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# Role of Stemloop D in Terminally Deleted Coxsackievirus B3 Replication

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# ROLE OF STEMLOOP D IN TERMINALLY DELETED COXSACKIEVIRUS B3 REPLICATION

by

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**ROLE OF STEMLOOP D IN TERMINALLY DELETED COXSACKIEVIRUS B3 REPLICATION**

Lee K. Jaramillo, M.S.

University of Nebraska, 2016

Advisor: Nora M. Chapman

Coxsackievirus B3 (CVB3) is an enterovirus with no known form of latency. However, assays designed to detect enteroviral RNA have shown that CVB3 RNA can persist for weeks beyond the acute infection both naturally and experimentally. Our previous work with coxsackievirus revealed an inhibited version of enteroviral replication where the progeny virus, termed terminally deleted (TD) virus, was missing a maximum of 49 nucleotides from the beginning of the 5' non-translated region (NTR). The largest terminally deleted virus, TD50, effaced stem a, stemloop b, and stemloop c from the secondary structure, the cloverleaf. We hypothesized that further deletion beyond those first 49 nucleotides (nt) would disrupt stemloop d and prevent alternative binding of replicative machinery to the point of lethality. Plasmid DNA designed to create functional wildtype CVB3, was used as a template to create two novel constructs: TD57 and TD78. The mutant plasmids would transcribe infectious viral RNA missing up to 56nt and 77nt from the start of the 5' NTR respectively. After transcription, the mutant RNA was transfected into HeLa cells and incubated for three days. Using RT-PCR we prove that despite the loss of stemloop d, these CVB3-TDs replicated and produced infectious encapsidated viral RNA.

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| | |
|---|---|
| PVR | poliovirus receptor |
| IRES | internal ribosomal entry site |
| VPg | viral protein genome linked |
| RdRp | RNA dependent RNA polymerase |
| PABP | poly A binding protein |
| CRE | cis-acting replication element |
| hnRNP C | heterogeneous ribonuclear protein C |
| RF | replicative form |
| RI | replicative intermediate |
| PCR | polymerase chain reaction |
| SAP | shrimp alkaline phosphatase |
| FBS | fetal bovine serum |
| RT | reverse transcription |
| CPE | cytopathic effect |

## Chapter 1: Introduction

### What is an Enterovirus

Enteroviruses belong to the family Picornaviridae. The enterovirus genus comprises 12 viral species including the prototypical enterovirus, Poliovirus. These viruses are classified according to serotype or the ability for neutralizing antibody to affect an isolated virus often identified by the most conserved genetic sequence of the capsid structure [1-3]. Enteroviruses contain only one copy of a single stranded positive sense RNA molecule within a naked icosahedral capsid [4]. The capsid structure consists of 60 protomers made from 3 viral proteins. The capsid structure includes a canyon depression which is the site of the virus interaction with the host cell receptor [5, 6]. Interaction with the receptor causes a conformational change allowing the genome to egress into the host cytoplasm where translation, replication, and assembly of the viral particle will occur.

### History of disease

Enterovirus infection can cause a range of diseases based on the infected organ, such as non-septic febrile illness, respiratory illness, acute hemorrhagic conjunctivitis, type 1 diabetes, myocarditis, meningitis, pancreatitis, paralytic poliomyelitis, and dilated cardiomyopathy to name a few [4]. In temperate regions of the world CVB infections mainly occur during the summer and fall, but can vary depending on the serotype [4, 7-11]. Coxsackievirus B 1, 3, and 4 exhibit an epidemic pattern where activity can increase at irregular patterns [12, 13]. Still, coxsackievirus infections are generally asymptomatic or subclinical [4].

The most notable disease, paralytic poliomyelitis, is contracted from poliovirus which is a virus with a long history in medicine. The first documented case of polio was by the physician Michael Underwood in 1789 when he was treating a young child for paralytic poliomyelitis [14].

Paralytic poliomyelitis is one of the more severe diseases that enterovirus can cause, but most of the research regarding the disease did not come until an epidemic of polio broke out in the late 19th century. It is believed that the cause of this outbreak was from an increase in public sanitation which in turn caused the general population to encounter the virus for the first time at a later age [15]. Prior to this time typical infection was between the age of 0 and 12 months old, a time when protective antibodies acquired from the mother were still circulating [15].

Research into the epidemic and the disease resulted in a number of landmark discoveries, one of which being the virus itself by Landsteiner and Popper [16]. Discovery of the viral agent furthered research in this area which would later bring about the polio vaccine. The first polio vaccine developed for human protection was an inactivated form of the virus by Salk [4]. This first vaccine was an incredible step on the road to polio eradication and was only bolstered by the discovery of a live form of the polioviruses by Sabin [4]. The live form of the virus was heavily attenuated, but still sufficiently replicated for a robust immune response. The normal processes of the virus could continue without affecting the host, which in turn generated a much more robust immune reaction that could last longer than the inactivated form of the vaccine could create [4]. Since advent of the vaccine there are now only three countries that have never interrupted transmission of the wildtype virus [17].

Despite our success in vaccination attempts, there are still many enteroviruses for which there are no vaccines and still affect millions of people annually. The virus is typically transferred via fecal-oral tract and can cause illness in the upper and lower respiratory system. Coxsackievirus A16 is the most common cause of hand, foot, and mouth disease in the United States and Coxsackievirus A24 along with enterovirus 70 have been associated with outbreaks of conjunctivitis. Echovirus, specifically 13, 18, and 30 have caused outbreaks of viral meningitis in the United States which can be life threatening. In regards to the rest of the world, enterovirus

71 has caused large outbreaks of hand, foot, and mouth, including cases of neurologic disease, worldwide and enterovirus D68 caused a nationwide outbreak in 2014 in the United States [18]. Infections with the virus are acute events, but under the right conditions can become chronic in a persistent infection [19]. In cases where myocarditis was ruled the cause of death in human patients, enteroviral RNA was often recovered when checked [20, 21].

**Enteroviral Infection and Life Cycle**

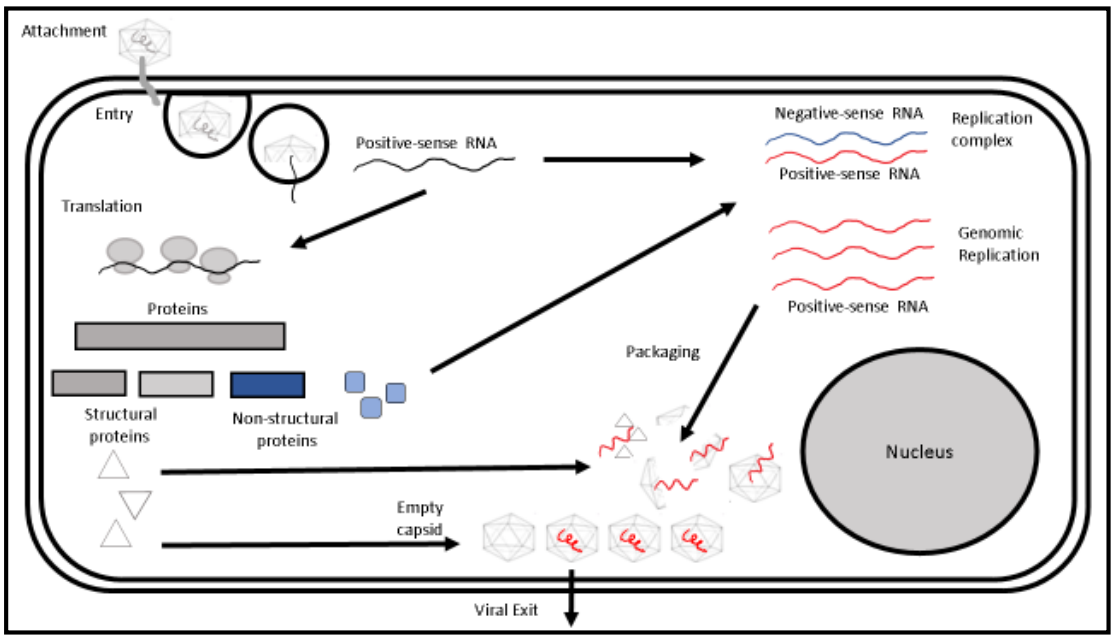



**Figure 1:** Brief overview of the enterovirus life cycle.

Receptor structure can vary from virus to virus, such as with poliovirus and coxsackievirus that use immunoglobulin like receptors, [5] but minor group human rhinoviruses can use VLDL-R for a receptor [22-24]. Each enterovirus species uses a host receptor that causes a conformational change of the capsid allowing the virus to be internalized via endocytosis and release the genome from the capsid. Rossman proposed that the receptor binds into a canyon structure found on the surface of the virus, as in coxsackievirus that encircle each twelve 5-fold vertex, each being inaccessible to the host immune system [6]. In the case of coxsackievirus B, binding of the CAR receptor can be preceded by binding of a secondary receptor known as DAF

or CD55 which can aid in the binding of CAR [25-27]. In poliovirus a similar interaction takes place using Poliovirus Receptor (PVR) [28].

The interaction with the enterovirus with its receptor leads to the formation of the A-particle that is characterized by a partial exposure of the RNA and release of the internal VP4 from the virus [29, 30]. Enteroviral particles have been shown to internalize using a number of methods. CVB3 has been shown to internalize using clathrin [31], whereas CVB4 has shown internalization using a lipid raft [32], and CVB3-RD variant that uses DAF, internalization using caveolin-1 [25]. It is believed that the egress of the genome into the cytoplasm is accomplished using the viral protein 4 that is internally located until the cellular receptor binding event [33-35]. Eventual translation and replication of the viral genome will allow for packaging and release from the host cell.

**Translation and Processing**

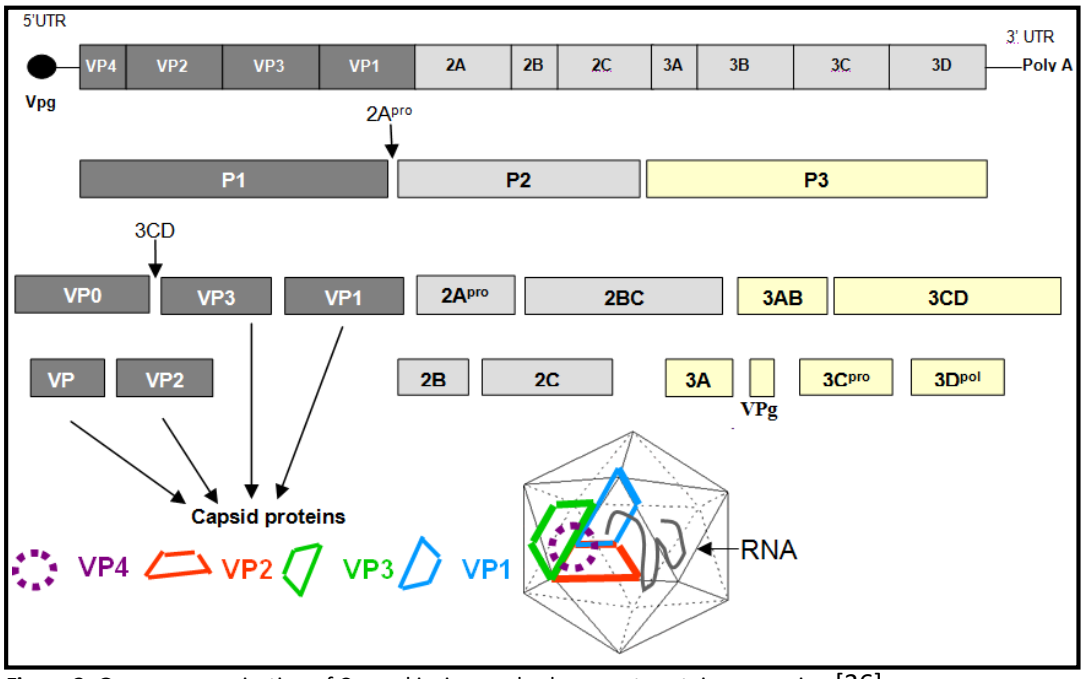



**Figure 2:** Genome organization of Coxsackievirus and subsequent protein processing [36].

Upon entry of the host cytoplasm, the RNA is translated via a cap-independent mechanism by host ribosomes using an Internal Ribosome Entry Sequence (IRES)[5]. This type of RNA structure is not unique to enteroviruses, varies from species to species, but all act as a way to guide the host ribosomal machinery to the site of initiation without the presence of a cap structure. Only one open reading frame exists in the small viral genome and from it a number of structural and nonstructural proteins are expressed. The open reading frame is divided into three general regions: P1, P2, and P3 with P2 broken down further to A-C and P3 A-D.

**P1**

From P1 come the capsid proteins, while P2 and P3 create a variety of nonstructural proteins used to replicate the genome. When translated, P1 will generate VP1, VP3, and VP0. VP0 will eventually be cleaved into VP4 and VP2 via a catalytic post-packaging step during viral assembly [5]. The structural proteins VP1, VP2, VP3, and VP4 are used to make the viral capsid. VP1, VP0, and VP3, will combine to make a protomer. This single protomer combines with other protomers to complete the capsid, which upon forming, undergoes an autocatalytic cleavage to then form VP2 and VP4 with VP2 on the outside and VP4 on the inside. The final infectious capsid contains the viral protein genome-linked (VPg) covalently linked to the viral genome, with which the RNA will be primed for transcription using a virally encoded RNA dependent RNA polymerase (RdRp)[5].

**P2**

P2 proteins mainly aid in the processing of the viral genome and remodeling of the host cell. The first P2 protein translated is protein 2A which is a cysteine protease that cleaves the junction between P1 and P2 after translation [37]. 2A also cleaves eIF4G, host nucleoporins, Poly A Binding Protein (PABP), and TBP: a number of host factors that naturally promote the creation

of host proteins and when cleaved can still be used by the enterovirus for translation and replication [38]. Viral protein 2B or 2BC disrupts the Golgi complex and secretory pathway, but also plays a role in virally produced membranous vesicles that has yet to be fully elucidated [39]. 2C has ATPase activity, RNA helicase activity, chaperone activity, and encapsidation involvement [40]. These functions of $2C^{ATPase}$ have been shown to be critical in vitro in enterovirus 71 and coxsackievirus A 16 [41, 42].

**P3**

The proteins of P3 are directly involved in the replication of the genome. The P3 region is processed in two ways: the major and minor pathway. The major pathway yields proteins 3AB and 3CD. The protein 3AB has been shown to stimulate the activity of the RNA dependent RNA polymerase [43-45], but also may play a role in the formation of vesicles. Protein 3CD is a protease used to cleave the polyprotein and an RNA binding protein PABP [46, 47]. Translation via the minor pathway yields proteins 3A and 3BCD. 3A has been shown to disrupt the trafficking of the ER to Golgi signaling, while 3BCD will be further processed into 3B, 3C, and 3D [39, 48-50]. While 3C is the major protease that processes P2 and P3 proteins, 3D the RdRp, and 3B (or VPg) are intimately involved in the replication of the viral genome.

**Wildtype Replication**

**VPg**

Protein 3B is the protein primer used in transcription of the RNA genome during replication. Before priming replication, VPg is first uridylylated using the RdRp and a Cis-acting Replication Element (CRE) as a template [51]. In coxsackievirus, 3CD will bind the stem of the CRE structure located in the 2C region and 3D with VPg attached will “read” the AA residue found in the loop of the CRE. The CRE structure can be located in a number of different areas,

taking on different shapes and sizes, but will always have a double adenine with which it can prime the addition of the uracil to VPg [52]. Addition of the uridine nucleotides to VPg is believed to be accomplished by 3D using a slide-back mechanism [51]. Once the UU is added to the VPg, it can be used as a primer and anneal to the 3' terminal AA of the negative strand viral RNA.

**3D**

Viral genome replication requires the function of the RNA dependent RNA polymerase or 3D. 3D is processed from P3 and again from 3CD [53]. 3D is essential for VPg uridylylation [54], initiation of the strands of viral RNA, and elongation of the viral RNA during viral RNA synthesis [55]. 3D polymerase is able to effectively transcribe the positive stranded viral RNA to create a negative strand that can then be used as a template for additional positive strands of viral RNA [56]. Enteroviral RdRp's have the same overall shape as other polymerases and have a right-hand conformation with a thumb, finger, and palm domain [57]. More to the point, rhinovirus, poliovirus, and coxsackievirus have very similar 3D structures [58]. The 3D fingers-domain is important for maintaining stable and processive elongation and is involved in binding incoming template RNA [59]. While flavivirus 3D can initiate polymerization de novo, poliovirus and coxsackievirus need VPg as a primer and by binding the protein primer with uridylylated 3B, the negative strand of viral RNA is first transcribed [60].

**Circularization**

VPg will prime the replication process, but both ends of the viral RNA and the interacting proteins are required to be in close proximity of one another and it is believed that this is accomplished by circularizing the positive strand [56]. Computer mapping of the 7500nt genome show complicated structures from beginning to end. Three locations in particular have

seen increased interest, in relation to replication, due to the complicated secondary structures that they form and the direct contribution to the efficient viral replication: 5' cloverleaf, 3' cloverleaf, and the CRE. A ternary complex composed of 3D, 3CD, Heterogeneous nuclear ribonuclear protein (hnRNP C), Poly-C Binding Protein, and domain I of the 5' end of the positive strand forms to allow interaction of the 5' end and 3' end PABP which binds multimers of adenine such as the polyA tract of the genome [61-63]. The VPg bound 3D associates with the 3' end of the positive stranded RNA and begins elongation of the negative strand. The first round of transcription yields the negative strand of viral RNA and will be the new template for a number of positive strands. The new negative strand and template positive strand form a heteroduplex of positive and negative stranded viral RNA [64, 65].

**Replicative Form**

This duplex creates the hypothetical heteroduplexed positive and negative strand of viral RNA termed the replicative form (RF) which is produced in the stage before exponential viral RNA replication [64, 65]. It is believed that a combination of host proteins and viral proteins are involved to unwind the RF, namely the ternary complex, 2C, and hnRNP C [56]. Newly uridylylated VPg attached to 3D, 2C, and hnRNP C proteins bind the newly synthesized 3' end of the negative strand RNA while the ternary complex reforms on the 5' end of the positive strand in the heteroduplexed RF [62, 66]. While the mechanism of action has yet to be fully elucidated, the RF is unwound making room for binding of the uridylylated VPg bound 3D to begin elongation [67].

**Replicative Intermediate**

Elongation of the new positive strand begins at this point, but before completion of the first strand of positive stranded RNA, it is hypothesized that multiple positive strands of RNA are

initiated on that same negative strand of RNA, creating a new structure of RNA referred to as the replicative intermediate (RI) [56]. Formation of the RI begins an exponential generation of newly synthesized positive stranded viral RNA. The ratio of positive to stranded RNA has been demonstrated to be 40:1 to 70:1 with the newly synthesized strands being used for transcription, translation, or packaging into empty capsids [68, 69].

**Terminally Deleted Viruses**

The wildtype enteroviral replication process described above is an acute event, with symptoms and viral titers both climaxing and clearing in a few days [70]. That being said, enteroviral RNA or protein has been detected in patients presented with heart maladies [71-74]. Furthermore, coxsackievirus infection in mice can persist for long periods of time, even months, when infected with cardiovirulent strains [75-78]. It is believed that this persistent infection is permitted due to low-levels of replication averting detection by the host immune system. These naturally occurring enteroviruses were first identified in coxsackievirus B and shown to have a number of deletions to domain I, more specifically, the cloverleaf [79].

**Cloverleaf**

The positive stranded EV RNA is flanked on each side by 5’ and 3’ non-translated regions that naturally form a number of secondary structures like the IRES and CRE, but also the aforementioned cloverleaf structure in domain I [80]. The cloverleaf, which typically is the binding site of the ternary complex in efficient replication, is modified in the case of the TDs. Terminally deleted viruses are shown to have a number of deletions on the cloverleaf structure with the largest being the removal of the first 49nt [79]. Because these viruses suffer the formation of an incomplete cloverleaf they are not found under normal experimental conditions possibly because they are outcompeted by wildtype replication. The initial replicative event

causing the creation of these TDs is thought to be an inaccurate initiation event during positive strand synthesis. Research has shown TDs generated in mice and in cell cultures lacking hnRNP C such as cardiomyocytes and pancreas islets [81].

**Cause of TDs**

As previously mentioned, hnRNP C is hypothesized to be involved in the initiation of the positive strand synthesis by unwinding of the RF. Inefficient priming of the new positive strands drops the ratio of positive to negative strands closer to 1:1 [79]. The positive to negative strand ratio is heavily unbalanced in efficient replication of the virus as stated earlier. The deletions found in these experiments affect only stem a, stemloop b, and stemloop c. Deletion into stemloop d has not been shown; possibly because stemloop d contains essential structural rearrangements critical to viral propagation. 3CD has already been shown to bind stemloop d and PCBP poly C region just beyond the opposite half of stem A [61-63]. This association led us to hypothesize that the structures such as the di-nucleotide bulge, the non-canonical base pairs, or the apical tetra loop are the minimum requirements for CVB replication. Our experiments show that deletion beyond the first 49nt produces viable virus: new TDs with further decreased replicative events. Removal of the di-nucleotide bulge and the complete elimination of stemloop d does not prevent CVB replication.

**Chapter 2: Methods and Results**

**Generating the Terminally Deleted Viruses**

<u>Hypothesis:</u>

The examination of size of TD genome deletions was assessed in several studies [79, 82, 83]and no larger deletions than 49nt were found.

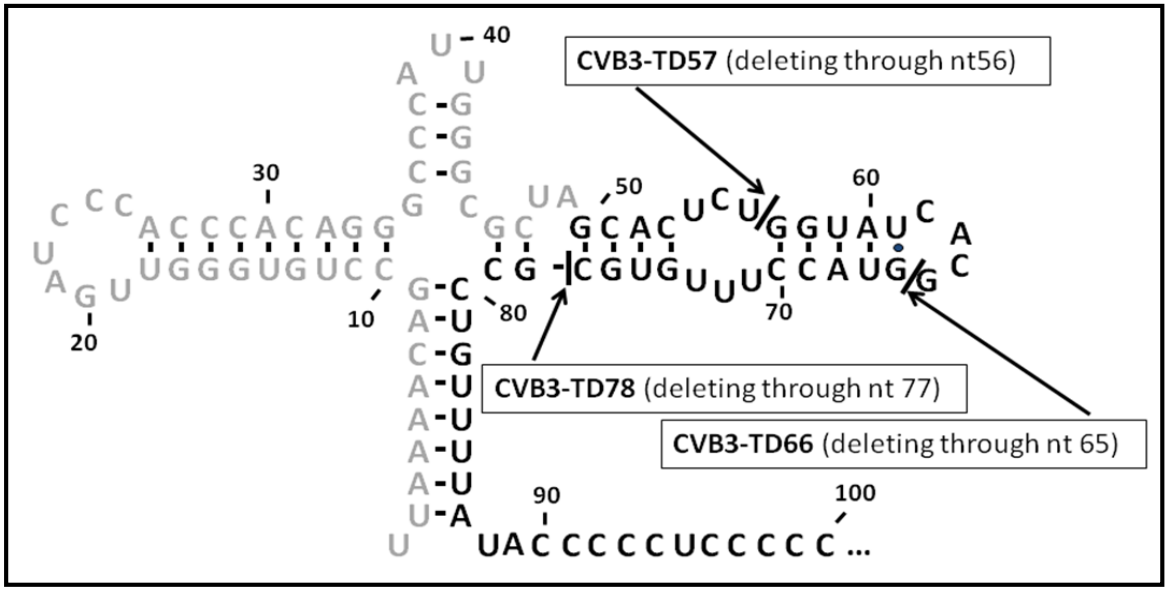
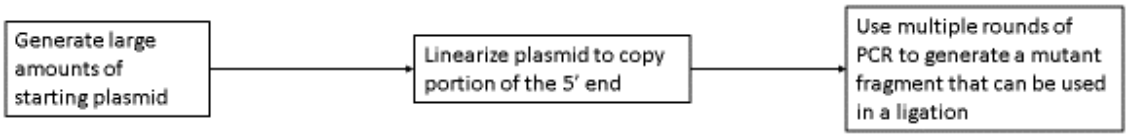



**Figure 3:** Stemloop d of domain I folded into the secondary structure known as the cloverleaf with the projected deletions noted by arrows.

As shown in the figure above, the 5' end of the CVB3 genome is shaped much like a cloverleaf, with three stem like structures formed by folding the RNA genome onto itself. Deletion of the first 49nt removes the stem and the first two leaves, but retains the third leaf, stem-loop D untouched. Mutational studies to the structures of the cloverleaf have targeted each of the three structures in stem-loop D [63, 84, 85] and in doing, showed a reduction in positive strand synthesis and a reduction in overall replication. With this in mind, we hypothesized that deletion beyond the first 49nt and into the cloverleaf, would be a lethal mutation. This is supported due to the findings that 3CD binds to stem-loop D [61] as part of the ternary complex. The research conducted as follows will show that deletion of the di-nucleotide bulge and the entire stem-loop D did not eliminate viral replication.

Growing the Starting Plasmid




To generate the deleted cDNA genomes with an upstream T7 RNA polymerase promoter and a ribozyme which would cleave the T7 transcripts correctly, the plasmid pCMVT7r28 [81] was used as the cDNA basis for mutagenesis. The smallest 5' terminal deletion was TD57, where the first 56nt from the 5' end of the genome would be deleted. It was suspected that deleting past the first 50nt would fail to generate any replicating virus. The pCMVT7r28 plasmid was used to create both the fragment and vector of the mutant. E. coli (strain DH5α) containing the plasmid was grown on LB Broth Miller (Luria-Bertani) plates (prepared according to Becton, Dickinson and Company, Franklin Lakes, New Jersey) coated with 50µg/mL ampicillin. Ampicillin was the selecting agent used to grow the pCMVT7r28 because the strain of *E. coli* used was not resistant to the drug and the plasmid provided the selective resistance. Single colonies would be picked from the plate and cultured in Terrific Broth, allowing for a higher density of bacteria in a shorter time period, (prepared according to Becton, Dickinson and Company, Franklin Lakes, New Jersey) again with 50µg/mL ampicillin. Cultures were processed using the QIAprep Spin Miniprep Kit (according to QIAGEN, Valencia, CA) where final elution was completed using 50µL DEPC treated sterile water. This kit is a streamlined version of an alkaline lysis where the target genome was purified from the remaining cellular debris using the QIAGEN columns. Plasmid concentration and purity was measured with a spectrophotometer and less than 1µg was run on a 1.0% agarose gel in 1xTAE stained with SYBR® Safe DNA Gel Stain (according to ThermoFisher

Waltham, MA) and visualized using a transilluminator. Two bands running at different positions was indicated relaxed and supercoiled plasmid DNA purified from the bacterial lysate versus bacterial genomic DNA. A larger 100mL Terrific Broth (ThermoFisher Waltham, MA USA) culture was grown from the single picked colony and processed using a QIAGEN Plasmid Purification Midi kit (according to QIAGEN) and eluted with 50µL DEPC treated sterile water. Again the samples were quantified using a spectrophotometer and observed on a 1% agarose gel before continuing. Using the highly concentrated pCMVT7r28, ClaI was used to linearize the circular plasmid (New England Biolabs Ipswich, MA) at a site following the poly A tract at the 3' end of the viral cDNA. Once linearized, the plasmid was then purified from enzymes and buffers using a standard ethanol precipitation with a final concentration of 0.625M ammonium acetate, 75% ethanol, and at least 2µL glycogen. The precipitation was then chilled in a -70°C freezer for 30min. The DNA was then pelleted in a centrifuge for 15min at 16,000xg. The supernatant was removed and the remaining pellet was washed twice with 100% Ethanol. DNAs were air dried and suspended in 50µL of DEPC treated water. This standard precipitation protocol was important to remove any buffers and enzymes leftover from the digest that may interact with additional digests or PCR. The final product from the precipitation was run on an agarose gel just as described before to verify that the DNA was cut and all debris was removed. Mobility of the cut DNA would run at approximately 10kbp. Our preparations often contained a smaller bacterial plasmid separate from our product which (uncut) electrophoresed at a mobility similar to a linear 50bp marker.

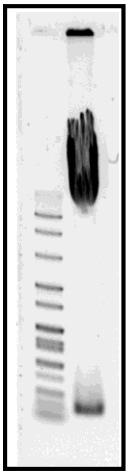


**Image 1:** Linearized pCMVT7r28 cut by ClaI run on an agarose gel.

The linearized plasmid was then further digested using Pst1 and Not1 (buffer and incubation as recommended by Invitrogen, Grand Island, NY). These two enzymes cut the genome in two places creating a smaller fragment with which to work. This template was used to amplify a product with the following primers (TD57RIBOZ1; E3) using Deep Vent DNA polymerase (New England Biolabs, Ipswich, MA) to decrease variation generated in polymerase chain reaction (PCR). Cycling followed this protocol: 94°C for 1 minute to denature, 60°C for 1 minute to anneal primers, 72°C for 1 minute to elongate, 35 cycles of 94°C for 30 seconds, 61°C for 30 seconds, 72°C for 40sec, followed by 10 minutes at 72°C to finish any amplified product. Products were analyzed on 2.0% agarose gels in 1X TAE and purified on silica matrix minicolumns (Zymoclean™ Gel DNA Recovery Kit, Zymo Research, Irvine, CA). The gel recovery kit was preferred over an ethanol precipitation as it was believed that by excising the correct band from the gel would allow for a more purified product.

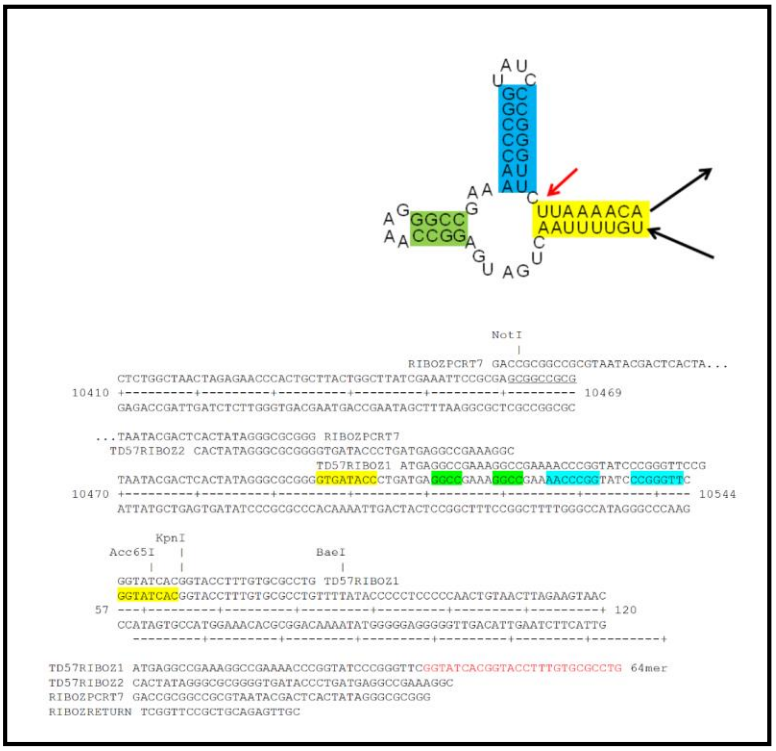



**Figure 4:** TD57 primer sequences as they apply to the 5' cloverleaf ribozyme.


**Figure 5:** TD78 primer sequences as they apply to the 5' cloverleaf ribozyme.

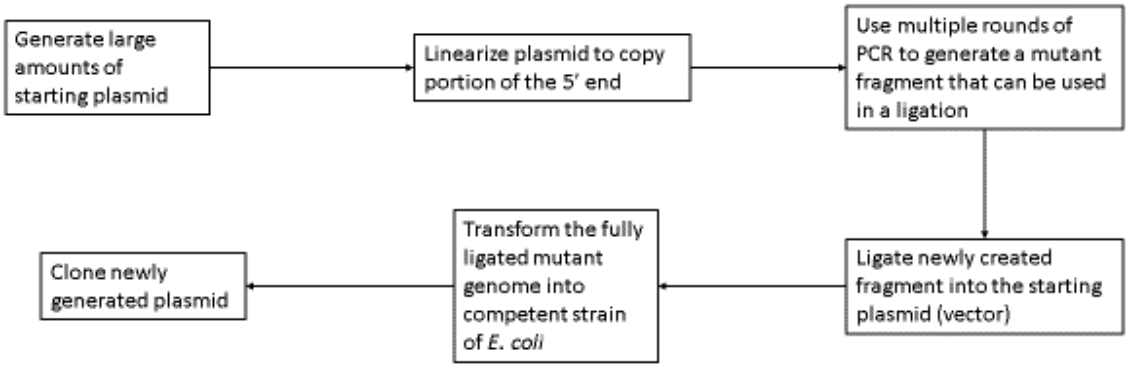
Subsequent amplification of the 0.55kb product with TD57RIBOZ2 and E3 followed the pervious protocol with the modification of using an annealing temperature of 56°C and eventual

purification using the DNA Clean & Concentrator™-5 (Zymo Research, Irvine, CA). The 0.55kb product of the second amplification was extended by amplification with RIBOZPCRT7 and RIBOZRETURN using an annealing temperature of 60.9°C and then purified. This product of 0.56kb contained a 5' terminal Not1 site and an internal PstI site allowing the generation of a 576bp product by digestion with these enzymes (Invitrogen and New England Biolabs). The digest was run on a 1% agarose gel and the vector band was excised and cleaned using the Zymoclean™ Gel DNA Recovery Kit with manufacturer recommendations. The vector DNA was then digested with shrimp alkaline phosphatase SAP according to factory recommendations to remove the phosphates still attached to the ends, making the fragment incapable of self-ligation. The DNA was then cleaned using the Zymogen DNA Clean & Concentrator™-5.

| Primer | Sequence |
|---|---|
| **TD57RIBOZ1** | ATGAGGCCGAAAGGCCGAAAACCCGGTATCCCGGGTTCGGTATCACGGTACCTTTGTGCGCCTG |
| **TD57RIBOZ2** | CACTATAGGGCGCGGGGTGATACCCTGATGAGGCCGAAAGGC |
| **TD78RIBOZ1** | ATGAGGCCGAAAGGCCGAAAACCCGGTATCCCGGGTTCGCCTGTTTTATACCCCCTCCCCCA |
| **TD78RIBOZ2** | CACTATAGGGCGCGGGAAACAGGCCTGATGAGGCCGAAAGGCCGAAAAC |
| **RIBOZPCRT7** | GACCGCGGCCGCGTAATACGACTCACTATAGGGCGCGGG |
| **RIBOZRETURN** | TCGGTTCCGCTGCAGAGTTGC |
| **PCMVPRIMER** | TATATAAGCAGAGCTCTCTGG |
| **E1** | CACCGGATGGCCAATCCA |
| **E3** | ACACGGACACCCAAAGTAGTCGGTTCC |
| **S5** | TATACCCCCTCCCCCAACTGTAACTTAG |
| **SReturn** | TACACTGGGGTAGTGCTGAGCG |

**Table 1:** A list of the primers used to create and verify the mutant viruses.

Cloning TD57




pCMVT7r28 was digested with NotI and PstI just as the fragment above, to create the appropriate ends for insertion of the newly generated fragment. This pCMVT7r28 vector and the newly generated TD57 fragment were ligated together using T4 DNA ligase (according to Promega Madison, WI). The ligated product was ethanol precipitated as described above. This plasmid was then transformed into *E. coli* cells using the *Mix & Go* Competent Cells - Strain Zymo 5α (according to Zymo Research Irvine, CA). DNA was prepared from cultures of individual colonies using the QIAprep Spin Miniprep Kit. The DNA isolated from the kit was then digested using HaeII (New England Biolabs Ipswich, MA) and observed by running a 2.0% agarose gel. HaeII was chosen as the restriction enzyme because the resulting agarose gel would show 4 bands (6.2kbp, 1.5kbp, 370bp, and 2.2kbp) while the TD57 and TD78 plasmids would show only 3 bands (1.5kbp, 370bp, and 8.5kbp). While some of the bands would be difficult to observe on a standard gel, the point at which both a 1.5kbp and a 2.2kbp band runs on a 2.0% agarose gel is easily determined.

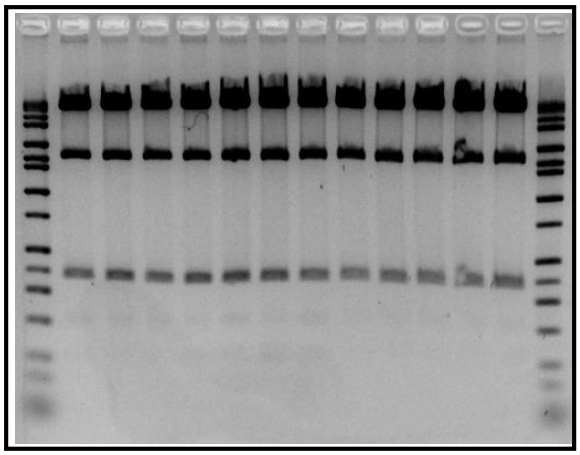


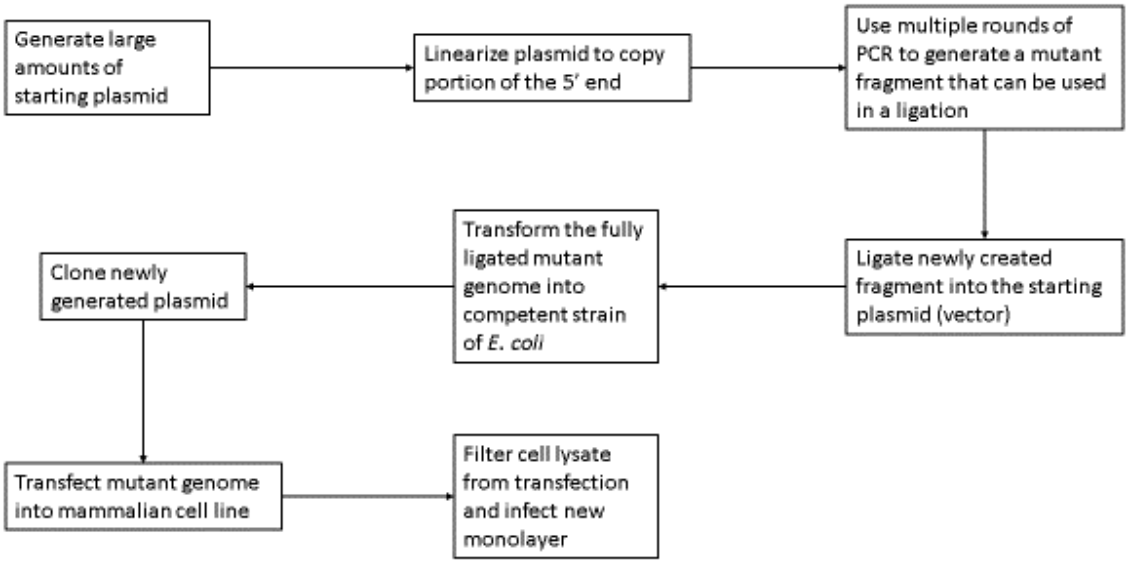
**Image 2:** A sample gel showing the results of the HaeII digest used to identify TD57 and/or TD78. This particular gel shows all plasmids cut are TD57.

Wells with bands at 10kbp, 1.5kbp, and 0.4kbp were sequenced with primer SReturn. When these samples returned with the correct sequence, the original colonies were grown in 100mL cultures and sequenced a final time using both pCMVPrimer and E1. Primer pCMVPrimer was used to read in the opposite direction and E1 is a primer that is further removed from the 5'NTR with a more familiar sequence.

Culturing HeLa cells

HeLa cells (American Type Culture Collection, Manassas, VA; CCL2) were cultured in 100mm culture dishes at 37°C, 5% CO2 in 10mL of DMEM with 10% Fetal Bovine Serum (FBS) and 1% gentamycin. FBS is used in media to provide the necessary nutrients needed for continued cell growth while gentamycin provides a selecting agent and means of preventing bacterial and fungal growth. Cells were also cultured in 6-well plates.

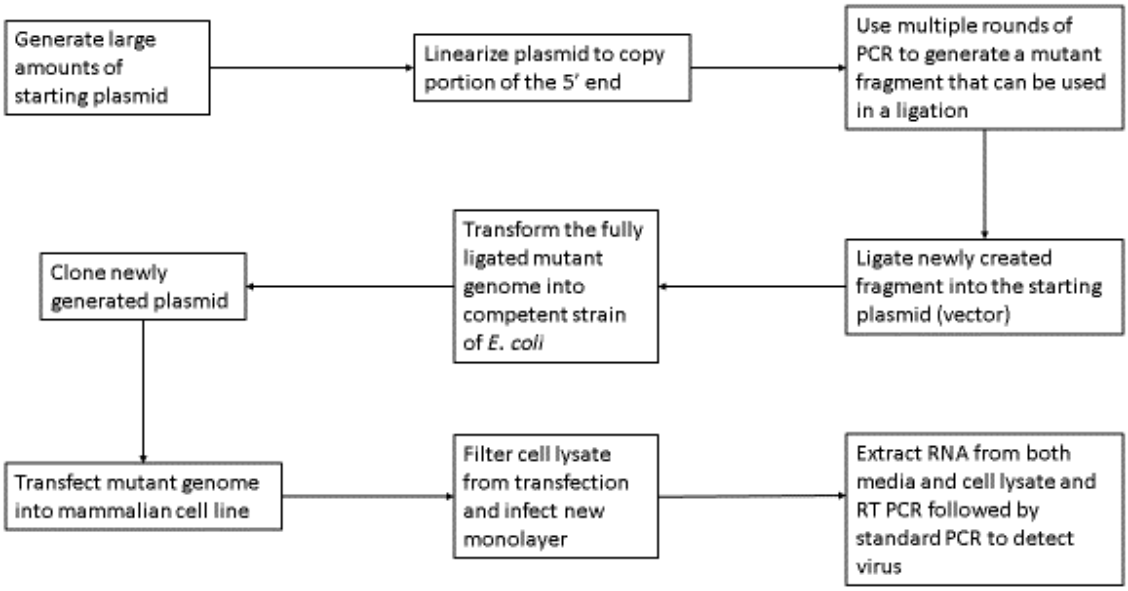
Transfection and Infection of TD57




The newly generated TD57 plasmid was linearized using a ClaI digest and purified with an ethanol precipitation. The linearized plasmid was then transcribed into RNA using T7 RiboMAX (according to Promega Madison, WI) and purified using a LiCl precipitation (addition of 2µL glycogen and 4M LiCl to a final concentration of 2M at -20°C for 1 hour). RNA was pelleted (16,000xg for 20min), the resulting pellet was washed with 100% EtOH, and finally suspended in sterile DEPC treated water. Transfecting 2µg of RNA into (approximately $3.0x10^5$ cells in each well of a 6-well plate) the HeLa cells was done using Transmessenger Transfection Reagent (according to QIAGEN) per manufacturer's instructions. After transfection, cells were incubated for 3 days. Alongside this transfection, three controls were also transfected: the original pCMVT7r28 plasmid (cut with ClaI) was transcribed, control MS2 bacteriophage RNA, and a water control. The transcribed pCMVT7r28 RNA would act as a positive control since it has been shown that the 28 plasmid generates infectious virus and kills the transfected monolayer. The MS2 RNA can't create bacteriophage without a prokaryotic host and is a control for toxicity from

introducing foreign RNA into HeLa cells. Two additional controls were also used, a negative control had no RNA in the transfection mix and the tissue culture control would be a well free from any transfection reagents.

**Detecting the Terminally Deleted Virus Post-Transfection**




Viral Preparation from HeLa Cells

Virus was prepared from cells by three rounds of freeze-thaw lysis in 500µL 0.1M NaCl. Freeze and thaw of the monolayer causes the cells to lyse. The lysed material was then passed through a 0.22µm filter that allows only the passage of virus and small organelles eliminating intact cells. After filtration, 50µg/mL RNase was added to each tube and incubated for 30min and spun down to degrade cellular RNA (non-encapsidated RNA). The supernatant was then processed with the Direct-zol™ RNA MiniPrep (according to Zymo Research Irvine, CA) without the addition of DNase. Because it was believed that very low levels of viral RNA would be produced additional cleaning steps could reduce the yield and we did not want to risk carry over

of DNase in the subsequent reverse transcription (RT) reactions. Trizol was the preferred method of RNA extraction and by using the kit we could eliminate some possible sources of contamination.

RT-PCR and PCR for Viral RNA

The RNA eluted from the column was used in an RT reaction facilitated by the Improm-II kit (according to Promega Madison, WI). RT PCR reactions were primed with E1 (Table) and the subsequent PCR using the Promega GoTaq® Green Polymerase Master Mix used primers S5 and SReturn to detect viral RNA. The same set of RT-PCR and PCR reactions were used for the cell lysate, media, and controls. A negative control using water and a positive control using pCMVT7r28 plasmid were cycled alongside the products from the transfection as well as an RT-PCR control with only water. The products from the S5/SReturn PCR were run on a 2.0% agarose gel alongside HiLo DNA ladder (Minnesota Molecular) and stained using SYBR® Safe DNA Gel Stain (Molecular Probes, Carlsbad, CA).

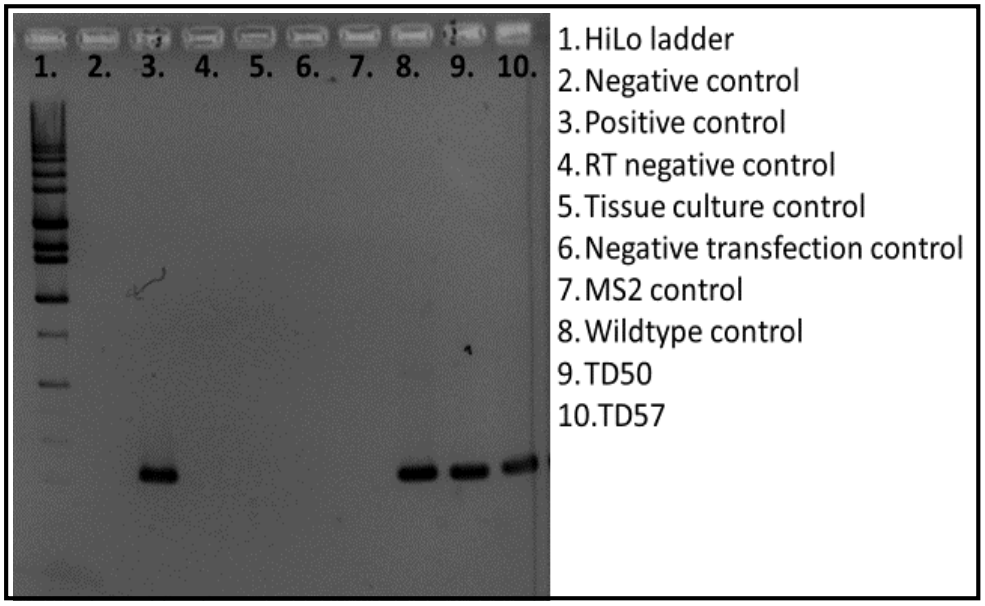



**Image 3:** The results from the S5/SReturn PCR used to identify TD57 and/or TD78. This gel verifies TD57 is present in culture.

The resulting gel showed that encapsidated RNA could be detected. A duplicate plate subjected to the same transfection conditions was fixed with glacial acetic acid and water solution (3:1) followed by staining with crystal violet.

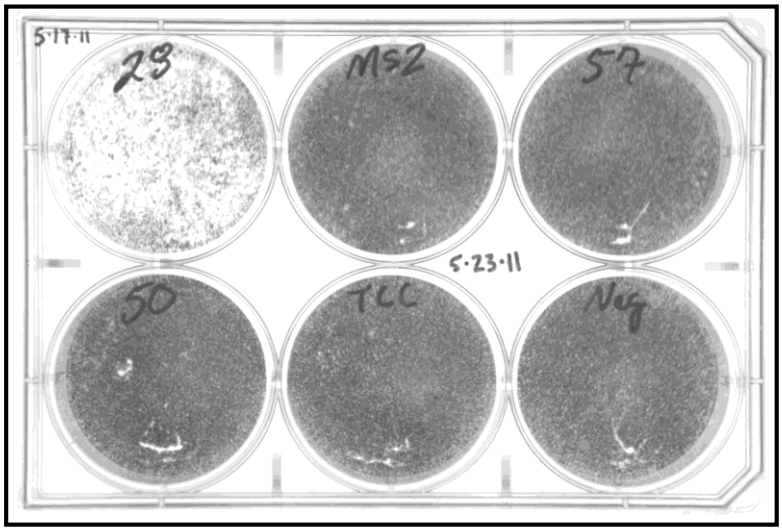



**Image 4:** A secondary 6-well plate created to show despite a successful transfection, TD57 fails to show CPE.

When dried, the plate was imaged above to show how wildtype virus created from the pCMVT7r28 showed cytopathic effect (CPE), whereas virus produced from TD50 and TD57 does not show CPE after three days of incubation despite being able to detect viral RNA via RT PCR.

TD78

The procedure for TD78 was similar to what was described above except the primers used for the initial creation of the fragment were TD78RIBOZ1, TD78RIBOZ2, TD78RIBOZRETURN. After creating the TD78 mutant the same procedures and PCR reactions were run and visualized on a 2.0% agarose gel. The same negative and positive controls were included when transfecting TD78 and the results are shown below.

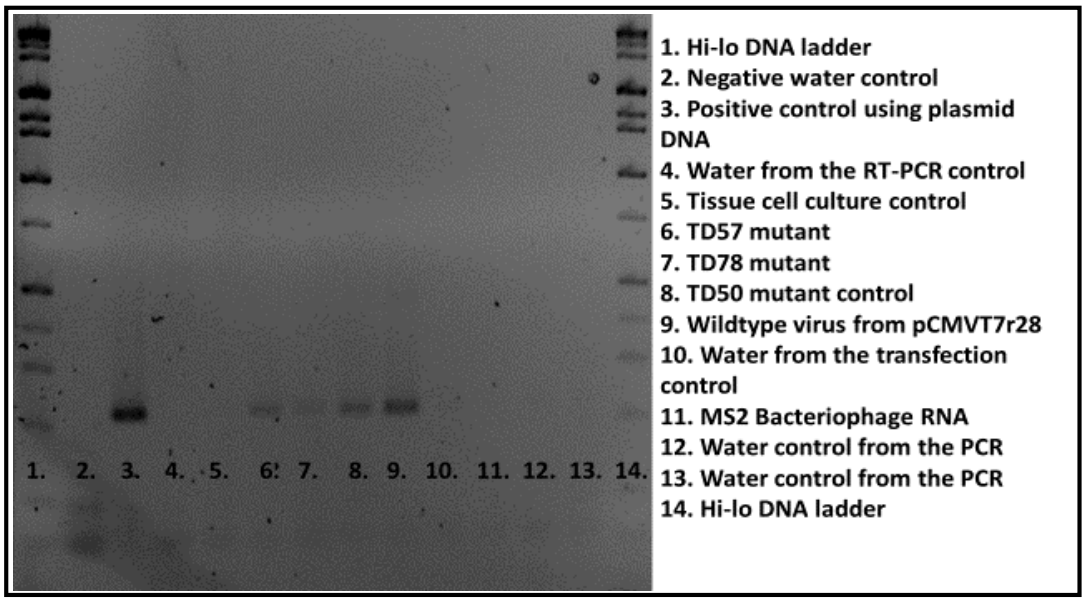



**Image 5:** Using equal amounts of reagent and input RNA, the above gel shows the results of the S5/SReturn PCR with TD78.

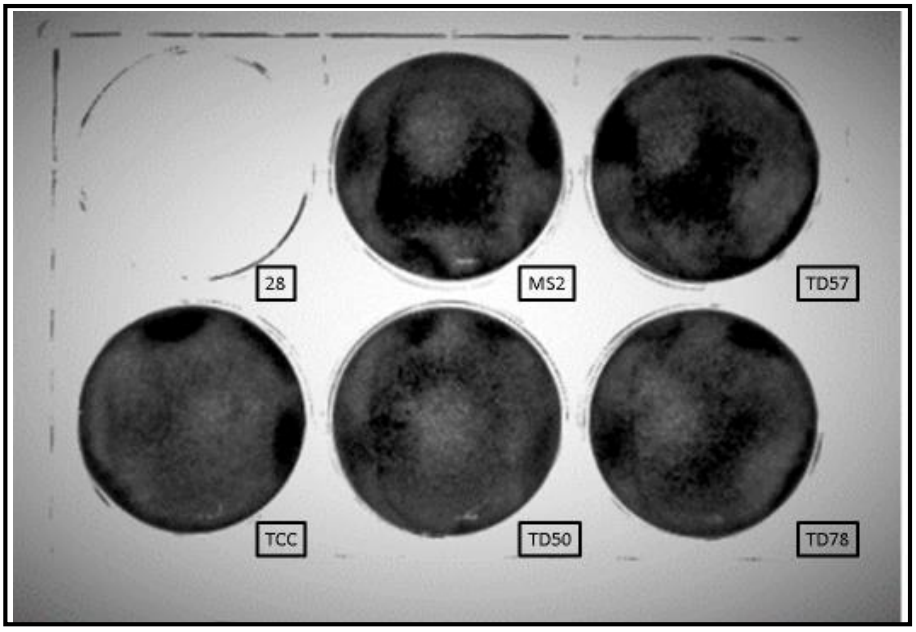



**Image 6:** The above image from a secondary 6-well plate, stained with crystal violet, shows that TD78 does not cause CPE.

## Chapter 3: Discussion

In this study, we have constructed two novel plasmids. We found that when these plasmids were transcribed into RNA and transfected into a susceptible cell line, they were capable of generating encapsidated virus. Furthermore, we showed that the virus produced from these RNA transfections did not regenerate the 5' terminal deletions cloned into the starting plasmids. The overall structure formed by the 5' cloverleaf in enteroviruses is critical to

efficient viral replication; continued replication despite the ability to form this structure could represent an alternate method used during viral replication or the current model of enteroviral replication is incomplete. It has been hypothesized by Smithee et al. that the alternate method is actually a randomized initiation event either by a lack of properly uridylylated VPg or an inability to form the three dimensional interaction between the 5' NTR and the CRE(2C) that provides the template for the addition of the two uridine residues [86].

Our initial hypothesis led with the assumption that the absolute minimal replication complex required for viability demanded that stemloop d in domain I remain intact. Our results herein show that this is not the case, but instead virus continues to replicate. This is an important distinction to make as the literature used to make our initial hypothesis believed that stemloop d was a critical structure to producing viable virus [87]. Based on our findings, we now know that the lack of stemloop d does not abolish the virus altogether.

The current literature believes that one of stemloop d's roles is the primary location for 3CD to bind. To explain the significance of this interaction, we must recall the formation of the ternary complex, where PCBP2 binds stemloop b, and 3CD binds the adjacent stemloop d. If stemloop b is removed, a secondary location has been shown to bind PCBP2 in its place: the stretch of cytosine residues directly following the opposite side of stem a [88]. An alternate binding location for 3CD has yet to be hypothesized, but formation of the ternary complex allows the 5' end of the viral genome to come into close proximity of the replication machinery bound to the CRE and the 3' poly A tail. With an altered location for 3CD to bind the 5' end of viral RNA, initiation is imperfect and cannot begin at the appropriate location let alone maintain the tertiary structure required to bring all necessary replicative proteins together. Subsequently, this type of replication argues a non-specific possibly random point of initiation, one where a uridylylated VPg is unnecessary. VPg must still be nucleotidylated [51] as it is the protein primer

required for 3D to begin polymerization, but the double uridine may be less important as Kim et al has shown that VPg (while still bound to the 5' end of genomes of TD viruses) would not have been uridylylated in priming these genomes [79].

We have shown that our mutated RNA is capable of producing virus and the end result still lacks the 5' end of the genome. This begs the question: how does the viral RNA continue to replicate enough of the genome to produce viable progeny? Further characterization of the mutated virus created herein and not addressed by this study would be necessary to fully verify the presence of both infectious viral particles and the encapsidated RNA. This could be accomplished by 1.) multiple passages demonstrating infectivity 2.) western blotting using antibodies specific to enterovirus to show capsid formation with enteroviral specificity 3.) reverse transcription PCR using various primers capable of polymerizing the 5' end before then sequencing the genome to look for changes. Assuming that the results mimic those shown in viruses lacking stem a, stemloop b, and stemloop c, the current replication model would need altering to account for the fact that 3CD has no place to bind and functionally attract the 3' end. A lack of the first 50 nucleotides of the viral genome has shown that positive strand replication is greatly affected, but not halted; this is thought to be because 3CD still has a location to bind to the 5' end and circularize the genome, but without the predicted location for 3CD to bind (as in TD78) it would seem that the genome either does not require circularization or 3CD has found a new point at which to bind.

The continued replication of viruses with deletions to the 5' end is not new. While the research and results herein is important, there is still much work to be done. Research in this field and particularly these viruses is extremely important as highlighted in Chapman et. al 2008 [19]. It is interesting to think that a human with dilated cardiomyopathy or acute myocarditis may not be accurately diagnosed until autopsy without specifically looking for terminally

deleted enteroviruses, since they replicate at such a low level, making them much harder to detect. How many cases, heart-related or enteroviral related do not screen for this possibility and therefore are diagnosed incompletely? Research into terminally deleted viruses and potential sources of vaccination should be explored more fully.

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