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MINIREVIEW

Parechoviruses

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Until recently, the family *Picornaviridae* consisted of five established genera (36). Enteroviruses, rhinoviruses, and hepatoviruses include human pathogens; foot-and-mouth disease viruses of ungulates are the most important aphthoviruses; and cardioviruses are found mainly in rodents (48). Following the early studies of Loeffler and Frosch on foot-and-mouth disease viruses (32), work on enteroviruses started in the early years of this century, when Landsteiner and Popper demonstrated, by injection of a filtrate into monkeys, that poliomyelitis is caused by viruses (31). However, it was 40 years before a new, related virus group was discovered (11). These were the coxsackieviruses, which characteristically could induce disease in newborn mice. Following the introduction of tissue culture systems (14) for virus propagation, several viruses which shared physicochemical properties (solvent-resistant, acid-stable particles) with polio- and coxsackieviruses, but grew exclusively in cell culture, were isolated. These were called ECHO (enteric, cytopathogenic, human, orphan) viruses (9). The three subgroups, plus the new enterovirus types 68 to 71, include more than 60 serotypes and, together with several simian, bovine, and porcine strains, comprise the *Enterovirus* genus (25). However, a wide range of evidence, detailed below, shows that two echoviruses have distinct biological and molecular properties. These, echoviruses 22 and 23, have recently been assigned to a sixth picornavirus genus, *Parechovirus*, and have been renamed human parechovirus 1 and 2 (HPEV1 and HPEV2), respectively (29). Here we review how parechoviruses relate to other members of the *Picornaviridae* family.

Previously studied picornaviruses have a single-stranded RNA genome of positive polarity, 7,100 to 8,500 nucleotides long, which is packaged into an icosahedral capsid made up of 60 copies of each of the capsid proteins VP1 through VP4 (48, 57). Genomic RNA is modified by the covalent attachment of a small protein (VPg; 20 to 25 amino acids) to the 5′ terminus. The genome can be considered to have four distinct domains. A 5′ untranslated region (5′UTR) precedes a single open reading frame, downstream of which there is a 3′UTR and a poly(A) tract. Ten, 11, or 12 proteins are encoded by different picornaviruses as shown in Fig. 1. The single polyprotein encoded by the genome is processed by a cascade of proteolytic events, instigated by virus-encoded enzymes, to give precursor molecules and then the final discrete proteins (40, 50). In several cases, the precursors themselves have important functional roles in virus replication. For instance, while the majority of processing events are brought about by $3C^{pro}$, cleavages in the capsid region appear to require $3CD^{pro}$, the precursor of 3C and 3D. Functions have been ascribed to most of the nonstructural proteins. In addition to its involvement with 3C in processing, 3D possesses the RNA-dependent RNA polymerase activity needed for replication. Some of the other proteins are also involved in the RNA replication complex, including 3A, 3B (VPg), and 2C (48).

In picornaviruses other than parechoviruses, four capsid proteins are observed. These are named 1A, 1B, 1C, and 1D in the systematic nomenclature based on genomic location, but they are commonly designated VP4, VP2, VP3, and VP1, respectively. The external polypeptides, VP1 to VP3, share the same core structure, an eight-stranded antiparallel β-barrel, and are of similar size, while VP4 is internal and is much smaller (47). Assembly involves VP1, VP3, and the precursor VP0. Cleavage of VP0 into VP4 and VP2, termed the maturation cleavage and brought about by an unknown mechanism, is the final assembly step and is associated with stabilization of the particle and acquisition of infectivity.

ISOLATION AND PRIMARY CHARACTERIZATION OF HPEV1 AND HPEV2

In 1956, during studies of summer diarrhea, Wigand and Sabin (62) isolated previously unrecognized viruses from rectal swabs of infants. Two of these viruses, originally classified as echovirus 22 (Harris strain) and echovirus 23 (Williamson strain), have recently been designated as prototypes of HPEV1 and HPEV2, respectively. Even during their original characterization, these viruses were found to exhibit growth properties distinct from those of other enteroviruses. These included difficulty in passage and adaptation to cultures of monkey kidney cells and the restriction of cytopathogenic effect to peripheral parts of the cell monolayer (62). Distinctive cytopathogenicity compared to other viruses in the enterovirus group (disappearance of the nucleolus and nuclear chromatin) was also reported on the basis of light and electron microscopy studies of cells infected with HPEV1 and HPEV2 (27, 56, 61). Evidence of exceptional secondary structure in the HPEV1 genome compared to poliovirus RNA was reported later (53, 54). Another difference from typical enteroviruses was the apparent lack of host cell protein synthesis shutoff (7, 58). One mechanism involved in such shutoff in enterovirus-infected cells is cleavage of p220, which has been shown not to occur in HPEV1-infected cells (8). Hybridization data showed that these two viruses are not recognized by cDNA probes originating from members of the enterovirus subgroups (1, 2, 22). These findings led to more detailed molecular analysis of HPEV1, and subsequently HPEV2, including determination of the genomic sequences (23, 58, 18, 39). These studies reveal a number of unusual features of the parechoviruses.

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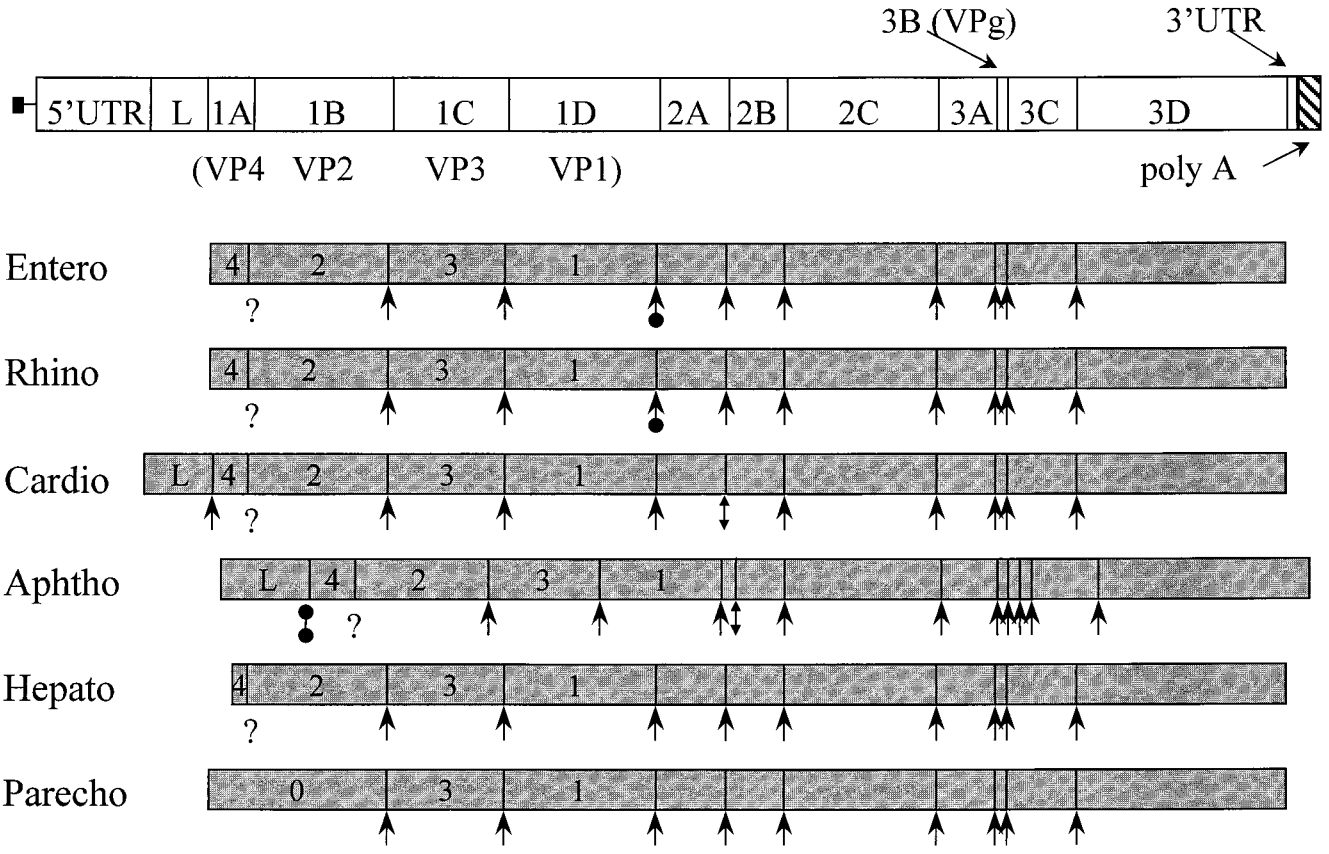

FIG. 1. The genome of a typical picornavirus, together with schematic maps of the polyprotein in each of the six picornavirus genera. VPg, the peptide covalently attached to the 5′ terminus of the RNA genome, is shown as a small solid square. The virus-encoded activities responsible for processing each protein boundary are indicated: vertical arrows, $3C^{pro}$ cleavages (also including those for which $3CD^{pro}$ are required); arrow with circle, $2A^{pro}$, the trypsin family member found in entero- and rhinoviruses; double-headed arrow, 2A-associated cleavages in cardioviruses and aphthoviruses; barbell, L^{pro} of aphthoviruses. The site of VP0 (maturation) cleavage, which occurs by an unknown, possibly autocatalytic mechanism, is indicated (?). For clarity, the positions of VP4 (1A), VP2 (1B), VP3 (1C), VP1 (1D), and VP0 (1AB) are indicated on the polyprotein maps as 4, 2, 3, 1, and 0, respectively. The aphthovirus foot-and-mouth disease virus encodes three tandem copies of VPg.

VIRAL PROTEINS

When purified HPEV1 was analyzed by polyacrylamide gel electrophoresis, a protein pattern different from that seen in typical enteroviruses was observed (58). To identify the capsid proteins, the three major bands with molecular sizes of 38, 30.5, and 30 kDa were analyzed by using N-terminal sequencing. By this means, VP1 (30.5 kDa) and VP3 (30 kDa) could readily be recognized in the predicted polyprotein by analogy with other picornaviruses. The 38-kDa polypeptide, however, gave no result in sequence analysis, suggesting that the N terminus is blocked. The purified protein was therefore subjected to trypsin digestion, followed by purification and sequencing of the separated peptides. Surprisingly, the sequences obtained represented both VP4 and VP2 regions in the predicted protein, and one of the peptides spanned the putative VP4/VP2 cleavage site. These observations strongly suggest that in HPEV1 most, if not all, of the VP0 molecules do not undergo the processing to VP2 and VP4 seen in other picornaviruses during the final maturation cleavage (48). This difference may have interesting consequences for the assembly of the virus particle.

The availability of the genomic sequence made it possible to compare the predicted viral proteins of HPEV1 with those of other representatives of the picornavirus family (23, 58). Similarities in the primary structures between HPEV1 and picornaviruses whose three-dimensional structure is known suggested that the common overall architecture of the major capsid proteins VP1 to VP3 is also found in HPEV1. Most of the β-strands could be relatively easily predicted in the primary structure, thus enabling further comparison of terminal regions and loops that link the β-strands (58). HPEV1 and both strains of HPEV2 studied (the reference strain, Williamson, and a 1986 isolate from Connecticut) have very similar sequences and presumably structures (18, 39). In VP1, the length of the termini corresponds to that in other picornaviruses, whereas the loops between β-strands D-E, E-F, and H-I are short, resembling those in the aphthovirus foot-and-mouth disease virus, and the G-H loop is shorter than in any other picornavirus analyzed. In the part of VP0 corresponding to VP2 in other picornaviruses, which is generally well conserved in terms of the length of the structural elements, the short E-F loop of HPEV1 and 2 also resembles that in foot-and-mouth disease virus. The brevity of these loops will probably give HPEV1 and 2 a flat appearance when compared to most other representatives of the picornavirus family. The most striking difference in the HPEV1 and 2 VP3, compared to that of any other picornavirus, is the approximately 30-amino-acid long N-terminal extension with a preponderance of positively charged amino acids.

Many of the HPEV1 and 2 nonstructural proteins could also be identified by using sequence alignments (18, 23). Both the RNA-dependent RNA-polymerase ($3D^{pol}$) and $3C^{pro}$, the trypsin family protease responsible for most processing of the picornavirus polyprotein, are easily recognized, and the critical

motifs thought to be involved in catalytic activity (YGDD and GXCGG, respectively) are conserved in these HPEV1 and 2 proteins. Polypeptide 2C, with proposed helicase activity in other picornaviruses, is also relatively well conserved between HPEV1 and 2 and other representatives of the family. On the other hand, 2A, 2B, and 3A exhibit only limited identity with those of other picornaviruses, thus making their precise identification in the polyprotein by sequence alignment more difficult.

PROTEIN PROCESSING

Proteolytic processing plays a dominant role in the functional expression of the picornavirus genome (40, 50). In addition, in some picornaviruses, proteases have another role, the inactivation of cellular proteins needed for translation of cellular mRNA or for transcription, thus bringing about host cell protein synthesis shutoff (40). An apparently important cleavage, since it occurs very early in the picornaviruses which have been well studied and involves a distinct activity in at least some cases, is that which separates the structural and nonstructural precursors. In entero- and rhinoviruses, this is brought about by $2A^{pro}$, another trypsin superfamily protease, which cleaves at its N terminus, liberating the capsid precursor P1 (Fig. 1). The 2A protein of aphthoviruses is extremely short and is required for a proteolytic event, brought about by a largely unknown mechanism, occuring at its C terminus (40, 50). Although significantly longer, the cardiovirus 2A has a homologous region at its C terminus which functions in an analogous manner. The hepatovirus 2A is distinct from those seen in these four genera and appears to lack any proteolytic activity (51). The L protein, found in aphthoviruses and cardioviruses, is a protease in the former genus, where it cleaves itself from the polyprotein, but not in the latter, where its removal is mediated by 3C. Proteolytic activity of L can also be presumed in the presently unclassified equine rhinovirus 2 (63).

These characteristics make the L and 2A proteins among the most diverse among picornaviruses, and so it is important to understand the nature of the corresponding proteins in parechoviruses. Originally, it was believed that HPEV1 and 2 have a short (12 amino acids) L peptide (23), a situation reminiscent of that proposed for hepatoviruses. However, in both cases the L peptide was proposed on the basis of the perceived requirement for an N-terminal myristoylation consensus sequence, since this modification occurs in other picornaviruses (6). The demonstration that the putative consensus in hepatoviruses is nonfunctional, and the finding that the HPEV1 VP0 contains the putative L region, imply that neither of these viruses has an L protein (60, 58). Thus, it appears that L is a feature only of cardioviruses and aphthoviruses.

The 2A protein of parechoviruses shows none of the characteristics of either of the picornavirus types described above, which are associated with P1-P2/3 cleavage. It is therefore unlikely to possess a proteolytic activity. This has been confirmed directly by a published report (52) and by our own unpublished observations. In vitro translation of subgenomic constructs containing the 2A region shows no evidence of autocatalytic processing. However, when exogenous $3C^{pro}$ is added, processing occurs. This, together with the possession of consensus sequences for $3C^{pro}$ processing at each end of 2A, suggests that 2A does not have a proteolytic role and that processing of this region, as is the case with hepatoviruses, is brought about entirely by $3C^{pro}$. It is interesting that the 2A protein is so variable between different picornaviruses and that some have evolved or acquired specific proteolytic activities to process this region. It appears that this region of the genome is highly plastic compared with most of the rest, which may be related to the need in other parts of the polyprotein to maintain protein order and identity, because of a requirement for functionally active precursors. The fact that the HPEV1 2A protein has no homology with any other known protein makes it difficult to ascribe a function. The 2A protein of enteroviruses appears to have several roles in replication, since there is evidence for an interaction with RNA during translation and for an involvement in RNA replication (33). It remains to be seen whether the parechovirus 2A protein possesses some equivalent functions, despite a lack of proteolytic activity.

5′UTR

The picornavirus 5′UTR has been extremely well studied, due to its critical involvement in both translation and in RNA replication, together with the mapping to this area in a number of picornaviruses of mutations which alter tropism or pathogenicity (48, 57). Its well-understood structure is also useful in the classification of picornaviruses (25, 42). The 5′UTR has a complex folding pattern, and a number of secondary and tertiary structure elements can be observed. Basically, two overall schemes are observed in the picornavirus genera: first, the closely similar structures seen in enterovirus and rhinovirus 5′UTRs; second, those seen in aphthoviruses and cardioviruses (59). The hepatovirus 5′UTR is a rather distant version of the latter, which could also be considered to be a third type (3). These overall structures correlate with differences among picornaviruses in the precise way in which ribosomes interact with the 5′UTR during translation initiation (35).

The existence of some primary sequence identity with the aphthovirus and cardiovirus 5′UTRs, together with a comparison of the HPEV1 and HPEV2 sequences which exhibit useful covariance, has enabled a secondary structure to be predicted for the HPEV1 and 2 5′UTR (18), shown in schematic form in Fig. 2. This follows closely the aphthovirus and cardiovirus scheme and is strikingly similar in the 3′ half of the 5′UTR, where it exhibits the major structural domains, including the prominent hammerhead and downstream secondary structures defined for cardio- and aphthoviruses. These are critical features of the internal ribosome entry site (IRES) that are involved in translation initiation, and it is likely that this process occurs in parechoviruses in a manner similar to that in cardio- and aphthoviruses (35). Studies using bicistronic constructs to direct in vitro translation, together with mutation of the virus genome, have defined the features of the 5′UTR which play a role in HPEV1 IRES function (55). These include the structural domains in the 3′ part of the 5′UTR which are highly similar to those of cardio- and aphthoviruses, the polypyrimidine tract and the AUG which initiates the open reading frame (Fig. 2).

GENETIC RELATIONSHIPS WITH OTHER PICORNAVIRUSES

In any region of the genome, with the exception of the 5′UTR, parechoviruses clearly constitute a separate molecular entity among picornaviruses. Figure 3 shows a dendrogram, based on the VP3 protein, illustrating the molecular relationships among the picornaviruses. The analysis shows that enteroviruses and rhinoviruses are closely related and that there is also some clustering of aphthoviruses and cardioviruses. This correlates well with overall similarities in genome organization between related genera, for example the nature of 2A and the presence or absence of an L protein (Fig. 1). In contrast, among currently recognized picornaviruses, both hepatoviruses and parechoviruses have no particularly close relatives and exemplify comparatively distinctive genetic lineages.

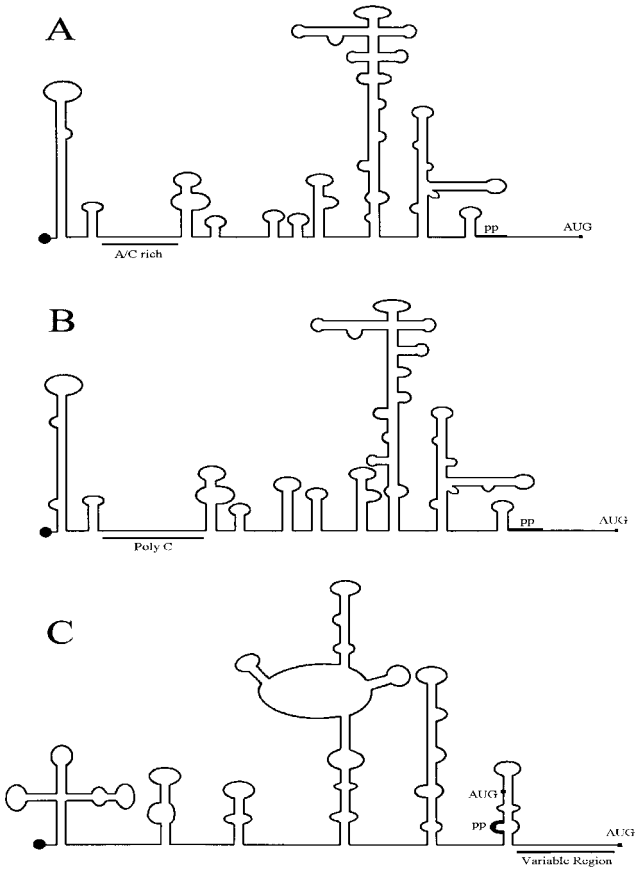

FIG. 2. (A) Schematic representation of the predicted 5′UTR secondary structure of the parechovirus HPEV1. The structure is strikingly similar to that predicted for encephalomyocarditis virus (B), a representative of the cardio- and aphthovirus 5′UTR group; it is dissimilar from that of poliovirus (C), a representative of the entero- and rhinovirus 5′UTR group. All picornaviruses have a polypyrimidine tract (shown as a heavy line and labelled pp) about 20 nucleotides upstream of an AUG (labelled with a square). In cardio- and aphthoviruses this AUG initiates the open reading frame, while in entero- and rhinoviruses a second AUG located downstream has this function. The variable region is seen in all enteroviruses, but is largely deleted in rhinoviruses. VPg, covalently attached to the 5′ terminus, is shown as a solid circle.

Again, this correlates with marked differences in genome and particle structure: for instance, in the case of hepatoviruses, an extremely short VP4 and the lack of a proteolytic activity associated with the 2A protein.

CELL SURFACE INTERACTIONS OF HPEV1

Sequence analysis of HPEV1 had already given an indication of possible mechanisms playing a role in host cell recognition, since the C terminus of VP1 contains an arginine-glycine-aspartic acid (RGD) motif (23, 58). This sequence is known to participate frequently in cell-cell and cell-matrix interactions (49) and to be utilized by several viral pathogens in their attachment and entry. Among picornaviruses, foot-and-mouth disease virus and coxsackievirus A9 (CAV9; an enterovirus) contain functional RGD motifs which react with cell surface integrins during early virus-cell interactions (4, 5, 17, 24, 45). Other picornaviruses recognize a variety of cell surface molecules, including members of the immunoglobulin superfamily, e.g., ICAM-1 recognized by rhinoviruses (15). In addition to the primary receptors, accessory molecules (coreceptors) are probably necessary for attachment, entry, or uncoating of picornaviruses.

The RGD motif in HPEV1 VP1 was shown to be functional by blocking experiments with RGD-containing synthetic peptides (58). Moreover, it was shown that HPEV1 competes for cell surface binding with CAV9, known to recognize the vitronectin receptor (αvβ3 integrin) on the cell surface (46). Receptor interactions of CAV9 are interesting in that the RGD-containing motif is not an absolute requirement for virus viability and can be deleted either by trypsin treatment (45) or mutation (20) without complete loss of infectivity. Growth properties of the mutant CAV9 are impaired in monkey kidney cells, but the attachment and entry steps in a rhabdomyosarcoma cell line (RD) appear to be independent of the interaction of the RGD motif with αvβ3 integrin (20). In contrast to the case with CAV9, removal of the HPEV1 RGD motif is lethal, suggesting that an obligatory RGD-integrin interaction is part of the entry process (2a).

When cell surface interactions of HPEV1 were studied by using phage display peptide libraries, it was shown that HPEV1 binds peptides containing an amino acid motif found, for example, in the integrin β1 subunit and in matrix metalloproteinase 9 (MMP-9). HPEV1 infection could be blocked by anti-αv, anti-β1, and, to a lesser extent, by anti-MMP-9 antibodies. This suggests that the virus might utilize αv integrins, in association preferably with a β1 chain, in cell attachment and that MMP-9 could also play a role in the process. Moreover, a previously described peptide interacting with the RGD se-

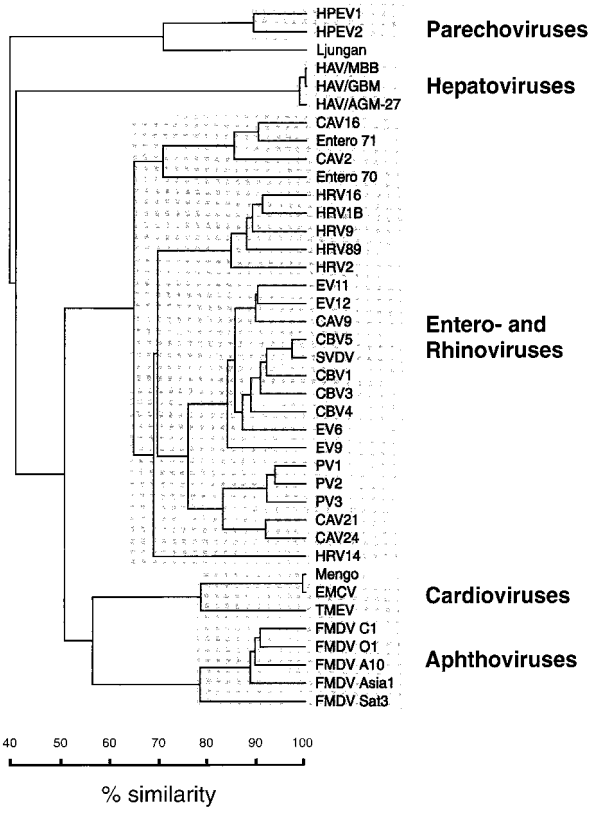

FIG. 3. Dendrogram, based on comparisons of the VP3 protein, illustrating the genetic relationships among selected representatives of each of the picornaviruses genera. Abbreviations: HAV, hepatitis A virus; HRV, human rhinovirus; EV, enterovirus; CBV, coxsackie B virus; SVDV, swine vesicular disease virus; PV, poliovirus; EMCV, encephalomyocarditis virus; TMEV, Theiler's murine encephalomyelitis virus; FMDV, foot-and-mouth disease virus.

quence (41) was able to inhibit the initiation of HPEV1 infection but did not affect CAV9 infection. These observations could be explained by differences in these two viruses in the utilization of cell surface molecules: the αv integrins may be a predominant receptor for HPEV1, whereas CAV9 is able to utilize two different strategies in cell surface interactions. In the case of foot-and-mouth disease virus, some strains appear to have an obligatory dependence on RGD-integrin interactions, whereas others have the ability to interact with heparan sulfate (26, 34).

CLINICAL MANIFESTATIONS AND EPIDEMIOLOGY

In view of the biological differences among parechoviruses, enteroviruses, and other human picornaviruses, it is of interest to compare the clinical manifestations in these infections. Data collected by the WHO Virus Unit between 1967 and 1974 showed that 60% of HPEV1 infections occurred in children under 1 year of age in contrast to, for instance, all typical echoviruses, where the corresponding figure was 15% (19). A seroepidemiological study of 110 individuals, representing different age groups in southwestern Finland, showed that 95% of neonates had HPEV1 antibodies, obviously of maternal origin, while only 20% of children between 2 and 12 months of age were seropositive (28). The proportion of seropositive individuals then increased significantly during the next year of life, and 97% of adults were HPEV1 seropositive, whereas less than 30% had antibodies to echovirus 30, one of the most prevalent enteroviruses. Thus, the incidence of HPEV1 infections seems to be extremely high, at least in the population surveyed, and the age distribution is distinct from that of typical enteroviruses. For a more detailed summary of clinical findings on HPEV1 infections, see reference 28.

Involvement of the central nervous system (CNS) appears to be more rare in HPEV1 infections (12%) than in enterovirus infections in general, whereas respiratory and gastrointestinal symptoms were frequently observed (26 and 29%, respectively) in HPEV1 isolation-positive patients (19). Similar findings were also reported from a study in Sweden (12), where HPEV1 infections occured in young children, with major peaks during late summer and autumn, resembling the epidemic pattern of enteroviruses, and less frequently, during the winter and early spring. Again, diarrhea was the most common clinical manifestation, followed by respiratory symptoms. Although CNS manifestations were rather rare in this patient group, an association of HPEV1 infections with encephalitis (30) and flaccid paralysis (16) has also been described. HPEV2 isolations have been reported less frequently, but these infections also seem to be associated with gastrointestinal symptoms (13). In conclusion, parechoviruses appear to be involved in diseases which resemble those caused by entero- and rhinoviruses and their occurrence also corresponds to the seasonal epidemic periods typical of enteroviruses and respiratory virus infections. However, there are differences in the age and symptom distribution.

APPEARANCE OF RELATED VIRUSES IN OTHER ANIMALS

After the two known members of the *Parechovirus* genus were isolated in 1956, no new serotypes belonging to this group have been identified and the other enteroviruses originally isolated from humans have been shown to be typical members of the *Enterovirus* genus (10, 21, 43). Similarly, there is no evidence that the currently known parechoviruses infect animals, although this has not been thoroughly studied. Interestingly, a new picornavirus, given the name Ljungan virus, was recently isolated from bank voles (*Clethrionomys glareolus*) in Sweden (38). This study was prompted by the observation of a correlation between the incidence of myocarditis in humans and bank vole population levels (37). Ljungan virus, and related strains isolated concurrently, has remarkable sequence homology with human parechoviruses in the capsid protein-encoding region (38). This is illustrated for the VP3 protein in Fig. 3. It can be seen that the degree of VP3 amino acid similarity between Ljungan virus and human parechoviruses exceeds 70%, which is greater than that seen, for instance, between some of the human enterovirus serotypes. Ljungan virus also possesses the basic N-terminal extension to VP3 previously seen only in human parechoviruses. The other Ljungan virus protein for which sequence data is available, VP0, has a correspondingly high degree of similarity to that of human parechoviruses, although the N terminus, a region of high variability between human parechoviruses, seems to be shorter by 38 amino acids. The virus further resembles human parechoviruses in lacking a VP0 N-terminal consensus sequence for myristoylation.

The isolation of these new viruses reflects our generally incomplete knowledge of viruses circulating in the environment and suggests that further studies may reveal a larger group of related parechoviruses in different species. The occurrence of such closely related viruses in species as diverse as humans and bank voles is highly interesting and suggests that there could be an animal reservoir for parechoviruses able to infect humans. Taken together with the high incidence of human parechovirus infections, this would have important implications for parechovirus epidemiology and human health.

CONCLUSION

It is clear that parechoviruses are common human pathogens and that, although typical picornaviruses, they represent distinctive molecular entities among members of this virus family. A number of questions remain to be answered. Notably, how do they apparently circumvent the need for VP0 cleavage, an essential step in virus maturation in other picornaviruses; what is the role of the unique N-terminal extension of VP3; what are the determinants of receptor binding, and what is the identity of the cell surface molecules involved; and what is the function of the 2A protein? However, the knowledge we already have has extended significantly our understanding of the molecular biology of picornaviruses.

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