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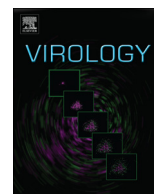
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# Domain I of the 5' non-translated genomic region in coxsackievirus B3 RNA is not required for productive replication



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## ABSTRACT

Domain I is a cloverleaf-like secondary structure at the 5' termini of all enterovirus genomes, comprising part of a *cis*-acting replication element essential for efficient enteroviral replication. 5' genomic terminal deletions up to as much as 55% of domain I can occur without lethality following coxsackie B virus infections. We report here that the entire CVB3 structural domain I can be deleted without lethality.

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## 1. Introduction

The six group B coxsackievirus serotypes (CVB1–6) are species B human enteroviruses (*Picornaviridae*) (Tracy et al., 2008). Domain I (Fig. 1A) of the 5' nontranslated region (NTR) RNA structure (Bailey and Tapprich, 2007) is located at the genome's 5' terminus and is required for efficient replication (Andino et al., 1990). It has been demonstrated that naturally-occurring deletions of 7–49 nucleotides from the 5' terminus of CVB3 genome are not lethal for the virus populations and occur following experimental inoculation of primary cell cultures (Kim et al., 2008) and of mice (Kim et al., 2005; Tracy et al., 2015) as well as after naturally-occurring human infections (Chapman et al., 2008). Group B coxsackievirus virions containing 5' terminally deleted genomes (CVB-TD) can be isolated, replicate productively in the absence of parental (wild-type) CVB genomes, and infections are inhibited using neutralizing antibody (Kim et al., 2005; Smithee et al., 2015). CVB3-TD genomes have 5' terminal sequences which can have any of the 4 ribonucleotides (Chapman et al., 2008; Kim et al., 2008; Kim et al., 2005) despite the presence of covalently attached VPg (Kim et al., 2005). CVB-TD populations demonstrate impaired replication with replication levels that are 100,000 fold lower than wildtype virus (Smithee et al., 2015).

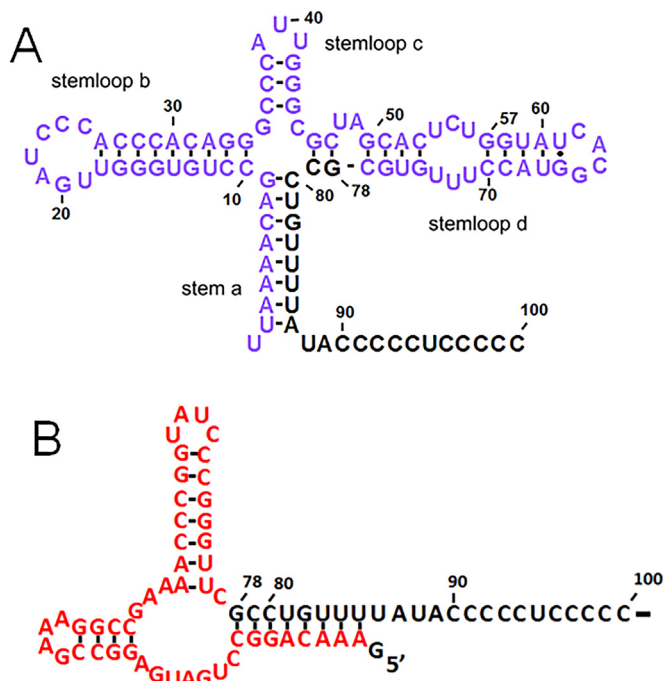
CVB3-TD populations with deletions greater than 49 nucleotides in length have not been detected in either cell culture experiments or from human CVB-TD isolates (Chapman et al., 2008; Kim et al., 2008; Kim et al., 2005). As domain I is one of 4 characterized *cis*-acting replication elements [CRE; (Paul and Wimmer, 2015)] which aid enteroviral replicational efficiency, we were curious to determine whether larger deletions of domain I were lethal, thus accounting for failing to detect them. We experimentally deleted nucleotides 1–77 from an infectious cDNA copy of a CVB3 genome (Tracy et al., 2002), thereby excising domain I through stem-loop d. This construct, CVB3-TD78, was viable and produced infectious progeny virus.

## 2. Results and discussion

A CVB3 genome with a deletion of the 5' 77 nucleotides (nt1–77) (Fig. 1A, C) was engineered to include an upstream T7 promoter and a ribozyme designed to generate transcripts with the 5' termini beginning at nt78 (Fig. 1B); this construct was termed pCVB3-TD78. HeLa cell monolayers were electroporated with full-length CVB3 RNA T7 RNA polymerase transcript from *Cla*I-digested pCVB3–28 (wt, positive control) or pCVB3-TD78 (Smithee et al., 2015). While electroporation with wt CVB3/28 RNA produced extensive cytopathic effect (CPE) at 48 h in cell cultures, no CPE was observed in cultures electroporated with CVB3-TD78 RNA or in control (non-electroporated) HeLa cell monolayers similar to

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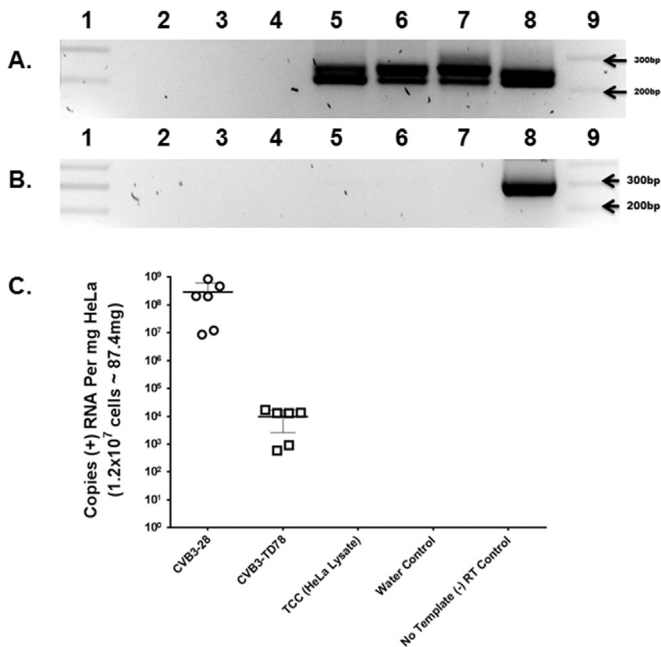
E-mail address: [nchapman@unmc.edu](mailto:nchapman@unmc.edu) (N.M. Chapman).



**Fig. 1. pCVB3-TD78 deletion and plasmid map.** (A) The structure of the 5' non-translated region of CVB3 indicating deleted sequence (blue) and conserved region (black) in TD78. (B) Ribozyme sequence (red) [as in (Wedekind and McKay, 1998)] of the 5' end of T7 RNA transcript. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

results with other CVB-TD strains (Kim et al., 2008; Kim et al., 2005; Smithee et al., 2015).

Virus was isolated from previously electroporated HeLa monolayers by freeze-thawing, followed by treatment of the cleared lysates with ribonuclease, and pelleting virions through a 30% sucrose cushion (Kim et al., 2005; Smithee et al., 2015). Viral RNA isolated from pelleted and resuspended wt and CVB3-TD78 virions was detected by semi-nested reverse transcription polymerase chain reaction (RT-PCR) (Smithee et al., 2015) using a 5' terminal primer annealing to the region after the RNA structure (S5, Table 1). Viral RNA was detectable in both samples, demonstrating that electroporation had induced productive viral replication as only newly synthesized viral RNA is encapsidated in progeny virions (Nugent et al., 1999) (Fig. 2A). RT-PCR mediated detection of sequences within the deleted region (Smithee et al., 2015) was consistent with the expected 78 nucleotide deletion (Fig. 2B) in which the S4 (Table 1) annealing site was deleted. Because CVB3-TD78 induced no apparent CPE, as expected from previous work characterizing CVB-TD strains (Kim et al., 2008; Kim et al., 2005; Tracy et al., 2015), RT-qPCR was used to quantify positive strand viral RNA (Smithee et al., 2015) with the titer expressed as positive strand viral RNA molecules per cell (Fig. 2C). Similar to results reported for other CVB-TD populations (Smithee



**Fig. 2. Replication of CVB3-TD78.** (A) RNA from purified virions of HeLa cultures 72 h post electroporation was reverse transcribed [primer E3; (Chapman et al., 1990)], amplified with S5 and E3Sub, reamplified with S5 and SReturn. (B) cDNA from virion RNA as in (A) was amplified with S4 (Kim et al., 2005) and E3Sub and reamplified with S4 and SReturn. Lanes 1 and 9, HiLo ladder (Minnesota Molecular); 2, no RNA; 3, no DNA; 4, untransfected HeLa preparation; 5–7, CVB3-TD78 transfected HeLa cell culture preparations; 8, CVB3-28 transfected HeLa cell culture preparation. (C) Quantitative RT-PCR on purified virion RNA from multiple transfected cultures. No signal above background was detected for negative controls.

et al., 2015), CVB3-TD78 replicated to approximately 100,000 times lower extent than did the wt virus, CVB3-28.

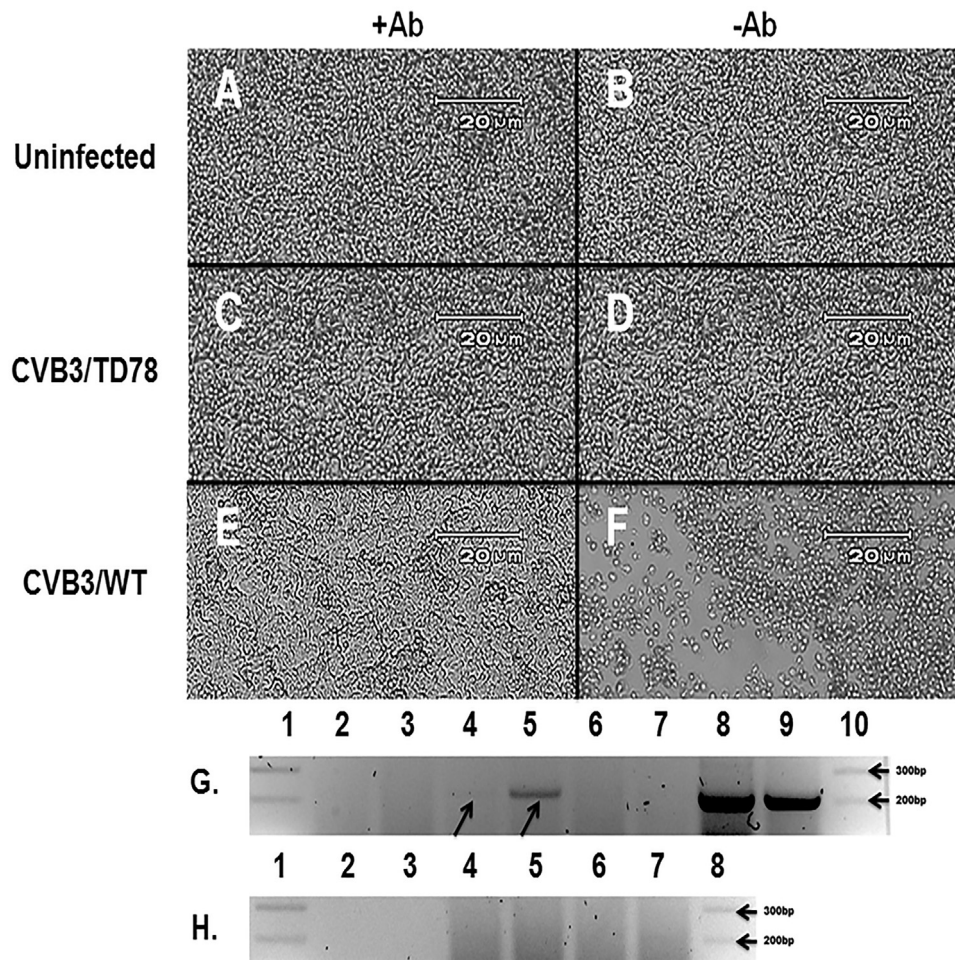
To test that the detected CVB3-TD78 viral RNA was encapsidated, virions were inoculated onto HeLa cell monolayers with (Fig. 3C) or without (Fig. 3D) prior incubation with anti-CVB3 neutralizing serum (ATCC; Manassas VA). No apparent CPE was observed for either CVB3-TD78 sample, while extensive CVB3/28-induced CPE was completely inhibited by neutralizing antibody (Fig. 3E,F). Then, using total RNA isolated from CVB3-TD78 cultures inoculated in the presence or absence of neutralizing serum to test for viral RNA by RT-PCR, viral RNA was detectable only in cultures which had not previously been treated with neutralizing antibody (Fig. 3G,H), indicating that a productive CVB3-TD78 infection was inhibited by CVB3-neutralizing antibody.

Results presented here demonstrate that an enteroviral genome which lacks all of domain I (nt1–77) still replicates productively, albeit to significantly lower titers than wt virus. It is known that domain I is required to form a ribonucleoprotein complex that promotes efficient replication of the viral genome (Andino et al., 1993). This complex forms with the binding of poly(C) binding protein 1 (PCBP) to stem-loop b and 3CD<sup>Pro</sup> to stem-loop d which

**Table 1**  
Primers used in this work.

Primer	Sequence (5'–3')	Annealing site	
E3	ACACGGACACCCAAAGTAGTCGGTTCC	Reverse Complement 537–563	(Chapman et al., 1990)
TD78RiboZ1	ATGAGGCCGAAAGCGCGAAACCCGGTATCCCGGGTTCGCCTGTTTATACCCCTCCCCCA		
TD78RiboZ2	CACATATAGGGCGCGCGAAACAGGCTGATGAGGCCGAAAGCGCGCGAAAC		
RIBOZPCRT7	GACCGCGCGCGCTAATACGACTCACTATAGGGCGCGCGG		
S4	CGCTAGCACTCTGGTATCACGGTACCTTTG	45–74	(Kim et al., 2005)
S5	TATACCCCTCCCCCACTGTAACCTAG	86–113	(Smithee et al., 2015)
E3Sub	AGTAGTCGGTTCGCG	Reverse Complement 535–549	(Smithee et al., 2015)
SReturn	TACACTGGGGTAGTGCTGAGCG	Reverse Complement 291–312	(Smithee et al., 2015)





**Fig. 3. Passage of CVB3-TD78.** (A–F) Purified CVB3-TD78 (C,D) or CVB3/28 (E,F) virions were inoculated onto HeLa cell cultures with (C,E) or without (D,F) prior incubation (1 h, 37 °C) with anti-CVB3 neutralizing serum diluted 1:400 in complete cell culture medium. Uninoculated HeLa culture (B) was also incubated with antibody (A). (G,H) RT-PCR of RNA from day 5 HeLa cultures inoculated with virus in the absence or presence of anti-CVB3 serum (RT using E3, 1st PCR S5/E3Sub, 2nd S5/SReturn). G1,10, H1,8: HiLo ladder; G2–5: CVB3/TD78 (G2,3 with antibody). G6–9: CVB3/28 (G6,7 with antibody); H4–7: HeLa culture (H4,5 with antibody); H2: no RNA; H3: no DNA.

in turn allows genome circularization (Barton et al., 2001; Gama-rnik and Andino, 2000; Herold and Andino, 2001). The ability of TD viruses without stem-loop b to replicate, as exemplified with CVB3-TD50 (which lacks the 5' nt1–49) (Kim et al., 2005), may be due to the binding of PCBP to the spacer region between domain I and II (Zell et al., 2008), a location that also remains intact in CVB3-TD78. Mutations in the poliovirus domain I (Andino et al., 1990; Trono et al., 1988) also showed loss of CPE (interpreted as lethal) when mutations disrupted stemloop d RNA structure. Interestingly, as observed with CVB-TD strains, such mutations also decreased RNA synthesis levels as well as the ratio of positive to negative strand RNA (Andino et al., 1990; Vogt and Andino, 2010). As our results for the TD78 deletion demonstrated a decrease in virus replication at a level similar to that observed for naturally-generated TD strains (which maintain the stemloop d of domain I) (Smithee et al., 2015), it is possible that some of the mutations of the cloverleaf reported as “dead” (Andino et al., 1990), might have actually replicated at similarly low levels of the CVB-TD viruses.

We previously demonstrated that the terminal deletion size of a CVB-TD genome can increase in size; this was observed following passage of CVB3-TD8 (which deleted the 5' 7 nt) in primary cell cultures or in mice (Kim et al., 2008; Kim et al., 2005). However, we did not observe an evolution of the terminal deletion populations of passaged CVB3-TD50 (containing a 5' 49 nt deletion) to larger deletions, which would erode or eliminate stemloop d (Kim et al., 2008; Kim et al., 2005). The observation that CVB3-TD78 was

replication competent in HeLa cell cultures at a level similar to CVB3-TD50 was surprising, for if CVB3-TD78 replicates as well as efficiently as CVB3-TD50, why was there no evolution to a population of larger deletions during passage of CVB3-TD50? We suggest this may be due to the non-selective (permissive) nature of HeLa cells for enterovirus replication. We know that CVB3-TD are not generated by passage of CVB3 in HeLa cell culture (Kim et al., 2008) but are selected in passage in hearts and pancreas of mice (Kim et al., 2005; Tracy et al., 2015) or in primary cell cultures (Kim et al., 2008). While the 5' terminal deletion of stemloop d may not be necessary for 5' terminally deleted enterovirus replication, it is possible that there is some loss of efficiency generated by deletion of this sequence, sufficiently so to prevent CVB-TD populations from accumulating a population without stemloop d in hearts or primary cells. This discrete loss of replicational efficiency may be sufficient to select against CVB-TD populations that delete the entire domain I. The loss of approximately 50 5' nt (as in CVB3-TD50) may represent the peak of fitness in the evolving TD populations and while larger 5' terminal deletions may transiently occur in a cell's quasispecies virus population, they never become dominant (Garcia-Arriaza et al., 2006).

The finding that the 5' NTR domain I, one of 4 CREs that promote replication efficiency, can be deleted from the CVB3 genome without lethal consequences for the virus, is novel and complements and extends others' studies showing that other CREs can also be deleted and are similarly unnecessary for enteroviral

replication, viz. the 3' NTR (Todd et al., 1997) and the CRE(2C) (Smithee et al., 2015). That modern enteroviruses can lose a CRE that, when present, confers greater adaptability upon the genome in a given environment through improved replication, is intriguing. These findings cumulatively beg the question how much of an enteroviral genome might be deleted or, as in the case of the CRE(2C), made non-functional, while maintaining the capacity to replicate productively. Assuming that the ancient enteroviral genomic ancestors were basic RNA replicons, how might, and in what order could, such beneficial genomic domains have been added as the genomic design evolved. Further studies employing enteroviruses with deletions of multiple CREs (such as recently reported in a CVB3 genome; Smithee et al., 2015), may shed light on pathways by which evolving evolutionary ancestor virus populations acquired these capabilities.

### 3. Materials and methods

Construction of CVB3 cDNA genome with deletion of nt1–77: Briefly, pCVB3–28 (Tracy et al., 2002) DNA was linearized by digestion with *Cl*I and amplified with 0.5  $\mu$ M of primers E3 (Chapman et al., 1990) and TD78RiboZ1 (Table 1) using Deep Vent DNA polymerase (New England Biolabs, Ipswich, MA) in 200  $\mu$ M dNTPs, 6 mM  $Mg_2SO_4$  and using 1 cycle at 94 °C for 5 min and 35 cycles (94 °C for 30 s, 62 °C for 30 s, and 72 °C for 60 s). This product was column purified (DNA Clean & Concentrator-5, Zymo Research, Irvine, CA) and amplified with E3 and TD78RiboZ2 (Table 1) with the same conditions and polymerase and again column purified prior to a final amplification with E3 and RiboZ17PCR (Table 1). A 0.54 kb *Not*I, *Pst*I restriction fragment of this product was ligated into a 9.8 kb *Not*I, *Pst*I pCVB3/28 restriction fragment to remove nt1–77 of the cDNA genome and to incorporate a TD78 specific ribozyme (Fig. 1B) (Chapman et al., 2008; Kim et al., 2005). pCVB3–28 and pCVB3-TD78 have the viral cDNA in opposite orientation to the ampicillin resistance gene, an orientation with increased stability of the viral cDNA insert. The sequence of the construct was confirmed (data not shown).

Generation of CVB3/TD78 and CVB3/28: *Cl*I-digested pCVB3–28 and pCVB3-TD78 plasmid DNA was transcribed with T7 polymerase (Ribomax T7, Promega, Madison, WI) for 1 h at 37 °C. T7 transcripts were digested with with RQ1 DNase (Promega) in the presence of 2 mM  $CaCl_2$  and concentrated by precipitation in 2 M LiCl at 4 °C, then pelleting the RNA at 12,000 rpm for 20 min. HeLa cell monolayers were electroporated with full-length CVB3 RNA T7 RNA polymerase transcripts of pCVB3/28 (wt, positive control) or pCVB3-TD78 (Smithee et al., 2015). Virions from CVB3-TD78 transfection cultures were prepared after 72 h, centrifugally cleared of cellular debris, incubated with ribonuclease A to degrade non-encapsidated RNA, and concentrated by ultracentrifugation through 30% w/v sucrose columns (Kim et al., 2005; Smithee et al., 2015). Viral RNA was isolated with Trizol (Ambion, Foster City, CA). Viral RNA concentration of stocks was determined with RT-qPCR (Smithee et al., 2015).

Detection of viral RNA in electroporated HeLa monolayers: Viral RNA from wt and CVB3-TD78 virions was reverse transcribed (Smithee et al., 2015) using the primer E3 (Chapman et al., 1990), then enzymatically amplified using primers S5 (nt 86–113) and E3Sub (Smithee et al., 2015) using GoTaq (Promega) in GoTaq 1X complete master mix (Promega; Madison, WI) for 1 cycle at 94 °C for 5 min and 35 cycles (94 °C for 10 s, 56 °C for 5 s, and 72 °C for 35 s) followed by amplification with primers S5 and SReturn or using S4 (nt 45–74) and E3Sub and S4 and SReturn (Smithee et al., 2015). Primers are shown in Table 1.

Detection of passage of CVB3 in HeLa monolayers: Virions (isolated as described) were inoculated onto HeLa cell monolayers

with or without prior incubation (1 h, 37 °C) with anti-CVB3 neutralizing serum diluted 1:400 (ATCC; Manassas, VA) (Smithee et al., 2015). Total RNA was isolated with Trizol at day 5 and viral RNA was detected using semi-nested RT-PCR using E3 (RT) and S5/E3Sub and S5/SReturn (PCR) as described.

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