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## Characterization of the Replication of Coxsackievirus B3 with a Mutationally Disrupted CRE(2C) and the use of Creatinine-Hydrochloride as an Antibacterial Agent

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**CHARACTERIZATION OF THE REPLICATION OF COXSACKIEVIRUS B3 WITH A  
MUTATIONALLY DISRUPTED CRE(2C) AND THE USE OF CREATININE-  
HYDROCHLORIDE AS AN ANTIBACTERIAL AGENT**

By

**Shane Edwin Smithee**

A DISSERTATION

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University of Nebraska Medical Center, 2015

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Following natural or experimental infection, and in cell culture, coxsackie B virus (CVB) RNA can persist for weeks in the absence of CPE yet with detectable viral RNA. Earlier studies in our laboratory demonstrated that this persistence produced viral RNA with up to 49 nucleotide deletions at the 5' genomic terminus, partially degrading the cloverleaf (or domain I), an RNA structure that is required for efficient viral replication. A *cis*-acting replication element (CRE) in the 2C protein coding region [CRE(2C)] templates the addition of two uridine residues to the virus-encoded RNA replication primer, VPg, prior to genomic replication. Because our previous work also demonstrated that 5' terminally deleted CVB (CVB-TD) genomes have VPg covalently linked despite rarely terminating in the canonical UU donated by CRE(2C) mediated uridylylation of VPg, it was hypothesized that a functional (uridylylating) CRE(2C) would be unnecessary for CVB-TD replication.

Using the same 16 mutations in the CVB3 CRE(2C) structure that were considered lethal for this virus by others, this work demonstrates that the wild type (wt) and the CVB3-TD virus carrying these mutations are viable with a non-uridylylating CRE(2C) in infected cell cultures and in mice. While the wt genome with the mutated

CRE(2C) displays suppressed replication levels similar to that observed in a CVB3-TD strain, mutation of CRE(2C) function in a CVB3-TD strain does not further decrease replication. Further, this work shows that replication of the parental CVB3 strain containing the mutated CRE(2C) drives *de novo* generation of 5' terminal genomic deletions, and that the 16 mutations introduced into the CRE(2C) region can revert completely to wt sequence after only eight days of replication in cell culture.

Cumulatively, these findings demonstrate that VPg can prime without being specifically uridylylated and that this priming is error prone, resulting in loss of 5' terminal sequence information. Additionally, these findings demonstrate that high selective pressure exists during replication which drives the complete reversion of the CRE(2C) to wt sequence, from which we propose that it has functions other than the experimentally defined VPg uridylylation template.

A final, unrelated finding of this dissertation shows that creatinine-hydrochloride effectively suppresses bacterial growth while allowing outgrowth of slower growing fungi.

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## **List of Abbreviations**

a.a. – amino acid

ANOVA – analysis of variance

cDNA – complimentary DNA

cfu – colony forming units

CKO – CRE-knock-out

CPE – cytopathic effect

CRE – *cis*-acting replication element

CRE(2C) – the HEV internal CRE located in the protein 2C coding region

CRN – creatinine

CRN-HCl – creatinine hydrochloride

CVB – coxsackie B virus

CVB3-CKO – CVB3 with a mutationally disrupted CRE(2C)

CVB3-TD50 – CVB3 with a 49 nucleotide deletion from the 5' genomic terminus

CVB3-TD50-CKO – CVB3-TD50 with a mutationally disrupted CRE(2C)

DNA – deoxyribonucleic acid

DNase - deoxyribonuclease

dNTPs – deoxynucleotide triphosphates

HEV – Human enterovirus

HIV – Human Immunodeficiency virus

i.p.- intraperitoneal

LBG - Luria broth supplemented with 1% w/v D-glucose

NBCS – newborn calf serum

PBS – phosphate buffered saline

PCR – polymerase chain reaction

p.i. – post infection

PV - poliovirus

RdRp – RNA-dependent-RNA-polymerase

RNA – ribonucleic acid

RNase - ribonuclease

rNTPs – ribonucleotide triphosphates

RPM – revolutions per minute

RT – reverse transcriptase

RT-qPCR – reverse transcription-quantitative polymerase chain reaction

SDA – Sabouraud's dextrose agar

TD – terminal deletion

VPg – viral protein genome linked

## Chapter 1

### Introduction

The human enteroviruses (HEV) are a diverse genus in the picornavirus family (*Picornaviridae*). The HEV have a variety of pathogenic members including the polioviruses, human rhinoviruses, and coxsackieviruses (3). The coxsackie B viruses have six serotypes (3) [CVB1-6] and cause, or have been etiologically linked to, a variety of important human diseases ranging from type I diabetes to myocarditis, among others (4-8). Extensive work has demonstrated that the CVB are able to persist after the acute phase of infection within the tissues of naturally infected humans or mice (9-12) and within experimentally inoculated primary cells (10, 13) through a mechanism in which sequence is deleted from the 5' end of the viral genome (termed CVB-TDs). As further discussed in chapter 2, these deletions cause loss of much of an important *cis*-acting replication element (CRE), termed the cloverleaf or (domain I), leading to an inefficient form of persistent replication in which both positive and negative strand are encapsidated (10), a phenomenon never observed in the wildtype (wt) virus. This work also showed that the CVB-TD still had VPg, the viral protein primer, attached to the 5' end of the viral genome though rarely terminating in the canonical UU donated by uridylylated VPg during initiation of replication (10).

The uridylylation of VPg (*i.e.*, the covalent linkage of two uridylic acid residues to the third amino-acid residue (Y<sub>3</sub>) in the VPg amino acid chain), also discussed in chapter 2, is mediated by the viral RNA-dependent-RNA polymerase (RdRp) and is templated on another important viral CRE (14-16), the location of which varies depending on the

picornaviral genome (16-23). This CRE in the species B HEV (HEV-B) is located within the 2C protein coding region of the genome (24) and so is termed CRE(2C). The CREs of the picornaviruses are RNA stem-loop secondary structures which function independently of the protein coding capacity of the region in which they are found (19, 22, 23). Discussed in detail in chapter 2, extensive work has demonstrated that mutational disruption of the sequence of either the stem or loop of the CRE(2C) leads to negative effects on VPg uridylylation and viral replication (24-29) and in general has been considered lethal. The findings of previous work from our laboratory characterizing the CVB-TD, showed CVB-TD strains packaged negative strand at a near 1:1 ratio compared to positive strand and rarely terminate in the canonical UU donated by VPg during initiation of replication though still having VPg attached to the 5' genomic terminus (10). These findings led to the hypothesis that CVB-TD populations did not require a functional CRE(2C) for replication. In testing this hypothesis, the 16 mutations previously published (24) were introduced into both CVB3-TD50 and our wt strain CVB3-28 [(30), termed CVB3-CKO] as described in chapter 2. Surprisingly, our preliminary studies showed that CVB3-CKO was replication competent in the absence of cytopathic effect (CPE). This novel finding led to the studies described in chapters 2 and 3 of this dissertation.

The findings discussed in chapter 2 of this dissertation show that CVB3-CKO is able to replicate without a functionally (*i.e.*, uridylylating) intact CRE(2C) in both HeLa cell culture and within mice following electroporation or transfection of viral RNA, respectively. Whereas wildtype CVB3 replicates efficiently, producing CPE within 48-72 hours (post electroporation of T7 transcribed RNA), and produces encapsidated positive strand on the order of  $10^8$  copies per milligram (wet weight) of HeLa cells (chapter 2), this is not the case when the CRE(2C) is mutationally disrupted. Indeed, CVB3-CKO

was found to replicate to a titer nearly 100,000 fold lower than that observed with wt, and produced no detectable CPE eight days after electroporation of HeLa cells (chapter 2). Similar findings were observed with both CVB3-TD50 and CVB3-TD50-CKO. Another novel finding reported in chapter 2 was that a population of viruses with 5' TDs evolved in CVB3-CKO after eight days post electroporation. This was an important finding that suggested that without an intact CRE(2C), CVB3-CKO initiates replication in a manner similar to that of the TD viruses discussed in chapter 2 and previously characterized (10). After characterizing the replication of CVB3-CKO, it was important to determine if the mutations introduced into the CRE(2C) region remained intact at various times leading up to the final day eight time-point. This was an important aspect of the work because it was hypothesized that reversions would occur within the CRE(2C) of CVB3-CKO after several rounds of replication.

As further discussed in chapter 3 of this dissertation, RNA viruses exist as heterogeneous populations, or quasispecies (31-33). As a whole, the RNA viruses are capable of rapid evolution due the highly error-prone nature of their RdRp (34-36) and through recombination of viral genomes (37-40). In addition to allowing for evolution and rapid selection of the fittest viral genomes in a given environment (33), these attributes also allow RNA viruses to rapidly revert mutated sequences which may be detrimental in a given environment; *i.e.*, mutations that would subsequently inhibit aspects of their replication or dissemination (41-44). This may be precisely the case when the CRE(2C) of CVB3 is mutationally altered. As discussed above and in chapter 2, others considered mutational disruption of the CRE(2C) of either poliovirus or CVB3 to be lethal (24, 45). However, the work presented in this dissertation indicates this is not the case, at least for CVB3. Indeed, not only has this work demonstrated that disruption of the CRE(2C) is not lethal for CVB3 (chapter 2), the results presented in chapter 3 of this

dissertation significantly extend these results and shows the novel finding that the 16 introduced mutations [(24); chapter 2] in the CRE(2C) revert completely to that of the wt sequence in the majority population, with this occurring after as few as eight days of replication in HeLa cells. This suggests that while loss of a functional CRE(2C) is not lethal to the virus, a high selective pressure exists which drives complete reversion to a wt CRE(2C) sequence. The data presented in chapter 3 also demonstrate that evolution of 5' TDs occurs prior to reversion of the CRE(2C). If this were not the case, and the CRE(2C) reverted without the evolution of 5' TDs, the presence of CPE would be expected because a cytopathic virus has both the intact 5' genomic terminus and an intact CRE(2C). This, however, was not observed. Together, these results are consistent with the CRE(2C) having another, as yet undefined, function(s) besides providing a framework and template for VPg uridylylation, and that the structure of the CRE(2C) is more important for viral replication than is most of domain I.

To provide education and a skill set outside of the field of virology, chapter 4, although unrelated to the work presented in chapters 2 and 3 of this dissertation, describes another novel exploration in the field of microbiology: the characterization of a small molecule that functions as an antibacterial, creatinine hydrochloride (CRN-HCl). Creatinine (CRN) is a vertebrate metabolic waste product normally found in blood and urine (46), the formation of which is further described in chapter 4. Previous work demonstrated the hydrochloride salt of creatinine, CRN-HCl, can be a potent inhibitor of bacterial replication (47). Interestingly, it was apparent that CRN-HCl did not inhibit the growth of yeasts or molds (*i.e.* fungi), making it a potentially useful addition to growth media to facilitate isolation of environmental or clinically relevant fungal species (47). In general and as elaborated on in chapter 4 of this dissertation, Sabouraud dextrose agar (SDA) is the current medium of choice for the detection and isolation of fungi, although it

does not offer optimal nutritional requirements for some fungi and can permit growth of bacteria which may subsequently inhibit fungal growth and/or obscure fungal isolation (48-54). The work presented in chapter 4 of this dissertation demonstrates a significant improvement for the process of identifying fungi by showing that CRN-HCl effectively suppresses bacterial growth in either liquid or solid agar media while allowing outgrowth of (slower growing) fungi from either experimentally prepared samples or environmental samples and that it can be used in addition to, or *in lieu* of, SDA.

Taken together, the data presented in this dissertation have important implications, not only for the field of virology but for microbiology as a whole. The results presented in chapters 2 and 3 change the way initiation of replication of the CVB, and perhaps the HEV, must be viewed, so forcing a re-examination of the currently understood HEV RNA replication mechanism. Further, these chapters offer a novel insight into how HEV may have replicated and evolved from more primitive forms. Chapter 4, while not related to the virology of chapters 2 and 3, further supports the antibacterial activity of a small molecule, creatinine, once considered to have no biological activity, and highlights its potential use in the clinical/research laboratory.

## Chapter 2

### Mutational disruption of the CRE(2C) in coxsackievirus B3 leads to 5' terminal genomic deletions<sup>1</sup>.

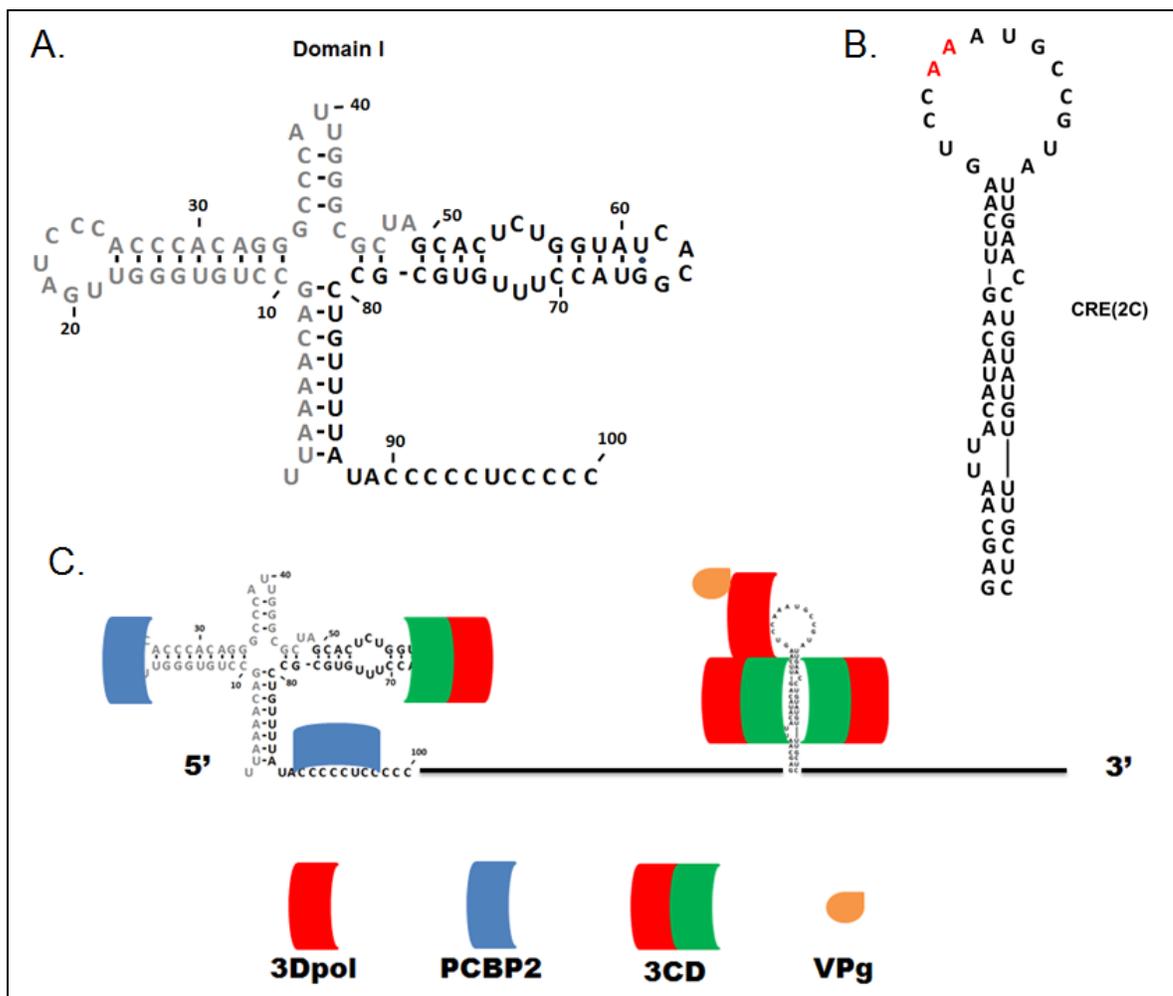
#### Introduction

Group B Coxsackieviruses (CVB, serotypes 1-6) are non-enveloped, single-stranded, positive sense RNA viruses classified as human enteroviruses (HEV), species B, within the viral family *Picornaviridae* (55). The CVB cause, or are etiologically associated with, a variety of human diseases including aseptic meningitis, myocarditis, pancreatitis, and type I diabetes (4-8). The small, single stranded, positive sense HEV RNA genome encodes 11 proteins from a single open reading frame (ORF). Upon successful viral infection of the host cell, the HEV genome is translated and then functions as a template for the synthesis of a negative-sense RNA strand, which serves in turn as the template for the replication of the positive-stranded genomic RNA (55-57).

Four *cis*-acting replication elements (CRE) which modulate replication and translation have been identified in the HEV genome; these are at the 5' and 3' termini of the genomic positive strand, within the open reading frame (ORF) of the positive strand, and at the 3' end of the negative RNA strand (a replicative intermediate) (17, 58). The 5' and 3' ends of the genomic RNA are non-translated regions (5' NTR and 3' NTR), the latter of which terminates in a poly-A tail. A clover-leaf secondary structure termed domain I (**Fig 1A**), is present at the 5' terminus of the HEV genome (59, 60) and is believed to be the site of

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<sup>1</sup> Submitted for publication to the Journal of Virology 05/19/2015



**FIG. 1. Domain I and the CRE(2C) of CVB3 and the location of protein binding to these structures.** (A) Domain I of CVB3 located at the 5' terminus of the genome (grey nucleotides indicate deleted sequence in CVB3-TD50). (B) The CRE(2C) of CVB3 located in the 2C protein coding region from nucleotides 4365-4425. The three red adenines at the apex of the loop represent the experimentally determined template for the uridylylation of VPg (C) During replication of the CVB3 genome, viral protein 3CD and host-cell protein poly(rC) binding protein 2 (PCBP2) bind in the region of domain I and viral proteins 3CD and 3Dpol bind to the CRE(2C) to mediate the uridylylation of VPg.

formation of the replication complex which includes both viral and host cell proteins required for negative strand synthesis (25, 60-64). Binding of the viral protein 3CD<sup>pro</sup> to stem-loop d of domain I and the host cell protein poly(rC)-binding protein 2 (PCBP2) to stem-loop b of domain I (or the poly-C tract in the spacer region between domain I and domain II of the internal ribosome entry site [IRES]) (**Fig 1C**), has been proposed to be required for negative strand synthesis (65-69). It has been demonstrated using a cell-free system that deletion of 4 nucleotides from stem-loop d of poliovirus RNA led to a loss of both negative strand synthesis and production of viral proteins (25), perhaps due to decreased, or loss of, binding of 3CD<sup>pro</sup>. Further studies demonstrated that, with domain I left intact, deletion of portions or all of the internal ribosome entry site (IRES) from poliovirus RNA did not affect the synthesis of either negative or positive genomic RNA in a cell-free system (70). These findings indicated that domain I, but not the IRES, of the 5'NTR was crucial for efficient replication of the viral RNA.

Our previous work identified a mechanism of CVB persistence in which domain I can largely be lost during replication in experimentally-inoculated cell cultures and mice (10, 13), as well as within heart tissue of naturally infected humans (9); more recent evidence suggests that HEV evolve 5' TD populations in cardiomyopathic human heart tissues at the terminal stage of disease (12) as well as within the pancreatic tissue of experimentally inoculated mice (11). The discovery of naturally-occurring enteroviral 5' terminal deletions (TD) modified our understanding of enteroviral biology not only by demonstrating that enteroviruses without an intact domain I were viable but that 5' CVB-TD populations replicated with a severe deficiency in positive strand RNA replication. This was readily apparent in the reduction of positive to negative strand viral RNA ratios to near unity in CVB-TD infected cells as well as by the demonstration of efficient encapsidation of the replication incompetent negative strand (10). The inefficient

replication observed with CVB-TD viruses (10, 13) confirmed and significantly extended the well-established role of domain I as contributing to efficient viral replication and positive-strand RNA synthesis (58, 60, 71). With much of domain I lost in TD viral RNA populations (**Fig 1A**), it is not surprising that CVB-TD strains replicate to low titers not detectable by standard cell culture assays using cytopathic effect (CPE), but require detection via enzymatic amplification. Further work by Sharma and colleagues demonstrated that CVB-TD viral genomic RNA was stable and replication competent with up to 49 nucleotides deleted from the 5' terminus, although at a minimal level in a cell free reporter system (69), supporting and extending our previous findings. Curiously, while we observed that CVB-TD genomes rarely terminated in the canonical UU derived from the uridylylated VPg primer during positive strand synthesis (9, 10, 13), VPg was still linked to the 5' end of the TD genome (10).

In addition to the 3' and 5' NTR CREs, picornaviruses also possess small hairpin structures within the genome necessary for efficient RNA replication which template the uridylylation of the viral protein primer VPg (14, 24, 72); VPg is subsequently used as the RNA replication primer. These hairpin structures are located at different sites within various picornaviral genomes (16-23) with that of the enterovirus genus (except rhinoviruses) located in the 2C coding region [CRE(2C) **Fig 1B**] (24). It has been firmly demonstrated that these CRE are location- independent (19, 22, 23): for example, poliovirus remains replication competent when the CRE(2C) is moved from the 2C protein coding region of the ORF to the 5' NTR (26). Despite diverse locations of picornaviral CREs, the location within a species is highly conserved (17). To determine the function and structural requirements of the HEV CRE(2C), both cell culture and cell free systems have been used to analyze the effects of mutations introduced into the CRE(2C) region. These studies defined the site of the template nucleotides for the

addition of uridine residues to VPg [in the loop of the predicted RNA structure (14, 73)], and defined the degree to which uridylation and replication are disrupted by mutations in the loop or stem of the structure (24-29). Studies of replication in cell culture not only demonstrated a loss of CPE upon transfection of some poliovirus (PV) CRE(2C) mutants but also demonstrated that some mutants were capable of a dominant-negative effect (74): replication of wt PV within the same cell was inhibited by the presence of these single site mutations. But this effect is not complete: in the cell free system, perhaps because of the non-compartmentalized nature of the replication, low level replication is observed in the mutated CRE(2C) genomes as evidenced by the presence of replicative form intermediate production and, unlike in cell culture assays, CRE(2C) mutations could be rescued *in trans* (75). In addition, passage of some initially non-cytopathic mutants (following transfection of T7 transcribed RNA) resulted in CPE, demonstrating that although the CRE(2C) mutations had a severely debilitating effect on virus replication, sufficient replication of the viruses had occurred permitting reversion to restore the wt sequence (19). Analysis of CRE(2C) mutations in cell free systems allows for quantitative assays of replication based on incorporation of radioactive nucleotides and demonstration of effects upon the generation of uridylylated VPg (24, 27, 29, 45, 75). The greatest effect upon uridylation was noted in mutations of the A5 and A6 nucleotides of the loop (**Fig. 1B; red nucleotides**) but considerable defects of uridylation and RNA replication in the cell free assays were observed with mutations in the stem or loop region (24, 45, 75). When 16 silent mutations were introduced into the CVB3 CRE(2C), creating the CRE(2C)-DM mutant (31), the virus was deemed unable to replicate based upon failure to detect both uridylylated VPg and positive strand synthesis and an inability to detect virus-expressed luciferase activity above background levels 10 hours after transfection of cell cultures.

Our findings (9, 10) that CVB3-TD strains replicated poorly compared to wt and that VPg was attached to CVB3 genomic termini despite a general lack of the 5'-UU consensus sequence, suggested the possibility that VPg was being non-specifically nucleotidylated, or nucleotidylated *in-situ*, instead of specifically uridylylated, in order to function as the primer for viral RNA replication. We therefore hypothesized that a functional (uridylylating) CRE(2C) would not be required for replication of TD viruses. Using the same 16 mutations described for the CRE(2C)-DM mutant (24), we generated the wt CVB3 genome with these mutations (termed here CVB3-CKO for CRE knockout) to serve as a replication control for studies of CVB3-TD replication; we also generated the CKO mutations in the CVB3-TD50 infectious cDNA clone (CVB3-TD50-CKO). Indeed, a non-functional CRE(2C) was not necessary for CVB3-TD50 replication but contrary to expectations, we also observed that CVB3-CKO was viable. In this report, we demonstrate that in cell culture or in mice, this specific disruption of CRE(2C) in CVB3, while detrimental to viral replication, is not lethal after all. Further, we demonstrate that when the native structure of CRE(2C) is altered in this way in a non-terminally deleted genome, the virus population rapidly evolves to become populations containing 5' terminal genomic deletions.

## **Materials and Methods**

**Cells and viruses.** Coxsackievirus B3, strain 28 (30), was used in this work as the wildtype (wt) strain. HeLa cell monolayer cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM/High Glucose [GE Life Sciences, Logan, UT,]) containing 10% newborn calf serum and 50 µg/mL gentamicin (GIBCO, Life Technologies, Grand Island, NY). Viral stocks were prepared in HeLa cells by

electroporation of  $2 \times 10^6$  cells at 100V and 1,980  $\mu$ F (Cell-PORATOR, GIBCO, Life Technologies) with 12  $\mu$ g of viral RNA (equivalent to  $2.96 \times 10^{12}$  genomes) transcribed from infectious cDNA clones of either wt CVB3, CVB3 in which CRE(2C) was mutationally disrupted with 16 mutations [(CVB3-CKO) as described below in mutational disruption of the CRE(2C) using overlap extension PCR], CVB3 with a 49 nucleotide 5' terminal deletion [CVB3-TD50; (10)] , and CVB3-TD50 in which the CRE(2C) was mutationally disrupted (CVB3-TD50-CKO). Cells were subsequently seeded into 100mm dishes. After eight days of incubation, with replacement of medium at day four, cultures were examined for cytopathic effect (CPE) using an Olympus CKX41 inverted microscope with a DP12 camera; complete CPE was observed with wt at 2 days post electroporation. After freezing and thawing three times, the lysates were cleared of cell debris by centrifugation at 30,000 RPM for 30 min at 8°C (SW28.1 rotor; Beckman, Brea, CA) and then prepared as described previously (10). This preparation was termed pass 1 as previously described (10).

**Mutational disruption of the CRE(2C) using overlap extension PCR.** The mutations in the CVB3 CRE(2C) were generated using overlap extension mutagenesis (76). Two overlapping fragments of CVB3 cDNA were generated by PCR from the infectious cDNA clone of CVB3/28 (30) using Deep Vent DNA polymerase (New England Biolabs; Ipswich, MA) and the primer pairs ID13 and CREKORev or CREKO and XBA (**Table 1**) with 4 mM  $\text{MgSO}_4$  and 200  $\mu$ M dNTPs. Cycling was carried out as noted (**Table 2**) with modified cycle denaturation and annealing times of 30 sec each. The two amplimers were gel purified, combined in Thermopol reaction buffer (20mM Tris-HCl, 10mM  $(\text{NH}_4)_2\text{SO}_4$ , 10 mM KCl, 4 mM  $\text{MgSO}_4$ , 0.1% Triton® X-100 pH 8.8; New England Biolabs) with 200  $\mu$ M dNTPs and repeatedly denatured and annealed 10 times

**Table 1. Names, annealing sites, and sequences of primers used in CKO studies.**

Primer Name	Region of CVB3/28 Genome	Strand	Nucleotide Sequence (5' to 3') <sup>b</sup>
S <sup>a</sup>	1-20	+	TTAAAACAGCCTGTGGGTTG
S4 <sup>a</sup>	45-74	+	CGCTAGCACTCTGGTATCACGGTACCTTTG
S5	86-113	+	TATACCCCTCCCCAACTGAACTTAG
S Return	291-312	-	TACACTGGGGTAGTGCTGAGCG
E2 <sup>a</sup>	450-464	+	TCCGCCCTGAATG
E3 <sup>a</sup>	537-563	-	ACACGGACACCCAAAAGTAGTCGGTTCC
E3Sub	535-549	-	AGTAGTCGGTTCCGC
P <sub>in</sub> E3	537-563	-	<u>GGAAATTCATCGATACCGGACACCGGACACCCAAAGTAGTCGGTTCC</u>
ID13	3601-3620	+	TGGATTTTCCGAACCAGGTG
CREKORev	4352-4417	-	CAAGCAAACCGGTTCTATCCTACACCTTACTTTTAAATTGAATAAATTGCTCATCTTCTTCTCAAG
CREKO	4375-4417	+	TATTCAA TTTAAAAGTAAAGTGATAGGATAGAACCAGGTTTGC TTGCTCCTGCACGGGAGGCC
XBA	4932-4958	-	GCATGTCTAGAGATATCTGACCTGTG
2B1	3746-3764	+	GGAGTGAAGGACTATGTGG
4523Rev	4504-4523	-	GGTCTGGCGGTAGTGAGTAC
2C2	4450-4469	-	CAATTAAGTTTGTGCCACC
2C7	4166-4186	+	GA AAAACACCGAATTCCTGAAC
P <sub>in</sub>	NA	NA	<u>GGAAATTCATCGATACCGG</u>
P <sub>in</sub> 5End1	1-10	+	<u>GGAAATTCATCGATACCGGTTAAAACAG</u>
P <sub>in</sub> 5End5	15-24	+	<u>GGAAATTCATCGATACCGGGTTGATCC</u>

<sup>a</sup>previously published in Kim *et al*, 2005

<sup>b</sup>underlined sequences are non-genomic cDNA

Table 2. Standard PCR cycle parameters and primer pair variations.

Table 2. Standard PCR cycle parameters and primer pair variations			
Primer Pairs	Annealing Temperature	Elongation Time	Expected amplicon size
Standard initial denaturation	5 min at 94°C		
Standard cycle denaturation	10 sec at 94°C		
Standard annealing time	5 sec		
Standard elongation temperature	72°C		
Standard reaction volumes	20µL		
Standard cycle repeats	35X		
Primer Pairs	Annealing Temperature	Elongation Time	Expected amplicon size
S5 and Pin	56°C	35 sec	490bp
S4 and E3Sub	56°C	45 sec	504bp
S5 and SReturn	60°C	35 sec	226bp
Pin5End1 and E3	63°C	35 sec	573bp
Pin5End5 and E3	63°C	35 sec	558bp
Pin and SReturn	53°C	25 sec	322bp
E2 and E3Sub	58°C	10 sec	113bp
4523Rev and 2B1	52°C	45 sec	777bp
2C2 and 2C7	54°C	30 sec	303bp
ID13 and CREKORev	55°C	2 min	816bp
CREKO and XBA	55°C	2 min	583bp
ID13 and XBA	55°C	2 min	1,357bp

(1 minute at 94°C / 50°C for 1 minute) followed by extension with Deep Vent polymerase (2,000 u/mL) at 72°C for 2 minutes. The mutated overlap extension product was subsequently directly amplified using the ID13 and XBA primers (**Table 1; 2**) and Deep Vent polymerase with the modified denaturation and annealing times as described above. The amplified product of this reaction was digested with BssH2 and XbaI, then ligated with the BssH2-XbaI restriction fragment of pCVB3 or pCVB3-TD50 (10) to produce pCVB3-CKO and pCVB3-TD50-CKO.

**Preparation of T7 RNA transcripts for electroporation or transfection.** Preparation of infectious cDNA copies of CVB3 and CVB3-TD50 genomes was described previously (10). Mutational disruption of the CRE(2C) region of these clones to generate CVB3-CKO and CVB3-TD50-CKO is described above in mutational disruption of the CRE(2C) using overlap extension PCR. Viral RNA was transcribed from 5 µg of ClaI-linearized cloned cDNA using the T7 RiboMAX System (Promega; Madison, WI) for one hour followed by removal of the DNA template by digestion with RQ1 DNase (6 units). RNA was precipitated overnight at -20°C in 120 µL containing 4mM EDTA, 80 µg of glycogen, and 2 M LiCl, pelleted and resuspended in RNase-free water. The expected 7.4 kb size of the viral RNAs was verified on a 1.4% native agarose gel and concentrations were determined spectrophotometrically.

**Cell culture of purified virus stocks (pass 2).** CVB3-CKO replicates to very low titers and does not produce observable CPE. Consequently, inoculation of cell cultures with both CVB3-CKO and wt CVB3 virions was performed as described previously (10) using virus titers based upon positive strand RNA concentration copy number in the virus

preparations which were derived from reverse transcription quantitative PCR (RT-qPCR) analyses (see titering viral RNA in purified virus stocks using RT-qPCR below). Thus, for passage of purified virions in HeLa cell cultures,  $3 \times 10^5$  HeLa cells (80-90% confluent) per well in 24-well plates were inoculated in duplicate with the equivalent of 3,190 positive RNA genomic strands. In addition, equivalent amounts of virus were incubated for 1 hour at 37°C with a 1:400 dilution of anti-CVB3 neutralizing antibody (ATCC; Manassas VA) prior to inoculation of cells. Plates inoculated in the presence of neutralizing antibody were incubated for 3 hours at 37°C, the monolayers were washed twice with phosphate buffered saline (PBS), then provided fresh medium and incubated with neutralizing antibody for 5 days. Plates inoculated without antibody were incubated overnight at 37°C prior to washing. The cells were harvested by direct TRizol lysis in the wells, followed by RNA isolation per manufacturer's protocol, isopropanol precipitation with glycogen as a carrier (77), and resuspended in nuclease free water. Total RNA was assayed for the presence of CVB3 RNA using nested RT-PCR as described below.

**Transfection of mice and detection of viral RNA in tissue homogenates using RT-PCR.** The use of mice in this study 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 25 µg (equivalent to  $6.2 \times 10^{12}$  genomes) of CVB3 or CVB3-CKO T7 transcripts (prepared as described in preparation of T7 RNA transcripts for electroporation or transfection above). Inocula (0.1 mL/mouse) were prepared in 0.4 mL aliquots containing 100 µg T7 transcribed RNA in 100 µL of nuclease free water, 100 µL of LIPID-based *In Vivo* Transfection Reagent (Altogen Biosystems, Las Vegas, NV), 20 µL transfection enhancer, and 80 µL of a 5% (w/v) sterile glucose solution. Hearts and spleens from

mice at 5 and 8 days post-transfection (wt CVB3) or 20 days post-transfection (CVB3-CKO and saline transfection control) were split into two approximately equal portions and one half weighed, then tissue was homogenized in 500  $\mu$ L TRizol (Invitrogen; Carlsbad, CA) and total RNA was extracted as described in cell culture of purified virus stocks above. To detect viral RNA, a series of four 10-fold dilutions were prepared to find optimal RNA concentration for reverse transcription. This method was required for all RNA preparations from mouse tissue, as the required dilution varied between 100 and 1,000 fold depending on the tissue and mouse. cDNA was then transcribed from each dilution with primer E3 (**Table 1**) at a final concentration of 2  $\mu$ M. Briefly, RNA was denatured for 5 min at 70°C and incubated for 2 min on ice. RNA was then reverse transcribed using the ImpromII reverse transcriptase system (Promega) with 0.8 mM dNTPs, 2 mM MgCl<sub>2</sub>, and 1  $\mu$ l enzyme (the number of units are proprietary and not provided by Promega) in 20  $\mu$ l reaction buffer for 60 minutes at 42°C. cDNA was ethanol precipitated as before, resuspended in water, and nested RT-PCR for viral RNA detection was performed as described below.

**Inoculation of cell cultures with spleen homogenates.** Murine spleen was homogenized in complete tissue culture medium, frozen and thawed 3 times, then centrifugally cleared at 16,000 x g for 10 min. These cleared homogenates were then treated with RNase A as described in cell culture of virus stocks above. Duplicate HeLa cell cultures (as described above) were inoculated with 100  $\mu$ L of cleared, RNased splenic homogenate from CVB3-CKO transfected mice (20 days post-transfection) or 1  $\mu$ L of splenic homogenate from wt CVB3 transfected mice (8 days post transfection; the lower volume for wt CVB3 was chosen based on the higher extent of replication in cell culture observed for wt CVB3 by RT-qPCR). Cell cultures were inoculated in the

presence or absence of a 1:400 dilution of anti-CVB3 neutralizing antibody; preparations containing neutralizing antibody were treated as described in cell culture of virus stocks above. After 3 hours of incubation (plates containing antibody) or overnight incubation (plates without antibody), cultures were washed 2 times with PBS and media was replaced with the presence or absence of neutralizing antibody. Cultures were observed daily by light microscopy for CPE. After 5 days, total RNA was extracted from each monolayer as described in cell culture of purified virus stocks above. RNA preparations were then assayed for viral RNA using nested RT-PCR (see “nested RT-PCR to detect viral RNA” below).

**Detection of positive strand viral RNA in purified pass 1 virus preparations.** Total RNA was isolated from purified virus preparations using TRizol as described in cell culture of purified virus stocks above, and cDNA was prepared (described above) using a tagged (78) antisense primer, PinE3 (**Table 1**), designed using a non-CVB3 specific oligo tag (Pin) 5' of nucleotides corresponding to a region of the 5' NTR that has not been found to delete (**Table 1**). Half of each cDNA reaction was amplified using the primers S5 and the tag primer Pin (**Table 1**) in GoTaq Green Master Mix (Promega). Reactions were cycled as noted for primer pairs (**Table 2**) followed by electrophoresis on 2% agarose gels for analysis.

**Enzymatic amplification of the CRE(2C) sequence from plasmids and T7 transcripts.** The CRE(2C) region of wt CVB3 and CVB3-CKO plasmids, and cDNA of T7 transcripts of cloned cDNA, was amplified using nested primers in 20  $\mu$ L reaction volumes containing GoTaq Green Master Mix (Promega). The primers for the first

enzymatic amplification were 4523Rev and 2B1 (**Table 1; 2**) at a final concentration of 0.5  $\mu$ M. After ethanol precipitation, a second round of PCR was carried out with the ethanol precipitated amplimers using primers 2C2 and 2C7 (**Table 1; 2**). PCR reactions were electrophoresed on 2% agarose gels and bands at the appropriate size (**Table 2**) were excised and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research; Irvine, CA) per manufacturer's protocol. Recovered amplimers were sequenced with primer 2C7 (**Table 1**) to verify that sequences were either wt (CVB3) or CRE(2C) mutants (CVB3-CKO) as expected prior to electroporation and virus preparation.

**Nested RT-PCR for detection of viral RNA in total RNA isolated from cell culture lysates and mouse tissues.** To test for viral RNA presence in cell culture or mouse tissue, nested RT-PCR was performed to detect the 5' NTR sequence in the CVB3 genome corresponding to nucleotides 45-564. Initial enzymatic amplification of all cDNA samples from viral RNA preparations (except for initial viral RNA detection in sucrose purified preparations; described in detection of positive strand viral RNA in purified pass 1 virus preparations above) was carried out using primers S4 and E3Sub (**Table 1; 2**) in GoTaq Green Master Mix (Promega). A second round of PCR was carried out using the ethanol precipitated product of the S4-E3Sub PCR amplification with S5 and SReturn (**Table 1; 2**). Final PCR products were analyzed on gels as described for "detection of viral RNA from purified virus preparations" above.

**Titring viral RNA in purified virus stocks using RT-qPCR.** Because CVB3-CKO, CVB3-TD50, and CVB3-TD50-CKO viruses do not produce detectable CPE upon infection of tissue culture monolayers, viral RNA copy number was quantitated using RT-

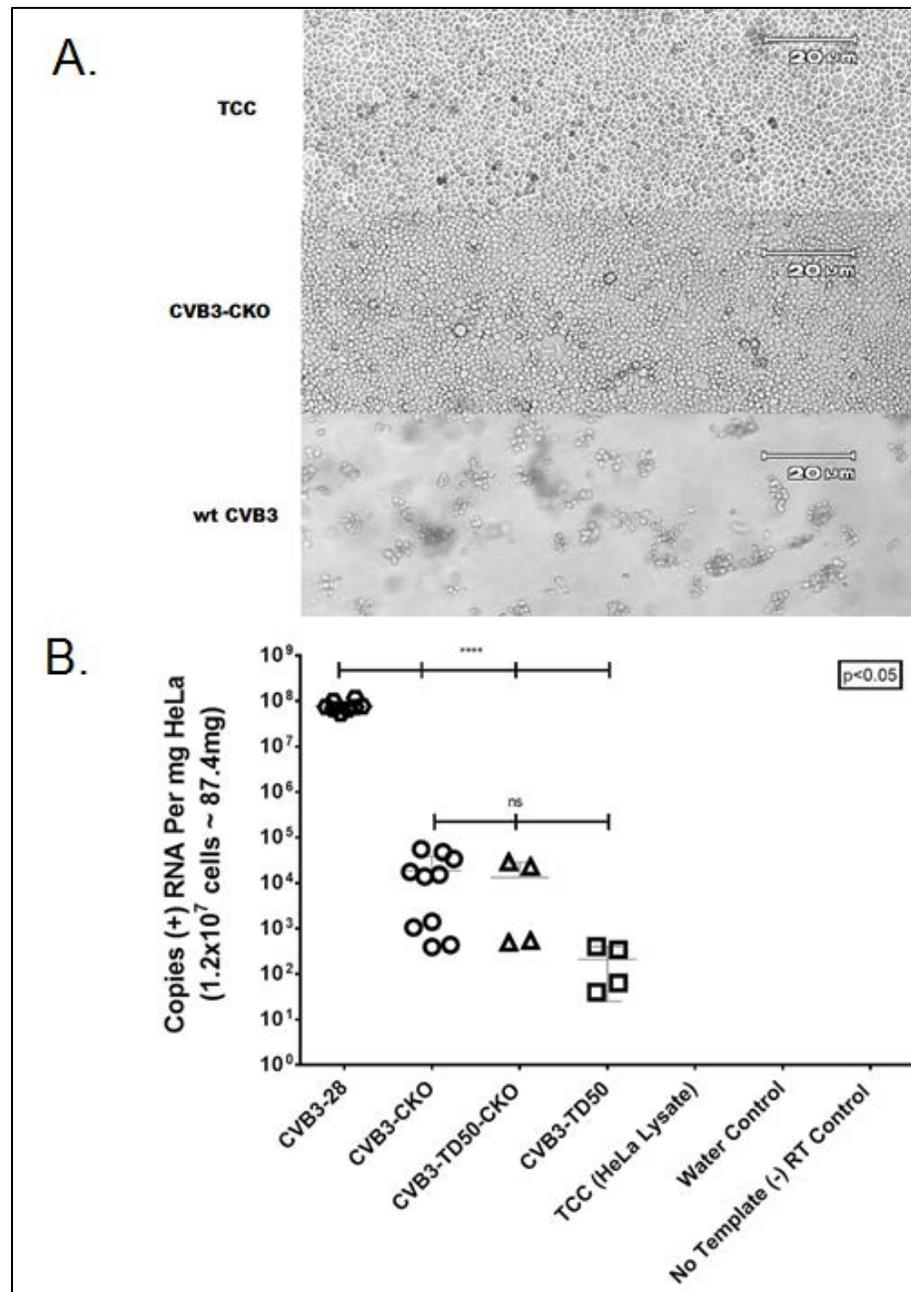
qPCR similar to that described previously (10). cDNA was transcribed using Improm II Reverse Transcriptase (Promega) and primer E3 (**Table 1**) as described in “transfection of mice and detection of viral RNA in tissue homogenates using RT-PCR” above. RT-qPCR was performed using an Opticon 2 DNA Engine (MJ Research; Waltham, MA) by combining cDNA reactions with primers E2 and E3Sub (**Table 1**) in Maxima SYBR Green/Fluorescein qPCR master mix (2X; Thermo Scientific, Waltham, MA). qPCR cycling was carried out as indicated for primer pairs (**Table 2**) with a modified annealing time of 10 sec. Standard curves were generated with 10 fold serial dilutions of a 500bp amplicon of CVB3 cDNA generated with primers S and E3 (**Table 1**); amplicon was at a known concentration previously calculated using spectrophotometry. Knowing the cDNA copy number generated from an RT reaction containing a specific amount of RNA, the efficiency of the cDNA transcription (molecules of cDNA/molecules of RNA) was calculated. Statistical analysis was carried out with GraphPad Prism software using one-way ANOVA with Bonferroni correction.

**RT-PCR to detect 5' terminal deletions in purified virus stocks.** cDNA from purified virion preparations was prepared using Improm II reverse transcriptase (Promega) and primer E3 (**Table 1**) as described in “transfection of mice and detection of viral RNA in tissue homogenates using RT-PCR” above. All reverse transcriptase reactions were assembled with an equal RNA copy number for comparison of presence of 5' terminal sequences. Copy numbers were calculated using either cDNA copy number determined by RT-qPCR (for virus preparations) or were calculated based on RNA concentration from spectrophotometric analysis (for T7 transcribed RNA controls). Tagged primers [(78) and detection of positive strand viral RNA in purified pass 1 virus preparations above] corresponding to regions of the 5' genomic terminus were used to detect 5'

terminal deletions. Initial enzymatic amplification was carried out using Pin5End1 and E3 (**Table 1; 2**) and 400 copies of E3 cDNA or Pin5End5 and E3 (**Table 1; 2**) with 1,400 copies (at the experimentally determined limit of detection for the respective primer pairs), in GoTaq Green Master Mix (Promega) with a modified annealing time of 10 sec. PCR products were ethanol precipitated. The second round of enzymatic amplification was performed with the first ethanol precipitated reaction product in GoTaq Green Master Mix with Pin and SReturn (**Table 1; 2**). PCR products were analyzed on agarose gels as described in “detection of positive strand viral RNA” above.

## **Results**

**CVB3-CKO replicates in HeLa cells.** The HEV CRE(2C) is required for efficient viral replication (24, 29, 45, 75) and it is established that various mutational disruptions of the CRE(2C) in either poliovirus or CVB3 are lethal based on the absence of radiographic evidence for positive strand (genomic) RNA replication, VPg uridylylation (24, 29, 45), or luciferase activity after 10 hours of incubation in cell culture (24). Our previous findings demonstrated that a CVB-TD genome rarely terminated in the canonical UU donated by uridylylated VPg upon replication, yet still had VPg linked to the genomic RNA (79). Therefore, we hypothesized that CVB-TD would not require a functional (that is to say, uridylylating) CRE(2C) in order to successfully replicate. To test this, we introduced the previously characterized 16 mutations [described as CVB3-CRE(2C)-DM; (24)] in both the wt CVB3 (initially intended as a negative control) and CVB3-TD50 infectious cDNA clones; the sequences of the CRE(2C) region in the DNA clones and in the T7



**FIG. 2. Virus without a functional CRE(2C) can be recovered in the absence of observable CPE following electroporation of HeLa cells with T7 RNA transcripts from infectious cloned genomes.** (A) CVB3-CKO does not produce observable CPE at eight days post electroporation with 18  $\mu$ g of RNA compared to the tissue culture control (TCC) electroporated with saline only. Wt CVB3 RNA induced complete CPE by 48 hours post electroporation. Images at 100x original magnification. (B) Quantitation of viral RNA in RNase treated and sucrose purified mutant virus preps (CVB3-CKO; CVB3-TD50, or CVB3-TD50-CKO). Loss of CRE(2C) function, deletion of 49 nucleotides from the 5' terminus in domain I, or both, does not lead to a lethal virus phenotype but deleteriously affects replication when compared to wt CVB3. Loss of CRE(2C) in CVB3-CKO lowers extent of replication to a level similar to that of CVB3-TD50, but loss of CRE(2C) in CVB3-TD50 (CVB3-TD50-CKO) does not further impact replication.

transcripts from the clones (after cDNA synthesis) were verified prior to use as described in methods.

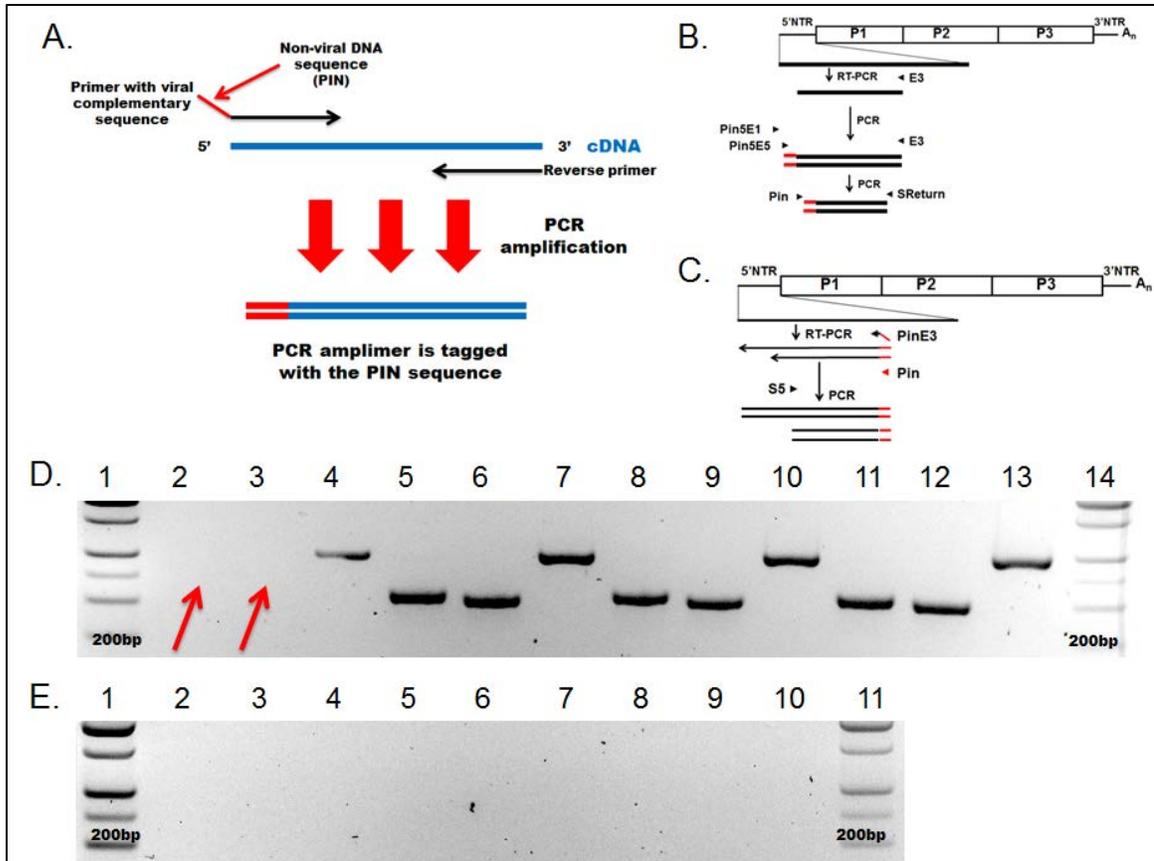
HeLa cells were electroporated with equivalent copy numbers of full-length wt CVB3, CVB3-CKO, CVB3-TD50, or CVB3-TD50-CKO T7 transcribed RNA, then cultured for 8 days. As expected from others' work (24), no CPE was observed in the CVB3-CKO electroporated monolayers (**Fig. 2A, CVB3-CKO**) when compared to the electroporation control cells (**Fig. 2A, TCC**), nor was CPE evident in cultures transfected with any of the other viral RNAs (data not shown) except wt CVB3 which completely lysed the culture by 48 hours post-electroporation (**Fig. 2A, wt CVB3**). The observed absence of CPE in cells electroporated with CVB3-CKO was consistent with studies indicating that a mutated CRE(2C) suppresses or ablates viral replication (24, 27, 45, 75).

However, because our previous work had demonstrated that CVB3-TD strains also showed no apparent CPE in cell cultures yet could be shown to be replicating using RT-PCR to detect viral RNA (10, 13), we also assayed all electroporated cultures for the presence of encapsidated CVB3 RNA. Viral RNA was extracted from concentrated, RNased virion preparations for the detection of viral RNA in pass 1 preparations. To determine whether and to what extent CVB3-CKO and CVB3-TD50-CKO were replicating (compared to wild-type CVB3 or CVB3-TD50), viral RNA was extracted from aliquots of the RNase treated and pelleted preparations and RT-qPCR performed (**Fig. 2B**). These results (based on five replicate plates and 10 data points [CVB3-CKO and wt CVB3] or two replicate plates and four quantitative data points [CVB3-TD50 and CVB3-TD50-CKO]) indicated that CVB3-CKO was indeed replication competent as the RNA was derived from pelleted viral particles. CVB3-CKO replicated to approximately five logs lower than wt CVB3 (**Fig. 2B, compare CVB3-28 [wt] to CVB3-CKO**), indicating that ablation of the CRE(2C) uridylylation function severely handicaps

replication of the parental virus. Interestingly, these results also showed that there is no significant difference in the extents of replication of CVB3-CKO compared to CVB3-TD50, and that the mutational disruption of the CRE(2C) in a CVB3-TD virus does not significantly alter its replication (**Fig. 2B, compare CVB3-CKO to CVB3-TD50-CKO and CVB3-TD50**). In summary, the ablation of CRE(2C) function is not lethal in either wt CVB3 or in a CVB3-TD strain but it does lower the level of replication to the level of CVB3-TD populations. Important to note is that the CVB3-TD50-CKO strain did not replicate at a lower level than either CVB3-CKO or CVB3-TD50, indicating that the functional loss of the two CREs [domain I and CRE(2C)] is not additive.

**Replication of CVB3-CKO is accompanied by generation of 5' terminally deleted**

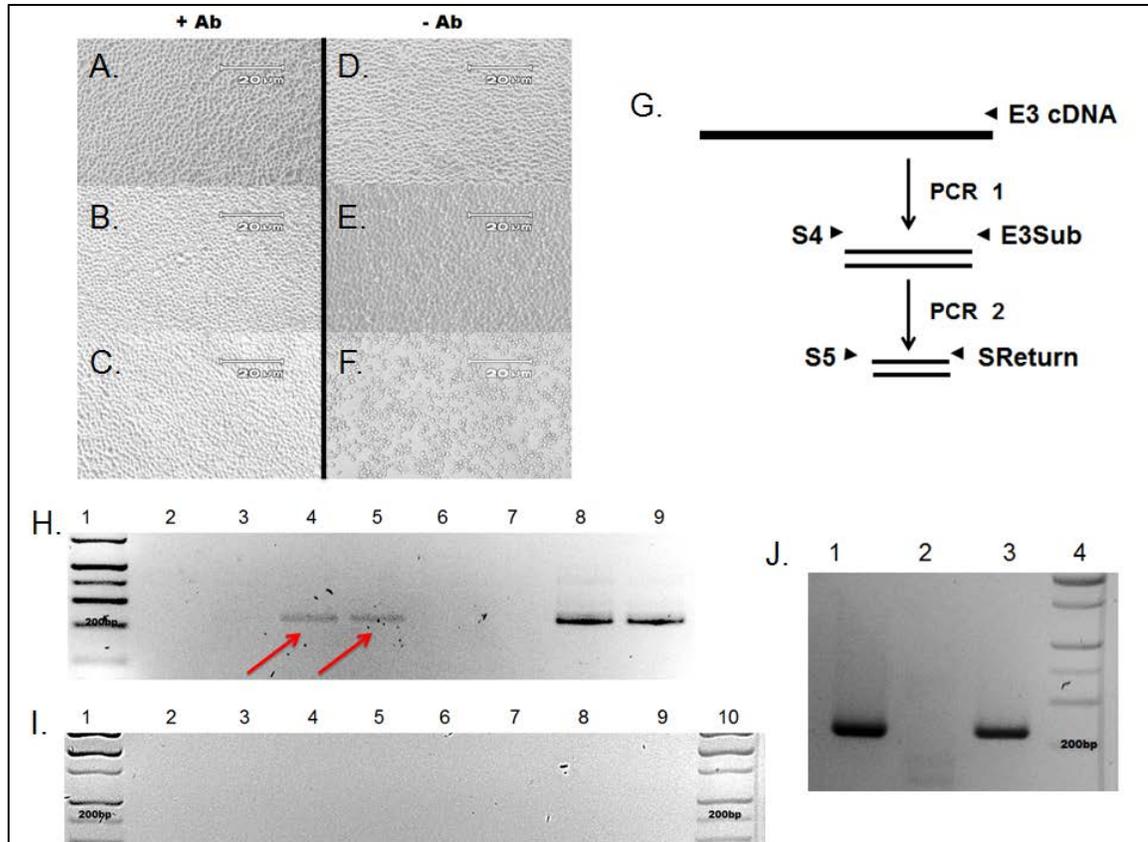
**CVB3-CKO genomes.** Because the replication of CVB3-CKO and CVB3-TD50 are similarly poor (*i.e.*, apparent absence of CPE and replication on the order of five logs lower than wt CVB3) and mutational disruption of the CRE(2C) does not affect overall replication of the CVB3-TD50-CKO strain more than that observed for CVB3-TD50, we hypothesized that alterations at either of these sites were likely to result in the same type of positive strand initiation and, consequently, 5' terminal genomic deletions could be occurring during the replication of CVB3-CKO in cell culture. We therefore used RT-PCR to test whether 5' terminal deletions had arisen in the CVB3-CKO population. Using equivalent numbers of positive sense viral RNA strands for wt CVB3 and CVB3-CKO, cDNA was transcribed using E3 primer (**Table 1**) followed by PCR using primers designed to detect sequences close to the 5' terminus (**Fig. 3A-C**). Absence of banding in the CVB3-CKO amplifications correlating to the 5' terminal 20 nucleotides (**Fig. 3D, arrows, lanes 2 and 3**) compared to wild-type CVB3 viral RNA (**Fig. 3C, lanes 5 and 6**) and controls (**Fig. 3C, lanes 8-9, 11-12**) indicated that the CVB3-CKO viral RNA had lost



**FIG. 3. Loss of a functional CRE(2C) leads to evolution of 5' terminal deletions in CVB3-CKO after 8 days of replication in HeLa cells.** (A) A schematic of how amplification with PIN primers works. (B-C) Relative position in the CVB3 genome of tagged primers used to amplify 5' terminal sequence (B) or to amplify sequence in the 5' NTR in a region where deletions do not occur (C). (D) Agarose gel analysis of E3 primed cDNA amplified with primers Pin5End1 and E3 or Pin5End5 and E3, then subsequently amplified with primers Pin and SReturn (lanes 2-3, CVB3-CKO purified virus preparation; lanes 5-6, CVB3-CKO T7 transcripts; lanes 8-9, wt CVB3 virus preparation; lanes 11-12, wt CVB3 T7 transcripts). To detect virus using a region of the 5' NTR that has not been found to delete, cDNA was made with primer PinE3 and amplified with primers S5 and Pin (lane 4, CVB3-CKO virus preparation; lane 7, CVB3-CKO T7 transcripts; lane 10, wt CVB3 virus preparations; lane 13, wt CVB3 T7 transcripts). 400 (Pin5End1) or 1,400 (Pin5End5) copies of cDNA prepared from purified viral RNA or T7 transcribed RNA (controls) were used in each PCR reaction. Arrows indicate the loss of sequence from the 5' terminus of CVB3-CKO (lanes 2 and 3) as compared to T7 or wt virus controls (lanes 5-6; 8-9; 11-12). However, CVB3-CKO virus could be detected by RT-PCR priming in a region of the 5' NTR that has not been found to delete (compare lane 4 to lanes 7; 10; and 13). (E) Controls (lanes 2-4, no cDNA PCR controls, lanes 5-7 no template RT-PCR controls, lanes 8-10 tissue culture controls) were negative.

5' terminal sequence. Water (no cDNA), no template RT-PCR, and tissue culture controls were all negative (**Fig. 3E, lanes 2-4 no cDNA PCR controls, lanes 5-7 no template RT-PCR controls, lanes 8-10 tissue culture controls**). However, bands were observed from cDNA amplified from both CVB3-CKO and wt CVB3 with primers that correlate to sequence in the 5' NTR of the genome (**Fig. 3C**) greater than 50 nucleotides downstream of the 5' terminus (**Fig. 3D, lane 4 [CVB3-CKO] and 7 [wt CVB3]**) demonstrating that virus was indeed present in the CVB3-CKO preparations. Thus, the majority population of CVB3-CKO RNA is lacking all or most of nt 1-24 of the wt CVB3 genome, similar to what was observed in persisting CVBs in human and murine infections (10, 11, 13).

**CVB3-CKO virions can be sequentially passaged in cell culture and productive infection is neutralized by anti-CVB3 neutralizing serum.** Previous work demonstrated that CVB3-TD viruses encapsidate RNA and could be passaged in cell culture and neutralized by anti-CVB3 neutralizing antibody (10). Because we had generated stocks of virions that had been partially purified using RNase A to degrade non-encapsidated RNA followed by ultracentrifugal pelleting through a 30% sucrose cushion (**Fig 2B**), we had evidence that viral RNA was encapsidated and RNA detection was not due to contamination with free viral RNA. This is in contrast to the previous observation that mutational disruption of the CRE(2C) in HEV was lethal based on the absence of CPE in cell culture, single-stranded RNA in autoradiographic assays, and the absence of luciferase activity after only ten hours of incubation in transfected cells (24, 29, 45). As a further test that CVB3-CKO was producing infectious virions, we used RNase-treated and ultracentrifugally-collected virus preparations to inoculate fresh cell cultures (termed pass 2) in the presence or absence of anti-CVB3 neutralizing antibody.



**FIG. 4. CVB3-CKO replicates without observable CPE and can be passaged from purified HeLa cell lysates and neutralized by anti-CVB3 neutralizing antibody.** (A-F) HeLa cell monolayers at five days post inoculation with wt CVB3 or CVB3-CKO with anti-CVB3 neutralizing antibody (A-C) or without antibody (D-E). CVB3-CKO does not produce CPE after infection. (B) Neutralizing antibody prevents CPE (C) with wt CVB3 at five days post inoculation (compare to without neutralizing antibody, panel F). Images at 100x original magnification. (G) Nested primers used to amplify sequence in the 5' NTR of CVB3 to detect viral RNA post inoculation of cell cultures. Primers are located in a region of the 5'NTR where deletions do not occur. (H) Total RNA was extracted from HeLa cells monolayers five days post inoculation and assayed for the presence of viral RNA by nested PCR and gel electrophoresis. Wt CVB3 was detected (lanes 8 and 9) as well as the non-cytopathic CVB3-CKO (arrows, lanes 4 and 5). The absence of bands when neutralizing antibody was present in the cell cultures (CVB3-CKO; lanes 2 and 3; wt CVB3; lanes 6 and 7) demonstrate that the antibody effectively suppressed productive infection. (I) Controls (lanes 2-4, no cDNA PCR controls; lanes 5-7, no RNA RT-PCR controls; lanes 8-10, tissue culture controls) were all negative. (J) Additional virus preparations were tested to verify observations made in H. CVB3-CKO can be passaged in cell culture (lane 3) as compared to the T7 RNA transcript control (lane 1) and can be neutralized with anti-CVB3 neutralizing antibody (lane 2).

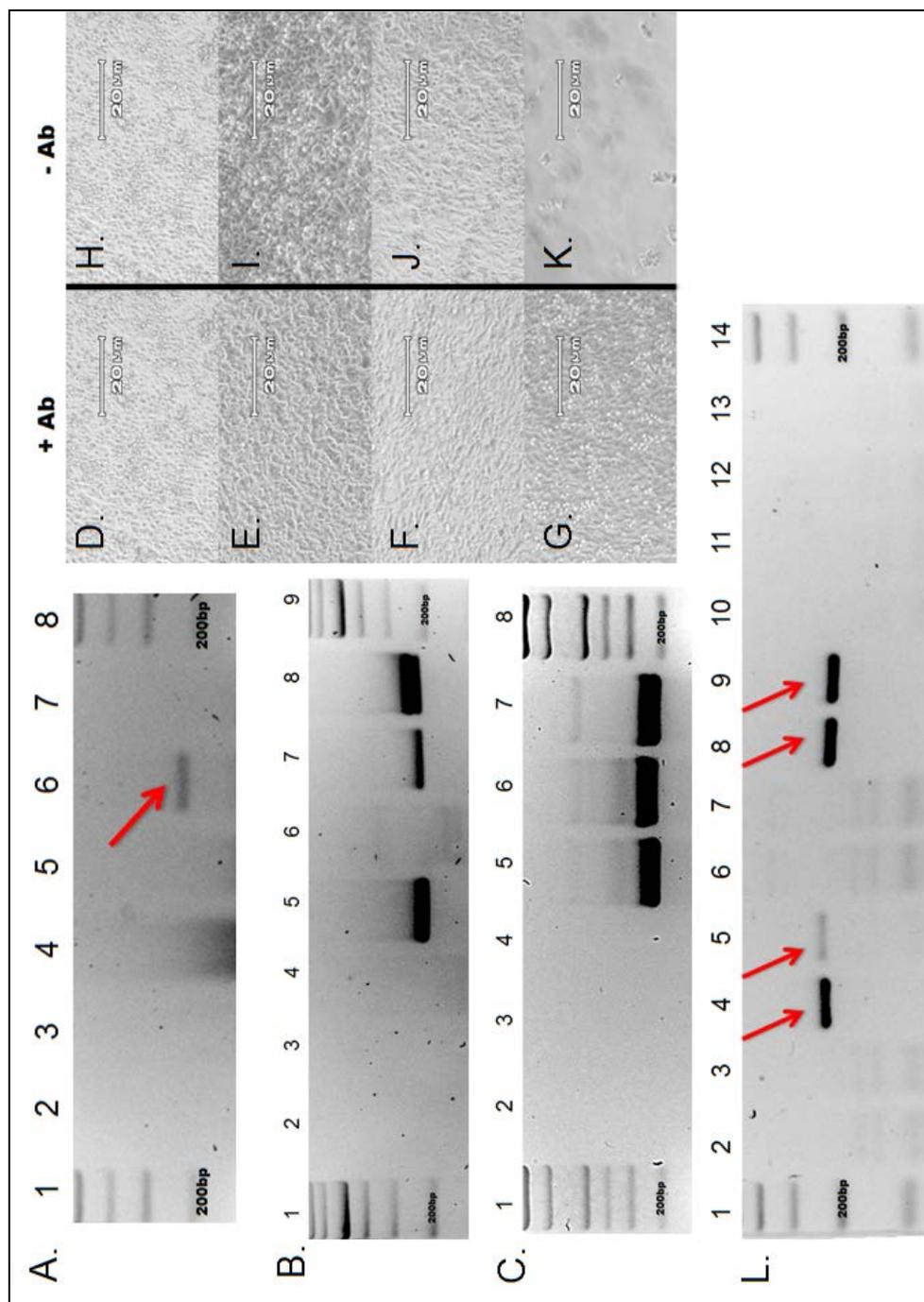
HeLa cells in 24-well plates were inoculated with CVB3-CKO (equivalent to 4,123 positive RNA strands, based on RT-qPCR quantitation of cDNA from virus preparations) with or without anti-CVB3 neutralizing antibody (**Fig. 4A-F, H**) as described earlier (10). After 5 days of incubation, cytopathic effects were present only in cultures inoculated with wt CVB3 in the absence of neutralizing antibody (**Fig. 4, compare panels A-E to panel F**), demonstrating that the amount of antibody used was sufficient to suppress a productive cytopathic infection.

Successful neutralizing antibody suppression of infection should be verifiable by an inability to detect viral RNA. To test whether viral RNA was present in the cultures with or without neutralizing antibody, nested RT-PCR for the detection of positive strand viral RNA was performed on total RNA prepared from cell cultures in two separate experiments (**Fig. 4G**). Viral RNA was detected in wells inoculated with CVB3-CKO and wt CVB3 in the absence of neutralizing antibody (**compare Fig. 4H, lanes 4 and 5 [arrows], and Fig 4J lane 3 [CVB3-CKO] with Fig. 4H lanes 8 and 9 [wt CVB3]**) but not in wells containing antibody (**compare Fig. 4H, lanes 2 and 3, and Fig. 4J lane 2 [CVB3-CKO] with Fig. 4H lanes 6 and 7 [wt CVB3]**). The water (no cDNA), no template RT-PCR, and tissue culture controls were negative (**Fig. 4I, lanes 2-4 no cDNA PCR controls, lanes 5-7 no template RT-PCR controls, lanes 8-10 tissue culture controls**). These results demonstrated that electroporation with CVB3-CKO T7 transcribed RNA produced infectious CVB3 virus particles that can be isolated and transferred to fresh cell cultures.

**CVB3 and CVB3-CKO RNA inoculated into mice generates virus.** Our observations that CVB3-CKO produced infectious virus and can be passaged in cell culture, led us to

ask if this were reproducible *in vivo*. A/J mice were chosen for this experiment as long-term CVB3 persistence has been demonstrated in this strain of mouse and this strain has been used regularly in our studies of CVB3-TD replication (10, 11, 80). Mice were inoculated i.p. with lipid conjugated CVB3-CKO or wt CVB3 T7 transcribed RNA and killed 8 days (wt CVB3) or 20 days (CVB3-CKO) post inoculation. Spleen and heart RNA preparations were assayed for the presence of viral RNA by RT-PCR. It was necessary to perform serial dilutions of RNA extracted from day 20 tissues of mice transfected with CVB3-CKO in order to detect the low level replication of this mutant virus against the high level background of the tissue RNA. Viral RNA was detectable only at higher dilutions (**Fig. 5A, compare lanes 4 [10 fold dilution], 5 [100 fold dilution] and 7 [10,000 fold dilution] to lane 6 [arrow; 1,000 fold dilution]**), though the required dilution varied slightly among different samples. Both CVB3-CKO and wt CVB3 RNA were detectable in spleen (**Fig. 5B, lanes 7 and 8 respectively**) and heart (**Fig. 5C, lanes 6 and 7 respectively**), demonstrating the infectivity in mice of T7 transcripts of CVB3 genomes.

To test whether CVB3-CKO RNA detected at day 20 post transfection in mice were encapsidated and infectious as would be expected of a true viral infection, cleared and RNase treated spleen homogenates were inoculated onto cell cultures either with or without prior incubation with anti-CVB3 neutralizing antibody. Spleen homogenates from mice inoculated with wt CVB3 RNA completely lysed the monolayers by two days post-exposure but for all other spleen homogenates, no CPE was evident by 5 days post-exposure (**Fig 5D-J**): no CPE was observed in cells exposed to homogenates of wt CVB3 transcript spleen tissue containing neutralizing antibody nor in control wells, nor in wells inoculated with CVB3-CKO spleen homogenates (**Fig. 5, compare panels D-J with panel K**). Lysis by wt CVB3 RNA demonstrated that inoculation of conjugated



**FIG. 5. T7 RNA transcripts from CVB3 and CVB3-CKO clones can be transfected into mice and virus replicates post transfection.** Nested RT-PCR could detect viral RNA using total RNA of hearts and spleens of mice transfected with wt CVB3 or CVB3-CKO T7 RNA transcripts: (A) A representative gel demonstrating the necessary 10 fold serial dilutions of total extracted tissue RNA to detect the low level replication of CVB3-CKO in mouse tissue 20 days post transfection (lane 1, HiLo marker; lane 2, no RNA RT-PCR control; lane 3, untransfected mouse RNA; lane 4, 10 fold dilution CVB3-CKO transfected mouse RNA; lane 5, 100 fold; lane 6, 1,000 fold; lane 7, 10,000 fold). CVB3-CKO RNA was detectable only at a 1,000 fold dilution of RNA (A, arrow, lane 6). (B) Detection of viral RNA in the spleens of transfected mice (lane 1, HiLo marker; lane 2, no cDNA PCR control; lane 3, no RNA RT-PCR control; lane 4, HeLa cell RNA control; lane 5, 1,000 copies CVB3 cDNA from T7 transcripts; lane 6, untransfected mouse RNA; lane 7, 1,000 fold dilution CVB3-CKO transfected mouse RNA at day 20; lane 8, 100,000 fold dilution wt CVB3 transfected mouse RNA at day 8). (C) Detection of viral RNA in the hearts of transfected mice (lane 1, HiLo marker; lane 2, no cDNA PCR control; lane 3, no RNA RT-PCR control; lane 4, untransfected mouse RNA; lane 5, 1,000 copies CVB3 cDNA from T7 transcripts; lane 6, 100 fold dilution CVB3-CKO transfected mouse RNA at day 20; lane 7, 10,000 fold dilution wt CVB3 transfected mouse RNA at day 4). (D-K) RNase treated homogenates of spleens were passaged onto HeLa cells at approximately 90% confluence and incubated overnight with (D-G) or without (H-K) neutralizing antibodies (D and H, no homogenate; E and I, control mouse homogenate; F and J, CVB3-CKO day 20 homogenate; G and K: CVB3 day 8 homogenate). Images at 100x original magnification. CPE was observed only in cultures inoculated with homogenates from mice transfected with wt CVB3 RNA (K). (L) Following five days of incubation, total RNA was tested with nested RT-PCR and analyzed on 2% agarose gels (lane 1, HiLo marker; lanes 2-3, CVB3-CKO plus neutralizing antibody; lanes 4-5, CVB3-CKO minus neutralizing antibody; lanes 6-7, wt CVB3 plus neutralizing antibody; lanes 8-9, wt CVB3 minus neutralizing antibody; lane 10, no cDNA control; lane 11, no RNA RT control; lane 12, uninfected control plus neutralizing antibody; lane 13, uninfected control minus neutralizing antibody) demonstrating that CVB3-CKO (arrows; lanes 4-5) and wt CVB3 (arrows; lanes 8-9) were detected in tissue homogenates following transfection of mice. Absence of amplicers in lanes 2 and 3 (CVB3-CKO) and 6 and 7 (wt CVB3) demonstrate that virus is neutralized by anti-CVB3 neutralizing antibody.

CVB3 RNA generated infectious virus in tissues of transfected mice. To assay for the presence of viral RNA in HeLa cultures that did not show CPE (CVB3-CKO and controls), total RNA was prepared from HeLa monolayers and assayed using nested RT-PCR for detection of positive strand RNA. In wells of HeLa cultures inoculated with tissue homogenates from mice transfected with either CVB3-CKO or wt CVB3 RNAs, viral RNA was detected at 5 days post inoculation (**Fig. 5L, arrows, CVB3-CKO lanes 4 and 5, wt CVB3 lanes 8 and 9**). However, when neutralizing antibody was present, no viral RNA was detected (**Fig. 5L, CVB3-CKO lanes 2 and 3, wt CVB3 lanes 6 and 7**). These results demonstrate that encapsidated, infectious virus was produced in tissues of mice following inoculation of wt CVB3 as well as CVB3-CKO RNA. Taken together, these results confirm that CVB3 is replication competent in the absence of a functional (uridylylating) CRE(2C).

## **Discussion**

Our hypothesis - that CVB3-TD viruses should be able to replicate without an uridylylating CRE(2C) - is firmly supported by the results presented here. Previously, a range of 5' terminal genomic deletions ranging from 7-49 nucleotides were characterized in CVB-TD populations which arose from both experimental and naturally occurring infections (9, 10, 13). In these CVB3-TD genomes, while VPg remained attached to the 5' terminus, only 1 (of 5) of the naturally-occurring deletions that were characterized, terminated in the UU that would normally be donated by uridylylated VPg at the start of replication (10). If CRE(2C)-uridylylated VPg were used in CVB3-TD virus positive strand initiation, CVB3-TD viral genomes would have been expected to have a higher proportion of 5' terminal uridine residues than has been observed. The results

presented here demonstrate that mutational disruption of the CRE(2C) structure using the 16 mutations previously shown to prevent uridylylation and inhibit replication (24), does not prevent replication of CVB3-TD, as shown by an insignificant impact on replication of TD viruses when assayed by RT-qPCR (**Fig. 2B**). We conclude that a functional CRE(2C) (defined as able to uridylylate VPg) is not necessary for CVB3-TD replication.

However, a most unexpected result from this work came from an intended control: the mutationally disrupted CRE(2C) structure in the wt CVB3 genome (CVB3-CKO). We fully expected this construct to show, as others had previously (24, 27, 45, 75), that an uridylylating CRE(2C) is necessary for CVB replication and that mutational disruption of the element would result in lost replication capacity. We were surprised, therefore, to observe that the strain termed CVB3-CKO, containing 16 silent mutations which ablate VPg uridylylation, was replication competent and able to be passaged in cell culture after isolation of viral particles. Our ability to isolate virus, and subsequently viral RNA, after RNase treatment and sucrose purification demonstrated that *de novo* replication was occurring following transfection of CVB3-CKO RNA, as only newly synthesized RNA is encapsidated (55). The additional finding that CVB3-CKO was viable in mice following inoculation of viral RNA transcribed from the infectious CVB3-CKO cDNA clone, confirmed and significantly extended findings derived from the cell culture system. These results are in sharp contrast to those of van Ooij and colleagues (24) who reported that these same 16 mutations when induced into CVB3 [albeit a different strain of CVB3 (81) than used in these studies CVB3/28 (30)], were lethal as demonstrated by a lack of observable CPE in BGM cell cultures, a lack of detectable luciferase activity 10 hours post transfection in BGM cells (when the P1 region had been replaced with a luciferase gene), and an undetectable level of single-stranded RNA

production in cell-free assays (24). The disparity between our results and those of van Ooij and colleagues (24) regarding the impact of the mutated CRE(2C) on CVB3 viability, can best be explained by the type and sensitivity of the assays used to assess the viability of these viruses and the ability of these viruses to replicate at detectable levels over an extended time (days) in culture. Differing effects may also be seen in various cell lines although this was not tested in the work reported here.

Yet another unexpected finding occurred while performing this work: the replication of the wt CVB3 genome with the mutationally disrupted CRE(2C) structure is associated with the development of a CVB3-CKO virus population possessing 5' terminal deletions. As in earlier studies (10), the CVB3-CKO-TD population is detectable in the complete absence of the wt (or parental) initiating genome population, and thus does not require the presence of the wt genome to replicate successfully. This novel finding demonstrates that while an important part of the normal and efficient initiation of wt virus positive strand synthesis requires the CRE(2C) structure and function to be intact, virus replication can still occur in the absence of both. This may occur by a lack of specific initiation of replication caused by two potential mechanisms. In the first, a lack of specific VPg uridylylation on a functional CRE(2C) could lead to VPg nucleotidylation at the site of RNA replication initiation. The absence of the 2 uridine residues linked to VPg during CRE(2C) mediated uridylylation would prevent annealing to the 2 adenine residues at the 3' terminus of the viral negative RNA strand. In the second scenario, perhaps the 3D interaction with VPg in the absence of the native CRE(2C) stem-loop structure prevents formation of the native initiation complex that would confer specificity to the 3' end of the negative strand (82, 83).

Our findings that CVB-TD genomes evolve successfully and replace the initiating wt population completely only in primary cell cultures and terminally differentiated tissues

(9, 10, 13) was hypothesized to occur due to a relative lack of the host protein heterogeneous nuclear ribonucleoprotein C1 (hnRNPC1) which is found at high levels in the cytoplasm only in transformed and dividing cells (84-86) and which is necessary for efficient positive strand RNA replication (87, 88). Alteration in the 3' end of the negative strand (which occurs in CVB-TD populations) should decrease positive strand synthesis because the viral proteins 2C and 2BC (89, 90), along with hnRNPC1, bind negative strand RNA in this region and, upon interaction with viral protein 3CD<sup>pro</sup>, are thought to mediate initiation of positive strand replication (88). It has been hypothesized that in quiescent or terminally differentiated cells and tissues, wherein hnRNPC1 becomes sequestered in the nucleus, an absence of cytoplasmic hnRNPC1 hinders specific (proper) initiation of enteroviral positive strand synthesis. Improper initiation at sites downstream of the 3' terminus, due to a lack of hnRNPC1 in the replication complex, then leads to generation of 5' terminally deleted (TD) genomes (10). However, in HeLa cells, a transformed and rapidly dividing cell line, hnRNPC1 is present in the cytoplasm and therefore, TDs have not been observed: our previous work showed that CVB3-TD virus populations were not detectable even after repeated passages of CVB3 in HeLa cells (13). But as replication of CVB3-CKO generates 5' terminal deletions (described above) a role for the CRE(2C) in the correct initiation of positive strand RNA can be strongly inferred, even in cells with high levels of cytoplasmic hnRNPC1. From the observations that deletions of the 5' end of the genome or mutations in the CRE(2C) result in a similar level of viral RNA replication, and the presence of both lesions in a single genome do not have an additive effect upon the extent of replication, it is clear that both these sites are required for the process of efficient (and correct) positive strand initiation. These findings also indicate that the "fall back method" of initiation (a non-specific initiation), seen in non-dividing cells in culture and in tissues when cytoplasmic hnRNPC1 is lacking (6, 10, 13) and in this study when CRE(2C) is disrupted, is one in

which the 5' terminal deletions are generated. This implies that even in transformed cells with an abundance of cytoplasmic hnRNP1, that a functional CRE(2C) is required or the virus replicates with a nonspecific and much less efficient initiation of positive strand RNA.

At this juncture, we have shown that CVB3, a typical enterovirus, can replicate with low efficiency (compared to wt CVB3) without two of the four known *cis*-acting elements required for replication (17, 58): namely, domain I in the 5' NTR and the CRE(2C). Thus, replication in the absence of both domain I and the CRE(2C) has not been seriously considered to date due to the experimentally verified importance of these structures for HEV replication (19, 24, 45, 62). That said, it has been demonstrated that a loss of another HEV *cis*-acting replication element, the 3' NTR, does not prevent replication either (91, 92). At the 3' end of the genomic RNA in the poly A tract, a ribonucleoprotein complex forms with the binding of viral protein 3CD<sup>pro</sup> and the host protein poly(A) binding protein (PABP) (68). Despite the apparent replication requirement for protein binding in the 3' NTR for viral replication, Todd and colleagues (1997) demonstrated that both poliovirus and human rhinovirus 14 were replication competent, albeit with delayed growth phenotypes, when the 3' NTRs were entirely deleted (92). A more recent study elaborated on this work, demonstrating an approximate five-log decrease in plaque forming units per microgram of RNA following transfection of poliovirus T7 transcripts with deleted 3' NTRs, but only an approximate one-log decrease in infectivity of recovered virions (91). These findings demonstrated that at least one of the required *cis*-acting replication elements could be deleted from a HEV genome without lethality and that it has a relatively small impact on infectivity in recovered virus. In addition, Goodfellow and colleagues (2000) demonstrated that after introducing two and five mutations into the poliovirus CRE(2C), a return to a wt lytic

phenotype occurred after serial blind passage despite the fact that no CPE was observed upon initial transfection (19). The ability of these viruses to revert demonstrates that replication was occurring in the absence of a wt CRE(2C) structure and lends support to our findings that mutational disruption of the CRE(2C) is not lethal.

The poor extents of replication of the CVB3-TD strains as well as the CVB3-CKO and CVB3-TD50-CKO strains led us to investigate the use of transfecting viral genomic RNA into mice as an alternative approach to generating virus. We used only one transfection reagent, LIPID-based *In Vivo* Transfection Reagent (Altogen Biosystems), but from the results obtained here (**Fig. 5**), it would appear that this approach has potential for studying diverse *in vivo* aspects of the biology of these inefficiently replicating virus strains, as well as the host response to infection by such viruses, without having to laboriously produce virus stocks from cell culture. To the best of our knowledge, this represents the first report of generating infectious virus by i.p. lipid based transfection of animals with a picornaviral RNA, though others have previously described methods to inoculate mice with viral DNA or RNA. Direct injection of mice with polyoma virus DNA suspended in saline or TNE buffer (93, 94) and direct injection of HBV DNA in chimpanzees (95) has been useful for studying these DNA viruses without the necessity of preparing infectious virions. Further, direct injection of the tibialis anterior muscle of mice with CVB3 RNA suspended in saline demonstrated that wt virus could be recovered several days post inoculation (96). However, the method described in this report may be more efficient than the methods previously reported, as it uses a reagent designed for *in vivo* RNA transfection.

Specific initiation at the 3' end of the negative strand template is critical to positive strand RNA viruses in order to avoid loss of genome during replication. We demonstrated an increase in the extent of loss of 5' terminal sequence by passage in

cell culture and in animals of CVB3 with a small terminal deletion (13) which led to the conclusion that the persisting CVB3-TD viruses are an evolutionary dead end despite the ability to prolong enterovirus infection in tissues. Based on our findings, it is tempting to speculate that the mutant virus genomes described in this work represent what may have been one of any number of primitive ancestors at one time in the evolutionary development of the HEV. The HEV ancestral genome would have likely been a basic replicon which evolved CRE structures to generate more efficient replication and dissemination in the face of increasingly effective host antiviral defenses. It remains to be seen what else might be deleted from an HEV genome in terms of critical elements that affect replication and still have replication competency.

### Chapter 3

#### A uridylylation incompetent CRE(2C) of coxsackievirus B3 is viable and reverts to wildtype following passage in cell culture or mice.

##### Introduction

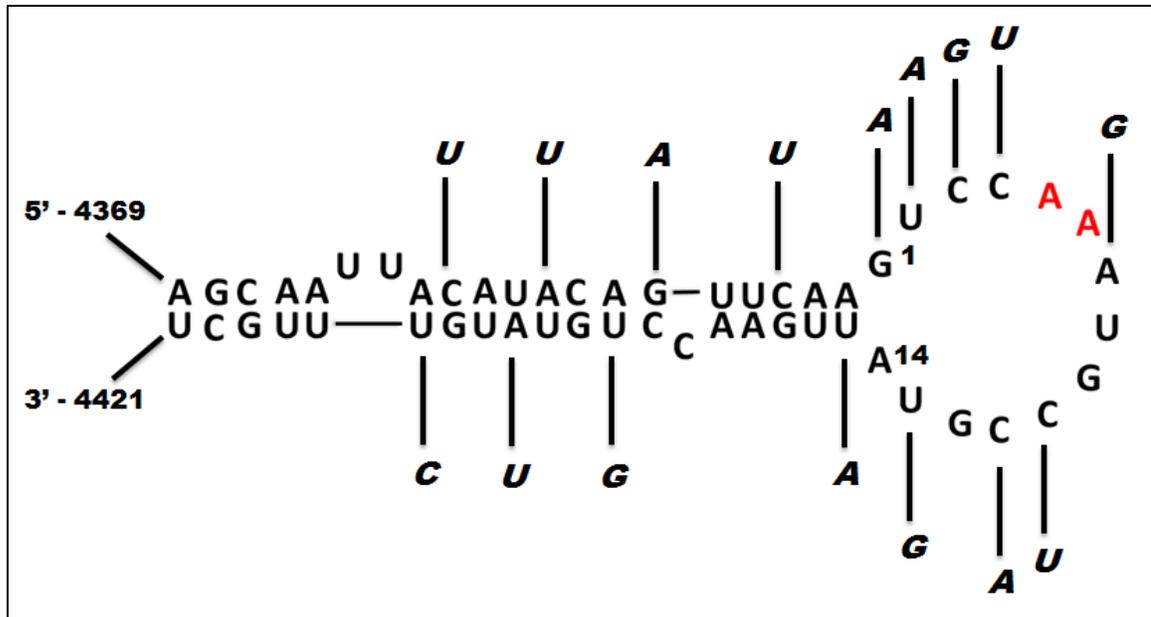
RNA virus populations exist as a quasispecies consisting of a spectrum (or swarm) of related genomes that are heterogeneous in nature (33). The variability of genetic sequence within the variant (mutant) spectrum allows for competition and selection of the fittest viruses in a given environment (33). These heterogeneous viral populations are generated within individual cells, groups of cells, and within infected organisms after experimental and natural infection (97-99) due to recombination and the error prone nature of the RNA-dependent RNA polymerase (RdRp) (34, 37, 100, 101). The error rate of the RdRp of RNA viruses is between 1,000,000-times higher than that of the DNA-dependent-DNA-polymerase replicating chromosomes in cells (55, 102).

The RNA viruses studied in our laboratory are the coxsackie B viruses (CVB; serotypes 1-6) which are small, non-enveloped, single-stranded RNA viruses classified in the human enterovirus genus of the *Picornaviridae* (55). The CVB have been etiologically associated with a variety of human diseases including type I diabetes, pancreatitis and myocarditis (4-8, 30). Previous work in this laboratory identified a persistence mechanism of the CVB *via* 5' terminal genomic deletions (CVB-TD) (10). The characterized populations of CVB-TD exhibited deletions ranging from 7-49 nucleotides, partially eroding domain I (10), the important *cis*-acting replication element (CRE) for efficient viral genome replication (25, 60-64). CVB-TD populations arise

following naturally-occurring human and experimental CVB infections of cell cultures or mice, thereby demonstrating that formation of TDs is a natural mechanism in the CVB (9, 10, 13). Poliovirus has also been found to persist for up to a year in the motor neurons of immunocompetent mice in the absence of cytopathic virus, suggesting (but not yet experimentally verified) that this HEV may also form TD populations (103).

Located within the 2C protein coding region of the HEV genome is a stem-loop structure termed CRE(2C), which has been shown to be the template for uridylylation of the viral protein primer VPg (14, 24, 73). Recently, we demonstrated that wildtype (wt) CVB3 was also replication competent when the CRE(2C) (**Fig. 6**) was mutationally disrupted [termed CVB3-CKO] (see chapter 2 of this dissertation). Prior to our studies, mutational disruption of the CRE(2C) in both poliovirus and CVB3 genomes had been considered to be lethal when studied using cell free assays to measure genome replication, VPg uridylylation through autoradiographic assays (24, 27, 45, 75), and by luciferase and cytopathic effect assays in BGM cells after short incubation times (24).

We demonstrated (chapter 2), however, that not only could infectious virus be isolated from cells electroporated with T7 RNA polymerase transcribed CVB3-CKO RNA but that recovered virions were inhibited by neutralizing antibody in cell culture, thus demonstrating the virions' infectivity (chapter 2). Another surprising finding from our cell culture work demonstrated that 5' terminal deletions (TDs) were occurring in CVB3-CKO after eight days of replication in HeLa cells (chapter 2); this was unexpected because generation of 5' terminal deletions do not occur during wt CVB infection of transformed cell lines such as HeLa cells (10). Further, we explored whether or not CVB3-CKO was infectious in mice using *in vivo* lipid transfection of infectious CVB3 T7 transcribed RNA (chapter 2). We demonstrated that CVB3-CKO RNA was detectable 20 days post transfection in both the spleens and hearts of mice and that infectious virions were



**FIG. 6. The CRE(2C) of CVB3 with mutations indicated.** The sequence of the CRE(2C) of CVB3 from nucleotides 4369-4421. Nucleotides changed to generate CVB3-CKO are indicated by italicized letters above the wt 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.

present using neutralizing antibody assays (chapter 2). The results of these mouse studies elaborated on and extended the cell culture findings, further illustrating that a functional CRE(2C) was not required by CVB3 for replication to occur (chapter 2).

The finding that CVB3-CKO was replication competent, and that 5' TDs were occurring at day eight post electroporation of HeLa cells (chapter 2), led us to question what was occurring to the mutated sequence in the CRE(2C) region of the genome: specifically, do reversions occur or do the mutations remain intact? In preliminary experiments to explore this question, we enzymatically amplified then sequenced across the CRE(2C) region of CVB3-CKO at day eight post electroporation of HeLa cells and observed that the CRE(2C) had completely reverted to a wt sequence. The finding that 16 mutations had completely reverted was so unexpected that we suspected an artifact and so repeated this same experiment numerous times, each time observing the same result. Clearly, the mutated CRE(2C) was reverting to the wt sequence.

Based on these findings, we hypothesized that during persistent replication in cell culture, high selective pressure drives the complete reversion of the mutated CRE(2C) to a wt sequence, thereby restoring the wt stem-loop structure, and that this occurs *following* evolution of 5' TDs in the majority population. Indeed, the data described below show that until eight days post electroporation, the 16 mutations introduced into the CRE(2C) largely remain intact in the majority of the genomes [(24); see chapter 2 of this dissertation]. Then, by 8 days post-electroporation, a majority population arises that has completely reverted to wt sequence. Further, we show by sequencing various ratios of complementary DNA (cDNA) clones that at a 4:1 ratio of wt:CVB3-CKO, only wt sequence can be detected. This control demonstrated that a relatively low ratio of wt revertants amongst a larger population of the mutated CKO would not be detectable by standard sequencing and thus a significant amount of reversion could be - and indeed is

- generated prior to the time that the reverted CRE(2C) was detectable. Finally, we show that TDs evolve in the majority population prior to the time of complete reversion of the CKO to wt sequence, thereby preventing the detectable presence of CPE that would be expected in cell culture were the CRE(2C) to revert with an intact 5' genomic terminus.

## **Materials and Methods**

**Cells and virus.** Coxsackievirus B3/28 (30) is the wt virus used in this work. Mutations were engineered in the CRE(2C) region of the wt or CVB3-TD50 cDNA clones as described previously (chapter 2) to create the CRE(2C)-knock-out (CKO) strains, CVB3-CKO and CVB3-TD50-CKO respectively. HeLa cell monolayer cultures were maintained as described previously (chapter 2). Viral stocks were prepared by electroporation of HeLa cells with T7 transcribed RNA from infectious cDNA clones of each viral genome and, following incubation for 1 to 8 days (CVB3-CKO) or 8 days (CVB3-TD50, CVB3-TD50-CKO, and wt CVB3), virus was biophysically purified as previously described (chapter 2).

**Transfection of mice with viral T7 transcribed RNA.** The University of Nebraska Medical Center Institutional Animal Care and Use Committee approved the protocol and use of mice for transfection studies. Male A/J mice (6-7 weeks old; Jackson Laboratory, Bar Harbor, ME) were inoculated intraperitoneally (i.p.) with CVB3-CKO T7 transcripts as previously described (chapter 2). Spleens and hearts were excised at 20 days post-transfection and prepared as previously described (chapter 2). Total RNA was extracted

from tissues by homogenization in 500  $\mu$ L TRizol (Invitrogen; Carlsbad, CA) and assayed as described below in “enzymatic amplification of the CRE(2C) sequence”.

**Enzymatic amplification of the CRE(2C) sequence from virus preparations and**

**mouse tissues.** To assay for reversions in the CRE(2C) region of CVB3-CKO and

CVB3-TD50-CKO, cDNA was transcribed from virus 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]; wt CVB3 [pass 3, 8 days post-

electroporation]) and from day 20 post-transfection mouse tissues (CVB3-CKO only)

with primer 4523Rev (**Table 1**) at a final concentration of 2  $\mu$ M using Improm II reverse

transcriptase (Promega, Madison, WI) as previously described (chapter 2). The

CRE(2C) region was amplified using nested primers in 20  $\mu$ L reaction volumes

containing GoTaq Green Master Mix (Promega) as described previously (chapter 2).

Briefly, cDNA was amplified in initial enzymatic reactions with primers 4523Rev and 2B1

at a final concentration of 0.5  $\mu$ M each (**Table 1; 2**), followed by ethanol precipitation and

amplification with primers 2C2 and 2C7 (**Table 1; 2**). For virus preparations at days 6, 7

and 8 post-electroporation, two concurrent amplifications with primers 2C2 and 2C7

were carried out in addition to, and in *lieu* of, the nested PCR described above; this

yielded better sensitivity at these time-points which had scant RNA for cDNA

preparation. PCR reactions were electrophoresed on 2% agarose gels and bands at the

appropriate size (**Table 2**) were excised and purified using the Zymoclean Gel DNA

Recovery Kit (Zymo Research; Irvine, CA) as previously described (chapter 2).

Reactions were sequenced at the UNMC core facility.

**Nested RT-PCR for detection of viral RNA in virus preparations.** To assay for viral RNA in ultracentrifuged sucrose purified virus preparations, nested RT-PCR was performed as previously described (chapter 2). Briefly, cDNA was primed with E3 (**Table 1**) as described in “enzymatic amplification of the CRE(2C) region” followed by enzymatic amplification using primers S4 and E3Sub (**Table 1; 2**) in GoTaq Green Master Mix (Promega). Reactions were ethanol precipitated and a second PCR was carried out using primers S5 and SReturn (**Table 1; 2**). Final PCR products were analyzed on 2% agarose gels.

**Quantitation of CVB3-CKO replication at various time points and titering of viral RNA in purified virus stocks using RT-qPCR.** Mutant viruses (CVB3-CKO, CVB3-TD50, and CVB3-TD50-CKO) do not cause CPE in tissue culture monolayers, therefore viral RNA copy number was quantitated using RT-qPCR as described previously [chapter 2; (10)] as a measurement of virion titer. cDNA was transcribed with primer E3 (**Table 1**) as described in “enzymatic amplification of the CRE(2C) region” above. As previously described (chapter 2), 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 (Thermo Scientific). Reaction parameters were as previously described (chapter 2) and as indicated (**Table 1; 2**)

**RT-PCR to detect 5' terminal deletions in purified virus stocks.** To determine the point at which 5' terminal deletions occur over a time-course of CVB3-CKO replication, cDNA was primed with E3 (**Table 1**) as described in “enzymatic amplification of the CRE(2C) region” above. To ensure results were comparable, reverse transcriptase

reactions were assembled with an equal RNA copy number calculated as previously described (chapter 2). Tagged primers [(78); chapter 2] were used to detect 5' terminal deletions. Briefly, initial enzymatic amplification was carried out using Pin5End1 and E3 (**Table 1; 2**) and 500 copies of E3 cDNA or Pin5End5 and E3 (**Table 1; 2**) with 1,500 copies (near the experimentally determined limit of detection for the respective primer pairs), in GoTaq Green Master Mix (Promega), followed by ethanol precipitation and a second round of enzymatic amplification using primers Pin and SReturn (**Table 1; 2**). PCR products were analyzed on 2% agarose gels.

## **Results**

**The 16 CKO mutations have completely reverted to the wt sequence by 8 days post-electroporation of HeLa cells.** Our previous findings (chapter 2) demonstrated that CVB3-CKO and CVB3-TD50-CKO were both viable in cell culture following electroporation of T7 transcribed RNA, and that CVB3-CKO was viable in mice following transfection of T7 RNA polymerase transcribed RNA. Further, it was demonstrated that CVB3-CKO from purified virus preparations, or from mouse tissue homogenates, could be passaged in cell culture and infection was inhibited by anti-CVB3 neutralizing antibodies (chapter 2). The fact that this virus was replicating despite the findings of others (24, 45), led us to question whether or not the 16 mutations introduced in the CRE(2C) region of CVB3-CKO remained present, or if reversions were occurring in this region after several rounds of replication in either cell culture or within the tissues of transfected mice. To answer this question, cDNA was prepared using viral RNA extracted from either purified virus preparations (CVB3-CKO or CVB3-TD50-CKO [wt CVB3 and CVB3-TD50 were tested as controls]) or from mouse tissues at 20 days post

transfection (CVB3-CKO) and the CRE(2C) region was enzymatically amplified and sequenced; wt and CVB3-CKO clones were used as controls. Alignments of amplified CRE(2C) sequences were subsequently conducted (**Fig. 7**).

The sequence of the total amplicon population indicated that at days 1 (**Fig. 7A row 5**), 3 (**Fig. 7A row 6**), and 5 (**Fig. 7A row 7**) post electroporation of HeLa cells with CVB3-CKO T7 transcribed RNA, the 16 mutations made in the CRE(2C) region remained intact in the purified CKO virus when compared to the wt cDNA clone and T7 transcribed RNA (**Fig. 7A row 1;2 respectively**) or the CVB3-CKO cDNA clone and T7 transcribed RNA (**Fig. 7A row 3;4 respectively**). These data demonstrated that reversions had not occurred in the majority CVB3-CKO population up to 5 days post-electroporation of HeLa cells; that is to say, the majority population still appeared to be mutant by sequence analysis. Surprisingly, by day 8 post electroporation, the 16 mutations had appeared to completely revert to that of the wt sequence (**compare Fig. 7A row 8;9 to Fig. 7A row 1;2 [wt] and Fig. 7A row 3;4 [CVB3-CKO]**). This was repeated using 5 different purified CVB3-CKO virus preparations from day 8 post-electroporation with the same results (two representative sample sequences are shown in **Fig. 7A**). To determine whether reversions were present in the tissues of mice at day 20 post transfection, the CRE(2C) region was amplified using the same method described for the purified virus preparations. Analysis of sequences from day 20 post-transfection spleen and heart demonstrated that complete reversion to the wt sequence had occurred in the CRE(2C) region of CVB3-CKO (**compare Fig. 7A row 10 and Fig. 7A row 11 respectively, to Fig. 7A row 1;2 and Fig. 7A row 3;4**). These data were repeated by assaying a different mouse heart and spleen (data not shown). A representative selection of sequencing chromatograms from days 1, 5, and 8 post-

A.

## CRE(2C) Loop

1. AAG AAG ATG AGC AAT TAC ATA CAG TTC AAG TCC AAA TGC CGT ATT GAA CCT GTA TGT TTG CTC CTG  
 2. AAG AAG ATG AGC AAT TAC ATA CAG TTC AAG TCC AAA TGC CGT ATT GAA CCT GTA TGT TTG CTC CTG  
 3. AAG AAG ATG AGC AAT TAT ATT CAA TTT AAA AGT AAG TGT AGG ATA GAA CCG GTT TGC TTG CTC CTG  
 4. AAG AAG ATG AGC AAT TAT ATT CAA TTT AAA AGT AAG TGT AGG ATA GAA CCG GTT TGC TTG CTC CTG  
 5. AAG AAG ATG AGC AAT TAR ATT CAA TTT AAA AGT AAG TGT AGG ATA GAA CCG GTT TGC TTG CTC CTG  
 6. AAG AAG ATG AGC AAT TAT ATT CAA TTT AAA AGT AAG TGT AGG ATA GAA CCG GTT TGC TTG CTC CTG  
 7. AAG AAG ATG AGC AAT TAT ATT CAA TTT AAA AGT AAG TGT AGG ATA GAA CCG GTT TGC TTG CTC CTG  
 8. AAG AAG ATG AGC AAT TAC ATA CAG TTC AAG TCC AAA TGC CGT ATT GAA CCT GTA TGT TTG CTC CTG  
 9. AAG AAG ATG AGC AAT TAC ATA CAG TTC AAG TCC AAA TGC CGT ATT GAA CCT GTA TGT TTG CTC CTG  
 10. AAG AAG ATG ASC AAT TAC ATA CAG TTC AAG TCC AAA TGC CGT ATT GAA CCT GTA TGT TTG CTC CTG  
 11. AAG AAG ATG AGC AAT TAC ATA CAG TTC AAG TCC AAA TGC CGT ATT GAA CCT GTA TGT TTG CTC CTG  
 12. AAG AAG ATG AGC AAT TAC ATM CAG TTC AAG TCC AAA TGC CGT ATT GAA CCT GTA TGT TTG CTC CTG  
 13. AAG AAG ATG AGC AAT TAC ATA CAG TTC AAG TCC AAA TGC CGT ATT GAA CCT GTA TGT TTG CTC CTG

1. wt plasmid clone
2. wt T7 transcribed RNA
3. CVB3-CKO plasmid clone
4. CVB3-CKO T7 transcribed RNA
5. CVB3-CKO 1 day postelectroporation
6. CVB3-CKO 3 days postelectroporation
7. CVB3-CKO 5 days postelectroporation
8. CVB3-CKO 8 days postelectroporation I
9. CVB3-CKO 8 days postelectroporation II
10. CVB3-CKO mouse heart 20 days posttransfection
11. CVB3-CKO mouse spleen 20 days posttransfection
12. CVB3-TD50CKO 8 days post electroporation
13. CVB3-TD50 8 days post electroporation

5'

3'



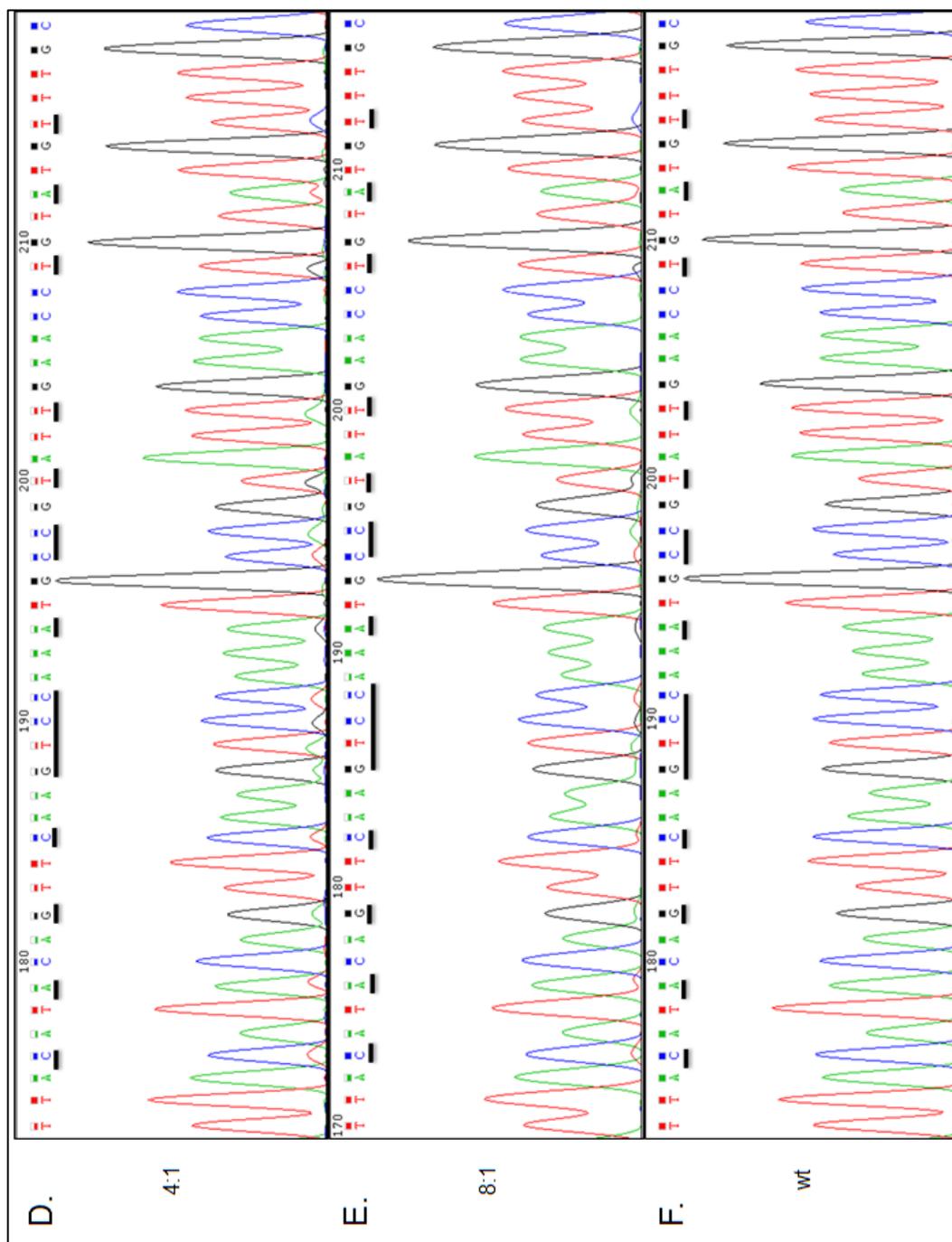
**FIG. 7. The CRE(2C) of CVB3-CKO completely reverts to a wt sequence by 8 days post-electroporation in HeLa cells and by 20 days post-transfection in mouse tissues.** (A) Sequence alignments of cDNA clones (1 [wt CVB3] and 3 [CVB3-CKO]), T7 transcribed RNA from cDNA clones (2 [wt CVB3] and 4 [CVB3-CKO]), 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 1], 9 [CVB3-CKO 8 days post-electroporation; virus preparation 2], 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 wt sequence and bold, dot underlined sequence indicates CKO mutations. By 8 days post-electroporation of HeLa cells with CVB3-CKO T7 transcribed RNA, the CRE(2C) sequence had completely reverted to wt (compare A5-7 [days 1, 3, and 5 post-electroporation] to A8 and A9 [two different 8 day virus preparations] or controls A1;2 [wt CVB3 clone and T7 transcript respectively] and A3;4 [CVB3-CKO clone and T7 transcript respectively]). This was also observed in mouse tissues at 20 days post-transfection with CVB3-CKO (A10 [heart] and A11 [spleen]) and at 8 days post-electroporation of HeLa cells with CVB3-TD50-CKO (A12). (B-D) Representative sequence chromatograms from CVB3-CKO at (B) 1 day post-electroporation of HeLa cells, (C) 5 days post-electroporation, and (D) 8 days post-electroporation demonstrating that sequence reads were clean.

electroporation of cell culture demonstrate that sequencing reads were clean (**Fig. 7B-C**). These results supported and extended the findings observed in cell culture, demonstrating that complete reversion to wt sequence was occurring in the CRE(2C) of CVB3-CKO. Because we observed a complete reversion of the CRE(2C) in CVB3-CKO after 8 days of replication in HeLa cells, and after 20 days in the tissues of transfected mice, we wished to determine if reversions were occurring in the CVB3-TD50 population when the CRE(2C) was mutated in the same way (CVB3-TD50-CKO). Using CVB3-TD50 as a control, analysis was conducted on CVB3-TD50-CKO and CVB3-TD50 as described for CVB3-CKO. Based on sequence analysis at 8 days post-electroporation of HeLa cells, a complete reversion of the CRE(2C) to wt sequence occurs in the CVB3-TD50-CKO population as well (**compare Fig. 7A row 12 to Fig. 7A row 13 and Fig. 7A row 12 to Fig. 7A row 1;2 [wt] and Fig. 7A row 3;4 [CVB3-CKO]**). These findings demonstrated that, regardless of whether the 5' terminus was intact or not when replication began, sequences within the CRE(2C) were indistinguishable from that of the wt CVB3 by 8 days post-electroporation in HeLa cells (CVB3-CKO and CVB3-TD50-CKO) or in the spleens and hearts of mice at day 20 post-transfection (CVB3-CKO).

**Standard Sanger sequence analysis is an inefficient assay for the presence of accumulating mutations in a mutant swarm.** We observed complete reversion to wt sequence in the CRE(2C) after 8 days of replication in HeLa cells, although prior to this time, the sequence of amplified cDNA showed only the induced mutations. We hypothesized that prior to the appearance of the complete reversion of 16 nt at 8 days, a growing population of revertants was present within the mutant cloud but that the dominant population masked detection of these changes. To test this hypothesis, a simple experiment was performed in which 2-fold increases of wt CVB3 cDNA clone

(from  $1.62 \times 10^9$  copies to  $1.30 \times 10^{10}$  copies) were mixed with a constant copy number of CVB3-CKO clone ( $1.62 \times 10^9$  copies) providing ratios of 1:1 to 8:1. Reaction mixtures were sequenced using primer 2C7 (**Table 1**), after which sequencing chromatograms were compared (**Fig. 8**). When comparing the CVB3-CKO clone (**Fig. 8A**) to the wt CVB3 clone (**Fig. 8F**), the sequences were as expected and confirmed that the expected CKO mutations were present in the CVB3-CKO clone used in these studies. At a DNA copy ratio of 1:1 (wt:CVB3-CKO [**Fig. 8B**]) it is also apparent that both sequences are detected equally as demonstrated by the presence of two peaks corresponding to two different nucleotides in the sequencing chromatogram. This indicates that at a copy ratio of 1:1 in an amplicon population, both sequences would be readily detectable. At a copy ratio of 2 wt to 1 CVB3-CKO (**Fig. 8C**), it is evident that the mutant sequence is already partially obscured by the two-fold majority wt sequence; this is indicated by the finding that 10 of the 16 mutations appear as wt in the chromatogram, although two peaks are still evident at each mutation (**Fig. 8C, arrows**). At a ratio of 4:1 (**Fig. 8D**), the 16 CKO mutations are nearly too completely masked by the wt sequence with only minor secondary peaks detected in the sequencing chromatogram in these regions. No difference in sequence chromatograms was observed when comparing a ratio of 4:1 and 8:1 (**compare Fig. 8D [4:1] to Fig. 8E [8:1]**). This indicates that when exploring a potentially mixed population, such as would be expected after days of replication in cells), only the majority population would be detectable using standard sequencing and that a majority population would need to be present at least at a 25% level in order to be detected.





**FIG. 8. 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 wt CVB3 and CVB3-CKO, with the concentration of CVB3-CKO being held constant and the concentration of wt CVB3 increasing by 2-fold to a final ratio of 8:1; underlined nucleotides indicate the 16 nucleotides mutated to generate CVB3-CKO from wt. (A) The DNA sequence of the CVB3-CKO CRE(2C) region using primer 2C7 (Table 1). (B) A 1:1 ratio of wt: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 wt sequence begins to obscure and mask the CKO sequence with only 6 of the 16 CKO mutations detectable (red arrows). (D-F) At a ratio of 4:1 wt:CVB3-CKO (D) and 8:1 wt:CVB3-CKO (E), the CKO mutations are no longer detectable as compared to the wt cDNA clone sequence chromatogram (F). Therefore, when 1 in 4 molecules contains the mutant sequence (CVB3-CKO) it is no longer detectable by standard sequencing.

**Contamination with wt virus or T7 transcribed RNA is unlikely.** To test the hypothesis that contamination may have been responsible for our observations, we reasoned that wt CVB3 genomes, if co-transfected with the replication impaired CVB3-CKO genomes, would result in rapid dominance of wt replication which would be apparent by the induction of CPE. This was tested by transfecting wt (cytopathic) CVB3 T7 transcribed RNA into HeLa cell monolayers in increasing amounts (10, 100, and 1,000 copies), while holding the copy number of (non-cytopathic) CVB3-CKO T7 transcribed RNA constant at  $1.97 \times 10^{11}$  copies. Previous work by Crowder and Kirkegaard (74) demonstrated that when one or two mutations were made in the loop of the CRE(2C) of poliovirus, a dominant negative effect occurred during co-infection with wt poliovirus. However, when multiple mutations throughout the stem and loop of the CRE(2C) were introduced, no inhibition of wt replication occurred (74). Based on these findings we did not expect a dominant negative effect on the replication of wt from a complete (16nt) disruption of the CRE(2C) as in CVB3-CKO. HeLa cell monolayers were observed for the presence of CPE every 24 hours for a period of 5 days. Complete CPE was observed in all monolayers transfected with 100 and 1,000 copies of wt CVB3 RNA in the presence of CVB3-CKO RNA, and in all wt RNA only control wells transfected regardless of the RNA copy number, after 24 hours of replication (**Table 3**). In contrast, CPE was not observed in wells transfected with CVB3-CKO RNA alone or in wells transfected with CVB3-CKO RNA and 10 copies of wt T7 transcribed RNA at 5 days post-transfection (**Table 3**). However, CPE was evident after two blind passages of frozen-thawed lysates to fresh cell cultures from the 10 wt CVB3 RNA copies plus CVB3-CKO RNA. In no case (1-3 blind passages) did lysates from CVB3-CKO RNA only cells cause CPE on fresh cell cultures. These results demonstrated that CVB3-CKO virus preparations which were assayed for reversions were not contaminated with wt CVB3, and supported our earlier observations that CVB3-CKO purified virus prepar-

**Table 3. Experimental contamination of CVB3-CKO T7 transcribed RNA transfections with wt CVB3 T7 transcribed RNA demonstrate wt CVB3 rapidly becomes dominant.**

T7 transcribed RNA Input (CVB3-CKO constant at $1.97 \times 10^{11}$ )	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 <u>only</u>	<u>NO</u>	<u>NO</u>	<u>NO</u>	<u>NO</u>	<u>NO</u>
CVB3-CKO + 10 copies wt CVB3	<u>NO</u>	<u>NO</u>	<u>NO</u>	<b>YES</b>	Not Tested
CVB3-CKO + 100 copies wt CVB3	<b>YES</b>	<b>YES</b>	<b>YES</b>	<b>YES</b>	Not Tested
CVB3-CKO + 1,000 copies wt CVB3	<b>YES</b>	<b>YES</b>	<b>YES</b>	<b>YES</b>	Not Tested
wt CVB3 <u>only</u> $1.97 \times 10^{11}$ copies	<b>YES</b>	<b>YES</b>	<b>YES</b>	<b>YES</b>	Not Tested
wt CVB3 <u>only</u> 10 copies	<u>NO</u>	<b>YES</b>	<b>YES</b>	<b>YES</b>	Not Tested
wt CVB3 <u>only</u> 100 copies	<b>YES</b>	<b>YES</b>	<b>YES</b>	<b>YES</b>	Not Tested
wt CVB3 <u>only</u> 1,000 copies	<b>YES</b>	<b>YES</b>	<b>YES</b>	<b>YES</b>	Not Tested

ations do not induce CPE upon passage (chapter 2). Further, previous work by de la Torre *et al.* suggested that, when studying rapid reversion in poliovirus, wt virus would have to contaminate viral preparations with equal efficiency and at very high levels for contamination to be a considerable explanation (43). Contamination of primers was also ruled out by conducting 10 replicate enzymatic amplifications containing only water, enzyme mix, and primers 2C2 and 2C7 (**Table 1;2**), all of which were negative when assayed on 2% agarose gels (data not shown).

**Compensatory mutations are extremely unlikely in the stem of CRE(2C) in CKO viruses.** The mutations introduced into the CRE(2C) were synonymous (third-base mutations within codons in the protein 2C coding region). However, those nucleotides which paired with the mutated bases held either first or second positions in codons within the 2C region and, therefore, we did not expect compensatory mutations to occur. To explore whether or not compensatory mutations could occur within the stem of the CRE(2C) in CVB3-CKO, our analysis of possible compensatory mutations in this region of all HEV-B genomes was conducted using GenBank (**Fig. 9**). The presence of compensatory mutations is unlikely in the stem of the CRE(2C) because all compensatory mutations would alter amino acid coding. This is supported by the finding that only one compensatory mutation has been reported in the HEV-B 2C protein, causing a E<sub>121</sub>K change (**Fig. 9A,B; boxes and arrows**). Compensatory mutations were not addressed in the loop region of the CRE(2C) as there are no defined binding partners for nucleotides in the loop (**Fig. 9B**); therefore, we were unable to predict possible compensatory mutations in this region of the CRE(2C). However, upon analysis of 35 random EV-B sequences in GenBank, no deviation from the sequence of



**FIG. 9. 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. Sequences are of cDNA copies of viral RNA deposited in GenBank. All compensatory mutations would affect the amino acid sequence of the 2C protein. Only 1 compensatory mutation was reported to exist in 429 HEV-B sequences examined (box and arrows). (B) Location of the compensatory mutation in the CRE(2C) secondary structure (box and arrow).

the CRE(2C) loop (**Fig 9B**) of wt CVB3/28 was found (data not shown).

**5' terminal deletions occur prior to complete CRE(2C) reversion during low-level persistent replication of CVB3-CKO.** Our previous work demonstrated that by day 8 post electroporation of HeLa cells, the CVB3-CKO had lost up to 20 nucleotides from the 5' genomic terminus in the majority (detectable at our level of sequencing sensitivity) population (chapter 2). This phenomenon has also been observed to occur naturally in the CVB after experimental inoculation of mice and primary cell cultures and after natural infection in humans, leading to a persistent, low-level infection (9, 10, 13). Replication of CVB3-CKO is also a low-level persistent replication (**Fig. 10A**) which results in complete reversion of the 16 mutations introduced in the CRE(2C) stem and loop overtime. Because, we observed that by day 8 post-electroporation in HeLa cells, the mutations introduced into the CRE(2C) of CVB3-CKO had completely reverted to wt in the majority population, we asked if 5' TDs occurred prior to reversion of the CRE(2C). We expected that this would be the case; if reversion occurred prior to the generation of 5' TDs, the population of CVB3-CKO would effectively become wt [with a natural CRE(2C) and intact 5' genomic termini] and CPE would then be detectable in cell culture. Notably, CPE was not observed with CVB3-CKO in either of these experimental conditions (chapter 2). Therefore, we analyzed the 5' genomic terminus of purified virus preparations of CVB3-CKO at days 1, 3, 5, 6, 7, and 8 post electroporation as described above and previously (chapter 2) to test if 5' terminal genomic deletions were occurring.

These results demonstrate that at days 1 and 3 post-electroporation, viral RNA from purified CVB3-CKO virus preparations still has an intact 5' genomic terminus from nucleotides 1-10 when compared to the wt CVB3 virus control or the CVB3-CKO T7



**FIG. 10. 5' terminal deletions occur between days 3 and 5 post electroporation of HeLa cells with CVB3-CKO T7 transcribed RNA.** (A) A time-course prepared using RT-qPCR and a minimum of 4 replicate points per time-point. The data demonstrate the low-level persistent replication of CVB3-CKO during which 5' terminal deletions occur prior to reversion of the CKO mutations in the CRE(2C) to wt sequence. (B-D) 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 (B-D lanes 2, 5, 8, and 11) Pin5End5 and E3 corresponding to nucleotides 15-25 (B-D 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 (B-D lanes 4, 7, 10, and 13), then subsequently amplified with primers S5 and SReturn was performed. 500 (Pin5End1; S4) or 1,500 (Pin5End5) copies of cDNA prepared from either viral RNA or T7 transcribed RNA (control) were used in each PCR reaction. Right facing arrows indicate the loss of sequence from the 5' terminus of CVB3-CKO by day 5 post-electroporation and later (lane B8;9 [5 days post-electroporation], lane B11;12 [6 days post-electroporation], lane C2;3 [7 days post-electroporation] and lanes C5;6 and C8;9 [2 different virus preparations at day 8 post-electroporation]) as compared to wt virus and T7 transcript controls (left facing arrows, lanes C11;12 [wt] and D11;12 [T7 transcript]) or to CVB3-CKO at days 1 and 3 post-electroporation (left facing arrows, lanes B2;3 [1 day post-electroporation] and B5;6 [3 days post-electroporation]). However, CVB3-CKO virus could be detected in all virus preparations by RT-PCR priming in a region of the 5' NTR not found to delete (compare lanes B4 [CVB3-CKO 1 day post –electroporation], B7 [CVB3-CKO day 3], B10 [CVB3-CKO day 5], B13 [CVB3-CKO day 6], C4 [CVB3-CKO day 7], C7 [CVB3-CKO day 8 preparation 1], and C10 [CVB3-CKO day 8 preparation 2] to lanes C13 [wt virus control] and D13 [T7 transcript control]). Controls (lanes C2-4, no cDNA PCR controls, lanes C5-7 no template RT-PCR controls, lanes C8-10 tissue culture controls) were negative.

transcript control (**compare left facing arrows Fig. 10B lane 2 [1 day] and Fig. 10B lane 5 [3 days] to Fig. 10C lane 11 [wt virus] and Fig. 10D lane 11 [T7 transcript control]**) and from nucleotides 15-15 (**arrows Fig. 10B lane 3 [1 day] and Fig. 10B lane 6 [3 days] to Fig. 10C lane 12 [wt virus] and Fig. 10D lane 12 [T7 transcript control]**) as expected. However, by day 5 post-transfection and later, the CVB3-CKO population has evolved to a TD population (**compare Fig. 10B lanes 8 and 9 [5 days], Fig. 10B lanes 11 and 12 [6 days], Fig. 10C lanes 2 and 3 [7 days], Fig. 10C lanes 5 and 6 [8 days preparation 1], and Fig. 10C lanes 8 and 9 [8 days preparation 2] to Fig. 10B lanes 2 and 3 [1 day], Fig. 10B lanes 5 and 6 [3 days], Fig. 10C lanes 11 and 12 [wt virus], and Fig. 10D lanes 11 and 12 [T7 transcript control]**) with the expected wt band no longer detectable. That virus was present in all RNA preparations, was verified using primers that anneal in a region of the 5' NTR that has not been found to delete, even when the 5' genomic terminus was not detected, using nested RT-PCR for detection of viral RNA in virus preparations (**compare Fig. 10B lane 4 [1 day], Fig. 10B lane 7 [3 days], Fig. 10B lane 10 [5 days], Fig. 10B lane 13 [6 days], Fig. 10C lane 4 [7 days], Fig. 10C lane 7 [8 days preparation 1], Fig. 10C lane 10 [8 days preparation 2], and Fig. 10C lane 13 [wt] to Fig. 10D lane 13 [T7 transcript control]**).

When compared to sequencing data (**Fig. 7**), these results show that 5' TDs are occurring prior to detection of complete reversion of the CRE(2C) to that of a wt sequence (**compare Fig. 10 demonstrating loss of 5' genomic terminus between days 3 and 5 post-electroporation [Fig. 10B lanes 5 and 6; Fig. 10B lanes 8 and 9 respectively] to Fig. 7 demonstrating that at 5 days post-electroporation the majority population remains a CKO population [Fig. 7 row 7]**).

## Discussion

The results described above present a surprising finding that was not expected when this work began: all 16 mutations introduced in the CVB3 CRE(2C) region (CVB3-CKO), revert completely to the wt sequence after a relatively short time of replication in HeLa cells. These reversions from the mutated CRE(2C) (CKO) to the wt CRE(2C) sequence could occur, at least in part, after several rounds of replication due to a high selective pressure on the structure in the full-length genome (CVB3-CKO); this would be due to the requirement of uridylylated VPg for replication of said full-length genome. However, from previous work (chapter 2), we know that by 8 days post-electroporation in HeLa cells, the majority CVB3-CKO population has developed into a TD population, and so reversions related to the requirement for VPg uridylylation are unlikely. The reason for this is that it has been demonstrated that while the CVB-TD still have VPg covalently linked to the 5' genomic terminus, these viruses can terminate in dinucleotides other than the canonical UU donated by VPg, suggesting that uridylylation of VPg is not occurring (10) or if it is, it is inefficient. Further, work has demonstrated that CVB-TD do not uridylylate VPg at detectable levels in cell free systems (J. B. Flanagan, personal communication). This finding further supports the hypothesis that CRE(2C) mediated uridylylation of VPg is not required by CVB-TD populations, and suggests that VPg is nucleotidylated either in-situ during replication of CVB-TD viruses, or is variably nucleotidylated at low levels using alternative templates. Cumulatively, these results suggest that selective pressure driving the complete reversion in the CRE(2C) sequence is likely related to another, and as yet undetermined, function of this structure in the life-cycle of TD viruses. The finding that the CRE(2C) reverts to a wt sequence in CVB3-TD50CKO after as few as 8 days of replication in HeLa cells (**Fig. 7-A row 12**), further supports the likelihood of other functions for this structure.

While it is difficult to accept that 16 mutations could suddenly revert *en masse*, the process must be understood as a kinetic process, not a sudden accretion of reversions (31, 102, 104, 105). The reversion of the 16 sites is extremely unlikely to be a saltatory jump but one that must occur over time, "suddenly" appearing (on day 8 post-electroporation) when one of the outliers in the quasispecies cloud of mutants attains complete reversion: at that point, it can replicate more rapidly than the others and so becomes the new master sequence. Several reports have demonstrated rapid mutation and reversion rates of RNA viruses in diverse environments. De la Torre and colleagues demonstrated that as many as 5 mutations introduced into the 3AB domain of poliovirus rapidly and completely reverted, after 5 days of incubation in HeLa cell culture, with no detection of intermediate populations in sequence space, a finding that could not be sufficiently explained at the time (43). Earlier work also demonstrated rapid reversion of single-nucleotide mutations in the 2C coding region which had caused guanidine-resistance in poliovirus, leading to a wt guanidine-sensitive phenotype in HeLa cells (44). Mutations in other RNA viruses have also been demonstrated to revert in various environments. Human immunodeficiency virus, though replicating differently than other RNA viruses *via* a DNA intermediate, was reported to revert (from a drug-resistant phenotype) a total of 52 mutations found in the reverse transcriptase and protease genes after a period of 50 days in infected persons undergoing therapy with drugs unrelated to those which induced the mutations (106). Another report demonstrated reversion to a consensus sequence in structurally conserved regions of HIV that was apparently more rapid than the forward mutation rate observed during chronic infection, occurring at an average of 1.2% per year (reversions) compared to 0.2% per year during the chronic stage of infection (forward mutations) (41). Interestingly, even in a very small RNA plant viroid (359nt; potato tuber spindle viroid), induced mutations that affected base pairing in hairpin structures found in the circular genomic molecule rapidly

reverted to wt after a single passage in the host (107). While likely a different mechanism than in RNA viruses, even a small DNA bacteriophage with a much less error prone replication than RNA viruses, due to the intrinsic exonuclease activity of DNA polymerases, has the capacity to naturally accrue as many as 14 mutations overall in a period of 10 days in bacterial culture (108). Together, these findings are consistent with, and support the validity of, our observation of rapid reversion of the 16 mutations introduced into the CRE(2C) of CVB3 which, when present, greatly decrease viral replication compared to wt (chapter 2).

Prior to our work (chapter 2), mutational disruption of the CRE(2C) was considered lethal for an HEV. In work done by van Ooij and colleagues exploring mutational disruption of the CRE(2C) of CVB3, both single and multiple mutations introduced in the CRE(2C) region of the genome deleteriously affected important aspects of viral replication (24). Mutations made in the loop of the CRE(2C) led in large part to a loss of detection of VPg uridylylation using autoradiographic assays and, further, led to a loss of luciferase activity in the construct used to explore the effect of these mutations (24); exceptions to this were changes to U<sub>8</sub> and A<sub>14</sub> (counting around the [CRE(2C)] loop from left to right [**see Fig. 6**]), suggesting that any mutations made in the CRE(2C) which affected function were lethal. Important to note is that the mutations assayed in these studies were not mutations that were part of the 16 nucleotide changes used in our experiments (reported here or in chapter 2) or by van Ooij and colleagues (24) when studying replication of CVB3 with a completely disrupted CRE(2C). Finally, work examining complete disruption of the predicted CRE(2C) secondary structure demonstrated that negative strand synthesis was occurring as evidenced by detection of double-stranded RNA (replicative form; RF) on autoradiographic assays from cell free systems (24, 29, 45). An altered positive to negative strand ratio during the replication of

mutant viruses was observed with the CVB-TD (10) and found to occur in CVB3-CKO (chapter 2). In a cell-free system, which lacks the natural and complete compartmentalization that occurs during viral replication in cells, this would generate equivalent amounts of positive (genomic) and negative (replicative intermediate) strand RNA leading to detection of only RF. This was not observed (24, 45) with wt replication in these systems due to the highly efficient transcription of positive strand RNA as would be expected. Despite the conclusions that CRE(2C) mutations were lethal (24), we suspect that replication was in fact occurring in CRE(2C) mutants studied in the cell free systems, and combined with our data demonstrating that CVB3-CKO is replication competent (chapter 2), would support our finding reported here that reversions are possible.

Others have explored the effects of mutational disruption of the CRE(2C) (19, 24, 74). For example, it has been demonstrated that individual, or few, mutations introduced into the loop of the CRE(2C) lead to a dominant negative effect, or cause a greater defect in either positive or negative strand synthesis, than do multiple mutations introduced into both the loop and stem. Goodfellow and colleagues demonstrated that mutating the loop of the CRE(2C) in poliovirus using either one synonymous mutation in varying locations, or 8 simultaneous mutations throughout the loop, led to loss of CPE in transfected HeLa cells (19). These mutations led to inhibition of viral replication based on autoradiographic assays, but CRE(2C) function could be rescued *in cis* by relocating an unaltered, wt CRE to the P1 region of the genome (19). Despite the negative effects observed, these studies also demonstrated that reversions were occurring in constructs containing 2 or 3 mutations in the loop of CRE(2C), with recovery of CPE after a single passage (2 mutations) or three blind passages (3 mutations) in HeLa cells (19). Sequence analysis conducted by Goodfellow and colleagues found sequence

conservation in the CRE(2C) to be significantly higher than would otherwise be expected (19), an important point suggesting that the virus jealously guards the sanctity of the wt sequence. Other studies conducted by Crowder and Kirkegaard found that two synonymous double mutations within the loop of the CRE(2C) of poliovirus led to a severe defect in viral replication, as indicated by the decrease or absence of plaque formation by mutant viruses (74); this work also demonstrated that these double mutants had a dominant negative effect, inhibiting wt poliovirus replication in co-transfected cells, suggesting that the CRE(2C), even with mutations in the loop, binds proteins required for viral replication (74). Importantly, when the CRE(2C) was disrupted entirely using 8 synonymous mutations throughout the stem and loop, no dominant negative effects were observed (74). Based on these studies, it becomes clear why it is unlikely that we see only single-base revertants in the CRE(2C) during sequence analysis, as, though reversion is stepwise in the absence of recombination, these mutants would be rapidly outcompeted by more fit replicons. Taken together, these data clearly indicate that the CRE(2C) structure is intimately linked to viral replication, possibly with functions outside that of uridylation. A putative multi-functional CRE(2C) sequence that is required by the virus, such as we propose here, may be why we do not see fewer reversions occurring but rather a complete reversion to wt the sequence.

The analysis of sequences in GenBank to explore the possibility of compensatory mutations within the stem of the CRE(2C), strongly suggests that it is unlikely for compensatory mutations to occur (**Fig. 9**), because any compensatory mutations in the region of the CRE(2C) would alter the amino acid sequence of the corresponding 2C protein. Further, all identified CRE structures within picornavirus genomes have a highly conserved stem structure (16, 18, 19, 22, 24) suggesting that compensatory mutations deviating from this should not occur. Of the possible compensatory mutations which

could occur due to base-pairing, only one has been reported, albeit rarely (1 in 429 sequences) in NCBI data bank, leading to an E<sub>121</sub>K change. This suggests that any other changes to the 2C protein are lethal mutations in the CVB population. Further work to explore this hypothesis is therefore warranted. Compensatory mutations in the loop of the CRE(2C) should also be very unlikely, as point-mutational analysis done by others demonstrated that single-nucleotide changes in the loop of the CRE(2C) inhibited the synthesis of the negative strand intermediate, and VPg uridylylation, while those in the stem did not (24). Further, it has been demonstrated that double-base changes in the loop of the structure are also highly deleterious to viral replication (as discussed above). These findings, coupled with the fact that the CRE(2C) is in a protein coding region, should prevent compensatory mutations that inhibit the function of the loop, or that alter the amino-acid sequence of the 2C protein. Because the bases in the loop region of the CRE(2C) structure are not expected to participate in base-pairing, we were unable to assess if compensatory mutations could occur. However, based on the absence of sequence variation in 35 random EV-B CRE(2C) loop sequences found in GenBank (data not shown), it is unlikely that deviations from the wt CVB3 CRE(2C) loop sequence would occur.

In entertaining other possibilities as to why wt sequence was detected in our sequencing assays, we concluded that contamination with wt RNA could be ruled out. This was based on transfection experiments in which few copies of wt CVB3 T7 transcribed RNA (from 10-1,000 copies) were transfected with a constant and higher concentration of CVB3-CKO T7 RNA ( $1.97 \times 10^{11}$  copies). Even when as few as 10 copies of wt RNA were present, wt virus replicated and produced detectable CPE on blind passage in HeLa cells (**Table 3**). Importantly, we never saw CPE even after three passages of purified CVB3-CKO virus in cell culture, indicating contamination with wt

CVB3 had not occurred. Further, subcloning and sequencing of 32 clones of the CRE(2C) sequence, prepared from four different CVB3-CKO purified virus preparations (8 clones each), demonstrated that all clones were of wt sequence (data not shown). This would be statistically impossible were contamination to have been a factor. Finally, we conclude from sequencing controls in which 2-fold increases of wt CVB3 cDNA clone were mixed with a constant concentration of CVB3-CKO clone, that with as low a ratio as 4:1 wt:CVB3-CKO, we would only detect wt sequence (**Fig. 8**). This suggested that during reversion only a 4:1 virus ratio would be required of the more efficiently replicating virus to completely mask the CKO mutants.

Of the discussed possibilities for complete reversion to occur, it is likely that reversions take place over time with those changes that led to decreased viral fitness being out competed. Standard Sanger sequence analysis being an inefficient detection assay, such changes are masked until the new master (wt) sequence is regenerated, whereupon it becomes the dominant (detectable) sequence, completely outcompeting viral genomes with mutations in the CRE(2C). It can also be expected that in some genomes, more than one reversion occurs at a time and, through events such as recombination between two partially reverted genomes, the wt sequence could eventually be recovered. When the wt sequence is regenerated, it rapidly dominates the population, with the CKO population collapsing and disappearing from detection. It can be concluded from the findings of others, as discussed above, that single base reversions would be less likely to replicate well when compared to multiple reversions, as they are more detrimental to viral replication than are larger numbers of mutations throughout the stem and loop of CRE(2C), having a dominant negative effect on other viruses replicating in the same cell (74). Therefore, it is highly unlikely that the observed 16nt reversion is an event re-creating the wt sequence. Far more likely is the gaining

over time of the correct changes, none of which alone would reconstitute function or structure, but would lead to it. As the viral population approached complete reversion, mimicking the lesser number of mutations made by others that slowed but did not eliminate replication, the reversions would eventually lead to a complete wt CRE(2C) and rapid re-ascendance of this more efficiently replicating population. Future work will be important to determine at what point reversion actually does occur during the replication of the CKO viruses. Because these viruses replicate at approximately 100,000-fold lower levels than wt, a large number of purified virus preparations will be required to generate enough RNA for methods such as deep sequencing or RNA-Seq.

## Chapter 4

### A novel, broadly applicable approach to isolation of fungi in diverse growth media.<sup>2</sup>

#### Introduction

There is a need for different and selective media for the propagation and isolation of fungi for identification in clinical settings and for the detection of fungal presence in the environment [14, 16, 17, 20]. The isolation of both yeasts and molds (*i.e.*, fungi) from clinical and environmental samples can be complicated by various factors. Fungal growth in clinical or environmental samples may often be inhibited due increased growth rates of bacteria as well as bacterial production of deleterious metabolites. These factors can result in failure to detect fungi in mixed cultures (109, 110). Additionally, fungi often have diverse and specific nutritional requirements which may limit the extent and robustness of the growth of a given organism in the absence of a specific nutrient (49, 54). Sabouraud dextrose agar (SDA), originally defined by Raymond Sabouraud in the late 1800s, remains the medium of choice for the isolation of fungi (50, 52). Sabouraud's formula, a basic glucose and peptone formulation adjusted to a pH of 5.6, uses the lower pH as the inhibitory growth factor for bacteria. Selective media such as SDA are used when a need exists to isolate fungi from mixed cultures containing bacteria. However, SDA does not inhibit all bacterial outgrowth: one study reported that in mixed clinical cultures containing both bacteria and yeast, yeast were detected on

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<sup>2</sup> The work presented in this chapter was previously published: Shane Smithee, Steven Tracy, Kristen Drescher, Lisa Pitz, and Thomas McDonald; A novel, broadly applicable approach to the isolation of fungi in diverse growth media, Journal of Microbiological Methods, 2014, 105:155-161. License for reuse granted by Elsevier 05.28.2015: 3637740371246.

SDA in only 77% of samples tested while others were obscured by bacterial growth (52). Further, selective media like SDA may not provide the necessary nutrients or be the most optimal media for isolation of all types of fungi from either clinical or environmental samples [3, 5, 15-18]. For example, when comparing SDA to other media, one study demonstrated that, in general, fungi associated with fungal keratitis grew more rapidly, and were detected more frequently, on media such as blood agar and chocolate agar versus SDA (48); this suggested that SDA did not provide optimal nutrients and growth requirements for these fungi. While this is not a universal finding (111), sufficient concern exists to identify a more efficient selective media than SDA.

The efficiency with which SDA functions for the isolation of fungi has been compared to other fungus identification media that employ antibiotics to suppress bacterial outgrowth. For example, after inoculation with clinical mixed cultures, more fungal isolates were propagated on inhibitory mold agar, a complex media containing chloramphenicol, than on SDA, although some genera were propagated only on SDA (53). Compared to modified Pagano-Levin agar (MPL) which contains both gentamicin and triphenyltetrazolium chloride, MPL proved to be more effective than SDA at isolating different yeast species from oral samples (51). It is important to note that the addition of antibiotics such as chloramphenicol to growth media may also adversely affect the growth of certain fungal species (112, 113). Other reports have explored chemically augmented SDA formulations to increase fungal isolation specificity. For example, the addition of gallium (III) nitrate to SDA effectively inhibited bacterial growth, although at concentrations greater than 16mM, gallium (III) nitrate also inhibited the growth of fungi as well as bacteria (114). Although these approaches are useful, they involve modifying different media with diverse reagents which increase costs for laboratories (53) in addition to increasing the difficulty in standardization of testing systems in clinical

settings. The various formulations of SDA can also limit the type of media on which specific or new fungi might best be propagated. The ideal reagent would be one that, if added to any growth medium, could function for the isolation of fungi while efficiently suppressing all bacterial outgrowths.

Creatinine (CRN) is a natural vertebrate metabolic waste product of the breakdown of the high-energy compound creatine phosphate [16]. This primarily non-enzymatic reaction leads to a steady production of CRN in the body which is subsequently voided in urine; CRN is considered to have no biological function [16]. However, our previous work demonstrated that the addition of the protonated form of creatinine [creatinine hydrochloride (CRN-HCl)] to growth media inhibits bacterial growth without affecting the outgrowth of fungi, even at concentrations greater than 200mM (47). The mechanism by which CRN-HCl selectively inhibits prokaryotes has not been elucidated, although it may overwhelm efflux pumps, causing an accretion of protons in bacterial cells, thereby lowering intracellular pH and disrupting metabolic pathways (47). Although the anhydrous form of CRN does not inhibit bacterial growth, the protonated form in either a hydrochloride or other salt, is highly effective as an antibacterial agent (47). Because of its demonstrated ability to inhibit bacterial replication and to kill bacteria, we tested the hypothesis that the addition of CRN-HCl to different solid and liquid media will facilitate isolation of fungi while completely inhibiting bacterial outgrowth.

## **Materials and Methods**

**Microbial strains and culture conditions.** Two bacteria and two yeast strains were used in this study: *Staphylococcus aureus* (ATCC 29213, Manassas, VA, USA), *Escherichia coli* strain DH5 $\alpha$ , a strain commonly used for molecular cloning (Invitrogen; Life Technologies, USA), *Saccharomyces cerevisiae* (common baker's yeast), and *Rhodotorula mucilaginosa* (an environmental isolate). Genus and species of microorganisms were verified as described below in enzymatic amplification of rRNA sequences. All microorganisms were grown routinely for 18 hours at 32°C shaking at 300 RPM in 100 mL cultures of LBG [Luria broth (LB; Amresco, Solon, OH, USA) supplemented with 1% w/v D-glucose (Fisher Scientific; Fair Lawn, NJ)]. Cultures were maintained on LBG agar. Cultures were individually prepared by inoculation with a single colony of each respective microorganism. Organism concentrations were determined by serial dilution and plating on LBG agar to determine counts of colony-forming units (cfu) per mL prior to subsequent experimentation.

**Enzymatic amplification of rRNA sequences for organism identification.** Genus and species were determined for microorganisms by amplifying the appropriate rRNA sequence from chromosomal DNA. DNA was isolated from 2 mL cultures inoculated originally with a single colony using the Zymo ZR Fungal/Bacterial DNA Miniprep Kit (Zymo Research; Irvine, CA, USA) per manufacturer's protocol. Primers specific to bacterial 16S rRNA and fungal 18S rRNA are shown in **Table 4 (primers specific to fungal rRNA (1), primers specific to bacterial rRNA (2))**. Twenty microliter ( $\mu$ L) reactions were assembled with GoTaq Green Master Mix (Promega) with primers included at a final concentration of 0.5  $\mu$ M, then cycled in a TC-312 thermal cycler (Techne; Duxford, Cambridge, UK) with the following conditions: initial denaturation for 5 min at 94°C followed by 20 cycles (16S primers) of 10 sec at 94°C, 20 sec at 39°C,

**Table 4. Primers used for sequencing of bacterial and fungal specimens. <sup>a</sup> (1), b (2)**

<b>Primer</b>	<b>Sequence</b>
<b>Fungal 18S Sequencing Primers</b>	
NS1 <sup>a</sup>	5'-GTA GTC ATA TGC TTG TCT C-3'
NS8 <sup>a</sup>	5'-TCC GCA GGT TCA CCT ACG GA-3'
<b>Bacterial 16S Sequencing Primers</b>	
F24 <sup>b</sup>	5'-GAG TTT GAT YMT GGC TCA G-3'
F25 <sup>b</sup>	5'-AAG GAG GTG WTC CAR CC-3'

and 2 min at 72°C or 40 cycles (18S primers) of 10 sec at 94°C, 20 sec at 43°C and 2 min at 72°C, finishing with 5 min at 72°C. Ten microliters of the 20 µL PCR reactions were analyzed on 1% agarose gels to verify successful amplification and expected size of the amplicon (data not shown). The remainder of the reaction was ethanol precipitated, resuspended in water, and submitted for sequence analysis at the UNMC sequencing core facility. Sequences were analyzed with Nucleotide BLAST using the National Center for Biotechnology Information (NCBI) GenBank.

**Culture media.** Liquid LBG was prepared as described previously (47) and as indicated above. Nutrient agar was prepared by adding 1.5% (w/v) bacto agar (BD; Sparks, MD) to liquid LBG prior to sterilization. Sabouraud dextrose agar (SDA) was prepared using Sabouraud dextrose broth (Sigma Aldrich; St. Louis, MO) per manufacturer's recommendations with 1.5% w/v bacto agar. Agar media containing 100 mM CRN-HCl was prepared by first cooling liquid agar medium in a water bath to 56°C, to which CRN-HCl (F.W. 149.6; Sigma-Aldrich St. Louis, MO, USA) was then added. Adding CRN-HCl to agar media prior to sterilization prevents cross-linking and solidification; after mixing to solubilize CRN-HCl, plates were poured. LBG or SDA agar media containing antibiotics were prepared by addition of a combined penicillin and streptomycin solution (Invitrogen) to a final concentration of 100 I.U./mL and 100 µg/mL respectively, and ampicillin solution (Fisher Bio Reagents) to a final concentration of 100 µg/mL with media below 56°C and immediately prior to pouring.

**Measuring effects of CRN-HCl concentration on microorganism growth at varying inocula.** Single colonies were inoculated into 50 mL LBG cultures in 250 mL

Erlenmeyer flasks and shaken overnight as described above. Absorbance at 600nm was measured for each culture using a spectrophotometer and cfu/mL determined for each culture (based on linear regression data constructed by plotting absorbance at 600nm against cfu/mL for each microorganism). A series of ten-fold dilutions were performed in LBG for final concentrations of ca.  $10^3$  -  $10^7$  cfu/mL of each microorganism. Cultures in sterile 16x125 mm glass culture tubes were prepared in duplicate containing 4.25 mL of LBG, 250  $\mu$ L CRN-HCl [at a concentration of 400 mM, 800 mM, 1.2 M, 1.6 M, or 2 M, (water instead of CRN-HCl was used as the control)], and 500  $\mu$ L of culture dilution (at either  $10^7$ ,  $10^5$ , or  $10^3$  cfu/mL). Cultures contained final concentrations of 20 mM, 40 mM, 60 mM, 80 mM, 100 mM or no CRN-HCl respectively, and  $10^6$ ,  $10^4$ , or  $10^2$  cfu per mL respectively in total volumes of 5mL. Tubes were placed at a slant and shaken at 300 RPM and 37°C for 18 hours. Following incubation, absorbances at 600nm were recorded, a series of tenfold dilutions of each culture in sterile water were made and plated on LBG agar in duplicate. Agar plates were incubated overnight at 37°C (with the exception of *R. mucilaginosus* plates which were incubated at 25°C for 48 hours due to better growth at lower temperatures) and colonies counted to determine counts of cfu/mL at each CRN-HCl concentration. These experiments were repeated with n=4 culture tubes for each of the four microorganisms at each inoculum, and n=8 plates inoculated and counted for each microorganism.

#### **Comparison of environmentally-obtained microbial growth on LBG and SDA agar.**

Five grams of soil were shaken in 50 mL of sterile 1x phosphate buffered saline (PBS) and allowed to settle for one hour. Fifty microliters of the supernatant were spread onto agar plates containing LBG, LBG + 100 mM CRN-HCl (LBG-CRN), LBG + antibiotics, SDA, SDA + 100 mM CRN-HCl (SDA-CRN), or SDA + antibiotics (prepared as

described above). Plates were incubated at 37°C and images of microbial growth on the plates were obtained at 24, 48, 72, and 96 hours post-plating using an AlphaMager HP MultiImage II imager (Protein Simple/Alpha Innotech; Santa Clara, CA, USA). To determine if the vigorous colonization observed at 24 hours on the SDA plate were bacterial and not fungal, two colonies with differing morphologies were picked after 24 hours of growth, inoculated into 5mL of Sabouraud dextrose broth, and incubated 18 hours at 32°C/300RPM. Following incubation, cultures were collected by centrifugation and DNA extracted as described above. Genus and species of microorganisms were verified following traditional PCR and sequencing as described above using primers specific to bacterial 16S ribosomal sequences as listed in **Table 4**. The bacteria were determined to be *Bacillus megatarium* and *Pasteurella pneumotropica* based on BLAST results using NCBI GenBank (results not shown). These experiments were done with three different soil samples processed as above and plated on each of the four different media described for n=3 of each plate type.

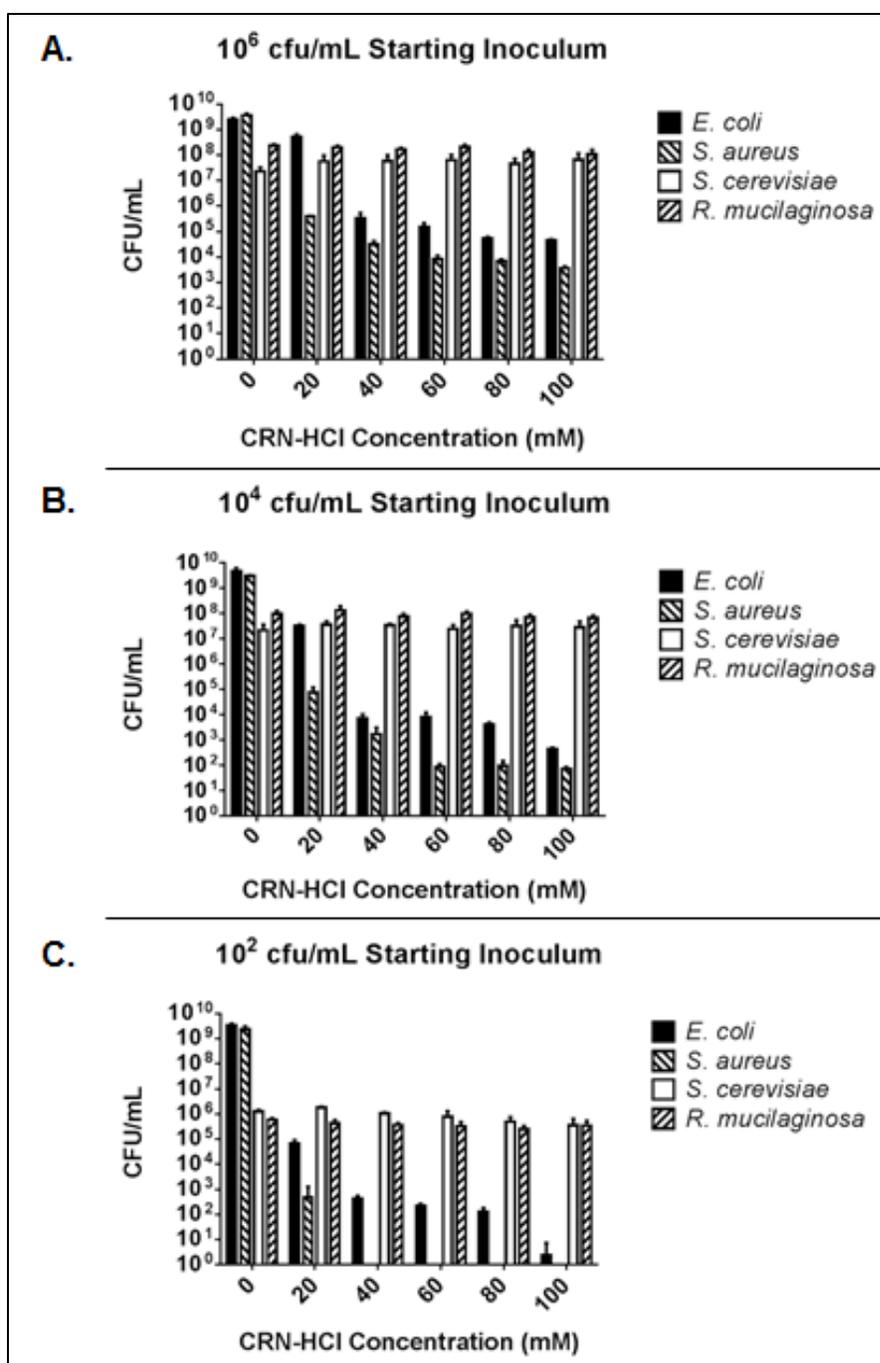
**Comparison of common bacterial growth on LBG and SDA.** Glycerol stocks of either *S. aureus* or *E. coli* were streaked for isolation onto LBG plates and incubated overnight at 37°C. A single colony from each plate was picked and streaked onto one half of LBG, SDA, LBG-CRN, or SDA-CRN plates. Plates were incubated overnight at 37°C and subsequently observed for growth. These experiments were repeated for n=2 plates of each type streaked with both *S. aureus* and *E.coli*.

**Isolation of an antibiotic-producing fungus using CRN-HCl.** Individual fungal colonies were selected from soil samples previously plated on LBG-CRN growth agar,

transferred to individual LBG plates without CRN-HCl, and incubated at room temperature; a total of greater than 50 fungi of varying morphologies were isolated. Fungal isolates were plated on medium without CRN-HCl in order to ensure that subsequent testing with bacteria could be accomplished. Fungal colonies were tested for antibiotic production when colonies were 2-3 cm in diameter. Cultures of *S. aureus*, *E. coli*, *S. cerevisiae*, and *R. mucilaginosa* to be used as the test target organisms were diluted to approximately  $1 \times 10^4$  cfu /mL. Using 25  $\mu$ L drops, each culture was spotted four times inward from the edge of the plate toward each fungal colony (termed "the drop-test" method) and plates were incubated overnight at 37°C. Plates were subsequently observed for microorganism growth and presence of zones of inhibition indicating antibiotic production by the fungus. Antibiotic production by an environmental fungal isolate is a representative of n=10 replicates.

## **Results**

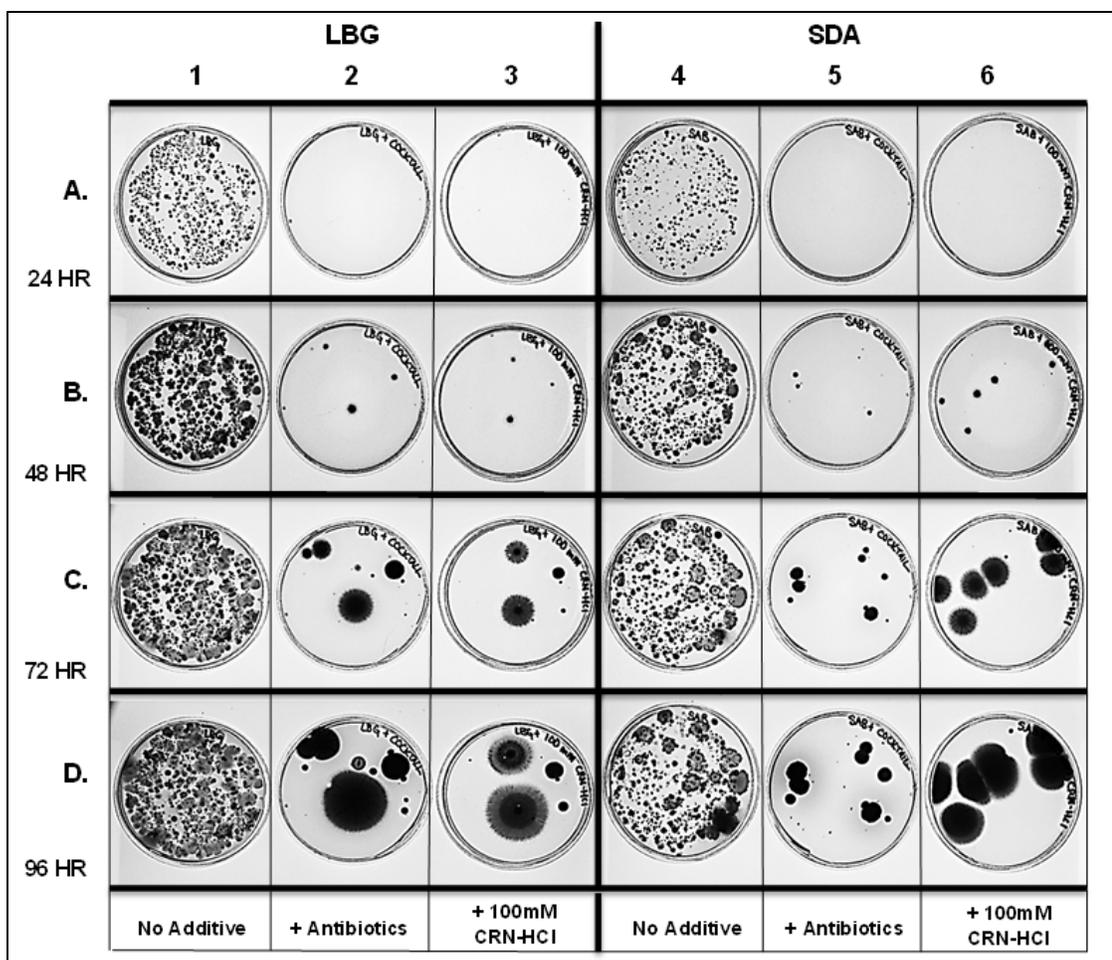
**Effect of CRN-HCl on microbial growth in liquid media.** We initially compared CRN-HCl concentrations using the 4 organisms listed above (section 2.1.) to determine impact upon growth. Cultures were inoculated with bacteria or yeast in various concentrations of CRN-HCl and incubated overnight with shaking. After measuring absorbance at 600nm, ten-fold serial dilutions were performed and plated to determine cfu/mL present. It is apparent that the growth of yeasts was uniform across the spectrum of CRN-HCl concentrations (**Fig. 11 A-C**). There was no difference observed in the growth of either yeast strain tested after 18 hours of incubation, confirming and extending similar results observed previously using absorbance at 600nm as a measure of replication [10]. The effects of the addition of CRN-HCl on bacterial growth varied depending upon the



**FIG. 11. Comparison of antibacterial effect of CRN-HCl at increasing concentrations on microorganisms at decreasing inocula.** Microorganisms (*S. aureus*, *E. coli*, *S. cerevisiae*, or *R. mucilaginosa*) were inoculated at three different concentrations into 5 mL cultures of LBG media containing increasing concentrations of CRN-HCl and cfu/mL as described in Methods. Cultures were inoculated at two different times in duplicate for  $n=4$  tubes for each microorganism and inoculum size. Each culture was plated in duplicate for  $n=8$  plates counted to determine cfu/mL. Inhibition of growth was observed in bacteria (*E. coli* and *S. aureus*) at increasing [CRN-HCl] but not in fungi (*S. cerevisiae* and *R. mucilaginosa*) (A-C). Panel A shows results for a  $10^6$  inoculum of each microorganism, panel B shows results for a  $10^4$  inoculum of each microorganism, and panel C shows results for a  $10^2$  inoculum of each microorganism.

bacteria. For example, CRN-HCl was bacteriostatic (*i.e.* after overnight incubation, approximately the same number of cfu/mL were present as were in the initial inoculum indicating inhibition of replication) for *S. aureus* at 20 mM but not for *E. coli*. Concentrations of 40-80 mM CRN-HCl were bacteriostatic to *E. coli* but bactericidal (*i.e.* after overnight incubation, fewer cfu/mL were present than were in the initial inoculum indicating cell death) to *S. aureus*. Concentrations of 100 mM CRN-HCl were bactericidal to both *S. aureus* and *E. coli* (results were consistent when comparing absorbance at 600nm; data not shown and (47)). Although *S. aureus* appeared to be more sensitive than *E. coli* to CRN-HCl overall (based on the CRN-HCl concentration required to reduce titer as measured by cfu/mL), there was no significant difference when analyzed using a two-tailed student t-test ( $p=0.8788 \cdot 10^6$  inoculum;  $p=0.7620 \cdot 10^4$  inoculum;  $p=0.8027 \cdot 10^2$  inoculum).

**Supplementation of diverse agar media with CRN-HCl.** Having shown that *E. coli* and *S. aureus* were killed in the presence of 100 mM CRN-HCl while fungi were unaffected in growth, we then asked whether similar results would be obtained using environmental samples containing a diverse population and unknown titer of microorganisms. Soil samples were therefore suspended in 1x PBS, spread onto LBG, LBG-CRN and LBG+antibiotic plates, incubated over a period of days, and observed for growth as described in section 2.5. above. Robust bacterial growth was observed within 24 hours of inoculation on the LBG plates containing no selective reagents (**Fig. 12, A-1**). In sharp contrast, no growth was observed at 24 hours post inoculation on LBG-CRN plates (**Fig. 12, A-3**) nor was growth observed on antibiotic containing LBG plates (**Fig.12, A-2**), confirming that CRN-HCl is as effective at suppressing growth of diverse, unknown bacterial species as an antibiotic cocktail. Growth on plates not containing



**FIG. 12. Comparison of CRN-HCl and antibiotic supplemented LBG to SDA for enhancement of fungal growth from environmental (soil) samples.** Plates were inoculated with soil samples suspended in 1x PBS prior to incubation. Three different soil samples were tested on each of the media described in methods for  $n=3$  plates of each type inoculated. Rows A-D, columns 1-3, show LBG plates either without addition of antibiotics or CRN (row 1), with the addition of antibiotics (row 2), or with the addition of 100 mM CRN-HCl (row 3). Rows A-D, columns 4-6, show SDA plates either without addition of antibiotics or CRN (row 4), with the addition of antibiotics (row 5), or with the addition of 100 mM CRN-HCl (row 6). Images were taken of plates at 24, 48, 72, and 96 hours after incubation at 37<sup>o</sup> C.

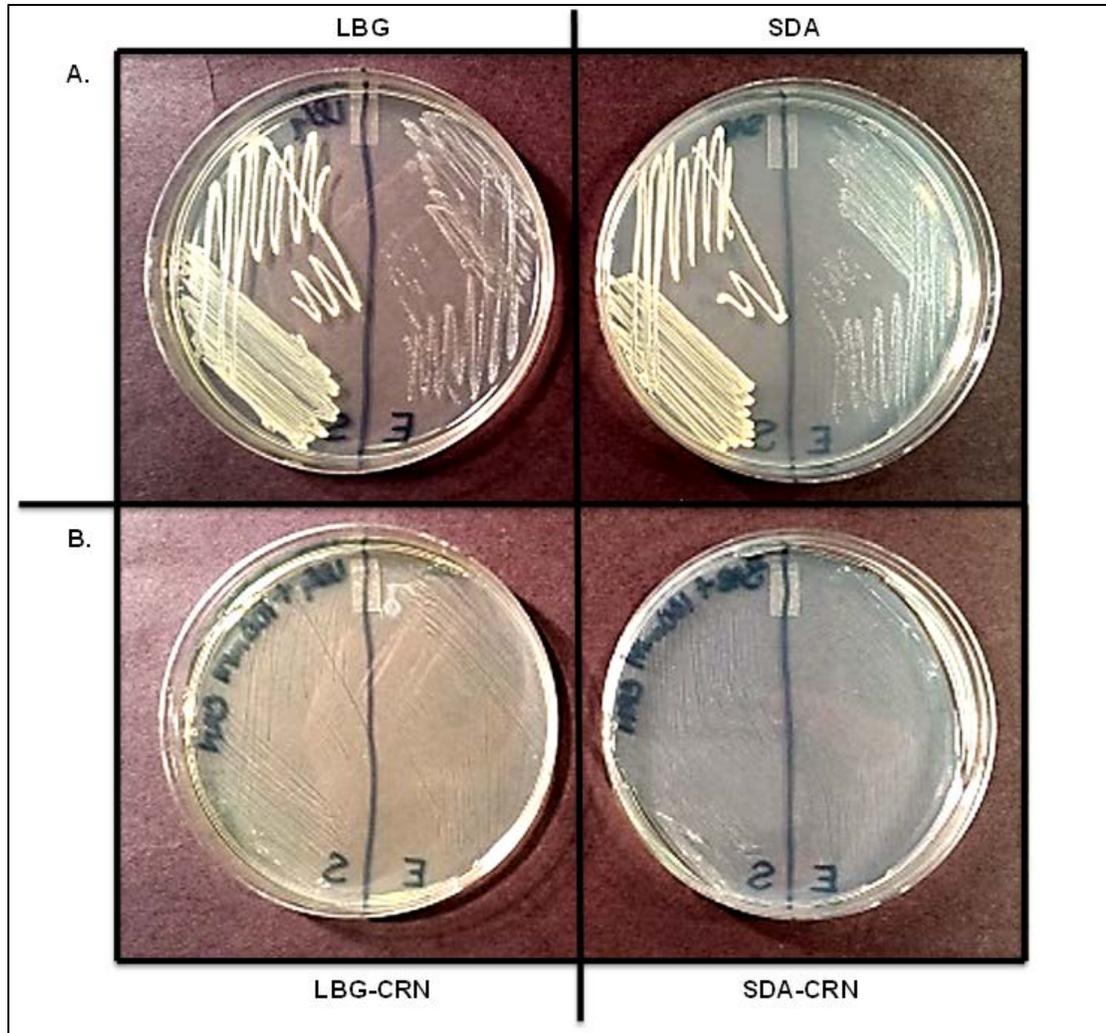
selective reagents was confirmed as bacterial by sequence analysis as described in Methods (data not shown). Similar results showing bacterial inhibition were also observed on both brain heart infusion plates and LBG plates containing 1% potato starch (data not shown). At 48 hours post inoculation, fungal colonies were observed on both LBG-CRN plates and LBG+antibiotic plates (**comparing Fig. 12, B-3 and B-2 respectively**). In several similar experiments, there was no significant difference between fungal outgrowth rates on antibiotic versus CRN-HCl plates when analyzed using a two-tailed student t-test ( $p=0.4885$ ). No apparent fungal growth was observed on LBG plates without addition of selective reagents, likely due to rapid outgrowth of bacteria (bacterial colonies observed at 24 hours increased in size throughout the time course) (**Fig. 12 column 1**); however, we did not assay the mixed population by sequence analysis for the presence of eukaryotic rRNA sequences.

**Comparison of fungal and bacterial outgrowth on Sabourad's agar with and without CRN-HCl to LBG and LBG-CRN inoculated with soil supernatants.**

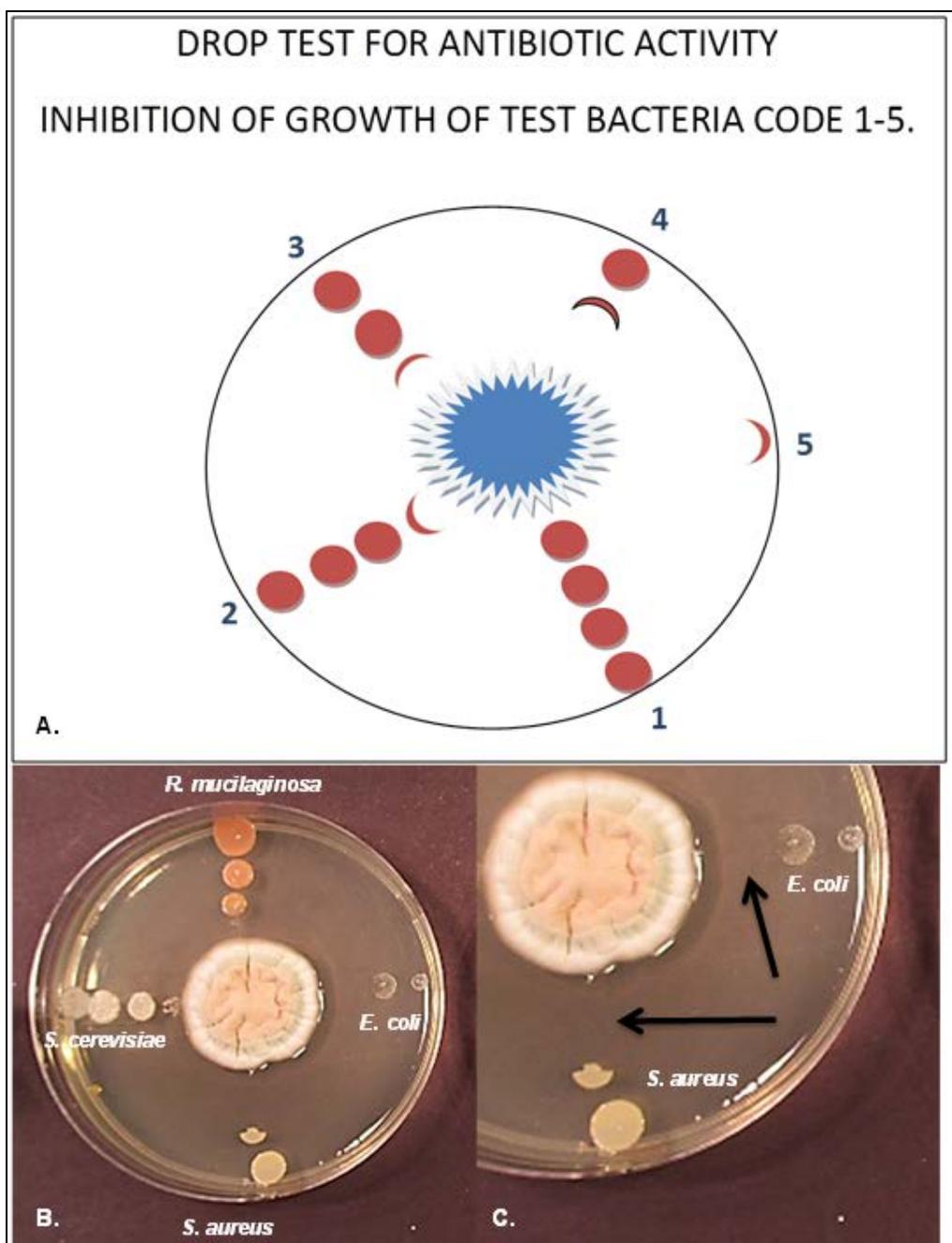
Sabourad's medium is used routinely to suppress the outgrowth of bacteria while isolating fungal specimens. To compare this selective medium with the bacteriostatic effect of CRN-HCl, we compared SDA to LBG with and without 100 mM CRN-HCl or antibiotics as a supplement. We inoculated the four different media with supernatants of soil samples as described above. Robust microbial growth was present on the SDA plates containing no selective reagents, similar to results observed on LBG plates (**comparing Fig 12, A-1 and A-4 respectively**). In contrast, and again as observed with LBG at 24 hours post inoculation, growth was observed neither on SDA-CRN plates (**Fig 12, A-6**) nor on SDA plates containing antibiotics (**Fig 12, A-5**). By 48 hours post inoculation, fungal colonies were present on both SDA-CRN plates (**Fig 12, B-6**) and

SDA+antibiotic plates (**Fig. 12, B-5**) similar to that observed for LBG plates containing either CRN-HCl or antibiotics. Further, and of potential clinical importance, *S. aureus* and *E. coli* were able to grow apparently as well on SDA as on LBG plates after 24 hours of incubation at 37° C (**Fig. 13A**). However, addition of CRN-HCl to these media effectively suppressed the growth of these bacteria (**Fig. 13B**).

**Using CRN-HCl to isolate antibiotic producing fungi.** The observation that media which contained CRN-HCl completely suppressed bacterial replication suggested to us that one application of this finding would be as an approach to facilitate new antibiotic discovery. We tested this hypothesis by screening environmental soil samples for fungi that produced anti-bacterial activity. Fungal colonies grown on various agar media containing 100 mM CRN-HCl were picked and transferred to LBG for growth in the absence of CRN-HCl. When fungal colonies were between 2-3cm in diameter, the two bacteria and two yeasts used for this study were prepared and spotted from the edge of the plate inward toward each fungal colony. Plates were incubated overnight as described in methods. Several hundred phenotypically different fungi were isolated, and then tested, in this manner for production of antibiotic activity against the two bacterial species and the two yeast species used in this project. This 'drop test' approach is diagrammed in **Fig. 14A**. No antibiotic activity (graded as number 1) is represented by each drop actively growing up to the fungal colony. Gradations of antibiotic output are signified by numbers 2-5 where number 5 represents the largest zone of inhibition produced by antibiotic output into the agar. Results from one fungal isolate that showed antibiotic activity is shown in **Fig. 14B, C**. There is clear inhibition of growth of the two bacterial species (graded as a 4 for significant antibiotic activity against both *S. aureus* and *E. coli*), but not the yeast species (graded as a 1 for no activity). **Figure 14C** shows



**FIG. 13. Comparison of common bacterial growth on LBG and SDA.** LBG and SDA (A; left and right respectively) and LBG-CRN and SDA-CRN (B; left and right respectively) were streaked with single colonies of *S. aureus* (left half of plates) and *E. coli* (right half of plates) then incubated overnight at 37° C.



**FIG. 14. A method for identification of antibiotic producing fungi using CRN-HCl for initial fungal isolation.** To demonstrate one use for CRN-HCl addition to media, fungi were picked from LBG-CRN plates (greater than 50 fungal colonies of varying morphologies), plated on LBG, and allowed to grow. Cultures of target organisms (*S. aureus*, *E. coli*, *S. cerevisiae*, or *R. mucilaginosa*) were diluted to approximately  $1 \times 10^4$  cfu /mL (in 25  $\mu$ L per drop) were then dropped inward from the edge of the plate toward the fungus and allowed to incubate (termed the “drop test” method). Panel A illustrates the “drop test” method with varying zones of inhibition (1 = no inhibition; 2 and 3 = mild inhibition; 4 and 5 = high inhibition). Panel B shows the results from one fungal isolate that showed antibiotic active (a representative of  $n=10$  replicates). Panel C shows an expanded view of the plate in panel B with arrows indicating zones of inhibition due to antibiotic production.

an enlargement of the inhibition zone against the Gram negative and Gram positive bacterial species. Although yeast growth was not inhibited, a slight negative effect on colony size at the fungal interface was noted which was attributed to nutrient competition, or other metabolic conditions, but was not considered anti-fungal activity. This fungus was submitted for sequence analysis and was identified as a *Penicillium* genus with the closest species association being *pupurogenum* based on 18S rRNA analysis. BLAST data (NCBI GenBank) gave an E-value of 0.0 which indicates a significant match for this organism.

## **Discussion**

Routine isolation of fungi from mixed samples can be complicated by a variety of factors; most commonly, fungal growth may be inhibited by bacteria (109, 110) or altered/inhibited by diverse and specific nutritional requirements (49, 54). There is a need for an agent that can be added to different and selective media for the propagation and isolation of fungi (52, 53, 111, 115) without the need for antibiotics. We demonstrate here that CRN-HCl is an alternative and cost-effective approach for the isolation of fungi in diverse media. On average, antibiotics cost between \$1-60 per gram (streptomycin and gentamicin, respectively) whereas the cost of CRN-HCl obtained commercially for laboratory use is on the order of \$0.50 per gram (Sigma Aldrich; St. Louis, MO). The addition of antibiotics to media can also adversely affect the growth of certain fungal species (112, 113). Previous work which demonstrated that the mechanism of bacterial killing by CRN-HCl was not due to decreased pH (47) led us to compare SDA to media supplemented with CRN-HCl. While SDA and CRN-HCl supplemented media function at a pH of 5-5.5, only media containing CRN-HCl kills

bacteria and completely suppresses bacterial replication while permitting fungal outgrowth.

The persistent value of SDA has been that it tends to inhibit bacterial growth while allowing fungal outgrowth but it has been noted that this selective medium is not as efficient as might be desired (48, 51-53, 114). Our data show that SDA failed to efficiently inhibit diverse bacterial growth compared to other media (**Fig. 12 and Fig. 13**) regardless of whether laboratory bacterial strains or, more convincingly, numerous unknown environmental species were tested. These results confirm and greatly extend previously published data (47) demonstrating the use of CRN-HCl in media as an effective means to suppress bacteria while allowing for the outgrowth of slower growing fungi. In this report, we screened diverse soil samples for fungi that might produce antibiotic-like activities to test whether CRN-HCl could function in a model more relevant to the goal of discovering new antibiotics. These results demonstrated that CRN-HCl is highly effective at suppressing the rampant outgrowth of diverse bacterial species found in the environment while permitting fungal outgrowth. Using this approach, several fungi were isolated that express an antibacterial activity (data not shown). This is an important point as many fungi grow much more slowly than bacteria [8, 13, 24]. Again, the need to screen numerous isolates for antibiotic activity accentuates the benefit of using low-cost CRN-HCl as the antibacterial in such tests. The use of CRN-HCl as an antibacterial agent should also be applicable to mixed clinical samples in which it is of interest to determine whether (slow growing) pathogenic fungi are involved in a disease course.

Creatinine hydrochloride has been shown to dramatically suppress bacterial growth. The addition of this inexpensive, readily available compound to several media demonstrated that while bacterial replication was ablated, fungal growth occurred similar

to growth on control media without CRN-HCl. Slow growing fungi can therefore be isolated without having first to remove associated bacterial populations and without any overgrowth of bacteria. Using a straightforward "drop test" to assay fungi isolated on CRN-HCl media, one can quickly determine whether the fungal isolate shows antibiotic activity. This assay could, if desired, be automated. Because fungi have notably different preferences for growth media (49, 54), results reported here importantly suggest the potential for the use of creatinine salts in diverse media that favor diverse fungal outgrowth. Protonated CRN should serve as a useful tool in the search for new antibiotics to address the growing problem of commonly encountered antibiotic resistant bacteria especially in clinical settings (116-119).

## Chapter 5

### Conclusions from the Characterization of the Replication of Coxsackievirus B3 with a Mutationally Disrupted CRE(2C) and the Use of Creatinine-hydrochloride as an Antibacterial Agent.

Chapters 2 and 3 of this dissertation describe novel, important, and heretofore unsuspected facets of HEV biology. As described in detail in the pages of this work, previous work in this laboratory demonstrated that the CVB persist in tissues and in cells after deletion of sequence from the 5' genomic terminus, a natural mechanism, leading to loss of much of domain I (9, 10, 13). Though these results contributed significantly to the field, little is still known about how the CVB-TD viruses initiate replication. Based on these previous findings which demonstrated that the CVB-TD rarely terminated in the canonical UU found at the 5' end of the wt virus (donated by uridylylated VPg after initiation of replication) but still had VPg attached, and packaged negative strand RNA (a replication intermediate) at a near 1:1 ratio with the (positive strand) genomic RNA (10), we hypothesized that the CVB-TD did not require a functional CRE(2C) in order to replicate. Mutational analysis by others had demonstrated that when the CRE(2C) [a structure and template for the uridylylation of VPg (14, 27)] of either poliovirus or CVB3 was disrupted by engineering in silent mutations, important steps in the replication of these viruses was affected: uridylylation of VPg did not occur to detectable levels using autoradiographic assays and positive strand synthesis was inhibited when compared to negative strand synthesis (24, 27, 45, 75). The combination of these findings led to the conclusion that without a functional (which is to say, uridylylating) CRE(2C), the HEV could not replicate (*i.e.*, disruption of the [CRE(2C)] was lethal).

Based on previous findings regarding the CVB-TD (9, 10, 13), and the findings of others regarding the CRE(2C) (24, 27, 45, 75), we introduced 16 mutations (24) in the region of the genome corresponding to the CRE(2C) of both CVB3-TD50 (CVB3-TD50-CKO) and wt CVB3 (CVB3-CKO; intended to be a negative replication control) to study the effects of mutational disruption of this structure on CVB-TD replication. These 16 mutations had been stated to be lethal in the CVB3 genome (24, 45). Because our preliminary data demonstrated, surprisingly and in counterpoint to others' findings (24, 27, 45), that CVB3-CKO in fact was replication competent, we examined the replication and evolution of the CVB3 viruses in which the structure of the CRE(2C) had been altered using wt CVB3 and CVB3-TD50 as positive controls for replication. The experiments outlined in chapter 2 of this dissertation illustrated the replication efficiency of the CVB3-CKO, demonstrating that these viruses replicated at near equal levels when compared to the CVB3-TD50 and CVB3-TD50-CKO virus populations. As a whole, the mutant viruses replicated at approximately 5 logs lower efficiency by day 8 post electroporation when compared to wt CVB3, but when compared to each other, were not significantly different (**Fig. 2**). This demonstrated that loss of a functional CRE(2C) affected the replication of wt CVB3 as much as loss of much of domain I, and supported the hypothesis that the CVB-TD viruses did not require a functional CRE(2C) for replication.

The observation that CVB3-CKO replicated at near equal efficiency to CVB3-TD50 and CVB3-TD50-CKO, coupled with the absence of observable CPE in cells electroporated or inoculated with this virus, suggested that loss of 5' genomic terminal sequence was occurring and that a population of TD viruses was evolving in cells electroporated with CVB3-CKO RNA. To confirm this, tagged primers [(78); **Table 1, 2**] were used to detect small deletions at the 5' genomic terminus. Indeed, when compared

to T7 transcript or wt CVB3 viral RNA controls, the majority CVB3-CKO population had evolved 5' TDs by day 8 post electroporation (**Fig. 3**). This demonstrated that when the CRE(2C) function was lost in wt CVB3, a non-specific initiation of viral RNA replication was occurring, generating a virus population that had lost 5' terminal genomic sequence. The experiments presented in chapter 2 also demonstrated that biophysically purified virus preparations isolated from cell culture at 8 days post electroporation were infectious as demonstrated by the ability to inhibit passage in the presence of anti-CVB3 neutralizing antibody (**Fig. 4**).

In an effort to demonstrate that the persistent replication of CVB3-CKO was not an artifact observed only in cell culture, it was important to demonstrate that replication of this virus was occurring in an animal model as well. In inoculated mice, many more cell types are available for infection by a CVB. Because of the replication defect present in the CVB3-CKO, it was difficult to acquire enough infectious virus to inoculate mice and, therefore, an *in vivo* transfection model was developed that allowed us to inoculate mice with T7 transcribed RNA of either CVB3-CKO or wt CVB3 (as a positive control). Using this method, we were able to demonstrate that CVB3-CKO virus was detectable in mouse tissues at day 20 post transfection and could be passaged in cell culture and inhibited by neutralizing antibody; the same results were observed with wt CVB3 transfected mice (**Fig. 5**). Together, these data extended the results observed in cell culture, and confirmed that a CVB could replicate in the absence of a functional CRE(2C). Further, these experiments confirmed that transfection of mice with T7 transcribed CVB3 RNA was a useful method for studying wt CVB and those viruses which replicate poorly in comparison (e.g., CVB3-CKO).

Further investigation of the mutations introduced into the CRE(2C) region of CVB3-CKO during replication led to another surprising and again, unanticipated, finding:

the introduced mutations had apparently completely reverted to the wt sequence in the majority population by day 8 post electroporation in cell culture and when assayed at day 20 post transfection in mouse tissues. In the experiments discussed in chapter 3 of this dissertation, we explored the aspects of this reversion through sequence analysis, as well as the relative point in time in which 5' terminal deletions occur relative to the CRE(2C) reversions. Initially, it was important to determine the ratio of wt to CKO at which we would no longer observe the mutant sequence by nucleotide sequencing. In analyzing sequences in which the ratio of wt:CKO increased by 2-fold to a final ratio of 8:1, we found that at a ratio as low as 4:1 or 8:1, we were no longer able to detect the 16 mutations that were introduced into the CRE(2C) region (**Fig. 8**). This was an important control which demonstrated that if CVB3 replicated more efficiently than those viruses which lacked a functional CRE(2C) in a mixed population, only a 4:1 ratio of revertants to mutants was required to render the mutant sequence undetectable by traditional sequencing techniques. Said differently, as long as a specific sequence did not exist at least as much as 25% of the total, it would not be detected by standard Sanger sequencing of the total population. This is entirely consistent with discussions of RNA virus quasispecies populations in which a master sequence is that which is dominantly detected, despite being surrounded by a 'swarm' of other sequences at lower and thus undetectable, concentrations (32, 33).

Based on preliminary analysis of virus preparations at 8 days post electroporation, which demonstrated complete reversion of the CRE(2C) of CVB3-CKO to that of wt, it was important to determine if we were able to detect an intermediate population at an earlier time-point or if the reversions appeared to occur simultaneously. If the reversions did appear to occur simultaneously, it would suggest that the structure provided by the CRE(2C) provided an increase in viral fitness within the cellular

environment which would allow for more efficient replication when compared to the CKO mutants. Indeed, when analyzing earlier time-points, it became clear that only after 8 days of replication post-electroporation in HeLa cells were we able to detect a complete reversion of the CRE(2C) to wt sequence (**Fig. 7**). Complete reversions were also found to occur in CVB3-TD50-CKO electroporated HeLa cells at day 8, and at day 20 post-transfection of CVB3-CKO T7 transcribed RNA in both the spleen and heart of mice (**Fig. 7**). Important to note at this juncture is that when considering the possibility of compensatory mutations, analysis of GenBank data demonstrated that only one compensatory mutation was reported out of 429 available HEV-B sequences (**Fig. 9**). This further supported the possibility of complete reversion of all 16 introduced mutations in the region of the CRE(2C), as well as the hypothesis that the structure is important for other functions besides that of VPg uridylylation. Finally, to complete these studies, it was important to determine at what point in replication 5' TDs evolved in the CVB3-CKO population. We expected that TD's were evolving prior to reversion of the CRE(2C) to wt: were reversions to occur prior to TD formation, we would have expected a return to a wt cytopathic phenotype but this was not observed. Indeed, 5' TDs were found to occur between days 1 and 8 post-electroporation of HeLa cells and this correlated with a slight decrease in viral RNA titer over time (based on RT-qPCR assays) characteristic of a low-level persistent replication (**Fig. 10**).

Taken together, the data presented in chapters 2 and 3 demonstrated that wt CVB3 is replication competent without a functional CRE(2C) and that this structure is not required for CVB-TD replication. Further, it is clear that 5' TDs form during the replication of CVB3-CKO and these TD mutations occur prior to a complete reversion of the 16 mutations introduced in the CRE(2C) to that of a wt sequence. These findings describe a new aspect of enteroviral biology and suggest that the CRE(2C) is important

in enteroviral replication with a function(s) other than providing a template for VPg uridylylation. Future work will be important to elucidate other functions of the CRE(2C) which may include, among other things, providing a structure for the binding and translocation of the initiation complex to the genomic termini for replication. It will be important to verify that VPg remains attached at the 5' genomic terminus of the CVB3-CKO viruses and to determine if an altered positive to negative strand ratio exists in these populations. Further, a more thorough investigation to determine the presence of intermediate populations during the reversion of the CRE(2C) to a wt sequence is warranted. This may be accomplished by techniques such as deep sequencing or RNA sequencing.

Chapter 4 of this dissertation, though unrelated to the work described in chapters 2 and 3, is connected to the others in that it presents the characterization of another novel finding in microbiology: the characterization of a small-molecule antibacterial agent, creatinine hydrochloride (CRN-HCl). The experiments discussed in chapter 4 enhance and extend work previously completed (47), and demonstrate the broad antibacterial activity of CRN-HCl in diverse media while simultaneously demonstrating its lack of activity on fungi (**Fig. 11**). Furthermore, the experiments presented in chapter 4 demonstrate that addition of CRN-HCl to growth media is more effective at preventing bacterial growth than is the traditional SDA (**Fig. 12**) and may be a cost effective alternative to use of this medium. Finally, the lack of antifungal activity makes CRN-HCl an excellent candidate for isolation of diverse yeasts and molds, some of which may have clinical importance or be producers of new antibiotics that can help combat the growing concern of antimicrobial resistance (**Fig. 13**). Future work exploring CRN-HCl should include experiments to understand the mechanism of action of this small molecule. Recent work in the laboratory (unpubl. data) indicates that bacteria can

develop resistance to CRN-HCl; whether this involves over-expression of proteins involved in the proton motive force as suggested in previous work (47, 120), or some other mechanism, remains to be studied. Once more thoroughly understood, many uses for CRN-HCl outside of the laboratory can be envisioned to include addition of this compound to topical antibiotics which should prevent infection and, perhaps, expedite wound healing.

Together, the data presented in this dissertation describe novel and important developments in the fields of virology and microbiology. The work outlined in chapters 2 and 3 of this dissertation demonstrate that not only is the replication of CVB3-TD50 not affected when the CRE(2C) was mutationally disrupted, but surprisingly, CVB3-CKO is replication competent and rapidly evolves 5' terminal deletions prior to a complete reversion of the mutated sequence back to that of wt. Therefore, the findings described in chapters 2 and 3 of this dissertation elegantly illustrate that there is still new knowledge to be gained in the field of enteroviral biology regarding the initiation and replication of the CVB-TDs, and the HEV in general, and further demonstrate that studying aspects of enteroviral biology using just one system (*e.g.*, the cell free system) can mask otherwise salient data. The work outlined in chapter 4 of this dissertation demonstrates that a small molecule, CRN-HCl, a natural vertebrate metabolic waste product once thought to have no biological function, is a potent inhibitor of bacterial growth. Creatinine-HCl is useful in the laboratory to suppress bacterial growth in mixed samples, allowing for the outgrowth of slower growing fungi, which presents a variety of applications such as isolation of antibiotic producing fungi. Therefore, the findings described in chapter 4 extend previous findings (47) and illustrate that the function of many molecules produced naturally should not be taken for granted. While there is still much work to be done in all aspects of this dissertation, the data presented here provide

an important framework for further experimentation in the fields of both enteroviral biology and microbiology.

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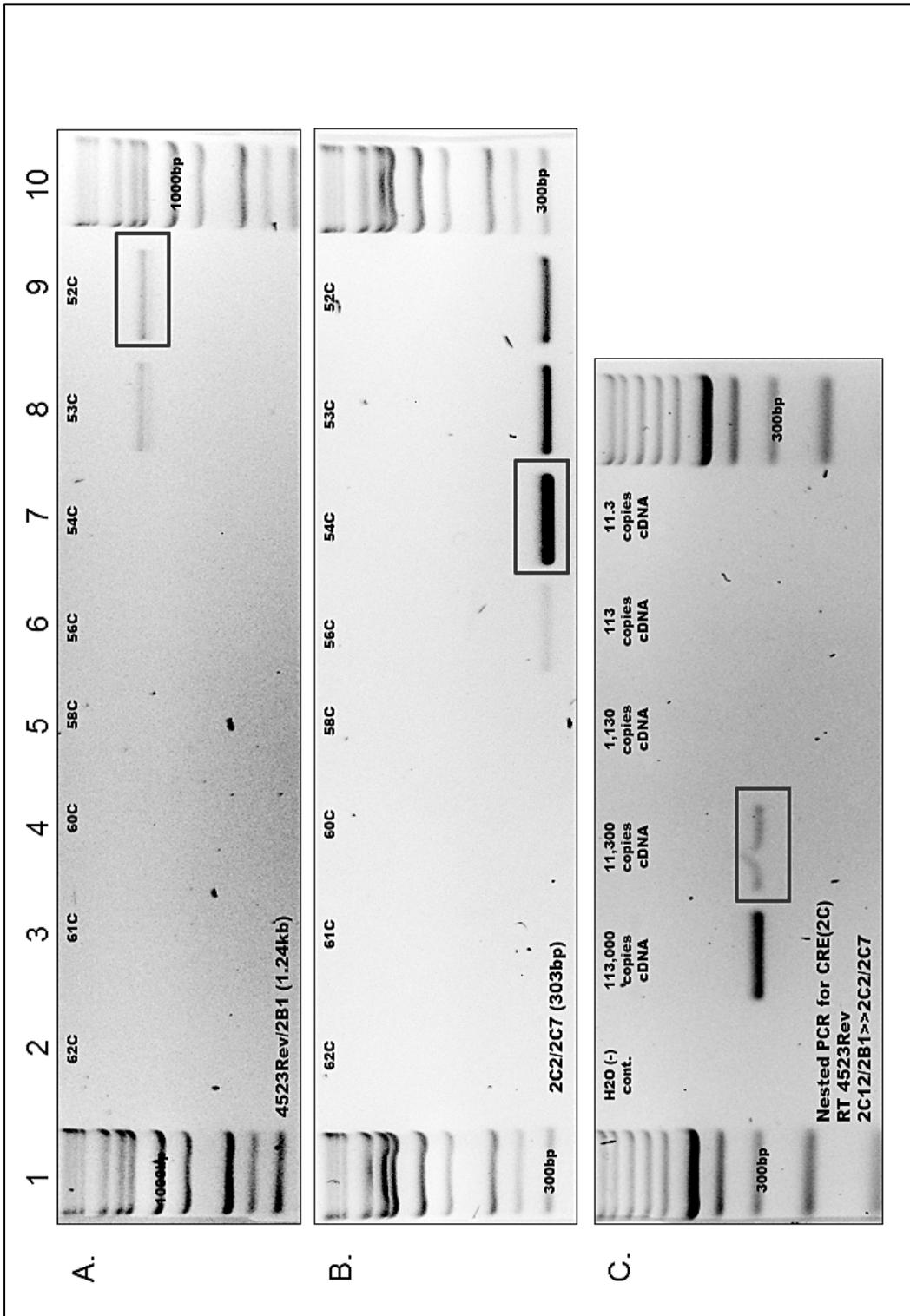
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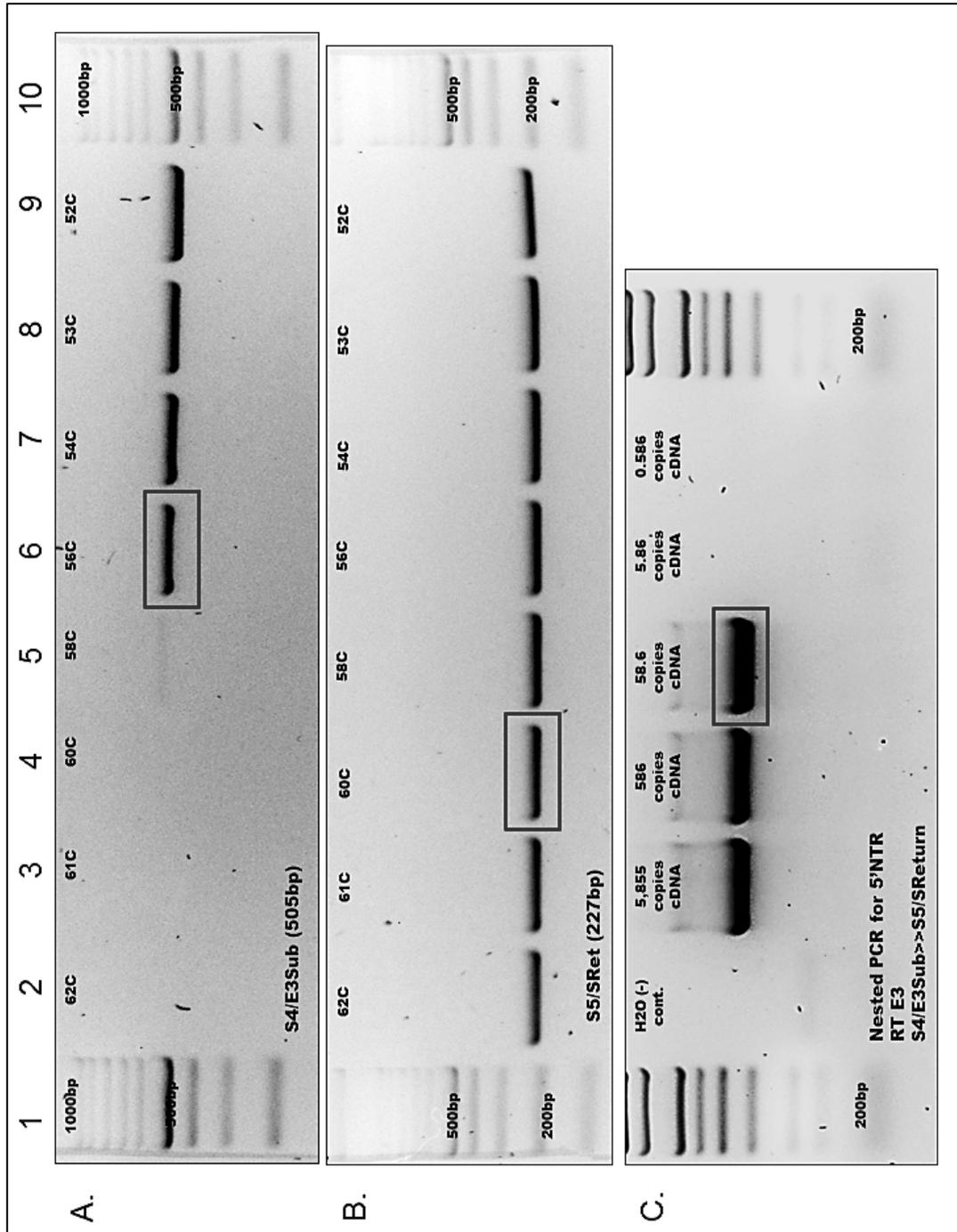
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### **Appendix A: Primer optimization and determination of RT-qPCR Efficiency**

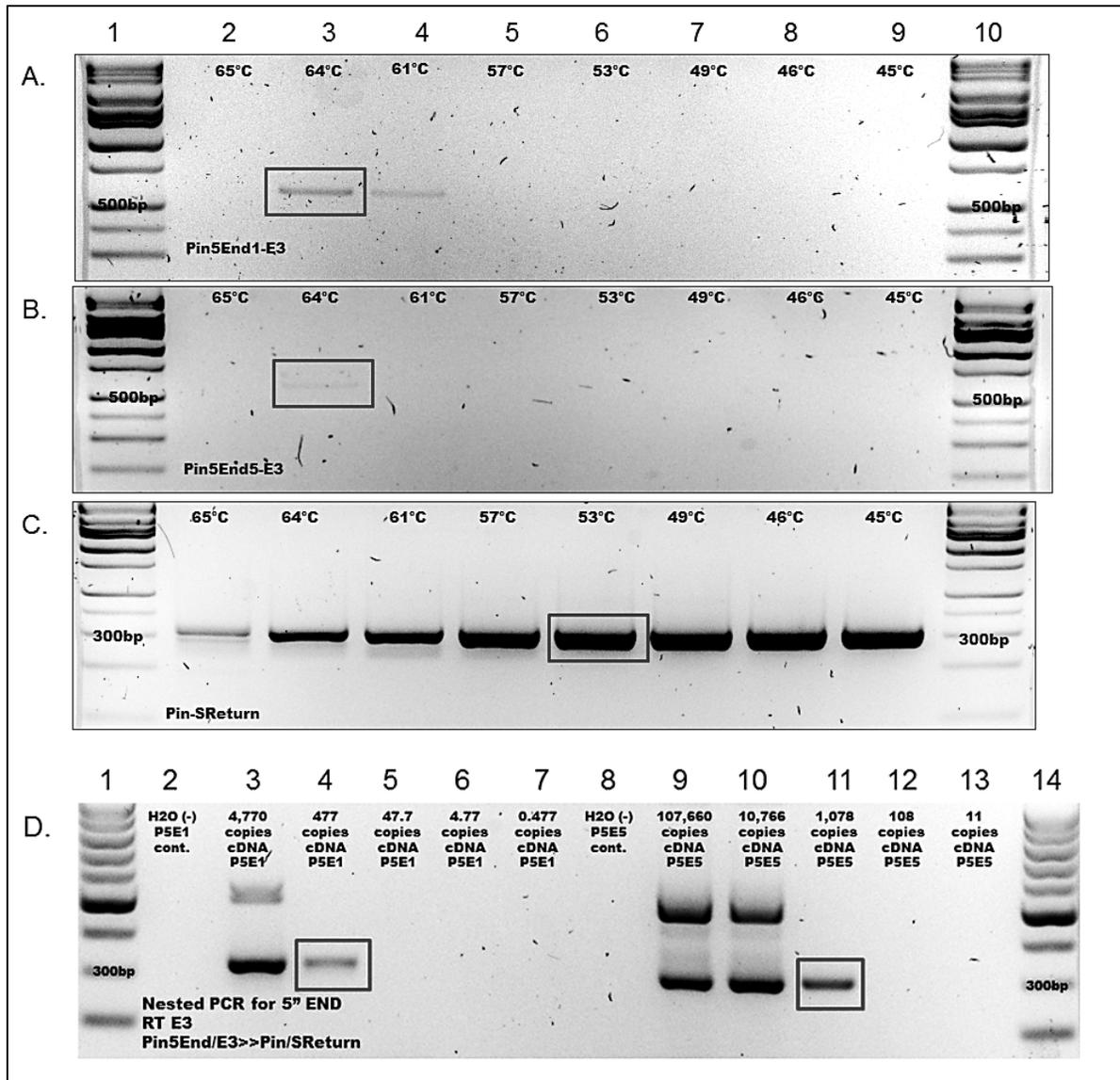
Primer optimization was done using temperature gradients to determine the optimal annealing temperatures for primer pairs 4523Rev and 2B1, 2C2 and 2C7, Pin5End1 and E3, Pin5End5 and E3, Pin and SReturn, S4 and E3Sub, and S5 and SReturn (**Table 1;2**). Following determination of optimal annealing temperatures, PCR was done with decreasing 10 –fold dilutions of cDNA clones of wt CVB3 to determine the limits of detection at the optimized temperatures. For RT-qPCR efficiency, 3 different known concentrations of T7 transcribed RNA prepared from infectious CVB3 cDNA clones was added to RT reactions with primer E3(**Table 1**). Following ethanol precipitation, q-PCR was run on each RT reaction and the efficiency was calculated to determine the number of cDNA copies detected per RNA molecule added to the initial reaction. The following pages contain the figures showing these optimizations.



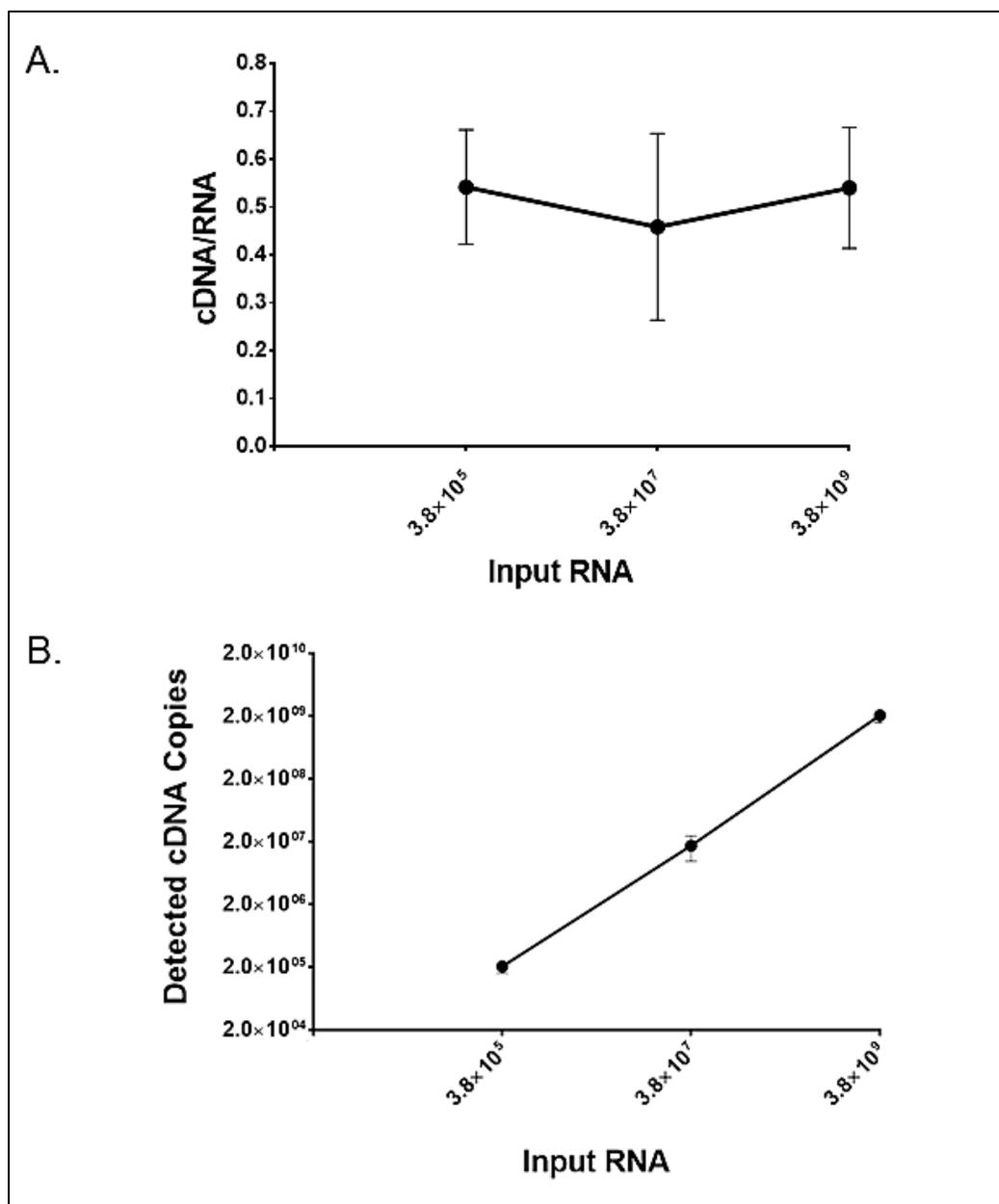
**FIG. A1. Primer optimization and efficiency of primers to amplify the CVB3 CRE(2C) genomic region.** Prior to amplifying the CRE(2C) region of the poorly replicating mutant viruses, optimization and limit of detection experiments were performed. (A) A temperature gradient was run with the primer pair 4523Rev and 2B1 in order to determine the optimal annealing temperature for these primers and amplimers were run on a 2% agarose gel. Lane A9 indicates the optimal annealing temperature for 4523Rev and 2B1 to be 52°C. (B) The process for 4523Rev and 2B1 was repeated for primers 2C2 and 2C7 to determine the optimal annealing temperature for this primer pair. Lane B7 indicates the optimal annealing temperature for 2C2 and 2C7 to be 54°C (C) Following determination of optimal annealing temperatures for both pairs, a limit of detection experiment was done using decreasing input of T7 RNA into RT reactions with primer E3, followed by nested amplification using primers 4523Rev and 2B1, followed by 2C2 and 2C7 to determine how many copies of cDNA could be detected using this method; between. Lane C4 indicates that the limit of detection for the nested PCR to amplify the CRE(2C) region is on the order of 10,000 copies of cDNA.



**FIG. A2. Primer optimization and efficiency of primers to detect 5' terminal deletions in RNA.** Prior to amplifying the 5'NTR to detect viral RNA in preparations, optimization and limit of detection experiments were performed. (A) A temperature gradient was run with the primer pair S4 and E3Sub in order to determine the optimal annealing temperature for these primers; amplimers were run on a 2% agarose gel. Lane A5 indicates the optimal annealing temperature for S4 and E3Sub to be 56°C (B) The process was repeated for primers S5 and SReturn to determine the optimal annealing temperature for this primer pair. Lane B6 indicates the optimal annealing temperature for primers S5 and SReturn to be 60°C; higher temperatures were acceptable as well (C) Following determination of optimal annealing temperatures for both pairs, a limit of detection experiment was done using decreasing input of T7 RNA into RT reactions with primer E3, followed by nested amplification using primers S4 and E3Sub, followed by S5 and SReturn to determine how many copies of cDNA could be detected using this method. Lane C5 indicates that the limit of detection of approximately 100 copies could be detected.



**FIG. A3. Primer optimization and efficiency of primers to detect 5' terminal deletions in RNA.** Prior to amplifying the very 5' termini to determine of deletions were occurring in CVB3-CKO, primers were optimized for efficient. (A) A temperature gradient was run with the primer pair Pin5End1 and E3, (B) primer pair Pin5End5 and E3, and (C) primers Pin and SReturn following amplification with Pin5End primers and E3. (A-C) Amplimers were analyzed on 2% agarose gels to determine optimal annealing temperatures for subsequent reactions. The optimal annealing temperature for primers Pin5End1 and E3 was determined to be 64<sup>o</sup>C (lane A3), 64<sup>o</sup>C for primers Pin5End5 and E3 (lane B3), and 53<sup>o</sup>C for primers Pin and SReturn (lane C6). (D) Following determination of optimal annealing temperatures for all pairs, a limit of detection experiment was done using decreasing input of T7 RNA into RT reactions with primer E3, followed by nested amplification using primers Pin5End1 and E3 or Pin5End5 and E3, followed by Pin and SReturn to determine how many copies of cDNA could be detected using this method; approximately 400 copies with P5End1 (lane D4) and 1,000 copies with Pin5End5 (lane D11).



**FIG. A4. Determination of the efficiency of RT reactions.** To determine the efficiency of the reverse transcription reactions with the primary primer used in the RT reactions, E3, RT reactions were set up with increasing concentrations of T7 transcripts prepared from the wt CVB3 infectious cDNA clone; concentrations cover a span of five logs from  $10^5$  to  $10^9$  copies of T7 RNA. Reactions were repeated three times each. (A) Following quantitative reactions, the ratio of detected cDNA to input RNA was calculated and graphed, demonstrating a near linear efficiency of 52% regardless of the input RNA. (B) Comparing detected copies of cDNA to input RNA again demonstrates a near linear efficiency of the RT reaction independent of input concentration.