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Sequence Alterations in Temperature-Sensitive M-Protein Mutants (Complementation Group III) of Vesicular Stomatitis Virus

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Sequences were determined of the coding regions of the M-protein genes of the Glasgow and Orsay strains of vesicular stomatitis virus (Indiana serotype) and of two group III (M-protein) mutants derived from each wild type. Synthetic primers were annealed with viral genomic RNA and extended with reverse transcriptase. The resulting high-molecular-weight cDNA was sequenced directly. Both Glasgow and Orsay wild types differed in 13 bases from a clone of the San Juan strain sequenced by J. K. Rose and C. J. Gallione (J. Virol. 39:519–528, 1981). Six of these base changes caused amino acid changes in each wild type, whereas seven were degenerate. The Orsay and Glasgow sequences resembled each other more closely than either resembled that of Rose and Gallione, differing in eight nucleotides and four amino acids. Each of the four mutants, however, differed from its parent wild type in only one or two point mutations. Every mutation caused a change either from or to a charged amino acid; the change for *ts*G31 was Lys (position 215) to Glu, the change for *ts*O23 was Gly (position 21) to Glu, the change for *ts*O89 was Ala (position 133) to Asp, the changes for *ts*G33 were Lys (position 204) to Thr and Glu (position 214) to Lys. The charge differences predicted from these amino acid changes was confirmed by nonequilibrium pH gradient electrophoresis for *ts*G31, *ts*G33, *ts*O23, and the two wild types. These mutations affect residues spanning nearly 85% of the linear sequence, although the mutants possess nearly identical phenotypic properties.

The M protein of vesicular stomatitis virus (VSV) is one of the three major proteins found in purified virions, accounting for nearly one-third of the total protein in purified viral preparations (1). M protein appears to be required for viral budding (21, 22, 25), implying that it must interact with both viral nucleocapsids and membranes. Interaction with the viral nucleocapsid is indicated by the M protein's ability to inhibit viral transcription, both in detergent disrupted viral preparations (3, 6, 26) and in infected cells (5, 11). M-protein binding to nucleocapsid depends largely on electrostatic interactions, since inhibition can be reversed by the addition of salt or polyanions such as polyglutamic acid (2, 3, 6, 26). The interaction of M protein with lipid membranes of intact virions has been demonstrated by cross-linking with radio-labeled lipids (15) and by labeling with membrane-specific probes (10, 27). The wild-type M protein does not appear to penetrate deeply into the membrane, however, since it does not react with a probe localized close to the center of the viral bilayer (24).

The temperature-sensitive mutants of VSV provide an important tool for the study of individual viral proteins. VSV has five transcribed genes, and its mutants fall into five complementation groups (16); group III corresponds to mutations in the M protein (7, 8, 16). Earlier studies from this laboratory have characterized some properties of the four group III mutants sequenced in the present study (9, 10, 17, 26). All four mutants behaved in a practically identical fashion. A weakening of electrostatic interactions between mutant M protein and nucleocapsid (as compared with the wild type) was indicated by (i) a decrease in the M protein's ability to inhibit polymerase activity in detergent-disrupted virions (9, 26), (ii) a decrease in spatial proximity between M protein and the nucleocapsid N protein in intact mutant virions as indicated by cross-linking experiments (9, 10), and (iii) an increase in RNA synthesis in cells infected with group III mutants at permissive temperature, as shown by others (5, 11). Most surprisingly, this decrease in M protein-nucleocapsid interaction was associated with a coordinate increase in the association of mutant M protein with membranes. This was demonstrated by (i) an increase in labeling of mutant as compared with wild-type M protein in intact virions by a membrane soluble photoactivated probe (9, 10) and (ii) a decrease in G-protein mobility on the surface of mutant as compared with wild-type-infected cells at the permissive temperature, measured by fluorescence photo-bleaching recovery (9, 17).

The present study was undertaken to determine the amino acid changes that underlie the altered properties of the mutant M proteins. We show that each mutant phenotype can be accounted for by a single amino acid substitution that decreases the M protein's positive charge or increases its negative charge. Since the mutated residues span nearly 85% of the linear sequence, it appears that most of the molecule is involved in its function.

MATERIALS AND METHODS

Cells and viruses. The Orsay and Glasgow variants of VSV Indiana and the group III mutants were obtained from A. Huang and A. Flamand. Viruses were grown in BHK-21 cells and isolated and purified as described previously (13). All stocks were plaque purified, and their temperature sensitivity was determined by plaque assay on Vero cells at 31 and 39°C.

Isolation of viral genomic RNA. Purified virus was pelleted and suspended (2 to 3 mg/ml) by brief sonication in 10 mM Tris–1 mM EDTA (pH 7.0). The virus was deproteinized with proteinase K (Sigma Chemical Co.; 0.04 to 0.5 mg/ml) in 1% sodium dodecyl sulfate and 0.25 M NaCl at 55°C for 10 min. The mixture was extracted twice with phenol-chloroform and once with chloroform, and the aqueous phase was precipitated twice with ethanol.

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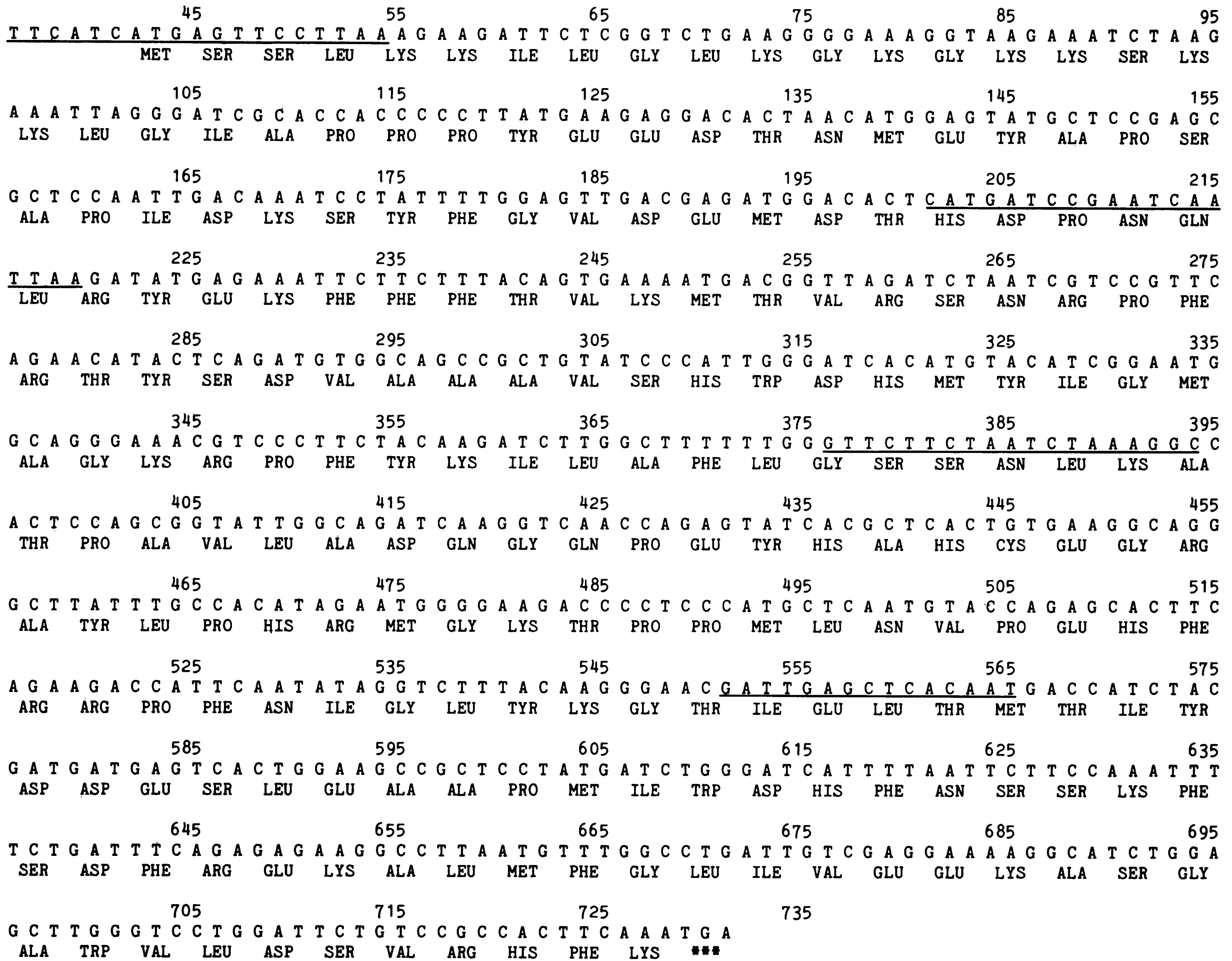

FIG. 1. Nucleotide sequence of cDNA in the translated region of the Glasgow M-protein gene and predicted amino acid sequence. The four synthetic primers used in this study are underlined. Numbering is from 3′ end of the M gene as reported by Rose and Gallione (19).

5′-End labeling of primers. Four synthetic primers were synthesized by D. H. L. Bishop, University of Alabama, Birmingham. The sequences were based on the sequence of M-protein mRNA published by Rose and Gallione (19) and chosen to hybridize with regions about 180 residues apart in the viral genome (see Fig. 1). The primers were treated with calf intestinal phosphatase (45°C for 45 min). After heat inactivation of this enzyme, the primers were labeled at their 5′ ends with T4 polynucleotide kinase (P-L Biochemicals) and [γ-^{32}P]ATP (ICN Pharmaceuticals Inc.).

Primer extension. Labeled primer was annealed to viral genomic RNA template by incubation at room temperature for 10 min in the presence of methylmercuric hydroxide and extended by using reverse transcriptase (Seikagaku America, Inc.). The reaction mixture (50 μl) contained 50 μg of viral genomic RNA, 50×10^6 cpm of end-labeled primer, 10 mM methylmercuric hydroxide, 10 mM dithiothreitol, 50 mM Tris hydrochloride (pH 8.3), 6 mM magnesium chloride, 110 mM potassium chloride, the four deoxynucleoside triphosphates at 1 mM each, and 40 U of reverse transcriptase. The reaction was incubated at 37°C for 2 to 3 h and terminated by the addition of 5 μl of 500 mM EDTA.

Sequence analysis. The template in the extended reaction product was hydrolyzed by the addition of 0.3 M sodium hydroxide (30 min at 50°C). After extraction with phenol and ether, the dried reaction product was taken up in 20 to 30 μl of 10 mM Tris–1 mM EDTA (pH 7.5) and passed through a column (30 by 0.9 cm) of Sephadex G-100 to remove shorter cDNA strands arising from specific stops, which were present in every reaction product. The void volume was pooled and precipitated with alcohol in the presence of 20 to 30 μg of tRNA as carrier. The dried material was dissolved in water and sequenced by the method of Maxam and Gilbert (12).

Electrophoresis. Nonequilibrium pH gradient electrophoresis (14) was carried out by a modification described by Sanders et al. (20) for resolution of very basic proteins. Virions were solubilized in buffer containing 0.3 M NaCl with protamine as described in footnote 4 of reference 20. Only the first (pH gradient) dimension was run, permitting side-by-side comparison of M protein from different mutants.

RESULTS

The nucleotide sequence of the M-protein gene of the Glasgow wild type of VSV, with the deduced amino acid

TABLE 1. Differences between M-gene sequences of Glasgow and Orsay strains of VSV Indiana and published sequence

Base number[a]	Triplet sequence[b] (19)	Glasgow	Orsay	Amino acid change (position)
136	AGC	AAC	AAC	Ser → Asn (32)
200	ACC	ACT	ACT	–[c]
201	TAT	CAT	CAT	Tyr → His (54)
210	AAT		CAT	Asn → His (57)
359	AAA	AAG	AAG	–
438	ACT	GCT	GCT	Thr → Ala (133)
446	TGC	TGT	TGT	–
470	CAT		CAC	–
473	AGG	AGA	AGA	–
552	ATT		GTT	Ile → Val (171)
575	TAC		TAT	–
596	GCA	GCC		–
620	TTC	TTT		–
681	AAA	GAA		Lys → Glu (214)
698	GCG	GCT	GCT	–
714	ATC	GTC	GTC	Ile → Val (225)
717	AGC	CGC		Ser → Arg (226)

[a] Numbering as in Fig. 1.
[b] Altered base is in boldface type.
[c] –, Identical to published sequence.

sequence, is shown in Fig. 1. It is important to note that the sequencing strategy used yields a consensus sequence directly; clones, each one necessarily derived from a single copy of the gene, were not used. This has proved fortunate in the light of the recent discovery by Schubert et al. (23) of extensive heterogeneity in cDNA clones prepared from the VSV L gene. The sequence shown in Fig. 1 differs from that reported for this region of the M gene of the San Juan strain by Rose and Gallione (19) in 13 locations, involving six amino acid substitutions (Table 1). The Orsay wild type also differs from that of Rose and Gallione by 13 nucleotides, resulting in six amino acid changes (Table 1). The Glasgow and Orsay wild types differ from each other somewhat less, by eight nucleotides and four amino acid changes (Table 1). The differences between our sequences and that of Rose and Gallione (19) may arise (i) from differences in the strains of VSV used or (ii) from the fact that Rose and Gallione reported the sequence of a single cDNA clone derived from M protein mRNA, whereas we obtained consensus sequences by using the genome as template.

The first primer was chosen to hybridize with a region encompassing the first 13 residues of the coding region to minimize the possibility of hybridization with undesired regions of the viral genome, e.g., sequences coding for the ribosome-binding regions. We have therefore been unable to verify directly the first five amino acids (Met-Ser-Ser-Leu-Lys) in the sequence deduced by Rose and Gallione (19). However, the sequence may reasonably be assumed to begin with Met in the mutants as well as in the wild type. In addition, the fact that extended products were obtained from all templates with this primer attests to the fact that its 3′-terminal nucleotide (A) is complementary to the viral genome in all the mutants; since this is the only nucleotide of the Lys triplet present in the primer and the other two nucleotides were sequenced in this study, Lys must be present as amino acid 5 in all the mutants. Only three amino acid residues, in positions 2 through 4, thus remain undetermined. However, in an early experiment carried out in collaboration with J. Perrault and M. McClure, Washington University Medical School, St. Louis, Mo., the sequence of the first 67 nucleotides of the *ts*O23 M gene, which included coding sequences for the first nine amino acids, was determined by extension of a 33-nucleotide primer (NS gene bases 757 through 790) originally used by Rose in sequencing the NS-M intergene region (18). The *ts*O23 sequence differed from that of Rose and Gallione in a single nucleotide (G at position 13 to A) in the noncoding region of the gene. Thus, no differences were found between the amino acid sequence of the N-terminal region of M protein deduced by Rose and Gallione and that deduced by us for *ts*O23.

TABLE 2. Base and amino acid changes in M-protein mutants of VSV

Mutant	Wild type	Base no.	Base change[a]	Amino acid change[a] (position)
*ts*G31	Glasgow	684	A → G	Lys → Glu (215)
*ts*G33	Glasgow	652	A → C	Lys → Thr (204)
		681	G → A	Glu → Lys (214)
*ts*O23	Orsay	103	G → A	Gly → Glu (21)
*ts*O89	Orsay	439	C → A	Ala → Asp (133)

[a] Relative to the appropriate wild type.

The sequence differences between the group III mutants and their respective wild types are shown in Table 2. *ts*G31 differs from the Glasgow wild type in only a single nucleotide, which changes lysine at position 215 to glutamic acid. Similarly, *ts*O23 and *ts*O89 each differ from the parent Orsay wild type in a single position, 21 and 133, respectively; in each an acidic amino acid substitutes for a neutral one. *ts*G33 has two changes, lysine at position 204 to threonine and glutamic acid at position 214 to lysine. However, in the wild-type sequence deduced by Rose and Gallione and in the Orsay wild type, a lysine residue is present at position 214. It may be, therefore, that substitution at this position does not give rise to the temperature-sensitive phenotype; the only relevant change would then be the lysine-to-threonine change at position 204.

A nonequilibrium pH gradient electrophoresis gel showing the relative migration of the two wild-type and four mutant M proteins used in this study is shown in Fig. 2. All six proteins could be compared in a single slab gel, since M is the only basic protein in the VSV virion. The identity of the

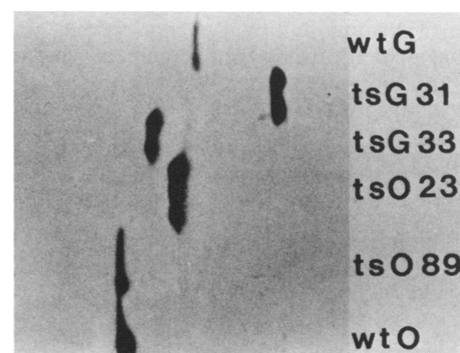

FIG. 2. One-dimensional nonequilibrium pH gradient electrophoresis gel comparing M proteins from wild-type and mutant strains of VSV, stained with Coomassie blue. The pH increases toward the bottom of the gel.

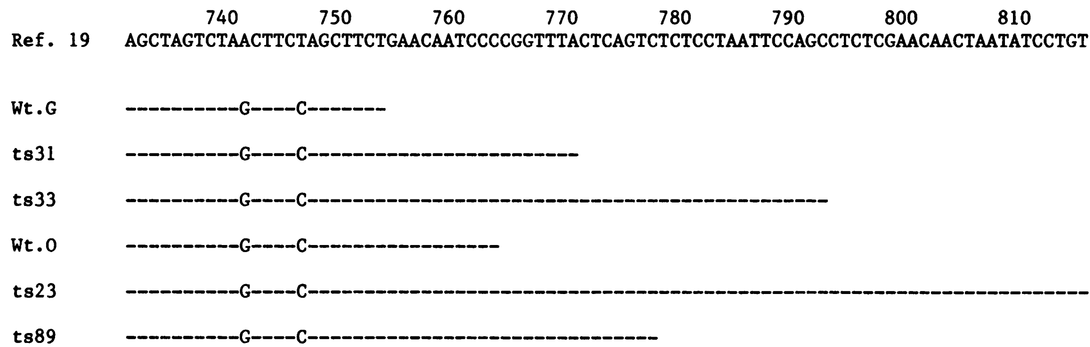

FIG. 3. Partial sequences of untranslated 3′ end of cDNA from VSV M-protein genes.

single prominent band in each lane as M protein was confirmed by two-dimensional electrophoresis (data not shown). The gel in Fig. 2 confirms several predicted relationships between M proteins: (i) the Orsay wild type is more basic than the Glasgow wild type, (ii) *ts*G33 is more basic than the Glasgow wild-type, (iii) *ts*G31 is less basic than Glasgow wild type, and (iv) *ts*O23 is less basic than the Orsay wild type. The M protein of *ts*O89, on the other hand, is identical to that of its parent Orsay wild type, whereas the deduced sequence predicts that it should instead be identical to *ts*O23; we have no explanation for this discrepancy. The finding of a single M-protein band in each lane is consistent with an earlier observation (4), suggesting that phosphorylation is restricted to a small fraction (5 to 10%) of viral M protein; the single M-protein bands in Fig. 2 therefore presumably correspond to the unphosphorylated form.

Partial sequences of the 3′ noncoding regions of the M-gene cDNA which were also obtained in this study are shown in Fig. 3. Two differences from the published sequence (19) were found in all six genes: A (position 741) to G, and T (position 746) to C.

DISCUSSION

In comparing the sequences for wild-type M protein of three different strains—the two in this paper and the one of Rose and Gallione (19)—it is noteworthy that of the eight amino acid changes observed, only two involved highly basic or acidic residues, and neither of these was shared by both Glasgow and Orsay wild types. In position 214, the Lys of Rose and Gallione is Glu in the Glasgow wild type but not in the Orsay wild type. Further, this residue is also Lys in *ts*G33 (which has an additional charge change; Table 2). It appears that a change from positive to negative charge in this position does not affect the wild phenotype. The other change was at position 226: Ser of Rose and Gallione becomes Arg in the Glasgow strain, but not in the Orsay strain. It may be that this position, only four residues from the C terminus, is not essential for protein function.

By contrast, all of the nucleotide changes in the mutants caused amino acid changes, and these all involved basic or acidic residues. All four mutants had one position at which a charge change of −1 or −2 occurred (Table 2). The one +2 change found, at position 214 in *ts*G33, is compatible with the wild phenotype, as discussed above. It is noteworthy that, in all the sequences examined, residues 214 and 215 were both charged; a charge of either +2 or 0 in these positions is apparently compatible with the wild phenotype, but a charge of −2 is not, as shown by *ts*G31 (Table 2).

All four mutants have previously been found to have very similar phenotypes, characterized by temperature sensitivity, decreased binding to nucleocapsids, and increased binding to membranes (7, 10, 16, 17, 24, 26). The fact that these two coordinate affinity changes arise from a single amino acid substitution suggests that the decreased binding to nucleocapsids may represent the primary effect of the mutation, since mutations more commonly decrease specific interactions than increase them. The mutations are so widely distributed along the polypeptide chain, however, that they are difficult to interpret in terms of a specific binding site. Since all of the changes involve charged residues, and since M protein binds nucleocapsids by ionic interactions (26), it is possible that all the mutations perturb the nucleocapsid binding site directly. In this case, the site must either be very large, or, more likely, arise from a specific folding of the protein that brings these widely separated residues together. Alternatively, the mutations may cause conformational changes that destabilize the protein conformation or decrease the accessibility of the binding site. In either case, the unusually high rate of spontaneous reversion found for the group III temperature-sensitive mutants (16) suggests that the specificity requirements for wild-type function may not be very stringent, since many different point mutations can evidently act equivalently to restore the wild (temperature-stable) phenotype. Experiments are in progress to characterize temperature-stable revertants of the mutants described in this paper.

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