

Comparative Genomics of the Coxsackie B Viruses and Related Enteroviruses

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Abstract Genomic analysis of the group B coxsackieviruses (CVB) has improved our understanding of CVB evolution, epidemiology, and pathogenesis. Comparison of capsid sequence alignments and virion structures allows correlation of capsid diversity with surface features, such as loops, the receptor canyon, and antigenic sites. Pairwise sequence comparisons and phylogenetic analyses can be used to rapidly identify and classify enteroviruses. Enteroviruses are monophyletic by type only within the capsid region. The CVBs as a group are monophyletic in the capsid region, probably due to their shared use of the coxsackievirus-adenovirus receptor (other members of HEV-B use different receptors). Outside the capsid region, enteroviruses are monophyletic only by species (not by type), reflecting a high frequency of intertypic recombination within a species. Further genomic studies, accompanied by well-characterized clinical outcome/disease data, will facilitate fine-scale mapping of genetic determinants that contribute to virulence.

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1 Overview

The coxsackie B viruses (CVBs) were identified as a unique enterovirus group on the basis of the characteristic disease they caused in suckling mice inoculated intracerebrally (Pallansch and Roos 2006). Sequence analyses have shown that echoviruses and many newer enteroviruses are closely related to the CVBs; as a result, these viruses are classified together in the species *Human enterovirus B* (HEV-B; genus *Enterovirus*, family *Picornaviridae*) (Stanway et al. 2005). Complete genome sequences are available for at least one representative of all 54 recognized types within HEV-B, except EV78, and multiple genomes are available for some types (total $n=96$) (Stanway et al. 2005) (<http://www.picornaviridae.com>). Comparison of capsid sequence alignments and virion structures allows correlation of capsid diversity with surface features, such as loops, the receptor canyon, and antigenic sites. Pairwise sequence comparisons and phylogenetic analyses can be used to rapidly identify and classify enteroviruses. Such analyses reveal that (1) enteroviruses are monophyletic by type only within the capsid region (Oberste et al. 1999); (2) the CVBs as a group are monophyletic in the capsid region (Hyypiä et al. 1997; Oberste et al. 1999; Pöyry et al. 1996), probably due to their shared use of the coxsackievirus-adenovirus receptor (other members of HEV-B use different receptors); and (3) outside the capsid region, enteroviruses are monophyletic only by species (not by type), reflecting a high frequency of intertypic recombination within a species (Andersson et al. 2002; Brown et al. 2003; Hyypiä et al. 1997; Lukashev et al. 2003, 2004, 2005; Oberste et al. 2004a, 2004b, 2004c, 2004d; Pöyry et al. 1996; Santti et al. 1999).

2 Picornavirus Genomics

While the genetic basis of complex phenotypes, such as transmissibility, host range, and receptor usage may not be clearly understood, all intrinsic properties of a picornavirus must ultimately derive from the viral genome. The Genomics Age for eukaryotic virology began in 1981, with the publication of the complete genome sequence of poliovirus type 1 (Mahoney strain) (Kitamura et al. 1981; Racaniello and Baltimore 1981). This accomplishment permitted the direct mapping of genetically and functionally defined viral proteins and facilitated the development of reverse genetic systems to help probe the molecular details of poliovirus replication, translation, and protein function (Racaniello and Baltimore 1981; Sarnow 1989; Semler et al. 1984; van der Werf et al. 1986). Similar approaches were quickly applied to studies of other virus families (Knipe et al. 2006), and other picornavirus genome sequences also followed soon afterward, representing all genera of *Picornaviridae*, and sometimes helping to define new genera (Cohen et al. 1987; Doherty et al. 1999; Forss et al. 1984; Hyypiä et al. 1992; Krumbholz et al. 2002; Oberste et al. 2003; Palmenberg et al. 1984; Stanway et al. 1984, 2005; Wutz

et al. 1996; Yamashita et al. 1998). The quantity and quality of picornavirus sequence data, and the ease with which it can be generated, have increased substantially with the introduction of PCR and improvements in sequencing technology over the last 25 years (Fig. 1) (<http://www.picornaviridae.com>).

This chapter will discuss lessons learned from studies on nucleotide and amino acid sequence conservation and divergence among the CVBs, and related enteroviruses, focusing on members of the species HEV-B, with reference to other enterovirus species to illustrate specific points when necessary. It must be borne in mind that the available enterovirus sequences are generally derived from prototype reference strains or a small number of more recent clinical isolates. Each of these isolates represents only a temporal and geographic snapshot in enterovirus evolution and may or may not be representative of their particular serotype or of enteroviruses as a whole. Generalizable patterns may be discerned only from the careful analysis of a large number of sequences obtained from viruses with a wide temporal and geographic distribution.

HEV-B is composed of 56 serotypes - approximately half of all known enterovirus serotypes - and includes coxsackievirus A9 (CVA9), the coxsackie B viruses (six types: CVB1-6), the echoviruses (E; 28 types: E1-7, 9, 11, 13-21, 24-27, 29-33), and most of the newer, numbered enteroviruses (EV; 21 types: EV69, 73-75, 77-88, 93, 97, 98, 100-101) (Stanway et al. 2005). Swine vesicular disease virus (SVDV) is also a member of HEV-B. SVDV infects and causes disease in pigs, but it is serotypically identical to CVB5 (Brown et al. 1973; Knowles and McCauley 1997; Zhang et al. 1999). The CVB genomes vary in length from 7,389 nucleotides (CVB1, strain Japan) to 7,403 nucleotides (CVB2, strain Ohio-1 and CVB5, strain 2000/CSF/KOR), with the typical picornavirus genome organization of a single, long, open reading frame flanked by 5'- and 3'-nontranslated regions (NTRs) that function in viral replication and translation (Racaniello 2001). The range of genome lengths within HEV-B is 7,389 (CVB1, strain Japan) to 7,453 (E9-strain DM) (Oberste et al. 2004a).

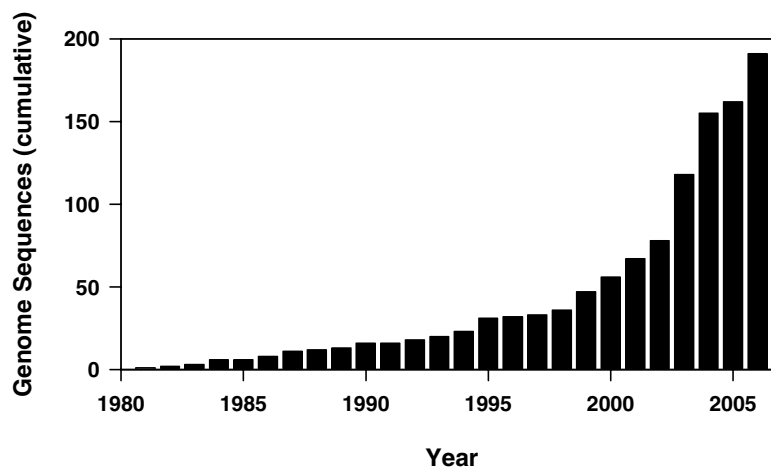


Fig. 1 The cumulative number of picornavirus complete genome sequences, by year

2.1 5'-NTR Diversity

The HEV-B 5'-NTR sequences are 738-750 nucleotides long and differ from one another by 5%-23% (Oberste et al. 2004a). Nearly 50% percent of the 5'-NTR residues are invariant among all of the viruses and almost 30% of the variable sites are concentrated in the hypervariable region, the 80-110 residues immediately upstream of the initiation codon (Oberste et al. 2004a). In the 5'-NTR, the viruses in HEV-A and HEV-B are all closely related to one another and intermix without regard to species, forming enterovirus 5'-NTR group II, whereas HEV-C (including the polioviruses) and HEV-D form group I (Brown et al. 2003; Hyypiä et al. 1997; Oberste et al. 2004a; Santti et al. 1999). A number of mutations that attenuate the cardiovirulent phenotype of certain CVB3 strains or the neurovirulence of polioviruses have been mapped to the 5'-NTR (Dunn et al. 2000, 2003; Evans et al. 1985; Kawamura et al. 1989; Macadam et al. 1991) (see also the chapter by K. Knowlton). Structural elements that are important for the function of the internal ribosome entry site are well conserved among all enteroviruses (see also the chapters by Sean and Semler and Marchant et al.). While RNA secondary structures are the primary functional units of the 5'-NTR, there also exist short segments of extraordinarily high primary sequence identity (Oberste and Pallansch 2005). These short segments have been exploited by numerous investigators as targets for molecular diagnostic assays, such as nucleic acid hybridization and RT-PCR (Oberste and Pallansch 2005; Rotbart and Romero 1995).

2.2 3'-NTR Diversity

The enterovirus 3'-NTR, the site of initiation of negative-strand RNA synthesis, is required for efficient genome replication (Brown et al. 2004; Mirmomeni et al. 1997; Rohll et al. 1995). The 3'-NTRs of the HEV-B viruses are similar in length, 102-109 nucleotides, and are 70%-99% identical to one another but only 42%-62% identical to those of representatives of other human enterovirus species (Oberste et al. 2006). While 3'-NTR sequences vary widely among the various enterovirus species (Brown et al. 2003; Oberste et al. 2004a, 2004b, 2004c), the existence of highly conserved secondary structures suggests that these structures, rather than the primary sequences, are the functional unit involved in replication (Mirmomeni et al. 1997; Pilipenko et al. 1992; Pilipenko et al. 1996). The predicted structures consist of three stem-loops termed X, Y, and Z in HEV-A and HEV-B, two stem-loops (X and Y) in HEV-C and HEV-D, and one stem-loop in the human rhinoviruses (Mirmomeni et al. 1997). Stem-loops X and Y form a tertiary structure through a so-called kissing interaction of their loop residues (Melchers et al. 1997; Mirmomeni et al. 1997; Pilipenko et al. 1992). The Z domain is apparently dispensable for replication in culture but may play a role in viral pathogenesis in vivo (Merkle et al. 2002).

2.3 *Polyprotein*

Picornavirus proteins are expressed from a single open reading frame, resulting in a polyprotein of approximately 2200 amino acids that is processed by viral proteases to yield the mature viral proteins (Racaniello 2001). The polyprotein is functionally divided into three regions: P1, P2, and P3 (Rueckert and Wimmer 1984). P1 encodes the virion structural proteins (capsid), while protein processing, replication, and host-cell interaction functions are encoded in P2 and P3 (see Sect. 6).

2.4 *Capsid Sequence Diversity*

The mature virion proteins, 1A-1D, are also known as VP4, VP2, VP3, and VP1, respectively (Rueckert and Wimmer 1984). The icosahedral capsid is composed of 60 copies of each of these proteins, five copies of VP1 at each fivefold axis of symmetry, and three copies each of VP2 and VP3 at each threefold axis, with VP4 internal to the capsid shell. The canyon surrounding the fivefold axis is the principal site of receptor interaction, with the dominant neutralizing epitopes arrayed on surface projections around the edges of the canyon. Most of the residues in these conformational epitopes are contributed by VP1 and VP2, but some are also contributed by VP3 (Huber et al. 1993; Mateu 1995; Reimann et al. 1991; Usherwood and Nash 1995). The capsid region is the most variable part of the polyprotein, both within and between species, whereas the non-capsid region sequences are much more highly conserved (Fig. 2). Despite the overall divergence, there are short conserved motifs throughout the capsid, often in structurally important regions. The HEV-B capsid protein (P1) sequences vary in length from 848 to 868 amino acids (Oberste et al. 2004a). Capsid sequences of a given serotype are collinear, but there are often insertions or deletions when comparing sequences of strains of different types. VP1, VP2, and VP3 vary in length, between types, and between species, but VP4 is collinear for all enteroviruses and rhinoviruses. The length differences tend to accumulate in regions of known diversity among the enterovirus capsid proteins - most of these variable regions are loops that are exposed on the surface of the virion, rather than being in the beta-barrel structural elements. The largest regions of high diversity are the VP2 puff region, the region surrounding the VP3 knob, both prominent surface projections, and the N- and C-terminal regions of VP1 (Fig. 2). The amino terminus of VP1 is buried in the native virion but changes conformation on virion uncoating, exposing an epitope that is highly conserved among all enteroviruses (Hovi and Roivainen 1993; Samuelson et al. 1994).

Within HEV-B, the complete P1 sequences are at least 68% identical to one another (Oberste et al. 2004a), and viruses of the same type are generally at least

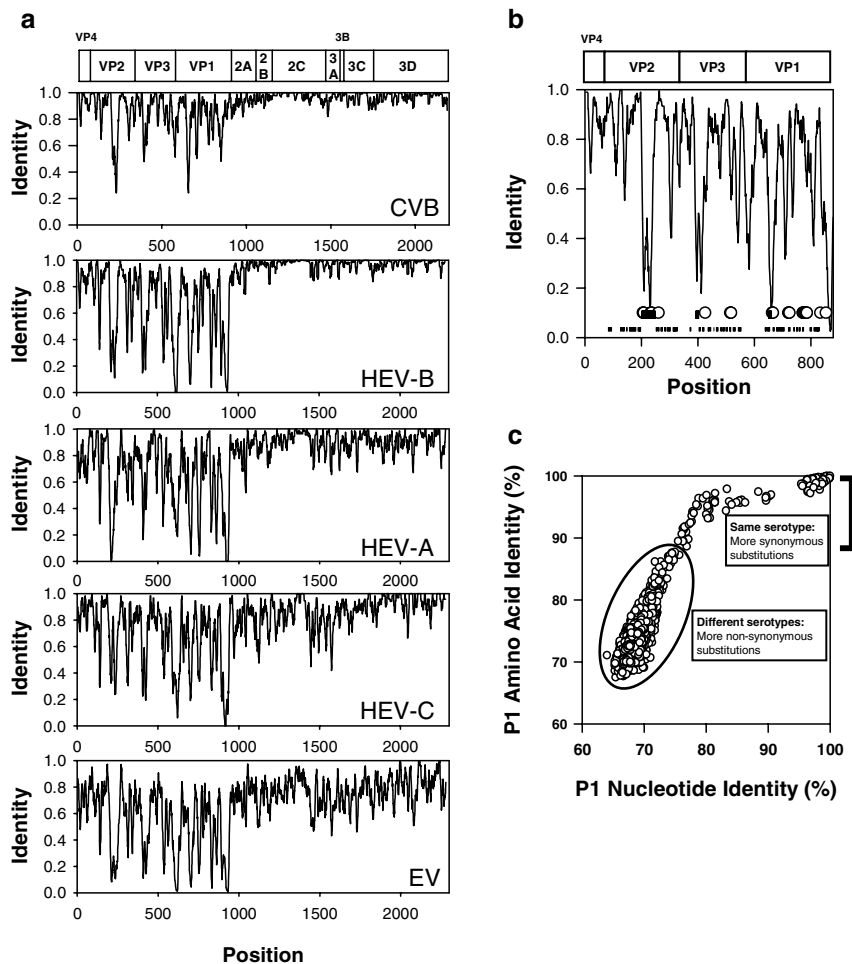


Fig. 2 Human enterovirus polypeptide amino acid sequence variation. **a** Overall polypeptide diversity among human enteroviruses (all EV, HEV-A-C, CVB); HEV-D is not shown because there are only two complete genome sequences available. Amino acid sequence identity in a sliding window of ten residues is plotted as a continuous curve. The individual plots depict diversity among (i) CVB + SVDV, (ii) all HEV-B, (iii) all HEV-A, (iv) all HEV-C, and (v) all enteroviruses. **b** HEV-B capsid diversity. Amino acid sequence identity in a sliding window of ten residues is plotted as a continuous curve. *Open circles* indicate regions that are in the receptor canyon of CVB3 (Muckelbauer et al. 1995). *Short vertical lines* indicate residues that form the α -helix and β -barrel structures of CVB3. **c** HEV-B P1 diversity. Amino acid identity is plotted vs nucleotide sequence identity for each pair of HEV-B sequences. The square bracket indicates the range of diversity among viruses of the same type

90% identical in complete capsid sequence (Oberste et al. 2001, 2005). The greatest sequence variation occurs in VP1, which varies by up to 43% among members of HEV-B (Oberste et al. 1999, 2004a). P1 sequences are monophyletic, both within

serotype and within species (Fig. 3a) (Brown et al. 2003; Oberste et al. 1999, 2004a, 2004b, 2004c). The HEV-B viruses differ from one another by up to 28% in VP2, 35% in VP3, and up to 30% in the VP4 sequences. The individual capsid proteins, VP1, VP2, and VP3, are also monophyletic by serotype and species, suggesting that recombination is rare within the capsid.

2.5 Nonstructural Region

Proteins derived from the P2 and P3 regions are involved in genome replication and protein processing, and some of these proteins are also involved in other important functions during viral replication, such as disruption of cellular processes. Most of these functions were determined using poliovirus, CVB3, or human rhinoviruses, but it is presumed that the proteins function similarly in most or all of the enteroviruses. Many of the proteins are discussed in greater detail elsewhere in this volume, but they will be briefly introduced here.

Protein 2A is a cysteine protease that cleaves *in cis* to liberate the P1 protein from the genome polyprotein (Ryan and Flint 1997; Toyoda et al. 1986) and is also involved in shutoff of host-cell protein synthesis (Kräusslich et al. 1987); however, the precise mechanism of host-cell shutoff has not been fully resolved (Belsham and Jackson 2000). The 2B protein plays a role in RNA replication by participating in the formation of membranous replication vesicles (Aldabe and Carrasco 1995) and intracellular transmembrane pores (van Kuppeveld et al. 2002); these membrane alterations may also contribute to release of mature virions (van Kuppeveld et al. 1995, 1997a, 1997b). Vesicle formation has also been attributed to 2BC and 2C (Aldabe and Carrasco 1995; Cho et al. 1994). 2C has RNA-binding, NTPase, and cysteine-rich sequence motifs that are highly conserved (Gorbalenya et al. 1988, 1989; Gorbalenya and Koonin 1989); the RNA-binding motif facilitates binding of 2C or 2BC to the 3' end of negative-strand RNA (Banerjee and Dasgupta 2001; Banerjee et al. 1997, 2001; Klein et al. 1999; Mirzayan and Wimmer 1992, 1994) and the cysteine-rich motif is involved in binding zinc (Pfister et al. 2000), but the role of the NTPase activity remains unknown.

Proteins derived from the P3 region provide the major enzymatic activities of the viral replication complex, contributing the primer protein, 3B (VPg), probably in the form of 3AB which is known to associate with intracellular membranes (Datta and Dasgupta 1994; Semler et al. 1982; Towner et al. 1996), as well as the RNA-dependent RNA polymerase (3D and/or 3CD) (Flanegan and Baltimore 1977), VPg uridylylation activity (3D) (Paul et al. 1998, 2000), and determinants involved in RNA binding and interaction with cellular proteins that are recruited into the viral replication complex (3C and/or 3CD) (Andino et al. 1990a, 1990b; Blair et al. 1998; Herrold and Andino 2001; Parsley et al. 1999). The 3C and 3CD proteins also provide the chymotrypsin-like serine protease activity that is responsible for the majority of viral protein processing (Ryan and Flint 1997).

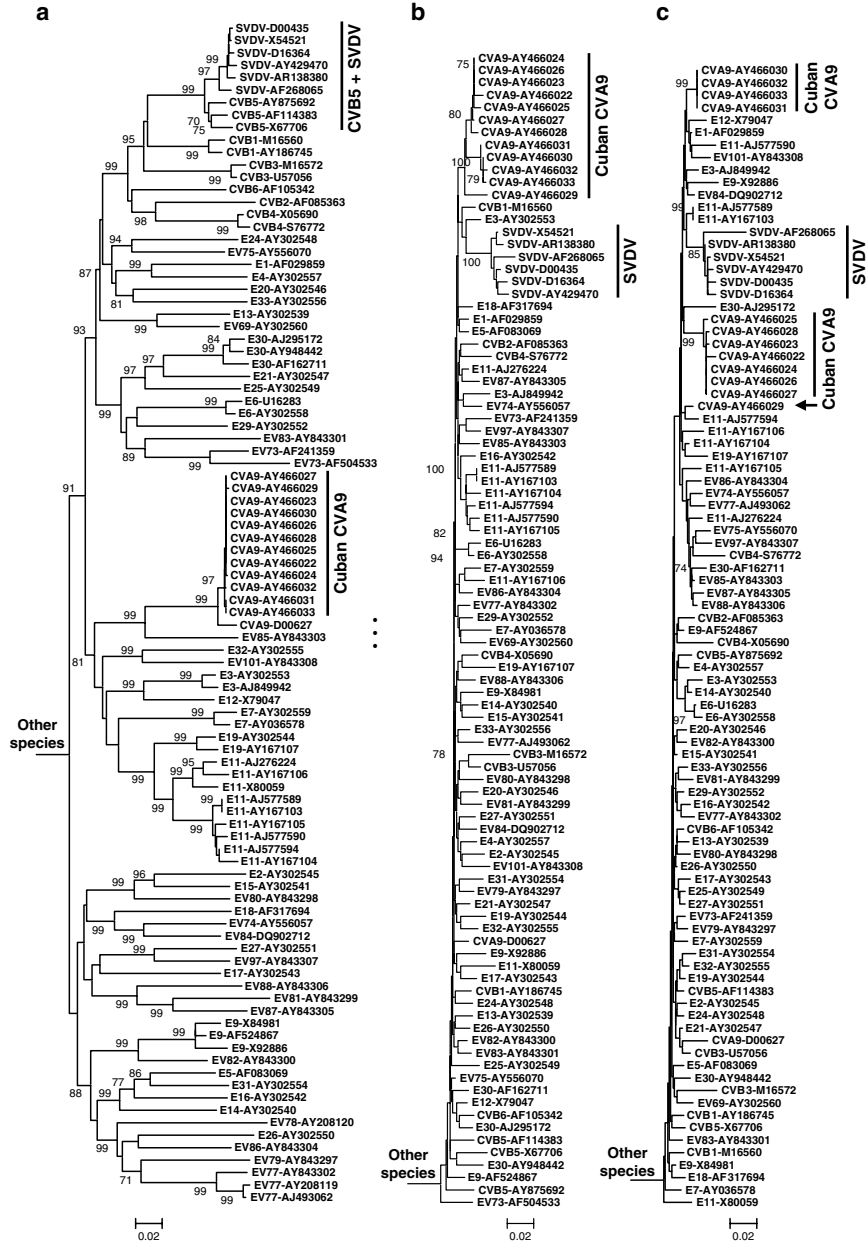


Fig. 3 Phylogenetic relationships based on aligned HEV-B amino acid sequences. Trees were constructed separately for P1 (a), P2 (b), and P3 (c), using the neighbor-joining algorithm implemented in MEGA, version 3.1 (Kumar et al. 2001), with the JTT substitution model (Jones et al. 1992); they are plotted to the same scale for each region. The scale bar indicates the number of amino acid substitutions per site

Because of the extensive RNA recombination that occurs outside the capsid-coding region, the CVB nonstructural proteins cannot be considered separately from those of the other viruses in HEV-B; that is, all members of HEV-B draw their P2 and P3 regions from a common genetic pool that is constantly exchanged by RNA recombination within a given capsid lineage (Andersson et al. 2002; Lindberg et al. 2003; Lukashev et al. 2003, 2004, 2005; Oberste et al. 2004a, 2004d; Santi et al. 1999). The non-capsid proteins are fully collinear among all of the HEV-B viruses (P2=578 aa; P3=756 aa). The P2 and P3 regions are highly conserved within HEV-B, more so than among members of other human enterovirus species (Fig. 2) (Brown et al. 2003; Oberste et al. 2004a, 2004b, 2004c). The 2A proteins are the most variable in P2-P3, differing by up to 19% within HEV-B. The 3B protein also varies by up to 18% (two amino acid differences, of 22 total), but the other mature nonstructural proteins (2B, 2C, 3A, 3C, and 3D) vary by no more than 14% (3A), and there are numerous examples of identical amino acid sequences for some nonstructural proteins among viruses of heterologous serotypes (Oberste et al. 2004a). The deduced 2C and 3D protein sequences are the most highly conserved, with no more than 6% variation in either protein.

Diversity of the nonstructural proteins is probably constrained by enzyme structure/function, as enzymes tend to be more sensitive to mutation than are structural proteins. In P2 and P3, the interspecies phylogenetic diversity (e.g., HEV-A vs HEV-B) is similar to P1, but the intraspecies diversity is much lower (Fig. 3). Unlike the P1 region, P2 and P3 sequences are not monophyletic by type and the CVBs are not monophyletic as a group. In P2 and P3, sequence monophyly can be taken as evidence of epidemiologic linkage, provided surveillance is sufficiently sensitive; that is, two viruses that share nearly identical sequences in this region must have diverged very recently from a common ancestor, as recombination has not yet occurred. For example, the SVDVs are monophyletic as a group in P2, but delinked from CVB5s (Fig. 3b), and SVDVs remain monophyletic in P3 (Fig. 3c), suggesting that they emerged as a swine pathogen following a single introduction (Zhang et al. 1999). Similarly, a group of Cuban CVA9 isolates are monophyletic in P1 and P2 but not in P3 (Fig. 3b,c), indicating that they are beginning to diverge from one another by recombination with other HEV-B strains that are cocirculating in Cuba (Fig. 3c).

2.6 *Cis-Acting Replication Element*

A distinct RNA structural element, the *cis*-acting replication element (*cre*), has been shown to be required for enterovirus replication (Goodfellow et al. 2000; Rieder et al. 2000). The poliovirus *cre* is a four-part stacked stem and conserved loop located in the region encoding 2C. The AAACA motif in the loop, which is required for *cre* function (Goodfellow et al. 2000; van Ooij et al. 2006), is completely conserved among all of the enterovirus 2C sequences (Brown et al. 2003; Oberste et al. 2004a, 2004b, 2004c). The expanded and generalized version of this

motif, RN₃AARN₆R, which models stem 1 of Goodfellow et al. (2000) as part of the loop (Yang et al. 2002), is also conserved. The structure of the predicted stem region is also well conserved among enteroviruses, with complete sequence conservation of a five-base-pair stem immediately adjacent to the 14-residue loop (Brown et al. 2003).

3 Conclusions

In all enterovirus species, nucleotide sequence evolution is largely the result of recombination and synonymous substitutions, resulting in relative conservation of the encoded polypeptide sequences, except in the capsid region where diversity is almost exclusively driven by nucleotide substitutions, with amino acid sequences relatively conserved within a type, but highly variable between viruses of different types. If recombination occurs at all within the capsid, the evidence is quickly obscured by rapid accumulation of nucleotide substitutions. With the exception of viruses of known epidemiologic linkage, all serotypes with multiple complete sequences show evidence of recombination; therefore, all enterovirus strains can be considered recombinants relative to nonlinked strains.

While non-capsid sequences may influence pathogenicity or tropism (e.g., by affecting replication or translation), the principal identity of an enterovirus (its antigenic structure, receptor binding, etc.) is controlled by the capsid. In general, an enterovirus might be viewed as a capsid sequence in search of non-capsid sequences of the highest fitness to provide a selective replicative advantage. The 5'-NTR and P2-P3-3'-NTR sequences of a given isolate represent only a snapshot of that particular isolate or of a closely related lineage, within a narrow temporal and geographic window. This view of the role of recombination in enterovirus evolution would predict that the specific genomic combinations and sequences in the P2-P3 regions of the prototype strains from 50 years ago are not likely to be present in currently circulating strains of the same serotype. Conversely, sequences related to those of a given prototype strain may be found in different serotypes within the same species among currently circulating enteroviruses. The observed genomic sequences agree well with these predictions. The designation of a serotype prototype strain is purely arbitrary, but it provides a context for the analysis of other clinical isolates of that serotype. That is, the prototype strains are simply a snapshot in time, arbitrarily chosen as a reference.

4 Future Directions

Despite recent progress in enterovirus genomics, there are many areas in which additional genomic studies can enhance our understanding of enterovirus basic biology and disease association. Genomic sequences from a large collection of

isolates of a given type (or related types, e.g., the CVBs) with well-characterized clinical outcome/disease will facilitate fine-scale mapping of genetic determinants that contribute to virulence. The combination of more capsid sequences and additional three-dimensional virion structures will permit comparative mapping of receptor interaction sites and broaden our understanding of virus-host interaction at the cell surface. Large-scale comparative genomics of wild strains, as well as directed cell-based and cell-free *in vitro* studies, will help develop a better understanding of the factors that facilitate and constrain enterovirus recombination. Finally, better methods to rapidly generate complete genome sequences, especially directly from original clinical material, will make all of these studies easier, cheaper, and more practical.

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