Identification and characterization of foot-and-mouth disease virus O₁ Burgwedel/1987 as an intertypic recombinant

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The foot-and-mouth disease virus field isolate Burgwedel/1987 subtype O₁ was found to differ genetically from the antigenically related strain O₁ Kaufbeuren within the region encoding the non-structural proteins. This genetic difference was indicated by the RNase mismatch cleavage method and confirmed by nucleotide sequencing. An alignment of sequences encoding proteinase 3C of the Burgwedel isolate and several other virus strains identified this isolate as an intertypic recombinant; the parent strains were O₁ Kaufbeuren and a subtype C₁ strain. Recombination occurred between nucleotide positions 5493 and 5521, within the region encoding peptide 3B₁. Thus, the 5' three-quarters of the O₁ genome were fused to the 3'-terminal quarter of the C₁ genome. Other contemporary isolates from the same district are not recombinants. Sequence alignment distinguished four patterns of proteinase 3C-coding sequences among the virus strains analysed: subtypes A₂₂, C₁ and O₁ exhibit one pattern each, and another pattern is common to subtypes A₅, A₁₆ and O₂.

Introduction

Foot-and-mouth disease virus (FMDV) has a high mutation rate owing to the imprecise mechanism by which its positive-sense ssRNA genome replicates (for reviews see Steinhauer & Holland, 1987; Smith & Inglis, 1987).

Another mechanism leading to genetic heterogeneity of FMDV is recombination between virus genomes (Pringle, 1965; King et al., 1982, 1985; McCahon & Slade, 1981; McCahon et al., 1985; Giraudo et al., 1987). This requires simultaneous infection of cells with two virus strains and occurs at a frequency of up to 10%. As with other picornaviruses, crossover occurs rarely in the region of the genome encoding the four structural proteins VP₁ to VP₄, but frequently in regions encoding non-structural proteins (for a review see Wilson et al., 1988). Usually, recombinants are produced experimentally using strains known to differ in biological properties and then selected by, for instance, the yield recombination test (Giraudo et al., 1987) or the infectious centre method (Pringle, 1965; McCahon & Slade, 1981).

In the present study, virulent FMDV, isolated from the field and designated O₁ Burgwedel/1987 according to its immunological reactivity and characterization of the VP₁-encoding sequence (Knowles et al., 1988) was identified as an intertypic recombinant. This finding is presented as an example of how to identify as a recombinant an FMDV strain that was not noticeable as such by serological methods, reduced virulence, altered plaque morphology or other properties. Further FMDV strains of this kind may exist. Their detection and analysis may provide new insights into FMDV phylogeny as well as the recombination mechanism.

Methods

Virus strains and RNA. Baby hamster kidney cells (BHK-21) were infected with the FMDV strains A₁ Bernbeuren/1984, C₁ No- ville/1965, O₂ Brescia/1947, O₁ Lombardy/1946, O₁ Lausanne/1965, O₁ Wettmar/1988, O₁ Kaufbeuren/1966 and its high passage variant, and two isolates of O₁ Burgwedel/1987. The latter two derived from different animals and were passaged in different laboratories up to four times to obtain a sufficient amount of virus. RNA of all virus strains was extracted together with total cellular RNA as described previously (Marquardt & Adam, 1988).

cRNA. Six FMDV O₁ Kaufbeuren cDNA fragments were excised from the expression plasmid pVP₁-Po [ (Klump et al., 1984), inserted into the multiple cloning site of the transcription vector pSPT₁₈ (Boehringer Mannheim) and subcloned to produce pSPT₁₈/₃ to pSPT₁₈/₈ (Fig. 1b). The restriction enzymes used for fragmentation of the viral cDNA and their cleavage sites are shown in Fig. 1. Transcript 3 represents most of the VP₁ coding region, and transcripts 4 to 8 are specific for the 3' half of the genome encoding the non-structural proteins 2B to 3D. Radiolabelled antisense cRNA was synthesized in vitro as described (Marquardt et al., 1991)

RNase mismatch cleavage method. Briefly, the method consists of the cloning of reference virus cDNA, subcloning of suitable cDNA restriction fragments into transcription vectors, synthesis of radiolabelled antisense RNA in vitro, hybridization of cRNA and RNA from the FMDV strain under investigation, incubation of the hybrids with RNase, and subsequent determination of the size of the cRNA in sequencing gels which are exposed to autoradiography.
RNA (25 μg) extracted from virus-infected cells was mixed with 1.5 μl of a transcription reaction mixture (about 105 c.p.m.) and then dried in a Speedvac centrifuge. The pellet was resuspended in 24 μl formamide, 3 μl H2O, and 3 μl 4 M NaCl, 10 mM EDTA, 0.4 M PIPES pH 6.7. The suspension was incubated for 10 min at 85°C and overnight at 45°C, and then diluted in 284 μl chilled H2O, 35 μl of 1 M NaCl, 50 mM EDTA, 0.1 M Tris-HCl pH 7.5 and 1 μl of RNase solution (5 μg RNase A and 1 μg RNase T1) and allowed to react for 30 min at 15°C. The conditions were milder than those required for the cleavage of single base mismatches in dsRNA (Marquardt et al., 1991), as the aim was to detect major differences and not microheterogeneity in the sequences. The digestions were terminated by the addition of 20 μg proteinase K in 2 μl and 20 μl 10% SDS and incubation at 37°C for 15 min. The solution was deproteinized with a mixture of phenol pH 8.3, chloroform and isooamyl alcohol, and desalted by repeated ethanol precipitations in the presence of 5 μg carrier tRNA. The dried precipitate was resuspended in the appropriate loading buffer for electrophoresis in standard 6% sequencing gels.

Sequence analysis. RNA extracted from virus-infected cells was used as template for oligodeoxynucleotide primer-dependent reverse transcription according to Sanger et al. (1977) as described previously (Marquardt & Adam, 1988; Krebs et al., 1991a, b). Six primers were synthesized which allowed sequencing of the genomes of different FMDV strains between the regions encoding proteins 3A and 3D. Sequences complementary to the oligodeoxynucleotide primers are underlined in Fig. 2.

Results

Comparison of FMDV genomes by the RNase mismatch cleavage method

FMDV of subtype O1 was isolated repeatedly in the field between October 1987 and January 1988 near Hannover, Germany. The isolates designated Burgwedel/1987, Wettmar/1988 and Burgdorf/1988 were propagated in cell culture to enable sequence analysis of their capsid protein VP1-encoding genome regions. The sequences were found to be identical to that of the vaccine production strain O1 Kaufbeuren (Knowles et al., 1988), except those of the Burgwedel isolates. These differed in VP1 gene-specific codons 43 and 101, resulting in amino acid changes. The changes may have enabled the virus to escape neutralization by the immune system of infected cattle (Parry et al., 1990).

Whether further differences between strains Burgwedel/1987 and O1 Kaufbeuren existed outside the VP1-encoding genome region was also analysed. As no differences within the region encoding the other capsid proteins were found (data not shown), the region encoding the non-structural proteins was analysed. This was done by the RNase mismatch cleavage method because it rapidly indicates whether a strain differs genetically from strain O1 Kaufbeuren (Marquardt et al., 1991). To this end, RNA extracted from cells infected with the FMDV strains O1 Kaufbeuren, O1 Burgwedel, O1 Lausanne, O1 Wettmar or O2 Brescia was hybridized to antisense cRNA transcribed from the O1 Kaufbeuren-specific cDNA fragments contained in vectors pSPT18/3 to pSPT18/7 (Fig. 1 b) and then incubated with different RNases.

Thereafter, each transcript was analysed for its size by gel electrophoresis and subsequent autoradiography. The results obtained are shown in Fig. 1(c). Owing to the mild conditions of RNA-mediated RNA hydrolysis, substrates containing only a few single base mismatches, for instance the hybrids of transcript 3 and RNA of strains Burgwedel or Lausanne, were inefficiently cleaved (lanes 6, 7 and 10). Therefore, the demonstration of significant cleavage with other transcript-template combinations implies extensive sequence mismatch. To demonstrate this, we used RNA of O2 Brescia, which is epizootically unrelated to O1 Kaufbeuren and differs by more than 10% in the capsid protein-encoding region (Krebs et al., 1991b). As expected, transcript 3 was poorly protected from RNase digestion by hybridization to O2 Brescia RNA (lane 8, bottom half). As it did not protect any other transcript, the existence of considerable differences throughout both virus genomes was indicated (other lanes 8).

Hybridization to both samples of O1 Burgwedel RNA protected transcripts from pSPT18/3 to -5, but not those from pSPT18/6 and -7 (lanes 6 and 7). In contrast, RNA from strains O1 Lausanne (lanes 10) or O1 Wettmar (panel 6, lane 11) protected the transcripts mentioned. This result suggested that the genome of O1 Burgwedel differed significantly in sequence from that of O1 Kaufbeuren downstream of the protein 3A-encoding region, whereas that of O1 Wettmar, isolated 3 months later than O1 Burgwedel in the same area, did not.

Fig. 1. FMDV O1 Kaufbeuren-specific transcripts in the RNase mismatch cleavage assay. (a) Genetic map of FMDV. The long open reading frame is marked by broadening of the open bar. It is segmented at the positions of the first nucleotide of each encoded polypeptide. Nucleotides are numbered according to the UWGCG database. (b) The cDNA restriction fragments subcloned into pSPT18. The sites of cDNA cleavage are indicated, as are the enzymes used for fragment excision. The length of each cDNA fragment, and hence the antisense RNA transcribed from it in vitro, is indicated by horizontal numbers. The transcripts are numbered 1 to 8. (c) The results obtained with transcripts 3 to 7 in the RNase mismatch cleavage assay. Lanes 1, untreated transcripts; lanes 2 and 4, no sample added; lanes 5, negative hybridization with RNA of uninfected cells as control; lanes 3, positive control with RNA of high passage O1 Kaufbeuren; lanes 6 and 7, two separate RNA preparations from O1 Burgwedel; lanes 8, O2 Brescia RNA; lanes 9, low passage O1 Kaufbeuren RNA; lanes 10 (except transcript 7), O1 Lausanne RNA; lane 11 (transcript 6 only), O1 Wettmar RNA. Autoradiograms of gels are shown.
different FMDV strains. The cDNA sequence of high passage FMDV Ol Kaufbeuren (Forss et al., 1984) between positions 5453 and 6250 is shown in the top line. Aligned with it are equivalent sequences of other FMDV strains (C. Santa Pau (Martlnez-Salas et al., 1985), A12 (Carroll et al., 1984), A12 (Robertson et al., 1985), and Ol Lausanne, Ol Burgwedel, Ol Wetmar, Ol Lombardy, Ol K. 3.KP, Ol Brescia and A1 Bernbeuren. Ol K. 3.KP shows the RNA sequence of passage 3 Ol Kaufbeuren virus. Dots indicate nucleotides identical to those in the high passage Kaufbeuren sequence, blanks mark unresolved or non-sequenced residues. Silent sequence differences are indicated by lower case letters, those causing amino acid changes by upper case letters. The beginning of the genome regions encoding 3Bt, 3B2, 3B3, 3C and 3D are marked by > above the top line. Sequences complementary to the oligodeoxynucleotide primers used for sequencing reactions are underlined.

Fig. 2. Alignment of sequences encoding 3B and protease 3C of different FMDV strains. The cDNA sequence of high passage FMDV Ol Kaufbeuren (Forss et al., 1984) between positions 5453 and 6250 is shown in the top line. Aligned with it are equivalent sequences of other FMDV strains (C. Santa Pau (Martinez-Salas et al., 1985), A12 (Carroll et al., 1984), A12 (Robertson et al., 1985), and Ol Lausanne, Ol Burgwedel, Ol Wetmar, Ol Lombardy, Ol K. 3.KP, Ol Brescia and A1 Bernbeuren. Ol K. 3.KP shows the RNA sequence of passage 3 Ol Kaufbeuren virus. Dots indicate nucleotides identical to those in the high passage Kaufbeuren sequence, blanks mark unresolved or non-sequenced residues. Silent sequence differences are indicated by lower case letters, those causing amino acid changes by upper case letters. The beginning of the genome regions encoding 3Bt, 3B2, 3B3, 3C and 3D are marked by > above the top line. Sequences complementary to the oligodeoxynucleotide primers used for sequencing reactions are underlined.
Sequence determination and alignment of 3BC-encoding regions

The implied genetic differences between strains Burgwedel and Kaufbeuren concern the 3'-terminal quarter of the viral genome. The quality of the differences was analysed by determining almost complete sequences of the region encoding peptides 3B1 to 3B3 and proteinase 3C of both isolates of Burgwedel virus. The sequences were indistinguishable (data not shown) and therefore combined to produce the O1 Burgwedel consensus sequence (Fig. 2), which was compared to the relevant sequences of highly passaged O1 Kaufbeuren (Forss et al., 1984), C1 Santa Pau (Martinez-Salas et al., 1985), A10 (Carroll et al., 1984) and A12 (Robertson et al., 1985). This comparison revealed a striking similarity to the C1 strain (Fig. 2) and no similarity with any other sequence.

To prove the suggested relationship of the 3C-encoding sequence of strain Burgwedel to that of a subtype C1 strain, the sequence of another C1 strain, Noville, was determined. It was found to be identical to that of Burgwedel, except at two positions. To exclude accidental sequence similarities, the 3BC-encoding regions of A5 Bernbeuren, O1 Lausanne, O1 Wettmar, O1 Lombardy, O2 Brescia and O1 Kaufbeuren of low and high passage number were also sequenced. All sequences were aligned (Fig. 2).

The sequence alignment identified strain Burgwedel as a recombinant of O1 and C1 genomes. The O1 strain contributed the part encoding the capsid proteins (Knowles et al., 1988) and 3' adjacent genome parts (Fig. 1c, panels 4 and 5, lanes 6 and 7). The C1 strain contributed the 3'-terminal quarter of the genome (Fig. 1c, panels 6 and 7, lanes 6 and 7). Furthermore, the alignment shows sequence identity for strains O1 Kaufbeuren and O1 Wettmar. Therefore, we conclude that O1 Burgwedel was not the ancestor of O1 Wettmar, despite the contemporary occurrence of both virus strains in the same district.

The crossover region in the recombinant virus genome

As suggested by the RNase mismatch cleavage method, strains Burgwedel and Kaufbeuren are genetically similar upstream of the 3B1-encoding region (Fig. 1c, panel 5, lanes 6 and 7). RNA sequencing, on the other hand, showed that these genomes differed downstream of this region (Fig. 2). Therefore, the recombination of O1 and C1 strains that generated the Burgwedel strain must have occurred somewhere in the 3B1-encoding region. The data suggest that positions 5493 and 5521 delimit the recombination site because the sequence of strain Burgwedel switched between that of O1 and C1 beyond these positions. As all three sequences were identical between these positions, a more precise determination of the recombination site was not possible.

Discussion

This report describes the identification of an intertypic recombinant FMDV, designated O1 Burgwedel/1987. Genetic differences from the immunologically related strain O1 Kaufbeuren were detected by the RNase mismatch cleavage method. Sequence alignments showed that the differences were due to recombination between subtype O1 and C1 strains. The site of genome recombination was identified within the region encoding peptide VPg1.

The identification of a subtype C1 strain as one of the parents of O1 Burgwedel was not possible by immunological diagnosis because the genome part acquired encodes non-structural proteins. At present, no antisera are available that distinguish between such proteins produced by different serotypes of FMDV. All non-structural virus proteins are efficiently recognized by heterologous antisera (Harris et al., 1981; Grubman et al., 1987; Berger et al., 1990). O1 Burgwedel could possibly have been identified as a recombinant by electrofocusing, a method shown to distinguish between some non-structural proteins of different strains (Giraud et al., 1987).

The mechanism proposed to be responsible for picornavirus RNA recombination requires that the viral RNA-dependent RNA polymerase 3D switches templates during RNA replication. The switch is not particularly site-specific, as suggested by the analysis of inter- and intratypic poliovirus recombinants (Kirkegaard & Baltimore, 1986). Additionally, it may require base pairing of different template strands. Therefore, the secondary structure score of FMDV genome sections with and without recombination zones has been calculated (Wilson et al., 1988). In general, this indicated crossover regions to be more likely to form secondary structures than other regions, but as no such coincidence is evident for the region encoding 3B1 to 3C, the crossover event here remains unexplained.

It may be no accident that recombination occurred within the region encoding three repeats of peptide 3B. They all appear to function equally (Sangar et al., 1986) despite some sequence heterogeneity. Recombination in this part of the genome may have less effect on virulence than recombination elsewhere in the region encoding non-structural proteins. Such a recombinant may grow without selection pressure as efficiently as its parents. The fact that 3B1 of all known recent FMDV strains is
one amino acid shorter than 3B₂ and 3B₃ may provide supporting evidence. This could have resulted from the loss of one codon during a recombination event earlier in FMDV evolution.

The alignment of 3C-encoding sequences of several FMDV strains showed that variation occurs mostly at wobbly codon positions. This may indicate that amino acid changes impair the enzymic activity of proteinase 3C, which mediates the majority of the maturation cleavages within the primary viral translation product (Vakharia et al., 1987).

Furthermore, the alignment revealed different patterns of 3C-encoding sequences. One was common to subtype O₁ strains isolated in 1965 and 1966 and their derivatives, and may represent the South American subtype O₁, the other by subtypes A₅ and A₁₀. The latter pattern is strikingly similar to that of the type O strains Lombardy and Brescia, being biochemically (Krebs et al., 1991a) but not serologically (Wittmann, 1964) related. This pattern may also be found in other strains of European origin. Nucleotides specific for this pattern are, among others, around position 6273. The significance of the different patterns to FMDV evolution is unclear at present. Analysis of 3C-encoding sequences of other isolates may enable the phylogeny of FMDV to be determined.

Both FMDV samples isolated at Burgwedel in October 1987 from different persons and by different persons are intertypic recombinants of high genetic similarity. This leads to the conclusion that recombination had occurred before the samples were isolated. As no type C antigens were detected in the samples collected from the field in 1987, there is no reason to assume co-circulation of O₁ and C₂ strains. Therefore the origin of the recombinant virus remains unclear. O₁ Wettmar, also isolated in the Hannover district but 3 months later than the Burgdorf isolates, was not a recombinant. Considering the genetic differences between the Burgwedel and Wettmar isolates, the outbreak in 1988 was probably not caused by virus which had persisted in the field since 1987, but by an independent isolate.

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References


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