Comparison of the 5' and 3' untranslated genomic regions of virulent and attenuated foot-and-mouth disease viruses (strains O₁ Campos and C₃ Resende)

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The complete 5' and 3' non-coding regions of two attenuated South American foot-and-mouth disease virus (FMDV) vaccine strains, O₁C-O/E and C₃R-O/E, and their corresponding virulent parental strains, O₁ Campos and C₃ Resende, have been cloned from polymerase chain reaction-amplified primary cDNA. Differences observed in the derived nucleotide sequences between attenuated and virulent viruses seem not to affect regulatory signal structures, supporting the theory that genetic variations, primarily in the 3' halves of the viral genomes, contribute to the attenuation phenotype of the vaccine strains. In addition, this is the first report on the complete sequence of the 5' untranslated region of a C-type aphthovirus. Approximately 10% of the nucleotides differ from the corresponding known sequences of serotypes A or O.

Although it has been possible to reduce the occurrence of foot-and-mouth disease in Central Europe to a small number of outbreaks per year by immunization with inactivated virus, there is a need for an alternative vaccine for several reasons. A major argument is the danger involved in handling large quantities of infectious material for vaccine production which has led frequently to spreading of the disease in the past (Beck & Strohmaier, 1987). Numerous attempts to develop safe recombinant subunit vaccines, mainly by the use of synthetic peptides corresponding to immunodominant regions on the structural protein VP1, resulted only in insufficient protection of cattle. In spite of several optimistic reports in the last few years, a positive outcome by this approach appears more and more unlikely. The construction of attenuated viruses for use as live vaccines could represent a possible way out of this situation.

There are, however, several obstacles in developing a safe, live foot-and-mouth disease virus (FMDV) vaccine. Due to the generally high genetic instability of RNA genomes, the attenuation phenotype may revert quickly to virulence as demonstrated for the Sabin strains of poliovirus (Evans et al., 1985). Although there is no doubt that the polio vaccine has been of great benefit in the past, the application of live viruses, whose attenuation relies on only a few single base exchanges, would no longer be permitted given our present knowledge on the low fidelity of RNA replication. This view is supported by numerous frustrating attempts to construct a stable, live FMDV vaccine. Conventionally attenuated FMDV strains, derived by serial passaging in non-natural hosts, reverted frequently to virulence. Only a few of these strains stably maintained the attenuation phenotype.

Another problem is the high serological diversity of the aphthovirus group. The great number of known subtypes can be divided into seven main serotypes. Immunization with a single strain does not confer protection against all subtypes of even one serotype. Present vaccines often contain mixtures of two or more subtypes for each serotype. Establishing live vaccines for all important FMDV types would therefore be a tremendous endeavour, especially in view of the low chance of their maintaining sufficient genetic stability.

The problem of genetic stability could be circumvented, at least in part, by the construction of attenuated strains from recombinant DNA. This has been shown to be possible, in principle, by the recent construction of an infectious cDNA clone of FMDV O₁ Kaufbeuren (O₁K) (Zibert et al., 1990). For this approach, however, several open questions have to be answered, e.g. what are the molecular mechanisms of attenuation in FMDV and which genetic changes would lead to a stable attenuation phenotype?

The nucleotide sequence data reported in this paper have been submitted to EMBL.
A major difficulty is the cloning of the 5' untranslated region (UTR) of the viral genome in a high copy number plasmid. This was the most critical step in establishing the full-length cDNA clone and has been managed only once (Zibert et al., 1990). Since cDNA clones from other serotypes may be infectious only with their authentic 5'-UTRs, cloning of this region from additional strains had to be demonstrated.

In order to study the molecular basis of attenuation of FMDV, we have already analysed biochemical and structural changes in the viral gene products between the two attenuated strains O1C-O/E and C3R-O/E and the corresponding virulent parental viruses O1 Campos (O1C) and C3 Resende (C3Res), respectively (Parisi et al., 1985; Sagedahl et al., 1987). We have shown that the dominant determinations of attenuation are located in both strains in the 3' portion of the viral genome (Giraudo et al., 1987, 1988) where a deletion in the 3A gene was detected as a major structural difference common to both attenuated strains (Giraudo et al., 1990). It was suggested that the 3A deletion could account for the attenuation; however, we could not fully exclude the contribution of exchanges in other regions of the viral genome. This holds especially for the extended UTRs, where changes involved in attenuation have been observed for poliovirus (Evans et al., 1985; Kawamura et al., 1989; Pollard et al., 1989).

To identify differences in the non-coding regions potentially contributing to the attenuation phenotype, we have now constructed cDNA clones from the 5'-UTRs of two South American vaccine strains and their respective wild-types, and have determined their nucleotide sequences, thereby also demonstrating the feasibility of cloning the genomic 5' regions of FMDV strains other than O1K. In addition, we have analysed the nucleotide sequences of the 3'-UTRs of these strains.

RNAs of the FMDV strains O1, C3Res, O1C-O/E and C3R-O/E were prepared as described by Sangar et al. (1980). For the 3'-UTRs, first strand cDNA was synthesized using oligo(dT) as a primer including 0.4 mM-CH3HgOH in the reaction for reverse transcription (Küpper et al., 1981). Template RNA was hydrolysed in 0.3 M-NaOH, 8 mM-EDTA for 1 h at 60°C, and the cDNA was recovered by ethanol precipitation. The single-stranded cDNA then served directly as the template for nucleotide sequence analysis using T7 DNA polymerase (Krawczak et al., 1989), were corrected by sequencing the corresponding region in several additional, independently isolated cDNA clones. In no case was any indication of a polymorphous virus population observed, which was not unexpected since the viruses had been plaque-purified before amplification. The sequences are shown in Fig. 1 and are compared with the nucleotide sequence of serotype O1K (Forss et al., 1984; Zibert et al., 1990). The O1K sequence of the 5'-UTR has been derived from a low passage (no. 7) of the virus virulent for cattle (Zibert et al., 1990).

The sequence of strain C3Res represents the first structural information on the 5'-UTR of a C-type aphthovirus. Comparable to other published genomic segments of non-structural protein coding regions of this serotype (Beck et al., 1983; Martinez-Salas et al., 1985), about 10% of the nucleotides differ from the O1K sequence (Table 1). Strain O1C differs also by some 10% from C3Res, whereas O1C and O1K are more closely related, as expected (2 to 5% nucleotide differences). Several base changes between the serotypes located in
complementary positions of presumed double-stranded sequences of the 5'-UTR compensate each other (positions 13/32 and 80/280 in 5'-UTR-S, and positions 481/586 and 714/733 in 5'-UTR-L), thus supporting the proposed secondary structure models for that region (Pilipenko et al., 1989, 1990).

The termini of the 5'-UTR-S fragment and the first 12 nucleotides following the poly(C) tract are imposed by the primer oligonucleotides used in PCR amplification and need not represent the true viral sequences. However, these regions are normally conserved between the different serotypes of FMDV (Harris, 1980; Newton et al., 1985; Clarke et al., 1987), and the fact that primer extension was successful with all of these oligonucleotides even under stringent conditions (70 °C hybridization temperature in standard PCRs) argues against differences between the primers and the original viral sequences.

In the 5'-UTR-S fragment, O,C-O/E differs in three positions (185, 186 and 280) from O1K, and O,C differs in a single position (86) from C3Res. These exchanges do not affect putative base-paired regions conserved between FMDV and encephalomyocarditis (EMC) virus (Pilipenko et al., 1990). In addition, three out of the four exchanges found in the attenuated strains are present in the corresponding positions of the (wild-type) O1K sequence. Interestingly, 12 out of 18 variations in O1K compared to O1K correspond to the C3Res sequence. This homology can be observed in the 5'-UTR-S fragment only and might reflect a recent recombination event between O1C and C3Res. Strain O1K was isolated during an epizootic in 1966 in Europe and is believed to derive from O1C from which it differs only by 1 to 5% of the nucleotides in other parts of the genome analysed so far (Beck & Strohmaier, 1987; compare also the fragments 5'-UTR-L and 3'-UTR).

A similar degree of variation was observed in the 5'-UTR-L fragment (Fig. 1b). Most base differences between O,C-O/E and O,C (seven out of 11) represent reversions to the O1K sequence. Of the remaining four exchanges, one is in a position (192) which varies between different strains, and another (position 729)

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**Table 1. Sequence homology in the 5'- and 3'-UTRs between O1K, O1C and C3Res**

<table>
<thead>
<tr>
<th>Serotype</th>
<th><strong>5'-UTR-S</strong>* (§)</th>
<th>**5'-UTR-L† (§)</th>
<th>**3'-UTR‡ (§)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1K</td>
<td>95 (18)</td>
<td>98 (10)</td>
<td>95 (5)</td>
</tr>
<tr>
<td>O1C</td>
<td>92 (28)</td>
<td>91 (65)</td>
<td>76 (22)</td>
</tr>
<tr>
<td>C3Res</td>
<td></td>
<td></td>
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</tbody>
</table>

* Region from 5' end to poly(C) (367 nucleotides).
† Region from poly(C) to the first translational start site of the polyprotein (position 805 of the O1K sequence; 714 nucleotides).
‡ Region between end of the polyprotein coding region and poly(A) (93 nucleotides).
§ Number of base exchanges in parentheses.
adopts the sequence of serotype A. The third (position 566) converts a G–C base pair of a conserved stem structure within the internal translation initiation region (Kühn et al. 1990; Belsham & Brangwyn, 1990) into a G–U base pair. The fourth exchange (position 218) is localized within a several-fold repeated sequence. Since the number of these repeats differs between FMDV strains, an influence of this exchange on virulence appears improbable.

Only two base changes were observed between C3R-O/E and C3Res in this fragment, one (position 646) converting the C3Res sequence into the O1K sequence, the other (position 591) leading to a G–U base pair instead of a G–C base pair in a stem structure of the translational control region, comparable to the exchange at position 566 of strain O1C-O/E mentioned above.

In the Sabin strains of poliovirus, essential base exchanges involved in attenuation act by destabilizing a base-paired structure of the internal translation initiation signal (Evans et al., 1985; Kawamura et al., 1989; Pollard et al., 1989). None of the changes in the FMDV vaccine strains seems to affect analogous structures in a similar way. It does not, therefore, appear very likely that the attenuation phenotype of these strains derives from the exchanges in the 5′-UTR, but rather may rely on variations elsewhere in the genome.

The data supporting this conclusion are consistent for C3R-O/E; however, for strain O1C-O/E some of our earlier results (Giraudo et al., 1987, 1988) do not exclude a possible contribution of the genomic 5′ part to complete attenuation. Intertypic recombinants containing the P3 region of the attenuated strain were fully attenuated, but an analogously constructed homotypic recombinant carrying the genomic 5′ half of the virulent parental strain O1C retained a low degree of virulence for cattle. Although the occurrence of an additional mutation during the construction of this recombinant cannot be excluded, the higher number of base exchanges in the 5′-UTR of this strain could explain a contribution of this region to the attenuation phenotype. In this case, the most likely candidates for important variations would be those at positions 218 and 566 mentioned above. A definite answer to this question can only be given by the construction of corresponding mutants in the infectious cDNA clone (Zibert et al., 1990).

No difference was found between the nucleotide sequences of the 3′-UTR of O1C-O/E and O1C, whereas four base exchanges and the deletion of a single nucleotide were observed between C3R-O/E and C3Res (Fig. 1c). Although not yet identified, regulatory elements for the initiation of minus-strand RNA replication could be located within the 3′-UTR of FMDV. The point mutations near the poly(A) tract could therefore contribute to the reduced virulence of strain C3R-O/E, in principle. The rate of RNA synthesis of this strain in primary bovine kidney (BK) cells is reduced compared with that of the wild-type virus (Sagedahl et al., 1987), whereas in baby hamster kidney cells the rate of RNA synthesis is not affected; this suggests no cis-effect of the exchanges at the level of RNA replication. With O1C-O/E, RNA synthesis is reduced in BK cells to a similar degree, but the 3′-UTR of this strain is conserved. The cell type-specific reduction of RNA synthesis is therefore probably due to a mutation elsewhere in the genome and not to exchanges in the 3′-UTR.

Restricted growth of the vaccine strains has also been observed for poliovirus in neuronal cells, whereas in HeLa cells parental and vaccine strains grow equally well (La Monica & Racaniello, 1989). In this case, the reduced replication is probably due to a reduced translation rate since the essential modifications are located in the 5′ part of the poliovirus genome and affect a translational control element. However, in the case of the two FMDV strains, genomic modifications connected with the attenuation phenotype have been allocated to the 3′ half of the genome (Giraudo et al., 1987, 1988). We therefore think that the cell type-specific reduction of RNA synthesis is rather due to the deletion in the 3A gene which occurs in a similar form in both strains. According to current models of viral RNA replication, 3A is thought to function as a membrane anchor for VPg during the initiation of the plus-strand synthesis (for a review, see Kuhn & Wimmer, 1987).

An additional argument against the contribution of the nucleotide exchanges in the 3′-UTR of strain C3R-O/E to the reduced replication rate is the fact that two of the four mutations (positions 7850 and 7869) represent the wild-type sequence of another C-type strain, C1 Santa Pau (Martinez-Salas et al., 1985). The remaining two base exchanges (positions 7871 and 7886) and the deletion of nucleotide 7868 are located in regions variable between serotypes.

In summary, we conclude from this structural analysis that the base exchanges in the 5′- or 3′-UTRs of the two attenuated FMDV strains are most probably not predominantly involved in the expression of the attenuation phenotype, although for strain C3R-O/E a minor contribution of the 5′-UTR-L fragment might be possible. This is in agreement with our earlier conclusions derived from the above mentioned homotypic and intertypic recombinants (Giraudo et al., 1987, 1988) suggesting that the major genetic variations determining the avirulent character are most probably located in the genomic 3′ halves of these two vaccine strains.

We greatly appreciate the support of the Alexander von Humboldt foundation to I.E.B. This work was supported by grant BCTR 381-5 from the Bundesministerium für Forschung und Technologie.
References


(Received 23 January 1991; Accepted 25 July 1991)

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