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Reversion to wildtype of a mutated and nonfunctional coxsackievirus B3CRE(2C)

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ABSTRACT

The *cis*-acting replication element (CRE) in the 2C protein coding region [CRE(2C)] of enteroviruses (EV) facilitates the addition of two uridine residues (uridylylation) onto the virus-encoded protein VPg in order for it to serve as the RNA replication primer. We demonstrated that coxsackievirus B3 (CVB3) is replication competent in the absence of a native (uridylylating) CRE(2C) and also demonstrated that lack of a functional CRE(2C) led to generation of 5′ terminal genomic deletions in the CVB3 CRE-knock-out (CVB3-CKO) population. We asked whether reversion of the mutated CRE(2C) occurred, thus permitting sustained replication, and when were 5′ terminal deletions generated during replication. Virions were isolated from HeLa cells previously electroporated with infectious CVB3-CKO T7 transcribed RNA or from hearts and spleens of mice after transfection with CVB3-CKO RNA. Viral RNA was isolated in order to amplify the CRE(2C) coding region and the genomic 5′ terminal sequences. Sequence analysis revealed reversion of the CVB3-CKO sequence to wildtype occurs by 8 days post-electroporation of HeLa cells and by 20 days post-transfection in mice. However, 5′ terminal deletions evolve prior to these times. Reversion of the CRE(2C) mutations to wildtype despite loss of the genomic 5′ termini is consistent with the hypothesis that an intact CRE(2C) is inherently vital to EV replication even when it is not enabling efficient positive strand initiation.

1. Introduction

The group B coxsackieviruses (CVB; serotypes 1–6), are small, non-enveloped, single-stranded RNA viruses classified as species B *enteroviruses* (EV) [order *Picornavirales*; family *Picornaviridae*; genus *enterovirus*; species *enterovirus B*] (Pallansch et al., 2013). Because the CVB cause, or are etiologically associated with, a plethora of human diseases including aseptic meningitis, type I diabetes, pancreatitis and myocarditis (Chapman and Kim, 2008; Drescher et al., 2004; Modlin and Rotbart, 1997; Pallansch et al., 2013; Tracy et al., 2011; Tracy et al., 2002), and because they are common and typical enteroviruses, understanding how these viruses replicate is especially relevant given current resurgence of interest in enteroviral diseases such as those caused by enterovirus A71 (Huang and Shih, 2014; Yip et al., 2013) and enterovirus D68 (Messacar et al., 2016; Tan et al., 2016).

Enteroviral persistence well after the acute infectious phase has been documented [reviewed (Chapman and Kim, 2008)]. Persistence of CVB RNA in the apparent absence of cytopathic virus populations in both tissues from experimentally-inoculated mice, as well as from naturally infected humans, can occur well after both the viremic phase and the rise of the type-specific adaptive immune response to the infection (Chapman et al., 2008; Kim et al., 2008; Kim et al., 2005; Leveque et al., 2012; Reetoo et al., 2000; Rey et al., 2001; Tam and Messner, 1999; Tracy et al., 2015). Our previous work identified the mechanism by which enteroviral persistence can occur in the apparent absence of replicating virus, showing it to manifest *via* naturally generated 5′ terminal genomic deletions (CVB-TD) (Kim et al., 2005). Once arisen, CVB-TD populations persist in the complete absence of intact wildtype virus populations (Kim et al., 2008; Kim et al., 2005). Characterized CVB-TD populations exhibit 5′ terminal genomic deletions ranging from 7 to 49 nucleotides that partially erode domain I (Chapman et al., 2008; Kim et al., 2008; Kim et al., 2005), a *cis*-acting replication element (CRE) that is crucial for efficient EV genome replication (Andino et al., 1993; Andino et al., 1990; Barton et al., 2001; Parsley et al., 1997; Sean and Semler, 2008; Xiang et al., 1995). Studies have demonstrated the generation of TD mutations in the heart and the pancreas of mice inoculated with wildtype CVB3 (Kim et al., 2005; Tracy et al., 2015), in the heart of a patient naturally infected with CVB2 (Chapman et al., 2008) and during CVB3 passage in primary cell cultures but not in cultures of immortal cell lines such as

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HeLa (Kim et al., 2008). CVB3-TD replicates in HeLa cells without cytopathic effect (Kim et al., 2008; Kim et al., 2005) and to a level 100,000 fold less than the wildtype CVB3-28 (Smithee et al., 2015) and produce infectious virion that can be neutralized with CVB3 specific antisera (Kim et al., 2005; Smithee et al., 2015). Preparations of CVB3-TD from HeLa cells demonstrate a nearly equal level of positive and negative strands and encapsidate both positive and negative strands (Kim et al., 2005). Passage of CVB3-TD with short terminal deletions demonstrate evolution to larger deletions in passage in mice (Kim et al., 2005) or in primary cell cultures (Kim et al., 2008) but no reversion to wildtype virus, that is, reacquisition of the deleted 5′ terminal sequence.

Located in the enteroviral protein 2C coding region is another of 4 known CREs [reviewed in (Paul and Wimmer, 2015)], a stem-loop secondary structure termed CRE(2C). This structure is the template for the viral RNA polymerase-mediated uridylylation of the viral protein VPg; the VPg-pUpU is subsequently used to prime positive strand viral RNA synthesis (Paul et al., 2003; Paul et al., 2000; van Ooij et al., 2006). The CRE(2C) is therefore also crucial to EV replication: several genetically-engineered mutational disruptions of the CRE(2C) of both poliovirus and CVB3 have been reported to be lethal when studied using cell free assays to measure genome replication and VPg uridylylation with autoradiographic analyses (Goodfellow et al., 2003a; Goodfellow et al., 2003b; Murray and Barton, 2003; van Ooij et al., 2006) or by luciferase and cytopathic effect assays in cell cultures after short incubation times (van Ooij et al., 2006).

After engineering 16 CRE(2C) mutations [as in the DM mutant shown by others (van Ooij et al., 2006) to be lethal for CVB3] into our infectious copy of a CVB3 genome (Tracy et al., 2002) for use as a negative control in other work, we were intrigued to find that this CRE knockout construct (hereafter termed CVB3-CKO) was, in fact, replication competent (Smithee et al., 2015). Ribonuclease-treated and ultracentrifugally isolated infectious virions from HeLa cells previously electroporated with T7 RNA polymerase-transcribed CVB3-CKO RNA caused productive infections in fresh cell cultures and were inhibited by anti-CVB3 neutralizing serum, confirming CVB3-CKO infectivity (Smithee et al., 2015). Interestingly, we observed that 5′ terminal genomic deletions (TDs) had also occurred in CVB3-CKO populations (Smithee et al., 2015). By inoculating mice with T7-transcribed CVB3-CKO RNA incorporated in a transfection reagent (Smithee et al., 2015), we further showed that infectious CVB3-CKO was generated in murine tissues by isolating infectious virions and neutralizing their infectivity in cell culture with anti-CVB3 serum. Together, these results demonstrated that a functional CRE(2C) in a CVB3 genome was not required for the induction of a productive CVB replication cycle.

The findings that CVB3-CKO is replication competent, although severely diminished in replication efficiency *vis-a-vis* wildtype CVB3, and that the CVB3-CKO had become 5′ terminally deleted as well, led us to ask whether the mutated CRE(2C) sequence (CKO) had remained intact or whether reversions in the sequence had occurred. In a series of experiments to preliminarily examine this question, we repeatedly sequenced amplimers encompassing the CRE(2C) region of CVB3-CKO isolated 8 days post-electroporation of HeLa cells and observed that all 16 of the mutated CKO nucleotides had reverted to wildtype. From these consistent results, we drew the conclusion that the CKO mutations were in fact reverting *in toto* to the wildtype CRE(2C) sequence. Here, we demonstrate that following electroporation of cells with CVB3-CKO RNA, a new CVB3 population arises with a reverted (that is, wildtype) CRE(2C) sequence. We also show that 5′ terminal genomic deletions evolve in this virus population before the CKO reverts. Because the loss of intact 5′ genomic termini lowers viral replication efficiency (Smithee et al., 2015), it prevents the detectable CPE that would be expected in cell culture had the CKO sequence reverted to the wildtype CRE(2C) in the presence of an intact 5′ genomic terminus. We suggest that the reversion to the wildtype CRE(2C) indicates that a functional CRE(2C) contributes to CVB-TD replication.

2. Materials and methods

2.1. Cells and virus

Coxsackievirus B3/28 [described previously; (Tracy et al., 2002)] is the (wildtype) virus strain used in this work. Mutations [as described previously (Smithee et al., 2015; van Ooij et al., 2006)] were made in the CRE(2C) region of the wildtype or CVB3-TD50 cDNA to create the CKO (cre knockout) strains, CVB3-CKO and CVB3-TD50-CKO respectively (Fig. 1). All cDNA clones used in this work were sequenced within the P-2C coding region to verify the expected sequence with primers 2C2 and 2C7 (Table 1) as described (Smithee et al., 2015). The CRE(2C) encoding region in T7 transcribed RNAs were also reverse transcribed, then similarly amplified and sequenced to verify the sequences were as expected (Smithee et al., 2015). HeLa cell monolayer cultures were maintained as described previously (Smithee et al., 2015). Viral stocks were prepared by electroporation of HeLa cells with T7 transcribed RNA from infectious cDNA clones of each viral genome using a GibcoBRL Cell Porator (Gathersburg, MD, USA) at 110 V, 1980 μF, and high ohms. Electroporated cell cultures were incubated for 1–8 days (CVB3-CKO) or 8 days (CVB3-TD50, CVB3-TD50-CKO, and wildtype CVB3). Following incubation with ribonuclease, virions were

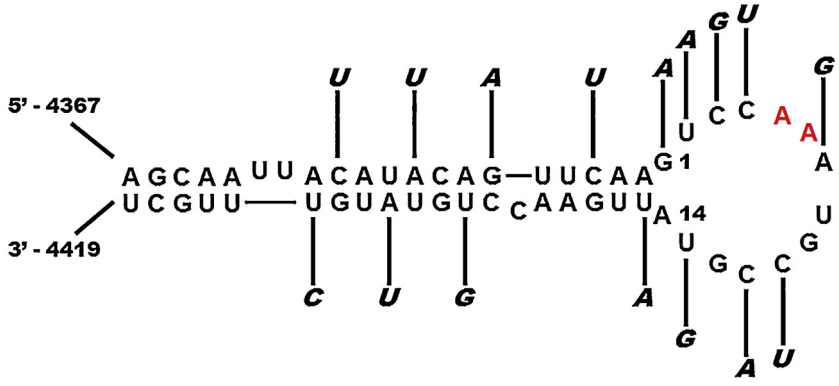

Fig. 1. The CRE(2C) of CVB3 with mutations indicated. The sequence of the CRE(2C) of CVB3 from nucleotides 4367–4419. Nucleotides changed to generate CVB3-CKO are indicated by italicized letters above the wildtype sequence. The numbering of the loop is indicated beginning with number 1 (G) and proceeding around the loop to number 14. Red nucleotides in the loop indicate the template nucleotides used for VPg uridylylation (Paul et al., 2003). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Primer names, annealing sites and sequences.

Primer Name	Region of CVB3/28 Genome	Strand	Nucleotide Sequence (5′ to 3′)
S[a]	1–20	+	TTAAAACAGCCTGTGGGTTG
S4[a]	45–74	+	CGCTAGCACTCTGGTATCACGGTACCTTTG
S5[b]	86–113	+	TATACCCCCTCCCCCAACTGTAACTTAG
SReturn[b]	291–312	–	TACACTGGGGTAGTGCTGAGCG
E2[a]	450–464	+	TCCGGCCCCTGAATG
E3sub[b]	535–549	–	AGTAGTCGGTTCCGC
E3[a]	537–563	–	ACACGGACACCCAAAGTAGTCGGTTCC
2B1[b]	3746–3764	+	GGAGTGAAGGACTATGTGG
2C1[b]	3944–3964	+	ACTGCCACACTAGCCCTTATC
2C7[b]	4166–4186	+	GAAAAACACGAATTCCTGAAC
2C2[b]	4450–4469	–	CAATTAAGTTTGTTGCCACC
4523Rev[b]	4523–4504	–	GGTCTGGCGGTAGTGAGTAC
XBA[b]	4932–4958	–	GCATGTCTAGAGAGTATCTGACCTGTG
PIN[b]	NA	NA	GGAATTCATCGATACGCG[c]
PIN5End1[b]	1–10	+	GGAATTCATCGATACGCGTTAAAACAGC[c]
PIN5End5[b]	15–24	+	GGAATTCATCGATACGCGGGGTTGATCC[c]
M13F(-20)	NA	NA	GTAAAACGACGGCCAG[c]

[a] previously published in Kim et al., 2005.
[b] previously published in Smithee et al., 2015.
[c] underlined sequence is not genomic cDNA.

purified by ultracentrifugal pelleting through 30% (w/v) sucrose as previously described (Smithee et al., 2015).

2.2. Transfection of mice with T7 transcribed viral RNA

The use and care of mice in these studies was approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Male A/J mice (6–7 weeks old; Jackson Laboratories, Bar Harbor, ME) were inoculated intraperitoneally (i.p.) with CVB3-CKO T7 transcripts as previously described (Smithee et al., 2015). Briefly, mice were inoculated i.p. using a 1 mL syringe with a 25 ga needle with 25 μg of CVB3 or CVB3-CKO T7 transcribed RNA in 100 uL volumes, previously prepared in 0.4 mL aliquots containing 100 μg T7 transcribed RNA in 200 μL of nuclease free water, 100 μL of LIPID-based *in vivo* Transfection Reagent (Altogen Biosystems), 20 μL transfection enhancer, and 80 μL of a 5% (w/v) sterile glucose solution. After euthanizing mice and removing spleens and hearts, total tissue RNA was extracted by homogenization in 0.5 mL TRizol (Invitrogen; Carlsbad, CA), then assayed as described for the enzymatic amplification of the CRE(2C) sequence (below).

2.3. Enzymatic amplification of the CRE(2C) sequence from virus RNA

To determine the sequence for reversions in the CRE(2C) regions of CVB3-CKO and CVB3-TD50-CKO, we transcribed cDNA, enzymatically amplified the DNA, after which the amplimers were treated as previously described (Smithee et al., 2015). Briefly, cDNA was transcribed from viral RNA preparations (CVB3-CKO [1, 3, 5, 6, 7, and 8 days post-electroporation]; CVB3-TD50-CKO [8 days post-electroporation]; CVB3-TD50 control [8 days post-electroporation]; wildtype CVB3 [pass 3, 8 days post-electroporation]) as well as from day 20 post-transfection mouse tissues (CVB3-CKO only) with primer 4523Rev (Table 1) at a final concentration of 2 μM using Improm II reverse transcriptase (Promega, Madison, WI). Reactions containing 0.8 mM dNTPs, 2 mM $MgCl_2$, and 1 μL enzyme (units not provided by supplier) in 20 μL reaction buffer were incubated for 60 min at 42 °C. The CRE(2C) region was amplified using nested primers in 20 μL reaction volumes containing GoTaq Green Master Mix (Promega). Briefly, cDNA was amplified in initial enzymatic reactions with primers 4523Rev and 2B1 at a final concentration of 0.5 μM (Table 1), with cycle times as follows: one cycle at 94 °C for 5 min; 35 cycles at 94 °C for 10 s, 52 °C for 5 s, and 72 °C for 45 s with a final extension at 72 °C for 5 min. PCR products were ethanol precipitated and resuspended in 12 μL of nuclease free water. A second enzymatic amplification was done with primers 2C2 and 2C7 (Table 1) [cycle times were as follows: one cycle at 94 °C for 5 min; 35 cycles at 94 °C for 10 s, 54 °C for 5 s, and 72 °C for 45 s; and a final extension at 72 °C for 5 min]. For virion RNA preparations at days 6, 7 and 8 post-electroporation, two concurrent amplifications with primers 2C2 and 2C7 were performed in addition to, and in *lieu* of, the nested PCR described above; this yielded better sensitivity at these time-points which had scant viral RNA for cDNA preparation. Bands of amplified cDNA, electrophoresed in 2% agarose, of the appropriate size (as predicted by the annealing sites of primers in Table 1) were excised and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research; Irvine, CA). Isolated DNA samples were sequenced at the UNMC Core Facility.

2.4. Subcloning of CRE(2C) amplimers

To test whether intermediate populations were detectable, the CRE(2C) region of CVB3-CKO at various time-points post electroporation of HeLa cells was enzymatically amplified as described above. Amplimers were then ligated with pCR2.1 Topo vector with a 3′-thymidine overhang and a covalently attached topoisomerase, and transformed into chemically competent *E. coli* (TOPO TA Cloning Kit; Invitrogen; Carlsbad, CA) per manufacturer's protocol. Insert-containing colonies were picked, inoculated into Terrific Broth (Difco, BD, Sparks, MD), and incubated overnight at 37 °C and 300 RPM. Plasmid DNA was isolated using Qiagen columns (QIAprep Spin Miniprep Kit, Qiagen; Valencia, CA). Sequencing was carried out with primer 2C7 (Table 1) at the UNMC High-Throughput DNA Sequencing and Genotyping Core Facility.

2.5. Nested RT-PCR for detection of viral RNA in virus preparations

To assay for viral RNA in ultracentrifugally isolated virion preparations, nested RT-PCR was performed as previously described (Smithee et al., 2015). Briefly, cDNA was primed with E3 (Table 1) as described in enzymatic amplification of the CRE(2C) region (above), followed by enzymatic amplification using primers S4 and E3Sub (Table 1) in GoTaq Green Master Mix (Promega). Cycle times for this reaction were as follows: one cycle at 94 °C for 5 min; 35 cycles at

94 °C for 10 s, 56 °C for 5 s, and 72 °C for 35 s with a final extension at 72 °C for 5 min. Reactions were ethanol precipitated and a second PCR carried out using primers S5 and SReturn (Table 1) using cycle times as follows: one cycle at 94 °C for 5 min; 35 cycles at 94 °C for 10 s, 56 °C for 10 s (based on gradient analysis), 72 °C for 35 s with a final extension at 72 °C for 5 min. Final PCR products were analyzed on 2% agarose gels. Amplimer bands of the expected size were purified, ligated with pCR2.1 Topo vector with a 3′-thymidine overhang and covalently attached topoisomerase (TOPO TA Cloning Kit, Invitrogen, Grand Island, NY), and amplified in DH5α *E. coli* (Invitrogen). Inserts in the plasmid DNA (QIAprep Spin Miniprep Kit, Qiagen) were sequenced with M13 Forward (-20) Primer (Table 1).

2.6. Titering viral RNA in purified virus stocks using RT-qPCR

Because the mutant viruses (CVB3-CKO, CVB3-TD50, and CVB3-TD50-CKO) do not cause CPE in tissue culture monolayers, viral RNA copy number was determined using RT-qPCR as described previously (Kim et al., 2005; Smithee et al., 2015). cDNA was transcribed with primer E3 (Table 1) as described in enzymatic amplification of the CRE(2C) above. As previously described (Smithee et al., 2015), RT-qPCR was performed using an Opticon 2 DNA Engine (MJ Research) using primers E2 and E3Sub (Table 1) in Maxima SYBR Green/Fluorescein qPCR master mix (2X; Thermo Scientific). Cycle times were as follows: one cycle at 94 °C for 5 min; 35 cycles at 94 °C for 10 s, 58 °C for 10 s, and 72 °C for 10 s with a final extension at 72 °C for 30 s. Standard curves were generated by performing 10 fold serial dilutions of a 500 bp amplimer of CVB3 cDNA which had been previously amplified from the CVB3/28 cDNA clone using primers S and E3 (Table 1) and purified. The concentration of this control DNA was determined using spectrophotometry at 260 nm.

2.7. RT-PCR to detect 5′ terminal deletions in RNA from purified virus stocks

To determine when 5′ terminal genomic deletions occurred over a time-course of CVB3-CKO replication, cDNA was primed with E3 (Table 1). To provide comparable results, reverse transcriptase reactions were each assembled with equal RNA copy numbers calculated as described earlier (Smithee et al., 2015). Tagged primers (Lanford et al., 1994) were used to detect 5′ terminal deletions (Smithee et al., 2015). Briefly, initial enzymatic amplification was carried out with E3 cDNA copy numbers determined to be over but near the experimentally determined limit of detection for each primer pair. For the primer pair, Pin5End1 and E3 (Table 1), this copy number was 500 copies of E3 cDNA and for the primer pair, Pin5End5 and E3 (Table 1) 1500 copies were used. Amplifications were carried out in GoTaq Green Master Mix (Promega), with cycle times as follows: one cycle at 94 °C for 5 min; 35 cycles at 94 °C for 10 s, 63 °C for 10 s, and 72 °C for 35 s; and a final extension at 72 °C for 5 min. Amplimers were ethanol precipitated, resuspended in 12 μL of nuclease free water and re-amplified in 20uL reactions containing 8 μL of the Pin5End1/5-E3 PCR product, 10 μL of GoTaq Green Master Mix and primers Pin and SReturn (Table 1) at a final concentration of 0.5 μM. Cycle times were as follows: one cycle at 94 °C for 5 min; 35 cycles at 94 °C for 10 s, 53 °C for 5 s, and 72 °C for 25 s; and a final extension at 72 °C for 5 min. PCR products were analyzed on 2% agarose gels.

2.8. Alignment of enterovirus B CRE(2C) protein and nucleotide sequences

Enterovirus B nucleotide sequences in GenBank were aligned with the *enterovirus* B reference nucleotide sequence (GenBank ID: 9626677) to compare the sequence encompassed by nt 4033–5019, which encodes protein P-2C, using BLASTN 2.2.32+ with megablast parameters (Morgulis et al., 2008; Zhang et al., 2000). The sequence was compared to the wildtype CVB3/28 and CVB3-CKO sequences; variations at positions with CKO mutations were noted. Similarly *enterovirus* B protein sequences in GenBank were aligned with the *enterovirus* B reference 2C protein sequence (GenBank ID: 25121824) using BLASTP 2.2.31+ (Altschul et al., 1997; Altschul et al., 2005). The sequence was compared to the wildtype CVB3/28 and CVB3-CKO aa sequences, and to the predicted aa sequences in which compensatory nucleotide mutations restored the ability of the CKO CRE(2C) stem to form had reversions not occurred, in order to determine if compensatory mutations were likely. The extent to which these variations in the CRE(2C) aa sequence existed in *enterovirus* B CRE(2C) sequences in GenBank was noted.

3. Results

3.1. The 16 CKO mutations revert to the wildtype CRE(2C) sequence

Earlier work (Smithee et al., 2015) demonstrated that CVB3-CKO and CVB3-TD50-CKO are both viable in cell culture following electroporation of T7 transcribed RNA; additionally, CVB3-CKO replicated in mice following transfection of CVB3-CKO T7 RNA polymerase-transcribed RNA. It was also demonstrated that CVB3-CKO virion preparations isolated from HeLa cell cultures post-electroporation, or from mouse tissue homogenates, could subsequently be detected after passage in cell culture, and that such infections were prevented when using anti-CVB3 neutralizing serum (Smithee et al., 2015). The fact that we observed replication of CVB3-CKO was contrary to previous results which indicated the mutations in the *enterovirus* CRE(2C) (Fig. 1) were lethal (Murray and Barton, 2003; van Ooij et al., 2006). Perhaps reversions were occurring, permitting this replication. We therefore asked whether the 16 mutations introduced in the CRE(2C) region of CVB3-CKO remained intact during replication, or whether reversions occurred in this region after replication in either cell culture or within the tissues of transfected mice. T7 transcribed full length RNA was electroporated into HeLa cell cultures to provide virion preparations (CVB3-CKO or CVB3-TD50-CKO [wildtype CVB3 and CVB3-TD50 were used as controls]) for preparation of viral cDNA. Viral cDNA was also prepared from RNA of mouse tissues at 20 days post transfection with CVB3-CKO RNA. The CRE(2C) region was enzymatically amplified and sequenced from these viral cDNAs; wildtype and CVB3-CKO clones were used as sequencing controls. Alignments of amplified CRE(2C) sequences were subsequently performed (Fig. 2).

Sequencing results from total amplimer populations indicated that at days 1, 3 and 5 (Fig. 2A, rows 5,6,7) post-electroporation of HeLa cell cultures with CVB3-CKO T7 transcribed RNA, the 16 mutations in the CRE(2C) region were intact: no reversions were evident in purified CVB3-CKO virion RNA when compared to the wildtype CVB3 cDNA clone and T7 transcribed RNA (Fig. 2A, rows 1,2 respectively) or the CVB3-CKO cDNA clone and T7 transcribed RNA (Fig. 2A, rows 3,4 respectively). However, by day 8 post electroporation, all 16 mutations had reverted to that of the wildtype sequence (compare Fig. 2A, rows 8,9 to rows 1,2 [wildtype] and rows 3,4 [CVB3-CKO]): we observed this result in 4 separate and repeat experiments using different and individually purified CVB3-CKO virion preparations from day 8 post-electroporation cell cultures (of which two representative sample sequences are shown in Fig. 2A, rows 8,9). To determine whether reversions were present in virion RNA isolated from murine tissues at day 20 post transfection, the CRE(2C) region was amplified using the same method described for the purified virion preparations. Analysis of sequences from day 20 post-transfection heart (Fig. 2A, row 10) and spleen (Fig. 2A, row 11) also demonstrated that reversion to the wildtype sequence

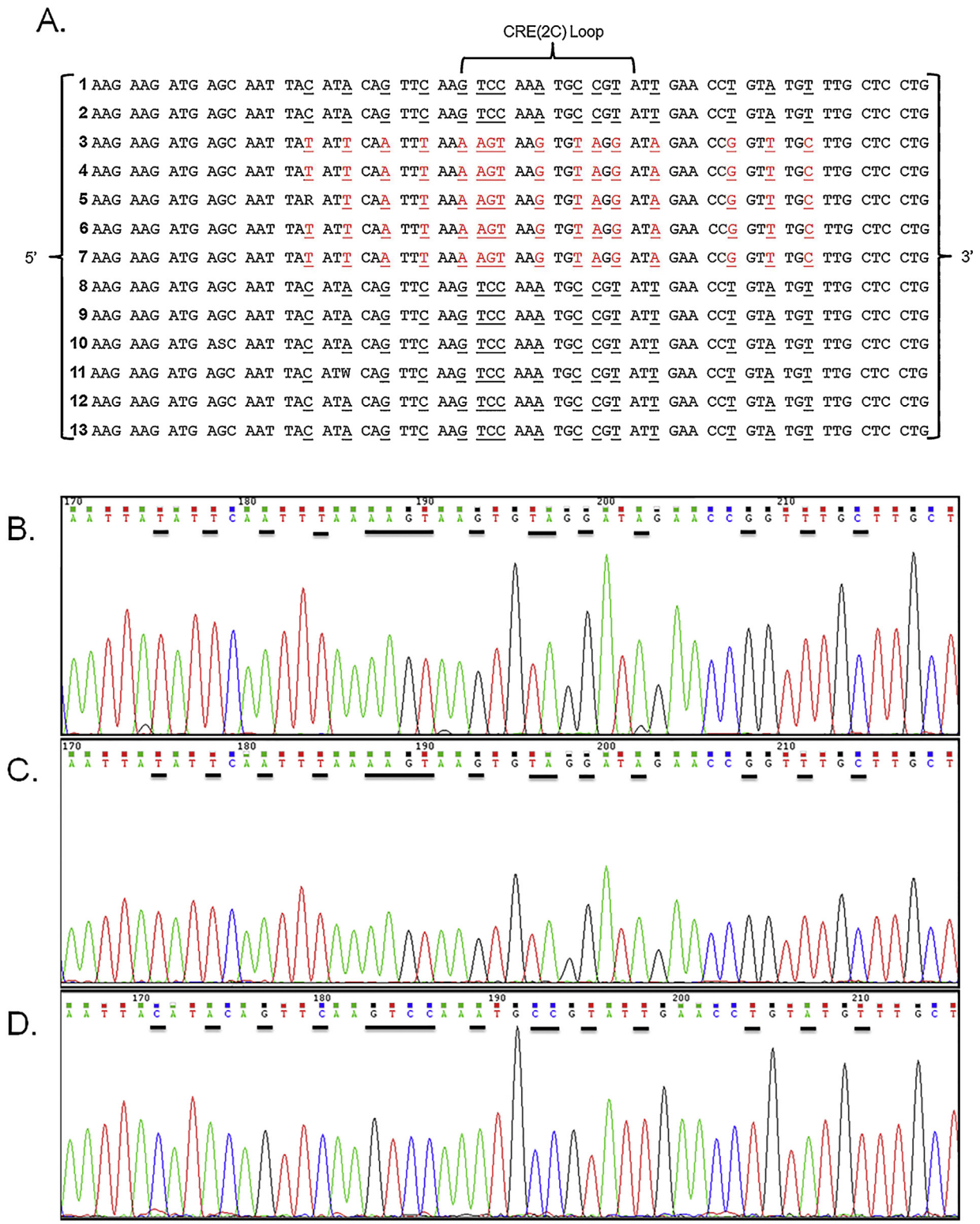

Fig. 2. The CRE(2C) of CVB3-CKO completely reverts to a wildtype sequence by 8 days post-electroporation in HeLa cells and by 20 days post-transfection in mouse tissues. (A) Sequence alignments of cDNA clones (1 [wildtype CVB3] and 3 [CVB3-CKO]), T7 transcribed RNA from cDNA clones (2 [wildtype CVB3] and 4 [CVB3-CKO]), RNA from purified virus preparations at various time-points post-electroporation of HeLa cells (5 [CVB3-CKO 1 day post-electroporation], 6 [CVB3-CKO 3 days post-electroporation], 7 [CVB3-CKO 5 days post electroporation], 8 [CVB3-CKO 8 days post-electroporation; virus preparation I], 9 [CVB3-CKO 8 days post-electroporation; virus preparation II], 12 [CVB3-TD50-CKO 8 days post-electroporation], and 13 [CVB3-TD50 8 days post electroporation]), and mouse tissues 20 days post-transfection (10 [CVB3-CKO; mouse heart] and 11 [CVB3-CKO; mouse spleen]). Underlined nucleotides indicate wildtype sequence and red, underlined sequence indicates CKO mutations. (B-D) Representative sequence chromatograms from (B) CVB3-CKO at 1 day post-electroporation of HeLa cells, (C) 5 days post-electroporation, and (D) 8 days post electroporation. 2A, line 5: R is the IUPAC designation for a mixture of purine at this position.

had occurred in the CRE(2C) region of CVB3-CKO (compare Fig. 2A, rows 10,11 respectively, to rows 1,2 and rows 3,4). The same result was obtained using another set of mouse tissues from different mice at the same time point (data not shown). A representative selection of sequencing chromatograms from days 1, 5, and 8 post-electroporation of cell culture demonstrate that sequencing reads were unambiguous (Fig. 2B,C,D). Together, these results indicated that reversion of CKO to wildtype sequence in the CRE(2C) encoding region had occurred and that this was detectable after 8 days in cell culture or following 20 days of replication in murine tissues.

3.2. CKO also reverts in a CVB3 genome lacking wildtype 5′ genomic termini

We then engineered the CKO mutations into an infectious cDNA cloned genome [CVB3-TD50; (Kim et al., 2005)] which lacked the 49 5′ terminal genomic nucleotides; this was termed CVB3-TD50-CKO (Smithee et al., 2015). We reasoned that CVB3-TD50-CKO should not require uridylylated VPg as a primer for RNA replication because unlike the wildtype genome, the genomic 5′ terminus is CG (Kim et al., 2005). As we had observed reversion of the 16 mutations in the CRE(2C) sequence of CVB3-CKO RNA after 8 days of replication in HeLa cells as well as at 20 days in the tissues of transfected mice, we asked whether reversions of the mutated sites also occurred in CVB3-TD50-CKO. Using CVB3-TD50 as a control, sequence analysis was conducted on CVB3-TD50-CKO and CVB3-TD50 as described above for CVB3-CKO. Based on sequence analysis at 8 days post-electroporation of HeLa cells, reversion of the 16 CKO mutations to wildtype sequence occurs in the CVB3-TD50-CKO population as well (compare Fig. 2A, row 12 to row 13 and row 12 to rows 1,2 [wildtype] and rows 3,4 [CVB3-CKO]). These findings indicated that whether or not the viral 5′ genomic termini were intact when replication was initiated, the CKO sequences reverted and were indistinguishable from those of the wildtype CVB3.

While we had observed reversion of CKO to the wildtype CRE(2C) sequence after 8 days of replication in HeLa cells, the sequence of total amplified cDNA populations prior to the 8 day time point showed only the expected genetically engineered CKO mutations. We hypothesized that prior to 8 days, populations of CRE(2C) revertants were continually forming and collapsing within each infected cell's mutant swarm but that the residual dominant sequence (CKO) in the total amplimer population effectively masked these changes (Borderia et al., 2010). This suggested that the wildtype (revertant) CRE(2C) population would have to be the dominant sequence before being able to be detected by sequence analysis. Thus, at some ratio of the mutant CKO sequence to the wildtype CRE(2C) sequence, the wildtype sequence (or any other variant of the mutant swarm) would not be detectable by Sanger sequencing analysis. We therefore determined the ratio of wildtype CRE(2C) to CKO sequences that would need to be present in a mixed population before the wildtype CRE(2C) sequence could, and the CKO sequence could not, be detected using Sanger sequencing. Increasing concentrations of wildtype CVB3 cDNA were mixed with a constant concentration of CVB3-CKO cDNA. Thus, two-fold increases of the wildtype CVB3 cDNA clone (from 1.62×10^9 copies [1:1] to 1.30×10^{10} copies [8:1]) were mixed with a constant copy number of the CVB3-CKO cDNA clone (1.62×10^9 copies). The CRE(2C) region was then amplified from these mixtures and sequenced using primer 2C7 (Table 1), after which sequencing chromatograms were compared (Fig. 3). The control CVB3-CKO and wildtype CVB3 clone DNA sequences were as expected (Fig. 3A, F), confirming that the expected CKO mutations were present in the CVB3-CKO cloned cDNA used in these studies. At a cDNA copy ratio of 1:1 [wildtype CRE(2C)/CVB3-CKO (Fig. 3B)] both sequences were detected, as shown by the presence of two peaks corresponding to two different nucleotides in the sequencing chromatogram. However, at a copy ratio of just 2:1 wildtype CRE(2C)/CVB3-CKO (Fig. 3C), the mutant CKO sequence is already partially obscured by the two-fold majority wildtype sequence: this is indicated by the finding that 10 of the 16 mutations may be read as wildtype in the chromatogram, although two peaks were still evident at each mutation site (Fig. 3C, arrows). At a ratio of 4:1 or 8:1, (Fig. 3D, E), all 16 CKO mutations are effectively masked by the wildtype sequence. Minor secondary peaks were detected in the sequencing chromatogram in these regions; such minor peaks are also observed in chromatograms of CVB3-CKO or wildtype plasmids alone (compare Fig. 3A, F to Fig. 3D, E). This indicated that when exploring a population of replicating RNA sequences 8 days post-electroporation with CVB3-CKO T7 transcribed RNA, only the majority population would be detectable at any time using standard Sanger sequencing. Intermediate populations would have to be present at a ratio of greater than 2:1 reverted:mutant in order to be detected using this approach. The strong inference is when reversion to wildtype of a CKO genome finally occurs, the new wildtype population rapidly predominates without the appearance of other stable and populous intermediates.

3.3. Reversion of CKO to wildtype CRE(2C) is not artifactual

Contamination with wildtype CVB3 RNA represented an obvious and trivial explanation for the observed reversion of 16 nt in the CKO to wildtype CRE(2C). Because we were using replication competent viral genomes, we used biological amplification (viral replication) as the most sensitive assay to test this hypothesis. A valid and sensitive assay to discriminate between wildtype CVB3 and the CKO strains or any of the CVB3-TD strains, is the induction of CPE for it has been clearly demonstrated that only wildtype CVB3 induces complete CPE in susceptible cell cultures, whereas neither CVB3-TD nor CVB3-CKO strains induce detectable CPE (Kim et al., 2008; Kim et al., 2005; Smithee et al., 2015). To test the hypothesis that contamination may have been responsible for our observations, we reasoned that if very few wildtype CVB3 genomes (which is to say, essentially undetectable levels by molecular means) were co-transfected with a gross excess of the replication impaired CVB3-CKO genomes, we would nonetheless observe the generation of CPE in the cell culture as the result of the dominance of the wildtype replication due to the 5 orders of magnitude difference in replication efficiency (Smithee et al., 2015) between these populations in HeLa cells. This was tested by transfecting mixes of T7-transcribed wildtype (cytopathic) CVB3 RNA and CVB3-CKO RNA into HeLa cell monolayers; the number of wildtype CVB3 RNA genomes ranged between 10 and 1000 copies while the copy number of (non-cytopathic) CVB3-CKO T7 transcribed RNA was held constant at 1.97×10^{11} copies. These mixes thus represented CVB3-CKO/wildtype CVB3CRE(2C) copy ratios of 2×10^{10}–2×10^8 to 1. Previous work by Crowder and Kirkegaard demonstrated that when one or two mutations were induced in the stem of the poliovirus CRE(2C), a dominant negative effect occurred during co-infection with wildtype poliovirus (Crowder and Kirkegaard, 2005); however, when multiple mutations throughout the stem and loop of the CRE(2C) were introduced (similar to those of CKO), no inhibition of wildtype virus replication occurred following co-infection (Crowder and Kirkegaard, 2005). Therefore, we did not anticipate a dominant negative effect on the replication of wildtype CVB3 from the structural disruption of the CRE(2C) sequence in CVB3-CKO. Following transfection of HeLa cell monolayers with the different viral RNA ratios, cultures were observed daily for the presence of CPE. In all cultures transfected only with wildtype RNA, complete CPE was observed after 24 h. Complete CPE was also observed in monolayers which had been transfected with both RNA types at 100 and 1000 copies of wildtype CVB3 RNA plus CVB3-CKO RNA (Table 2). Importantly, CPE was never observed in wells transfected

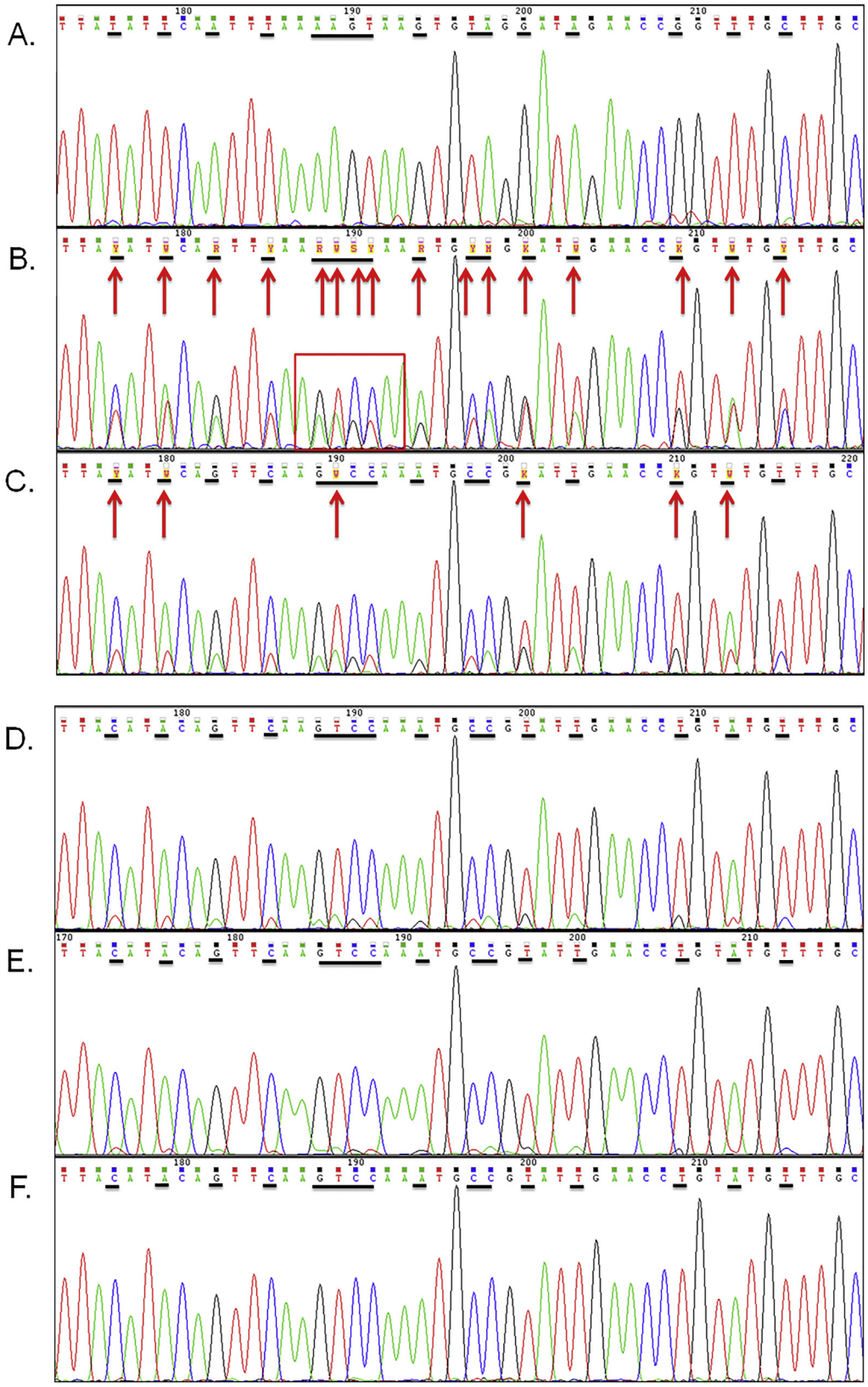

Fig. 3. Standard Sanger sequence analysis is inefficient at detecting mutations in a mixed population. (A-C) Sequencing was conducted on mixed population of cDNA clones of wildtype CVB3 and CVB3-CKO, with the concentration of CVB3-CKO being held constant and the concentration of wildtype CVB3 increasing by 2-fold to a final ratio of 8:1; underlined nucleotides indicate the 16 nucleotides mutated to generate CVB3-CKO from wildtype. (A) The DNA sequence of the CVB3-CKO CRE(2C) region using primer 2C7 (Table 1). (B) A 1:1 ratio of wildtype:CVB3-CKO demonstrates that both sequences are detected at near equal efficiency as indicated by double peaks (the box highlights a representative region with double peaks) and nucleotide ambiguity codes at positions corresponding to variable regions (red arrows). (C) At a ratio of 2:1, the wildtype sequence begins to obscure and mask the CKO sequence with only 6 of the 16CKO mutations detectable (red arrows). (D-F) At a ratio of 4:1 wildtype:CVB3-CKO (D) and 8:1 wildtype:CVB3-CKO (E), the CKO mutations are no longer detectable as compared to the wildtype cDNA clone sequence chromatogram (F).

Table 2
Experimental contamination of CVB3-CKO T7 transcribed RNA transfections with wildtype CVB3 T7 transcribed RNA demonstrate wildtype CVB3 rapidly becomes dominant.

T7 transcribed RNA Input (CVB3-CKO constant At 1.97×10^{11})	**CPE Present 1 day post transfection**	**CPE Present 5 days post transfection**	**CPE Present 1 blind passage**	**CPE Present 2 blind passages**	**CPE Present 3 blind passages**
CVB3-CKO **only**	*NO*	*NO*	*NO*	*NO*	*NO*
CVB3-CKO + 10 copies wildtype CVB3	*NO*	*NO*	*NO*	**YES**	Not Tested
CVB3-CKO + 100 copies wildtype CVB3	**YES**	**YES**	**YES**	**YES**	Not Tested
CVB3-CKO + 1000 copies wildtype CVB3	**YES**	**YES**	**YES**	**YES**	Not Tested
wildtype CVB3 **only** 1.97×10^{11} copies	**YES**	**YES**	**YES**	**YES**	Not Tested
wildtype CVB3 **only** 10 copies	*NO*	**YES**	**YES**	**YES**	Not Tested
wildtype CVB3 **only** 100 copies	**YES**	**YES**	**YES**	**YES**	Not Tested
wildtype CVB3 **only** 1000 copies	**YES**	**YES**	**YES**	**YES**	Not Tested

only with CVB3-CKO RNA alone or in mock-transfected cultures. In wells transfected with just 10 copies of wildtype T7 transcribed RNA and the excess of CVB3-CKO RNA, CPE was not observed by 5 days post-transfection (Table 2). However, when freeze-thawed lysates of cells in these wells were passaged to fresh cell cultures, CPE was evident after two passages (5 days of incubation following each passage). This delayed CPE was likely due to the relative difficulty of repeatedly measuring 10 RNA molecules with absolute precision for the initial transfection. It is important to note with respect to the latter finding that in no case (1–3 passages of 5 days each) did lysates from the controls (cultures transfected only with CVB3-CKO RNA) cause CPE on cell cultures. These results demonstrated, along with previous observations that passage of CVB3-CKO purified virus preparations do not induce CPE (Smithee et al., 2015), that CVB3-CKO virus preparations which were assayed for reversions were not contaminated with wildtype CVB3 and further, did not revert to a wildtype phenotype (ability to induce CPE) during passage. Contamination of PCR primers was also ruled out by conducting 10 replicate enzymatic amplifications containing only water, enzyme mix, and primers 2C2 and 2C7 (Table 1), all of which were negative for amplified cDNA when assayed on 2% agarose gels (data not shown).

3.4. Stable compensatory mutations are not predicted to occur in the stem of CRE(2C) in CKO viruses

We were curious to determine whether stable, partially reverted CVB3-CKO populations might arise. The mutations in the stem of the CKO mutant (Smithee et al., 2015; van Ooij et al., 2006) (Fig. 1**)** were synonymous, third-base mutations within codons in the protein 2C coding region. However, those nucleotides which pair with the mutated nucleotides are either in first or second positions within the 2C codons. Thus, any single compensatory change in the opposite strand to allow the CRE(2C) structure with the CKO mutation would result in an amino acid coding alteration. We aligned predicted 2C protein amino acid sequences of available *enterovirus* species-B in GenBank to identify all variations that have been reported. Of the seven predicted compensatory mutations which could restore the stem of CRE(2C), six would produce amino acid variants not reported to GenBank and only one had a variation which was reported; it was present in just one of 429 reported 2C amino acid sequences (Fig. 4A,B; boxes and arrows), suggesting much lower fitness of the viruses carrying these variants. This analysis indicated that it would be highly unlikely that stable compensatory mutations could be selected in the stem of the CRE(2C).

We also aligned all available CRE(2C) loop sequences of EV-B (more than 400 in GenBank). Of the 8 loop mutations engineered in the CKO construct, two were found just once in 425 aligned EV-B loop sequences and one was found in 3 of 416 EV-B aligned loop sequences. The high degree of CRE(2C) loop sequence conservation may not be surprising given the observed deleterious effects on uridylylation of mutations at several positions in the loop (van Ooij et al., 2006). Because the CRE(2C) loop is also part of a coding sequence with a defined secondary structure, the adoption of stable nucleotide changes which alter coding capacity and/or structure of this region would be even less likely. Taken together, these analyses indicated that viral genomes with partially reverted CRE(2C) sequences would not have high fitness and would likely not be a major component of the population.

3.5. Inability to capture intermediate forms of the reverting CKO sequence by PCR amplification

Total amplimer sequencing failed to show revertants prior to observation of reversion of all 16 mutations (above). Control experiments (above) demonstrated that at a 4:1 ratio of one sequence to another divergent sequence, sequence analysis of the dynamic population was an insufficiently sensitive detection assay for minor variants. Analyses of known CRE(2C) sequences had indicated that changes from the wildtype CRE(2C) would be inconsistent with a viable and stable virus population. However, we attempted to isolate and characterize intermediate reversion forms of the sequence through sequencing of amplimers derived from viral RNA 5, 6, and 7 days post-electroporation to test whether one or more minor, yet stable, populations might inapparently exist below the level of our detection. Nested PCR was performed on cDNA from four different CVB3-CKO RNA preparations as described above (Smithee et al., 2015) and 8 individual insert-positive colonies were assayed at each time point for each PCR. No intermediate (*i.e.*, altered from CKO) sequences were observed: the 16 CKO mutations remained intact at each time point for all clones analyzed. We performed similar experiments on viral RNA from cultures at day 8: in these, all sequences were wildtype (data not shown). No CKO or partial revertant sequences were present in any of the 32 sequences analyzed, which corresponded to four different, ribonuclease treated and biophysically purified 8 day CVB3-CKO virion preparations. These results suggest that despite it being certain that intermediate revertant populations are constantly being generated (*e.g.*, 3 of 22 clones of an 5′NTR region of similar size from these virus preparations had variations from the original plasmid cDNA sequence; data not shown), the consensus sequence detected prior to 8 days

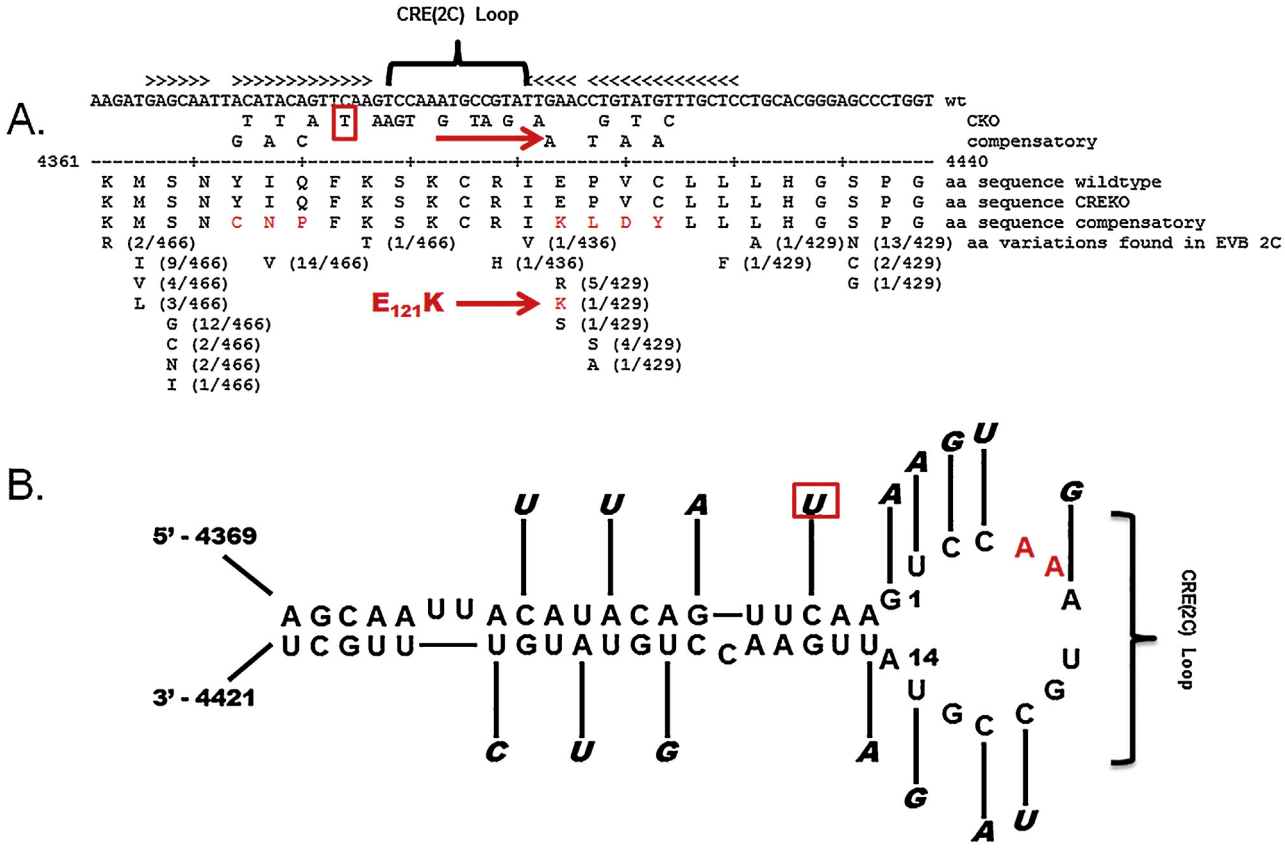

Fig. 4. Compensatory mutations are unlikely in the stem of the CRE(2C). (A) Sequence analysis using NCBI was used to explore the possibility of compensatory mutations in the CRE(2C) of CVB3. Potential amino acid variations resulting from compensatory mutations to restore the CRE(2C) RNA structure are noted. All variations in the CRE(2C) encoding sequence that would affect the amino acid sequence of the 2C protein are noted. Only 1 amino acid variation identical to those resulting from compensatory mutations was reported to exist in 429 EV-B 2C aa sequences examined (box and arrows). (B) Location of this compensatory mutation in the CRE(2C) secondary structure (box).

post-transfection was CKO. This is consistent with the hypothesis that none of the intermediates were able to establish a temporary stable dominance until complete reversion of a CKO genome to the wildtype CRE(2C) sequence occurred, whereupon that genome was strongly selected. The possibility of exploring this interesting finding using more advanced sequencing techniques, such as RNAseq (Wilhelm and Landry, 2009) or CirSeq (Acevedo and Andino, 2014), was also considered; however this was impractical due to the low level of RNA available from virus preparations. For example, a single 100 mm dish of HeLa cells at 8 days post electroporation yielded on the order of 100,000 copies of viral RNA (Smithee et al., 2015) or the equivalent to approximately 4.0×10^{-7} μg. With the average RNAseq experiment requiring greater than, or equal to, 5 μg of RNA, we would have required the viral RNA yield from approximately $1–2 \times 10^{7}$ 100 mm dishes per experiment or 2.5×10^{4} dishes for more sensitive next generation sequencing requiring 10 ng RNA.

3.6. 5′ terminal genomic deletions occur before the CKO sequence reverts to wildtype

Previous work demonstrated that the CVB3-CKO population had lost as many as 20 nucleotides from the 5′ genomic terminus in the majority population by day 8 post electroporation of HeLa cells (Smithee et al., 2015). This phenomenon of 5′ genomic terminal sequence loss in CVB populations can occur naturally after experimental inoculation of mice and primary cell cultures, as well as after natural infection in humans, leading to persistent, low-level infections (Chapman et al., 2008; Kim et al., 2008; Kim et al., 2005; Tracy et al., 2015). Although the engineered 16 mutations in the CRE(2C) that results in the CKO sequence is not a natural occurrence, the replication of CVB3-CKO is a persistent low-level replication similar to populations with 5′ terminally deleted genomes. We wanted to determine whether 5′ terminal deletions occurred prior to reversion of the CRE(2C): we anticipated this would be the case because, were reversion of the CKO occurring prior to the generation of 5′ terminally deleted genomes, the population of CVB3-CKO would become wildtype [with a natural CRE(2C) and intact 5′ genomic termini] and CPE would therefore be detectable in cell culture. Of note, however, CPE was not observed with CVB3-CKO (Smithee et al., 2015). Therefore, we analyzed the 5′ genomic termini of RNA from purified virion preparations of CVB3-CKO at days 1, 3, 5, 6, 7, and 8 post electroporation, as described above and previously (Smithee et al., 2015), to test whether 5′ terminal genomic deletions were occurring. These results demonstrated that at days 1 and 3 post-electroporation, purified CVB3-CKO virion RNA showed intact 5′ genomic termini when compared to the wildtype CVB3 virus control or the CVB3-CKO T7 transcript control (compare Fig. 5A lanes 2 and 3 [1 day] and lanes 5 and 6 [3 days] to Fig. 5B, lanes 11 and 12 [wildtype virus] and Fig. 5C, lanes 11 and 12 [T7 transcript control]). However, by day 5 post-electroporation, the CVB3-CKO population had evolved into a 5′ terminally deleted population (compare Fig. 5A, lanes 8 and 9 [5 days], lanes 11 and 12 [6 days], Fig. 5B, lanes 2 and 3 [7 days], lanes 5 and 6 [8 days preparation 1], and lanes 8 and 9 [8 days preparation 2] to Fig. 5A, lanes 2 and 3 [1 day], lanes 5 and 6 [3 days], Fig. 5B, lanes 11 and 12 [wildtype virus], and Fig. 5C, lanes 11 and 12 [T7 transcript control]). As a control to determine whether virus was indeed present in all preparations, RT-PCR with primers that anneal in a region of the 5′ NTR that has

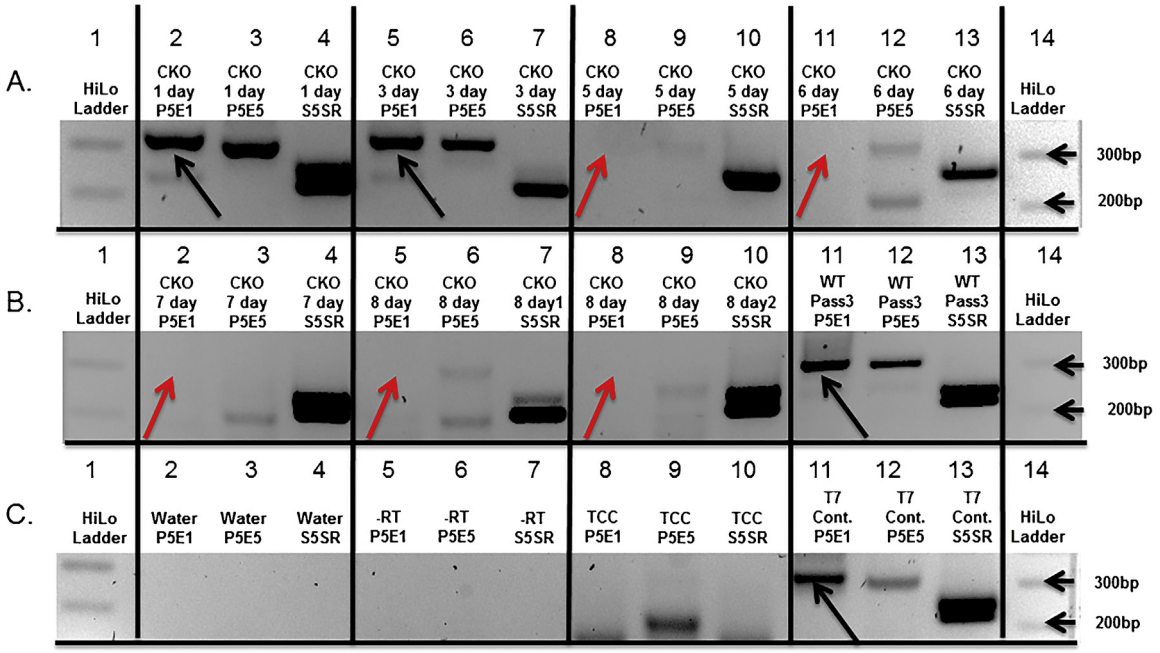

Fig. 5. 5′ terminal deletions occur between days 3 and 5 post electroporation of HeLa cells with CVB3-CKO T7 transcribed RNA. To detect 5′ terminal deletions agarose gel analysis of E3 primed cDNA amplified with primers Pin5End1 and E3 corresponding to nucleotides 1–10 and the 5′ genomic terminus (A-C lanes 2, 5, 8, and 11) Pin5End5 and E3 corresponding to nucleotides 15–25 (A-C lanes 3, 6, 9, and 12), then subsequently amplified with primers Pin and SReturn was performed. To detect virus using a region of the 5′ NTR not found to delete, gel analysis of E3 primed cDNA amplified with primers S4 and E3Sub (A-C lanes 4, 7, 10, and 13), then subsequently amplified with primers S5 and SReturn was performed. 500 (Pin5End1; S4) or 1500 (Pin5End5) copies of cDNA prepared from either viral RNA or T7 transcribed RNA (control) were used in each PCR reaction. Right facing (red) arrows indicate the loss of sequence from the 5′ terminus of CVB3-CKO by day 5, 6, 7, 8 post-electroporation as compared to wildtype virus and T7 transcript controls or to CVB3-CKO at days 1 and 3 post-electroporation (left facing (black) arrows]. Controls: lanes C2-4, no cDNA PCR controls; lanes C5-7 no template RT-PCR controls; lanes C8-10 tissue culture controls.

not been found to delete, even when the 5′ genomic terminus was not detected (E3, E3sub, E2, S5 and SReturn; Table 1), were used with nested RT-PCR for detection of viral RNA in virus preparations (compare Fig. 5A, lane 4 [1 day], lane 7 [3 days], lane 10 [5 days], lane 13 [6 days], Fig. 5B, lane 4 [7 days], lane 7 [8 days preparation 1], lane 10 [8 days preparation 2], and lane 13 [wildtype] to Fig. 5C, lane 13 [T7 transcript control]). When compared to sequencing data (Fig. 2), these results show that 5′ TDs were occurring between day 3 and 5 post-transfection, well before detection of the reversion of CKO to that of the CRE(2C) wildtype sequence at day 8 [compare Fig. 5, which demonstrates loss of 5′ genomic terminus between days 3 and 5 post-electroporation (Fig. 5A, lanes 5 and 6; lanes 8 and 9 respectively) to Fig. 2 demonstrating that at 5 days post-electroporation the majority population remains a CKO population (Fig. 2, row 7)].

4. Discussion

Genetically engineered mutational disruptions of enteroviral CRE(2C) sequences are reported as either significantly deleterious or lethal for replication (Goodfellow et al., 2000; Goodfellow et al., 2003a; McKnight and Lemon, 1998; Morasco et al., 2003; Murray and Barton, 2003; van Ooij et al., 2006). Both single and multiple mutations hamper important aspects of CVB3 replication (van Ooij et al., 2006). A 16 nucleotide mutant [termed DM by van Ooij et al. (24) or CKO in this report], as well as single nucleotide mutants, demonstrated diminished levels of VPg uridylylation and single-stranded viral genomes (but not of the double-stranded replicative form or RF). Virus-encoded luciferase also showed a decrease to background levels in transfected cells, indicating that the expression of proteins encoded by viruses with a disrupted CRE(2C) was extremely low (van Ooij et al., 2006). However, previous work (Smithee et al., 2015) demonstrated that CVB3-CKO virus is indeed viable, replicating in cell culture and in animals, but at a level similar to that observed in naturally occurring CVB-TD virus populations (Smithee et al., 2015), a level that permits persistence in tissues but not enough to achieve detectable CPE. Clearly detection of CPE is an insufficiently sensitive method to assess viability of such virus populations.

The observed generation of 5′ terminal genomic deletions in replication of the CKO genomes was surprising, but given the low level of replication, suggested a strong effect of the CRE(2) mutations upon positive strand initiation (Smithee et al., 2015). The complete reversion of the mutations in the context of 5′ terminal deletions was also unexpected. Contamination with infectious wildtype CVB3 cDNA or RNA does not explain our findings. Had even a minor population of wildtype CVB3 (that is, 1–10 infectious units) been present, the rapidity of wildtype virus population replication would have produced detectable CPE: this was not observed when tested. Transfection experiments were performed in which 10-fold increases in wildtype CVB3 T7 transcribed RNA (from 10–1000 copies) were transfected with a constant concentration of CVB3-CKO T7 transcribed RNA (Table 2). Importantly, CPE was not observed even after three passages of purified CVB3-CKO virus in HeLa cell culture, indicating contamination with wildtype CVB3 had not occurred. Sequence analysis also argues against contamination. By sequencing mixed populations of wildtype CVB3 cDNA and CVB3-CKO cDNA, we determined that a wildtype CRE(2C) must represent about 25% of a mixed population in order to be detected by standard Sanger sequencing employed in this study. Thus, by the time the wildtype CRE(2C) sequence was detectable in a mixed virus population, it is reasonable to assume the wildtype population would have been well on the way to becoming dominant given the advantage of more rapid replication. This rapid collapse (Garcia-Arriaza et al., 2006) of one (CKO) quasispecies population and the rapid succession of the other (wildtype) population, not contamination, explains the inability to detect intermediate CRE sequences by cloning: stable populations (beginning with CVB3-

CKO and ending with wildtype CVB3) are the only significantly populous genomic groups. With standard Sanger sequence analysis being a relatively inefficient detection assay, such changes were masked until the new wildtype master sequence was generated, whereupon it became the dominant (detectable) sequence. As we observed that CVB3-CKO generated a TD population days before the reversion to wildtype CRE(2C) was apparent, the increase in fitness of the CRE(2C) reversion was not as apparent as reversions in non-TD genomes. Notably, this occurred in HeLa cells, a transformed cell line in which CVB-TD populations have not been observed to arise (Kim et al., 2005): therefore, for contamination to have validity as an explanation for the results reported here, it would have required that the input virus population were also a CVB3-TD strain. It is clear that contamination is not a rational explanation for these data.

Genetic changes at numerous loci are not saltatory, which is to say it is not immediate: there is a period of time during which such changes occur. However, this does not necessarily imply that the time required is directly related to the number of changes: this process is affected by diverse forces (Garcia-Arriaza et al., 2006). If, as in the case we report here, a new multi-locus sequence seems to 'suddenly' arise, it may give the (mistaken) impression that the various reversions occurred in one or very few replication cycles. The chain of events leading to this reversion was subject to numerous pressures. de la Torre and colleagues, for example, observed something similar with a smaller set of mutations, finding it remarkable that ". . .four silent mutations reverted together or not at all": 4 sites in the 3AB domain of poliovirus had rapidly and completely reverted after 7 days of incubation in HeLa cell culture with no detection of intermediate populations, a finding that could not be adequately explained at the time (de la Torre et al., 1992). Previously, de la Torre et al. also demonstrated rapid specific reversion of single-nucleotide, guanidine-resistance inducing mutations in the poliovirus 2C coding region which resulted in a wildtype guanidine-sensitive phenotype in HeLa cells (de la Torre et al., 1990). The rapid emergence of a fit virus strain following mutation of a single nucleotide is perhaps more readily understood than a similar event involving numerous loci which must all be different in order for a fit population to arise. Nonetheless, postulating that enteroviral replicative error continually tries out numerous possibilities in an outstandingly large number of potential variations [with 16 mutated nucleotides, this is theoretically 4^{16} potential variations!] on a theme during virus passage, it can be seen that at some point, a completely reverted wildtype sequence could reoccur providing that the environment in which this process occurs is selective for the wildtype population.

This argument then begs the question: would not intermediate sequence populations be detectable? We did not detect them. However, this is not equivalent to stating that intermediate (partially reverted) CRE(2C) sequences did not occur or were not constantly appearing and being discarded as non-competitive. Reversion of the mutated CKO sequence to the wildtype CRE(2C) sequence was constrained in at least three ways. Successful reversion to wildtype must not only have regenerated an intact stem-loop secondary structure but not just any stem-loop; it must have, as well, regenerated the structure's function (*i.e.,* the ability to enable uridylylation). Reversion was further impacted by the requirement to retain an intact reading frame for the protein 2C. To this point, CRE(2C) sequence alignments (Fig. 4) revealed that non-wildtype compensatory mutations which might have restored stem structure, would also have altered the protein 2C coding sequence. Further, maintenance of the CRE(2C) loop region has been previously demonstrated to be under a stricter selection pressure than expected for other regions of the genome (Goodfellow et al., 2000). Our analysis of available EV-B 2C sequences to explore the possibility that non-wildtype compensatory mutations might arise within the stem of the CRE(2C) (Fig. 4), together with the fact that all identified uridylylation CRE structures within EV genomes have a highly conserved stem structure (Goodfellow et al., 2000; Paul et al., 2000; van Ooij et al., 2006), are consistent with those findings and indicate that sequences with compensatory mutations (which would be expected in intermediate populations) would be less fit. Mutations in the CRE(2C) loop have been infrequently reported in enterovirus isolates (from analyses of sequences in GenBank; data not shown) and point-mutational analysis done by others (van Ooij et al., 2006) have demonstrated that single nucleotide changes in the loop of the CRE(2C) inhibited the synthesis of the negative strand intermediate and VPg uridylylation. Thus, it appears that altering the CRE(2C) loop, even on a small scale, prevents efficient viral replication: the strong inference is that alterations in the EV 2C protein coding sequence, including those in the CRE(2C) sequence, are not advantageous to the virus.

These arguments are further strengthened by work (Crowder and Kirkegaard, 2005), that may further illuminate why stable viral populations containing large amounts of intermediate (partially mutated) CRE(2C) structures do not arise: some intermediate CRE sequences generated during replication in any given cell may actually hinder, rather than aid, reacquisition of the wildtype CRE(2C) sequence. For example, two synonymous double mutations within just the stem of the poliovirus CRE(2C) structure expressed a dominant negative effect in *trans*, inhibiting wildtype viral replication in co-transfected cells, indicated by the decrease or absence of CPE by the mutant strains (Crowder and Kirkegaard, 2005). However, when the CRE(2C) structure was disrupted with 8 synonymous mutations in both the stem and loop, not just the stem, dominant negative effects were not observed (Crowder and Kirkegaard, 2005). We hypothesize that in our population of replicating TD-CVB3-CKO, the very low level of RNA replication which occurs is further affected by individual reversions that restore partial CRE(2C) structure but which also exert a dominant-negative effect, thereby lowering the replication of any variants which have sufficient reversion of the structure to have any CRE(2C) function. Such mutants cannot exert this dominant-negative effect on the CKO variant, despite its reduced fitness, because it does not have a functional CRE(2C) structure. Only when the population has enough complete reversions of the CRE(2C) can the fitness of this population allow it to more efficiently replicate than the genomes with the complete CKO mutations. In becoming the dominant population, it is to be recalled that a successfully competitive viral population need only have a slight advantage (rate of replication, virion stability, *etc.*) in order to completely replace another population (Clarke et al., 1994; Martinez et al., 1991).

Yet another complication to the generation of a reverted CRE(2C) population in any given cell, is that the CVB3 genomes lose the wildtype 5′ genomic termini well before reversion of the CKO to wildtype, thus becoming TD. This, by itself, dramatically slows viral RNA replication although, as we have previously shown (Smithee et al., 2015), the TD and the CKO lesions are not significantly additive in their impact on suppressing replication. It is apparent that a genome such as CVB3-TD50-CKO, which replicates with similar efficiency as either CVB3-TD50 or CVB3-CKO (Smithee et al., 2015), indicates that the pressure to revert CKO to wildtype is not only due to a requirement for more efficient positive strand RNA replication. Because of this 5′ terminal deletion, reversions at the CRE(2C) site do not generate a significant increase in replication in the viral population. For this reason, the amplified cDNA in these experiments does not reflect a large pool of template viral RNAs and so, the probability of detecting the reverted CRE(2C) is low until the completely reverted CRE(2C) dominates the population. Because the CVB3 genome lost the wildtype 5′ genomic termini prior to detection of the reverted (wildtype) CRE(2C) and because full reversion to wildtype CRE(2C) still occurred when the CKO was engineered into a CVB3-TD genome, we infer that there is a strong

natural selection for a wildtype CRE(2C) in a CVB3 genome even in the context of a positive strand RNA initiation that does not depend upon VPg uridylylation.

One hypothesis to account for the drive to revert the CRE(2C) site is that in a CVB-TD genome, CRE(2C) uridylylated VPg may sufficiently increase negative strand initiation over that which occurs with the mutated CRE(2C) sequence. If true, this would imply that the CRE(2C) has a role in for negative strand initiation in systems in which positive strand initiation is not efficient. It is known from work with poliovirus type 1 and with CVB3 that negative strand initiation requires the 5′ end (domain I) and the CRE(2C) in addition to the wildtype 3′ end in order to replicate the negative strand (Paul and Wimmer, 2015). Studies verified the importance of the CRE(2C) for generation of the uridylylated protein primer (VPg or precursors) for initiation of positive strand RNA but not for negative strand RNA replication (Barton and Flanegan, 1997; Goodfellow et al., 2003b; Morasco et al., 2003; Murray and Barton, 2003). Cell free replication systems for analysis of enterovirus RNA replication have been developed using guanidine hydrochloride which inhibits initiation of negative strand RNA and the uridylylation of the protein primer for initiation (VPg or precursors) to isolate preinitiation replication complexes (PIRCs) (Barton et al., 1995; Barton and Flanegan, 1997). Using these PIRCs with capsid deleted replicons, it has been shown that deletion or alteration of structure in domain I can cause a loss of efficiency of negative strand replication (Barton et al., 2001; Herold and Andino, 2001) but alterations to the sequence of the presumed template for positive strand initiation (the 3′ end of the negative strand) has strong effects upon the efficiency of the generation of positive strand RNA (Sharma et al., 2009; Vogt and Andino, 2010). Consistent with these findings, previous work with CVB3-TD viruses has demonstrated a significant decrease in positive strand RNA replication (Kim et al., 2005; Smithee et al., 2015).

The study of the CVB3 CRE(2C) demonstrated that while negative strand replication (as measured as RF generated from positive strand RNAs) is not affected by the DM mutations (identical to the CKO mutations used in our study), single point mutations in the CRE(2C) have the ability to lower negative strand replication (van Ooij et al., 2006). Under limiting concentrations of UTP or CTP, CRE(2C) generated uridylylated VPg increases efficiency of negative strand initiation *in vitro* (Steil and Barton, 2008). Many assays of RF as an assay of negative strand initiation use limiting concentrations of UTP or CTP. These findings suggest that in limiting NTP concentrations, replication in general is greatly lowered for the CKO enterovirus and what is present is largely negative strand. The conditions of our work with HeLa cells and in the mouse have a greater concentration of these NTPs (Traut, 1994) which is likely to increase the efficiency of negative strand RNA synthesis even in the context of these CVB3-TD-CKO genomes.

As we know that the CKO replication generates 5′ terminal deletions prior to the stage at which the CKO reversion to wildtype occurs and occurs also in virus generated from a genome with both a deletion of the 5′ terminal 49 nt and the CKO mutations, the context in which reversion is occurring is in the mode of TD virus replication. The CVB3-TD viruses do not have an intact structure of stem a of domain I but replicate both strands at nearly equal levels (Kim et al., 2008; Kim et al., 2005) suggesting that the greatly decreased production of positive strands in viral progeny also results in a decrease in negative strand replication. The difference between a NTP-limiting cell free *in vitro* environment and a living HeLa cell may affect extent and type of both positive and negative strand initiation but it must be acknowledged that cells and animals are the natural environment for the replication of the enterovirus. As the replication of TDs does not favor uridine residues in the 5′ ends of positive strands, despite the fact that these genomes have covalently attached VPg (Kim et al., 2005), positive strand initiation does not occur *via* CRE(2C) uridylylated VPg. In these conditions, the selection of a reverted CRE(2C) in replication suggests that the functional CRE(2C) contributes to the fitness of TD genomes *via* enhancing negative strand initiation. Alternatively, a functional CRE(2C) may have an as yet unidentified role in positive strand initiation. Having shown that 5′ terminal genomic deletions occur during replication of the CVB3-CKO genome, it is apparent that the lack of a functional CRE(2C) is affecting the site and efficiency of initiation of the positive strand and potentially may play a role other than in production of uridylylated protein primer. Further work to examine levels of each RNA strand during the process of reversion might distinguish whether the selective pressure to revert the CKO mutations in the CVB3-TD is due to a decrease in negative strand replication alone and/or positive strand initiation.

Considering the foregoing discussion, we suggest a theoretical mechanistic pathway for the observations reported here. Beginning with the initial replication cycles of transfected infectious CVB3-CKO RNA, errors are introduced into all new viral RNA strands at an average rate between 1 and 3 nucleotide changes per each new RNA molecule transcribed (Domingo et al., 2008; Holland et al., 1982; Levi et al., 2010). Due to the input CKO mutations, viral RNA replication is severely retarded relative to what is observed with wildtype virus, significantly lowering the number of viral RNA molecules in each infected cell. In the process of replication of this mutated genome, 5′ terminal deletions occur, presumably due to altered positive strand initiation. At the site of the CRE(2C), as each new strand is replicated, only those errors that do not disrupt the viral open reading frame will permit progression toward a new and viable viral RNA generation. The mechanism by which the 16 CKO mutations revert to wildtype must clearly be a progressive, albeit stochastic, process (Andino and Domingo, 2015; Domingo and Holland, 1997; Holland et al., 1982; Jenkins et al., 2002). As well, any mutations that act in trans with a dominant negative impact (Crowder and Kirkegaard, 2005) will further narrow the bottleneck through which viral genomes with accumulating correct CRE(2C) reversions must pass. In the meantime, although the TD-CVB3-CKO genome is replication-impaired, it nonetheless remains among the most fit (able to replicate and package) of the nascent variations in the population. That is, the TD-CVB3-CKO will proceed (albeit inefficiently) to new generations while, at least initially, few variants of this sequence will survive to build upon the previously tolerated mutations. Taken together, this reasonably accounts for the observation that CKO is the CRE(2C) sequence that is repeatedly detectable upon analysis following introduction to cells. Indeed, it is also intriguing to consider that this may occur because these CKO genomes do not have a CRE(2C) structure which can be inhibited by a *trans*-dominant negative effect from partially reverted genomes. Yet this halting process changes once an intact wildtype CRE(2C) is regenerated: this iteration is able to outcompete all others in the population including those with the CKO mutations. Despite having lost the 5′ genomic termini, this new population with an intact CRE(2C) appears to have a slight but definite selective edge, and so appears to occur ‘all at once’ rather than as in fact having endured an exceptionally arduous journey through sequence space. As there is selection at the CRE(2C) site for the wildtype sequence despite the 5′ terminal deletion uridylylated VPg-independent positive strand initiation, this RNA structure plays a role in RNA replication in the naturally occurring TD viruses.

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References

Acevedo, A., Andino, R., 2014. Library preparation for highly accurate population sequencing of RNA viruses. Nat. Protoc. 9 (7), 1760–1769.
Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25 (17), 3389–3402.
Altschul, S.F., Wootton, J.C., Gertz, E.M., Agarwala, R., Morgulis, A., Schaffer, A.A., Yu, Y.K., 2005. Protein database searches using compositionally adjusted substitution matrices. FEBS J. 272 (20), 5101–5109.
Andino, R., Domingo, E., 2015. Viral quasispecies. Virology 479–480, 46–51.
Andino, R., Rieckhof, G.E., Baltimore, D., 1990. A functional ribonucleoprotein complex forms around the 5′ end of poliovirus RNA. Cell 63 (2), 369–380.
Andino, R., Rieckhof, G.E., Achacoso, P.L., Baltimore, D., 1993. Poliovirus RNA synthesis utilizes an RNP complex formed around the 5'-end of viral RNA. EMBO J. 12 (9), 3587–3598.
Barton, D.J., Flanegan, J.B., 1997. Synchronous replication of poliovirus RNA: initiation of negative-strand RNA synthesis requires the guanidine-inhibited activity of protein 2C. J. Virol. 71 (11), 8482–8489.
Barton, D.J., Black, E.P., Flanegan, J.B., 1995. Complete replication of poliovirus in vitro: preinitiation RNA replication complexes require soluble cellular factors for the synthesis of VPg-linked RNA. J. Virol. 69 (9), 5516–5527.
Barton, D.J., O'Donnell, B.J., Flanegan, J.B., 2001. 5' cloverleaf in poliovirus RNA is a cis-acting replication element required for negative-strand synthesis. EMBO J. 20 (6), 1439–1448.
Borderia, A.V., Lorenzo-Redondo, R., Pernas, M., Casado, C., Alvaro, T., Domingo, E., Lopez-Galindez, C., 2010. Initial fitness recovery of HIV-1 is associated with quasispecies heterogeneity and can occur without modifications in the consensus sequence. PLoS One 5 (4), e10319.
Chapman, N.M., Kim, K.S., 2008. Persistent coxsackievirus infection: enterovirus persistence in chronic myocarditis and dilated cardiomyopathy. Curr. Top. Microbiol. Immunol. 323, 275–292.
Chapman, N.M., Kim, K.-S., Drescher, K.M., Oka, K., Tracy, S., 2008. 5′ terminal deletions in the genome of a coxsackievirus B2 strain occurred naturally in human heart. Virology 375 (2), 480–491.
Clarke, D.K., Duarte, E.A., Elena, S.F., Moya, A., Domingo, E., Holland, J., 1994. The red queen reigns in the kingdom of RNA viruses. Proc. Natl. Acad. Sci. U.S.A. 91 (11), 4821–4824.
Crowder, S., Kirkegaard, K., 2005. Trans-dominant inhibition of RNA viral replication can slow growth of drug-resistant viruses. Nat. Genet. 37 (7), 701–709.
de la Torre, J.C., Wimmer, E., Holland, J.J., 1990. Very high frequency of reversion to guanidine resistance in clonal pools of guanidine-dependent type 1 poliovirus. J. Virol. 64 (2), 664–671.
de la Torre, J.C., Giachetti, C., Semler, B.L., Holland, J.J., 1992. High frequency of single-base transitions and extreme frequency of precise multiple-base reversion mutations in poliovirus. Proc. Natl. Acad. Sci. U.S.A. 89 (7), 2531–2535.
Domingo, E., Holland, J.J., 1997. RNA virus mutations and fitness for survival. Annu. Rev. Microbiol. 51, 151–178.
Drescher, K.M., Kono, K., Bopegamage, S., Carson, S.D., Tracy, S., 2004. Coxsackievirus B3 infection and type 1 diabetes development in NOD mice: insulitis determines susceptibility of pancreatic islets to virus infection. Virology 329 (2), 381–394.
Garcia-Arriaza, J., Ojosnegros, S., Davila, M., Domingo, E., Escarmis, C., 2006. Dynamics of mutation and recombination in a replicating population of complementing, defective viral genomes. J. Mol. Biol. 360 (3), 558–572.
Goodfellow, I., Chaudhry, Y., Richardson, A., Meredith, J., Almond, J.W., Barclay, W., Evans, D.J., 2000. Identification of a cis-acting replication element within the poliovirus coding region. J. Virol. 74 (10), 4590–4600.
Goodfellow, I.G., Kerrigan, D., Evans, D.J., 2003a. Structure and function analysis of the poliovirus cis-acting replication element (CRE). RNA (New York, N.Y.) 9 (1), 124–137.
Goodfellow, I.G., Polacek, C., Andino, R., Evans, D.J., 2003b. The poliovirus 2C cis-acting replication element-mediated uridylylation of VPg is not required for synthesis of negative-sense genomes. J. Gen. Virol. 84 (Pt 9), 2359–2363.
Herold, J., Andino, R., 2001. Poliovirus RNA replication requires genome circularization through a protein–protein bridge. Mol. Cell 7 (3), 581–591.
Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S., VandePol, S., 1982. Rapid evolution of RNA genomes. Science 215 (4540), 1577–1585.
Huang, P.N., Shih, S.R., 2014. Update on enterovirus 71 infection. Curr. Opin. Virol. 5, 98–104.
Jenkins, G.M., Rambaut, A., Pybus, O.G., Holmes, E.C., 2002. Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. J. Mol. Evol. 54 (2), 156–165.
Kim, K.S., Tracy, S., Tapprich, W., Bailey, J., Lee, C.K., Kim, K., Barry, W.H., Chapman, N.M., 2005. 5'-Terminal deletions occur in coxsackievirus B3 during replication in murine hearts and cardiac myocyte cultures and correlate with encapsidation of negative-strand viral RNA. J. Virol. 79 (11), 7024–7041.
Kim, K.S., Chapman, N.M., Tracy, S., 2008. Replication of coxsackievirus B3 in primary cell cultures generates novel viral genome deletions. J. Virol. 82 (4), 2033–2037.
Lanford, R.E., Sureau, C., Jacob, J.R., White, R., Fuerst, T.R., 1994. Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT/PCR. Virology 202 (2), 606–614.
Leveque, N., Renois, F., Talmud, D., Nguyen, Y., Lesaffre, F., Boulagnon, C., Bruneval, P., Fornes, P., Andreoletti, L., 2012. Quantitative genomic and antigenomic enterovirus RNA detection in explanted heart tissue samples from patients with end-stage idiopathic dilated cardiomyopathy. J. Clin. Microbiol. 50 (10), 3378–3380.
Martinez, M.A., Carrillo, C., Gonzalez-Candelas, F., Moya, A., Domingo, E., Sobrino, F., 1991. Fitness alteration of foot-and-mouth disease virus mutants: measurement of adaptability of viral quasispecies. J. Virol. 65 (7), 3954–3957.
McKnight, K.L., Lemon, S.M., 1998. The rhinovirus type 14 genome contains an internally located RNA structure that is required for viral replication. RNA (New York, N.Y.) 4 (12), 1569–1584.
Messacar, K., Abzug, M.J., Dominguez, S.R., 2016. 2014 outbreak of enterovirus D68 in North America. J. Med. Virol. 88 (5), 739–745.
Modlin, J.F., Rotbart, H.A., 1997. Group B coxsackie disease in children. Curr. Top. Microbiol. Immunol. 223, 53–80.
Morasco, B.J., Sharma, N., Parilla, J., Flanegan, J.B., 2003. Poliovirus cre(2C)-dependent synthesis of VPgpUpU is required for positive- but not negative-strand RNA synthesis. J. Virol. 77 (9), 5136–5144.
Morgulis, A., Coulouris, G., Raytselis, Y., Madden, T.L., Agarwala, R., Schaffer, A.A., 2008. Database indexing for production MegaBLAST searches. Bioinformatics (Oxford, England) 24 (16), 1757–1764.
Murray, K.E., Barton, D.J., 2003. Poliovirus CRE-dependent VPg uridylylation is required for positive-strand RNA synthesis but not for negative-strand RNA synthesis. J. Virol. 77 (8), 4739–4750.
Pallansch, M.A., Oberste, M.S., Whitton, J.L., 2013. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. 6th edn. Fields Virology, Howeley, D.M.K.a.P.M., 1. 2 vols. Wolters Kluwer Philadelphia.
Parsley, T.B., Towner, J.S., Blyn, L.B., Ehrenfeld, E., Semler, B.L., 1997. Poly (rC) binding protein 2 forms a ternary complex with the 5'-terminal sequences of poliovirus RNA and the viral 3CD proteinase. RNA (New York, N.Y.) 3 (10), 1124–1134.
Paul, A.V., Wimmer, E., 2015. Initiation of protein-primed picornavirus RNA synthesis. Virus Res. 206, 12–26.
Paul, A.V., Rieder, E., Kim, D.W., van Boom, J.H., Wimmer, E., 2000. Identification of an RNA hairpin in poliovirus RNA that serves as the primary template in the in vitro uridylylation of VPg. J. Virol. 74 (22), 10359–10370.
Paul, A.V., Peters, J., Mugavero, J., Yin, J., van Boom, J.H., Wimmer, E., 2003. Biochemical and genetic studies of the VPg uridylylation reaction catalyzed by the RNA polymerase of poliovirus. J. Virol. 77 (2), 891–904.
Reetoo, K.N., Osman, S.A., Illavia, S.J., Cameron-Wilson, C.L., Banatvala, J.E., Muir, P., 2000. Quantitative analysis of viral RNA kinetics in coxsackievirus B3-induced murine myocarditis: biphasic pattern of clearance following acute infection, with persistence of residual viral RNA throughout and beyond the inflammatory phase of disease. J. Gen. Virol. 81 (Pt 11), 2755–2762.
Rey, L., Lambert, V., Wattre, P., Andreoletti, L., 2001. Detection of enteroviruses ribonucleic acid sequences in endomyocardial tissue from adult patients with chronic dilated cardiomyopathy by a rapid RT-PCR and hybridization assay. J. Med. Virol. 64 (2), 133–140.
Sean, P., Semler, B.L., 2008. Coxsackievirus B RNA replication: lessons from poliovirus. Curr. Top. Microbiol. Immunol. 323, 89–121.
Sharma, N., Ogram, S.A., Morasco, B.J., Spear, A., Chapman, N.M., Flanegan, J.B., 2009. Functional role of the 5' terminal cloverleaf in Coxsackievirus RNA replication. Virology 393 (2), 238–249.
Smithee, S., Tracy, S., Chapman, N.M., 2015. Mutational disruption of cis-acting replication element 2C in coxsackievirus B3 leads to 5'-Terminal genomic deletions. J. Virol. 89 (23), 11761–11772.
Steil, B.P., Barton, D.J., 2008. Poliovirus cis-acting replication element-dependent VPg Uridylylation lowers the Km of the initiating nucleoside triphosphate for viral RNA replication. J. Virol. 82 (19), 9400–9408.
Tam, P.E., Messner, R.P., 1999. Molecular mechanisms of coxsackievirus persistence in chronic inflammatory myopathy: viral RNA persists through formation of a double-stranded complex without associated genomic mutations or evolution. J. Virol. 73 (12), 10113–10121.
Tan, Y., Hassan, F., Schuster, J.E., Simenauer, A., Selvarangan, R., Halpin, R.A., Lin, X., Fedorova, N., Stockwell, T.B., Lam, T.T.-Y., Chappell, J.D., Hartert, T.V., Holmes, E.C., Das, S.R., 2016. Molecular evolution and intraclade recombination of enterovirus D68 during the 2014 outbreak in the United States. J. Virol. 90 (4), 1997–2007.
Tracy, S., Drescher, K.M., Chapman, N.M., Kim, K.S., Carson, S.D., Pirruccello, S., Lane, P.H., Romero, J.R., Leser, J.S., 2002. Toward testing the hypothesis that group B coxsackieviruses (CVB) trigger insulin-dependent diabetes: inoculating nonobese diabetic mice with CVB markedly lowers diabetes incidence. J. Virol. 76 (23), 12097–12111.
Tracy, S., Drescher, K.M., Chapman, N.M., 2011. Enteroviruses and type 1 diabetes. Diabetes Metab. Res. Rev. 27 (8), 820–823.

Tracy, S., Smithee, S., Alhazmi, A., Chapman, N., 2015. Coxsackievirus can persist in murine pancreas by deletion of 5' terminal genomic sequences. J. Med. Virol. 87 (2), 240–247.
Traut, T.W., 1994. Physiological concentrations of purines and pyrimidines. Mol. Cell. Biochem. 140 (1), 1–22.
van Ooij, M.J., Vogt, D.A., Paul, A., Castro, C., Kuijpers, J., van Kuppeveld, F.J., Cameron, C.E., Wimmer, E., Andino, R., Melchers, W.J., 2006. Structural and functional characterization of the coxsackievirus B3 CRE(2C): role of CRE(2C) in negative- and positive-strand RNA synthesis. J. Gen. Virol. 87 (Pt 1), 103–113.
Vogt, D.A., Andino, R., 2010. An RNA element at the 5'-end of the poliovirus genome functions as a general promoter for RNA synthesis. PLoS Pathog. 6 (6), e1000936.
Wilhelm, B.T., Landry, J.R., 2009. RNA-Seq-quantitative measurement of expression through massively parallel RNA-sequencing. Methods 48 (3), 249–257.
Xiang, W., Harris, K.S., Alexander, L., Wimmer, E., 1995. Interaction between the 5'-terminal cloverleaf and 3AB/3CDpro of poliovirus is essential for RNA replication. J. Virol. 69 (6), 3658–3667.
Yip, C.C., Lau, S.K., Woo, P.C., Yuen, K.Y., 2013. Human enterovirus 71 epidemics: what's next? Emerg. Health Threats J. 6, 19780.
Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol.: A J. Comput. Mol. Cell Biol. 7 (1-2), 203–2214.