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# An infectious cDNA copy of the genome of a non-cardiovirulent coxsackievirus B3 strain: its complete sequence analysis and comparison to the genomes of cardiovirulent coxsackieviruses

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**Summary.** The genome of the non-cardiovirulent coxsackievirus B3 (CVB3) strain CVB3/0 was cloned and sequenced to aid in the elucidation of the viral genetic basis for the CVB3 cardiovirulent phenotype. Reverse-transcribed sub-genomic complementary DNA (cDNA) fragments were enzymatically amplified using generic oligonucleotide primers and were assembled as a complete infectious genomic copy (pCVB3-0) downstream of the T7 RNA polymerase promoter. Positive-strand viral RNA transcribed from pCVB3-0 using T7 RNA polymerase and transfected into HeLa cells produced infectious virus (CVB3/0c). No differences in phenotype were observed comparing growth of CVB3/0c to the parental CVB3/0 in HeLa single-step growth curves, virus yields, or plaque size. When inoculated into C3H/HeJ mice, CVB3/0c achieved cardiac titers equivalent to the parental CVB3/0 and like the parental virus, demonstrated a non-cardiovirulent phenotype. The nucleotide sequence of the cloned CVB3/0 genome was determined and compared to the genomes of infectious cDNA clones of cardiovirulent CVB3 strains. Two consistent differences among nucleotides in non-translated regions and 8 amino acid differences relative to two well-characterized infectious cDNA copies of genomes from cardiovirulent CVB3 strains were identified.

## Introduction

Coxsackievirus B3 (CVB3) is an established etiologic agent of human inflammatory heart disease or myocarditis [20, 48, 54], a disease which is often fatal in neonates and young children [5, 6], and can result in significantly decreased heart function and/or dilated cardiomyopathy in adults [29]. Experimental murine models provide a relevant approach to the study of CVB3-induced inflammatory heart disease [38, 48, 54, 55]. Coxsackievirus B3 is one of six

coxsackieviruses in the B serogroup of human enteroviruses in the family *Picornaviridae*. A typical human enterovirus, CVB3 possesses a single-stranded, message sense RNA genome of 7400 nucleotides which is polyadenylated at the 3′ terminus [43, 48, 51]. The single open reading frame is bounded on the 5′ and 3′ termini by non-translated regions (NTRs) which represent about 10% and 1.5% of the genome, respectively. The long open reading frame codes for 2,185 amino acids beginning at nt 743 from the 5′ terminus and is divided into three sections [40]: capsid protein (P1) and non-structural protein (P2, P3) coding regions.

Unlike the related polioviruses (PVs) in which the genetic basis for neurovirulence has been mapped, the genetic loci which determine cardiovirulent and non-cardiovirulent CVB3 phenotypes have not yet been described. Specific strains of CVB3 have been described which are either cardiovirulent (capable of inducing acute inflammatory heart disease and cardiomyocyte necrosis in mice) or non-cardiovirulent (inducing no murine heart disease) [10–12, 17, 51]. Cardiovirulence in mice also reflects a cardiovirulent phenotype in humans, for CVB3 strains isolated from hearts of pediatric cases of fulminant myocarditis also induce myocarditic lesions in mice (S. Tracy, unpubl. data; [5, 49]). Two infectious cDNA copies of cardiovirulent CVB3 genomes and their complete nucleotide sequences have been reported [17, 51]. One other CVB3 sequence exists [21] but this strain has not been cloned as an infectious cDNA copy, nor has the cardiovirulence phenotype of the parental virus been reported.

The virulence phenotype in other picornaviruses is predominantly associated with the 5′ half of the viral genome. In the closely-related coxsackievirus B4 (CVB4), virulence for the murine pancreas has been reported to map to the 5′ half of the viral genome [33]. In addition, primary attenuating genetic loci have been mapped in poliovirus (PV) vaccine strain genomes [2, 23, 36]: a single transition in the 5′ NTR in the region of nt 472–481 accounts for a significant amount of attenuation in all three serotypes of PV Sabin strains and other major sites of attenuation map to amino acid changes in the capsid proteins. Virulence phenotypes of the more distantly related cardioviruses are also affected by alterations in the 5′ NTR nucleotide sequence and/or capsid proteins [8, 37, 45].

We report here for the first time the cloning and sequence analysis of the genome of the well-characterized, non-cardiovirulent CVB3 strain, CVB3/0 [11], and its comparison to the genomes of CVB3 strains expressing cardiovirulent phenotypes. Molecular cloning was greatly accelerated and simplified by enzymatic amplification of viral cDNA using enterovirus-generic PCR primers [50] designed for analysis of related but divergent enterovirus genomes. The progeny virus (CVB3/0c) is indistinguishable phenotypically from the parental strain. Ten genetic differences have been identified in both coding and non-coding regions of the CVB3 genome with potential significance for determination of the cardiovirulence phenotype.

## Materials and methods

### *Viruses and cells*

Viruses were propagated in HeLa cell monolayer cultures [51]. The non-cardiovirulent parental CVB3 strain, CVB3/0 [11], was plaque purified three times on HeLa cell monolayers. Viral stocks from a single large plaque were grown at a multiplicity of infection of 1 infectious unit of virus per cell and stored at −75 °C. Derivation of the infectious molecular clone and propagation of the cardiovirulent CVB3/20 strain was previously described [51].

### *Molecular cloning of CVB3/0 complementary DNA (cDNA)*

Viral RNA was isolated and purified from viral stocks using standard techniques [47]. Random hexameric oligonucleotides or oligo $dT_{12}$ were used to prime cDNA synthesis using ribonuclease H-free murine leukemia virus reverse transcriptase (SuperScript; Gibco/BRL, Gaithersburg, MD, U.S.A.). Polymerase chain reactions (PCRs) were performed using 40 cycles of amplification, *T. aquaticus* (Taq) DNA polymerase, 50 μM each dNTPs, and the cDNA yield from 1 μg of viral RNA (approximately 10–50 ng) to minimize mutations which can occur using the Taq DNA polymerase [15, 18, 46, 50]. The primers V0 and V2 (Table 1) were used in a PCR to obtain a 770 bp sequence containing the 5′ non-translated region (NTR) of CVB3/0. A portion of the cloned cardiovirulent genome, pCVB3-20 (51), was used to place the T7 RNA polymerase promoter one nucleotide upstream of the CVB3/0 cDNA genome, replacing as well the 5′ terminal 69 bp of CVB3/0 with those from CVB3/20 (Fig. 1). A PCR with oligo $dT_{12}$-primed cDNA and the primers PAN and L (Table 1) generated a fragment with the 3′ terminus of the CVB3/0 genome and a polyA tract. Partially overlapping, internal fragments of the CVB3/0 genome were obtained using the primer pairs D and G, 2AS and K, and J and M (Table 1). Assembly of the full-length, infectious cDNA copy of the CVB3/0 genome was accomplished by ligation and insertion of restriction endonuclease-cleaved cDNA fragments (Fig. 1) into pSVN (previously generated from pSVA13 [19] by insertion of a Not I linker in the blunted EcoR I site).

### *Nucleotide sequence analysis*

Concurrent to being assembled into the full-length clone, the sub-genomic clones containing fragments of the CVB3/0 genome were sequenced using the dideoxynucleotide chain termination method with CVB3-sepecific primers and modified T7 DNA polymerase (Sequenase, USBC Inc., Cleveland, OH, U.S.A.) [44, 51]. Following assembly of the full-length clone and demonstration of its infectivity in cell culture, sequences were confirmed across all overlapping and flanking regions of the genome used for ligations. Although cleavage sites in the CVB3 polyprotein can be inferred by comparison to PVs, specific polyprotein cleavage sites for CVB3 have not been determined. In all following discussions, the amino acid number of a CVB3 viral protein is numbered from the methionine residue (aa 1) encoded by nt 743–745, the first codon of the open reading frame.

### *Virus characterization in cell culture*

Single-step growth curves, virus yields, and plaque assays using CVB3/0- or CVB3/0c-infected monolayer HeLa cell cultures were performed essentially as described [52]. Plaques were visualized after fixing the cultures with acetic acid/ethanol at 50 h post-inoculation and staining with crystal violet.

*RNA transfection*

Positive strand CVB3/0 RNA (7–8 kb on neutral agarose minigels) was transcribed from linearized pCVB3-0 using T7 RNA polymerase as described [53]. HeLa cells were transfected with 1–5 μg of RNA transcripts using Lipofectin (Gibco/BRL, Gaithersburg, MD., U.S.A.) under conditions described by the supplier. Cytopathic effects due to virus replication were observed with 48–72 h post-transfection. The titer of infectious virus in the supernatant medium was determined on monolayer HeLa cell cultures. Stock virus for further experimentation was propagated by infecting HeLa cells at a multiplicity of infection of 1 $TCID_{50}$ per cell. Stock virus (CVB3/0c) was titered and stored in aliquots at −75 °C.

*Inoculation of mice with virus*

Male C3H/HeJ mice (21–24 days old) (Jackson Laboratory, Bar Harbor, ME, U.S.A.) were inoculated by intraperitoneal route (i.p.) with 0.1 ml unsupplemented MEM medium containing $1–2 \times 10^5$ $TCID_{50}$ of CVB3/0c, parental CVB3/0, or cardiovirulent CVB3/20c derived from the infectious cDNA copy, pCVB3-20 [51]. At 3 and 10 days post-inoculation (p.i.), hearts of 6 mice from each group were bisected: one half was frozen for later determination of virus titer as described [51], and the other half fixed in 10% buffered formalin, stained with hematoxylin and eosin and 2–4 longitudinal sections per heart examined for pathologic alterations [51].

## Results

*Cloning of the CVB3/0 genome*

The genome of CVB3/0 was reverse-transcribed, then enzymatically amplified using specific pairs of primers to produce a series of overlapping, sub-genomic double-stranded cDNA fragments. The location of the sub-genomic fragments and the assembly strategy are shown in Fig. 1. The complete construct (pCVB3-0) was obtained by transformation in *E. coli* strain *SURE* (Stratagene, San Diego, CA, U.S.A.), selection of positive colonies in colony hybridization using oligonuleotide probes, and verification on neutral agarose gels of expected insert length of about 7.4 kbp by restriction endonuclease digestion. The construct pCVB3-0 was re-transformed into *E. coli* strain *DH1* for preparation of DNA stocks.

Conditions for the PCR [50] were used which have been demonstrated to minimize the error rate of Taq polymerase [15, 18, 46]: all four dNTP's at 50 μM in the reaction, total cDNA from one reverse transcription reaction (approximately 10–50 ng) used as template for each individual subgenomic fragment, a low number of amplification cycles (40 cycles), and the annealing temperature of the oligonucleotide primers optimized. Although other thermal stable DNA polymerases are available, none proved to be as efficient as Taq polymerase in initial trials for the generation of fragments of 1 kbp or greater in length.

*Characterization of CVB3/0c obtained from transfection of RNA transcripts*

The construct pCVB3/0c placed the CVB3/0 genome one G residue downstream from the T7 RNA polymerase promoter, permitting transcription of positive-

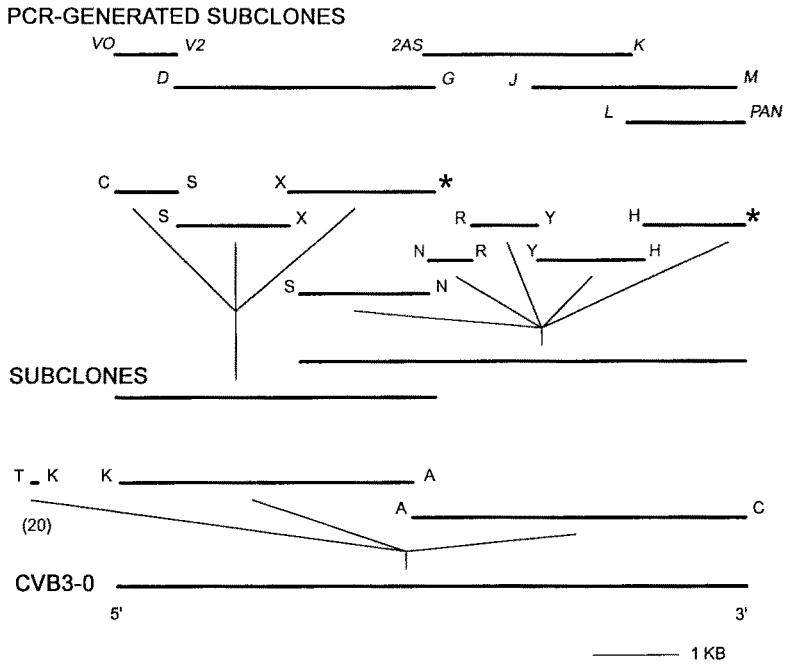


**Fig. 1.** Location of sub-genomic fragments and the assembly strategy of the full-length genomic cDNA copy. *V0, V2, 2AS, D, G, J, K, L, M* and *PAN* are primers used to generate subgenomic CVB3-0 fragments ([50]; Table 1). Restriction endonuclease cleavage sites used in construction of pCVB3-0 are shown: *A* ApaL 1; *C* Cla 1; *H* Hae 2; *K* Kpn 1; *N* Nco I; *R* EcoR I; *S* Sst 1; *T* Not 1; *X* Xho 1; *Y* Xba 1. * denotes restriction sites contributed by the plasmid vector. *20* is pCVB3-20 [51]

strand viral RNA for transfection. Transcription using T7 RNA polymerase and pCVB3-0 linearized by cleavage with the restriction endonuclease *Cla I* downstream of the 3′ terminal poly A tract was performed as described previously [53].

CVB3/0c obtained from transfection of HeLa cells with T7 RNA polymerase transcripts was compared to the parental virus, CVB3/0, in single-step growth curves and by plaque assay. Both viruses replicated with equivalent kinetics and to equivalent yields in HeLa cells (Fig. 2a). In addition, CVB3/0c plaques were indistinguishable from the parental CVB3/0 on HeLa cells (Fig. 2b).

### *Cardiovirulence phenotype and cardiac replication of CVB3/0c in C3H/HeJ mice*

The cardiovirulence phenotype of CVB3/0c was tested in comparison to the parental CVB3/0 by inoculating the viruses into young C3H/HeJ male mice and examining the murine hearts for histopathologic evidence of myocarditis [3]. The cardiovirulent CVB3/20c virus [51] was used as the positive control for induction of disease.

Titers of infectious virus in heart tissues on day 3 p.i. were equivalent for both CVB3/0c and its parental virus, CVB3/0. Virus titers in murine hearts at 3 days

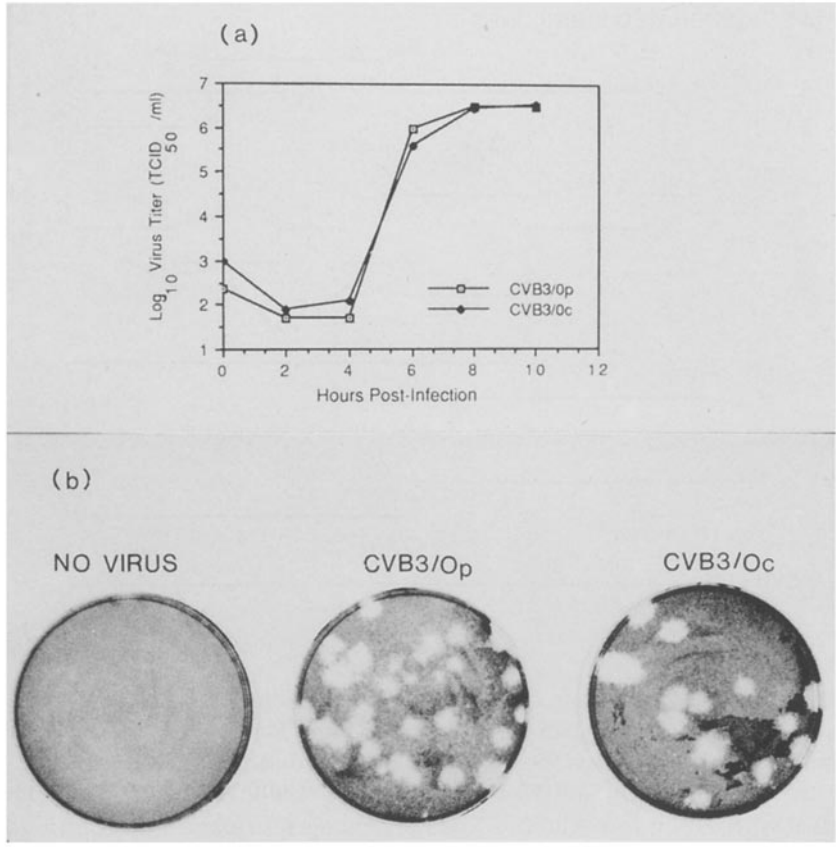


**Fig. 2.** Characterization of CVB3/0c in HeLa cell cultures. **a** Single-step growth curves of CVB3/0 and CVB3/0c. Equivalent numbers of HeLa cells in monolayers were inoculated at a 100/1 virus/cell ratio. After incubation for 30 min and washing, plates were harvested at the times shown and titers of infectious virus in supernatant media. *CVB3/0c* CVB3/0 derived from pCVB3-0; *CVB3/0p* CVB3/0 parental strain. **b** Plaque morphology of CVB3/0 and CVB3/0c. HeLa cell monolayers were inoculated with either virus, overlaid with 0.5% agar in medium and incubated for 50 h, then fixed as described in Materials and methods

post-inoculation averaged $8.8 \times 10^2$ $TCID_{50}$ ($\pm 7.0 \times 10^2$) for both parental CVB3/0 virus and progeny CVB3/0c. No change in cardiovirulence phenotype was apparent in the recombinant-DNA derived CVB3/0c virus when compared to the parental CVB3/0 virus. Examination of the hearts from mice inoculated with CVB3/0c for inflammatory lesions and/or necrotic foci revealed no pathologic changes (Fig. 3a), similar to hearts from mice inoculated with the non-cardiovirulent parental CVB3/0 (Fig. 3b). The extensive lesions induced by CVB3/20c in siblings (Fig. 3c) demonstrated that these mice were indeed susceptible to inflammatory heart disease when inoculated with a cardiovirulent CVB3 strain.

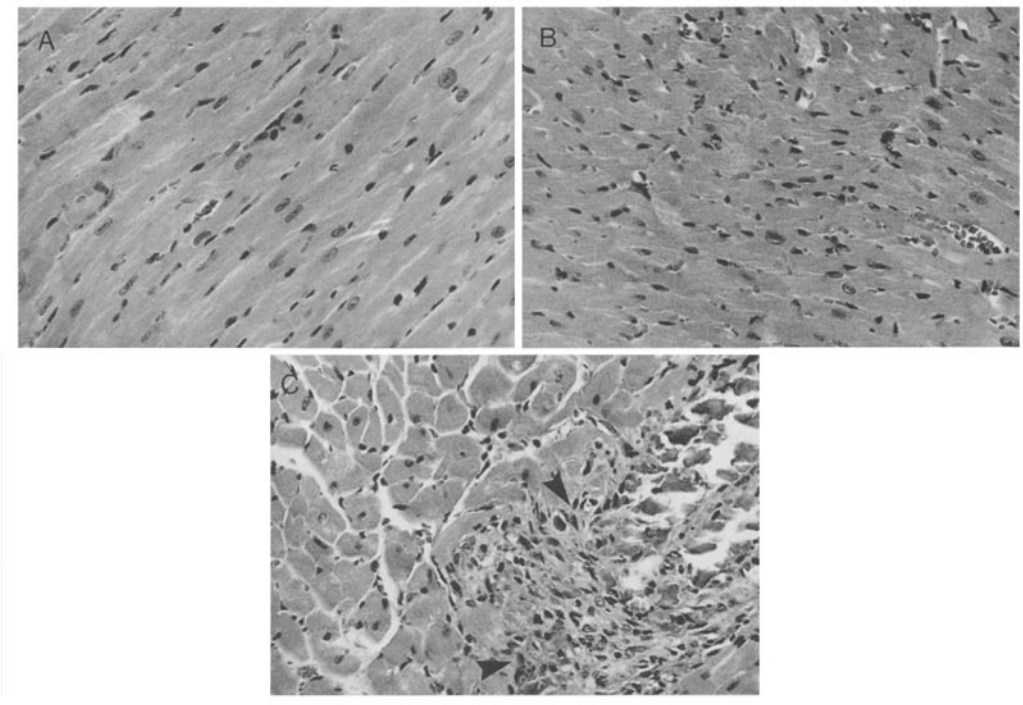


**Fig. 3.** Cardiac pathology in murine hearts infected with CVB3/0 and CVB3/0c. Thin (6–8 micron) sections were cut from formalin-fixed, paraffin-embedded hearts taken from mice 10 days p.i. Heart sections were stained with hemotoxylin and eosin and examined by light microscopy. Sections shown are typical and are from mice **A** inoculated with $1–2 \times 10^5$ $TCID_{50}$ CVB3/0c, **B** inoculated with $1–2 \times 10^5$ $TCID_{50}$ of parental CVB3/0, or **C** inoculated with $1–2 \times 10^5$ $TCID_{50}$ cardiovirulent CVB3/20 [51]. Note absence of pathologic changes in hearts infected with CVB3/0c or parental CVB3/0 (**A**, **B**) versus the extensive cardiac damage induced by CVB3/20 (**C**). Arrowheads designate lesions in **C**. Original magnification × 100

### *Nucleotide sequence analysis of CVB3/0 genome and comparison to the genomes of cardiovirulent CVB3 strains*

The nucleotide sequence of the infectious cDNA copy of the non-cardiovirulent CVB3/0 genome, pCVB3-0, was determined completely and in both directions with no ambiguities. Sites at which sub-genomic fragments were ligated for construction of the full-length, infectious cDNA copy of the genome were sequenced again to verify that no changes had occurred at the restriction endonuclease sites or in nearby flanking sequences.

The genome of CVB3/0 is similar to other CVB3 genomes which have been constructed as infectious cDNA clones [17, 51]: both the nucleotide and predicted amino acid sequences of the CVB3/0 genome are greater than 99% identical to these cardiovirulent CVB3 cDNA genomes (Table 1). The CVB3/0c genome is 7,400 nucleotides in length (excluding the poly A tract). The 5′ NTR, 742 nucleotides in length, precedes the start of a long open-reading frame

**Table 1.** Oligonucleotide primers used to amplify the coxsackievirus B3 genome

| Primer name | Sequence[a] | Location in genome[b] |
|---|---|---|
| VO[c] | 5′-CATCGATTAAAACAGCCTGTG[d] | 1–15 |
| V2[c] | 5′-GCGTTGATACTTGAGCTCCC | 764–745 |
| D[c] | 5′-GAGCTCAAGTNTCAWCRCA | 747–765 |
| 2AS | 5′-GGNGAYTGYGGNGGGATCCT | 3617–3636 |
| G[c] | 5′-TCTAGACCTGTTCCATGGCNTCNTCTTC[d] | 3746–3725 |
| J[c] | 5′-GYTGYCCACTAGTRTGTG | 4884–4901 |
| L[c] | 5′-ARGAACCAGCTGTNCTCAG | 6024–6042 |
| K[c] | 5′-TCTAGAATATTGCCTCYTCAAA[d] | 6081–6071 |
| M[c] | 5′-TCTAGAARGARTCCAACCAYTTCCT[d] | 7300–7277 |
| PAN[c] | 5′-GCGGCCGC($T_{12}$) | 3′-terminal poly A |

[a] In some primer sequences, primers were synthesized partially degenerate. Per IUPAC-IUB nomenclature, N is either A, T, C, or G; Y is T or C; R is A or G; W is A or T

[b] Map positions are referenced to the CVB3 genome [51] given in nucleotide numbers, where 1 is the 5′ terminal nucleotide of the CVB3 genome. Some primers are the reverse complement of the CVB3 genome sequence. Oligonucleotides have 0–2 mismatches with the CVB3–20 genome

[c] See [50]

[d] Underlined sequence represents sequence of the CVB3 genome or reverse complement of the genome; the remainder of the oligonucleotide sequence is present to generate a restriction site

consisting of 2,185 amino acids. The open reading frame ends in a single AGA termination codon followed by a 100 nucleotide 3′ NTR and a 44 nucleotide poly A tract. The T7 RNA polymerase transcript of the cDNA genome in the *Cla I*-linearized pCVB3-0 contains an 11 nucleotide residue (5′-ATCAAGCTTAT) derived from the pBluescript polylinker (Stratagene Inc., San Diego, CA, U.S.A.) immediately following the poly A tract.

We compared the complete sequence of CVB3/0 to the sequences derived for the infectious cDNA clones of cardiovirulent CVB3 strains [17, 51] in order to identify genetic loci potentially critical for determination of the cardiovirulence phenotype. We considered as significant only those loci which are the same in both cardiovirulent genomes but which vary in the cloned non-cardiovirulent pCVB3-0 genome. We used sequence information from the two infectious clones of proven cardiovirulence phenotype [17, 51] for this comparison, both of which represent cardiovirulent CVB3 phenotypes in mice. Another CVB3 sequence [21] has never been reported as an infectious clone. Our analysis (Table 2) identified 1 nucleotide difference in the 5′ NTR (nt 234), 8 amino acid differences in the viral polyprotein (5 in structural and 3 in non-structural proteins), and 1 nucleotide difference in the 3′ NTR (nt 7334) which differentiate genomes of non-cardiovirulent and cardiovirulent CVB3 strains (Fig. 4).

Three nucleotide changes in the 5′ NTR were identified which varied as a function of the viral cardiovirulent phenotype, but only one may have significance

**Table 2.** Nucleotide and predicted amino acid sequence differences between CVB3 genomes

| Nucleotide Number | CVB3 strain 20 | M1 | 0 | Nucleotide number | CVB3 strain 20 | M1 | 0 | AA number | CVB3 strain 20 | M1 | 0 | Nucleotide number | CVB3 strain 20 | M1 | 0 | AA number | CVB3 strain 20 | M1 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5'NTR (N1–742) | | | | 1A (N743–949) | | | | | | | | 2A (N3296- or 3305–3745*) | | | | | | | |
| 31 | C | C | T/C | 788 | A | A | G | 16 | R | R | G | 3324 | T | T | C | 7/10*(861) | V | V | A |
| 35 | G | – | G | 830 | A | A | G | 30 | I | I | V | 3346 | A | G | A | | | | |
| 234 | T | T | C | | | | | | | | | 3434 | A | G | G | 44/47*(898) | T | A | A |
| 598 | C | C | T/C | 1B (N950–1738) | | | | | | | | | | | | | | | |
| 610 | T | C | T | 987 | T | C | C | 13(82) | V | A | A | 2B (N3746–4042) | | | | | | | |
| 647 | C | T | C | 1401–1402 | GC | CG | GC | 151(220) | S | T | S | 3782 | C | T | T | 13(1014) | L | F | F |
| 667 | T | C | T | 1408 | A | G | A | | | | | 3804 | A | A | G | 20(1021) | Q | Q | R |
| | | | | | | | | | | | | 4028 | A | A | T | 95(1096) | M | M | L |
| 3'NTR (N7301–7400) | | | | 1C (N1739–2452) | | | | | | | | | | | | | | | |
| 7334 | T | T | C | 1861 | A | A | G | | | | | | | | | | | | |
| 7400 | – | G | G | 1963 | T | C | T | | | | | 2C (N4043–5029) | | | | | | | |
| | | | | 2182 | C | T | C | | | | | 4078 | G | A | G | | | | |
| | | | | 2201 | G | A | G | 155(487) | V | I | V | 4165 | G | A | G | | | | |
| | | | | 2271 | T | T | A | 178(510) | F | F | Y | 4177 | A | G | A | | | | |
| | | | | 2438 | C | C | G | 234(566) | Q | Q | E | 4585 | C | T | T | | | | |
| | | | | 1D (N2453–3295 or –3304*) | | | | | | | | 3A (N5030–5296) | | | | | | | |
| | | | | 2560 | A | G | A | | | | | 5119 | C | C | T | | | | |
| | | | | 2593 | C | T | C | | | | | | | | | | | | |
| | | | | 2632 | T | C | C | | | | | 3B (N5297–5362) | | | | | | | |
| | | | | 2690 | A | A | G | 80(650) | K | K | E | | | | | | | | |
| | | | | 2851 | C | T | C | | | | | 3C (N5363–5911) | | | | | | | |
| | | | | 2881 | C | T | T | | | | | 5383 | T | C | C | | | | |
| | | | | 3243 | G | A | A | 264(834) | R | Q | Q | 5412 | G | C | C | 17(1557) | R | T | T |
| | | | | | | | | | | | | 5735 | T | C | C | | | | |
| | | | | | | | | | | | | 3D (N5912–7300) | | | | | | | |
| | | | | | | | | | | | | 7026 | T | C | T | 372(2095) | V | A | V |

CVB3/20 is the sequence of Tracy et al. [51]. CVB3/M1 is the sequence of Klump et al. [17]. CVB3/0 is the name of the non-cardiovirulent CVB3 strain. – designates a deleted nucleotide relative to the other sequences. Amino acid number in parentheses is the position in the polyprotein, in which the N-terminal Met is as 1. *Uncertainty in numbering is due to two candidate 1D/2A cleavage sites

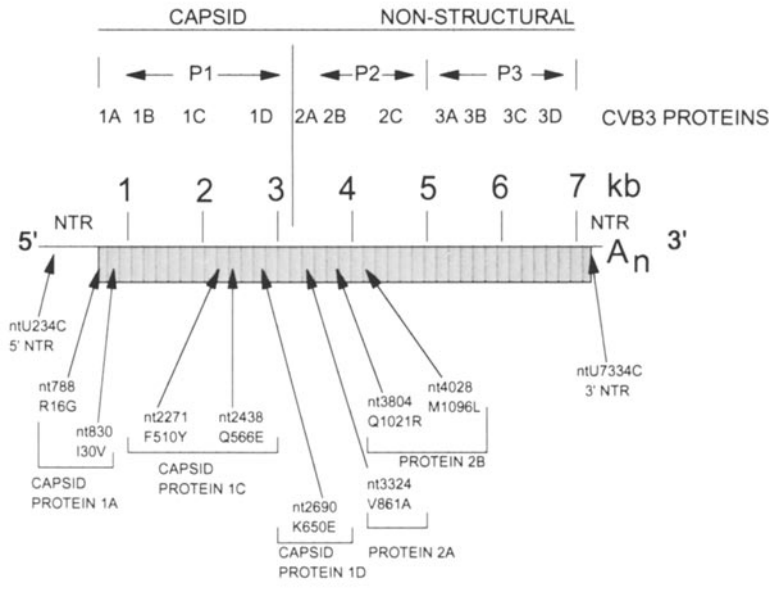


**Fig. 4.** Significant differences between the cardiovirulent and non-cardiovirulent CVB3 genomes. Cardiovirulent CVB3 nucleotide or amino acid precedes non-cardiovirulent CVB3/0 nucleotide or amino acid. $A_n$ Polyadenylate sequence; *kb* kilobase; *NTR* non-translated region

in this context. The change at nt 31 (C in cardiovirulent CVB3/20; [51]) was incorporated into the pCVB3-0 construct along with nt 1-69 from pCVB3-20 in order to place the T7 RNA polymerase promoter upstream of the viral 5′ terminus. This did not alter the resultant CVB3/0c phenotype (Figs. 2, 3) from that of the parental CVB3/0. Although nt 234 is not within predicted higher order structures, the change at nt 598 does alter a G–C base pair to G–T in such a higher-order structure [42]. Another pCVB3-0 construct contained another cloned CVB3/0 5′ NTR which was identical to the cardiovirulent genomes at nt 598; transcripts from this latter plasmid produced virus identical to CVB3/0c in both HeLa cell culture and in demonstrating a non-cardiovirulent phenotype in mice (data not shown). The single difference in the 3′ NTR at nt 7334 is located within a region of predicted secondary and tertiary structure [16, 31].

Comparative analysis of the amino acid sequence in the predicted pCVB3-0 polyprotein with predicted polyproteins from two other infectious cardiovirulent CVB3 cDNA clones delineated 8 potentially significant amino acid differences (Table 2, Fig. 4). Two amino acid changes (R16G, I30V) occur in protein 1A, the smallest of the capsid proteins and the one which is located entirely in the viral capsid interior [14, 39]. Amino acid 510 in capsid protein 1C changes from tyrosine in CVB3/0 to phenylalanine in both cloned cardiovirulent CVB3 genomes. Alignment of the amino acid sequences of the capsid proteins of many enteroviruses [30] and comparison of these alignments to known near atomic structures for PVs and human rhinoviruses [13, 14, 26, 39] suggest this change is in the G–H loop of the 1C protein which is located at the interface between

capsid protomers. The other locus in protein 1C (Q566E) is 5 residues upstream of the putative 1C/1D cleavage site [17, 51]. The sole change in capsid protein 1D (K650E) substitutes an acidic glutamic acid residue for a basic lysine in cardiovirulent CVB3 variants. This site is located, again by best alignment analysis with crystallographic data from solved three-dimensional enterovirion capsid structures [14, 26, 39, 56], in the external B–C loop of the protein.

Three amino acids in two non-structural proteins also vary as a function of phenotype (Table 2). A single amino acid change (V861A) occurs in the protein 2A, one of the two enteroviral proteases responsible for polyprotein processing and associated with the shut-off of host cell protein synthesis characteristic of enteroviruses [9]. Two amino acid differences (Q1021R, M1096L) are located in protein 2B, a viral protein which has been associated with the establishment and maintenance of membrane-associated RNA replication complexes in PV-infected cells [4].

## Discussion

We report here the molecular cloning and complete genomic sequence analysis of an infectious cDNA copy of the genome of the non-cardiovirulent and well-characterized CVB3 variant, CVB3/0 [11]. The use of the PCR and generic enterovirus primers ([50]; Table 1) to generate overlapping, sub-genomic fragments from reverse-transcribed cDNA greatly facilitated the cloning and rapid assembly of an infectious, full-length cDNA copy of the CVB3/0 genome. The characterization of the progeny CVB3/0c in both cell cultures and in mice demonstrated that primary measurable phenotypic traits of the parental CVB3/0 strain were not altered in the recombinant DNA-derived CVB3/0c. These data suggest that the PCR can be used with care to facilitate the cloning of sub-genomic enterovirus sequences for assembly into infectious cDNA genomic clones without alteration of phenotype.

The complete sequence analysis of the CVB3/0 genome represents the first comprehensive examination of a CVB3 genome which codes for a non-cardiovirulent phenotype and provides the first comparative data base to aid in the identification of candidate genetic loci which determine CVB3 cardiovirulence. All other reports of infectious cDNA copies of CVB3 genomes have been based on cardiovirulent strains [17, 51]. Ten potentially significant genetic differences have been identified (Fig. 4) by comparison of the sequence of CVB3/0 to two other infectious cDNA clones of cardiovirulent phenotypes of CVB3 [17, 51]; Table 1), some of which are likely to be involved in the determination of the cardiovirulent phenotype.

One nucleotide difference with potential significance for the cardiovirulence phenotype was noted at nt 234 (Table 2). The change at nt 31 was obviated as significant because this region was actually taken from the cardiovirulent pCVB3-20 clone [51] and the resultant CVB3/0c virus is non-cardiovirulent in the mouse (Fig. 3). An alternate construct of pCVB3-0 utilizing another cloned CVB3/0 5′ NTR (C598C; Table 2) produced virus which is non-cardiovirulent in

the mouse (data not shown), arguing that nt 598 itself also is not critical for cardiovirulence. Although nt 234 is not located in any proposed 5′ NTR secondary structures or in regions found critical for translation in the polioviruses [25, 32], it is possible that tertiary structures necessary for efficient function of the 5′ NTR may be affected by changes at this site [1]. The site U7334C in the 3′ NTR of CVB3/0 is in a region which may form secondary and tertiary structures [16, 31]. Alterations in the pseudoknot in this region of PV1 RNA are linked to temperature sensitivity of viral RNA replication [16, 41]. Although this change would not alter the pseudoknot, nt 7334 is between two predicted stem structures and may contribute to, or slightly alter, tertiary structures [32, 41].

These data contrast with those derived for the non-translated regions of the 3 PV serotypes. No virulence marker has been identified in the PV 3′ NTR [2], although mutations have been induced in this region which inhibit PV RNA synthesis [41]. While the primary attenuating locus in PV RNA is located in a region of significant higher order structure (nt 472–480; [2, 23, 36, 42]), the nucleotide change nt U234C in the CVB3 5′ NTR appears to be between two stem structures, in a region devoid of predicted secondary structure [1, 42]. The possibility exists that this region may interact with other regions of the CVB3 genome not predicted by structural analyses. Furthermore, interaction of host cell proteins in this region must also be considered a possibility; it is clear that host cell proteins do bind to enteroviral RNA in the 5′ NTR [7].

Eight loci were identified in the CVB3 polyprotein which correlated with the cardiovirulence phenotype (Table 2, Fig. 4), five of which are located in the capsid proteins. Two differences occur in protein 1A (R16G, I30V), the innermost and most conserved of all enterovirus capsid proteins. Neither of the loci is near the predicted cleavage site of protein 1A from 1B [30]. While it is not readily apparent what role these changes in the primary structure of protein 1A might play in the determination of cardiovirulence, it is interesting to note that the change R16G is a site which also varies among CVB4 strains as a function of pancreatic virulence [34]. In both virulent CVB3 and CVB4 strains, arginine is present in this position in protein 1A. Although the Val/Ile change I30V is conservative, the homologous PV1 residue is part of a *beta*-sheet structure formed by regions of capsid proteins 1A and 1C on the inner surface of the poliovirus capsid [13]. Furthermore, interactions of residues in this *beta* strand with the myristoylate residue of the N-terminal glycine of protein 1A appear to affect assembly and stability of the PV1 capsid [28].

Alignments of enterovirus capsid amino acid sequences demonstrate that the change F510Y in capsid protein 1C (Table 2, Fig. 4) occurs at the homologus position as a second site mutation in the PV3 capsid. Located at the interface between adjacent protomers, it acts to suppress the temperature-sensitivity phenotype [22] of the PV3 Sabin (neurovirulence attenuated) strain [26]. The change Q566E in capsid protein 1C is located near the putative $3C^{pro}$ cleavage site between capsid proteins 1C and 1D [17, 51]. The change K650E in protein 1D is located (by best alignment of the primary structure with those of

established enterovirion structures; [14, 26, 39, 56]) in the external B–C loop of capsid protein 1D. In some enteroviruses, this loop contains antigenic sites [27, 35] and is a prominent feature in the external surface topography of the closely-related PVs [14, 56]. This external region of the PV capsid can be critical in interactions with some receptors [24, 56].

Three amino acids changes (V861A, Q1021R, M1096L) were identified in the non-capsid viral proteins which varied as a function of CVB3 cardiovirulence phenotype (Table 2, Fig. 4). For both CVB3 proteins 2A and 2B, the only information available is by inference with results obtained from studies of PVs, the most closely related and well-studied of enteroviruses. The single change in CVB3 protein 2A (V861A) maps within 7 to 9 residues of the putative amino terminus of protein 2A, and thus relatively close to the self-cleavage site of protein 2A, a protease implicated in the termination of host protein synthesis by PVs [9]. Elegant immune electron microscopy studies have demonstrated that PV proteins 2B and 2C help to form key endoplasmic reticulum membrane-associated viral replication complexes which are generated in the host cell following virus infection [4].

The non-cardiovirulent CVB3/0 is a robust virus which is essentially indistinguishable in HeLa cell cultures from the cardiovirulent virus, CVB3/20. The alteration(s) conferred by one or more of the genetic differences described here is evidenced only in vivo by the ability of cardiovirulent strains to cause murine inflammatory heart disease. The high degree of identity of the sequences of two cardiovirulent CVB3 genomes [17, 51] and CVB3/0 suggests that relatively few loci affect the cardiovirulence phenotype. Nevertheless, it is possible that changes at multiple sites could be necessary to generate the cardiovirulence phenotype, or that changes at secondary sites may act to suppress a phenotype. Further sequence analyses of other non-cardiovirulent CVB3 genomes will be necessary to fully determine whether all primary loci determining the cardiovirulence phenotype have been identified.

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## References

1. Agol VI (1991) The 5′-untranslated region of picornaviral genomes. Adv Virus Res 40: 103–180
2. Almond JW (1987) The attenuation of poliovirus neurovirulence. Annu Rev Microbiol 41: 153–180
3. Beck MA, Tracy SM (1989) Murine cell-mediated immune response recognizes an enterovirus group-specific antigen(s). J Virol 63: 4148–4156
4. Bienz K, Egger D, Pfister T, Troxler M (1992) Structural and functional characterization of the poliovirus replication complex. J Virol 66: 2740–2747

5. Burch GJ, Sun S-H, Chu K-C, Sohal RS, Colcolough HL (1968) Interstitial and coxsackievirus B myocarditis in infants and children. JAMA 203: 55–62.
6. Cherry JD (1987) Enteroviruses: polioviruses (poliomyelitis), coxsackieviruses, echoviruses, and enteroviruses. In: Feigin RD, Cherry JD (eds) Textbook of pediatric infectious diseases. Saunders, Philadelphia, pp. 1729–1790
7. del Angel RM, Papavassiliou AG, Fernandez-Tomas C, Silverstein SJ, Racaniello VR (1989) Cell proteins bind to multiple sites within the 5′ untranslated region. Proc Natl Acad Sci USA 86: 8299–8303
8. Duke GM, Osorio JE, Palmenberg AC (1990) Attenuation of Mengo virus through genetic engineering of the 5′ noncoding poly (C) tract. Nature 343: 474–476
9. Etchison D, Milburn SC, Edery I, Sonenberg N, Hershey JWB (1982) Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220 000–dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. J Biol Chem 257: 14806–14810
10. Gauntt CJ, Godeny EK, Lutton CW, Arizpe HM, Chapman NM, Tracy SM, Revtyak GE, Valente AJ, Rozek MM (1989) Mechanism(s) of coxsackievirus-induced acute myocarditis in the mouse In: de la Maza LM, Peterson EM (eds) Medical virology 8. Plenum, New York, pp 161–182
11. Gauntt CJ, Trousdale MD, LaBadie DRL, Paque RE, Nealon T (1979) Properties of coxsackievirus B3 variants which are amyocarditic or myocarditic for mice. J Med Virol 3: 207–220
12. Gauntt CJ, Trousdale MD, Lee JC, Paque RE (1983) Preliminary characterization of coxsackievirus B3 temperature-sensitive mutants. J Virol 45: 1037–1047
13. Filman DJ, Syed R, Chow M, Macadam AJ, Minor PD, Hogle JM (1989) Structural factors that control conformational transitions and serotype specificity in type 3 poliovirus. EMBO J 8: 1567–1579
14. Hogle JM, Chow M, Filman JD (1985) The three-dimensional structure of poliovirus at 2.9 A resolution. Science 229: 1358–1365
15. Innis M, Gelfand D (1990) Optimization of PCRs. In: Innis M, Gelfand D, Sninsky J, White T (eds) PCR protocols. Academic Press, San Diego, pp 3–12
16. Jacobson SJ, Konings DAM, Sarnow P (1993) Biochemcial and genetic evidence for a pseudoknot structure at the 3′ terminus of the poliovirus RNA genome and its role in viral RNA amplification. J Virol 67: 2961–2971
17. Klump WM, Bergmann I, Muller BC, Ameis D, Kandolf R (1990) Complete nucleotide sequence of infectious coxsackievirus B3 cDNA: two initial 5′ uridine residues are regained during plus-strand RNA synthesis. J Virol 64: 1573–1583
18. Krawczak M, Reiss J, Schmidtke J, Roesler U (1989) Polymerase chain reaction replication errors and reliability of gene diagnosis. Nucleic Acids Res 17: 2197–2201
19. Kuhn RJ, Wimmer E, Semler BL (1987) Expression of the poliovirus genome from infectious cDNA is dependent upon arrangements of eukaryotic and prokaryotic sequences in recombinant plasmids. Virology 157: 560–564
20. Leslie K, Blay R, Haisch V, Lodge A, Weller A, Huber S (1989) Clinical and experimental aspects of viral myocarditis. Clin Microbiol Rev 2: 191–203
21. Lindberg AM, Stalhandske POK, Pettersson U (1987) Genome of coxsackievirus B3. Virology 156: 50–63
22. Macadam AJ, Ferguson G, Arnold C, Minor PD (1991) An assembly defect as a result of an attenuating mutation in the capsid proteins of the poliovirus type 3 vaccine strain. J Virol 65: 5225–5231
23. Macadam AJ, Pollard SR, Ferguson G, Dunn G, Skuce R, Almond JW, Minor PD (1990) The 5′ non-coding region of the type 2 poliovirus vaccine strain contains determinants of attenuation and temperature-sensitivity. Virology 181: 451–458

24. Martin A, Wychowski C, Couderic T, Crainic R, Hogle J, Girard M (1988) Engineering a poliovirus type 2 antigenic site on a type 1 capsid results in a chimeric virus which is neurovirulent for mice. EMBO J 7: 2839–2847
25. Meerovitch K, Nicholson R, Sonenberg N (1991) In vitro mutational analysis of *cis*-acting RNA translational elements within the poliovirus type 2 5′ untranslated region. J Virol 65: 5895–5901
26. Minor PD, Dunn G, Evans DMA, Magrath DI, John A, Howlett J, Phillips A, Westrop G, Wareham K, Almond JW, Hogle JM (1989) The temperature-sensitivity of the Sabin type 3 vaccine strain of poliovirus: molecular and structural effects of a mutation in the capsid protein VP3. J Gen Virol 70: 1117–1123
27. Minor PD, Ferguson M, Evans DMA, Almond JW, Icenogle JP (1986) Antigenic structure of polioviruses of serotypes 1, 2, 3. J Gen Virol 67: 1283–1291
28. Moscufo N, Chow M (1992) Myristate-protein interactions in poliovirus: interactions of VP4 threonine 28 contribute to the structural conformation of assembly intermediates and the stability of assembled virions. J Virol 66: 6849–6857
29. Olsen EG (1992) The pathogenesis of dilated cardiomyopathy. Postgrad Med J 68 [Suppl 1]: S7–10
30. Palmenberg AC (1989) Sequence alignments of picornaviral capsids. In: Semler BL, Ehrenfeld E (eds) Molecular aspects of picornavirus infection and detection. American Society for Microbiology, Washington, pp 211–242
31. Pestova TV, Hellen CUT, Wimmer E (1991) Translation of poliovirus RNA: role of an essential *cis*-acting oligopyrimidine element within the 5′ nontranslated region and involvement of a cellular 57-kilodalton protein. J Virol 65: 6194–6204
32. Pilipenko EV, Maslova SV, Sinyakov AN, Agol VI (1992) Towards identification of *cis*-acting elements involved in the replication of enterovirus and rhinovirus RNAs: a proposal for the existence of tRNA-like terminal structures. Nucleic Acids Res 20: 1739–1745
33. Ramsingh A, Hixson A, Duceman B, Slack J (1990) Evidence suggesting that virulence maps to the P1 region of the coxsackie-virus B4 genome. J Virol 64: 3078–3081
34. Ramsingh A, Araki H, Bryant S, Hixson A (1992) Identification of candidate sequences that determine virulence in coxsackievirus B4. Virus Res 23: 281–292
35. Reimann BY, Zell R, Kandolf R (1991) Mapping of a neutralizing antigenic site of coxsackievirus B4 by construction of an antigen chimera. J Virol 65: 3475–3480
36. Ren R, Moss EG, Racaniello VR (1991) Identification of two determinants that attenuate vaccine-related type 2 poliovirus. J Virol 65: 1377–1382
37. Rodriguez M, Roos RP (1992) Pathogenesis of early and late disease in mice infected with Theiler's virus, using intratypic recombinant GDVII/DA viruses. J Virol 66: 217–225
38. Rose NR, Neumann DA, Herskowitz A, Traystman MD (1988) Genetics of susceptibility to viral myocarditis in mice. Pathol Immunopathol Res 7: 266–278
39. Rossman MG, Arnold E, Erickson JW, Frankenberger EA, Griffith AG, Hecht HJ, Johnson JE, Kamer G, Luo M, Mosser AG, Rueckert RR, Sherry B, Vriend G (1985) Structure of a human common cold virus and functional relationship to other picornaviruses. Nature 317: 145–153
40. Rueckert R, Wimmer E (1984) Systematic nomenclature of picornavirus proteins. J Virol 50: 957–959
41. Sarnow P, Bernstein HD, Baltimore D (1986) A poliovirus temperature-sensitive RNA synthesis mutant located in a noncoding region of the genome. Proc Natl Acad Sci USA 83: 571–575
42. Skinner MA, Racaniello VR, Dunn G, Cooper J, Minor PD, Almond JW (1989) New model for the secondary structure of the 5′ non-coding RNA of poliovirus is supported

by biochemical and genetic data that also show that RNA secondary structure is important in neurovirulence. J Mol Biol 207: 379–392
43. Stanway G (1990) Structure, function and evolution of picornaviruses. J Gen Virol 71: 2483–2501
44. Tabor S, Richardson CC (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc Natl Acad Sci USA 84: 4767–4771
45. Tangy F, McAllister A, Aubert C, Brahic M (1991) Determinants of persistence and demyelination of the DA Strain of Theiler's virus are found only in the VP1 gene. J Virol 65: 1616–1618
46. Tindall KR, Kunkel TA (1988) Fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase. Biochemistry 27: 6008–6013
47. Tracy S (1985) Comparison of genomic homologies in the coxsackievirus B group by use of cDNA: RNA dot-blot hybridization. J Clin Microbiol 21: 371–374
48. Tracy S, Chapman NM, Beck MA (1991) Molecular biology and pathogenesis of coxsackie B viruses. Rev Med Virol 1: 145–154
49. Tracy S, Chapman N, Pallansch M, Coller B, Beck M, Kolbeck M, Woronoff Y (1991) Characterization and molecular cloning of a coxsackie B virus from a human myocarditic heart. J Cell Biochem 15E: M342
50. Tracy S, Chapman NM, Pistillo JM (1992) Detection of human enteroviruses using the polymerase chain reaction. In: Becker Y, Darai G (eds) Frontiers of virology. Springer, Berlin, Heidelberg, New York, Tokyo, pp 331–344
51. Tracy S, Chapman NM, Tu Z (1992) Coxsackievirus B3 from an infectious cDNA copy of the genome is cardiovirulent in mice. Arch Virol 122: 398–409
52. Trousdale MD, Paque RE, Gauntt CJ (1977) Isolation of coxsackievirus B3 temperature-sensitive mutants and their assignment to complementation groups. Biochem Biophys Res Commun 76: 368–375
53. van der Werf S, Bradley J, Wimmer E, Studier FW, Dunn JJ (1986) Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. Proc Natl Acad Sci USA 83: 2330–2334
54. Woodruff JF (1980) Viral myocarditis: a review. Am J Pathol 101: 425–483
55. Woodruff JF, Kilbourne ED (1970) The influence of quantitative post weaning undernutrition of coxsackievirus B3 infection of adult mice. I. Viral persistence and increased severity of lesions. J Infect Dis 121: 137–163
56. Yeates TO, Jacobson DH, Martin A, Wychowski C, Girard M, Filman DJ, Hogle JM (1991) Three-dimensional structure of a mouse-adapted type 2/type 1 poliovirus chimera. EMBO J 10: 2331– 2341

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