnight.

6.6. Chemical Modification Data Interpretation

Nucleotide modifications appear in the 12% urea sequencing gels as dark bands. Nucleotides are modified when the structure is solvent exposed or not complexed with RNA, allowing the nucleotide to be methylated. The relative darkness or lightness of the band reflects the amount of modification, and thus the degree to which the nucleotide was solvent exposed.

D. Results

1. DNA Extraction

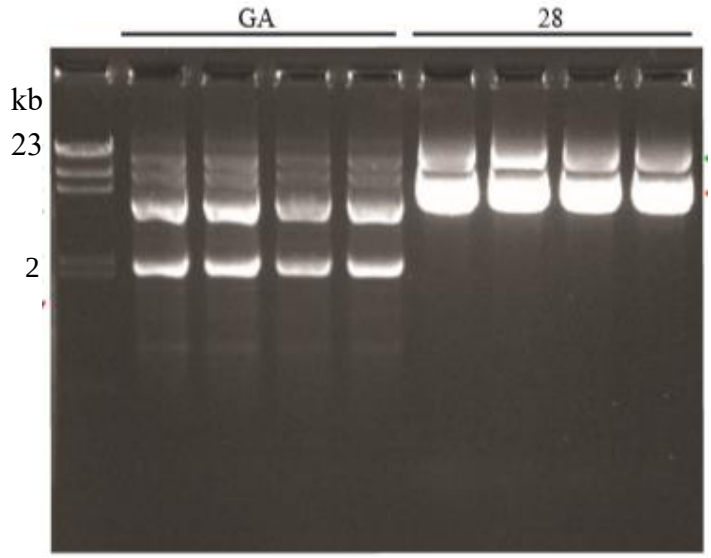

Figure 4. Plasmid DNA Extraction. 1% Agarose gel demonstrating the extraction of plasmid DNA from E. coli Sure cells. Gel electrophoresed for 60 minutes at 70 V. Image from Luhr, 2016, figure 13.

2. DNA Digestion

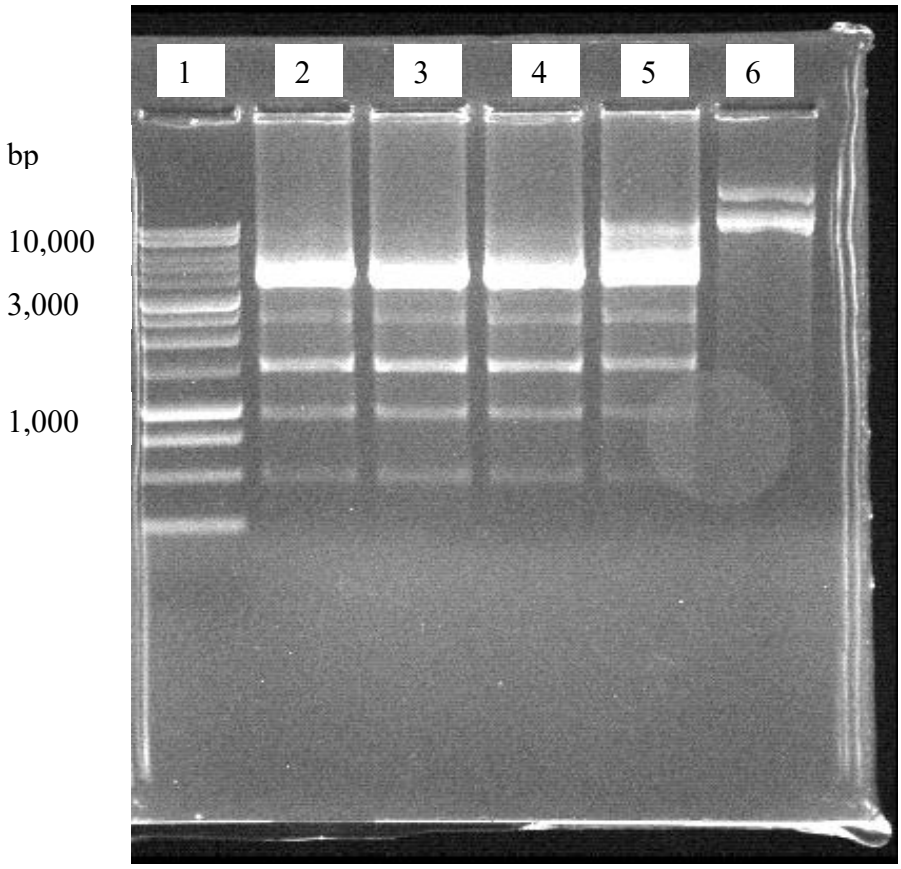

Figure 5. Plasmid DNA Digestion. 1% Agarose Gel confirming the digestion of plasmid DNA. Lane 1 is a 1 kB ladder. Lanes 2-5 demonstrate digested plasmid DNA. Lane 6 shows the uncut DNA plasmid. Gel was run for 60 minutes at 70V.

3. In vitro Transcription of 5' UTR

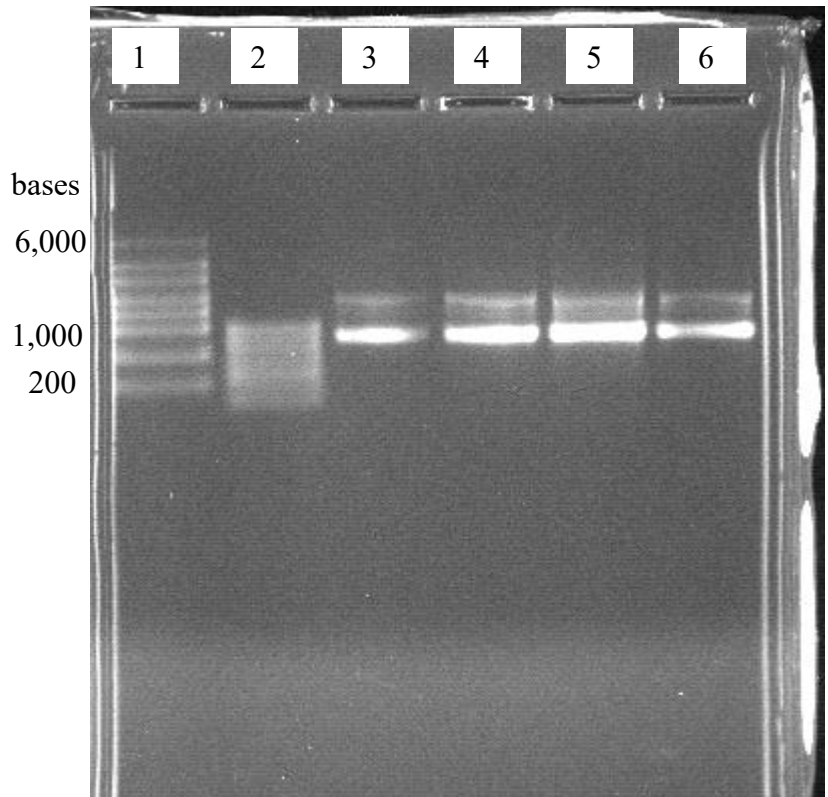

Figure 6. RNA Product. 1.5% Agarose gel confirming the desired 746 base-pair RNA product. Lane 1 is a high range riboruler. Lane 2 is a low range riboruler. Lanes 2-6 confirm the presence of RNA. Gel was run for 90 minutes at 70V.

4. Chemical Modification

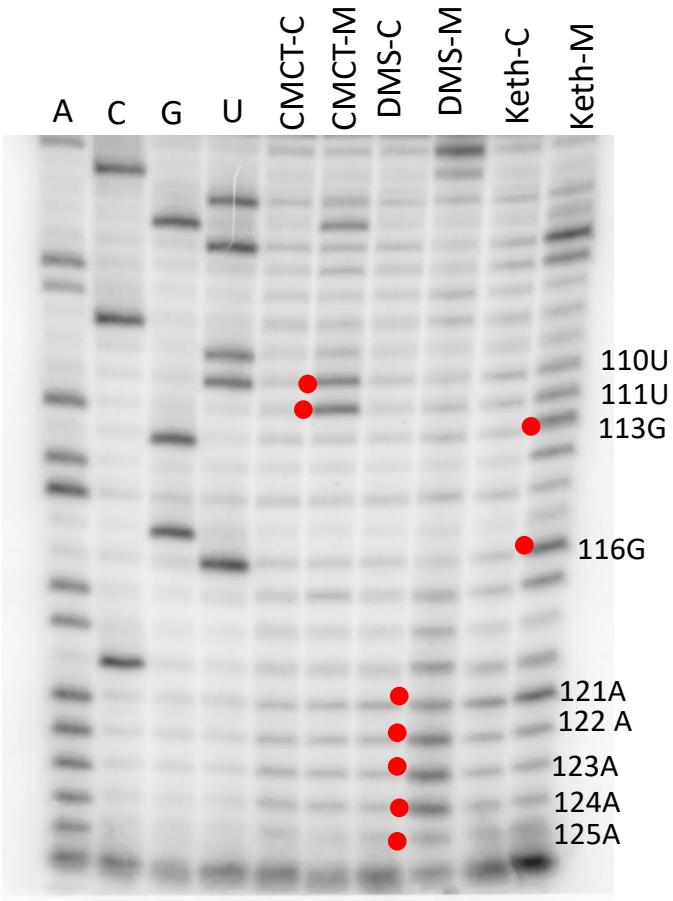

Figure 7. 12% Urea sequencing gel confirming chemical modifications through domain II. The strain used for this gel possess mutations at 122 and 124, C to A. This strain is a mutant of CVB3/28. Labels on the right indicate nucleotide position corresponding to sequencing tracks (A,C,G,U). Results for the 3 mutagenic chemicals (M) and control (C) are shown, CMCT, DMS, and Kethoxal (Keth).

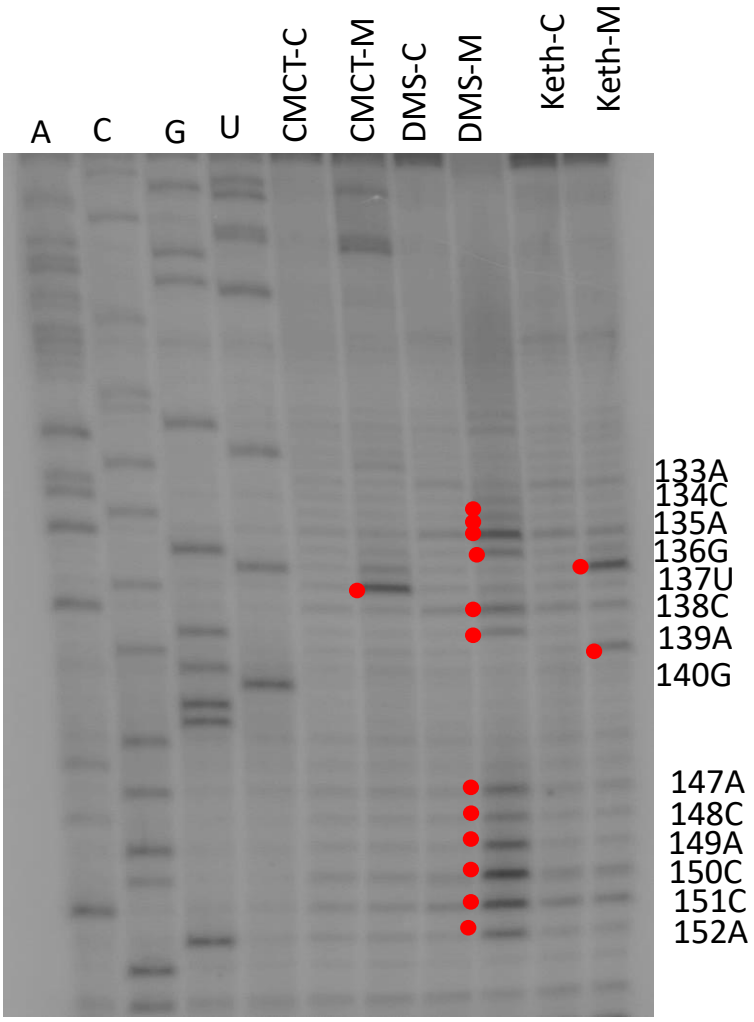

Figure 8. 12% Urea sequencing gel confirming modifications through domain II. Labels on the right indicate nucleotide position according to the sequencing tracks (A,C,G,U). Lanes 6-10 contain control (C) and modified (M) samples for CMCT, DMS, and Kethoxal (Keth). Strong modifications are annotated with a red, filled circle. The strain imaged here possess mutations at 122 and 124, C to A. This is a mutant strain of CVB3/28.

5. Proposed Structures Generated via SHAPE

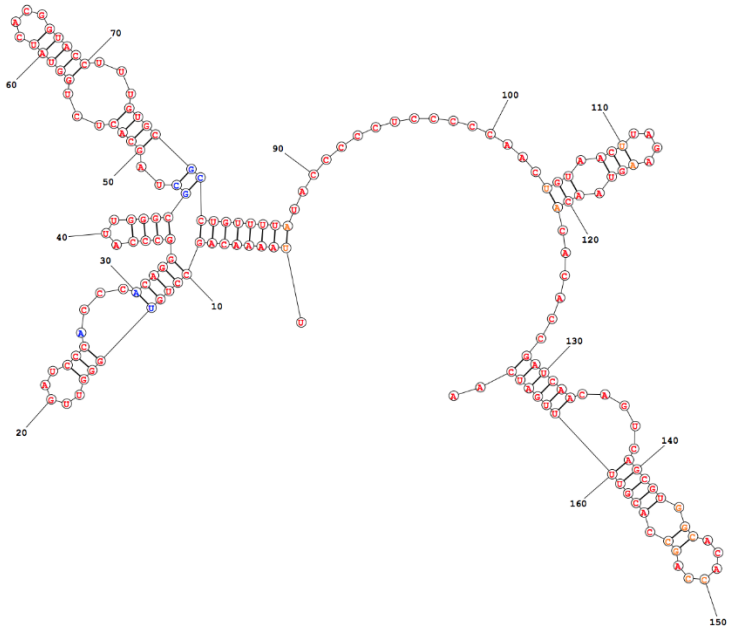

Figure 9. Proposed model for domains I and II (CVB3/28) generated via SHAPE protocol and RNA Shapefinder. Image provided by Tapprich Lab Members.

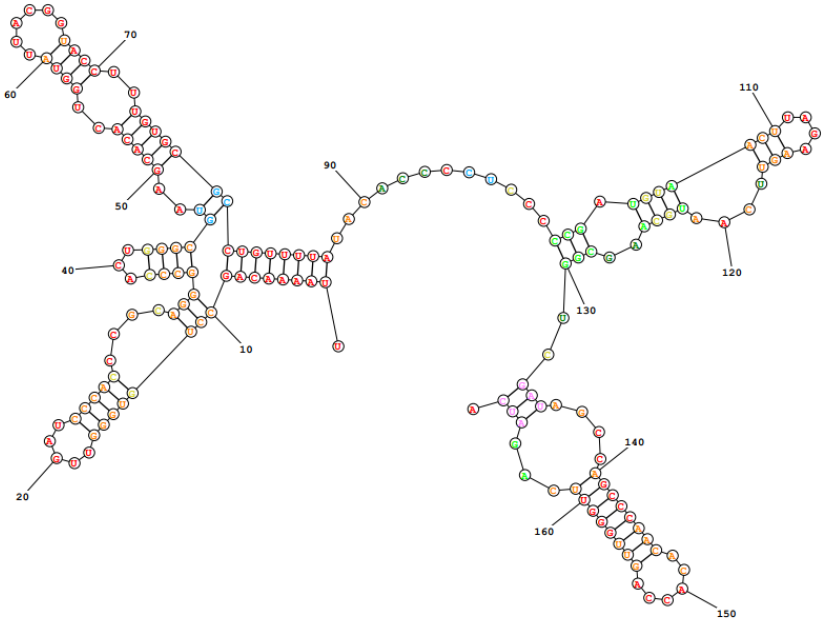

Figure 10. Proposed model for domains I and II (CVB3/GA) generated via SHAPE protocol and RNA Shapefinder. Image provided by Tapprich Lab Members.

E. Discussion

Understanding viral structure and its ability to determine virulence is essential to improve the knowledge surrounding human health and viruses. The purpose of this work was to present information generated using chemical modification protocols and compare it to results generated using SHAPE analysis. Nucleotide associations responsible for higher level RNA folding patterns and structure haven't been defined.

1. Domain II in Mutant Strain

The sequencing gel found in figure 7 confirms that the strain of CVB3 analyzed does indeed possess the mutations at nucleotide positions 122 and 124 from C to A. Figure 7 also demonstrates that nucleotides 110,111, 113, 116 and 121-125 are heavily modified. Figure 8 of the mutant CVB3 strain shows nucleotides 147-152 and 122-140 to be heavily modified. Heavily modified nucleotides have been highlight with a red dot on the 5' UTR map shown below.

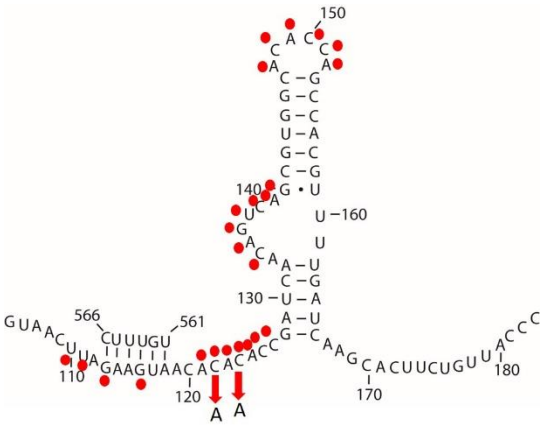

Figure 11. Annotated domain II 5' UTR map of CVB3/28.

Modification at positions 113 and 116 indicate interruption of the proposed Watson-Crick base pairing occurring in this region. The structure may be altered from the known model because of the mutations at 122 and 124 are causing structural changes. The region spanning nucleotides 121-125 appears to be modified indicating that domain II is becoming more open to

modification. Nucleotides from positions 133-140 and 127-152 are modified, but this modification is not novel. In the known structure this nucleotide sequence appears to be un-complexed with RNA, allowing it to be solvent exposed.

The chemical modification method is limited in its scope because it depends upon visual inspection of the relative darkness of bands.

2. Proposed Models for 5' UTR

The proposed models for domain I and II of CVB3/28 and CVB3/GA are similar but different. These differences between the strains provide quantitative information surrounding the flexibility of the phosphodiester backbone. Structural differences are analyzed below.

Positions 13-17 show different Watson-Crick base pairing between the proposed structures. CVB3/GA proposed displays 5 correctly matched base pairs, while CVB3/28 proposed only shows 3 correctly matched base pairs leading to the loop at nucleotides 18-22. In the Bailey and Tapprich model the loop also differs by including more nucleotides, 19-25.

Positions 57-61 are in a loop structure. In CVB3/28 proposed there are 5 base pairs leading to the loop, while CVB3/GA proposed has only 4 nucleotide pairs leading to the loop. Additionally, positions 62-65 of CVB3/28 proposed match that of the Bailey and Tapprich Model. While CVB3/GA proposed differs from the Bailey and Tapprich model. The proposed structure includes 2 additional nucleotides, increasing the size of the loop to nucleotides 61-66.

Positions 70-77 vary between proposed structures, as well as varying from the known model. CVB3/GA proposed appears to be slightly more structured than CVB3/28 proposed as noted by the additional nucleotide base pairing and deceased number of nucleotides in the loop. From the known structure this region is fairly structured as nucleotides appear complexed with other nucleotides.

Positions 111-140 appear very different between the proposed structures. This region includes the interconnecting region between domains I and II. This stretch of nucleotides possess many nucleotide differences between the known structures of CVB3/GA and CVB3/28. Primary differences or differences in sequence are likely the reason for the drastic structural differences seen.

Positions 140-147 are base paired in CVB3/GA proposed, while two nucleotides within this range in the CVB3/28 proposed not base paired, but are instead in the loop structure. Thus, this structure is more open.

The region spanning positions 160-170 vary significantly between the proposed structures. In the primary sequence there are 2 nucleotide differences. In CVB3/28 proposed there is more RNA complexed with RNA in the form of base pairing than in CVB3/GA proposed.

The nucleotide differences described above confirm that the virulent and avirulent strains do have different amounts of flexibility in the backbone. Thus, the strains have unique structures leading to differences in virulence phenotype.

3. Future Studies

The next step in SHAPE analysis consists of analyzing RNA bound to protein, poly (rC) binding protein 2 (PCBP2). These studies will allow us to gather quantitative data about how RNA changes structure by folding to either increase or decrease the amount of backbone exposed. Also, analyzing chimeric constructs with swapped SLII regions would greatly improve our understanding of how the SLII confers virulence and the structure it adapts.

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