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# Identification of Potential Recombination Breakpoints in Human Parechoviruses[▽]

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**Based on a comparison of the phylogeny of two distant regions, evidence has been found for recombination within parechoviruses. However, recombination breakpoints could not be detected in this way. We searched for potential recombination breakpoints in parechovirus by analysis of complete parechovirus sequences, including a newly isolated strain. Bootscan analysis demonstrated that parechoviruses are mosaic viruses build of regions related to corresponding genomic regions of other parechoviruses. With a genetic algorithm for recombination detection, sites for recombination were found. Analysis of partial sequences, as defined by recombination breakpoints, showed phylogenetic segregation between regions.**

Parechovirus is a widespread pathogen associated with mild gastroenteritis and respiratory symptoms but is also a major cause of viral sepsis and meningitis in young children (31).

Human parechoviruses (HPeVs) form a species in the *Parechovirus* genus of the *Picornavirus* family. The HPeV species is subdivided into six different types, parechoviruses 1 to 6 (3, 5, 16, 29, 30). The genomic RNA is approximately 7.5 kb in length and contains a single large open reading frame (ORF) that codes for a single polyprotein that is processed by the viral 3C protease into mature viral proteins (4). Three main regions are recognized within the polyprotein, P1, P2, and P3. The P1 region encodes the structural proteins. The P2 and P3 regions deliver the nonstructural proteins involved in virus replication (4). The ORF is flanked by untranslated regions (UTRs) that are involved in RNA translation and replication (24, 32).

Homologous recombination plays an important role in the evolution of most positive-strand RNA viruses, including picornaviruses (2, 7, 12, 14, 28). Recombination events can be detected by comparisons of small parts of the viral genomes, usually the regions encoding the structural protein VP1 and the nonstructural protein $3D^{pol}$ (6, 19, 20, 25, 26). Recent reports describe widespread recombination within the HPeVs (6). However, recombination hot spots cannot be detected by analysis of partial genome sequences. Multiple-sequence analysis of complete viral genome sequences is necessary to find potential recombination breakpoints. In this report, we describe the complete genome sequence of a recent parechovirus type 1 isolate. Sequence data from this isolate and complete genome sequences of parechoviruses available in GenBank were used for extensive phylogenetic analysis and for a search for recombination events between recent isolates.

Parechovirus strain 7555312 was isolated from a throat swab obtained from an immature fetus sent in for routine diagnostics. The virus was identified as HPeV type 1 by virus neutralization. The complete genomic RNA was amplified as partially overlapping fragments of 1 to 1.5 kb with oligonucleotide primers selected from conserved HPeV sequences. The full genomic sequence was obtained by a genome walking method from the amplified products. The genome of strain 7555312 was 7,336 nucleotides long, excluding the 3′ poly(A) tail. The genomic RNA contained a 5′ UTR of 709 nucleotides, an ORF of 6,539 nucleotides, and a 3′ UTR of 91 nucleotides, followed by a poly(A) tract.

Phylogenetic analysis was performed with available complete genome sequences of parechovirus strains as described in the legend to Fig. 1. Nucleotide and amino acid sequences were aligned by using ClustalW (13). The overall nucleotide and amino acid sequence identities between HPeV type 1 strain 7555312 and other HPeV strains are shown in Table 1. For the complete genome sequences, we observed overall nucleotide identities of 87% with recent HPeV type 1 strain BNI-788St, compared with an observed identity of approximately 80% with other HPeV strains. Comparison of specific areas already produced a first indication of recombination events between various virus isolates. The amino acid identities within the capsid region between type 1 virus strain 7555312 and type 1 strains Harris and BNI-788St were 91 and 96%, respectively. On the other hand, the amino acid identities within the P2 and P3 regions were the highest between type 1 strain 7555312 and recent type 4 strain K251176-02. A similar pattern was observed for the 5′ UTR, in which a similarity of 95% was observed between type 1 strain 7555312 and type 4 strain K251176-02, in contrast to a similarity of 92% with recent type 1 strain BNI-788St. Alignments were analyzed by using the maximum-likelihood algorithm as implemented in PHYLIP version 3.67 (10) and SimPlot version 3.5.1 (21). Figure 1A shows a similarity plot of all of the complete parechovirus genomic sequences in which type 1 strain 7555312 was used as the query sequence. The SimPlot graph clearly shows the high degree of similarity within the UTRs and the regions encoding the nonstructural proteins, whereas the most divergent region is the capsid protein-encoding part of the genome.

Two different approaches were used for the detection of

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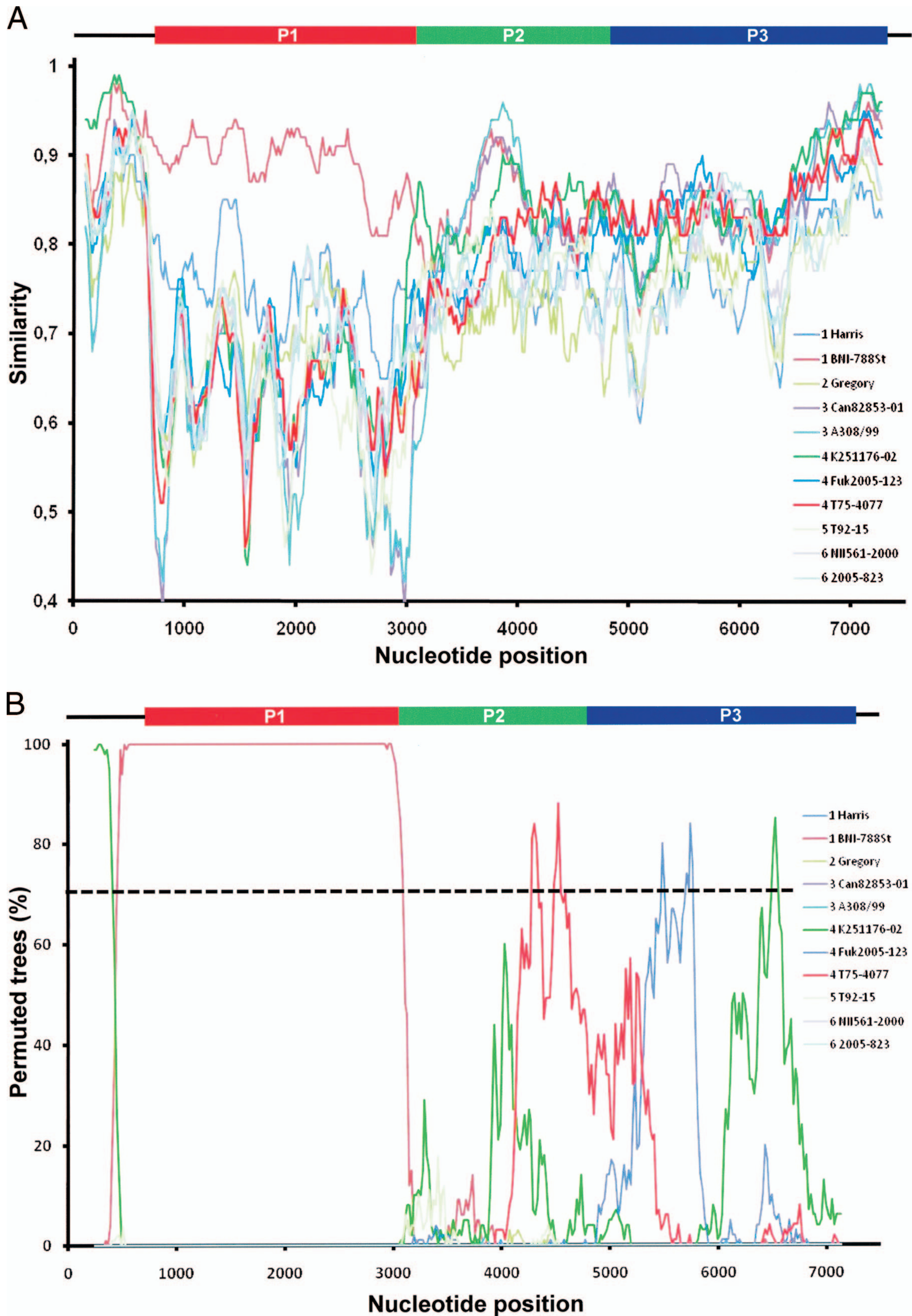


FIG. 1. Phylogenetic analysis of complete parechovirus genomes with SimPlot software (21). Analysis was performed with HPeV type 1 strain 7555312 (EMBL accession number FM178558), which is described in this paper, in combination with available sequences of HPeV type 1 strains

TABLE 1. Nucleotide and amino acid sequence identities between HPeV type 1 strain 7555213 and other parechoviruses

| Type, strain | % Nucleotide (amino acid) sequence identity | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5′ UTR | VP0 | VP3 | VP1 | 2A | 2B | 2C | 3A | 3B | 3C | 3D | 3′ UTR |
| 1, Harris | 86 | 82 (94) | 78 (90) | 76 (88) | 77 (89) | 80 (97) | 79 (94) | 76 (88) | 73 (92) | 80 (97) | 82 (94) | 87 |
| 1, BNI-788St | 93 | 92 (99) | 91 (98) | 87 (96) | 82 (93) | 89 (100) | 85 (97) | 82 (94) | 78 (88) | 86 (97) | 88 (96) | 93 |
| 2, Gregory | 86 | 74 (83) | 76 (84) | 71 (76) | 75 (86) | 76 (96) | 76 (87) | 76 (84) | 72 (92) | 82 (97) | 82 (93) | 90 |
| 3, Can82853-01 | 87 | 72 (74) | 70 (76) | 68 (71) | 79 (85) | 89 (100) | 85 (98) | 83 (97) | 80 (92) | 87 (97) | 89 (96) | 95 |
| 3, A308/99 | 87 | 72 (74) | 69 (75) | 68 (71) | 78 (85) | 91 (100) | 85 (98) | 81 (94) | 80 (88) | 86 (97) | 89 (96) | 95 |
| 4, K251176-02 | 94 | 72 (79) | 71 (79) | 73 (76) | 83 (93) | 84 (100) | 87 (99) | 81 (97) | 83 (96) | 83 (97) | 90 (97) | 93 |
| 4, Fuk2005-123 | 89 | 72 (79) | 70 (78) | 72 (76) | 79 (89) | 82 (99) | 83 (99) | 83 (96) | 85 (88) | 87 (97) | 87 (95) | 93 |
| 4, T75-4077 | 89 | 71 (79) | 72 (79) | 71 (74) | 76 (89) | 81 (100) | 85 (98) | 85 (95) | 82 (92) | 86 (97) | 88 (96) | 90 |
| 5, T92-15 | 89 | 72 (79) | 70 (77) | 67 (70) | 79 (89) | 83 (98) | 80 (95) | 78 (92) | 73 (92) | 81 (96) | 82 (93) | 91 |
| 6, NII561-2000 | 88 | 73 (79) | 75 (82) | 72 (75) | 78 (89) | 80 (98) | 79 (93) | 76 (91) | 68 (88) | 82 (98) | 85 (95) | 84 |
| 6, 2005-823 | 87 | 74 (79) | 74 (83) | 73 (75) | 77 (87) | 81 (98) | 78 (93) | 76 (91) | 70 (88) | 83 (98) | 85 (94) | 83 |

possible recombination events between various parechovirus strains. First, bootscan analysis was performed on the same data set of parechovirus sequences. Figure 1B shows the results of the bootscan analysis in which type 1 strain 7555312 was used as the query sequence. A sliding window of 500 nucleotides instead of the default value of 200 nucleotides was used in order to improve the signal-to-noise ratio. Signals of 70% or more of the observed permuted trees indicate potential recombination events. Bootscan analysis demonstrated that parechovirus type 1 strain 7555312 is a mosaic virus built of a capsid coding region related to the corresponding part of recently isolated type 1 strain BNI-788St, a 5′ UTR related to the corresponding region of recently isolated type 4 strain K251176-02, and a nonstructural protein-encoding region parts of which are related to the corresponding regions of parechovirus type 4 strains T75-4077, Fuk2005-123, and K251176-02 and parechovirus type 3 strain A308/99. Phylogenetic relationships within the 3′ UTR are less well defined. Bootscan analysis performed on reversed parechovirus sequences and a sliding window ranging from 200 to 500 nucleotides resulted in bootscan signals of approximately 30% of the observed permuted trees with HPeV type 4 strain K251176-02 (data not shown). Second, recombination was further analyzed by GARD, a genetic algorithm for recombination detection (17, 18). This method makes use of a statistical approach to search recombination breakpoints from multiple-sequence alignments of homologous sequences. Potential breakpoints are identified by improvement of the small-sample corrected Akaike information criteria for phylogenetic trees constructed of individual recombinant fragments. Based on the outcome of the analysis, a level of support is assigned and expressed as a breakpoint placement score (17, 18). By using GARD on a set of complete genome sequences of HPeVs, we found three major potential breakpoints for recombination (Fig. 2A). The first potential breakpoint is found near the 5′ end of the ORF that forms the border between the internal ribosome entry site- and VP0-encoding regions. The next potential recombination hot spot is found at the border between the P1 and P2 regions and includes the 5′ part of the 2A protein-encoding region between the nucleotides at positions 3025 and 3117. Finally, a major potential breakpoint can be recognized around nucleotide position 5166, just upstream of the protein 3B-encoding part of the viral genome. The results of the GARD analysis are supported by the SimPlot and bootscan analyses described above (Fig. 1). Analysis of partial sequences as defined by recombination breakpoints found by GARD showed a phylogenetic segregation between the individual recombination fragments (Fig. 2B). In addition, a weaker signal was found by GARD indicating a possible breakpoint within the 3D-encoding region (Fig. 2A). This observation is supported by bootscan analysis (Fig. 1B). It indicates that the 5′ part of the P3 region is related to type 4 strain Fuk2005-123, whereas the 3′ part may be more related to type 4 strain K251176-02. However, recombination at this position might disrupt the correct folding of the $3D^{pol}$ protein. In combination with the low breakpoint placement score, a recombination breakpoint within the 3D-encoding region might be unlikely.

In conclusion, in this report we describe an approach to the detection of recombination breakpoints in the HPeV species. The results described above correlate with the findings of other investigators describing recombination in other picornavirus genera. The formation of mosaic viruses from building blocks related to the corresponding regions of related viruses demonstrates the independent evolution of regions encoding either the structural or nonstructural proteins or the UTRs of the

Harris (GenBank accession number L02971) (15) and BNI-788St (GenBank accession number EF051629) (8); HPeV type 2 strain Gregory (GenBank accession number NC_001897) (11); HPeV type 3 strains Can82853-01 (GenBank accession number AJ889918) (1) and A308/99 (GenBank accession number AB084913) (16); HPeV type 4 strains K251176-02 (GenBank accession number DQ315670) (5), Fuk2005-123 (GenBank accession number AB433629) (29), and T75-4077 (GenBank accession number AM235750) (3); HPeV type 5 strain T92-15 (GenBank accession number AM235749) (3); and HPeV type 6 strains NII561-2000 (GenBank accession number AB252582) (30) and 2005-823 (GenBank accession number EU077518) (9). (A) Similarity plot analysis with HPeV type 1 strain 7555312 as the query sequence. The analysis made use of a sliding window of 200 bases and a step size of 20 bases. The *y* axis shows the percent similarity between the selected HPeV sequences and the query sequence. (B) Bootscan analysis with HPeV strain 7555312 as the query sequence. The analysis was done with a sliding window of 500 bases and a step size of 20 bases. The *y* axis shows the percentage of the permuted tree in which the selected HPeV strains cluster with the query sequence.

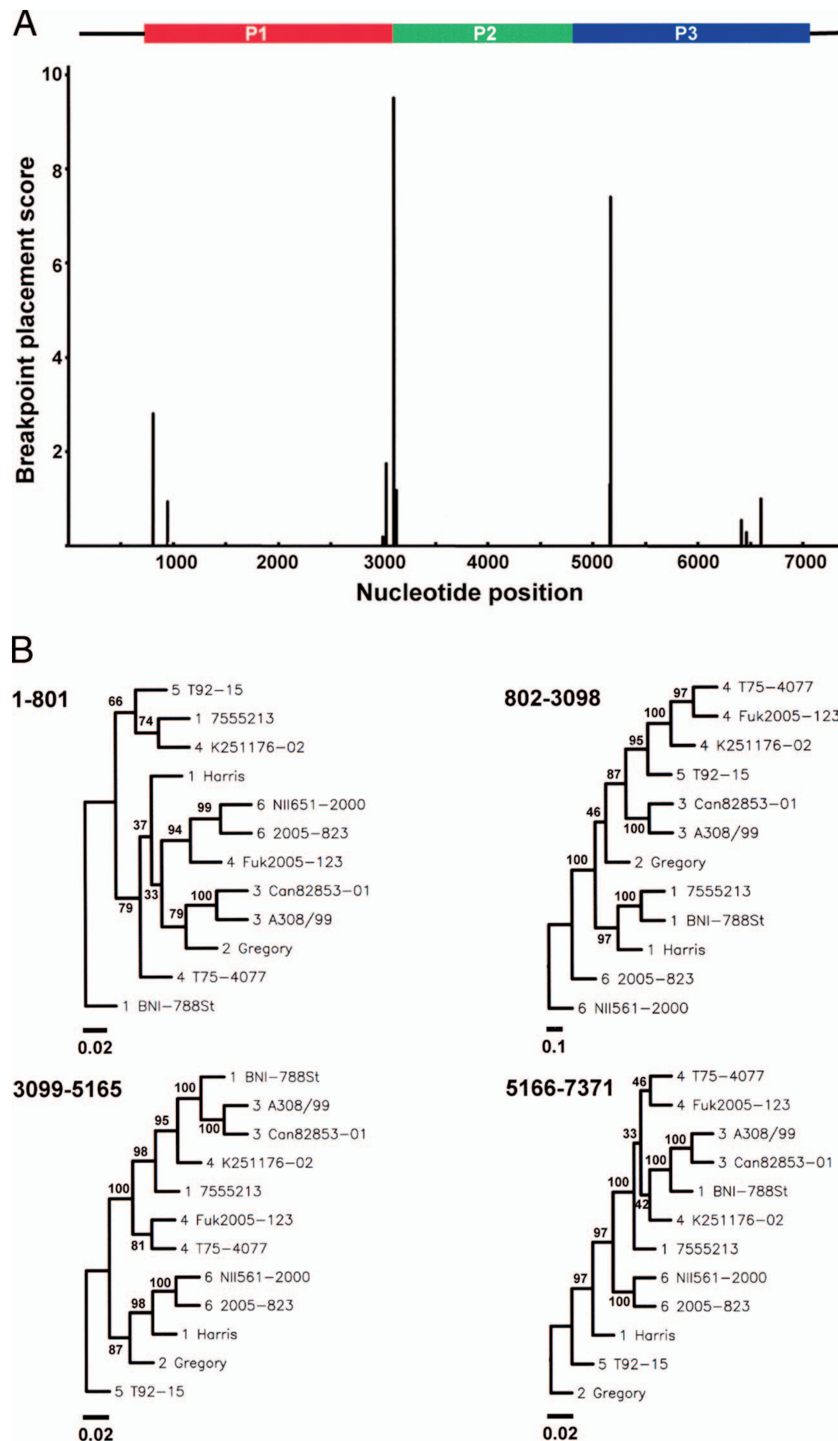


FIG. 2. Detection of recombination breakpoints with GARD (17, 18). (A) GARD plot displaying potential recombination breakpoints within the parechovirus genome. The probability of the breakpoints is indicated by corrected Akaike information criterion values as defined in references 17 and 18. (B) Phylogenetic analysis of the sequence fragments defined by the potential recombination breakpoints.

viral RNA. Similar observations were reported for other picornaviruses such as human enteroviruses (22).

The bootscan analysis described above indicates that newly described HPeV type strain 7555312 is the result of recombination in which the 800 proximal nucleotides of the 5′ UTR are evolutionarily related to a recent parechovirus type 4 strain. The breakpoints that were found in the GARD analysis correspond to recombination hot spots described for other picornaviruses. Simmonds and Welch (27) described the presence of sharply defined boundaries between the VP2/VP3/VP1 block and flanking sequences in phylogenetic compatibility. This corresponds well to the finding described here of potential break-

points within the VP4- and 2A-encoding regions of the parechoviruses. As in other nonenveloped positive-strand RNA viruses, phylogenetic segregation of parechovirus genotypes is limited to structural protein-encoding regions. Selective pressure is predominantly found in regions encoding structural proteins that are exposed on the virion surface and are thereby accessible to the immune system of the infected host. This might explain the presence of a major breakpoint within the region encoding capsid protein VP4, a protein found exclusively on the interior side of the virion. Lukashev and coworkers described a model for the evolution of enterovirus species (22, 23). It was found that recombination takes place strictly between members of the same species. It was stated that a whole range of nonstructural protein genes recombine frequently, producing new virus variants. In accordance with this model, we showed here that circulating parechovirus strains are the products of frequent recombination events. Previous reports stated that, based on the comparisons of VP1 and partial 3D sequence data, there is no evidence for recombination between parechovirus type 3 strains and other parechovirus types (6). Parechovirus type 3 lacks the RGD motif in the capsid and consequently requires a receptor that differs from the receptor of the other parechovirus types. This results in the genetic isolation of the parechovirus type 3 strains. By the analysis of full-length parechovirus genomic sequences, we were able to detect parechovirus type 3 sequences in the 3′ part of the genome. This might indicate that all HPeVs, as a single species, are able to recombine with each other.

**Nucleotide sequence accession number.** The nucleotide sequence determined in this study was deposited at EMBL under accession number FM178558.

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