

5' terminal deletions in the genome of a coxsackievirus B2 strain occurred naturally in human heart

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ABSTRACT

Enteroviruses can induce human myocarditis, which can be modeled in mice inoculated with group B coxsackieviruses (CVB) and in which CVB evolve to produce defective, terminally deleted genomes. The 5' non-translated region (NTR) was enzymatically amplified from heart tissue of a fatal case of enterovirus-associated myocarditis in Japan in 2002. While no intact 5' viral genomic termini were detected, 5' terminal deletions ranged in size from 22 to 36 nucleotides. Sequence of the 5' third of this viral genome is of a modern strain, closely related to CVB2 strains isolated in Japan in 2002. A CVB3 chimera containing the 5' NTR with a 22 nt deletion produced progeny virus upon transfection of HeLa cells. When the 5' 22 nucleotide deletion was repaired, the virus induced myocarditis in mice and replicated like wild type virus in murine heart cells. This is the first report of these naturally-occurring defective enteroviral genomes in human myocarditis.

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Introduction

The group B coxsackieviruses (CVB, serotypes 1–6) are typical human enteroviruses (HEV) classified within HEV species B (Stanway et al., 2005). The genome is a single molecule of positive sense RNA, encoding 11 proteins from within a single open reading frame (ORF). The ORF is flanked on both the 5' and 3' ends by non-translated regions (NTR) (Racaniello, 2007) with both NTRs essential for maximal virus replication (Brown et al., 2004). Numerous studies in which either the 5' or 3' NTR was altered by experimental mutational or deletional analysis have shown that viral replication is deleteriously, usually lethally, affected (Barton et al., 2001; Brown et al., 2005; Murray et al., 2004; Trono et al., 1988).

Myocarditis (inflammation of the heart muscle) has been frequently associated with an HEV etiology (Kim et al., 2002). Acute myocarditis is believed to be a precursor to dilated cardiomyopathy [DCM; (Mason, 2003; Spotnitz and Lesch, 2006)], a serious disease that can lead to death in lieu of transplantation. Human enteroviral RNA has also been detected in dilated cardiomyopathic hearts and in myocarditic hearts (Andreoletti et al., 2000; Archard et al., 1998; Bowles et al., 1989; Kammerer et al., 1994; Rey et al., 2001; Satoh et al., 1994; Tracy et al., 1990). Despite the detection of HEV RNA in adult myocarditis and DCM, there has been a near complete inability to isolate infectious enterovirus in cell culture from these tissues (Rey et al., 2001) with only rare reports in the literature (Longson et al., 1969; Monaldi et al., 1963; Soutar, 1971). This is

quite different from HEV-associated pediatric myocarditis in which infectious virus can usually be readily isolated (Fechner et al., 1963; Gear and Measroch, 1973; Piraino et al., 1982; Porres et al., 1985). Similar observations have been made in mouse models of myocarditis (Gauntt and Huber, 2003; Kim et al., 2001; Woodruff, 1980), in which viral RNA can often be detected in heart tissue of mice experimentally inoculated with CVB3 long after lytic virus is not detectable using cell culture (Kim et al., 2005; Klingel et al., 1992; Reetoo et al., 2000).

The likely mechanism underlying the disparity between the detection of enteroviral RNA in the absence of culturable virus, was discovered by studying CVB infections in the mouse heart (Kim et al., 2005). In these studies, following inoculation of mice with CVB3, heart tissue was screened at various times post-infection for infectious virus. When these viral RNA populations were examined, no intact 5' genomic termini were detected and a variety of deletions existed with sizes ranging from 7 to 49 nt in length. These 5' terminal deletions (TD) had profound impacts upon the viral biology. As virus replication was so slowed that cytopathic effects (cpe) were not observed, virus titers had to be quantitated by RT-mediated qPCR analysis of RNA genomes in infected cells. Measurements of the positive to negative strand viral RNA ratio in infected cell cultures showed them to be close to unity rather than the high positive to negative ratios normally seen in wild type virus-infected cells (Kim et al., 2005). Interestingly, this finding extended into virion-encapsidated RNA as well, with CsCl-purified virion preparations showing the presence of negative as well as positive strand RNA. Clearly, packaging of negative strand RNA which cannot be translated upon infection of a cell, also served to enhance the attenuation of replication

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brought about by the TD state. Recently, we have demonstrated that similar TD mutations can occur in primary cell cultures (Kim et al., 2008). These findings have defined a novel and unsuspected aspect of HEV biology.

We were curious, therefore, to test the hypothesis that TD mutations occurring naturally during HEV replication in the human could similarly be associated with human heart disease in the absence of a detectable cytopathic virus population. Oka et al. published a case

report on two patients with fulminant myocarditis (Oka et al., 2005), one of whom showed evidence of enterovirus involvement. We used formalin-fixed, paraffin-embedded heart tissue from this adult case of fatal, HEV-associated myocarditis in Japan in 2002, to demonstrate that TD genomes with deletions of up to 36 nt were detected in the myocardium in the absence of detectable wild type viral genomes. Sequence analysis of the 5' third of the genome identified the virus as a CVB2 with high identity to CVB2 strains circulating in Japan in 2002.

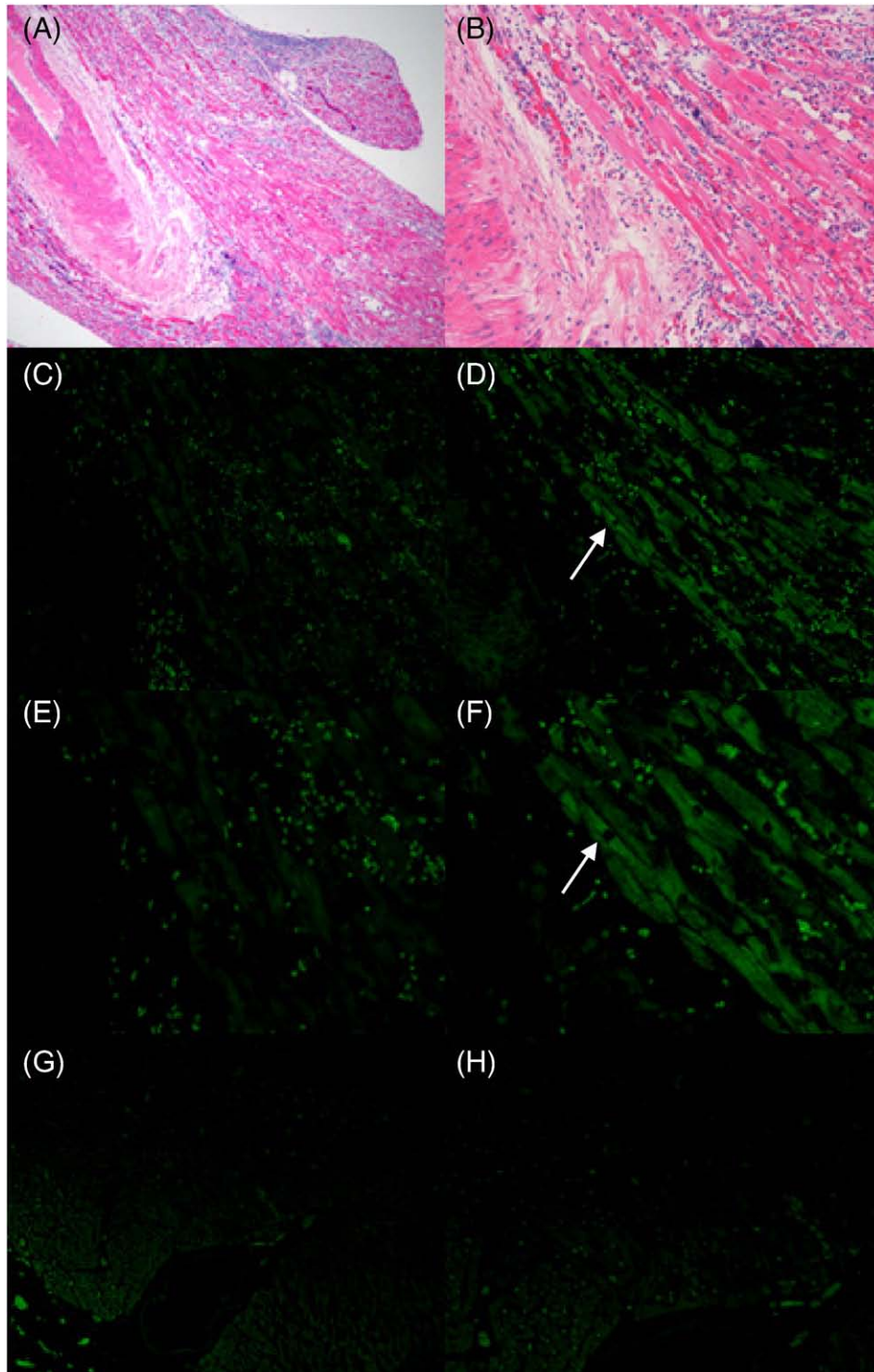


Fig. 1. Enterovirus protein in heart from case of fulminant myocarditis. Serial sections of formalin-fixed, paraffin-embedded heart were stained with hematoxylin and eosin (A, B), or with an isotype negative control antibody (C, E), or with an antibody against a conserved epitope in HEV capsid protein VP1 (D, F). A non-infected human heart was also stained with the HEV capsid antibody as a negative control (G, H). Original magnification, $\times 40$ (A), $\times 100$ (B–D, G), $\times 200$ (E, F, H). Arrows in D and F indicate the same position.

To our knowledge, this is the first report to show that HEV TD genomes can also naturally occur in human beings. As persistent expression of CVB proteins and 2Aprotease alone are sufficient for induction of cardiomyopathy in the mouse (Wessely et al., 1998a; Xiong et al., 2007) and as detection of CVB TD genomes in a diseased human heart disease is likely due to persistent, defective HEV replication (Chapman and Kim, 2008), there is now a mechanism to link of acute viral myocarditis with postviral DCM (Mason, 2003; Spotnitz and Lesch, 2006).

Results

Extent of myocarditis and enterovirus infection in the heart muscle

In 2002, an adult male died of myocarditis in Mito, Japan; the details of this enterovirus-associated fatal adult myocarditis case have been described previously (Oka et al., 2005). Using formaldehyde-fixed, paraffin-embedded heart muscle samples from which to cut sections, we used immunohistochemistry to confirm and extend an earlier work (Oka et al., 2005) that enteroviral capsid protein was detectable in the tissue. Diffused lymphocytic infiltrates were observed throughout sections from all tissue samples used in this study together with localized myocarditic lesions and muscle damage (Fig. 1A,B), consistent with the published diagnosis of fulminant myocarditis (Oka et al., 2005). Immunohistochemical staining for HEV capsid protein VP1 demonstrated widespread infection in the heart muscle (Fig. 1D, F). Detection of viral protein was adjacent to but seldom

overlapped the localized inflammatory infiltrates, a finding that has been observed previously by others in cases of HEV-associated myocarditis and DCM (Zhang et al., 2000) as well as in persistent viral infections in a murine model of CVB1-induced myositis (Tam et al., 1991). Immunohistochemical stainings of sections from a clinically normal human heart sample (Fig. 1G,H) for HEV capsid protein VP1 were negative.

Detection of 5' terminal deletions in the viral RNA in the myocarditic heart muscle

We tested for the presence of deleted 5' termini in RNA samples derived from heart sections that had been initially assayed for the presence of HEV RNA using enterovirus B specific primers (Table 1) designed from conserved sequences [data not shown, (Chapman et al., 1990)]. Complementary DNA (cDNA) generated from total heart RNA using the conserved HEV primer E1 (Chapman et al., 1990) was used for PCR amplification with primers specific for 5' terminal sequences of HEV-B cDNA and the primer E3Sub (Table 1). The orientation of these primers and location within the generic HEV 5' non-translated region (5' NTR) is outlined in Fig. 2A. Each of the 5' terminus primers amplified cDNA from the wild type CVB3/28 positive control RNA (Fig. 2B). However, only S3EntB (nt 33–58) and S4EntB (nt 50–79) efficiently amplified sequences in the heart cDNA (Fig. 2B), although a weak signal was obtained using S2EntB (nt 21–46). The inability to amplify heart cDNA with primers S and S1EntB (nt 1–25) and only weakly using a primer located further in from the 5' end (S2EntB), recalled previous

Table 1

Primer ^a	Genomic location ^a , strand ^b	Nucleotide sequence (5' to 3') ^c
<i>Primers for detection of cDNA</i>		
E1	644–627, –	CACCGGATGGCCAATCCA
E2	450–464, +	TCCGGCCCTGAATG
E3	563–537, –	ACACGACACCCAAAGTAGTCGGTTC
E3Sub	549–535, –	AGTAGTCGGTCCCG
E3REV	537–562, +	GGAACCGACTACTTTGGGTGCCGTG
E5	378–361, –	GCAGGCCCAACGCAGC
S	1–20, +	TTAAAACAGCCTGTGGGTTG
S1ENTB	1–25, +	TTAAAACAGCCTGTGGGTTGTTCC
S2ENTB	21–46, +	TTCCACCCACAGGGCCACTGGGGC
S3ENTB	33–58, +	GGGCCACTGGGCGCTAGCACTCTGG
S4ENTB	50–79, +	GCACTCTGGTATCACGGTACCTTTGTGCC
<i>Primers for generation of cDNA</i>		
With E1		
E8	65–82, +	GGTACCTTTGTGCCCTG
With E2		
DREVENTB	764–745, –	GCGTTGACACTTGAGCTCCC
With Mito5NTR	644–672, +	GACCAATAGAGCGATCGTCTATTTG
VP4REV	1046–1025, –	CGACCAATTTGGCACTCTTG
With MitoVP4	916–942, +	GGACATTATGATAAARTCTATG
VP2REVC	1537–1514, –	GGTGCGCAAGTTKATCCACTGRTG
With MitoVP4		
VP2REVA	1582–1557, –	GGCACACTGTTTATRTATGGCATBAC
With MitoVP2	1444–1468, +	GACCGATGTCCAAACTGCAGTGTGC
CVB2VP1REV	2605–2582, –	CTGGTTTGCATCGTRTCACTRGG
<i>Primers for cloning</i>		
22RIBOZ1	22–44, +	ATGAGGCCGAAAGGCCGAAACCCGGTATCCCGGGTTCGCCACCCACAGGGCCCACTGGGC
22RIBOZ2		CACTATAGGGCCGGGTGGGTGGGCTGATGAGCCGAAAGGCCGAAACCCGGTATC
TD22RIBOZPCRT7		<u>GACCCGGCCCGGTAATACGACTCACTATAGGGCCGGGTGGGTGGGCTGATGAGG</u>
DREV	764–745, –	GCGTTGACTTGAGCTCCC
ENTBSENSUS	1–42, +	TTAAAACAGCCTGTGGGTTGTTCCACCCACAGGGCCCACTG
28RIBOZ1	1–20, +	TGAGGCCGAAAGGCCGAAACCCGGTATCCCGGGTTCCTAAAACAGCCTGTGGGTTG
28RIBOZ2		<u>GACACTGATCCGGGGTTTTAACTGATGAGGCCGAAAGGCCGAAACCCGGTATC</u>
28RIBOZPCRT7		<u>GACCCGGCCCGGTAATACGACTCACTATAGGGCCGGGTGTTTAACTGATGAGG</u>

^a Numbering as in CVB3/28 (GenBank accession no. AY752944).

^b Positive is same sense as genomic RNA, negative is antisense to genomic RNA.

^c Underlined sequence is not genomic cDNA in cloning primers.

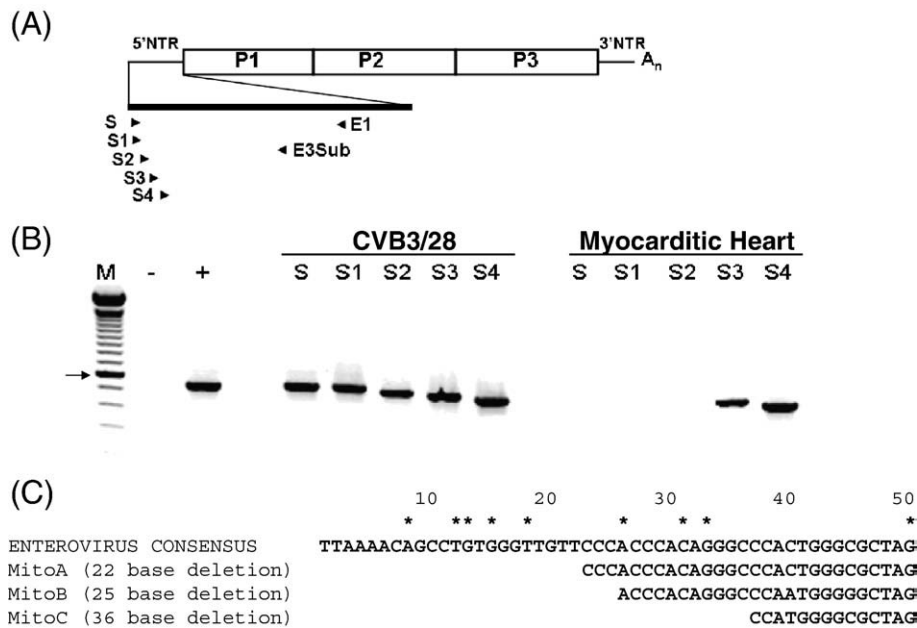


Fig. 2. cDNA from human myocarditic heart was not amplified with 5' terminal enterovirus-specific primers. (A) Orientation of primers within the 5' NTR. (B) E1-primed cDNA of total RNA from human myocarditic heart or purified CVB3/28 was amplified with 5' terminal primers S, S1, S2, S3, S4 and E3Sub in separate PCRs and then electrophoresed in 1.5% agarose. M, 100 bp ladder (Invitrogen); -, PCR with S and E3Sub without template; +, PCR with S and E3Sub with pCVB3/28. Abbreviations for S1EntB-S4 EntB are S1-S4. Arrow indicates 600 bp band. (C) Alignment of the 5' terminal sequences of CVB2/Mito with the enterovirus B consensus sequence. Three sizes of 5' terminal deletions were cloned and sequenced (22, 25 and 36 nt). Numbering begins from the 5' terminus. Asterisks indicate positions of previous terminal deletions detected in hearts of mice (Kim et al., 2005) and in cell culture passage (Kim et al., 2008).

results obtained from mouse hearts (Kim et al., 2005) and cell cultures (Kim et al., 2008) that indicated 5' terminal sequences were missing from the viral RNA.

We then tested directly for 5' terminal deletions using sequence analysis. Heart cDNA primed with E1 was tailed with dC₁₀, amplified with E5 and a primer specific for the oligo dG tail [DC-Tail which has a 3' oligo dC₁₀; (Kim et al., 2005)], and cloned. Only four unique clones were obtained due to the low yield of tailed cDNA. Two clones showed deletions of the 5' nt 1–22, one had nt 1–24 deleted, and one had nt 1–35 deleted relative to consensus 5' terminal sequence derived from 90 HEV-B 5' terminal sequences in the GenBank database (Fig. 2C). Control experiments using this approach with cDNA from wild type CVB3/28 stocks generated clones with 10 C residues at the 5' terminus upstream of the authentic HEV-B consensus 5' terminus (Kim et al., 2005). One of the 4 clones had a 5' terminal C₁₀ tract followed by sequence that aligned with nt 26–360 of the enterovirus B sequence (Fig. 2C, MitoB) while in two clones, the C₁₀ tract was followed by sequence aligning to nt 23–360 (Fig. 2C, MitoA). Because nt 23–25 are CCC in the HEV-B consensus, it is possible that the deletions in these genomic termini might be 22–25 nt in length, although 25 and 22 nt are the most likely deletions based upon the C₁₀ tract. Similarly, the final clone (Fig. 2C, MitoC) most likely had a deletion of 36 nt, although 35–38 nt deletions are similarly possible. Like the previously described CVB3-TD genomes isolated from mouse hearts (Kim et al., 2005) or cell cultures (Kim et al., 2008), the deletions disrupt or delete regions in the secondary structure termed domain I (Bailey and Tappich, 2007): the loss of nt 1–22 and 1–25 delete stem a, while the greater deletion (nt 1–36) delete stems a and b of domain I. Together, these results demonstrated that the population of HEV RNA in the human heart contained 5' terminal genomic deletions.

The nucleotide and amino acid sequences of the heart virus identify it as a modern strain of CVB2

Sequence analysis using overlapping cloned cDNAs was carried out to determine the identity of the virus in the heart muscle. The sequence of the 5' 2,563 nt of the heart HEV RNA (GenBank accession no.

EU177671) was determined as outlined in Materials and methods. A BLAST search of GenBank using this sequence revealed closest identity to the genomes of the six CVB serotypes, with the sequences showing highest identity (92–93%) being partial sequences of CVB2 isolates from 2002 in Kanagawa, Japan (GenBank accession nos. AB162751, AB162752). Alignment of the 2,563 nt sequence to the prototype CVB genomes of serotypes 1–6 (GenBank accession nos. AF081485, M16560, AY752944, X05690, AF114383, AF114384) revealed that the heart virus sequence shared highest nt identity (84%) with that of CVB2, strain Ohio-1 (Polacek et al., 1999) with significantly lower identities shared by genomes of the other 5 CVB serotypes (74–75%). The relatively low identity with the prototype CVB2 sequence (and to other prototype CVB genomes) in comparison to the 2002 Kanagawa CVB2 isolates, was not surprising given that the prototype strains circulated more than 50 years ago (Melnick et al., 1950). However, a recently published study of an HEV in type I diabetes patients (Dotta et al., 2007) generated a sequence which shared >99% overall nt identity with the CVB4 prototype strain Benschoten originally isolated in 1951 (Sickles et al., 1955), with just one nt mismatch in the 5' NTR. As RT-PCR has a risk of contamination with laboratory strains [see, for example, (Giacca et al., 1994)], it can be concluded that the CVB4, "Tuscany" strain (Dotta et al., 2007), was derived from contamination with the 58 year old prototype strain. The 84% identity of the Mito sequence with the CVB2 prototype and higher identity with sequences of CVB2 isolates from the same time and geography demonstrates that the Mito sequence is not due to contamination.

Oberste and colleagues (Nix et al., 2006; Oberste et al., 1999a,b) have demonstrated that sequence analysis of the HEV VP1 genomic region correlates with HEV serologic identity; virus strains within a specific serotype share >75% nucleotide identity or 85% amino acid identity. The VP2 puff region [aa2129–2180 (Muckelbauer et al., 1995)] also has a similar level of intraserotype (but not interserotype) identity and can be used reliably to type HEV strains (Nasri et al., 2007). As we were unable to sequence further than 2563 nt, we analyzed the sequence encoding the entire VP2 capsid protein (nt 950–1721), comparing it to those from other CVB genomes. The closest identity again occurred with CVB2

(84–85%; GenBank accession nos. EF174468, EF174469, AF081485, AF085363), with greater divergence from other CVB serotypes (72–74%). We then examined the VP2 Puff sequence (aa197–250), a structure that contributes to the determination of serotype (He et al., 2001). When compared to those of modern and prototype CVB2 strains, the heart virus sequence revealed highest identity with other CVB2 strains (Table 2). Taken together, these results indicated that the heart virus was a modern (ca. 2002) strain of CVB2 and was subsequently termed CVB2/Mito.

Terminally-deleted CVB2/Mito 5' NTR in a CVB3 genome produces non-cytopathic virus but restoration of the 5' terminus to the consensus HEV-B sequence produces a cytopathic virus

We replaced the 5' NTR of the infectious cDNA copy of the cardiovirulent strain CVB3/28 genome (Tracy et al., 2002) with the CVB2/Mito 5' NTR containing the 22 nt deletion to study how the CVB2/Mito 5' NTR would function in an intact CVB genome. The 5' NTRs of enteroviruses have been shown to be functionally interchangeable, producing infectious progeny viruses (Chapman et al., 2000a; Lukashev et al., 2003; Semler et al., 1986). No cpe was observed following transfection of this cDNA, termed CVB3/5NTRMitoTD23 [the TD23 genome is named for the first nt of the genome relative to the enterovirus consensus; (Kim et al., 2005)], into HeLa cell cultures (Fig. 3A), although transfection of the control CVB3/28 cDNA caused widespread cpe within 48 h (Fig. 3A). However, upon restoration of the 22 nt 5' terminal deletion (CVB3/5NTRMito), a lytic phenotype was restored (Fig. 3A).

To verify that the transfection which did not generate cpe was in fact productive and that transfected cDNA genomes produced viral RNA, this experiment was repeated with HeLa cultures transfected with RNA from CVB3/28, CVB3/5NTRMitoTD23, CVB3/5NTRMito, or mock transfected as described above. Total RNA was isolated from each culture, DNase treated, and assayed for the presence of viral RNA

by RT-PCR using E1 and primers specific for the 5' terminus. Despite the lack of cpe in CVB3/5NTRMitoTD23 infected cultures, viral RNA was readily detected (Fig. 3B) with 5' end primers outside of the deleted sequence. Both the wild type (positive control) CVB3/28 RNA and the RNA from the cultures infected with the CVB3/5NTRMito (repaired deletion) strain were detected with all of the primers as expected (Fig. 3B); these strains both induced complete cpe (Fig. 3A).

When supernatants of the CVB3/5NTRMitoTD23 infected cultures were treated with ribonuclease, passed through 0.2 µm filters, and placed on fresh HeLa cell monolayers, no cpe resulted but viral RNA remained detectable (data not shown), indicating that infection was due to a normal infectious process by virions and not by adventitious contamination with free viral RNA. Measurement of the ratio of positive to negative strand viral RNA in a purified CVB3/5NTRMitoTD23 stock by real time RT-mediated quantitative PCR was carried out. This revealed that both negative and positive strand viral RNA was detected in RNA isolated from CsCl-purified virions with a positive/negative strand ratio of 1.6:1. This finding with the chimeric CVB3/Mito5NTRTD23 strain was consistent with known CVBTD biology established with murine viral isolates (Kim et al., 2005), confirming that CVBTD strains can encapsidate either negative or positive stranded RNA. By comparison, stocks of parental strain CVB3/28 showed no detectable negative strand RNA as expected from prior work (Kim et al., 2005; Novak and Kirkegaard, 1991). Together, these results showed that the CVB2/Mito 22 nt deletion incurred the same type of replication lesion in the chimeric CVB3/5' NTRMitoTD23 strain as did other TD strains described previously (Kim et al., 2005).

Analysis of CVB3/5NTRMito in cell culture

Stocks of the chimeric CVB3/5NTRMito strain were analyzed in single step growth curves together with control virus strains, cardiovirulent

Table 2
Alignment of the predicted amino acid sequence of the VP2 Puff from the heart virus (Mito) with CVB serotype strains

	Year	197	250
MITO	2002	PEAEMGCTNKENTPLFEKLCGQDNAKEFSREGPTVSEGATDVQTAVCNAGMGVG	
CVB2/SE-96-72087	1996I.....	
CVB2/SE-05-90025	2005A.....	
CVB2/Ohio (prototype)	N.....T.....I.K.....	
CVB4/SE-04-85460	2004V..A TYGD...GET..Q.EQN---AAT.E.A.....	
CVB4/SE-04-84828	2004V..A TYGD...GET..Q.EQN---AAT.E.A.....	
CVB4/Benschoten (prototype)	A..A AYGD...GET..S.EQN---AAT.K.A.....	
CVB4/E2	1958A..A TYGD...GET..Q.EQN---AVT.E.A.....	
CVB6/Schmitt (prototype)	S.LN.A AAD.SAGEV.RQ.TV.P---AN.QNQ.....H..A..A	
CVB1/SE-02-71582	2002S.LN...E AE.S.G.T..M.TDTKVG-ESN.KK.....W.....	
CVB1/SE-99-94869	1999S.LN...E AE.S.G.T..M.TDTKVG-ESN.KK.....W.....	
CVB1/Tucson (prototype)	S.LD...E VE.S.G.S.RL.TDTQVG-ESNEKK.....W.....	
CVB1/Conn-5 (prototype)	S.LN...K AE.S.G...RM.TDTEVG-TSNDKK.....W.....	
CVB5/SE-03-79895	2003ATLA.K.DLKS.SNGET.SM.ESQN---T.Q.A..AN.I.....	
CVB5/Peterborough	1954ATLA.K.DQKS.SNGET.NT.DSQN---TT.Q.A..AN.I.....	
CVB5/Faulkner (prototype)	ATLA.K.DQKS.SNGET.NV.ESQ---N.S.Q.A..AN.I.....I.	
CVB3/SE-94-51301	1994ATLD...SS.E.L.G.A...ADK-.VA.GSNKL..R.Y.....I.	
CVB3/AS	1977ATLD...SSAE.L.G.S...ADK-.VA.GSNKL..RV.Y.....I.	
CVB3/CO	1978ATLD...SS.E.L.G.A...ADK-.VA.GSNKL..RV.Y.....I.	
CVB3/GA	1956ATLD...SSAE.L.G.S.M...TDK-.VAAGSNKL..RV.H.....	
CVB3/Nancy (prototype)	ATLD...SSAE.L.GDS...ADK-.VA.GSNKL..RV.Y.....	

GenBank accession nos: CVB2/SE-96-72087, ABI31471; CVB2/SE-05-90025, ABI31477; CVB2/Ohio, AAD46138; CVB4/SE-04-85460, ABI31513; CVB4/SE-04-84828, ABI31533; CVB4/Benschoten, P08292; CVB4/E2, Q86887; CVB6/Schmitt, AAF21972; CVB1/SE-99-94869, ABI31571; CVB1/SE-02-71582, ABI31508; CVB1/Tucson, AA084299; CVB1/Conn-5, AAC00531; CVB5/SE-03-79895, ABI31514; CVB5/Peterborough, Q03053; CVB5/Faulkner, AAF21971; CVB3/SE-94-51301, ABI31532; CVB3/AS, AAD53727; CVB3/CO, AAD53726; CVB3/GA, AAT79531; CVB3/Nancy, AAA42931; CVB3/20, AAV34213; CVB3/31-1-93, AAG23918; CVB3/28, AAV34212. VP2 "Puff" sequence of strains selected based on alignment with CVB3 2129-2180. Dot indicates conserved sequence relative to Mito; - indicates deletion.

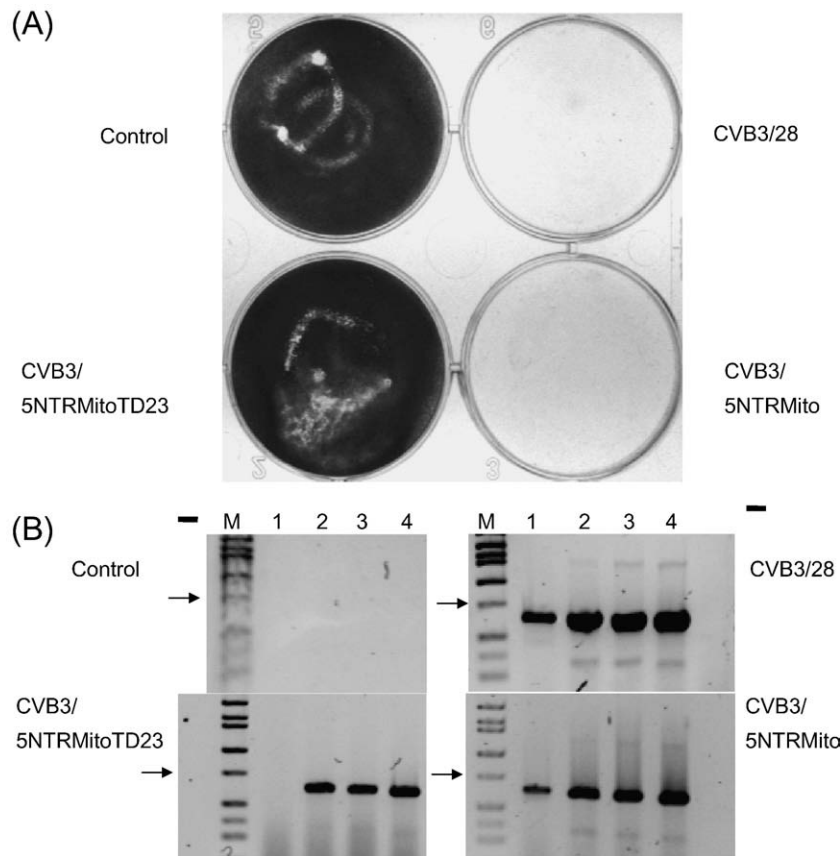


Fig. 3. CVB3/5NTRMitoTD23 replicates without cytopathic effect but restoration of 5' terminal sequence to HEV-B consensus results in cytopathic replication. (A) HeLa cell monolayers were transfected with T7 RNA polymerase transcripts of pCMVT7r5NTRMitoTD23 (CVB3/5NTRMitoTD23), pCMVT7r5Mito (CVB3/5NTRMito), pCVB3-28 (CVB3/28) or mock transfected, control. Monolayers were fixed at 72 h and stained with crystal violet. (B) cDNA from RNA of 72 h cultures of transfected HeLa monolayers was amplified with primer E1 and primers S1ENTB (lane 1), S2ENTB (lane 2), S3ENTB (lane 3), S4ENTB (lane 4) and electrophoresed in 1.5% agarose. Lane M, Hi-Lo DNA Marker (Minnesota Molecular, Minneapolis, MN); lane -, PCR with S4ENTB and E1 without template cDNA. Arrow indicates 750 bp.

CVB3/28 and non-cardiovirulent CVB3/GA (Lee et al., 2005), in HeLa cell and MHC cultures. The chimeric CVB3/5NTRMito strain replicated with similar initial kinetics in HeLa cultures as the two wild type CVB3 strains, reaching a similar yield of infectious particles 2 h later (Fig. 4A). HeLa cultures are a rich environment for the growth and replication of HEV, making it difficult to differentiate virus strains that differ by replication

phenotype (Agol et al., 1989; Lee et al., 2005). However, when these virus strains were similarly analyzed in cultures of MHC, a cell culture system in which cardiovirulent CVB3 strains replicate to higher levels than non-cardiovirulent strains (Kim et al., 2008), CVB3/5NTRMito and CVB3/28 replicated similarly in rate and extent, while the non-cardiovirulent CVB3/GA replicated more slowly to 3 orders of magnitude lower titer

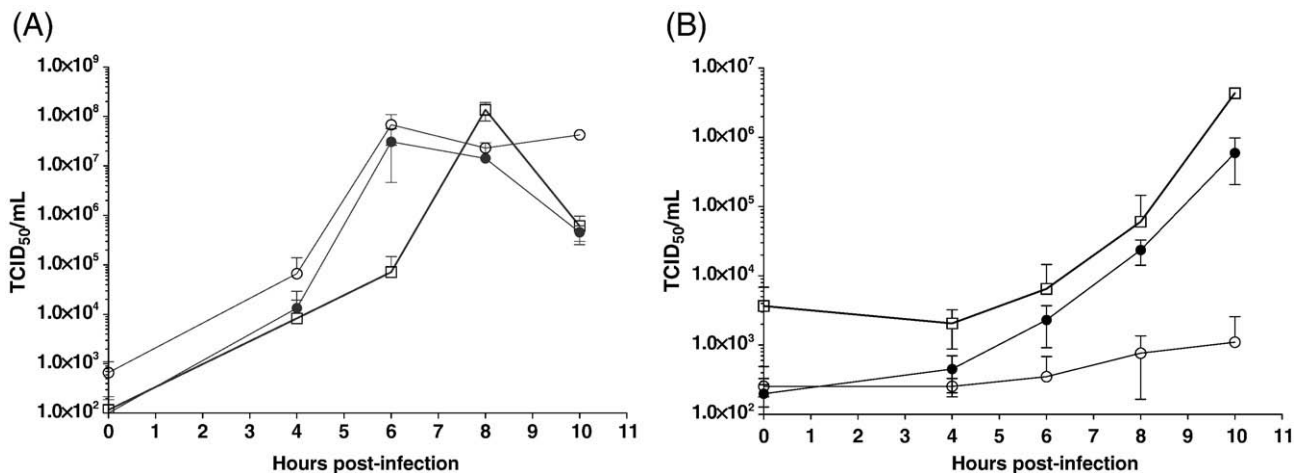


Fig. 4. CVB3/5NTRMito replicates in murine heart cells like virulent CVB3/28 and but not avirulent CVB3/GA. Single step growth curves were carried out in HeLa (A) and MHC (B) monolayers using triplicate wells initially seeded with 100,000 cells per well. Monolayers were inoculated with CVB3/5NTRMito, CVB3/28, or CVB3/GA at an MOI of 25. Virus titers were determined on HeLa cell monolayers. Square, CVB3/5NTRMito; filled circle, CVB3/28; open circle, CVB3/GA.

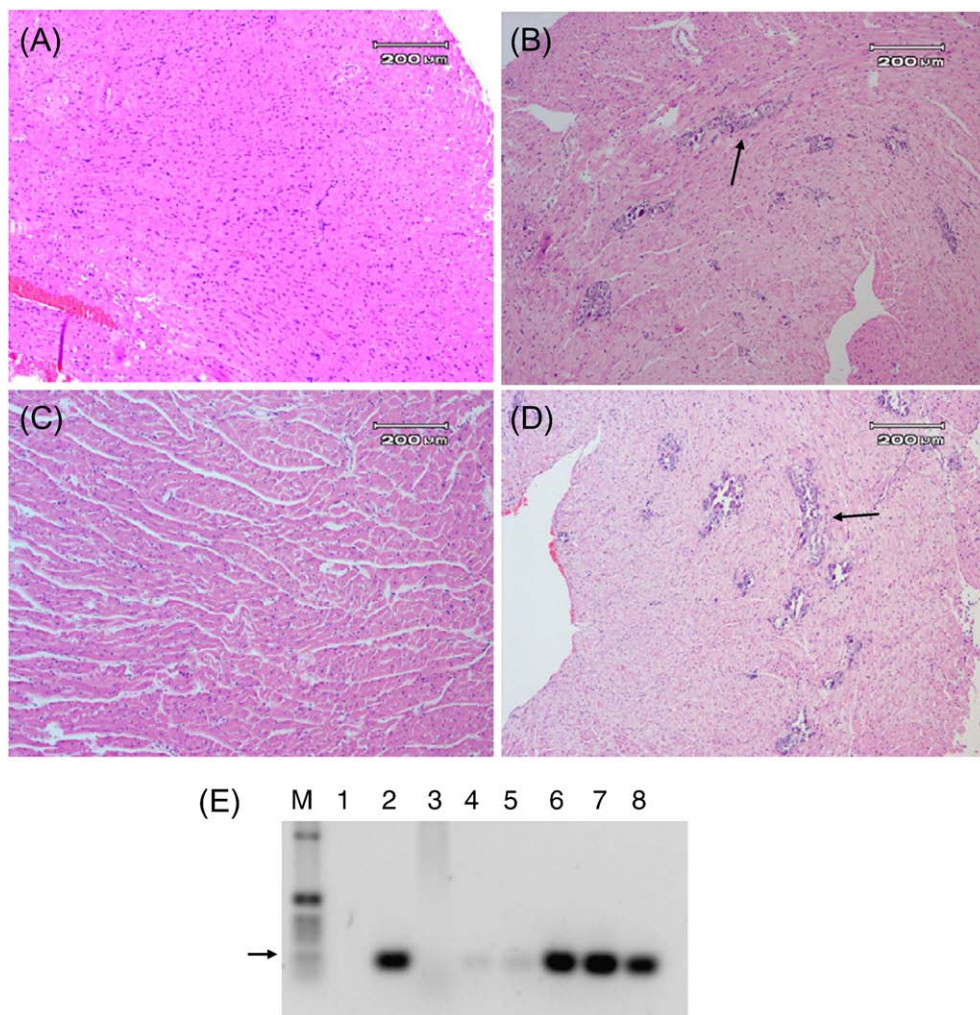


Fig. 5. CVB3/5NTRMitoTD23 shows no pathogenic effects in mouse heart but CVB3/5NTRMito with a restored 5' terminus causes myocarditis. Male A/J mice were mock infected (saline) (A), or inoculated with 1×10^5 TCID₅₀ units of CVB3/28 (B), or the equivalent of 1×10^5 TCID₅₀ units of CVB3/5NTRMitoTD23 (C), or 1×10^5 TCID₅₀ units of CVB3/5NTRMito (D). At day 14 post-inoculation hearts were fixed in 10% buffered formalin and sections stained with hematoxylin and eosin. Typical lesions indicated by arrows. Original magnification, $\times 100$. E. Heart homogenates of mock-infected, CVB3/5NTRMitoTD23, CVB3/5NTRMito, or CVB3/28 inoculated A/J mice were used to inoculate HeLa cell monolayers. cDNA from RNA of 72 h cultures of transfected HeLa monolayers was amplified with primer E1 and E2 and electrophoresed in 1.5% agarose. Lane M, 1 kb ladder (Invitrogen); lane 1, PCR with E1 and E2 without template cDNA, lane 2 with CVB3/28 viral cDNA, lane 3 with cDNA of mock-infected heart homogenate culture, lanes 4, 5 with cDNA from CVB3/5NTRMitoTD23 heart homogenate cultures, lanes 6, 7 with cDNA from CVB3/5NTRMito heart homogenate cultures, and lane 8 with cDNA from CVB3/28 heart homogenate culture. Arrow indicates 510 bp.

(Fig. 4B). These results demonstrated that with an intact 5' terminal sequence, the 5' NTR of CVB2/Mito permitted the chimeric virus to replicate like a cardiovirulent strain.

Inoculation of mice with CVB3/5NTRMito induces myocarditis

To investigate whether the chimeric virus with the restored 22 nt would induce myocarditis, male A/J mice (4–5 weeks old) were inoculated with 1×10^5 TCID₅₀ equivalents of the defective strain (CVB3/5NTRMitoTD23), the restored strain (CVB3/5NTRMito), or the control strain (CVB3/28). Hearts from control mice, inoculated with 100 mM saline (virus diluent) (Fig. 5A) were normal, and indistinguishable from the hearts from mice inoculated with the defective strain CVB3/5NTRMitoTD23 (Fig. 5C), despite the presence of viral RNA in the myocardium (as determined by RT-PCR; data not shown). Inoculation with CVB3/5NTRMito with the restored 5' genomic terminus, induced myocarditis (Fig. 5D) to an extent similar to that induced by the control, cardiovirulent strain, CVB3/28 (Fig. 5B). These results demonstrate that the CVB2/Mito 5' NTR is capable of working in synergy with the rest of a cardiovirulent CVB genome to generate myocarditis.

Discussion

This report documents for the first time that group B coxsackieviruses can generate heretofore unsuspected 5' terminal genomic deletions during replication in the human host subsequent to a naturally-occurring infection. Using RNA isolated from formalin-fixed, paraffin-embedded heart muscle samples from a case of fulminant myocarditis in 2002 in Mito, Japan (Oka et al., 2005) in which HEV protein and RNA were detected, we have identified the virus as a CVB2 strain with close identity to the Kanagawa strain of CVB2 which circulated in Japan in 2002. Importantly, while we were unable to detect intact 5' viral genomic termini, deletions at the 5' termini of at least 22–36 nt were present. These data from a human sample closely mirror results first obtained from mouse heart tissue following experimental CVB3 inoculation (Kim et al., 2005) and more recently, from CVB3-inoculated cell cultures (Kim et al., 2008), thereby demonstrating that this newly-discovered aspect of HEV biology has clinical relevance in addition to the basic description of a new aspect of the enterovirus replication cycle.

The discovery that HEV can delete the 5' terminal genomic sequences during replication and thereby can persist in the host for long

periods of time [as has been shown in the mouse; (Kim et al., 2005)], has led to the demonstration of dramatic changes in the replication cycle of such viruses. Terminally-deleted CVB3 strains isolated from mouse heart or tissue culture (Kim et al., 2008; 2005), similar to the CVB2/Mito genome population characterized here, replicate very slowly and to low titers. CVB3TD strains persisted for months in murine heart tissue but did not induce cytopathic effects when heart homogenates were inoculated onto HeLa cell monolayer cultures (Kim et al., 2005). Because progeny infectious virus can nonetheless be detected in such cultures, cell lysis undoubtedly occurs; however, due to the very slow replication rate of the CVBTD strains, cpe in cell cultures is not observable by light microscopy. No viral or genomic RNA replication was noted in a recent study (Hunziker et al., 2007) of a CVB3 genome with an engineered deletion of the 5' terminal 32nt. In that study, viral translation was assessed by labeling transfected cells for 30 min, while RNA replication was assayed by slot-blot. However, previous work which required accumulation of viral proteins for 24 h for detection by Western blot and enzymatic amplification for detection of viral RNA (Kim et al., 2005), strongly suggests that these assays would not have been sufficiently sensitive to detect the low levels of virus found in cultures of CVB3TD.

The slow replication of CVBTD strains is linked to the deletions in the domain I RNA structure, a structure that is known to be crucial for HEV replication (Andino et al., 1990; Lyons et al., 2001; Sharma et al., 2005). The interesting observation that the normally high positive/negative viral RNA strand ratios in wild type infected cells (Novak and Kirkegaard, 1991) decrease to near unity with CVB3TD viruses, further indicates that 5' terminal deletions deleteriously affect positive strand viral RNA synthesis. The discovery of TD genomes has also provided a mechanistic explanation for the detection of nearly equimolar levels of positive and negative strand RNA that have been reported during persistence of CVB in mice (Klingel et al., 1992; Tam and Messner, 1999). Because CVBTD (but not wild type) virions can contain either negative or positive stranded viral RNA with nearly equal probability (Kim et al., 2005), CVBTD attenuation is further enhanced because negative strand viral RNA cannot be used as an mRNA upon infection. We have also shown that passage of wild type CVB3 in cardiac primary cell cultures can also generate TD strains (Kim et al., 2008), although this was not observed in immortal cell cultures such as HeLa. These latter findings indicate that the type of host cell and tissue plays a key role in determining whether TD genome populations can displace wild type virus. We have not yet explored whether other organs may harbor similar populations of CVBTD strains following experimental inoculation of mice.

Our discovery that TD genomes occur in human tissue raises the question of their impact upon human health. Following experimental inoculation of mice with CVB3, we (Kim et al., 2005) and others (Klingel et al., 1992; Reetoo et al., 2000) have shown that viral RNA can be detected in mouse heart muscle for weeks, well past the time that cytolytic virus can be isolated in susceptible reporter cell cultures. Long-term persistence of an HEV infection in the apparent absence of lytic virus, has similarly been observed in human hearts by analysis of sequential endomyocardial biopsies (Jin et al., 1990; Kuhl et al., 2005; Stille-Siegener et al., 1993). Although the myocarditic heart used in this study was from an acute stage of enteroviral myocarditis, no non-deleted 5' ends of viral genomes were detected by cloning or by amplification of 5' NTR sequence, indicating that in this tissue the process of selection of deleted genomes is rapid. Although the possibility of repeated HEV infections cannot be rigorously disproven in clinical studies, we propose here that HEV persistence which occurs via generation of a TD genome population, is the more likely mechanism. How could a slowly-replicating, persisting TD virus population damage the heart? A plausible mechanism was suggested by work in which a CVB3 genome was cloned in a transgenic mouse line under the cardiac myosin promoter (Wessely et al., 1998b). The VP4/VP2 autocatalytic cleavage site (Wessely et al., 1998a) was engineered to prevent correct cleavage of the VP0 protein, a lesion that prevented production of infectious virus

particles. These transgenic mice developed enlarged hearts, reminiscent of dilated cardiomyopathy, a pathologic outcome that was subsequently demonstrated to be due solely to expression of 2Apro (Xiong et al., 2007), one of two CVB proteases, in the host. It is well established that the expression of HEV proteins can alter host cell protein expression and secretion (Belov and Ehrenfeld, 2007; Kundu et al., 2005; Kuyumcu-Martinez et al., 2004; Lloyd et al., 1987). CVB proteins can cleave and disrupt host cell proteins such as dystrophin (Badorff et al., 1999), a cytoskeletal protein altered in X-linked dilated cardiomyopathy (Towbin et al., 1993), and host translation factors such as poly A binding protein (Joachims et al., 1999; Kerekatte et al., 1999). Such changes can cause cell and tissue dysfunction in the infected host. In the current fatal case of fulminant myocarditis described by Oka et al. (2005), the CVB2 population had already evolved to a TD form and viral proteins were expressed and accumulating in many intact cardiomyocytes at the time of death (Fig. 2). Had the patient survived such an extensive myocardial infection (which produced sufficient virus for verification of the 5' terminal deletion), there would have been the potential for extensive alteration of myocardial function by expression of the viral proteins.

The development of DCM as a consequence of viral myocarditis has been discussed extensively in the medical literature (D'Ambrosio et al., 2001; Figulla, 2004; Kawai, 1999; Mason, 2003) and has been reviewed and summarized recently (Spotnitz and Lesch, 2006). A question frequently cited in these reviews has been the role that HEV infections play in the development of DCM; whether symptoms are due to direct viral-mediated effects on cell function during persistent infection as discussed above or due to inflammation and autoimmunity persisting post-infection (Fairweather et al., 2005; Huber, 2006). Although we have not yet documented the presence of TD forms in DCM, this study demonstrates that HEV in human heart infections can rapidly evolve to the TD form, a form capable of persisting far beyond the acute stage (Kim et al., 2005). The results presented here and elsewhere (Kim et al., 2008; 2005) have now documented a natural and previously unsuspected mechanism by which cardiotropic HEV strains can persist as a productive infection of the heart, providing an environment in which cleavage of and damage to host cell proteins by viral proteases expressed from the persistently infectious viral genome, can accumulate with time with deleterious outcomes.

How does a TD genome population persist in the face of an activated adaptive immune response? We theorize that TD genomes arise through RNA polymerase error; in support of this, while the HEV 3Dpol has been shown to be a processive enzyme, small amounts of prematurely terminated transcripts have been detected (Rodriguez-Wells et al., 2001). We propose that TD genomes are present in any replicating HEV population and that this defective quasispecies population can rapidly become dominant if the environment changes to favor them over lytic wild type HEV replication (Domingo et al., 2006). At present, we cannot assay for the presence of TD genomes in the presence of intact wild type genomes, and can therefore demonstrate TD RNA only when the TD RNA population has become dominant. In experimentally-inoculated mice (Kim et al., 2005), we have shown that TD genomes become detectable in the latter part of the acute viral replication period when the adaptive immune system has been activated and has largely cleared the wild type virus from the host. Activation of the serotype-specific adaptive immune response against the infecting virus is a prime candidate for the significant change in the environment that leads to clearing of fast-replicating wild type virus without eradication of the TD population. In support of this, inoculation of *scid* mice (which lack functional B and T cells) with poorly pathogenic CVB strains results in long-term persistence of infectious, cytopathic virus (S Tracy, unpublished data).

But why aren't TD populations also eradicated in the immunocompetent host? We theorize that it is related to the TD virus population's slow replication rate, which may be linked with low antigen levels or expression. Following inoculation of immunocompetent mice with cloned CVB3TD strains (Kim et al., 2008; 2005), we have been unable to

detect neutralizing antiviral antibodies (lowest dilution, 1:6), although antibodies that bind purified virions used as target antigens in ELISA are detectable, albeit at lower concentrations than in sera of mice inoculated with wild type CVB3 (N. Chapman, S. Tracy, unpublished data). Together with the profound defects in TD virus replication, these observations suggest that TD virus populations are poorly antigenic, most likely due to low virus antigen loads in the host. These results may also define why areas of extensive lymphocytic infiltration border but seldom overlap with, areas of viral protein expression found in this study (Fig. 1) and in work of others (Tam et al., 1991; Zhang et al., 2000). Thus, even under the scrutiny of a thoroughly activated adaptive immune system, HEV persistence can occur via a significantly restrained lifestyle compared to the acute, high-titer, cell-damaging replication normally associated with HEV infections (Pallansch and Roos, 2001).

Materials and methods

Cell culture and viruses

CVB3/28 (Tracy et al., 2002) and CVB3/GA (Lee et al., 2005) have been cloned, sequenced and described previously. Virus stocks were prepared in HeLa cell monolayers as previously described (Kim et al., 2005) or propagated in cultures of primary murine heart cells (MHC). MHC cultures were derived from minced ventricular tissue of a male C3H/HeJ mouse at 6 weeks of age as described (Toraason et al., 1989) with minor modification. Briefly, mouse heart tissue was finely chopped, washed in saline, and trypsinized (50 µg/ml; Worthington Biochemical Corp., Lakewood, NJ) at 50 µg/ml in calcium and magnesium-free Hanks balanced salt solution with 30 mM 2,3 butanedione 2-monoxime for 20 h at 6 °C. This was then treated with collagenase (100 U/ml; Worthington Biochemical Corp.) for 1 h at 37 °C in Claycomb medium (SAFC Biosciences, Inc.; Lenexa, KS), and passed through a 70 µm cell strainer (BD Falcon, San Jose, CA). The filtrate was placed into culture in Claycomb medium supplemented with 10% fetal bovine serum, 1 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM norepinephrine and 2 mM L-glutamine at 37 °C, 5% CO₂. MHC cultures were used between passages 4 and 15.

Detection of viral capsid protein in human heart tissue by immunohistochemistry

Formalin-fixed, paraffin-embedded heart muscle samples (approximately 9 cm³) were used to cut sections for immunohistochemistry and RNA isolation (below). Immunohistochemical staining for viral capsid protein was performed essentially as described (Drescher et al., 2004; Tracy et al., 2002). Briefly, following deparaffinization, rehydration, and steaming in 10 mM sodium citrate (pH=6.0) to expose epitopes, slides were blocked with normal goat serum (Sigma; St. Louis, MO). Detection was performed using a mouse monoclonal antibody against a common antigen in the enteroviral capsid protein VP1 (Clone 5-D8/1; DAKO Corp., Carpinteria, CA). Control slides received an isotype control sera (mouse IgG2a isotype control; eBioscience, San Diego, CA). Antibody was detected with an Alexa UO 488-labeled goat anti-mouse IgG (H+L) antibody (Invitrogen; San Diego, CA).

Isolation of human heart RNA

About 40–50 sections (6 µm) from a tissue block were cut for each RNA preparation. Sections were deparaffinized in xylene, washed successively with 100%, 90% and 70% ethanol, washed once in 10 mM Tris-HCl, pH 8.0, then mixed with 6000 U/ml proteinase K (Ambion; Austin, TX) in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, 1% w/v SDS, for 18 h at 56 °C. Total RNA was then isolated on silica-based matrix columns (ZR Viral RNA Kit; Zymo Research, Orange, CA). Over the course of this work, enteroviral RNA was isolated from three different samples of the myocarditic heart muscle. RT-PCR for enteroviral RNA (below) was performed prior to using the RNA for characterizing the viral genome.

Virus identification strategy

Because the serotype of the HEV strain in the myocarditic heart had not been previously identified (Oka et al., 2005), we proceeded to amplify the viral genome in overlapping fragments for sequence analysis. Purified heart RNA was short in length after extraction from the formalin-fixed heart and it was necessary to use numerous reverse transcription and PCR steps to acquire overlapping amplicons for progressive sequence analysis of the HEV genome inward from the 5' end. We first amplified the viral 5' non-translated region (NTR) because 5' NTR sequences are well-conserved among the HEV (Racaniello, 2007), followed by amplicons within the capsid protein coding region of the genome. All primers used are listed in Table 1. HEV-conserved primers targeting the 5' NTR [E8 (Kim et al., 2005) with E1 (Chapman et al., 1990) and E2 (Chapman et al., 1990) with DRevEntB (reverse complement of nt 745–766, Table 1)] were used to amplify and sequence 662 nt of the 5' NTR. Heart virus-specific sense primers (Mito5NTR, MitoVP4, MitoVP2, Table 1) were designed using this 5' NTR sequence as well as subsequently amplified cDNA sequences. cDNA was amplified using sense and antisense primers (VP4REV, VP2REVC, VP2REVA, CVB2VP1REV; Table 1) based on a human enterovirus B (HEV-B) consensus sequence from alignment of 90 complete HEV-B genomes in GenBank (November, 2005). We used an HEV-B consensus sequence because of the established association of the CVB (classified as HEV-B) with myocarditis (Kim et al., 2001).

cDNA was transcribed in 20 µl reactions containing 100–200 ng of heart RNA, 10 pmol of primer E1 (Chapman et al., 1990), DREVENTB, VP4Rev, VP2REVC, VP2REVA, or CVB2VP1REV (Table 1) and 1 U Improm-II reverse transcriptase (Promega; Madison WI), 3 mM MgCl₂ and 0.5 mM each dNTP in Improm-II buffer. The cDNA was either diluted 10 fold with water prior to PCR or concentrated by ethanol precipitation. cDNA was amplified using *Taq* polymerase (Promega) (Kim et al., 2005). For the detection of the viral genome, amplification was carried out using primers E1 and E2 (Chapman et al., 1990). For characterization of 5' terminal sequences, the primer E3Sub (Table 1) was paired with S (Kim et al., 2005) or any of the SENTB primers (Table 1). For cloning of the viral 5' NTR, E1-primed cDNA was tailed with dG₁₀ residues using terminal deoxynucleotidyl transferase (Promega), amplified with a primer annealing to the 3' G₁₀, DC-Tail (Kim et al., 2005) and E5 (Table 1), then ligated into pPCR-Script-Amp (Strataclone PCR; Stratagene, La Jolla, CA) as described previously (Kim et al., 2005). Colonies with insert-containing plasmids were identified by PCR using primers that flank the polylinker (M13forward and reverse; Stratagene). Plasmid DNA was purified and both strands sequenced using the same primers.

Generation of a chimeric infectious CVB3 cDNA with the Mito 5' NTR

Overlap extension (Horton et al., 1989) was used to generate a complete 5' NTR from smaller fragments. The cDNA of the 22 nucleotide deletion in the CVB2Mito genome was amplified with DC-Tail (Kim et al., 2005) and E5. A clone containing the E8–E1 fragment of the viral sequence (nt 65–644) was used as a template to amplify with primers E8 and E1. These amplicons were purified from an agarose gel, mixed in equimolar ratios in water, denatured at 94 °C for 1 min, cooled to room temperature, and amplified by PCR with terminal primers DC-Tail and E1. Similarly, cDNA amplified from cloned cDNA containing nt 644–1046 with Mito5NTR and VP4REV was denatured with the product of the first overlap PCR, cooled to room temperature, and amplified with DREV and 23RIBOZ1 (Table 1). Subsequent amplification with DREV and 23RIBOZ2 (Table 1) followed by amplification with DREV and TD23RIBOZPCRT7 (Table 1) was used to generate a 5' NTR with an added NotI site, T7 RNA polymerase promoter, a ribozyme which cleaves RNA transcripts at nt 23 (Herold and Andino, 2000) and a SacI site at nt 751. Using the NotI and SacI sites, the deleted 5' NTR was substituted for the CVB3/28 5' NTR in a subclone containing nt 1–2011 of cDNA of CVB3/28 to generate pBST7rTD235NTR. This recombinant Mito/CVB3 sequence was subsequently ligated into the pCVB3-28 plasmid which has a full length

infectious cDNA (Tracy et al., 2002) using the NotI site and an XhoI site (at nt 2011) to generate pCMVT7r5NTRMitoTD23.

To generate a chimeric CVB3/28 genome with the CVB2/Mito 5' NTR and a restored 5' terminal sequence (nt 1–22), cDNA was amplified from pBST7rTD235NTR with successive PCRs using DREV and ENTBCONSENSUS (Table 1), then with DREV and 28RIBOZ1 (Kim et al., 2005), with DREV and 28RIBOZ2 (Kim et al., 2005) and finally with DREV and 28RIBOZPCRT7 (Table 1) to generate a NotI site, a ribozyme and the consensus 5' terminal 22 nucleotides generated by alignment of 90 5' terminal containing enterovirus B sequences from GenBank. This cDNA was cloned into the pCMVT7r28 infectious cDNA as described above to generate pCMVT7r5NTRMitoCVB3.

Generation of progeny virus from infectious cDNA clones

RNA polymerase T7 was used to transcribe RNA from pCMVT7r5NTRMitoCVB3, pCMVT7r5NTRMitoTD23, and pCMVT7r28 (Ribomax; Promega). HeLa cells (1×10^6 per well) growing as a monolayer in 6 well plates, were transfected with 10 μ g T7 transcript RNA of pCMVT7r28 or pCMVT7r5NTRMitoCVB3 (Transmessenger; Qiagen, Valencia CA), then incubated at 37 °C for 72 h or until 90% cytopathic effect was observed. Plates were harvested by freezing and thawing three times, after which the lysate was centrifugally cleared of cellular debris. Virus stocks (CVB3/5NTRMito, CVB3/28) were prepared on HeLa cells and titered as described previously (Kim et al., 2005). Virus was generated from pCMVT7r5NTRMitoTD23 (termed CVB3/5NTRMitoTD23) by transfection of HeLa and concentration as described previously (Kim et al., 2005).

Determination of positive to negative strand viral RNA ratios

To determine positive to negative polarity ratios of viral RNA, RNA was purified from virus-infected cells (ZR Viral RNA Kit, Zymo Research, Promega). T7 (positive strand) and SP6 (negative strand) transcripts from a subclone of CVB3/28 (pBSCVB3NXh containing nt 1–2011 of the CVB3 genome) were prepared (Ribomax) for positive and negative strand controls. RNA was purified by annealing a biotinylated strand-specific primer (E3 for annealing to positive strand and E3REV for negative strand) to RNA and purified using streptavidin-labeled magnetic beads (Dynabeads M-280 streptavidin; Dynal Biotech, New York NY) as described previously (Kim et al., 2005). cDNA was reversed transcribed from purified positive strand using primer E1 and from negative strand with primer E2 (Chapman et al., 1990). For quantitative PCR, ten percent of cDNA was used with E1 and E2 at 0.125 OD₂₆₀/ml in DyNAmo SYBR Green qPCR reaction mix (Finnzyme; Finland). Ten-fold dilutions of 2.16×10^{11} copies through 2.16×10^3 copies of T7 or SP6 transcripts of pBSCVB3NXh were used for the generation of a standard curve. Cycling times were 1 cycle at 95 °C for 15 s, 45 cycles at 95 °C for 20 s, 55 °C for 20 s and 72 °C for 20 s and finishing with a final extension at 72 °C for 10 min. qPCRs were carried out using an Opticon 2 DNA Engine (MJ Research, Waltham, MA). The copy number of viral RNA samples was determined based on a standard curve of *C*(t) level versus copy number of the T7 (positive strand) or SP6 (negative strand) RNA polymerase transcripts.

Single step growth curves

Single step growth curves were performed as previously described (Chapman et al., 2000b) with CVB3/5NTRMito, cardiovirulent CVB3/28 (Tracy et al., 2002) and nonvirulent CVB3/GA (Lee et al., 2005) using a multiplicity of infection (MOI) of 25 TCID₅₀ per cell on HeLa and MHC monolayer cultures. Time points were taken by freezing cultures at –70 °C, after which infectious titers were assayed on HeLa cell monolayers.

Inoculation of mice

Five 3–4 week old A/J male mice (Jackson Laboratory; Bar Harbor, ME) were inoculated intraperitoneally with 1×10^5 TCID₅₀ units of

CVB3/28 or CVB3/5NTRMito, or with 0.1 ml concentrated CVB3/5NTRMitoTD23 [the concentration of positive strand RNA by quantitative PCR was equivalent to 10^4 TCID₅₀ (Kim et al., 2005)]. At day 14 post-inoculation, the pancreas and half of each heart from each mouse were formalin-fixed, embedded, sectioned and stained with hematoxylin and eosin for pathology. HeLa cell cultures (1×10^5 cells in 24 well plates) were inoculated with the homogenate made from the other heart half and incubated for 72 h (Kim et al., 2005). Total RNA was then prepared from these cell cultures and viral cDNA amplified with E1 and E2 primers as described above.

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