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## Multiple sites of recombination within the RNA genome of foot-and-mouth disease virus

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### Summary

Recombinant foot-and-mouth disease viruses were isolated from cells infected with a mixture of temperature-sensitive (*ts*) mutants belonging to different subtype strains. In order to select for recombination events in many different regions of the genome, crosses were performed between various pairs of mutants, with *ts* mutations in different regions of the genome. *ts*<sup>+</sup> progeny were analysed by electrofocusing virus-induced proteins and RNase T<sub>1</sub> fingerprinting of their RNA. All but 5 out of 43 independent isolates, from nine crosses, proved to have recombinant RNA genomes. Maps of these genomes, based on a knowledge of the locations of the unique oligonucleotides, were constructed. Most could be interpreted as being the products of single genetic cross-overs, although three recombinants were formed by two cross-overs each. Cross-overs in at least twelve distinct regions of the genome were identified. This evidence of a large number of recombination sites suggests that RNA recombination in picornaviruses is a general, as opposed to a site-specific, phenomenon.

picornavirus, foot-and-mouth disease virus, recombination, RNA fingerprint

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### Introduction

The ability to exchange genetic information between RNA molecules by recombination has been demonstrated definitively in only two viruses, poliovirus and foot-and-mouth disease virus (FMDV or aphthovirus). Recombination was first

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detected in these picornaviruses by measuring the increase in the number of resistant viruses produced when a mixture of sensitive mutants was grown in the same cell (Hirst, 1962; Ledinko, 1963; Pringle, 1965). Genetic maps, based on this measure of recombination frequency, were constructed for both viruses (Cooper, 1968; Lake et al., 1975; McCahon et al., 1977). Only recently, however, has the existence of RNA recombination been confirmed biochemically. Recombination between different subtype strains of FMDV was demonstrated by electrofocusing virus-induced proteins and RNase T<sub>1</sub> fingerprinting of the genomic RNA (King et al., 1982; Saunders et al., 1985). Different types also recombined with each other, though at a lower frequency (McCahon et al., 1985). In the case of poliovirus, inter-type recombinants have been recovered from a contact of a human subject who had received trivalent live vaccine (Kew and Nottay, 1984). Recombination has also been shown to occur between poliovirus types 1 and 3 in tissue culture (Agol et al., 1984).

To date, few recombinants of either poliovirus or FMDV have been characterised, and nothing is known of the mechanism by which they are formed. The purpose of the work described here was to determine whether RNA recombination is a general or a site-specific phenomenon. The O<sub>1</sub> and O<sub>6</sub> subtype strains of FMDV were chosen to be parents in this study, since they were closely related to each other, yet had readily distinguishable RNA fingerprints. Previous crosses between these two strains (King et al., 1982; Saunders et al., 1985) revealed only a limited number of recombination sites (just two from the fingerprints of eleven independent recombinants), suggesting that the mechanism of RNA recombination may, like RNA splicing, be highly sequence-specific. However, the results of a more extensive series of genetic crosses, described in this paper, show that there are a large number of recombination sites in the picornavirus genome.

## Materials and methods

### *Viruses*

The origin of the wild-type (*ts*<sup>+</sup>) O<sub>1</sub> strain, Pacheco, and of its chemically induced *ts* mutants, has been described previously (Lake et al., 1975), and likewise for the wild-type O<sub>6</sub> strain, V1, and its spontaneous mutants (King et al., 1982). *ts*<sup>+</sup> recombinants were isolated by the infectious centre method described by McCahon and Slade (1981).

### *RNA fingerprints*

These were done on RNA induced in infected cells as described by La Torre et al. (1982), except that approximately 10<sup>7</sup> cells were labelled with 0.2–0.5 mCi ortho[<sup>32</sup>P]phosphoric acid, and the LiCl fractionation step was omitted. Two-dimensional electrophoresis was as described by Harris et al. (1980).

## Results

### *Isolation of recombinants*

Recombinants between the two FMDV subtypes, O<sub>1</sub> and O<sub>6</sub>, were isolated by

TABLE 1  
SUMMARY OF GENETIC CROSSES AND CLASSIFICATION OF RECOMBINANTS

Cross	Parents		Percent of infected cells yielding <i>ts</i> <sup>+</sup> virus			Recombinant type <sup>a</sup> (No. of isolates)
	O <sub>1</sub> (location)	O <sub>6</sub> (location)	O <sub>1</sub> only	O <sub>6</sub> only	O <sub>1</sub> + O <sub>6</sub>	
A	<i>ts</i> 13 (capsid <sup>b</sup> )	<i>ts</i> 302 (P56a <sup>c</sup> )	< 0.06	2.8	13.8	1 (2), 2 <sup>d</sup> (5)
B	<i>ts</i> 13 (capsid)	<i>ts</i> 304 (P34 <sup>c</sup> )	< 0.06	1.9	9.4	1 (4), 8 (2), 12 (1)
C	<i>ts</i> 16 (P34 <sup>b,c</sup> )	<i>ts</i> 302 (P56a)	5.6	2.8	26.1	2 (2), 6 (2)
D	<i>ts</i> 16 (P34)	<i>ts</i> 303 (capsid <sup>c</sup> )	5.6	2.5	7.1	3 (2), 10 (1), 11 <sup>e</sup> (2), 15 (1), 16 (1), 17 (1), R <sup>f</sup> (1)
E	<i>ts</i> 16 (P34)	<i>ts</i> 304 (P34)	5.6	1.9	3.9	5 (1), 13 (1), R <sup>g</sup> (4)
F	<i>ts</i> 22 (P56a <sup>b,c</sup> )	<i>ts</i> 303 (capsid)	1.1	2.5	16.9	3 (1), 7 <sup>h</sup> (4)
G	<i>ts</i> 22 (P56a)	<i>ts</i> 304 (P34)	1.1	1.9	16.0	4 (1), 7 <sup>i</sup> (1)
H	<i>ts</i> 33 (VP2 <sup>b,c</sup> )	<i>ts</i> 302 (P56a)	0.1	0.2	4.4	1 (1), 2 (1)
I	<i>ts</i> 03 (P56a <sup>b</sup> )	<i>ts</i> 303 (capsid)	0.3	0.1	0.8	9 (1)

<sup>a</sup> Defined by protein and oligonucleotide composition.

<sup>b</sup> Based on genetic map locus (McCahon et al., 1977).

<sup>c</sup> Based on covariation with a protein or oligonucleotide: *ts*16, Saunders et al., (1985); *ts*22, Lowe et al. (1981); *ts*33, King and Newman (1980); *ts*302, *ts*303, King et al. (1982).

<sup>d</sup> Protein P20b altered in one isolate.

<sup>e</sup> Oligonucleotide O<sub>6</sub>-46 missing in one isolate.

<sup>f</sup> Revertant of O<sub>6</sub>.

<sup>g</sup> Revertants of O<sub>1</sub>.

<sup>h</sup> Oligonucleotide O<sub>1</sub>-32 altered in one isolate.

<sup>i</sup> Identical to Rec 7 except for one new oligonucleotide.

infecting cells with a mixture of *ts* mutants, and selecting *ts*<sup>+</sup> progeny in an infectious centre assay (McCahon and Slade, 1981). A variety of *ts* mutants was used, with the aim of generating as many different types of recombinant as possible. Table 1 lists the nine inter-subtype crosses (A to I) that have been performed to date. The results of four of these crosses, A, F, G and H, have been described in part previously (King et al., 1982; Saunders et al., 1985). The approximate location of the *ts* lesion in each parent is given in Table 1.

As Table 1 shows, the percentage of mixedly infected cells that gave rise to virus plaques at the non-permissive temperature was, in most cases, higher than in the singly infected controls. However, there was no significant increase in two of the crosses, D and E, although a low recombination frequency could have been obscured in these crosses by the high background of one of the parents, *ts*16. The *ts*<sup>+</sup> progeny of the genetic crosses were initially analysed by electrofocusing their induced proteins, after which a selection was examined in more detail by RNase T<sub>1</sub>, fingerprinting of the RNA. This paper describes the compositions of 43 progeny viruses that have been analysed by both techniques.

#### *Inheritance of proteins*

Only a limited variety of recombinant protein patterns was observed, and, with the exception of Rec 9, all fell into the four types, exemplified by Rec 1, 2, 3 and 4,

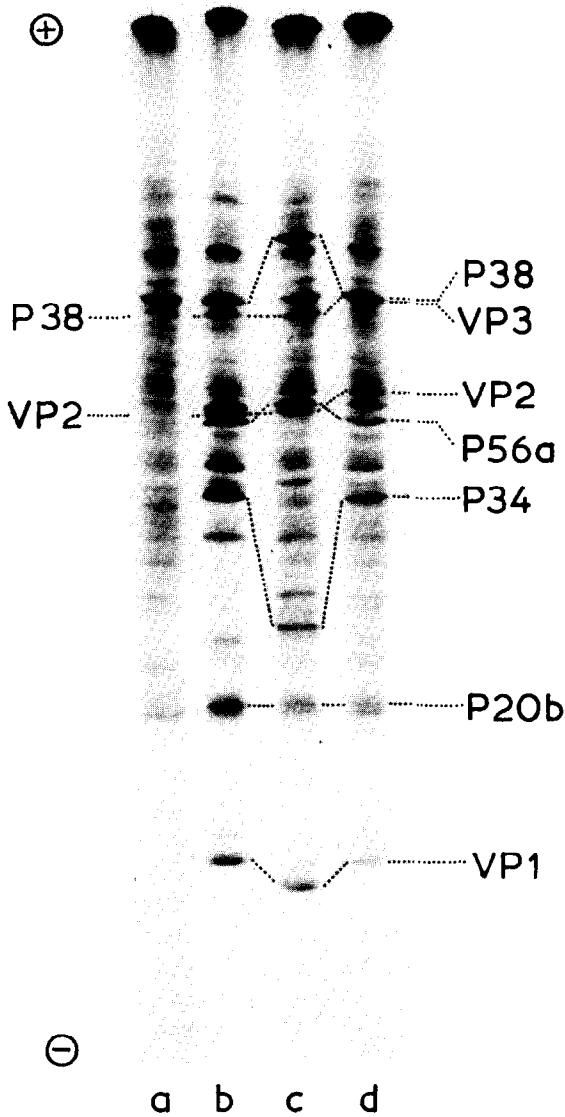


Fig. 1. A genetic cross-over within the region of the genome coding for the coat proteins, detected by electrofocusing virus-induced proteins. Baby hamster kidney cells, (a) uninfected or infected with (b) recombinant Rec 9, (c) O<sub>1</sub>-ts03, or (d) O<sub>6</sub>-ts303, were labelled with [<sup>35</sup>S]methionine for 30 min, followed by a 30 min chase with unlabelled methionine. Electrofocusing was performed in gels containing pH 3.5–10 Ampholine ampholytes as described by King et al. (1982).

described previously (Saunders et al., 1985). As Fig. 1 shows, Rec 9 possessed a VP2 like that of the O<sub>1</sub> strain whereas all its other proteins resembled those of the O<sub>6</sub> strain. This was the only example of a capsid with a hybrid protein composition. In general, protein charge, as measured by electrofocusing, behaved as a stable inherita-

ble character, although one recombinant, described previously, possessed a P20b that resembled neither parent (Saunders et al., 1985).

In addition to recombinants, several crosses yielded a high proportion of  $ts^+$  viruses that appeared to have inherited a complete set of proteins from one parent. These were originally assumed to be revertants. However, the production of so many revertants was unexpected, especially in crosses like F (Table 1), in which there was an enhanced yield of  $ts^+$  progeny. Several of these viruses were therefore examined by RNA fingerprinting. This showed that most (12/17) were not revertants, but recombinants, and these are also included in the analyses described below.

### *Inheritance of oligonucleotides*

We have previously shown that the parental strains,  $O_1$  and  $O_6$ , differ from each other in most of their large unique oligonucleotides (King et al., 1982). Among the 43  $ts^+$  progeny that have been examined to date, a variety of fingerprints were found, all but five of which were recombinant. Fig. 2 illustrates an example of each of the fifteen different types of recombinant fingerprint that were seen, except for Rec 1, 2, 3 and 4, which were described previously (Saunders et al., 1985). In conjunction with the protein data, above, a total of 17 different types of recombinant, excluding minor variants described below, could be distinguished. These were called Rec 1–17. A complete list of the recombinants isolated from each cross is given in Table 1.

The subtype origin of each oligonucleotide was assigned by reference to the oligonucleotide pattern of the parental mixture (Fig. 2), shown diagrammatically in Fig. 3. The genome locations of 37 subtype-specific oligonucleotides (18  $O_1$  and 19  $O_6$ ), have previously been determined from their sequence (Saunders et al., 1985). Maps, summarising the protein and oligonucleotide compositions of Rec 5–15, are shown in Fig. 4. (Those of Rec 1–4 were published previously.) Most of the recombinants can be interpreted simply as the products of one, or at most two, homologous recombinational events. However, some features of Fig. 2 and Fig. 4 require explanation. Two of the  $ts$  mutants used as parents,  $ts$  13 and  $ts$  16, carried mutations affecting individual oligonucleotides (Saunders et al., 1985), which were passed on to their progeny. This explains the absence of spot  $O_1$ -20 from the fingerprint of Rec 12, and of spot  $O_1$ -31 from Rec 10 and 11. For the same reason, no significance regarding the location of the genetic cross-overs in Rec 16 and Rec 17 can be attributed to the absence of spot  $O_1$ -31.

At least two oligonucleotide changes appear to have been caused by mutations that arose, not in the parents, but during the isolation of the recombinants themselves. Thus, oligonucleotide  $O_6$ -21 and its homologue,  $O_1$ -20, were both missing from Rec 15. This cannot be explained by homologous recombination within this region of the genome, the oligonucleotide sequences being highly conserved (Saunders et al., 1985). Moreover, the change occurred at a site remote from the only known genetic cross-over in Rec 15. For the same reasons, recombination is an unlikely explanation for the substitution of a new spot in the fingerprint of Rec 12 (arrowed in Fig. 2) in place of the homologous oligonucleotides  $O_1$ -21 and  $O_6$ -25. Other fingerprint alterations are listed in footnotes to Table 1. The alteration in spot  $O_1$ -32

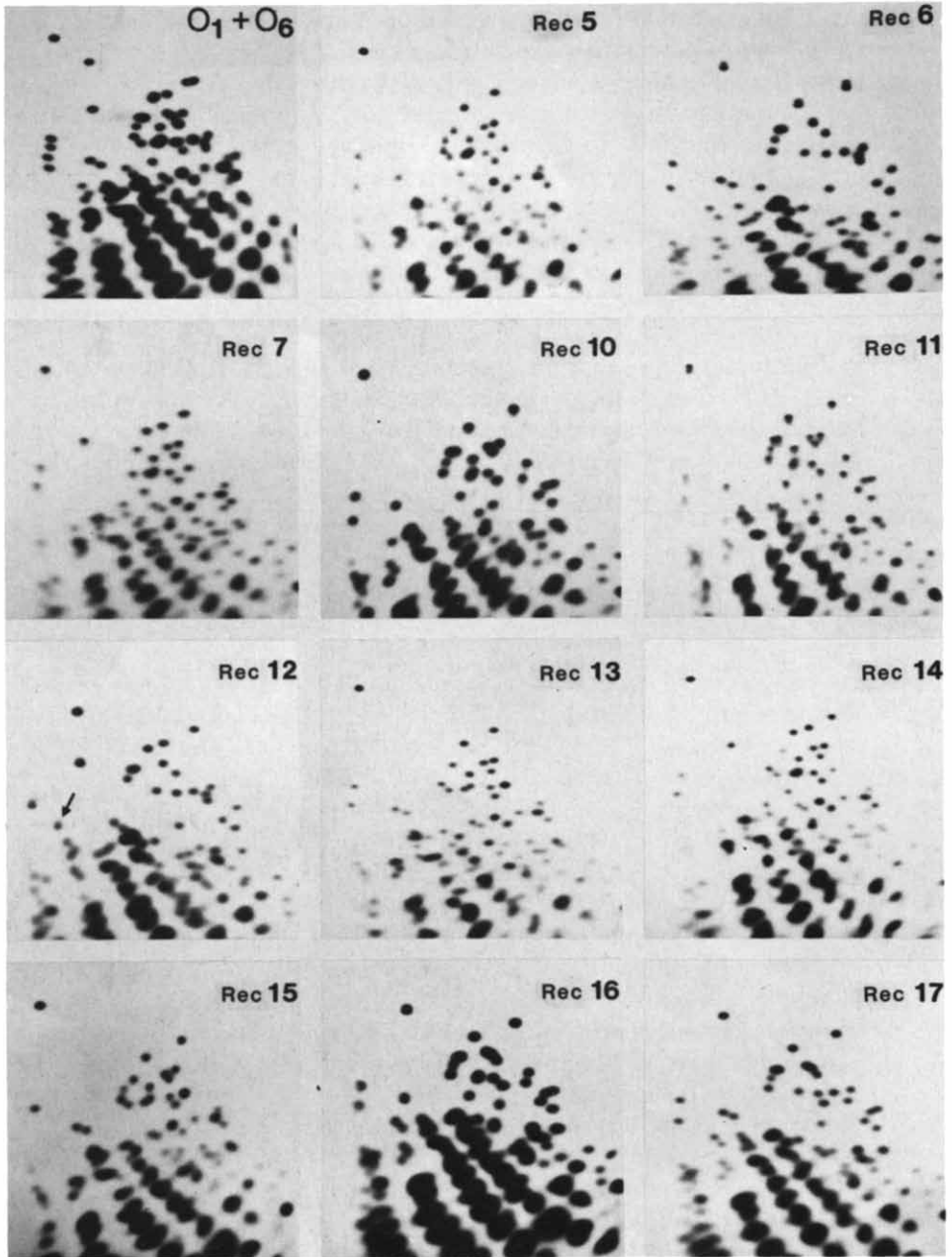


Fig. 2. RNase  $T_1$  fingerprints of a mixture of RNAs of the two wild-type parental strains,  $O_1 + O_6$ , and of a representative of each of the recombinant types 5-17. Not shown are Rec 8 (identical in oligonucleotide composition to Rec 1, King et al., 1982), and Rec 9 (identical to Rec 10). To save space, the poly(A) and poly(C) tracts, at the top of each fingerprint, are omitted.

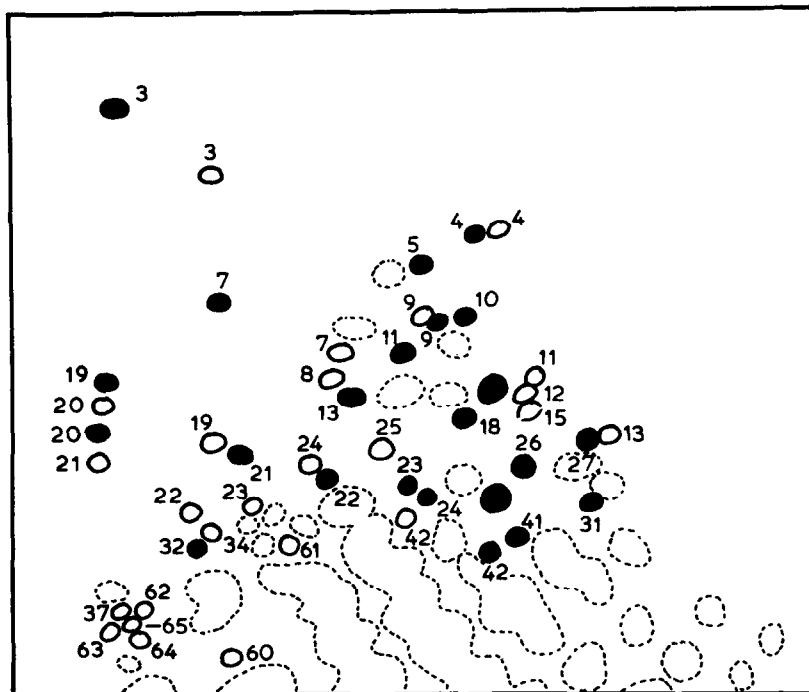


Fig. 3. Diagram of the  $O_1 + O_6$  mixture in Fig. 2, showing the oligonucleotides specific to the  $O_1$  strain (filled), and  $O_6$  (empty). Regions of the fingerprint in which the two parents could not be distinguished are shown as dotted lines.

in one of the type 7 recombinants probably represents a third example of a mutation, since this oligonucleotide was also located far from the known cross-over region. The other alterations involve oligonucleotides that have not been mapped, and their cause is unknown. Since the oligonucleotides on which this genetic analysis is based represent only about 5% of the total viral genome, the occurrence of three oligonucleotide changes in 43 isolates implies a high frequency of spontaneous base substitutions (of the order of one per genome) among the progeny of these crosses.

Most recombination events would be expected to occur within, rather than between, polypeptide-coding regions, and the oligonucleotide maps of Fig. 4 illustrate many examples of recombinant genes. It is remarkable that none of these genes gave rise to a novel recombinant protein; in all cases the gene product exhibited the same isoelectric point ( $pI$ ) as one or other parent. It is unclear whether the distribution of charged amino acid residues in the parental protein sequences was too conserved for proteins of novel  $pI$  to be generated, or whether such hybrid proteins were non-functional. In two instances (VP2 of Rec 10 and the P56a of Rec 14), the parental origin of a protein appeared to differ from that of the oligonucleotides in that region of the genome. In both cases, however, a genetic cross-over occurred near one end of the gene in question. The protein and oligonucleotide data



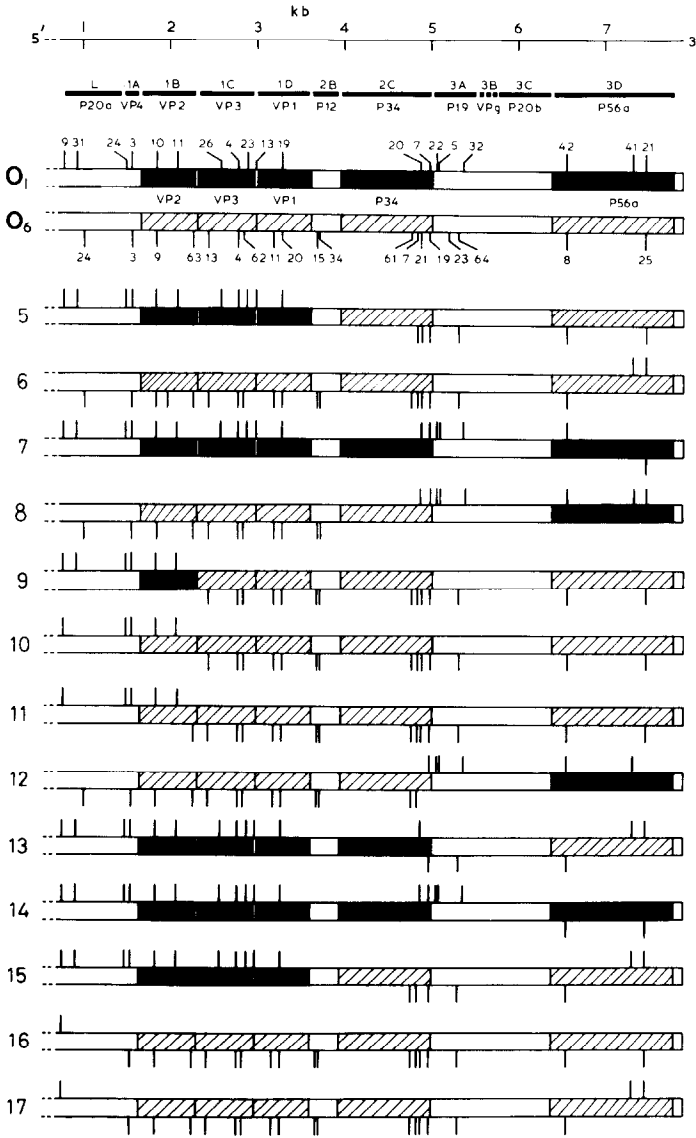


Fig. 4. Maps of the genomes of recombinant types 5-17. Lines pointing upwards indicate O<sub>1</sub> oligonucleotides, and downwards, O<sub>6</sub> oligonucleotides. Filled areas indicate O<sub>1</sub> proteins, and shaded, O<sub>6</sub> proteins. Coding regions of the viral proteins, shown at the top of the figure, are identified in two ways: names above each gene are based on the unified nomenclature for picornaviruses (Rueckert and Wimmer, 1984), and, below, using the FMDV nomenclature.

can be reconciled by postulating that the subtype determinant of *pI* is located near the carboxy-terminus of VP2, whereas that of P56a is located near the amino-terminus.

## Discussion

As in many other studies of recombination in picornaviruses, the recombinants described in this paper were isolated using high temperature as the selective pressure. The RNA fingerprints verify that this biological assay does primarily detect genetic recombination, rather than some other process, such as complementation. Thus, nearly all (38/43) of the independent *ts*<sup>+</sup> isolates, including many that were originally assumed to be revertants on the basis of their protein patterns (King et al., 1982; Saunders et al., 1985), did indeed possess recombinant genomes. Only in crosses D and E were revertant-like viruses isolated, and it is significant that these were the only crosses in which there was no enhancement in the yield of *ts*<sup>+</sup> progeny over that of the singly infected controls (Table 1). The results in Table 1 further indicate that recombinants are generated by a high proportion of infected cells, showing that recombination is a normal concomitant of virus replication.

Maps of all the different kinds of recombinant that have been distinguished to date are shown in Fig. 5. The regions of the genome in which cross-overs occurred, indicated by dotted lines, are deduced from the protein and RNA compositions summarized in Fig. 4 and results published previously (Saunders et al., 1985), taking account of both the presence and absence of oligonucleotides, and also of any differences between *ts* parents and wild-type. In contrast to our previous studies, which distinguished only two cross-over regions, Fig. 5 shows that recombination took place in many different regions scattered throughout the genome. Indeed, the only region in which recombination is known not to have occurred is the region coding for VP1 and most of the adjoining VP3 gene. Since VP1 and VP3 are the most poorly conserved picornaviral proteins (Forss et al., 1984; Stanway et al., 1983), it is possible that recombination in this region was prevented by nucleotide sequence heterogeneity, or that recombination did occur but gave rise to non-functional gene products.

Fig. 5 also shows the loci, deduced from recombination analysis, of the *ts* mutants that were used as parents. *ts*16 is of particular interest since this mutation also confers partial resistance to guanidine (gr), a property often associated with changes in P34 (Saunders and King, 1982). The fact that the possible locus of *ts*16 overlaps the 3'-end of the P34 gene supports the conclusion of a previous study (Saunders et al., 1985) that guanidine-resistance mutations are carried by a small carboxy-terminal region of P34. The loci of the other mutants in Fig. 5 are consistent with independent information summarised in Table 1. One parent, *ts*304, is missing from Fig. 5 because no single locus is consistent with all the recombination data. A possible explanation is that recombination within P34 suppressed, rather than eliminated, the *ts* phenotype of *ts*304 in either Rec 5 or Rec 12; independent evidence suggests that *ts*304, like *ts*16, is located in the P34 gene (Table 1).

Examination of Fig. 5 shows that recombination took place in no less than ten separate, i.e. non-overlapping, regions of the RNA genome. In addition, the difference between the protein patterns of Rec 1 and Rec 8, implies that their cross-overs occurred at distinct sites, even though their cross-over regions overlap. The same is also true of Rec 9 and Rec 10. Thus, a minimum of 12 different

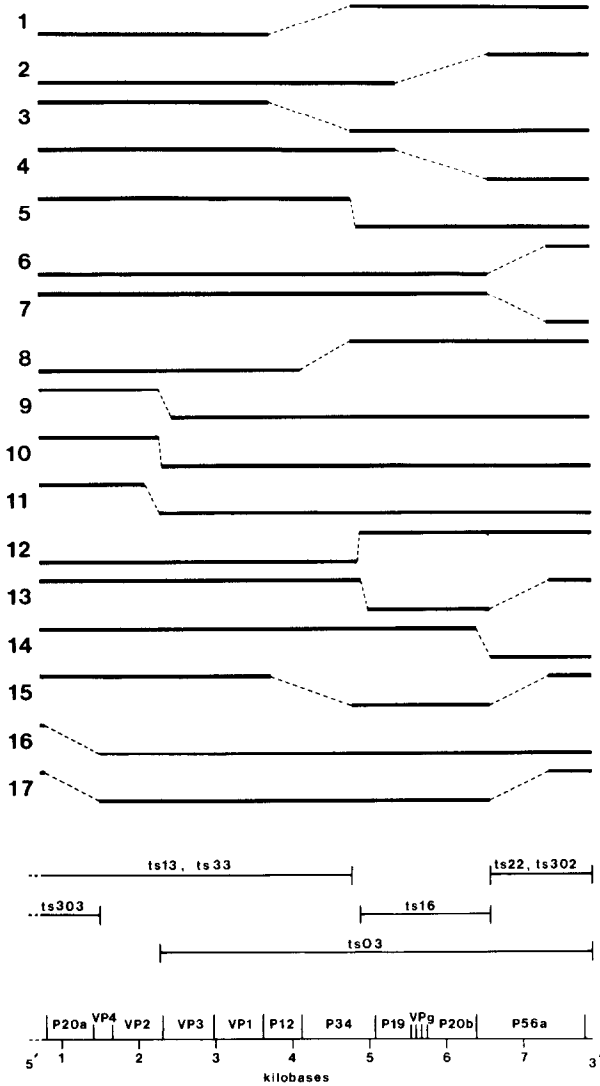


Fig. 5. Seventeen different types of recombinant genome produced by crossing O<sub>1</sub> and O<sub>6</sub> subtypes of FMDV. Regions of the genome of known parentage are represented by solid lines (O<sub>1</sub>, upper; O<sub>6</sub>, lower); dotted lines linking them indicate cross-over regions. Loci of the parental *ts* mutations, as determined by these recombination studies, are shown at the bottom of the figure.

cross-over sites are needed to explain the variety of compositions observed. The actual number of recombination sites may have been much larger than twelve, since the estimate ignores several variants whose significance is unclear (Table 1), and is based on only a limited number of biochemical markers. It is noticeable that the commonest types of cross-over (e.g. Rec 1, Table 1) occurred in the least well-de-

fined locations (Fig. 5), suggesting that more detailed analysis of such groups of recombinants would reveal additional cross-overs.

The existence of so many cross-over sites tends to favour a general mechanism of RNA recombination, with a virtually infinite number of potential recombination sites, rather than a site-specific mechanism. Information at the level of nucleotide sequence will be needed to confirm this conclusion. Recombination in RNA can be conceived as being either an homologous or non-homologous process. The production of defective interfering (DI) particles exemplifies the latter type of RNA sequence rearrangement, since it requires little or no homology at the site of the cross-over (Jennings et al., 1983). Sequence rearrangements of DI RNAs are frequently complex (Lehtovaara et al., 1981; O'hara et al., 1984), and 'mosaic' DI RNAs, involving cross-overs between different genome segments of the same virus (Jennings et al., 1983) or between viral and host RNAs (Monroe and Schlesinger, 1984), have also been described. A viable recombinant might therefore be produced by a non-homologous mechanism if, by chance, the cross-over were to occur between two RNA molecules at an homologous site. However, it seems unlikely that most recombinants are generated in this way, since rearranging sequences at random is an inefficient way of generating equal cross-overs unless the number of potential donor and acceptor sites in the RNA is small; as we have seen, recombination occurs at many sites in the FMDV genome, and it is particularly difficult to explain the high proportion of double cross-overs observed (3 out of 38) on the basis of independent non-homologous events.

We therefore conclude that genetic recombination in picornaviruses is primarily an homologous process, that entails proper alignment of the parental RNA sequences. In this respect, recombination appears to differ from other forms of specific sequence rearrangement in RNA, such as splicing (Cech, 1983) and the synthesis of coronavirus messenger RNAs containing a common leader sequence (Baric et al., 1983), which have been studied exclusively as intra-molecular events.

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