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Short Communication

Molecular characterization of M1146, an American isolate of Ljungan virus (LV) reveals the presence of a new LV genotype

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Ljungan virus (LV) is a suspected human pathogen recently isolated from bank voles in Sweden. This study describes the genetic characterization of a virus, M1146, which was isolated in 1962 from another vole species (*Microtus montanus*), trapped in Oregon, USA. Based on antigenic properties, M1146 was postulated previously as a putative member of the family *Picornaviridae*. The near complete genomic sequence verifies that M1146 is a member of the *Picornaviridae*, most closely related to LVs isolated in Sweden. The strain M1146 possesses typical LV genomic organization, including a cluster of two 2A homologues. There are significant differences throughout the capsid protein region, while the non-structural region of M1146 is closely related to the Swedish LV genomes. Genetic and phylogenetic analyses show that M1146 represents a new genotype within the distinct LV cluster. Isolation of LV from both Swedish and American voles trapped over a period of 30 years suggests a continuous worldwide presence.

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The family *Picornaviridae* comprises a wide range of human and animal pathogens. The positive-stranded picornaviral RNA genome contains one long open reading frame encoding a single polyprotein that is preceded by a 5′ untranslated region (UTR), followed by a 3′UTR and a poly(A) tail (Racaniello, 2001). Traditionally, picornaviruses have been identified, differentiated and classified on the basis of biophysical/antigenic properties and on pathogenicity in experimental animals. More recently, sequence comparisons of viral genomes have supplemented this information and aided in the establishment of several new genera (Doherty *et al.*, 1999; Hyypiä *et al.*, 1992; Kaku *et al.*, 2001; Wutz *et al.*, 1996; Yamashita *et al.*, 1998; Zell *et al.*, 2001). The family *Picornaviridae* is divided into nine genera, of which six are currently recognized (*Aphthovirus*, *Cardiovirus*, *Enterovirus*, *Hepatovirus*, *Parechovirus* and *Rhinovirus*) and three are tentatively proposed (*Erbovirus*, *Kobuvirus* and *Teschovirus*) (King *et al.*, 2000; Pringle, 1999).

The GenBank accession number of the sequence reported in this paper is AF538689.

Three serologically related strains of Ljungan virus (LV) isolated recently from bank voles (*Clethrionomys glareolus*) are proposed as aetiological agents of myocarditis (Niklasson *et al.*, 1998, 1999). Sequence analysis of the genomes of three Swedish LV isolates, 87-012 (prototype strain), 174F and 145SL, identified LVs as novel members of the *Picornaviridae* with several distinctive molecular features (Johansson *et al.*, 2002). In particular, the LV capsid protein VP1 contains a unique C-terminal extension and the 2A region encompasses a cluster of two diverse 2A homologues. Phylogenetic analysis revealed that the three LV isolates constitute a distinct monophyletic group, which, together with the genus *Parechovirus*, is separated from other members of the *Picornaviridae* (Johansson *et al.*, 2002; Lindberg & Johansson, 2002).

In the early 1960s during a routine sentinel arbovirus survey in New York, USA, two infectious viruses were isolated from voles (Whitney *et al.*, 1970). Mice inoculated intracerebrally with these strains exhibited either paralysis or convulsions and died within 14 days. Continual attempts to propagate

these isolates in cell culture failed. By employing relatively crude biophysical assays, these agents were postulated as putative members of the families *Parvo-* or *Picornaviridae*. A third infectious agent, M1146 (Johnson's *Microtus montanus* enterovirus USA M-1146), isolated in 1962 from voles trapped in Oregon, USA, was shown later to be serologically related to the two New York strains (Johnson, 1965; Main *et al.*, 1976). In the present study, the sequence of the M1146 viral genome was determined and analysed for relationships to other members of the *Picornaviridae*. Molecular characterization confirmed that M1146 is a bona fide member of the *Picornaviridae* and most closely related to LVs isolated from bank voles in Sweden (Johansson *et al.*, 2002). On the basis of sequence similarities and predicted genomic organization presented herein, we propose that M1146 should be recognized as a new genotype of LV.

Generation of sufficient quantities of virus for molecular characterization was undertaken initially by intracerebral inoculation of M1146-infected mouse brain suspension and later by two *in vitro* passages on rhabdomyosarcoma (RD) cells (ATCC), characterized by a mild cytopathic effect. Viral RNA extraction, RT-PCR and nucleotide sequencing strategy were performed as described previously (Johansson *et al.*, 2002). On most occasions, a nested PCR amplification approach was required to obtain sufficient DNA to determine the nucleotide sequence, probably reflecting a low efficiency of virus replication in cell culture. The final genomic sequence was derived from virus propagated in two separate passages in RD cells, extracted and amplified separately to represent the dominant genotype present during propagation in RD cells. Sequences were aligned using the CLUSTAL X program (Thompson *et al.*, 1994, 1997) and edited manually using DAMBE [Data Analysis in Molecular Biology and Evolution (Xia, 2000)]. Prior to phylogenetic analysis, datasets were investigated for the presence of phylogenetic signals corresponding to tree-like evolution using the likelihood mapping method (Strimmer & von Haeseler, 1997). Phylogenetic reconstruction was conducted by employing the maximum-likelihood method using quartet puzzling, as implemented in TREE-PUZZLE, version 5.0 (Strimmer & von Haeseler, 1996), based on 1000 puzzling steps. The resulting trees were visualized using the TREEVIEW program (Page, 1996).

Analysis of the M1146 genome revealed a typical LV-like genomic organization (Fig. 1A). The viral genome is predicted to encode a polyprotein of 2254 aa, followed by a 96 nt long 3′UTR and a poly(A) tail. Despite several attempts with various 5′RACE (rapid amplification of cDNA ends) protocols and amplifications with primers derived from the Swedish LV isolates, only 634 nt of the 3′-proximal region of the M1146 5′UTR was amplified successfully and sequenced. This may be due to a unique 5′UTR sequence of M1146, or to a stable secondary structure of the 5′-terminal region, as predicted previously for the Swedish LVs and human parechoviruses (HPEVs) (Ghazi *et al.*, 1998; Johansson *et al.*, 2002). An initiator codon in an optimal Kozak context (Kozak, 1986) is present at position 635. In the three LV genomes published previously, a second in-frame methionine is encoded 5 aa further downstream from the putative initiating methionine residue (Fig. 1C) (Johansson *et al.*, 2002). A corresponding alternate initiator codon is not present in the M1146 genome, supporting previous predictions concerning the location of the start codons in the Swedish LV genomes.

Comparison of the M1146 sequence with representative picornaviruses identified the Swedish LVs as the nearest taxa. The deduced M1146 polyprotein region exhibits 80–81 % amino acid identity to the Swedish LVs, which is lower than the pairwise identities between the Swedish isolates (89–99 %, values calculated using the GAP program, Genetics Computer Group) (Johansson *et al.*, 2002). Complement fixation assays failed to detect antigenic identity between M1146 and the Swedish LV strain 342SL (unpublished data), a LV isolate which exhibits $>99{\cdot}7$ % amino acid identity in the P1 region to LV strain 145SL (Johansson *et al.*, 2002). Alignment of the four deduced LV polyproteins revealed that the predicted cleavage sites are strongly conserved between the Swedish LVs and M1146 (Fig. 1C). No maturation cleavage is predicted to occur in M1146, as has been shown for LV 87-012 (E. S. Johansson, D. R. Shafren, G. Frisk, T. Hyypiä, K. Edman & A. M. Lindberg, unpublished results). The P1 region, encoding the structural capsid proteins, is the most divergent genomic region, with 73 % pairwise amino acid identity between M1146 and the Swedish LVs. The majority of the variable positions in the P1 region were located outside the predicted β-barrel structures. Two regions with increased variability are the BC-loop with the adjacent knob region of VP3 and the BC-loop of VP1. This is consistent with these regions of the structural proteins of picornaviruses identified frequently as major neutralizing antigenic sites (Mateu, 1995; Racaniello, 2001). In contrast to the capsid protein region, the non-structural protein region was much more conserved between M1146 and the Swedish LVs (84–86 % amino acid identity). Amino acid motifs conserved among picornaviral $2C^{ATPase}$, $3C^{pro}$ and $3D^{pol}$ proteins were identified in the M1146 proteins without any substitutions known to compromise respective functions (Gorbalenya & Koonin, 1993).

Phylogenetic relationships of the $3D^{pol}$ protein sequences with LVs and representative members of the *Picornaviridae* revealed a close relationship between M1146 and the Swedish LVs (Fig. 2A). All LVs (M1146, 87-012, 174F and 145SL) cluster together in a distinct monophyletic group that is more related to the genus *Parechovirus* than to other picornaviruses. Analysis of the relationship between LVs and HPEVs using the capsid protein precursor (P1) showed further that, although LVs and HPEVs are related, these clades are clearly distinct from each other (Fig. 2B). Furthermore, this analysis indicated that the four LVs characterized to date cluster geographically and by vole species, with M1146 being more distant to the closely related Swedish LV isolates.

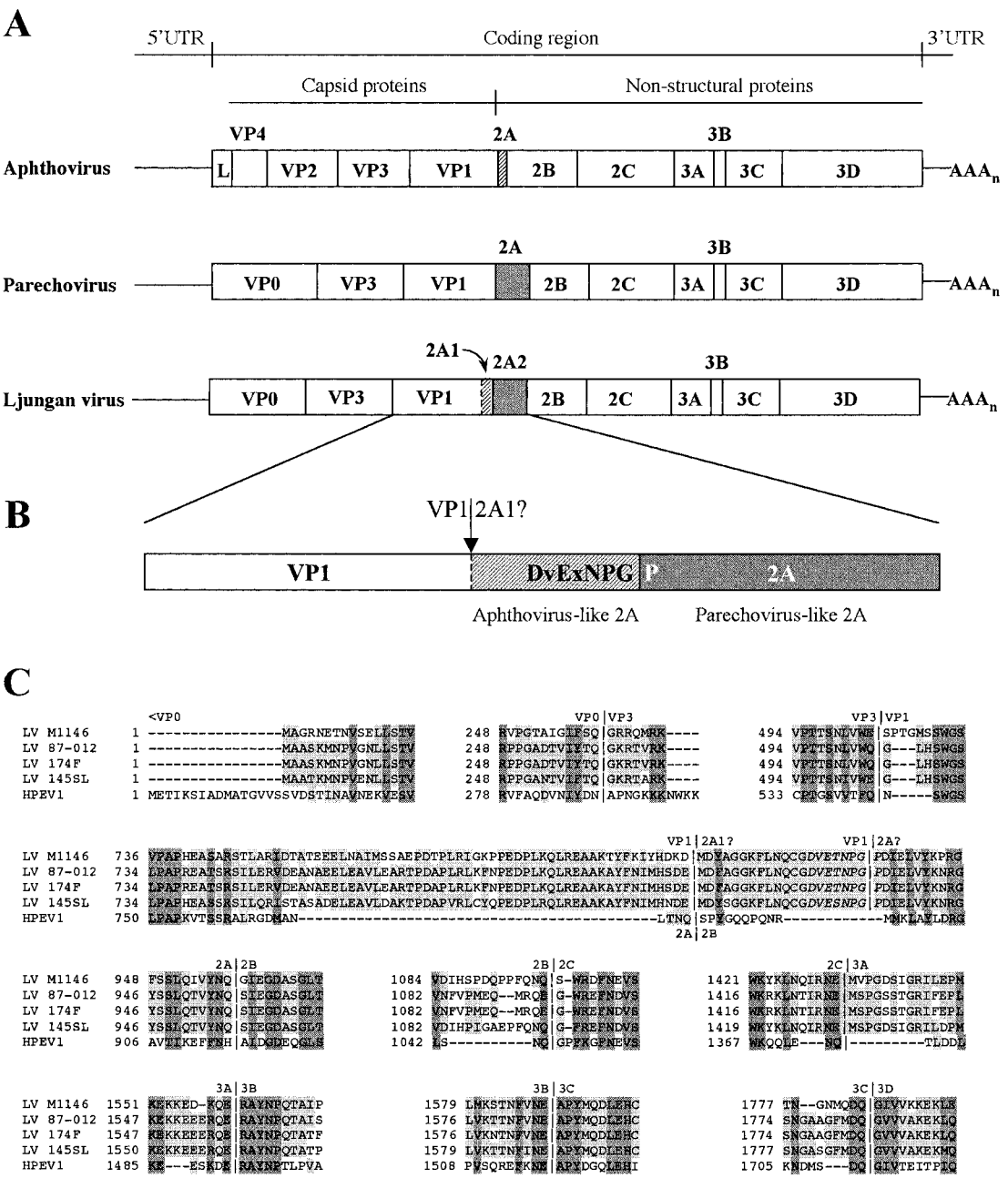

Fig. 1. Overview of the M1146 viral genome. (A) Schematic representation of the genomic organization of aphthovirus, parechovirus and LV M1146. The polyprotein-encoding region (boxed) is flanked by the 5′UTR and the 3′UTR, which contains a poly(A) tail at the end. The protein products within the polyprotein region are indicated. (B) Enlargement of the VP1–2A region of the M1146 genome. The solid vertical line indicates the herein and previously predicted N terminus of the parechovirus-like 2A protein and the dotted line indicates the potential C terminus of VP1, previously proposed based on genetic analysis of the Swedish LV genomes (Johansson *et al.*, 2002). The DvExNPG|P motif (indicated) may either constitute the C terminus of a separate aphthovirus-like 2A protein or, more likely, be included at the C terminus of a VP1–2A1 'fusion' protein. (C) Comparison of the terminal region of VP0 and the predicted polyprotein cleavage sites of four LVs (M1146, 87-012, 174F and 145SL) with HPEV1. The conserved DvExNPG|P motif is highlighted in italics. The 2A|2B and 3B|3C cleavage sites for HPEV1 are as previously proposed based on sequence comparisons of LV and HPEV (Johansson *et al.*, 2002). Amino acid numbering is from the first residue of the polyprotein. Dark and light grey backgrounds highlight alignment columns with 100 and 60 % conserved residues (Nicholas *et al.*, 1997).

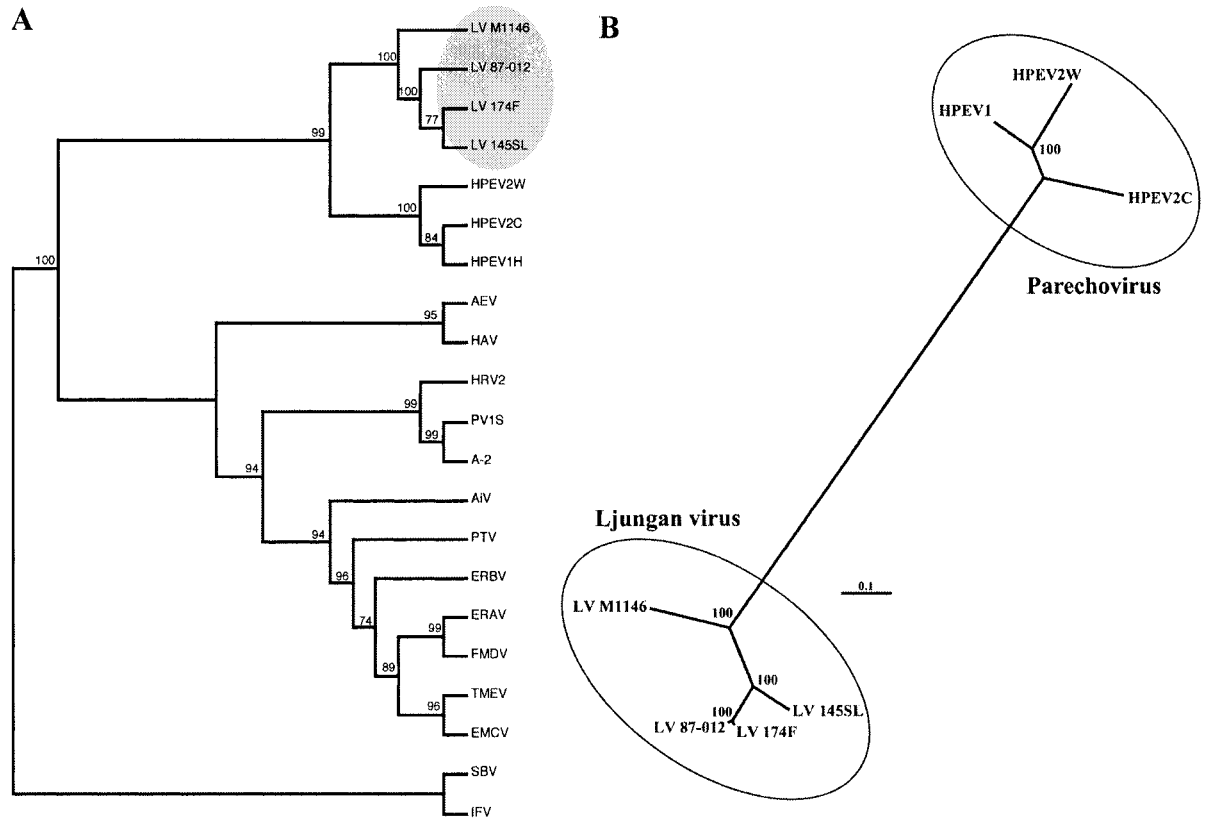

Fig. 2. Phylogenetic relationship of M1146 with representative picornaviruses were inferred by employing the maximum-likelihood method using quartet puzzling as implemented in TREE-PUZZLE, version 5.0, software (Strimmer & von Haeseler, 1996) with the JTT substitution model for amino acid sequences (Jones *et al.*, 1992). Numbers at nodes represent percentage reliability values for the internal branch; values above 70 % are indicated. (A) Phylogenetic relationship based on $3D^{pol}$ protein sequences. The $3D^{pol}$ tree was rooted using two 3D-like sequences encoded by the picorna-like viruses, infectious flacherie and sacbrood viruses. (B) Unrooted quartet puzzling tree based on the capsid precursor P1 protein sequences. The bar represents substitutions per site. GenBank accession numbers of viruses used are: Ljungan virus, strains M1146 (AF538689), 87-012 (AF327920), 174F (AF327921) and 145SL (AF327922); *Aphthovirus*, foot-and-mouth disease virus (FMDV, MJ10975) and equine rhinitis A virus (ERAV, L43052); *Cardiovirus*, encephalomyocarditis virus (EMCV, M22457) and Theiler's murine encephalomyelitis virus (TMEV, M20301); *Enterovirus*, poliovirus type 1 strain Sabin (PV1S, V01150) and A-2 plaque virus (A-2, AF201894); *Erbovirus*, equine rhinitis B virus (ERBV, X96871); *Hepatovirus*, hepatitis A virus (HAV, M59810) and avian encephalomyelitis virus (AEV, AJ225173); *Kobuvirus*, Aichi virus (AiV, AB010145); *Parechovirus*, human parechovirus type 1 strain Harris (HPEV1H, S45208), human parechovirus type 2 strain Williamson (HPEV2W, AJ005695) and human parechovirus type 2 strain CT86-6760 (HPEV2C, AF055846); *Rhinovirus*, human rhinovirus type 2 (HRV2, X02316); *Teschovirus*, porcine teschovirus type 1 (PTV, AJ011380); picorna-like insect viruses, infectious flacherie virus (IFV, AB000906) and sacbrood virus (SBV, AF092924).

The UTRs of picornaviruses are characterized by complex secondary structures, which serve for initiation of translation and RNA replication (Racaniello, 2001). Initiation of translation is dependent on the tertiary structure in the 5′UTR referred to as the internal ribosome entry site (IRES). The LV 5′UTR has been predicted previously to form a type II IRES (Johansson *et al.*, 2002), also found in aphtho-, cardio-, erbo- and parechoviruses (Ghazi *et al.*, 1998; Hinton & Crabb, 2001; Racaniello, 2001). The nucleotide sequence of the M1146 5′UTR (634 nt) shares only 68–72 % identity to the Swedish LVs. The majority of the observed variances are compensatory nucleotide changes, supporting the predicted folding. The folding pattern thus appears to be very similar among all LV 5′UTRs.

The 3′UTRs of the Swedish LVs have been predicted previously to fold into two hairpins, structurally similar to those identified in the 3′UTR of poliovirus (Auvinen & Hyypiä, 1990; Johansson *et al.*, 2002; Pöyry *et al.*, 1996). M1146 possesses a shorter 3′UTR (96 nt) than the Swedish LVs (107–111 nt). The predicted RNA secondary structure of M1146 3′UTR [MFOLD 3.1 (Zuker *et al.*, 1999) and STAR (Abrahams *et al.*, 1990) programs] was shown to vary compared to the stem–loops in the Swedish LVs (Fig. 3B, C).

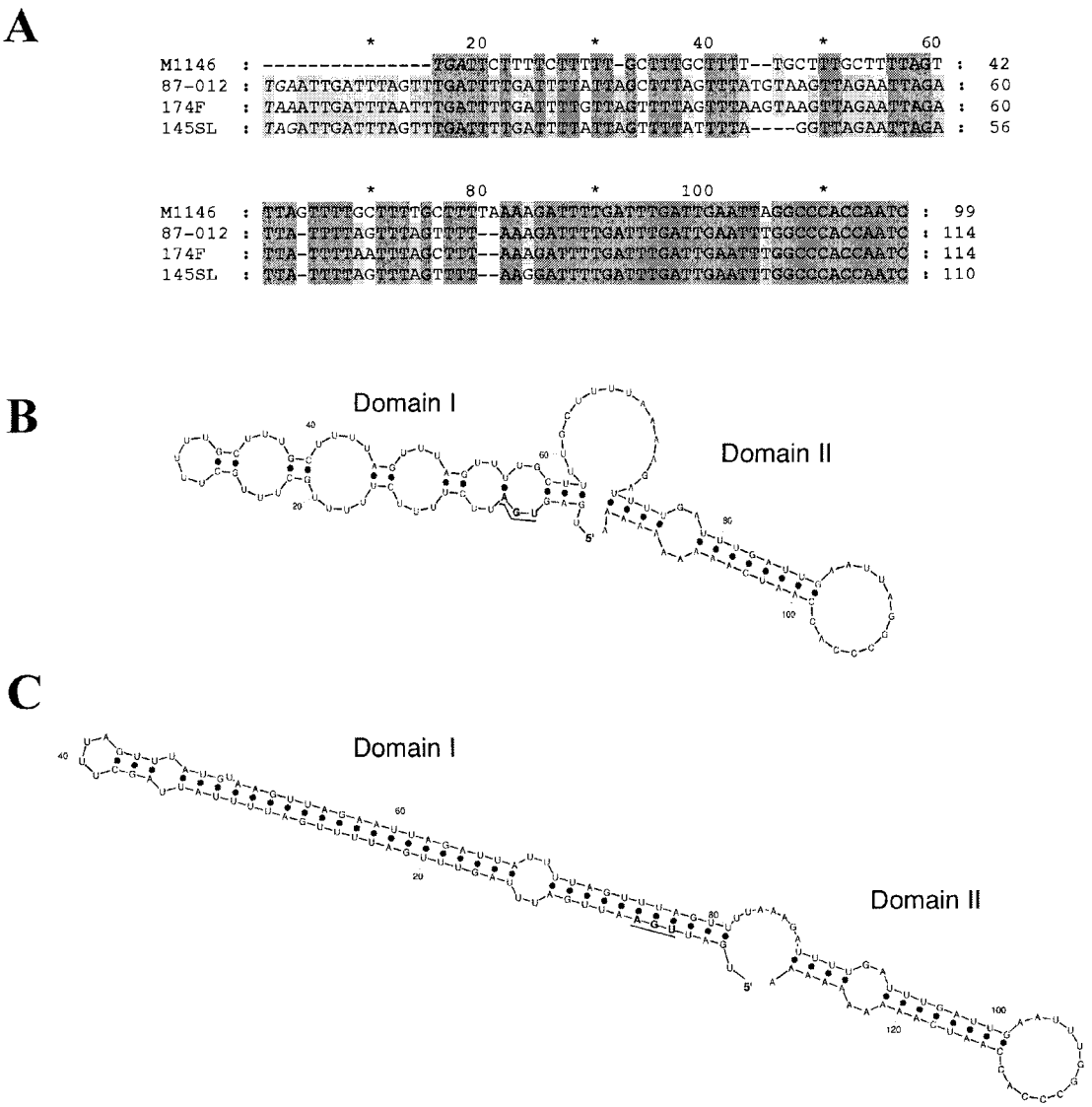

Fig. 3. Predicted secondary structures for the LV 3′UTR. (A) Alignment of complete 3′UTRs of M1146 and three Swedish LVs (prototype strain 87-012, 174F and 145SL). Dark and light grey backgrounds highlight alignment columns with 100 and 60 % conserved residues (Nicholas *et al.*, 1997). Predicted secondary structure of LV M1146 (B) and 87-012 3′UTR (C). Energies for optimal folds by MFOLD at 37°C, 1 M NaCl were $\Delta G = -17{\cdot}8$ kcal mol^{-1} and $\Delta G = -30{\cdot}4$ kcal mol^{-1} for M1146 and 87-012, respectively. Stop codons are underlined. Analyses of the 3′UTR foldings included part of the 3D-encoding region and the poly(A) tail to simulate authentic viral RNA molecules. *, Units of 10 aa.

Interestingly, both the primary sequence of the 3′ one-third of the 3′UTR and the predicted stem–loop structure thereof (domain II) are highly conserved among all four LVs (Fig. 3). In contrast, the primary sequence of the 5′-proximal two-thirds of M1146 3′UTR is only distantly related to the corresponding region of the 3′UTR of the Swedish LVs, with no specific affinity found with any picornavirus or sequence of other origin (data not shown). This region of M1146 3′UTR is predicted to fold into a less stable and more open stem–loop structure that differs considerably to the more stable domain I predicted for the closely related Swedish LVs. The functional significance of these different predicted structures on the LV replication should be revealed in future studies.

Recently, we have predicted several unique molecular features in VP1–2A region of the Swedish LV isolates (Johansson *et al.*, 2002). The LV-specific C-terminal 43-residue insertion of VP1 was identified also in the M1146 polyprotein (Fig. 1C). The insertion in M1146 exhibits a more diverged amino acid sequence compared to the same region in the Swedish LVs. Furthermore, the predicted cluster of two diverse 2A protein motifs was observed in M1146, a feature unique for the LV clade. The C-terminal

2A protein is homologous to the H-NC family of 2As present in parecho-, kobu- and avian encephalomyelitis virus (Fig. 1B) (Hyypiä *et al.*, 1992; Marvil *et al.*, 1999; Yamashita *et al.*, 1998). This H-NC type of 2A protein contains conserved motifs that are characteristic of a family of cellular proteins involved in the control of cell proliferation (Hughes & Stanway, 2000). The N-terminal 2A protein motif is a homologue of the 2A protein present in aphtho-, cardio-, erbo- and teschoviruses and contains the conserved DvExNPG|P (uppercase letters denote absolutely conserved residues) motif at the C terminus (Beard & Mason, 2000; Cohen *et al.*, 1988; Doherty *et al.*, 1999; Pevear *et al.*, 1988; Wutz *et al.*, 1996). The 2A protein in these viruses (18–21 aa in aphtho-, erbo- and teschoviruses) is proposed to mediate the primary polyprotein processing at its own C terminus in a co-translational manner (Donnelly *et al.*, 1997, 2001a, b; Hahn & Palmenberg, 2001; Palmenberg *et al.*, 1992). The C terminus of VP1 in the Swedish LVs has proved to be the most difficult border to predict due to lack of conformity of this region with other picornaviruses. Molecular analysis of the Swedish LVs suggested a cleavage site ($H^S/_N$DE|M), consistent with the unique LV $3C^{pro}$ specificity located 20 aa upstream of the DvExNPG|P motif (Fig. 1C) (Johansson *et al.*, 2002). Processing of this site would generate a cluster of two distinct 2A proteins (2A1 and 2A2). However, the HDKD|M sequence encoded by M1146 in this position does not conform to $3C^{pro}$ sites located elsewhere in LV genomes (Fig. 1C), suggesting that the M1146 polyprotein, and presumably also the Swedish LV polyproteins, are not processed at this position.

In agreement with this finding, recent analysis of the protein composition of LV 87-012 by SDS-PAGE revealed a slightly larger molecular mass of VP1 than if the VP1|2A1 site was processed as predicted previously by sequence analysis (E. S. Johansson, D. R. Shafren, G. Frisk, T. Hyypiä, K. Edman & A. M. Lindberg, unpublished results). However, the observed molecular mass corresponds to the size of a potential VP1–2A1 'fusion' protein. Hence, the DvExNPG|P motif may be included at the C terminus of VP1. Aphthovirus 2A-like sequences encoding this motif have not only been identified in several members of the *Picornaviridae* but also at various locations in picorna-like insect viruses (Govan *et al.*, 2000; Isawa *et al.*, 1998; Johnson & Christian, 1998; Pringle *et al.*, 1999; Wilson *et al.*, 2000; Wu *et al.*, 2002). In some of these viruses, the aphthovirus 2A-like sequence appears to mediate polyprotein processing both between individual capsid proteins and between the structural precursor and the non-structural protein region (Donnelly *et al.*, 2001a; Wu *et al.*, 2002). Taken together, these data imply that the VP1|2A1 border proposed previously may not be processed in any of the LV genomes. Consequently, the aphthovirus 2A-like homologue in the LV polyproteins may be the C terminus of capsid protein VP1 and mediate polyprotein processing in a manner similar to that suggested for the picorna-like insect viruses.

In conclusion, we have employed molecular characterization to confirm that the virus, M1146, isolated from voles in the early 1960s in the USA indeed is a member of the *Picornaviridae*. Despite being isolated on two continents from different rodent species spanning more than 30 years, molecular comparison revealed that M1146 is most closely related to LVs isolated from bank voles in Sweden (Johansson *et al.*, 2002). Identification of LVs in both Swedish and North American voles suggests a continued presence of LVs over a wide geographical range throughout numerous vole populations. The M1146 non-structural proteins exhibited a high degree of similarity to the Swedish LV proteins, while the deduced protein sequence of the P1 region was more divergent. Therefore, M1146 represents a new genotype of the LV clade, prototyped by LV 87-012. Data presented herein together with distinct molecular features of LV reported previously (Johansson *et al.*, 2002) strongly support the establishment of a new genus of picornavirus.

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