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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Cells and virus

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

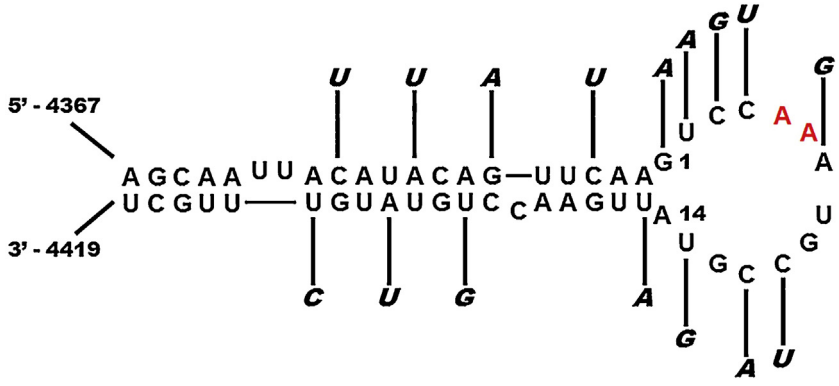

Fig. 1. The CRE(2C) of CVB3 with mutations indicated. The sequence of the CRE(2C) of CVB3 from nucleotides 4367–4419. Nucleotides changed to generate CVB3-CKO are indicated by italicized letters above the wildtype sequence. The numbering of the loop is indicated beginning with number 1 (G) and proceeding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