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Complete genome sequence analysis of Seneca Valley virus-001, a novel oncolytic picornavirus

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The complete genome sequence of Seneca Valley virus-001 (SVV-001), a small RNA virus, was determined and was shown to have typical picornavirus features. The 7280 nt long genome was predicted to contain a 5′ untranslated region (UTR) of 666 nt, followed by a single long open reading frame consisting of 6543 nt, which encodes a 2181 aa polyprotein. This polyprotein could potentially be cleaved into 12 polypeptides in the standard picornavirus L-4-3-4 layout. A 3′ UTR of 71 nt was followed by a poly(A) tail of unknown length. Comparisons with other picornaviruses showed that the P1, 2C, 3C and 3D polypeptides of SVV-001 were related most closely to those of the cardioviruses, although they were not related as closely to those of encephalomyocarditis virus and Theiler's murine encephalomyelitis virus as the latter were to each other. Most other regions of the polyprotein differed considerably from those of all other known picornaviruses. SVV-001 contains elements of an internal ribosome entry site reminiscent of that found in hepatitis C virus and a number of genetically diverse picornaviruses. SVV-001 is a novel picornavirus and it is proposed that it be classified as the prototype species in a novel genus named '*Senecavirus*'.

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INTRODUCTION

The family *Picornaviridae* currently comprises nine genera: *Enterovirus*, *Rhinovirus*, *Aphthovirus*, *Cardiovirus*, *Hepatovirus*, *Parechovirus*, *Erbovirus*, *Kobuvirus* and *Teschovirus* (Stanway *et al.*, 2005), although the two human rhinovirus species are to be moved to the genus *Enterovirus* and five novel genera have been proposed (Krumbholz *et al.*, 2002; Oberste *et al.*, 2003; Tseng & Tsai, 2007; Tseng *et al.*, 2007; Kapoor *et al.*, 2008; http://www.picornastudygroup.com/). Prominent members of the family *Picornaviridae* include *Poliovirus*, rhinovirus, *Hepatitis A virus* (HAV) and *Foot-and-mouth disease virus* (FMDV). Picornaviruses are non-enveloped viruses that are small in size (27–30 nm). The icosahedral capsid is formed from 60 protomers of three or four polypeptides. These viruses have a single-stranded, positive-sense RNA genome that encodes a single polyprotein, which is processed co- and post-translationally by virus-encoded proteases. Both ends of the genome are modified: the 5′ end has the viral protein VPg covalently attached, and the 3′ end has a poly(A) tail. Structural proteins are encoded towards the 5′ end of the genome and non-structural proteins at the 3′ end. These viruses have a rapid replication cycle that occurs in the host-cell cytoplasm. The presence of an internal ribosome entry site (IRES) element in the 5′ untranslated region (UTR) allows for RNA translation in a cap-independent manner. There are four different IRES structures found in picornaviruses: type I in entero- and rhinoviruses, type II in cardio-, aphtho-, parecho- and erboviruses (and possibly kobuviruses), type III in hepatoviruses and type IV in teschoviruses, 'sapeloviruses', 'tremoviruses', duck hepatitis virus 1 and seal picornavirus 1 (Hellen & de Breyne, 2007). The type IV IRES is also found in hepaciviruses, GBV-B viruses and pestiviruses (family *Flaviviridae*) (Brown *et al.*, 1992).

Here, we report the discovery and genetic analysis of the complete genome of a novel picornavirus, Seneca Valley virus isolate 001 (SVV-001), and propose that it be designated the prototype species in a novel genus, '*Senecavirus*', in the family *Picornaviridae*. The complete genome sequence analysis of SVV-001 presented here supports the classification of the virus as a picornavirus, with the most closely related members of the family being cardioviruses.

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The GenBank/EMBL/DDBJ accession number for the complete genome sequence of SVV-001 is DQ641257.

METHODS

Cells and virus. PER.C6 cells (Fallaux *et al.*, 1998), obtained through a licence with Crucell, were used for cultivation of SVV-001. The PER.C6 cells were cultivated in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10 % fetal bovine serum (Biowhitaker) and 10 mM $MgCl_2$ (Sigma). Plaque-purified SVV-001 was amplified in PER.C6 cells. The infected cells were harvested when complete cytopathic effect (CPE) was observed and were used as the source of the virus. The virus was purified by two rounds of CsCl-gradient centrifugations: a step gradient (density of CsCl, 1.24 and 1.4 g ml^{-1}) at 24 000 r.p.m. for 1 h, followed by continuous gradient centrifugation (density of CsCl, 1.33 g ml^{-1}) at 65 000 r.p.m. overnight. The concentration of the virus was determined spectrophotometrically, assuming that an A_{260} of 1 was equal to 9.5×10^{12} particles (Scraba & Palmenberg, 1999). The virus titres were also determined by standard plaque and $TCID_{50}$ assays using PER.C6 cells.

Electron microscopy. Purified SVV-001 was mounted onto carbon-coated Formvar grids by using the direct application method, stained with uranyl acetate and visualized with a JEOL 1200 EX transmission electron microscope (Electron Microscopy BioServices). Additionally, PER.C6 cells were infected with SVV-001 at an m.o.i. of 100 and infected cells were collected at 2, 4, 8, 24 and 36 h post-infection. The cells were stained en bloc with 2 % aqueous uranyl acetate, dehydrated in a graded ethanol series and infiltrated and embedded in Spurr's plastic resin. The samples were allowed to polymerize overnight at 70 °C. Ultrathin sections, 60–80 nm in thickness, of SVV-001-infected PER.C6 cells were cut from embedded blocks and mounted onto 200-mesh copper grids. The grids were then post-stained with uranyl acetate and Reynolds' lead citrate (Reynolds, 1963) and examined by using a JEOL 1200 EX transmission electron microscope. Representative micrographs were taken at ×10 000–25 000 magnifications.

SDS-PAGE and N-terminal sequence analyses. Purified SVV-001 was subjected to electrophoresis using a 10 % NuPAGE pre-cast Bis–Tris polyacrylamide mini-gel electrophoresis system (Novex). Half of the gel was visualized by silver staining using a Dodeka silver stain kit (Bio-Rad), whilst the other half was used to prepare samples for amino acid sequencing of the amino (N) termini of the capsid proteins. Prior to transfer of proteins to membranes, the gel was soaked in 10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer, pH 11, for 1 h, and a PVDF membrane (GE Healthcare) was wetted in methanol. Proteins were then transferred to the PVDF membrane. After transfer, proteins were visualized by staining with Amido black for approximately 1 min, and bands of interest were excised with a scalpel and air-dried. The proteins were subjected to automated N-terminal sequence determination by Edman degradation using a pulsed-phase sequencer. The N-terminal sequences of the three viral proteins were subjected to similarity searches using the BLAST program at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/).

Genome analysis of SVV-001. The genomic RNA of SVV-001 was extracted by using TRIzol reagent (Invitrogen). Briefly, 250 µl purified virus (approx. 2.5×10^{13} virus particles) was mixed with 3 vols TRIzol and 240 µl chloroform. The RNA present in the aqueous phase was precipitated by adding 600 µl 2-propanol. An aliquot of RNA was resolved on a 1.25 % denaturing agarose gel and the band was visualized by ethidium bromide staining (data not shown). Synthesis of SVV-001 cDNA was performed under standard conditions using 1 µg RNA along with random 14-mer oligonucleotides, oligo-dT or viral-specific primers, and avian myeloblastosis virus reverse transcriptase, Thermo-X (Invitrogen) or the Transcriptor Reverse Transcriptase system (Roche Applied Science). DNA sequences of cDNA subclones in pCRII (Invitrogen) or of PCR products were determined at Lofstrand Laboratories (Gaithersburg, MD, USA), Commonwealth Biotechologies Inc. (Richmond, VA, USA) or Cleveland Genomics (Cleveland, OH, USA). The 5′ end of the genome was cloned by using PCR with degenerate oligonucleotides encoding 5′ sequences with similarities to cardiovirus sequences. Sequence data were compiled to generate the complete genome sequence of SVV-001 and were then analysed by using sequence-analysis programs [Clone Manager (Sci Ed Software), Vector NTI (Invitrogen) and Lasergene (DNASTAR)]. The predicted amino acid sequence was compared with entries in the NCBI protein sequence database by using BLAST.

Secondary-structure predictions. The program PSIPRED v. 2.5 (Jones, 1999) was used to predict the secondary structures of SVV-001 and other picornavirus 2B proteins. The program MEMSAT3 (Jones *et al.*, 1994), run from the PSIPRED protein structure prediction server (McGuffin *et al.*, 2000; http://bioinf.cs.ucl.ac.uk/psipred/), was used to predict transmembrane topology of the 3A polypeptides of SVV-001, encephalomyocarditis virus (EMCV) and Theiler's murine encephalomyelitis virus (TMEV).

Phylogenetic analysis. The program SimPlot v. 3.5.1 (Lole *et al.*, 1999) was used to compare the genomes of SVV-001 and other cardioviruses. The following parameters were used: window, 200 bp; step, 20 bp; GapStrip, on; Kimura (2-parameter); T/t ratio, 2.0. The GenBank accession numbers of the sequences used in this analysis were EMCV-R (M81861), EMCV-Mengo (L22089), TMEV-GDVII (M20562), Theiler-like virus (TLV) of rats NGS910 (AB090161) and Saffold virus (SAF-V; EF165067). For this analysis, the entire genome was used except for the 5′ UTR sequences upstream of the EMCV poly(C) tract, as the alignments are poor in this region. The programs BioEdit v7.0.1 (Hall, 1999) and CLUSTAL X v. 1.83 (Thompson *et al.*, 1997) were used to compile the alignments of SVV-001 sequences with those of other viruses. Phylogenetic analyses were conducted by using MEGA version 3.1 (Kumar *et al.*, 2004). Mid-point-rooted neighbour-joining trees (Saitou & Nei, 1987) were constructed by using an amino acid difference matrix based on a Poisson-corrected distance. Confidence levels on branches were estimated by bootstrap resampling using 1000 pseudoreplicates.

RESULTS

Discovery of SVV-001

SVV-001 was isolated at Genetic Therapy Inc. (Gaithersburg, MD, USA) in 2002. While cultivating adenovirus-5 (Ad5)-based vectors in PER.C6 cells, uncharacteristically rapid CPE at <24 h post-infection was observed. During CsCl (1.33 g ml^{-1}) purification, a very faint band at the same location as adenovirus was obtained. Upon SDS-PAGE analysis, three protein bands corresponding to 36, 31 and 27 kDa were observed (data not shown). To obtain an initial identification of SVV, the N-terminal sequences of the excised structural polypeptides were determined. Two of these sequences were related most closely to those of cardioviruses (see below). To analyse the virus further, a purified sample and virus-infected PER.C6 cells were subjected to electron microscopy. The purified virus sample revealed icosahedral particles of about 27 nm in diameter, appearing singly or as small aggregates on the grid (data not shown). The small virion size was consistent with the virus belonging to the family *Picornaviridae*.

Ultrastructural studies of infected PER.C6 cells were conducted at 2, 4, 8, 24 and 36 h post-infection. At early time points, no virus was observed in infected cells (data not shown). However, crystalline, lattice-like structures were observed in the cytoplasm of cells at 24 h post-infection (data not shown). Based on the similarity results, electron microscopic features and the observed replication of the virus in the cytoplasm, the unknown virus was suspected to be a picornavirus. The identified cytopathic agent was plaque-cloned and designated Seneca Valley virus-001 (SVV-001).

Sequencing of SVV-001

The complete nucleotide sequence of the SVV-001 genome was determined from several overlapping cDNA clones. However, the first 10 nt at the 5′ end were derived cardiovirus consensus sequence and do not necessarily represent the actual 5′-end sequence of SVV. The RNA genome of SVV-001 consists of 7280 nt, excluding a 3′ poly(A) tail, and has a G+C content of 51.6 mol%, a 666 nt 5′ UTR and a shorter 3′ UTR (71 nt). The organization of SVV-001 genome was determined by alignment of its nucleotide sequence and deduced amino acid sequence of the open reading frame (ORF) with those of other picornaviruses (data not shown). This analysis revealed a large, single ORF with the potential to encode a polyprotein precursor of 2181 aa. The genomic features of SVV-001 are related most closely to those of members of the genus *Cardiovirus* (Table 1; Fig. 1; and discussed below).

In order to examine the possibility that SVV-001 arose as a recombinant virus derived from cardioviruses, the genomic sequence of SVV-001 was compared with those of other cardioviruses, and a similarity plot (SimPlot) was constructed (Fig. 2). If recombination had occurred, one or more of the viruses would have been closer to SVV-001 than the rest in the part of the graph representing the recombined sequence. In fact, all of the viruses analysed are approximately the same distance from SVV-001 along the length of the genome, indicating that SVV-001 is probably not a recombinant derived recently from any of the known cardioviruses.

The 5′ and 3′ UTRs

Our initial database searches revealed that part of the SVV 5′ UTR (nt 406–625) had a nucleotide sequence identity of 57.3 % with that of hepatitis C virus (HCV) (GenBank accession no. DQ140286), suggesting that SVV may contain a type IV IRES. Subsequently, Hellen & de Breyne (2007) published predicted secondary structures of various flaviviruses and picornaviruses, including SVV-001. Their analysis supported the hypothesis that the SVV IRES was of type IV. An analysis of nt 1–400 of the SVV-001 5′ UTR, using RNAdraw (Matzura & Wennborg, 1996), predicted a high level of secondary structure, with seven simple and two complex stem–loop structures (data not shown).

Folding of the 3′ UTR of SVV revealed two stem–loops with the potential to form a kissing-loop structure (Fig. 3). This type of structure has been shown to be important in enterovirus replication (Mirmomeni *et al.*, 1997).

The SVV-001 polyprotein

The polyprotein of SVV-001 was analysed for potential protease-cleavage sites based on alignment with the cardioviruses. The cleavage sites of the SVV-001 polyprotein were mostly predicted readily by this method (Table 2). However, the N-terminal sequences of the three major (36, 31 and 27 kDa) structural proteins of purified SVV-001 were determined directly by amino acid sequencing and were DHNTEEMENSADRVTTQTAGNTAINTQSSLGVLCAY, STDNAETGVIEAGNTDTDFSGELAAP and GPIPTAPRENSLMFLSTLPDDTVPAYGNVRTPPVNY, respectively. These polypeptides were identified as VP2, VP1 and VP3, respectively, from their position on the genome sequence and similarity to cardiovirus sequences. Thus, from the predicted SVV-001 polyprotein sequence, the VP4/VP2/VP3/VP1 cleavage sites were shown to be K/D, Q/G and H/S, respectively (Table 2). As the start of the P1 region was predicted to occur 71 residues upstream of the VP2 sequence (where a GxxxT/S myristoylation motif occurs), this implies the presence of VP4 and thus a VP0 maturation cleavage.

Primary cleavage events were predicted to occur in a fashion similar to that in cardio-, aphtho-, erbo- and teschoviruses, involving separation of P1–2A from 2BC–P3 by a novel ribosome-skipping mechanism involving the sequence NPG↓P (Donnelly *et al.*, 2001) and a traditional cleavage event by 3C between L and P1 and between 2BC and P3. Most predicted SVV cleavages were typical of picornavirus 3C proteinases, being at Gln/Gly, Gln/Ser or Glu/Asn pairs (Table 2). There were two unusual cleavage sites: His/Ser between VP3 and VP1 and either Gln/Gln or Gln/Pro between 3B and 3C (Table 2).

The sizes of the predicted polypeptides of SVV-001 were then compared with those of two members of the cardioviruses, EMCV and TMEV (Fig. 1), the most noticeable difference being the size of the 2A protein; the SVV-001 2A was considerably smaller than that of EMCV or TMEV.

Leader protein

The only picornaviruses to possess leader polypeptides preceding the capsid region are members of the genera *Cardiovirus*, *Aphthovirus*, *Erbovirus*, *Kobuvirus*, *Teschovirus* and the proposed genus '*Sapelovirus*'. In aphthoviruses and erboviruses, the leader proteins are papain-like cysteine proteinases that are able both to self-cleave carboxy-terminally and also to cleave the eukaryotic initiation factors (eIF) 4GI and 4GII, leading to shut-off of host-cell

Table 1. Comparison of predicted SVV proteins and UTRs with those of other picornaviruses

Values are percentage amino acid sequence identity, or percentage nucleotide sequence identity for UTRs.

Genus/species	Serotype	Strain	GenBank accession no.	Identity (%) in region:												
				5′ UTR	L	1AB	1C	1D	2A*	2B	2C	3A	3B	3C	3D	3′ UTR
Cardiovirus																
Encephalomyocarditis virus	EMCV	R	M81861	31.5	20.3	41.0	41.7	18.4	36.0	15.2	41.3	11.9	5.9	37.1	58.2	32.4
Theilovirus	TMEV	GDVII	M20562	29.6	16.2	41.2	38.4	19.2	28.0	18.1	39.3	14.3	23.5	36.4	58.0	32.4
Theilovirus	VHEV	Siberia/55	M80888, M94868†	29.8	14.7	42.7	39.1	19.1	28.0	18.1	39.9	17.9	23.5	35.9	56.3	32.4
Theilovirus	Rat TLV	NGS910	AB090161	28.4	13.2	41.5	39.1	19.2	36.0	15.1	41.6	15.5	35.3	34.5	56.7	32.4
Theilovirus	SAF-V	California/81	EF165067	29.1	11.9	40.8	41.7	20.8	36.0	14.3	41.3	14.6	29.4	35.0	55.4	35.0
Aphthovirus																
Foot-and-mouth disease virus	FMDV-O	V1/UK/24	AY593829	28.3	6.5	29.1	32.3	14.5	44.4	8.7	35.0	11.1	19.0‡, 13.6‡, 22.7‡	22.7	45.8	23.9
Equine rhinitis A virus	ERAV	PERV	DQ272578	29.2	7.9	31.0	33.8	13.2	33.3	10.4	35.1	4.8	22.7	20.6	48.3	22.9
Erbovirus																
Equine rhinitis B virus	ERBV-1	P1436/71	X96871	31.6	11.5	33.5	41.7	9.6	36.8	8.6	35.6	12.2	16.7	21.7	41.7	38.0
Teschovirus																
Porcine teschovirus	PTV-1	Talfan	AF231769	35.8	11.8	24.6	30.6	12.4	33.3	3.3	30.9	4.9	27.3	28.6	40.0	15.8
Enterovirus																
Poliovirus	PV-1	Mahoney	V01149	25.9	–	25.8	31.6	14.3	–	6.5	29.9	8.4	23.8	18.6	32.4	25.0
Human enterovirus C	CV-A1	Tompkins	AF499635	25.0	–	26.8	31.0	14.5	–	10.8	28.0	10.7	23.8	17.5	31.9	24.6
Human enterovirus A	CV-A16	G-10	U05876	26.5	–	25.4	26.5	11.5	–	5.3	31.5	8.5	23.8	14.1	29.6	31.0
Human enterovirus B	CV-B5	Faulkner	AF114383	26.0	–	26.6	30.3	12.4	–	8.4	29.0	8.2	23.8	15.3	29.6	25.0
Human enterovirus D	EV-70	J670/71	D00820	23.7	–	26.8	28.0	14.3	–	11.6	30.6	9.4	19.0	16.4	31.8	32.4
Simian enterovirus A	SEV-A1	A2PV	AF201894	24.5	–	28.3	30.7	13.7	–	11.6	27.7	10.8	14.3	14.1	31.2	17.5
Bovine enterovirus	BEV-1	VG/5/27	D00214	26.2	–	25.2	26.0	12.5	–	7.4	27.0	5.9	19.0	17.5	29.9	19.4
Bovine enterovirus	BEV-2	BEV-261	DQ092770	27.4	–	28.0	26.8	11.5	–	8.4	28.6	7.1	14.3	16.4	29.4	19.7
Porcine enterovirus B	PEV-9	UKG/410/73	AF363453	25.7	–	27.7	30.7	11.3	–	3.2	26.1	4.7	23.8	19.8	31.9	19.7
Rhinovirus																
Human rhinovirus A	HRV-1B	B632	D00239	25.6	–	23.4	26.3	13.2	–	11.0	28.0	8.2	20.0	18.6	30.2	13.2
Human rhinovirus B	HRV-3	FEB	DQ473485	25.0	–	23.8	26.5	12.9	–	7.5	30.2	7.4	23.8	17.0	32.2	17.4
Unassigned	HRV-?	HRV-QPM	EF186077	25.5	–	24.7	25.2	14.4	–	11.8	30.3	2.8	14.3	19.2	30.9	32.7
‘*Sapelovirus*’																
‘Porcine sapelovirus’	PSV	V13	AF406813	31.5	6.6	30.4	30.4	13.3	–	15.7	29.0	10.5	19.0	15.3	30.3	22.5
‘Simian sapelovirus’	SSV	SV2-2383	AY064708	32.4	13.4	29.3	36.0	12.4	–	11.5	27.9	8.2	19.0	17.0	31.8	25.8
‘Avian sapelovirus’	ASV	TW90A	AY563023	27.8	15.2	28.3	30.7	15.6	–	8.5	29.8	4.6	23.8	15.4	30.9	11.8
Kobuvirus																
Aichi virus	AiV	A846/88	AB040749	28.4	11.5	22.2	26.8	14.9	–	8.1	26.4	4.4	22.7	16.9	33.1	29.6
Bovine kobuvirus	BKV	U-1	AB084788	27.8	9.3	21.2	28.2	14.1	–	10.8	23.5	6.9	27.3	13.6	32.4	19.7

Table 1. cont.

Genus/species	Serotype	Strain	GenBank accession no.	Identity (%) in region:											
				5′ UTR	L	1AB	1C	1D	2A*	2B	2C	3A	3B	3C	3D	3′ UTR
Hepatovirus																
Hepatitis A virus	HAV	HM-175	M14707	30.0	–	24.1	15.9	9.4	–	9.4	24.3	13.9	18.2	17.0	23.6	9.3
'Tremovirus'																
'Avian encephalomyelitis virus'	AEV	Calnek	AJ225173	36.8	–	20.2	16.1	8.9	–	7.9	25.3	10.8	15.0	14.8	24.2	20.0
Parechovirus																
Human parechovirus	HPeV-1	Harris	L02971	28.2	–	17.1	14.5	8.3	–	5.9	21.3	9.0	13.6	12.6	22.4	22.5
Ljungan virus	LV	87-012	AF327920	28.9	–	19.5	15.1	5.2	44.0	6.0	21.0	7.9	14.3	10.8	22.4	12.3
Unassigned																
	DHV-1	H	DQ249300	30.1	–	16.6	12.3	6.7	20.0	11.6	23.4	6.0	9.1	18.3	22.5	31.0
	SePV-1	HO.02.21	EU142040	26.2	–	12.9	12.7	7.6	30.8	7.9	24.1	15.7	23.8‡, 22.7‡	15.9	25.6	45.9

*A 27 aa sequence (WCPSMLPFRSYKQKMLMQSGDIETNPG), which included the predicted 9 aa SVV 2A, was used for the comparison.
†H. Lipton, personal communication.
‡FMDV has three copies of 3B (VPg) and SePV-1 has two copies.

protein synthesis (Devaney *et al.*, 1988; Gradi *et al.*, 2004). The cardiovirus leader polypeptide binds zinc, is phosphorylated during infection and plays a role in the regulation of viral genome translation (Dvorak *et al.*, 2001). The functions of the kobuvirus, teschovirus and sapelovirus leaders are not known. The SVV-001 leader polypeptide lacks the catalytic residues necessary for proteolytic activity and does not contain either a zinc-finger motif [C-x-H-x(6)-C-x(2)-C] in the leader amino-terminal region or a tyrosine-phosphorylation motif [K-x(2)-E-x(2)-Y] approximately 14 residues downstream, possibly indicating a function distinct from that of leader peptides of aphthoviruses/erboviruses and cardioviruses.

The P1 region proteins

In picornaviruses, the P1 polypeptide is cleaved by the 3C protease to give VP0, VP3 and VP1. Sixty copies of these three polypeptides form the capsid. In most picornaviruses (the exceptions being parechoviruses, kobuviruses, duck hepatitis virus 1 and seal picornavirus), a maturation cleavage of VP0 occurs to give VP2 and the internally located VP4 (reviewed by Leong *et al.*, 2002). The capsid proteins of SVV-001 were all similar in size to those of cardioviruses (Fig. 1), and a VP0 maturation cleavage was predicted to occur.

The P2 region proteins

The 2A protein in cardioviruses performs two distinct functions. One is a ribosome-skipping function to enable P1–2A to be separated from the elongating polyprotein (at the conserved NPG↓P motif), and the second is inhibition of cap-dependent mRNA translation (Aminev *et al.*, 2003a) and cellular mRNA transcription (but not rRNA transcription) (Aminev *et al.*, 2003b). Notably, the 2A protein of SVV-001 is significantly different in size from those of cardioviruses. The cardioviruses have a long (approx. 150 aa) 2A protein, whereas SVV-001 is predicted to have a short (9 aa) 2A protein (discussed below). Thus, the 2A protein of SVV-001 would be predicted to perform only the P1–2A/2BC–P3 ribosome-skipping function. This is also the case in aphthoviruses, erboviruses and teschoviruses, which have very short 2A sequences ending in NPG↓P. It is also possible that the 2A protein of SVV-001 could remain attached to the carboxy terminus of VP1, as it probably does in another picornavirus, Ljungan virus (Johansson *et al.*, 2002). However, unlike SVV-001, Ljungan virus has a second 2A polypeptide of different function immediately downstream of VP1.

The 2B protein of poliovirus is a viroporin that functions to enhance membrane permeability and plays a role in the formation of intracellular virus replication vesicles (Agirre *et al.*, 2002). The 2B protein of SVV-001 had no primary sequence similarity to those of other known picornaviruses. The secondary structures of the 2B proteins of representatives of all of the picornavirus genera, including SVV-001,

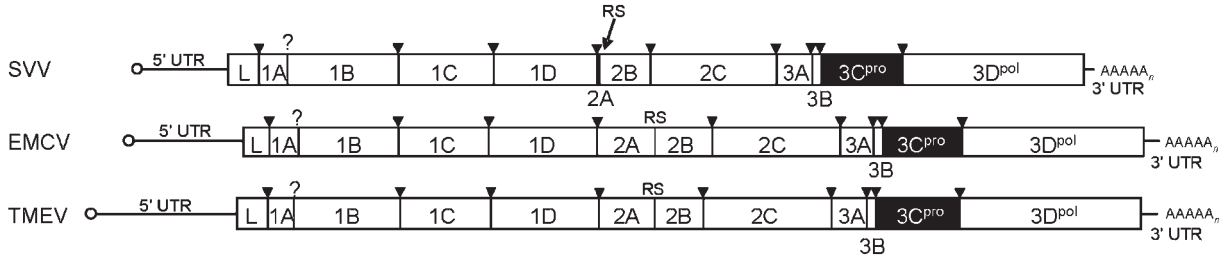

Fig. 1. Genome organization of SVV-001, EMCV and TMEV. The ORFs are flanked on either side by UTRs. Gene regions (drawn to scale) are presented. Gene regions encoding proteases are highlighted in black. The genome-linked 3B peptides at the 5′ end (○) and the poly(A) tails at the 3′ ends are indicated. The processing sites of the 3C protease (arrowheads) and the 2A ribosome-skipping (RS) NPG↓P sequences are also shown. Question marks indicate unknown proteolytic activities responsible for the maturation cleavage of the 1AB precursor.

were predicted by using the PSIPRED server (data not shown). This analysis revealed that, despite being very different in primary sequences, all picornavirus 2B proteins may be very similar in their secondary structure, being composed almost exclusively of helix–coil–helix structures, consistent with their possible role as viroporins.

The 2C protein is a helicase-like polypeptide involved in RNA synthesis (Gorbalenya *et al.*, 1990; Tanner & Linder, 2001). The Hel-like domains of all picornaviruses viruses fall into superfamily III and contain motif 'A' [GxxGxGK(S/T)], followed about 35 aa downstream by motif 'B' (ΦΦΦxxDD, where Φ is any hydrophobic residue), and about 30 aa downstream of motif 'B' by motif 'C' [KgxxΦxSxΦΦΦx(S/T)(S/T)N]. In SVV-001, these motifs are represented by GKPGCGKS, FVTLMDD and KGRPFTSNLIIATTN, with spacing of 36 and 32 aa, respectively.

The P3 region proteins

Little is known about the function of the 3A polypeptide; however, all picornavirus 3A proteins contain a putative transmembrane α-helix, which is characterized by a region of high hydrophobicity (aa 41–62 in SVV-001). Primary sequence identity between SVV-001 and cardioviruses is low for this protein; however, the 3A polypeptides are predicted to be of similar lengths and the putative transmembrane α-helix lies in the same region of the protein of SVV-001 compared with those of cardioviruses (data not shown).

The genome-linked polypeptide, VPg, which is encoded by the 3B region, shares few amino acids in common with the other picornaviruses; however, the third residue is a tyrosine, consistent with its linkage to the 5′ end of the virus genome (Rothberg *et al.*, 1978).

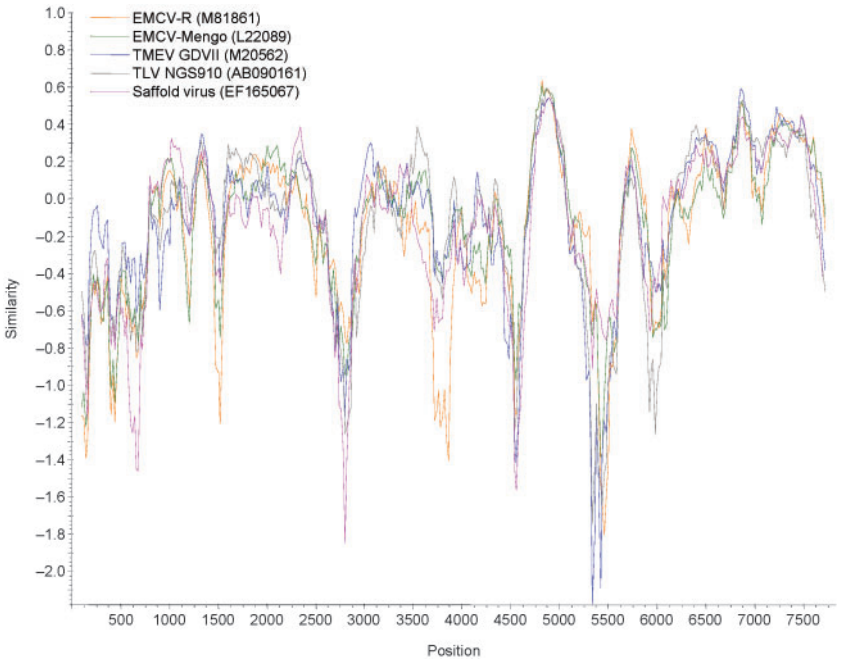

Fig. 2. Relationship of SVV-001 to the cardioviruses. The complete genome sequences of EMCV-R, EMCV-Mengo, TMEV-GCVII, TLV-NGS910 and SAF-V [not including sequences upstream of the EMCV poly(C) tract] were aligned with that of SVV-001 and a SimPlot graph was constructed.

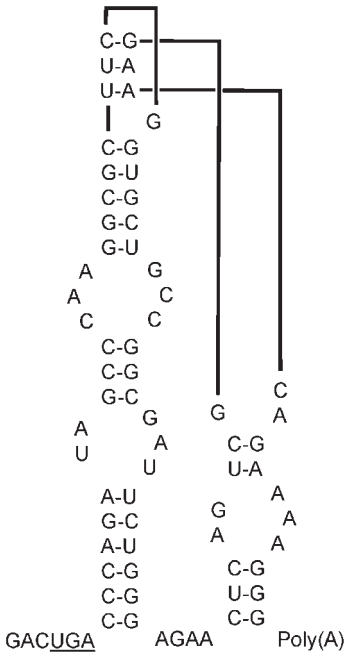

Fig. 3. Prediction of a potential 'kissing-loop' tertiary structure at the 3′ end of the SVV genome. The polyprotein stop codon is underlined.

The picornavirus 3C proteinase is a chymotrypsin-like enzyme with a cysteine in place of a serine in the catalytic site (Gorbalenya *et al.*, 1986; Bazan & Fletterick, 1988). A 'catalytic triad' (Bazan & Fletterick, 1988) is made up of a histidine (SVV $^{H}3C^{48}$), an aspartate/glutamate (SVV $^{D}3C^{84}$) and the conserved cysteine (SVV $^{C}3C^{160}$). The catalytic cysteine is typically followed 10–20 aa downstream by a GΦH motif (SVV $^{GLH}3C^{176–178}$) that seems to be involved in substrate recognition. The active-site residues have been confirmed by analysis of the known three-dimensional structures of 3C in other picornaviruses [HAV, Allaire *et al.*, 1994; Bergmann *et al.*, 1997; PV-1, Mosimann *et al.*, 1997; human rhinovirus (HRV)-14, Matthews *et al.*, 1994; HRV-2, Matthews *et al.*, 1999)]. The active-site residues are conserved in the 3C sequence of SVV-001 and all other known picornaviruses.

The 3D polypeptide interacts with the 3AB protein and can also act as a component of the 3CD protein. As such, it functions in virus replication and VPg uridylylation, and is the major component of the RNA-dependent RNA polymerase (RdRp). SVV-001 contains amino acid motifs conserved in the 3D protein of picornaviruses, i.e. KDEL/IR, PSG, YGDD and FLKR (Argos *et al.*, 1984).

Phylogenetic comparison of SVV-001 polypeptides with those of other picornaviruses

Those SVV-001 polypeptides that could be aligned with those of the cardioviruses (P1, 2C, 3C and 3D) were compared with the same proteins of representative members of each of the picornavirus species. Distance matrices and unrooted neighbour-joining trees were constructed and confidence limits on branches were accessed by bootstrap resampling (1000 pseudoreplicates). Phylogenetic trees comparing the P1, 2C, 3C and 3D polypeptides of SVV-001 with those of other representative picornaviruses show that, whilst SVV-001 is clearly different from EMCV and theiloviruses, it is related most closely to the members of the genus *Cardiovirus* (Fig. 4).

DISCUSSION

SVV-001 was first isolated from cell-culture medium, presumably from either contaminated porcine trypsin or fetal bovine serum. Over a 20 year period, 12 other virus

Table 2. Protease-cleavage sites of SVV-001 and cardioviruses

Cleavage between	SVV-001	EMCV	TMEV	TLV	VHEV	SAF-V
L/VP4	LQ/GN	LQ/GN	PQ/GN	PQ/GN	PQ/GN	PQ/GN
VP4/VP2	LK/DH*	LA/DQ	LL/DQ, LM/DQ	LL/DQ	LL/DE	LM/DQ
VP2/VP3	EQ/GP*	RQ/SP	AQ/SP	PQ/SP	PQ/SP	AD/SP
VP3/VP1	FH/ST*	PQ/GV	PQ/GV, PQ/GI, PQ/GS	PQ/GV	PQ/GV	PQ/GV
VP1/2A	MQ/SG	LE/SP	LE/NP	LQ/NP	LE/NP	LQ/DP
2A/2B	NPG/P†	NPG/P†	NPG/P†	NPG/P†	NPG/P†‡	NPG/P†
2B/2C	MQ/GP	QQ/SP	PQ/GP	AQ/SP	PQ/GP‡	QQ/SP
2C/3A	LQ/SP	AQ/GP, AQ/AP	AQ/SP	AQ/SP	AQ/SP‡	AQ/SP
3A/3B	SE/NA	EQ/GP	EQ/AA	EQ/AA	EQ/AA‡	EQ/AA
3B/3C	MQ/Q/P§	IQ/GP, VQ/GP	IQ/GG	LQ/GG	IQ/GG‡	VQ/GG
3C/3D	MQ/GL	PQ/GA	PQ/GA	PQ/GA	PQ/GA‡	PQ/GA

*Determined by amino-terminal sequencing.
†Ribosome-skipping sequence.
‡H. Lipton, personal communication.
§Alternative cleavage sites are proposed for 3B/3C.

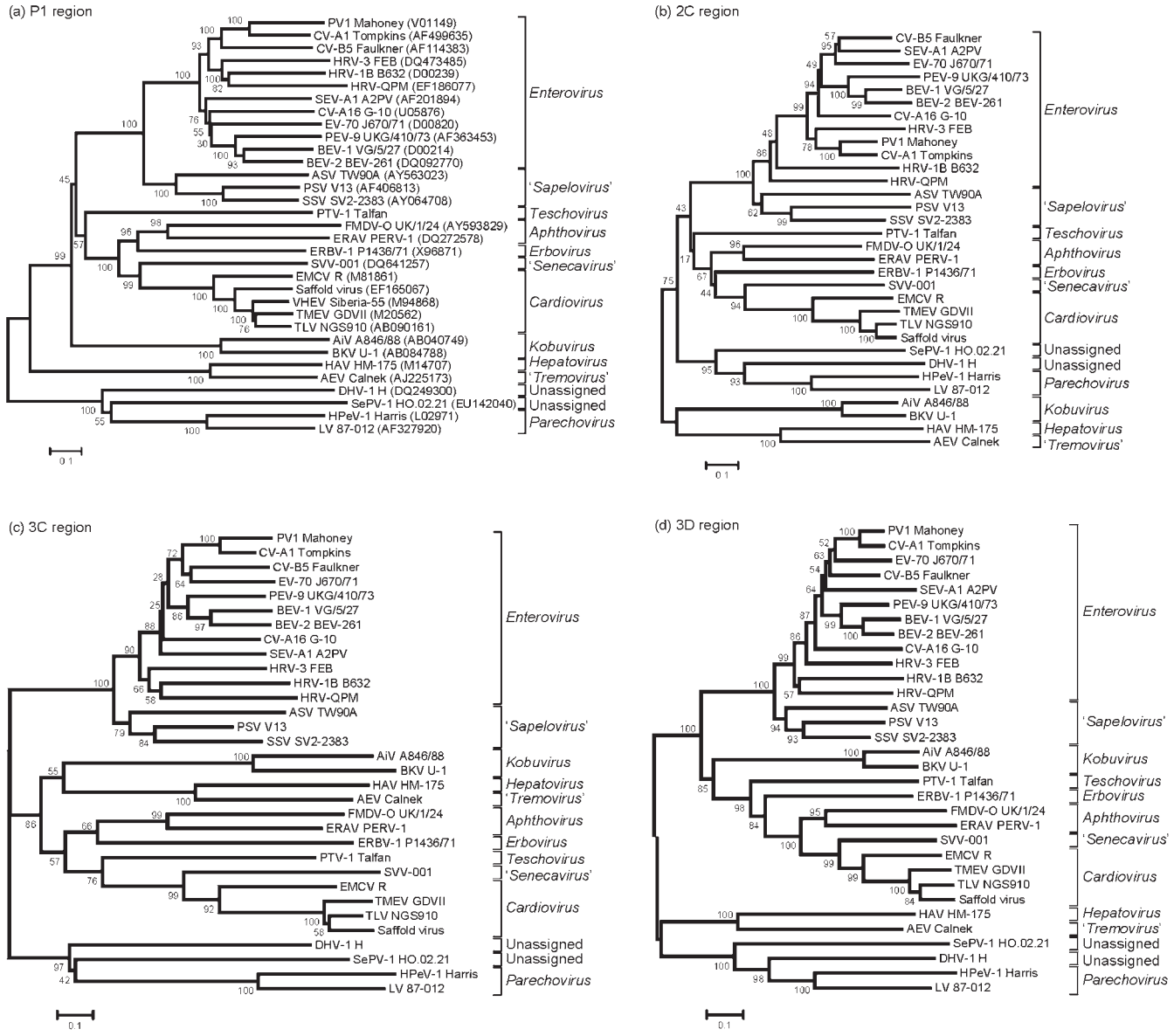

Fig. 4. Mid-point-rooted neighbour-joining trees. These trees show the relationship of SVV-001 to other picornaviruses for (a) the P1 capsid; (b) the 2C protein; (c) the 3C proteinase; and (d) the 3D polymerase. GenBank accession numbers are shown in (a). The two human rhinovirus species are to be reassigned to the genus *Enterovirus*. Proposed novel genus names are shown between quotation marks. Abbreviations: AEV, avian encephalomyelitis virus; AiV, Aichi virus; ASV, avian sapelovirus; BEV, bovine enterovirus; BKV, bovine kobuvirus; CV, coxsackievirus; DHV, duck hepatitis virus; EMCV, encephalomyocarditis virus; ERAV, equine rhinitis A virus; ERBV, equine rhinitis B virus; EV-70, enterovirus; FMDV, foot-and-mouth disease virus; HAV, hepatitis A virus; HPeV, human parechovirus; HRV, human rhinovirus; LV, Ljungan virus; PEV, porcine enterovirus; PSV, porcine sapelovirus (formerly PEV-8); PTV, porcine teschovirus; PV, poliovirus; SePV, seal picornavirus; SEV, simian enterovirus; SSV, simian sapelovirus; SVV, Seneca Valley virus; TLV, Theiler-like virus of rats; TMEV, Theiler's murine encephalomyelitis virus; VHEV, Vilyuisk human encephalomyelitis virus.

isolates that were serologically very similar to SVV-001 were isolated at the National Veterinary Services Laboratory (NVSL) in Ames, IA, USA (L. M. Hales, B. H. Jones, N. J. Knowles, J. Landgraf, J. House, K. L. Skele, K. D. Burroughs, P. S. Reddy & P. L. Hallenbeck, unpublished). These viruses were isolated from pig specimens submitted to the NVSL from several different states of the USA, suggesting that SVV-001 and its close relatives are relatively common and distributed widely, both temporally and geographically.

The complete genome sequence of SVV-001 was determined and was shown to have a typical picornavirus L-4-3-4 genome layout. The principal genome regions that are conserved well enough across all of the picornaviruses to allow a meaningful phylogeny to be constructed are the IRES, P1, 2C, 3C and 3D regions. Other genome regions are often very different between genera and sometimes even between virus species. Comparisons with other picornaviruses showed that the P1, 2C, 3C and 3D polypeptides of SVV-001 were related most closely to those of the cardioviruses, whilst other regions differed considerably from those of all other picornaviruses. In the non-structural polypeptides 2C, 3C and 3D, which are generally considered to be relatively conserved in picornaviruses, SVV-001 is also related most closely to the cardioviruses, although it is not related as closely to EMCV and TMEV as they are to each other. SVV-001 diverges greatly from the cardioviruses in the 2B and 3A polypeptides and has no detectable relationship with any known picornavirus in these regions. Recently, these types of difference have been used to propose that avian encephalomyelitis virus (AEV), currently assigned to the genus *Hepatovirus* (Marvil *et al.*, 1999), be classified in a novel genus, provisionally named '*Tremovirus*' (http://www.picornastudygroup.com/).

Several characteristics of SVV-001 differ from those of cardioviruses: (i) the SVV-001 IRES appears to be type IV, not type II like cardiovirus IRES sequences (Hellen & de Breyne, 2007); (ii) the cardioviruses have a long (150 aa) 2A protease, whereas that of SVV-001 is predicted to be much shorter (9 aa), if it is indeed cleaved from VP1; and (iii) the amino acid sequences of the leader, 2B and 3A polypeptides do not share sequence similarity with those of cardioviruses. In FMDV and human rhinoviruses, these proteins have been shown to be involved in host-cell tropism and virulence (Lomax & Yin, 1989; Beard & Mason, 2000; Knowles *et al.*, 2001; Pacheco *et al.*, 2003; Harris & Racaniello, 2005).

The complete genome sequence and phylogenetic analyses assume significance in the light of recent findings that the virus has very potent oncolytic properties. *In vitro* and *in vivo* studies of the virus revealed its tropism towards tumour cells with neuroendocrine properties (Reddy *et al.*, 2007). Currently, the virus is being evaluated in phase I clinical trials for treatment of neuroendocrine cancers. A few other members of the family *Picornaviridae* have been found to possess cell-killing activity against certain human cancers (Au *et al.*, 2005; Shafren *et al.*, 2005; Adachi *et al.*, 2006; Ochiai *et al.*, 2006; Toyoda *et al.*, 2007).

The data presented here demonstrate clearly that SVV-001 is a member of the family *Picornaviridae* and is related most closely to, but differs from, members of the genus *Cardiovirus*. Recognizing the unique properties of SVV-001, the *Picornaviridae* Study Group (PSG) recommended that the virus be classified as a novel species, '*Seneca Valley virus*', placed in a novel picornavirus genus, '*Senecavirus*' (http://www.picornastudygroup.com/). The taxonomic position of SVV-001 within the family *Picornaviridae* will be decided by the International Committee on Taxonomy of Viruses (ICTV) following recommendations by the PSG and supporting published material.

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