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Temperature-Sensitive Mutants of Foot-and-Mouth Disease Virus with Altered Structural Polypeptides

II. Comparison of Recombination and Biochemical Maps

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The structural polypeptides of foot-and-mouth disease virus were digested with *Staphylococcus aureus* V8 protease in the presence of sodium dodecyl sulfate. The protease-resistant peptides derived from temperature-sensitive mutants were compared with those of the wild type by electrofocusing in a polyacrylamide gel. Covariation between the charge shifts of different peptides indicated that they shared common sequences: only five independent peptides in all were derived from VP1, VP2, and VP3, accounting for approximately 50% of the polypeptide sequences. In two instances, amino acid substitutions that caused similar shifts in the isoelectric point were found to be located in different peptides. However, 15 mutants that possessed identical shifts in VP2 could not be distinguished by peptide analysis. The polypeptides of revertants able to grow at the nonpermissive temperature were compared with those of the parental mutants. By this test, 6 of the 12 distinguishable classes of coat protein mutations were found to covary with temperature sensitivity. In addition to true revertants, several phenotypic revertants which possessed a second charge change, either in a different structural polypeptide or in a different region of the same polypeptide, were isolated. The orientation of the recombination map was deduced from the loci of the coat protein mutations.

Picornaviruses provide a unique example of genetic recombination between RNA molecules. Much work was devoted to quantitating recombination frequencies measured in crosses between markers for temperature sensitivity or inhibitor resistance. This work culminated in recombination maps for poliovirus and foot-and-mouth disease virus (FMDV or aphthovirus) which were approximately additive and linear (4, 12, 15).

The problem of relating a gene function to a map locus is that, thus far, no genetic marker in either the poliovirus or the FMDV map has ever been correlated with a physicochemical character in the viral RNA or in a virus-coded polypeptide. In the case of poliovirus, considerable progress was made by observing physiological defects of mutants under restrictive conditions (5). Most of the map's mutations were assigned to that region of the genome, approximately 40% of the total, that codes for structural protein. However, many members of this class of mutant were also defective for other functions, for example, RNA synthesis.

The FMDV map appeared to be similar to that of poliovirus in both respects. A selection of 18 of the total 70 temperature-sensitive (*ts*) mutants at various map loci were found to be either acid labile or cystine dependent (13; McCahon, unpublished observations). Since both characters indicate defects in structural protein (14), this result implied either that the entire map represents only the coat protein region of the genome or that many of the *ts* mutants have second mutations affecting the coat proteins. Twenty-nine mutants were examined for viral RNA and antigen syntheses at the restrictive temperature. Differences were demonstrated among mutants, but there was no clear correlation with the map locus (R. A. J. Priston, unpublished observations). These results expose two limitations of the physiological approach: (i) it is imprecise in that, for example, individual structural polypeptides cannot be distinguished; (ii) pleiotropic effects make it difficult to map nonstructural functions.

Electrofocusing the mutant gene products offers a solution to the problem of physically locating missense mutations. The accompanying paper (9) describes the application of electrofocusing to the structural polypeptides of FMDV and its mutants. In this paper we describe the properties of revertants and the electrofocusing of peptide fragments to differentiate among mutations that cause similar charge changes. Twenty *ts* markers of the FMDV recombination map are identified as missense mutations affecting structural polypeptides. Since the poly-

peptide-coding positions are known (6, 17), we have thereby determined the orientation of the map.

MATERIALS AND METHODS

The origin of the FMDV mutants was described previously (11-13). *ts* mutants numbered less than 100 were induced, and those numbered more than 100 and *ts* O_3 were spontaneous. Growth, purification, and trypsin treatment of FMDV were as described in the preceding paper (9).

Peptide fingerprints. Virus pellets containing 50 μg of protein were disrupted by heating to 100°C for 1 min in 50 μl of 1% sodium dodecyl sulfate (SDS)-2% (vol/vol) 2-mercaptoethanol-0.01 M Tris-10% (vol/vol) glycerol. Ten microliters of 1-mg/ml *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc.) in 0.01 M Tris-hydrochloride (pH 7.4)-1 mM EDTA was added, and the mixture was incubated for 1 h at 37°C before the reaction was stopped by heating to 100°C for 1 min. Half the preparation was loaded per gel.

Electrofocusing. Electrofocusing was as described for method (ii) of reference 9, except the electrophoresis was continued for the times indicated in the figure legends.

Determination of molecular weight. Molecular weights of peptides were determined from a plot of electrophoretic mobility in SDS-polyacrylamide gel versus the logarithm of molecular weight. Standards used were the intact forms of VP1, VP2 and VP3 (each with a molecular weight of 30,000), soybean trypsin inhibitor (molecular weight, 22,500), myoglobin (molecular weight, 16,400), cytochrome *c* (molecular weight, 12,400), and insulin (molecular weights, 2,600 and 3,200). A mixture of [^{35}S]methionine-labeled and unlabeled FMDV polypeptides was fingerprinted as described above, and the gel was stained. Peptide bands were cut out of the gel and washed twice in water for 10 min and then in 0.125 M Tris-hydrochloride (pH 7.4)-2% 2-mercaptoethanol-0.1% SDS-10% glycerol for 30 min. Each slice was loaded, with markers in the same solvent, onto an SDS-polyacrylamide gel (85 by 6 mm) containing 17.15% acrylamide and 0.35% *N,N'*-methylenebisacrylamide and electrophoresed with the Laemmli (10) discontinuous buffer system for 6.5 h at 40 V. The SDS-polyacrylamide gels were stained, sliced longitudinally, dried, and autoradiographed to determine the positions of the labeled peptides.

Isolation of revertants. Two methods for isolating revertants were used. In the first, which was used with *ts* 12, 16, 58, 107, and 109, test tube cultures of BHK cells were infected at 37°C for 30 min and then washed with acid buffer, 0.1 M $NaPO_4$ (pH 6.2), to destroy inoculum virus. After fresh medium was added, the cultures were incubated at 37°C for a further 75 min, followed by 4 h at 41°C. The virus yields were plaque assayed at 41°C. Plaques were picked as presumptive revertants and cloned at 41°C. No more than one revertant was isolated per culture. The second method, which was used for the other mutants listed in Table 1, except for *ts* 3, was a modification of the procedure of Lake and Mackenzie (11) for isolating mutants. Each mutant was assayed by the agar-cell suspension technique (3), and plaques were allowed to form at 37°C for 16 to 20 h. An agar layer containing tetrazolium stain was then added to the plates, and the incubation was continued for 20 to 24 h at 41°C. Plaques with clearly defined halos were picked and cloned at 41°C. Seed stocks were prepared in small monolayer cultures at 41°C. Working stocks were prepared by a single passage of the seed stocks in monolayer cultures at 37°C.

RESULTS

Location of amino acid substitutions. Table 1 lists the 24 *ts* mutants of FMDV (strain O Pacheco) that possessed variant structural polypeptides. All 70 *ts* mutants of the recombination map were screened by electrofocusing for shifts in the isoelectric points of VP2, VP3, VP4 and the major fragment of VP1, VP1L, produced by trypsin treatment of the virus (1). For 29 of these mutants, the search was extended to intact VP1, but no additional variants were found. Also screened were three *ts* mutants not on the map, one of which, *ts* 3, was found to be variant; its properties are also included in Table 1. Both of the mutants that carried the guanidine resistance (gs^+) marker, $tsgs^+$ O_3 and $tsgs^+$ E_{18}, had normal structural polypeptides. The accompanying paper (9) describes all of the classes of charge shifts that could be distinguished on the

TABLE 1. *Summary of electrofocusing mutations observed in FMDV structural polypeptides*

Class of mutation	Mutant	Location of amino acid substitution[a]	Score of revertants with wild-type polypeptide
a	*ts* 12	VP1β[b]	0/3
b	*ts* 16	VP1β[b]	0/3
c	*ts* 40	VP1α	2/4
d	*ts* 58	VP1α	0/3
e	*ts* 103	VP1α	1/4
f	*ts* 109	VP1α	0/3
g	*ts* 3	VP2γ[b]	ND[c]
h	*ts* 65, 63, 33, 58, 62, 47, 59, 37, 42, 23, 61, 66, 44, 41, 67	VP2β	11/16[d]
i	*ts* 22	VP2β	0/8
j	*ts* 109	VP2α	3/3
k	*ts* 28	VP3α	1/7[e]
l	*ts* 107	VP3α	2/3

[a] α, β, and γ specify peptides produced by the action of *S. aureus* V8 protease in the presence of SDS.

[b] These substitutions are located in polypeptide segments that are absent from peptide fingerprints.

[c] ND, Not determined.

[d] Total for this class of mutation. In addition, four suppression reversions were found that affected the same polypeptide.

[e] Also four suppression reversions affecting VP1.

basis of magnitude or direction. In several cases, different mutants exhibited identical charge shifts. Thus, the variant VP2 of *ts* 109 was indistinguishable from that of *ts* 22, the difference between the VP1L of *ts* 16 and that of *ts* 109 was uncertain, and, most notably, there were 15 identical cases of altered VP2, designated "class h" in Table 1.

To discriminate among as many different classes of mutational events as possible, the variant polypeptides were subjected to a simple form of peptide fingerprinting, adapted from the method of Cleveland et al. (2). Virus samples were denatured with SDS and, before electrofocusing, were digested with *S. aureus* V8 protease. The resulting fingerprints are shown in Fig. 1.

A total of seven prominent and reproducible peptides were derived from VP1, VP2, and VP3. The origin of each peptide could be inferred by an inspection of mutant fingerprints. For example, only one peptide, VP1α, is derived from VP1, and it is clear which peptide when the fingerprints of *ts* 103 (gel f) and wild-type *ts*$^+$ (gel b) are compared. This technique enabled us to distinguish those VP1 mutants that are located within this peptide (*ts* 40, 58, 103, and 109) from those that are not (*ts* 12 and 16). Figure 1 also shows that the 6,000-dalton VP1α is contained entirely within VP1L.

Three peptides are derived from VP2: the α peptide is altered in the case of *ts* 109 (Fig. 1m), whereas the other two bands are shifted coordinately in *ts* 22 (gel k) and class h mutants (gel i). These two homologous peptides are therefore designated VP2β. Thus, there are three distinguishable locations for amino acid substitutions in VP2: the α and β peptides and segments of VP2 that are not represented at all in the fingerprints of Fig. 1. *ts* 3 (gel h) and a suppressor of *ts* 37 (see below) are in the third category.

ts 28 (gel n) and 107 (gel o), the two mutants altered in VP3, have amino acid substitutions in the same (α) pair of homologous peptides, although the shifts were of differing magnitude. The more acidic (lower) of the pair co-runs with the lower of the two VP2β bands. A prominent peptide toward the low-pH end of the gradient was also assigned to VP3: in Fig. 1 it is designated VP3β. None of the O Pacheco mutants exhibited an altered VP3β; however, VP3 mutants of two other serotype O strains of FMDV were found to have a variant peptide at this isoelectric point (data not shown).

Molecular weights were determined for the peptides derived from VP1, VP2, and VP3 by electrophoresis on an SDS-polyacrylamide gel (Table 2). The seven peptides leave much of VP2, VP3, and, especially, VP1 unaccounted for. Electrofocusing the SDS-peptide complexes toward the cathode after preparing the sample as described by O'Farrell et al. (16) resulted in fingerprints that were similar to those of Fig. 2, but inverted (data not shown). However, no

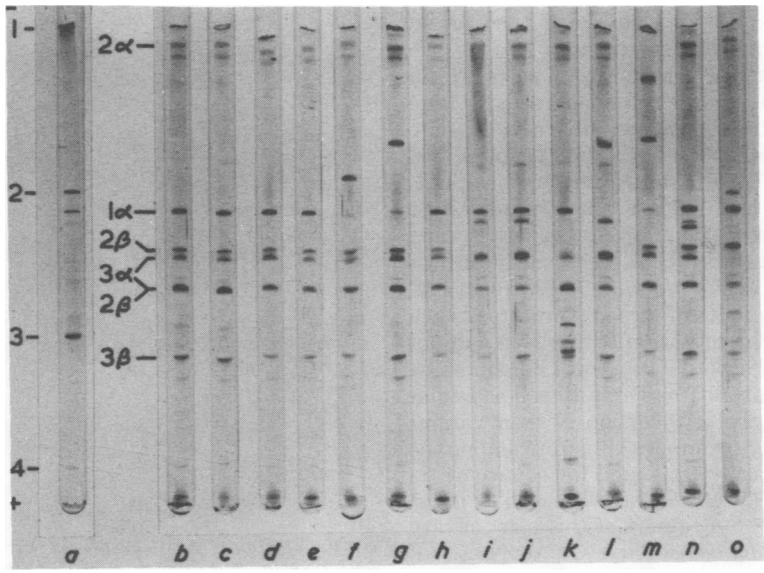

FIG. 1. *Peptide fingerprints of FMDV mutants with altered structural polypeptides. Virus polypeptides were disrupted in SDS and electrofocused toward the anode for 1 h at 200 V, followed by 6.5 h at 400 V, after either (a) no treatment or (b to o) digestion with S. aureus V8 protease. (a and b) ts*$^+$*; (c) ts*$^+$ *previously treated with trypsin to cleave VP1 to VP1L; (d) ts 12; (e) ts 16; (f) ts 103; (g) ts 40; (h) ts 3; (i) ts 37; (j) ts 37R50; (k) ts 22; (l) ts 58; (m) ts 109; (n) ts 107; (o) ts 28.*

TABLE 2. *Molecular weights of peptides generated by proteolysis in SDS solution*

Peptide	Mol wt
VP1α	6,000
VP2α	10,000
VP2β (upper)[a]	10,000
VP2β (lower)	10,000
VP3α (upper)	11,000
VP3α (lower)	16,000
VP3β	6,000

[a] Upper (most basic) of the two VP2β bands in Fig. 1.

additional peptides were found. Furthermore, no diffusible peptides were revealed by rapidly staining gels immediately after electrofocusing. The small polypeptide, VP4, was comparatively resistant to proteolysis under the conditions used for fingerprinting.

Peptide fingerprints show that the variant forms of VP2 in *ts* 22 and 109, although indistinguishable in the intact form, have mutations affecting different peptides. Hence, the two mutations cannot be sisters and are listed separately in Table 1. Similarly, the VP1 mutations of *ts* 16 and 109 were shown to affect different regions of VP1. However, fingerprints failed to discriminate among any of the class h mutations of Table 1: all 15 exhibited identical shifts in the same 10,000-dalton peptides derived from VP2. Two examples, *ts* 37 and 58, are shown in Fig. 1. Thus, the 26 coat protein mutations were resolved into 12 classes (Table 1, a through l), distinguishable by electrofocusing the variant polypeptides and their fragments.

Revertants. Revertants were selected for their ability to grow at the nonpermissive temperature. Electrofocusing was then performed to determine whether the variant polypeptide had also reverted. Table 1 shows that for the double mutants *ts* 58 and 109 it is the VP2 alteration that covaries with temperature sensitivity and not the alteration in VP1. No covariation was observed for three *ts* mutants, *ts* 12, 16, and 22, although a minimum of three revertants of each was studied. The mutation within *ts* 3 had not been mapped and was not tested. The remaining classes of *ts* mutation in Table 1 each scored at least one instance of reversion in their variant polypeptide. Five examples only from among the class h mutations were tested for covariant reversion. They all scored positive, and it is assumed that all 15 members of this class covary with temperature sensitivity. On the basis of these results, 20 of the 26 electrofocusing mutations listed in Table 1 were assigned to the coat protein region of the genome. These 20 mutations fell into six physically distinguishable classes (Table 1, c, e, h, j, k, and l).

This test does not distinguish between a true reversion and a suppression reversion that happens to restore the wild-type charge by a substitution elsewhere in the polypeptide. The distinction is not important since both provide positive evidence for covariation. Such compensating charge shifts might be revealed by fingerprinting the revertant polypeptide. However, when this was done (e.g., revertants of class h and k mutations), no hidden suppressors were found (data not shown).

It is of interest that several suppression revertants were found among those revertants in which the wild-type charge on the polypeptide was not fully restored. The suppressors were of two types, examples of which are shown in Fig. 2. *ts* 37R50 possessed a mutation that suppressed *ts* 37 and that compensated only partly for the charge shift in VP2. Fingerprints of *ts* 37 and *ts* 37R50 were identical (Fig. 1), showing that the *ts* mutation and its suppressor affect different regions of the polypeptide. For the VP3 variant, *ts* 28, suppression revertants that possessed a charge shift in another polypeptide, VP1, were isolated (Fig. 2).

DISCUSSION

A novel method of peptide fingerprinting was used to distinguish among similar electrofocusing mutations. The technique was also useful for identifying suppression reversions and for setting a limit of 0.3 kilobase on the physical separation of the 15 class h mutations. A determi-

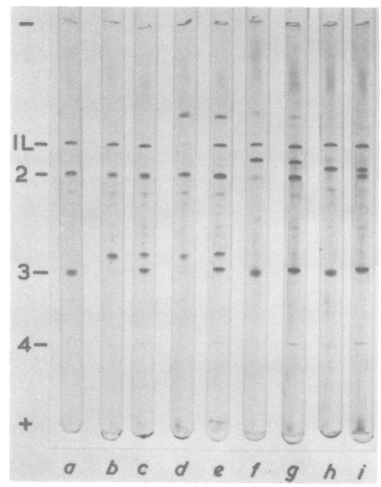

FIG. 2. *Suppression reversions. Virus was treated with trypsin, and after disruption with SDS the polypeptides were electrofocused toward the anode for 1 h at 200 V, followed by 5 h at 400 V. (a) ts⁺; (b) ts 28; (c) ts 28 and ts⁺, co-electrofocused; (d) ts 28R30; (e) ts 28R30 and ts⁺; (f) ts 37; (g) ts 37 and ts⁺; (h) ts 37R50; (i) ts 37R50 and ts⁺.*

nation of the position of each peptide in the polypeptide sequence would open the way to "protease cleavage mapping" of missense mutations by electrofocusing. However, it should be noted that approximately half of the informational content was lost by digestion to fragments that were too small to detect or too acidic or basic to focus.

It might be expected that phenotypic reversions, in which a second mutation compensates for the *ts* defect, would predominate over true reversions (8). Hence, a lack of covariation between *ts* and electrofocusing mutations does not prove that the mutations are independent, whereas a single instance of covariation was taken as sufficient evidence that the two characters were caused by a single mutation.

For several phenotypic revertants, suppression mutations were revealed as a second charge change in one of the structural polypeptides. One class of suppressors that altered the α peptide of VP1 suppressed the *ts* defect of *ts* 28, located in VP3α. This suggests that these two regions of the polypeptide are functionally associated.

Five charge change mutations listed in Table 1 failed to covary with temperature sensitivity in reversion studies and are therefore omitted from the discussion of the recombination map. In four of these cases, there are additional reasons for believing that their alterations are caused by second mutations, independent of the *ts* character. *ts* 58 and 109 each have two variant polypeptides, only one of which covaried with the *ts* lesion. Preliminary recombination studies show that *ts* and charge change markers of *ts* 16 and 22 can be separated. For example, ts^+ recombinants, produced by crossing *ts* 22 with viruses having mutations that map to its left, invariably possess the altered VP2 of *ts* 22 (data not shown). The status of one mutant, *ts* 12, is still uncertain, but its inclusion would not affect the interpretation which follows.

Comparison of biochemical and recombination maps. FMDV RNA has a total length of 8.0 kilobases. All of the known gene products are encoded along a 7.5-kilobase sequence bounded on one side by a polycytidylic acid tract near the 5′ end of the virus RNA and on the other side by the 3′-terminal polyadenylic acid tract (6, 7, 17). Reversion studies identified 20 *ts* mutations that covaried with alterations in VP1, VP2, or VP3 (Table 1). These three polypeptides are encoded together near the 5′ end of the genome, as shown in Fig. 3. Within this set of mutations, there is no apparent correlation between individual map loci and polypeptide-coding regions. However, the distribution of the 20 charge change loci is significantly nonrandom in that they all lie at, or to the left of, a recombination frequency of 1.25%, near the center of the map. It follows that the recombination map represents the genome oriented with its 5′ terminus on the left.

The recombination data from which the maps of both FMDV and poliovirus were constructed are notoriously subject to variation. Of the 70 *ts* mutations of the FMDV map, five loci at the extreme left, including two charge change mutants, *ts* 28 and 65, were singled out by McCahon et al. (15) as being particularly speculative. If these are eliminated, a relationsbip between the recombination frequency and the genomic distance becomes clear. Figure 3 shows the map loci of the five different classes of charge change mutation. The h class, represented by mutants with identical substitutions in the β peptide of VP2, is designated *h* in Fig. 3. gs^+ and *ts* O_3 are standard reference points of the map (15). *h* specifies a position between 0.8 and 1.6 kilobases on the biochemical map, and since it was mapped independently 14 times, its locus is accurately known. The standard deviation of the *h* loci, ±0.2% recombination frequency, is a measure of the mapping precision for individual markers in the coat protein region.

Figure 3 shows a possible alignment of the biochemical and recombination maps. Recombination frequency values coincide with polypeptide-coding regions for all of the mutants except *ts* 103, which lies approximately one standard deviation from the boundary of the VP1-

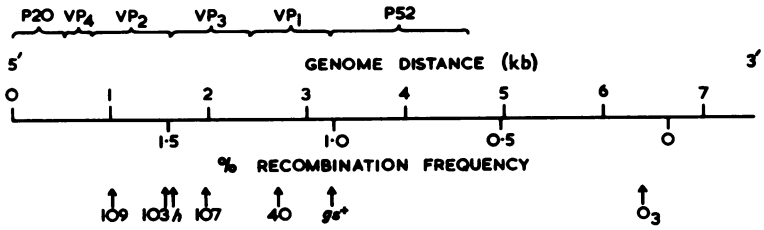

FIG. 3. *Tentative alignment of the recombination map (lower scale) with the biochemical map (upper scale) of the FMDV genome. Genetic markers are plotted at the loci previously determined by McCahon et al. (15). h is plotted at the mean locus of the class h mutations (see text). kb, Kilobase.*

coding region. Since our information is limited to a group of proteins encoded at one end of the genome, it is not possible to estimate precisely how much of the genome is represented by the recombination map.

Our physical interpretation of the FMDV recombination map shown in Fig. 3 must be regarded as tentative. However, it provides a useful theoretical basis for future recombination studies, which we hope will lead to a better description of genetic recombination in RNA at the molecular level.

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