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Recombination and Oligonucleotide Analysis of Guanidine-Resistant Foot-and-Mouth Disease Virus Mutants

KEITH SAUNDERS,[1]† ANDREW M. Q. KING,[1]* DAVID McCAHON,[1] JOHN W. I. NEWMAN,[1] WILLIAM R. SLADE,[1] AND SONJA FORSS[2]‡

Animal Virus Research Institute, Pirbright, Woking, Surrey GU24 0NF, United Kingdom,[1] and Mikrobiologie, University of Heidelberg, 6900 Heidelberg, Federal Republic of Germany[2]

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Guanidine resistance (*gr*) mutations of foot-and-mouth disease virus were mapped by recombining pairs of temperature-sensitive mutants belonging to different subtypes. In each cross, one parent possessed a *gr* mutation. Recombinants were isolated by selection at the nonpermissive temperature and assayed for the ability to grow in the presence of guanidine. From the progeny of three crosses, four different types of recombinant were distinguished on the basis of protein composition and RNA fingerprint. The sequences of the RNase T_1-resistant oligonucleotides were determined and located in the full-length sequence of foot-and-mouth disease virus. The resulting maps show that (i) each recombinant was generated by a single genetic crossover, and (ii) both of the *gr* mutations studied were located within an internal 2.9-kilobase region which spans the P34 gene. This supports our hypothesis that guanidine inhibits the growth of foot-and-mouth disease virus by acting on nonstructural polypeptide P34. Additional evidence was provided by RNA fingerprinting *gr* mutants. In two of four cases the *gr* mutation was associated with a change in an oligonucleotide located near the 3′ end of the P34 gene; in one of these the nucleotide substitution was identified.

Guanidine inhibits the multiplication of several picornaviruses, including foot-and-mouth disease virus (FMDV) (or aphthovirus) and poliovirus, at concentrations that have little effect on the host cell. This inhibition is characterized by a rapid cessation of free virion RNA synthesis, whereas other forms of viral RNA, replicative form and replicative intermediate, continue to be synthesized for a considerable time (3, 8). Little is known about the mechanism of action of guanidine, since it acts only on whole cells or on crude, detergent-free extracts of infected cells (30), more purified forms of the viral RNA polymerase being unaffected by the inhibitor (4, 6).

We reported previously that 4 of 10 guanidine-resistant (*gr*) mutants of FMDV were altered in the isoelectric point of polypeptide P34 (28), implying that the primary target of guanidine is the major product of the middle region of the picornavirus genome, P34 in FMDV, or "2C" in the unified nomenclature of picornavirus proteins (27). However, there remained several uncertainties about the proposed link between guanidine resistance and P34. First, the results provided no information about those *gr* mutants that synthesized an apparently normal P34. Second, it was impossible to check the singularity of our *gr*/P34 mutants by applying the covariant reversion test since there was no way of selecting guanidine-sensitive (*gs*) revertants. Finally, similar studies on poliovirus have produced conflicting evidence for the site of action of guanidine, most implicating the capsid proteins (10, 11, 20, 31), although a recent study by Anderson-Sillman et al. (2) shows that some *gr* mutants of poliovirus also produce an altered 2C.

For these reasons it was important to find an independent method of mapping *gr* mutations. We have shown that different subtype strains of FMDV, with readily distinguishable proteins and RNA fingerprints, have the ability to undergo genetic recombination (17). This paper describes a series of genetic crosses in which one subtype carried a *gr* mutation and the other did not. Analysis of the recombinant progeny shows that, although neither of the *gr* mutants chosen for this study was detectably altered in P34, their mutations were both located in, or near, the P34 gene. Furthermore, one *gr* mutation has been located by sequence analysis to a single base substitution within the P34 gene.

MATERIALS AND METHODS

Viruses. The origin of the wild-type (*ts*$^+$ *gs*) O_1 strain, Pacheco, and its chemically induced *ts* mutants has been described previously (21). Spontaneous *gr* derivatives of *ts*22 and *ts*13 were isolated by the first method described in reference 28. The origin of the O_6 strain, V1, and its spontaneous *ts* mutants was as described previously (17). *ts*$^+$ recombinants were isolated by the infectious-center method described by McCahon and Slade (24).

RNA fingerprints. RNA fingerprints were done on RNA induced in infected cells as described by La Torre et al. (22), except that approximately 10^7 cells were labeled with 0.2 to 0.5 mCi of $^{32}P_i$ and the LiCl fractionation step was omitted. Two-dimensional electrophoresis was as described by Harris et al. (16).

Oligonucleotide sequencing. A 1-μg portion of viral RNA, prepared as described previously (18), was incubated with 3 U of RNase T_1 (Calbiochem) and 25 U of polynucleotide kinase (P-L Biochemicals) in an 80-μl reaction containing 0.1 mCi of [γ-^{32}P]ATP in 0.01 M Tris-hydrochloride (pH 7.5)–12 mM $MgCl_2$–5 mM dithiothreitol for 1 h at 37°C. The reaction was stopped by adding 10 μl of 0.1 M EDTA, and the solution was extracted twice with 1:1 phenol-chloroform. A 20-μg amount of carrier tRNA and ammonium acetate to a final concentration of 2 M were added before the oligonucleotides were precipitated with 3 volumes of ethanol. The 5′-end-labeled oligonucleotides were separated by two-

* Corresponding author.
† Present address: Biophysics Laboratory, University of Wisconsin, Madison, WI 53706.
‡ Present address: Scripps Clinic and Research Foundation, La Jolla, CA 92037.

TABLE 1. Results of genetic crosses

Cross	O_1 parent (location)[a]	O_6 parent (location)[a]	% of infected cells yielding ts^+ virus		
			O_1 only	O_6 only	$O_1 + O_6$
A	*ts*13*gr* (capsid)	*ts*302*gs* (P56a)	<0.06	2.8	13.8
B	*ts*22*gr* (P56a)	*ts*303*gs* (capsid)	1.1	2.5	16.9
C	*ts*22*gr* (P56a)	*ts*304*gs* (P34)	1.1	1.9	16.0

[a] Approximate location of *ts* mutation (see text).

dimensional electrophoresis (see above). The gel was exposed to X-ray film at 4°C, and each unique spot was cut out of the gel and soaked in 0.3 ml of 0.01 M Tris-hydrochloride (pH 7.5)–1 mM EDTA–0.3 M ammonium acetate containing 0.3 μg of carrier tRNA for 48 h at 4°C. After precipitation in 3 volumes of ethanol, the oligonucleotides were redissolved in water and sequenced, using the partial enzyme digestion method described by Harris (15).

RESULTS

Genetic crosses. Pairwise crosses were performed between *ts* mutants of FMDV strains O_1 Pacheco and O_6 V1. The *ts* mutations were used as selectable markers, and the parents were chosen with the aim of generating a variety of recombination events on both sides of the *gr* locus and in both directions. A spontaneous *gr* derivative of the *ts* mutant was used as the O_1 parent in each cross, the O_6 parent being wild type (*gs*) for this character.

Three crosses, A, B, and C, were performed as shown in Table 1. The location of the *ts* mutation of *ts*13*gr* was based on its map locus (25), which corresponded to the region of the genome encoding the capsid proteins (19). The other parent in cross A, O_6-*ts*302*gs*, had a *ts* mutation that covaried with an electrophoretic change in P56a, encoded at the 3′ end of the genome (17). In cross B, the *ts* mutations were reversed in position; the O_1 parent, *ts*22*gr*, has been shown by a variety of means to have a *ts* RNA polymerase, polypeptide P56a (23), whereas the *ts* mutation of the other parent, O_6-*ts*303*gs*, is believed to be located in the coat protein genes (17). In cross C, the same O_1 parent was crossed with O_6-*ts*304*gs*; the latter is unusual in having an electrophoretic alteration in P34 (see Fig. 1C). Two ts^+ revertants of *ts*304*gs* were found to induce a wild-type P34 (not shown), indicating that the protein alteration and temperature sensitivity were caused by the same mutation.

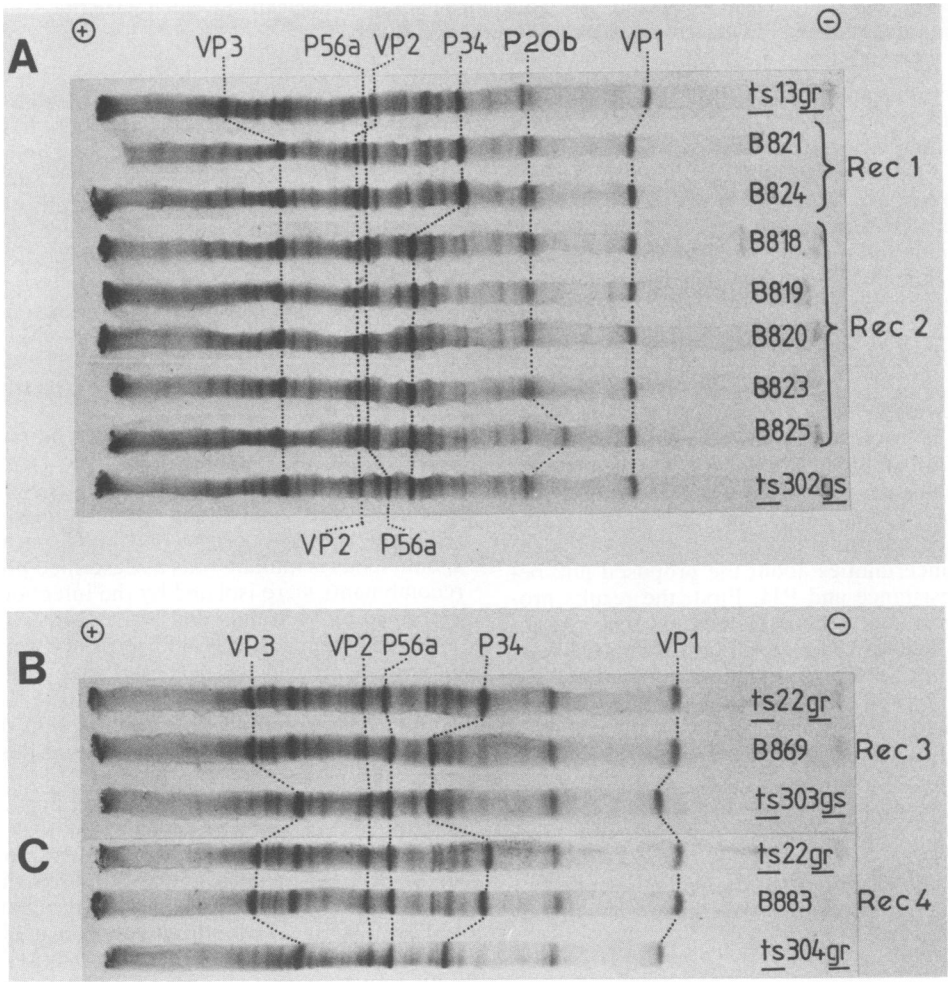


FIG. 1. Detection of recombinants by electrofocusing virus-induced proteins. Infected baby hamster kidney cells were labeled with [^{35}S]methionine for 30 min, followed by a 30-min chase with unlabeled methionone. Electrofocusing was performed in gels containing pH 3.5 to 10 Ampholine ampholytes as described by King et al. (17). The identification of the viral proteins, using two-dimensional gels, is also described in reference 17.

The ts^+ recombinants were isolated by using the infectious-center method (24). In all three crosses the proportion of mixedly infected cells that gave rise to virus plaques at the nonpermissive temperature (13 to 17%) was considerably higher than that of the singly infected controls (0 to 3%; Table 1). Indeed, these values were only slightly lower than those obtained in crosses between mutants of the same strain, carried out at the same time (data not shown). Presumptive recombinant plaques were picked from each cross and cloned at the nonpermissive temperature, and stocks were prepared at the permissive temperature. The entire procedure was carried out in the absence of guanidine.

Analysis of induced proteins. We have shown previously that subtypes O_1 and O_6 can readily be distinguished in five of the major induced proteins by electrofocusing in polyacrylamide gel (17). Recombinants were initially identified among the ts^+ progeny by using this method, and the protein patterns of recombinants isolated from crosses A, B, and C are shown in Fig. 1.

(i) Cross A: O_1-*ts*13*gr* × O_6-*ts*302*gs*. From the locations of the parental *ts* lesions, detailed above, we should expect the 5′ end of a recombinant ts^+ genome to be derived from the O_6 parent and the 3′ end to be derived from the O_1 parent. Seven of the eight ts^+ progeny analyzed possessed these characteristics, having a set of coat proteins resembling those of the O_6 subtype and a P56a resembling that of the O_1 subtype (Fig. 1A). The remaining progeny virus was indistinguishable from O_6ts^+ and was therefore assumed to be a revertant of O_6-*ts*302*gs*. The recombinant patterns were of two types which differed according to the parental origin of P34. Two recombinants, Rec B821 and B824, induced a P34 like that of the O_1 parent, whereas the P34 of the other recombinants in Fig. 1A resembled that of the O_6 parent. The former type of recombinant, termed Rec 1, appeared to have been generated by a genetic crossover approximately in the middle of the genome, between the coat protein and P34 genes, whereas the latter type of recombinant, Rec 2, appeared to have been formed by a crossover nearer to the 3′ end, between the P34 and P56a genes. It can be seen that the protein pattern of one of the Rec 2 recombinants, B825, differs from both parents in a minor band, P20b, situated between P34 and VP1. This could have been caused by either a mutation or a recombinational event within the P20b gene. With this exception, the proteins of all the progeny resembled one or the other parent.

(ii) Cross B: O_1-*ts*22*gr* × O_6-*ts*303*gs*. Of nine ts^+ progeny examined by electrofocusing, only one, B869, gave rise to a recombinant protein pattern (Fig. 1B), the others being indistinguishable from revertants of the O_1 parent. B869 had O_1 coat proteins and O_6 nonstructural proteins P34 and P56a, and thus had the inverse protein composition to that of Rec 1 (Fig. 1B). This type of recombinant is called Rec 3. It may be noted that the protein pattern of the O_1 parent, *ts*22*gr*, was slightly different from that used in cross A, owing to a *ts* mutation affecting P56a. This made the parental origin of P56a more difficult to identify in the recombinants of crosses B and C than in those of cross A. However, our interpretations were confirmed by RNA fingerprinting described below.

(iii) Cross C: O_1-*ts*22*gr* × O_6-*ts*304*gs*. Again, only one, B883, of the eight ts^+ progeny examined gave rise to a recombinant protein pattern (Fig. 1C). This recombinant, type Rec 4, resembled the O_1 parent in all five major proteins except P56a, which resembled that of the O_6 parent. Thus, Rec 4 had the inverse protein composition to that of Rec 2. It completed a set of four different types of inter-subtype recombinant in which reciprocal crossovers on both sides of the P34 gene are represented.

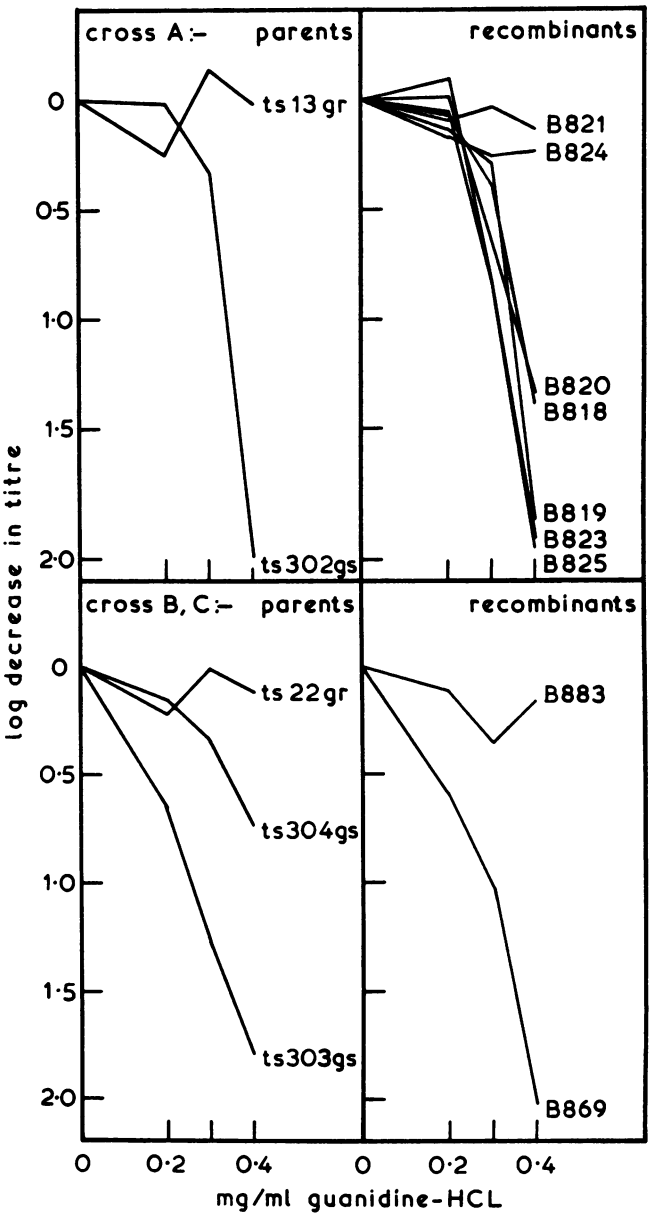
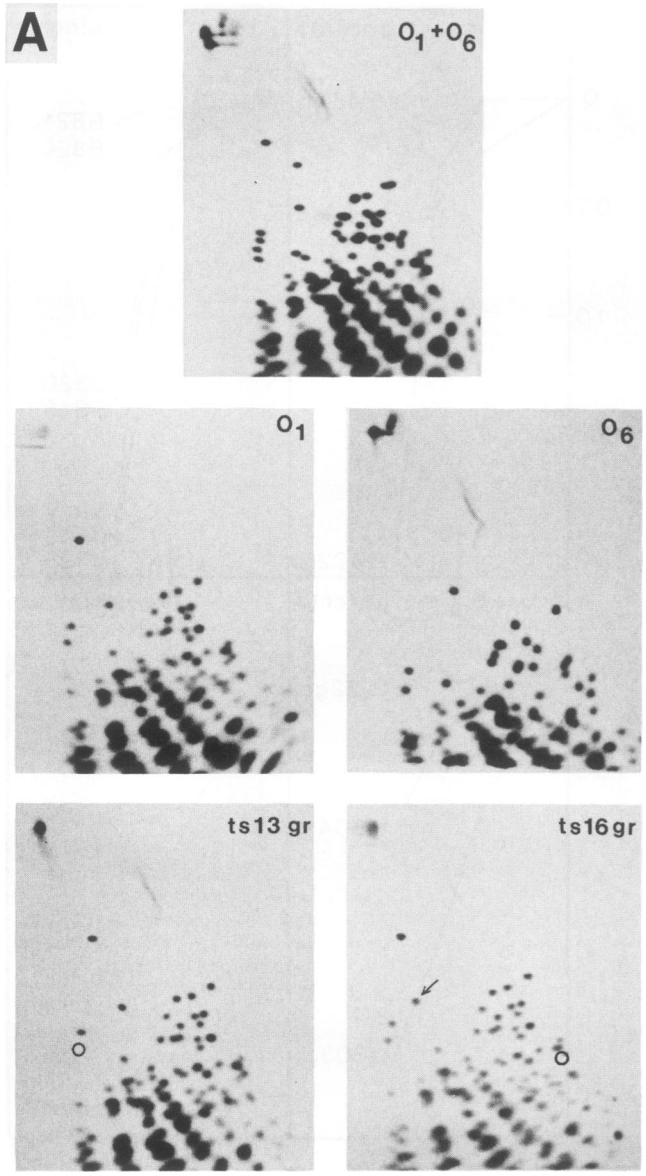


FIG. 2. Assays of guanidine resistance. Cell monolayers were infected in duplicate with virus at a multiplicity of infection of approximately 100 and incubated in the presence of guanidine-HCl for 4 to 6 h. The virus titer was assayed on monolayers in the absence of guanidine.

Guanidine resistance of recombinants. Recombinants, identified by their protein patterns, were assayed for the ability to grow in the presence of three different concentrations of guanidine (Fig. 2).

(i) Cross A. The seven recombinants fell into two distinct groups. Two, B821 and B824, were resistant to guanidine like the O_1 parent, whereas yields of all of the other recombinants declined sharply with increasing concentrations of guanidine, their response being similar to that of the O_6 parent (the slight difference between the O_6 parent and two recombinants, B280 and B818, was not regarded as significant). These results can be correlated with the protein



analyses; both *gr* recombinants were of the Rec 1 type, whereas all *gs* recombinants were Rec 2. Since the only difference between Rec 1 and Rec 2 revealed by electrofocusing was in P34, the results of this cross are consistent with the hypothesis that P34 determines resistance to guanidine.

(ii) Crosses B and C. The parents used in cross B, O_1-*ts*22*gr* and O_6-*ts*303*gs*, responded to guanidine like their *gr* and *gs* counterparts of cross A. In contrast, O_6-*ts*304*gs*, used in cross C, was somewhat less sensitive to guanidine than the other *gs* parents, having an inhibition curve intermediate between *gr* and wild-type *gs*. Despite this, the difference in sensitivity between the two parents of cross C was sufficient to permit the inheritance of the guanidine character to be determined unequivocally; B869 (type Rec 3) was clearly *gs*, whereas B883 (Rec 4) was *gr*.

Since Rec 4 has an O_1-like P34 and Rec 3 has an O_6-like P34, their patterns being otherwise identical, the results of crosses B and C confirm the genetic linkage between guanidine resistance and P34 that was revealed by cross A. However, caution should be exercised in interpreting electrofocusing evidence. A resemblance in a protein between a recombinant and its parent does not imply that the whole of the protein was inherited from that parent, but merely that part of it must have been. For this reason more precise information on the sites of recombination was obtained by fingerprinting the recombinant RNAs.

Oligonucleotide compositions of recombinants. The RNA fingerprints of the two FMDV subtypes are compared in Fig. 3A. The patterns are identical to the O_1 and O_6 fingerprints published previously (17), despite the fact that the latter were done on the virion RNAs of *ts* mutants, whereas those shown here were obtained by fingerprinting RNAs extracted from cells infected with the equivalent ts^+ (wild-type) strains. The fingerprint of the mixture of O_1 and O_6 RNAs shows that these two subtypes can be distinguished in most of the large oligonucleotides. Excluding doublets, there were 21 O_1-specific oligonucleotides and 25 O_6-specific oligonucleotides that could be used as genetic markers for studying recombination. Each set of oligonucleotides was numbered as shown in Fig. 3B.

The RNAs of the five parental mutants were also fingerprinted. Each was identical to the wild type, except for

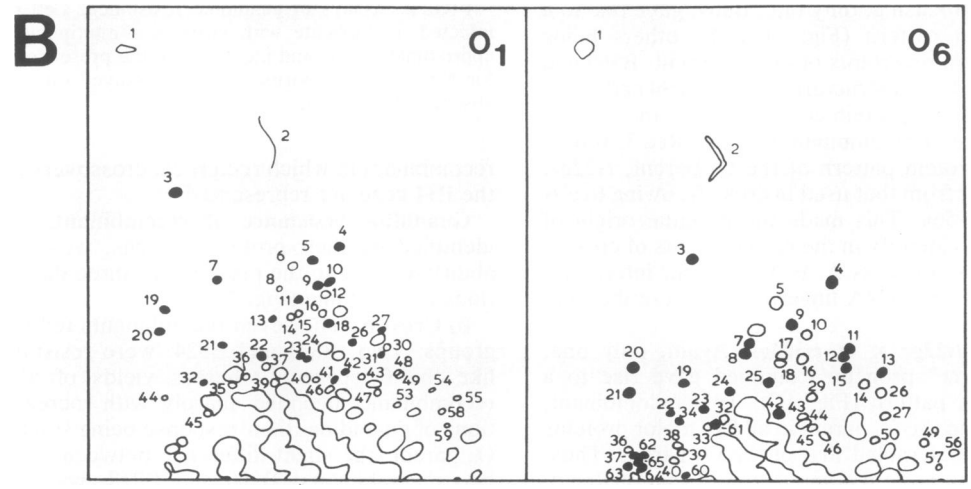


FIG. 3. RNase T_1 fingerprints of the RNA of the wild-type strains, O_1 and O_6, and of two *gr* mutants derived from the O_1 strain (A) and diagrams (B) of these fingerprints. (A) Circles in the fingerprints of *ts*13*gr* and *ts*16*gr* indicate the positions of missing spots 20 and 31, respectively. The arrow in *ts*16*gr* indicates the altered spot 7. (B) Subtype-specific oligonucleotides are shown as filled symbols, whereas regions in which the two strains could not be distinguished are shown as empty symbols.

O_1-*ts*13*gr*, in which spot 20 (indicated by a circle in Fig. 3A) was missing. The remaining fingerprint in Fig. 3A, that of *ts*16*gr*, will be discussed later. The RNAs of all nine recombinant viruses that had been identified by electrofocusing among the ts^+ progeny of crosses A, B, and C were fingerprinted. Four different types of fingerprint were obtained, corresponding to the four different types of protein pattern defined earlier: Rec 1, 2, 3, and 4. Thus, the oligonucleotide patterns of the two Rec 1 viruses were indistinguishable from each other, as were those of the five Rec 2 viruses. A representative of each type of fingerprint is shown in Fig. 4.

The oligonucleotide compositions of Rec 1, 2, 3, and 4 were determined by comparing their fingerprints with that of the subtype mixture shown in Fig. 3. All oligonucleotides appeared to be inherited from one or the other parent, there being no evidence of aberrant spots. The subtype-specific oligonucleotides are listed in Table 2. It can be seen that the compositions of Rec 1 and Rec 3 RNAs were essentially the reciprocal of each other, in that all subtype-specific oligonucleotides present in Rec 1 RNA were missing from Rec 3, and vice versa. The only exception was O_1 oligonucleotide 20, which was missing in both types of recombinant. However, this can be explained by the fact that the O_1 parent of Rec 1, *ts*13*gr*, lacked this oligonucleotide. Rec 2 and Rec 4 were also reciprocals of each other. Hence, on the basis of these four types of recombinant, the oligonucleotide markers of both parents could be classified into three linkage groups: (i) those oligonucleotides invariably inherited with coat proteins of the same subtype, (ii) those similarly linked to polypeptide P34, and (iii) those linked to P56a (Table 2).

Location of oligonucleotides. Nucleotide sequences were determined for the oligonucleotides of both O_1 and O_6 parental strains and are given in Table 3. Since the sequence of neither strain was known, the oligonucleotides were located by searching for homologous regions in the sequences of other FMDV strains. Most of the oligonucleotides were located in the sequence of O_1 Kaufbeuren (O_1K; 14a), although three possessed significant homology with strain A_{10} (9) only. The test of significance applied in each case was that there should be less than 5% probability of an equal or better match arising by chance in a random 8-kilobase sequence. A total of 38 oligonucleotides (19 of each subtype) were located in the FMDV genome. Eight subtype-specific oligonucleotides could not be mapped with 95% confidence, either owing to sequence divergence between the parental and reference strains or because the oligonucleotides were located in the noncoding region on the 5′ side of the polycytidylic acid tract, for which no sequence was available. The locations of the oligonucleotides are given in Table 3. It can be seen that eight of the O_1 oligonucleotides were homologous to O_6 oligonucleotides. The average degree of homology in nucleotide sequence between these pairs of oligonucleotides was 89%.

TABLE 2. Inheritance of oligonucleotides by recombinants

Recombinant type	Oligonucleotides linked to:		
	Coat proteins	P34	P56a
Rec 1	O_6: 3, 4, 9, 11, 12, 13, 15, 20, 22, 24, 34, 42, 60, 62, 63	O_1: 5, 7, 22, 32	O_1: 21, 41, 42
Rec 2	O_6: 3, 4, 9, 11, 12, 13, 15, 20, 22, 24, 34, 42, 60, 62, 63	O_6: 7, 19, 21, 23, 61, 62, 64, 65	O_1: 21, 41, 42
Rec 3	O_1: 3, 4, 9, 10, 11, 13, 18, 19, 23, 24, 26, 27, 31	O_6: 7, 19, 21, 23, 61, 62, 64, 65	O_6: 8, 25, 37
Rec 4	O_1: 3, 4, 9, 10, 11, 13, 18, 19, 23, 24, 26, 27, 31	O_1: 5, 7, 20, 22, 32	O_6: 8, 25, 37

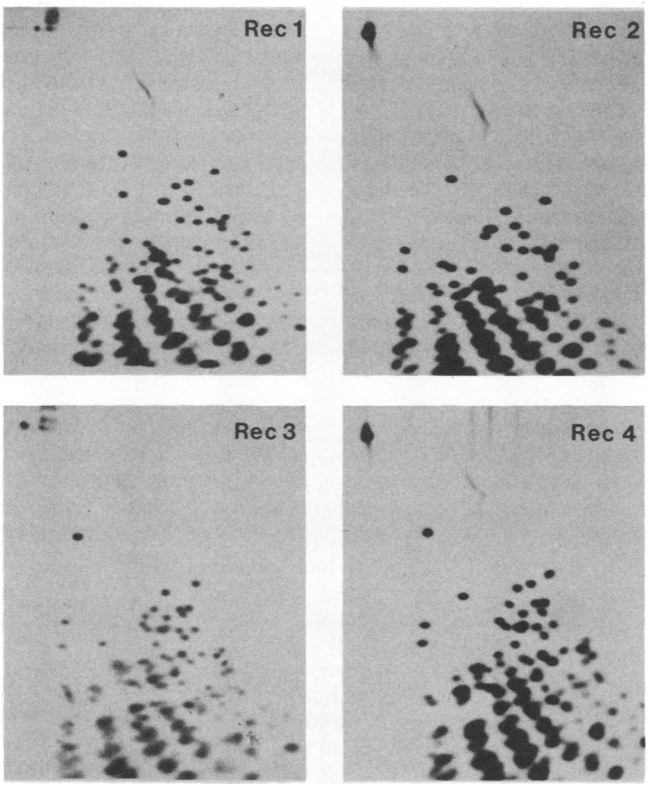


FIG. 4. Representatives of the four types of recombinant RNA fingerprint.

Sites of genetic recombination. Figure 5 shows the genome locations of the oligonucleotide and protein markers of the two parental strains. From the compositions listed in Table 1, biochemical maps were constructed for each of the four types of recombinant, and these are also shown in Fig. 5. It may be noted that the physical locations deduced from sequence data agreed with the genetic analysis of Table 1, in that proteins and oligonucleotides belonging to the same linkage group invariably occupied neighboring positions in the genome.

Each of the four types of recombinant appeared to have been produced by a single recombinational event, and Fig. 5 indicates the regions in which these events occurred. Since Rec 1 possessed oligonucleotide O_6-34 but lacked the neighboring oligonucleotide O_6-61, recombinants of this type must have been produced by genetic crossovers somewhere within the 1.0-kilobase region between these two markers, a region spanning the 3′ end of the P12 gene and the 5′ end of the P34 gene. Rec 3, being the reciprocal of Rec 1, resulted from a crossover in the same region, but in the opposite direction to that of Rec 1. Similarly, recombinants of the Rec 2 and Rec 4 types were generated by crossovers in the region between oligonucleotides O_1-32 and O_1-42, which lies to the 3′ side of the P34 gene.

Knowledge of crossover sites permits limits to be placed on the possible locus of the *gr* mutations of O_1-*ts*13*gr* and O_1-*ts*22*gr*. Rec 1 and Rec 4 recombinants carried the O_1

TABLE 3. Sequences and locations of oligonucleotides

Oligonucleotide	Sequence[a]	Position[b]
O_1	1 11 21 31	
3	ACACAACCUC CACCCACACA ACCAAC-CC -AAAC---G	1565–1603
4	UUCACCUUUU CCA-CCCCUA CCAC--G	2779–2805
5	AUCUCAAUUC CUUCUCAAAA A-C-G	5080–5104
7	CCUCAACCAC CCCUCCAAAA CAUG	4981–5004
9	CACCUUUCCU UUACAAACAC -G	777–799[c]
10	UUUCUUCAAA ACACACC-C- -CG	1842–1864
11	-UCACACUUU UCC--CACC- G	2080–2100
13	CACACAAACC ACUUCUA-G	2970–2988[d]
18	CACUAUUACC ACUCUC-G	
19	AAAACACCAC CAACCCAACG	3272–3291
20	ACACCCACAC CAACCC-G	4883–4900
21	CCAAACCAUC ACUCC-G	7473–7489
22	CCACCCAAUC UUCA-G	5061–5076
23	UU-CAAAUCA CACACG	2875–2890
24	UACCAAAA-- CCA-G	1495–1509
26	UCAAACACCU UUCU-G	2575–2590
27	AACACUUUUU CUAC-G	
31	ACCUUUUACU CU-G	922–935
32	CAAACAUCAC CA-G	5369–5382
41	AACAACAUUU A-G	7321–7333
42	UCAUCUUCUC CA-G	6554–6567
O_6		
3	ACACAACUUC CAAUC-C-AA AAC-AC-C-C -G	1565–1596
4	-UCACUUUCU CCAUCCCCUA -C---CG	2779–2805
7	UACAAAAUC- -C--C---C- G	4840–4860
8	CCA-CUUCUC CAAACACA-G	6554–6573
9	-UUCUUCAAA ACCCACCUA- --G	1842–1864
11	UCCACUUACU AC--C-C-G	3181–3199
12	AAACCUUUUC CUUUCACG	
13	UUUUACCAAU CUCCUUG	2436–2452
15	UCAAAUUUCU CCAAAUUG	3688–3705
19	CCUCAACCAC CCC-CC-G	4981–4998
20	AACACCACCA ACCCAAC-G	3274–3292
21	ACACCCACAC CAACC-G	4883–4899
22	ACCUCAACAA CAC-G	
23	ACCCCUCAUC CAACG	5196–5210
24	UCUACAACUC ACCCC-G	1016–1032[d]
25	UCAAACCAAU AC-CC-G	7473–7489
34	AAACCAUCAA CC-G	3710–3723
42	CUC-UCCCCC ATCAG	
60	UUAUCAAAC- G	
61	CAACCAC-AA CAUG	4736–4749
62	AAACCACAAA -G	2843–2854
63	-CCCAACCAA CG	2273–2284
64	AAACCCACAA G	5312–5322[d]
65	CAAACAACAA -G	

[a] Bases underlined indicate positions at which the sequences of the oligonucleotides differed from that of strain O_1K (14a).
[b] Positions are with reference to the O_1K sequence.
[c] Sequence match assumes a one-base deletion as in strain C_1 (5).
[d] Significant sequence homology at this locus observed with the A_{10} strain (9).

determinant of guanidine resistance, whereas Rec 2 and Rec 3 did not. The only region of the O_1 genome common to the former pair of recombinants, but lacking in the latter, was the central region between oligonucleotides O_6-34 and O_1-42. Both *gr* mutations must therefore lie within this region. The possible locus includes the regions coding for P34, P19, and P20b, but excludes all of the capsid protein genes and nearly all of the polymerase (P56a) gene.

Oligonucleotide alterations in *gr* mutants. It was noted earlier that one of the five parental mutants used in this study, O_1-*ts*13*gr*, had an altered RNA fingerprint, with spot 20 missing (Fig. 3). Its parent, *ts*13*gs*, had a normal RNA fingerprint (not shown), and that *ts*13*gr* was isolated as a spontaneous, single-step derivative of *ts*13*gs* suggests that guanidine resistance may have been conferred by the mutation in oligonucleotide 20. This oligonucleotide belongs to the group of oligonucleotides that are genetically linked to guanidine resistance and is located within the P34-coding region.

Of three other *gr* mutants examined in this way, one, a 5-fluorouracil-induced mutant called *ts*16*gr*, was also found to have an altered RNA fingerprint. Spot 7 was shifted and spot 31 was missing (Fig. 3A). *ts*16*gr* is unusual in that its *ts* and *gr* characters are conferred by the same mutation (28), thus permitting the isolation of *gs* revertants. Four independent ts^+gs revertants of *ts*16*gr* were examined, and in three

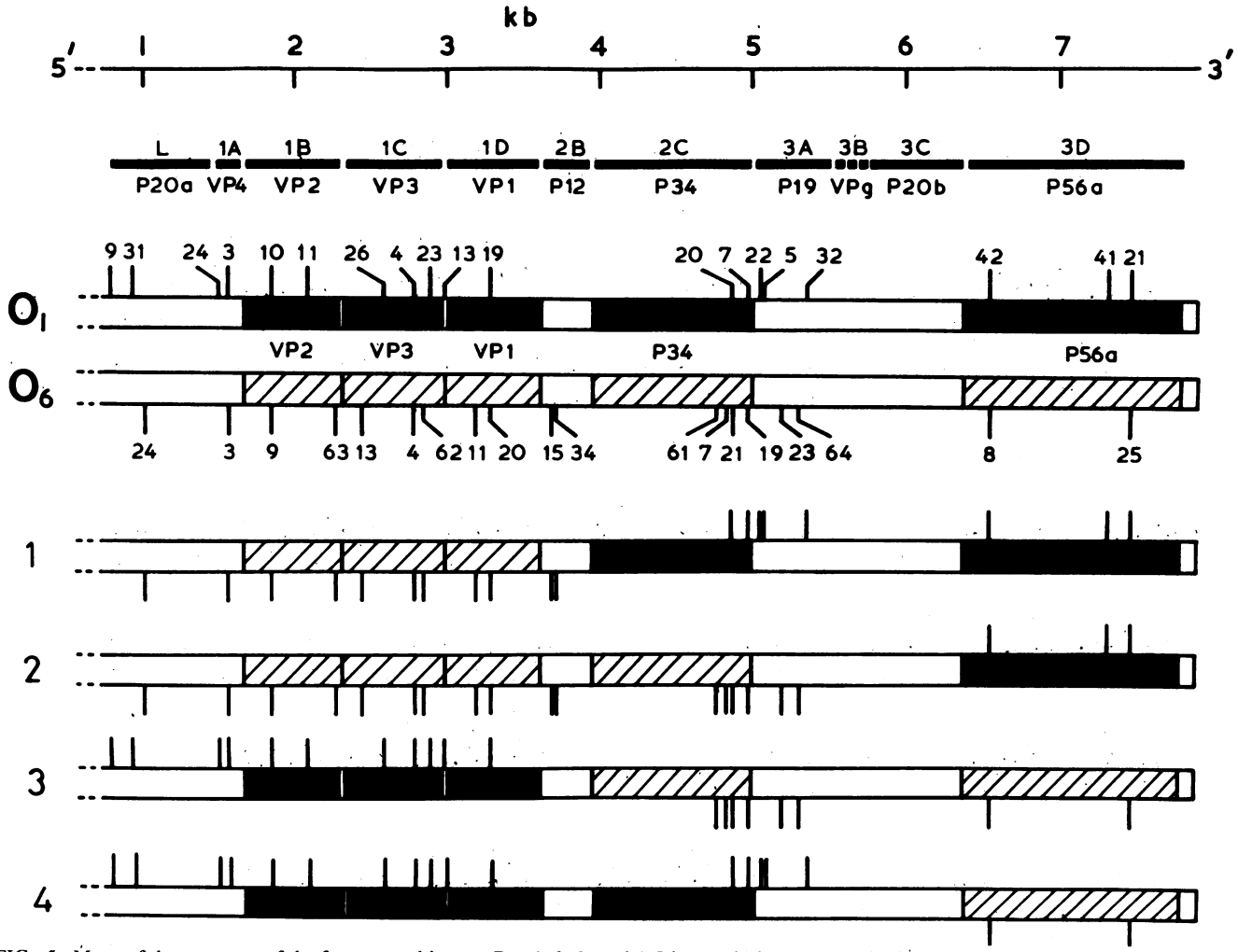


FIG. 5. Maps of the genomes of the four recombinants, Rec 1, 2, 3, and 4. Lines pointing upwards indicate O_1 oligonucleotides, and those pointing downwards indicate O_6 oligonucleotides. Filled regions indicate O_1 proteins, and shaded regions indicate O_6 proteins. Coding regions of the viral proteins, shown at the top, are identified in two ways: names above each gene are based on the new unified nomenclature for picornaviruses (27), and those below were designated by the traditional FMDV nomenclature. kb, Kilobases.

of them spot 7 was found to have reverted to its wild-type position (not shown). Other mutations possessed by *ts*16*gr*, which caused the loss of spot 31 (Fig. 3A) and an electrophoretic shift in VP1 (18), were retained by the revertants.

The positions of the wild-type and mutant forms of spot 7 in the fingerprint indicated that the former contained three U residues and the latter contained only two. The site of the mutation was determined by comparing their sequences (Fig. 6). This revealed a U-to-C change at the penultimate nucleotide. Alignment of the sequence with the long open reading frame of FMDV indicates that the *gr* mutation was located at position 5,003 and resulted in the substitution of a methionine residue by a threonine 25 amino acid residues from the carboxy terminus of P34.

DISCUSSION

Locus of *gr*. Recombination analysis places both *gr* mutations between 3.7 and 6.6 kilobases on the physical map of FMDV shown in Fig. 5. That P34 is encoded within this region, and was itself genetically linked to *gr*, is consistent with our previous observation that *gr* mutations are frequently accompanied by changes in P34 (28). Thus, the recombination experiments support the conclusion of our previous study (28) that *gr* mutations are carried by P34 even when, as in the mutants used here, no alteration could be detected in that protein. Although the possible locus of *gr*, defined by these recombination studies, includes parts of several nonstructural protein genes, it does lie entirely outside the region of the genome that codes for the capsid proteins; there is no evidence from any of our biochemical studies on *gr* mutants of an involvement of capsid protein in guanidine resistance, such as has been proposed by several workers for poliovirus (10, 11, 20, 31). However, our results agree with two recent studies (1, 13) in which poliovirus recombinants were selected by using a combination of specific antiserum and guanidine. The locus of *gr* is defined most precisely by the recombinant RNA fingerprints published by Agol et al. (1); examination of these shows that *gr* lies to the 3' side of oligonucleotide 3, which is located near the 5' end of the P2-X gene.

Oligonucleotide changes in the RNAs of *ts*13*gr* and *ts*16*gr* provide clues to the precise locus of *gr*. The two oligonucleotides involved, O_1-20 and O_1-7, were located within 100 bases of each other near the 3' end of the P34 gene. In the case of *ts*16*gr*, coreversion of oligonucleotide 7 and guanidine resistance confirms that the same mutation was respon-

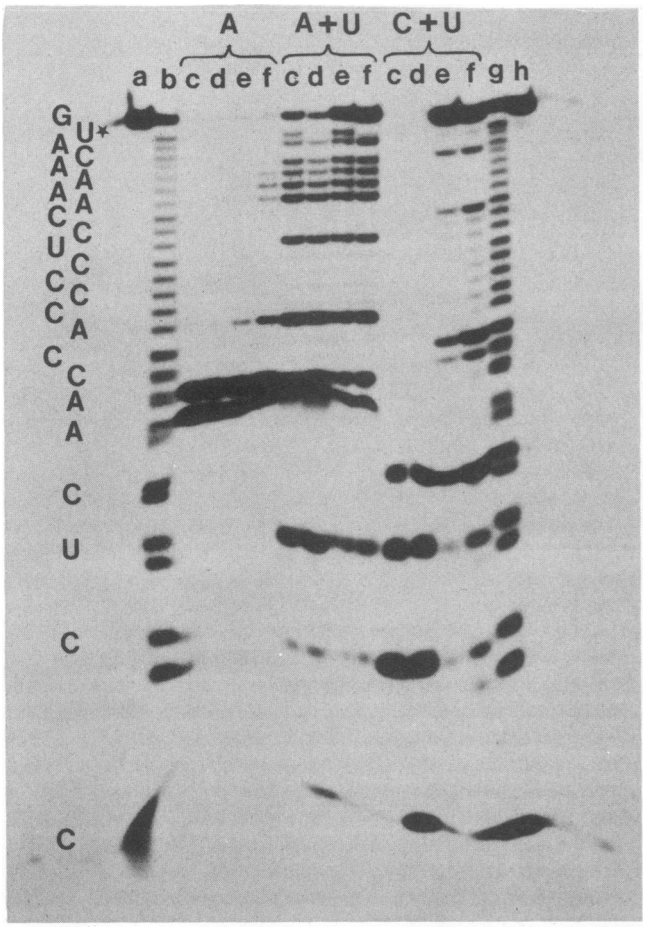


FIG. 6. Comparison of the sequence of oligonucleotide 7 of *ts*13*gr* (lanes d, f, g, and h) with that of the wild type (lanes a, b, c, and e). The site of the single base substitution (U to C) is starred. Sequencing was by partial digestion of 5'-end-labeled oligonucleotide with the following RNases: U2 (A specific), Phy M (A and U specific), and *Bacillus cereus* (C and U specific). Digestions were done with a high (lanes c and d) and a low (lanes e and f) concentration of each enzyme; for details, see text. Untreated oligonucleotide was run in lanes a and h, and alkali-treated oligonucleotide was run in lanes b and g.

sible for both characters. A high frequency of primary, as opposed to second-site, revertants has been a notable feature of previous studies of FMDV mutants (19).

The results of this and our previous study (28) bring the number of independent *gr* mutations that have been mapped in or near the P34 gene to eight. O_6-*ts*304, though used here as a "*gs*" parent, represents yet another example, the ninth, of a link between *gr* and P34, since it possessed an electrophoretically altered P34 (Fig. 1C) and was significantly less sensitive to guanidine than the wild type was (Fig. 2). The variety of biochemical phenotypes exhibited by these nine mutants suggests that guanidine resistance can result from a change in one of several amino acid residues in P34. We therefore conclude that there are probably several sites, limited to a small carboxy-terminal region of P34, at which mutations may confer resistance to guanidine.

Function of 2C. Guanidine should prove to be a useful probe for elucidating the function of picornavirus protein 2C (the systematic name for P34 and its poliovirus equivalent, P2-X). The effects of the inhibitor on viral RNA replication indicate that 2C is required for some aspect of RNA synthesis other than chain elongation. In considering what that function might be, it should be remembered that the actual target of inhibition need not be 2C itself, since picornaviruses produce replicative proteins in vast excess over RNA molecules. The functionally active form could be any protein containing the carboxy terminus of 2C.

Two replicative processes have recently been described, either of which could be tested as possible targets of guanidine. First, an enzymatic activity that adds two uridylate residues to VPg, so generating a primer suitable for initiating the synthesis of either strand of poliovirus (or FMDV) RNA, has been discovered in a cell membrane fraction (29); uridylated VPg has also been identified in infected cells (12). Second, an investigation of the effect of psoralen cross-linking on the poliovirus replication complex (26) shows that the in vivo form of the replicative intermediate is predominantly single stranded, implying that an unwinding activity exists for separating the newly synthesized RNA strand from its template. The suggestion that protein 2C performs the latter function is especially attractive, since failure to unwind growing RNA chains from their templates would explain the altered sedimentation coefficient of the replicative intermediate synthesized in the presence of guanidine (3, 8) and the relative increase in the amount of replicative form.

It is noteworthy that 2C is a membrane-bound protein (7, 29) and some of the effects of guanidine on RNA replication in infected cells are similar to those of nonionic detergents on cell extracts (14). We have speculated previously that the integrity of the 2C-membrane complex is likely to be essential for 2C function (28).

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