JOURNAL OF VIROLOGY, Apr. 1980, p. 59-66
0022-538X/80/04-0059/08$02.00/0

Vol. 34, No. 1

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

I. Identification by Electrofocusing

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The structural polypeptides of foot-and-mouth disease virus were analyzed by electrofocusing in a polyacrylamide gel containing 9 M urea. Three versions of the technique were used to accommodate the widely differing isoelectric points of the four polypeptides. VP2 was identified by comparing mature virus with procapsids. The selective actions of proteases on virions of two serotypes and on their 12S particles were examined. From this emerged a simple test for distinguishing the similarly sized polypeptides: VP1, VP2, and VP3. The effects of carbamylation and succinylation on the charge of the polypeptides were investigated. From the properties of polypeptides modified either chemically or by mutation, it was concluded that all amino acid substitutions that might be expected to cause a charge change would be detected except for neutral-to-histidine substitutions in the most basic polypeptide, VP1. In a sample of 73 temperature-sensitive mutants, 11 classes of variant polypeptides were distinguished on the basis of charge. Their molecular weights were unchanged. Alterations were found in all structural polypeptides except VP4. Mutations affecting VP2 caused similar shifts in the precursor, VP0.

Many temperature-sensitive (*ts*) mutants of foot-and-mouth disease virus (FMDV or aphthovirus) have been isolated in this laboratory (9-11, 14). Most mutations to temperature sensitivity belong to the missense class (4), which causes the substitution of a single amino acid. More drastic types of mutational change tend to destroy the biological activity of the affected protein. When the substituted amino acid carries a different charge, the resulting shift in the isoelectric point of the polypeptide may be detected by electrofocusing in a polyacrylamide gel. This paper describes the application of electrofocusing to the structural polypeptides of FMDV and of 73 *ts* mutants. This approach for locating missense mutations has advantages of sensitivity and simplicity since (i) it should detect an acceptable proportion of amino acid substitutions, approximately 30% if they arise at random (17), and (ii) the four structural polypeptides of FMDV can be analyzed in one dimension so that many isolates can be screened together.

Electrofocusing has many important applications. In the accompanying paper (7), the technique provided the basis for a physical interpretation of the FMDV genetic recombination map (11). Another application is the study of charge shift substitutions that are accepted in the evolution of FMDV (15). Here, we describe procedures for electrofocusing the highly acidic and basic polypeptides of the FMDV capsid and their identification. We also demonstrate the ability of the technique for resolving single charge changes in such polypeptides by examining the shifts produced either by chemical modification of the polypeptides or by mutation in the genes coding for them.

MATERIALS AND METHODS

Virus growth and purification. The origin of the FMDV parental strain, O Pacheco, and its mutants was described previously (9-11). FMDV type A10 (isolate 61) was kindly provided by D. V. Sangar. Viruses were grown in baby hamster kidney (clone 21) monolayers in Roux bottles at 37°C for 12 to 16 h before freezing. To the thawed lysate was added an equal volume of saturated ammonium sulfate, pH 7.6, at 0°C. After centrifugation for 30 min at 5,000 × *g*, the pellet was suspended in 0.04 M $NaPO_4$ (pH 7.6)-0.1 M NaCl and clarified by centrifugation at 20,000 rpm for 15 min in a Beckman SW27 rotor at 20°C. The virus was pelleted by centrifugation at 27,000 rpm for 2 h and resuspended by sonication in 0.04 M $NaPO_4$ (pH 7.5)-0.1 M NaCl. Insoluble matter was removed by centrifugation for 10 min at 1,500 × *g*, and 0.1 volume of 10% sodium dodecyl sulfate (SDS) was added to the supernatant before layering on a linear 15 to 45% sucrose gradient in the same solvent and centrifugation for 70 min at 41,000 rpm at 20°C in an SW41 rotor. The gradient was fractionated, and the virus was assayed by absorbance at 260 nm, assuming a specific absorbance of 10 for a virus solution containing 1 mg of protein per ml (1). Virus was concentrated

by pelleting at 50,000 rpm in an SW50.1 rotor for 1 h at 20°C.

[^{35}S]methionine-labeled viruses were prepared in the same manner with the following modifications. Cell monolayers were washed twice in methionine-free Eagle medium and then infected at a high multiplicity. Medium was replaced by fresh methionine-free medium at 30, 60, and 90 min postinfection. At 2 h, 100 to 200 μCi of [^{35}S]methionine (1.3 Ci/μmol; Radiochemical Centre, Amersham, England) per Roux bottle was added, and incubation was continued until 5 to 7 h postinfection before freezing. After sucrose density gradient centrifugation, virus was detected by liquid scintillation counting of samples on glass fiber disks. Mature virus has a sedimentation coefficient of 146S, whereas procapsids (naturally occurring empty particles) peak at 70S. Before any further treatment, labeled virus was mixed with unlabeled FMDV type O Pacheco.

Proteolytic treatments. Virus was treated in two forms, whole (146S) particles or partially disrupted (12S) particles. Pelleting disrupted some of the virus to a form that had the protease sensitivity characteristic of 12S particles. For whole virions, therefore, proteolytic treatments were performed on sucrose gradient fractions of purified virus. Trypsin-tolylsulfonyl phenylalanyl chloromethyl ketone (Worthington Biochemicals Corp., Freehold, N. J.) was added to give a concentration of 33 μg/ml, and the mixture was incubated for 30 min at 37°C before excess soybean trypsin inhibitor was added and the virus was pelleted. Treatment with bromelain (BDH, Poole, England) was done at 1 mg/ml in the same manner, except no inhibitor was added. Pellets were stored at −70°C until needed.

12S particles (20) were produced by heating the virus in 0.01 M Tris-hydrochloride (pH 7.4)–1 mM EDTA at 60°C for 1 min. Samples were treated with trypsin at a concentration of 10 μg/ml for 10 min at 37°C before the reaction was stopped with excess trypsin inhibitor.

Preparation of virus polypeptides for electrophoresis. For SDS-polyacrylamide gel electrophoresis and for electrofocusing toward the anode, virus polypeptides were denatured by heating at 100°C for 1 min in 1% SDS–0.01 M Tris–2% 2-mercaptoethanol–10% glycerol. For electrofocusing toward the cathode, virus pellets were resuspended in 0.01 M Tris-hydrochloride (pH 7.4)–1 mM EDTA and heated to 60°C for 1 min. Each 100 μl of solution was treated with 5 μl of 0.2-mg/ml pancreatic RNase and incubated for 1 h at 37°C. Polypeptides were denatured by adding 105 mg of urea (Aristar; BDH) and 3.7 μl each of mercaptoethanol and Nonidet P-40. Approximately 20 μl, containing 10 μg of protein, was loaded per gel. Preparations were stored at −70°C.

SDS-polyacrylamide gel electrophoresis. Cylindrical gels (65 by 5 mm) contained 12.25% (wt/vol) acrylamide and 0.25% *N,N'*-methylenebisacrylamide. Electrophoresis was for 5 h at 40 V, using the discontinuous buffer system of Laemmli (8). Gels were stained with Coomassie brilliant blue.

Electrofocusing. Methods for electrofocusing were based on those of O'Farrell (12). Cylindrical gels (130 by 2.5 mm) were prepared from a solution of 9.5 M urea, 4.0% acrylamide (recrystallized), and 0.2% *N,N'*-methylenebisacrylamide (recrystallized) that had been deionized by stirring with Amberlite monobed resin BM-1. For 10 ml of gel solution, 9.5 ml of the above solution was mixed with 0.5 ml of LKB Ampholine and 10 μl of 10% (wt/vol) ammonium persulfate. After degassing, 7 μl of *N,N,N',N'*-tetramethylethylenediamine was added, and the solution was sucked up into the tubes. Gels were left overnight before running. Samples were loaded on top of the gels. After electrofocusing, gels were washed three times in 30% (vol/vol) 2-methoxyethanol to remove Ampholine and stained in 0.1% Kenacid blue R (BDH). Gels to be autoradiographed were dried down with the polypeptide bands of the carrier virus aligned.

The pH at which each polypeptide focused was estimated from pH profiles. These were measured for duplicate gels sliced into 5-mm pieces and soaked in 10 volumes of degassed water.

Three variations of the electrofocusing technique were used. (i) For focusing VP1, VP2, and VP3, gels contained pH 3.5 to 10 Ampholine. A 2.5-μl amount of 0.1 M 2-mercaptoethylammonium chloride was added to each sample. The upper (anodic) reservoir was filled with 0.1 M H_3PO_4, and the lower (cathodic) reservoir was filled with 0.2 M KOH. Under these conditions, those polypeptides that bear a net positive charge migrate into the gel toward the cathode. Electrofocusing was for 1 h at 200 V, followed by 4 h at 400 V. (ii) For focusing VP2, VP3, and VP4, the method was as for (i), except the polarity and reservoir solutions were reversed, and a mixture of Ampholines, pH 3.5 to 10, pH 5 to 7, and pH 2.5 to 4, was used in the ratio 5:3:2. Electrofocusing was for 1 h at 200 V, followed by 5 h at 400 V. (iii) For high-resolution electrofocusing of VP2 and VP3, gels contained a mixture of pH 3.5 to 10 and pH 5 to 7 Ampholine in the ratio 2:1 and 2% (vol/vol) Nonidet P-40. This nonionic detergent was omitted from the other methods since it caused streaking of VP4. The upper (cathodic) reservoir solution was 0.02 M NaOH, and the lower (anodic) reservoir solution was 0.01 M H_3PO_4. Electrofocusing was for 1 h at 200 V, followed by 16 h at 400 V.

Chemical modification of polypeptides. A virus pellet containing 40 μg of [^{35}S]methionine-labeled protein was suspended in 0.2 ml of 1.75 M *N*-ethylmorpholine acetate, pH 8.0, and incubated for 10 min at 55°C. Two microliters of RNase (2 mg/ml) was added, and the mixture was incubated for 1 h at 37°C. Urea (0.2 g) was added, and the solution was treated in the following three different ways. (i) For a zero time control, a 30-μl sample was added to 15 μl of 20% (vol/vol) 2-mercaptoethanol–6% (vol/vol) Nonidet P-40–6 M urea. (ii) For carbamylation, 0.2 ml was added to 14.8 mg of KCNO, and the reaction mixture was incubated at 50°C. Thirty-microliter samples were quenched as in (i) at the times indicated in the legend to Fig. 2. (iii) For succinylation, to 80 μl of polypeptide solution was added 0.7 μl of 5% (wt/vol) succinic anhydride freshly dissolved in dimethyl sulfoxide. After 3 min at room temperature, a 30-μl sample was treated as in (i). Five microliters of each sample was electrofocused with untreated structural polypeptides as markers.

RESULTS

Electrofocusing FMDV structural polypeptides. The four polypeptides of FMDV

cover such an extreme range of isoelectric points that we were unable to electrofocus all of them together. Electrophoresis toward the cathode, i.e., from low to high pH, focused VP1, VP2, and VP3 (Fig. 1B). The system was not allowed to reach equilibrium, but was stopped before VP1 reached the bottom of the gel. The approximate pH's at which each polypeptide of the parental strain, type O Pacheco, banded were as follows: for VP1, 8.3; for VP2, 6.2; and for VP3, 5.1. VP4 remained at the origin during this mode of electrofocusing. Poor yield and resolution, especially of the most basic components of a polypeptide mixture, often resulted from the cathodic mode of operation unless both acrylamide and urea were deionized and a cationic thiol-containing scavenger, mercaptoethylammonium chloride, was added to the sample.

When the polarity and pH gradient were reversed (Fig. 1C), VP2, VP3, and VP4 could be focused, provided that a more acidic pH range was used: approximate banding pH's were, respectively, 6.6, 5.6, and 4.7. VP1 remained at or near the origin and failed to focus. This is also a non-equilibrium procedure. Thus, neither method yields true values of the isoelectric point. A third method was sometimes used, which provided an improved resolution of VP2 and VP3, but at the expense of information about VP4. This was achieved by using a narrower pH range and running to equilibrium (see Fig. 4).

Identification of electrofocused polypeptides. Figure 1A shows the electrophoretic pattern of FMDV polypeptides on SDS-polyacrylamide gels. The three large polypeptides, VP1, VP2, and VP3, all have molecular weights of approximately 30,000 (5). They run so closely together that, instead of identifying them by molecular weight, we define VP1 as the polypeptide that is sensitive to trypsin in the intact virus (3) and VP2 as the polypeptide that, with VP4, is generated by cleavage of the precursor, VP0, found in procapsids (2, 16).

These criteria were used to distinguish VP1, VP2, and VP3 after electrofocusing, as follows. The most basic polypeptide was cleaved specifically (Fig. 1B) when virus was treated with trypsin. Therefore, this is VP1. To distinguish VP2 from VP3, type A10 was studied because this strain of FMDV accumulates procapsids. The preparation of procapsids shown in Fig. 1D contained less VP2 or VP4 than did mature virus, but instead contained VP0 at an intermediate position in the pH gradient.

It remained to be shown that the electrofocusing order of the structural polypeptides was the same for the two serotypes, O and A. This was done by making use of the finding that VP1 and VP2 are both cleaved by trypsin treatment of virus that has been disrupted to 12S particles. Thus, VP1, VP2, and VP3 are intact in all of the untreated (a samples) preparations shown in

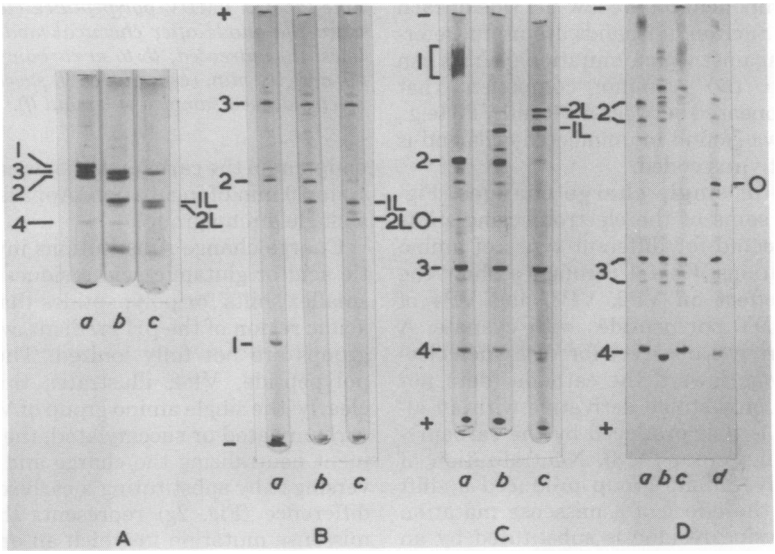

FIG. 1. *Identification of FMDV structural polypeptides after electrofocusing. Sensitivity to trypsin was used to distinguish VP1, VP2, and VP3: (a) untreated; (b) trypsin-treated virus; (c) trypsin-treated 12S particles. Type O Pacheco (ts$^+$) polypeptides were analyzed by: (A) SDS-polyacrylamide gel electrophoresis; (B) electrofocusing toward the cathode; (C) electrofocusing toward the anode. (D) Autoradiograph of [^{35}S]-methionine-labeled polypeptides from mature virions (a, b, and c) and untreated procapsids of type A10 FMDV (d), electrofocused toward the anode. Polypeptides are identified on the figure by numerals.*

Fig. 1A, B, C, and D. Of these three polypeptides, VP2 and VP3 remain intact after treatment of the virus with trypsin (b samples), whereas only VP3 is resistant to trypsin treatment of 12S particles (c samples). VP4 was identified by its molecular weight (approximately 10,000 [5]) measured by SDS-polyacrylamide gel electrophoresis.

The large trypsin-resistant fragments of VP1 and VP2, VP1L and VP2L, respectively, have isoelectric points far removed from those of the intact polypeptides. The site at which trypsin cleaves VP1 is evidently the same in both the virion and 12S particle. The molecular weight of VP2L is estimated to be 17,000, assuming a value of 18,000 for VP1L (5).

Electrofocusing patterns are often complicated by the presence of minor and multiple bands. These can arise in four ways. (i) Multiple bands at the positions of the large polypeptides of the A10 strain (Fig. 1D) represent examples of polymorphism. After the stock of A10 was recloned, only a single major component was found at each position (data not shown). (ii) Preparations of mature virions contain traces of VP0. That of type O Pacheco focuses between VP2 and VP3 (Fig. 1C). It was identified by comparing its molecular weight with that of type A10 and also by observing that its position is always shifted by mutations affecting VP2 (see Fig. 3). (iii) As has been noted by others (6, 19), single charge shift variants are always observed as a trace component on the low-pH side of each major band. Such minor bands are useful reference points against which mutational shifts can be measured. (iv) A minor component that sometimes appeared between VP0 and VP3 (e.g., see Fig. 31) was found in uninfected cells and is therefore not virus coded.

Detection of single charge changes. Figure 2 shows some of the electrofocusing shifts that are expected for different types of amino acid substitutions. Gels a through e show the progressive effect on VP2, VP3, and VP4 of reacting FMDV polypeptides with cyanate. A similar pattern was observed for VP1 when electrofocusing was toward the cathode (data not shown). The only stable derivative with an altered charge is that produced by the carbamylation of amino groups (18). Neutralization of each successive amino group produced a shift representing the effect of a missense mutation in which a lysine residue is substituted by an uncharged residue. Since the ϵ-NH_2 of lysine is fully protonated below pH 7, we can equate these incremental shifts in VP2, VP3, and VP4 with unit charge changes. Identical shifts would be expected for charge changes involving highly basic arginine residues. Figure 2 shows that an

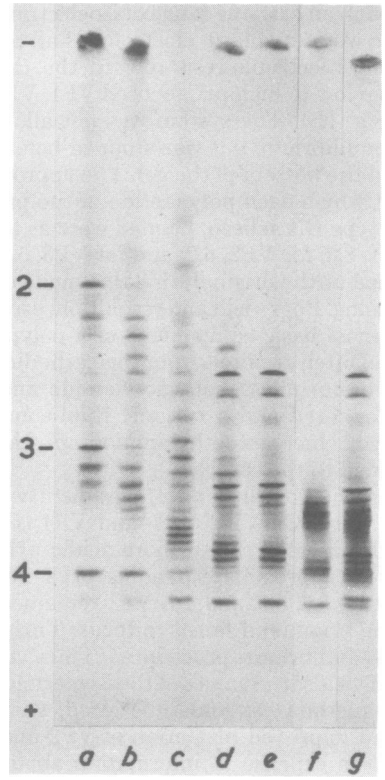

FIG. 2. *Detection of single charge changes and discrimination among different classes of the group that can be ionized. Autoradiograph of [^{35}S]methionine-labeled FMDV polypeptides electrofocused toward the anode after chemical modification as follows: (a) untreated; (b to e) carbamylated for 1, 10, 30, and 100 min, respectively; (f) succinylated; (g) coelectrofocused samples of (e) and (f).*

endpoint in the carbamylation was attained after 10 to 30 min of incubation. Apparently, VP4 has a single amino group.

Charge change substitutions involving aspartic acid or glutamic acid residues may produce smaller shifts for polypeptides that focus in the acidic region of the pH gradient, where carboxyl groups are not fully ionized. The most acidic polypeptide, VP4, illustrates this point very clearly: the single amino group of VP4 was either carbamylated or succinylated, the former treatment neutralizing the charge and the latter reversing it by substituting a carboxyl group. The difference (Fig. 2g) represents the effect of a missense mutation in which an uncharged residue is substituted by an aspartic acid or glutamic acid residue. Although small when compared with a unit charge shift, this class of mutation in VP4 appears to be detectable by electrofocusing.

FMDV mutants with altered structural polypeptides. Of the 73 *ts* mutants that were

examined, 24 manifested charge changes in one or more structural polypeptides. Examples of all of the variant forms of polypeptides are shown in Fig. 3, 4, and 5. The variant polypeptides appeared to have unchanged molecular weights, as judged by electrophoretic mobility on SDS-polyacrylamide gels.

Compared with *ts*$^+$ (Fig. 3a), the parental strain, *ts* 3, 37, and 22 (Fig. 3b to g) showed shifts of varying magnitude in the isoelectric point of VP2. In the case of *ts* 22, the shift was exactly one unit of charge, whereas for *ts* 37 the shift was slightly smaller and in the opposite (basic) direction. Two mutants, *ts* 109 (Fig. 3h and i) and 58 (Fig. 4A, gels b and c), were altered in both VP1 and VP2. The VP2 shift of *ts* 109 appeared identical to that of *ts* 22, and the VP2 shift of *ts* 58 appeared identical to that of *ts* 37 (data not shown). Figure 3j to m illustrate two different examples, *ts* 28 and 107, of mutants with shifts in the isoelectric points of VP3.

Six mutants showed basic shifts of varying magnitude in VP1. The variant VP1 of *ts* 109 is shown in its intact form in Fig. 3h and i, and in Fig. 4A (gels f and g) after proteolytic cleavage to VP1L. Under the latter conditions, a much higher resolution was obtained. Figure 4A shows that the mutational shifts in VP1L of *ts* 16, 40, and 58, although very similar, appeared to be different from each other, the shift in *ts* 16 corresponding to one unit of charge. The difference between the VP1L of *ts* 16 and 109 (gels f and g) is uncertain.

The comparisons of Fig. 4A were facilitated by using bromelain instead of trypsin. The two proteases produce VP1L fragments that are indistinguishable in size but which differ by one unit of charge. Thus, in Fig. 4B, the VP1L of trypsin-treated *ts*$^+$ (gel a) has an isoelectric point identical to that of bromelain-treated *ts* 16 (gel e), whereas the VP1L of trypsin-treated *ts* 16 (gel b) has an isoelectric point identical to that of bromelain-treated *ts* 12 (gel f), which has a double shift. Chymotrypsin and trypsin are alike in their effects on VP1 (data not shown).

ts 103 showed an interesting charge change in VP1 (Fig. 5). The ionization of the residue involved is such that VP1L, which focuses at pH 6.5, is fractionally shifted by approximately 0.4 unit of charge, whereas intact VP1 at pH 8.3 is indistinguishable from *ts*$^+$. The alteration in VP1L is purely a charge effect since mutant and parental peptide fragments comigrated on SDS-polyacrylamide gels. These properties of *ts* 103

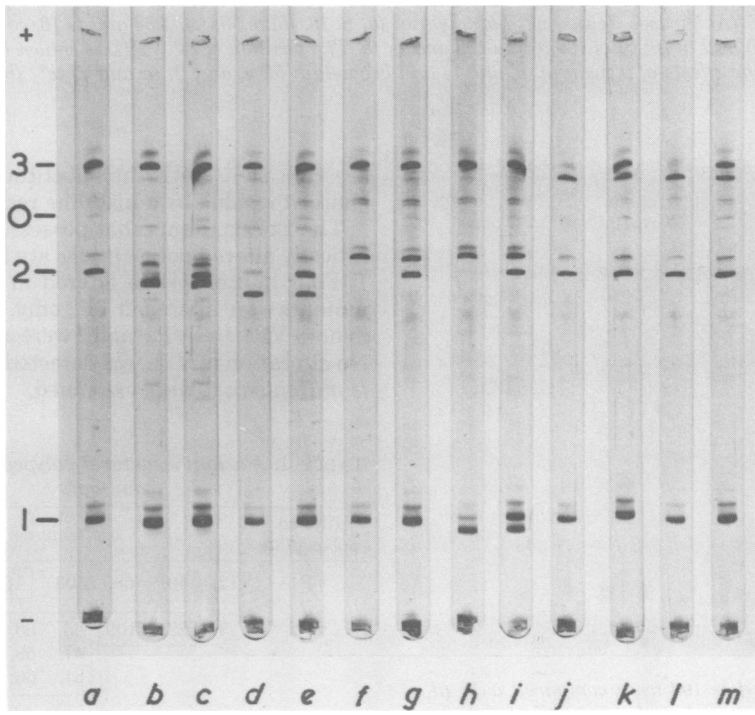

FIG. 3. *Examples of FMDV mutants with altered structural polypeptides: (a) ts*$^+$*; (b) ts 3; (c) ts 3 and ts*$^+$*, co-electrofocused; (d) ts 37; (3) ts 37 and ts*$^+$*; (f) ts 22, (g) ts 22 and ts*$^+$*; (h) ts 109; (i) ts 109 and ts*$^+$*; (j) ts 107; (k) ts 107 and ts*$^+$*; (l) ts 28; (m) ts 28 and ts*$^+$*. Electrofocusing was toward the cathode.*

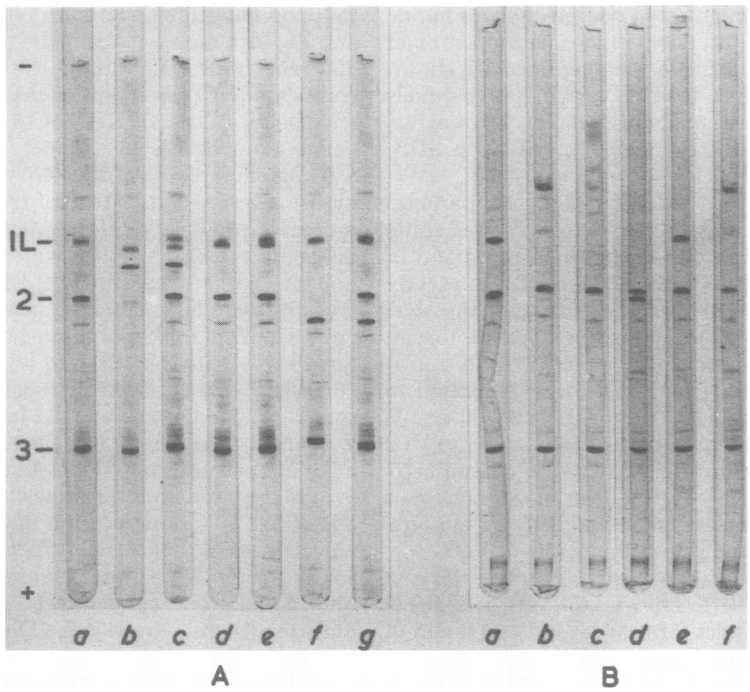

FIG. 4. *High-resolution electrofocusing of structural polypeptides from mutants altered in VP1L. Viruses that had been treated with protease were analyzed by electrofocusing in the anodic mode, using a modification in which the Ampholine mixture was at a pH 3.5 to 10/pH 5 to 7 ratio of 2:1, and electrofocusing was continued for 16 h at 400 V. (A) Viruses treated with bromelain: (a) ts 16; (b) ts 58; (c) ts 58 and ts 16, co-electrofocused; (d) ts 40; (e) ts 40 and ts 16; (f) ts 109; (g) ts 109 and ts 16. The position of ts⁺ VP1L is shown in (B), gel d. (B) Comparison of the effects of trypsin (a, b, and c) and bromelain (d, e, and f): (a and d) ts⁺; (b and e) ts 16; (c and f) ts 12.*

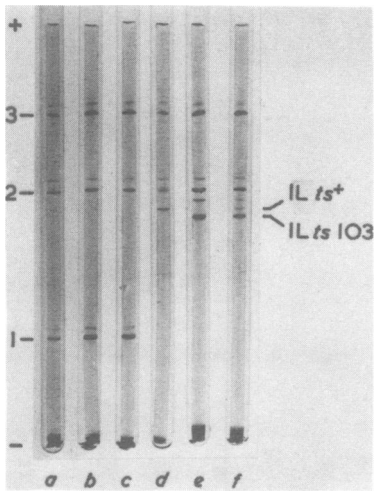

FIG. 5. *ts⁺ and ts 103 were compared with (d, e, and f) and without (a, b and c) prior tryptic cleavage of VP1: (a and d) ts⁺; (b and e) ts 103; (c and f) ts⁺ and ts 103, co-electrofocused. Electrofocusing was toward the cathode.*

are consistent with the substitution of an uncharged residue by a histidine residue.

The 24 *ts* mutants that possessed electrophoretically altered polypeptides are listed in Table 1. Four mutants were altered in VP1 only, 16 mutants were altered in VP2 only, 2 were altered in both VP1 and VP2, and 2 were altered in VP3. No alteration in VP4 was detected among the 73 *ts* mutants that were examined.

TABLE 1. *Variant structural polypeptides of FMDV ts mutants*

Altered polypeptide	Mutant no.[a]
VP1	12, 40, 58, 103 [16, 109]
VP2	3 [22, 109] [65, 63, 33, 58, 62, 47, 59, 37, 42, 23, 61, 66, 44, 41, 67]
VP3	28, 107

[a] Mutants grouped within a box exhibit a similar polypeptide alteration.

DISCUSSION

Two problems concerning the electrofocusing of FMDV structural polypeptides were the extreme isoelectric points of VP1 and VP4 and the identification of VP1, VP2, and VP3. VP1 is so basic that it could only be focused in its positively charged form before reaching its isoelectric point. This technique, first described by O'Farrell et al. (13), has also been used by Hamann and Drzeniek (6) for electrofocusing the structural polypeptides of poliovirus. In contrast, we were unable to perform electrophoresis on positively charged VP4 under any circumstances; therefore, a minimum of two experiments was needed to examine all four polypeptides. The second problem, identification, was caused by the very similar rates of electrophoretic migration of VP1, VP2, and VP3 in SDS-polyacrylamide gels. The sequential numbering of the virion polypeptides was originally based on this measure, although it obviously provides a useless test of identity, especially for an unfamiliar strain of virus. We therefore took care to identify these three polypeptides by criteria that define their coding sequence. The homology between VP0 and VP2 in type O FMDV was confirmed by the observation that mutations affect them coordinately. There is a need for a simple diagnostic test for distinguishing VP2 from VP3. A good candidate for such a test is the selective resistance of VP3 to proteolytic attack in the partially disrupted (12S) form of the virion.

Controlled carbamylation of virus polypeptides produced a series of bands in which each incremental shift represents one unit of charge. The shifts displayed by the variant polypeptides of *ts* mutants, in most cases, corresponded closely to unit charge shifts, suggesting that such polypeptides are altered in a single amino acid residue. It was concluded from the chemical modification studies that electrofocusing can detect any charge change substitution involving a residue of lysine, arginine, glutamic acid, or aspartic acid in any of the four structural polypeptides. The properties of *ts* 103 suggest that we can add the fifth charged residue, histidine, to that list for any polypeptide except VP1 in its intact (basic) form. None of the 26 instances of mutational shift affected VP4, which is also conserved in evolution (15). The ability to detect substitutions in VP4 involving glutamic and aspartic acid residues is, therefore, significant. In a polypeptide as acidic as VP4, acidic residues must outnumber basic ones, and it was important to know whether the apparent absence of any mutations causing charge changes in VP4 was due to an insensitive method for detecting them or to the fact that few, if any, *ts* mutations are located in the gene for VP4. Figure 2 shows that the method is sensitive to such a shift, even though it is extremely small.

The shifts produced by single-charge changes tend to decrease with the increasing size of the polypeptide, as can be seen by comparing the shifts in VP2 with those of its large precursor, VP0. However, electrofocusing has sufficient powers of resolution to enable amino acid substitutions to be detected in polypeptides that are much larger than those studied here, provided that they are not modified in ways that result in excessive charge heterogeneity. Some polypeptides do not band sharply unless Nonidet P-40 is included in the gel, whereas others, especially acidic polypeptides such as VP4, focus only in the absence of this nonionic detergent.

Chemical modification of VP4 showed that when a polypeptide gains or loses a residue that is only partially ionized at the electrofocusing pH, the resulting shift is smaller than a unit charge shift. Of the 11 distinguishable shifts that were observed, 7 were nonunit shifts. This provides valuable information for enabling us to discriminate among as many independent mutational events as possible and in some instances to infer which type of charged residue is involved. The six mutants with an abnormal VP1 illustrate this. To account for the double charge shift in the VP1 of *ts* 12 by a single base substitution, the genetic code requires a glutamic acid residue to be substituted by a lysine residue. The smallest shift, that of *ts* 103, has all of the properties expected of a neutral-to-histidine substitution. Since a neutral-to-lysine or a neutral-to-arginine substitution represents a unit charge shift, we interpret the slightly smaller shifts of *ts* 40, 58, and 109 as being due to the substitution of an aspartic acid or glutamic acid residue in VP1 by a neutral residue. Moreover, since these three shifts are apparently different from each other, it follows that the mutations are located at different positions in the gene coding for VP1.

All of the alterations in isoelectric points that we observed can be accounted for by single amino acid substitutions. Since the variant polypeptides were not altered in size, it is likely that they all represent the effects of missense mutations.

ACKNOWLEDGMENT

We thank D. McCahon for advice and encouragement.

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