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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 CVB structural domain I can be deleted without lethality.

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 *Cla1*-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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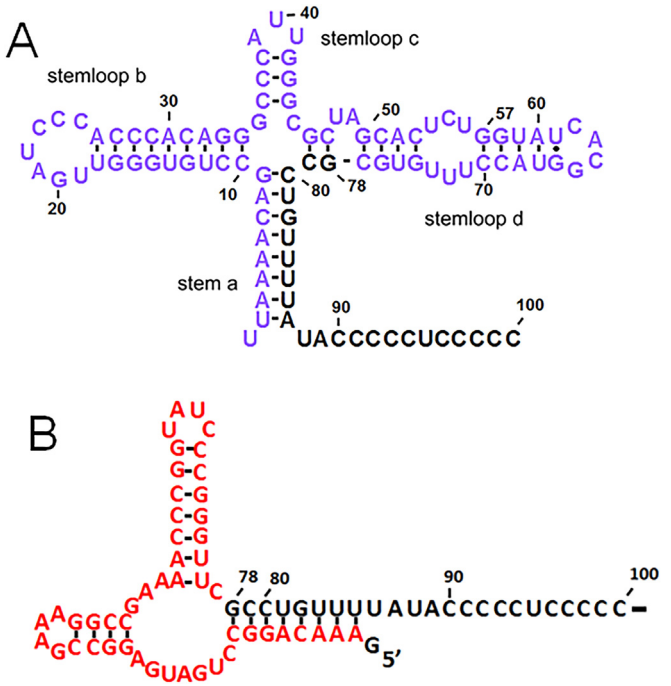

Fig. 1. pCVB3-TD78 deletion and plasmid map. (A) The structure of the 5′ non-translated region of CVB3 indicating deleted sequence (blue) and conserved region (black) in TD78. (B) Ribozyme sequence (red) [as in (Wedekind and McKay, 1998)] of the 5′ end of T7 RNA transcript. (For interpretation of the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

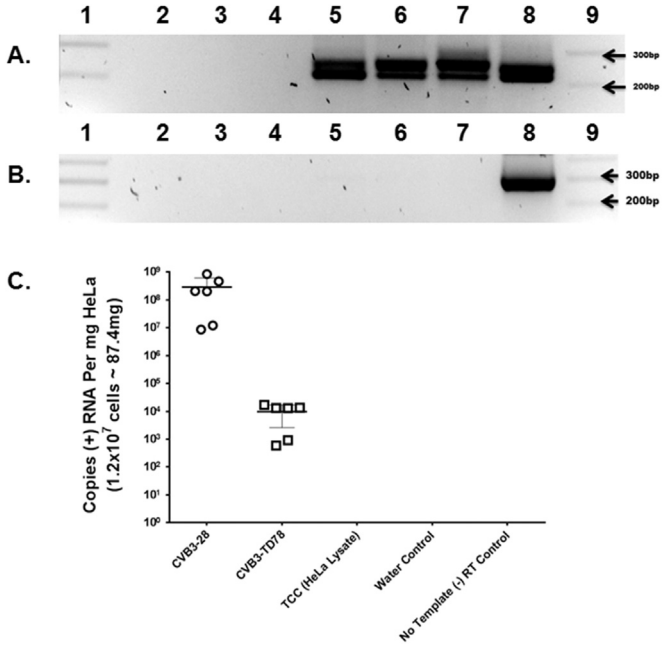

Fig. 2. Replication of CVB3-TD78. (A) RNA from purified virions of HeLa cultures 72 h post electroporation was reverse transcribed [primer E3; (Chapman et al., 1990)], amplified with S5 and E3Sub, reamplified with S5 and SReturn. (B) cDNA from virion RNA as in (A) was amplified with S4 (Kim et al., 2005) 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.

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 quantitate 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 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 $3CD^{pro}$ 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	ATGAGGCCGAAAGGCCGAAAACCCGGTATCCCGGGTTCGCCTGTTTTATACCCCCTCCCCCA		
TD78RiboZ2	CACTATAGGGCGCGGGAAACAGGCCTGATGAGGCCGAAAGGCCGAAAAC		
RIBOZPCRT7	GACCGCGGCCGCGTAATACGACTCACTATAGGGCGCGGG		
S4	CGCTAGCACTCTGGTATCACGGTACCTTTG	45–74	(Kim et al., 2005)
S5	TATACCCCCTCCCCCAACTGTAACTTAG	86–113	(Smithee et al., 2015)
E3Sub	AGTAGTCGGTTCCGC	Reverse Complement 535–549	(Smithee et al., 2015)
SReturn	TACACTGGGGTAGTGCTGAGCG	Reverse Complement 291–312	(Smithee et al., 2015)