JOURNAL OF VIROLOGY, Feb. 1987, p. 256–263
0022-538X/87/020256-08$02.00/0

Vol. 61, No. 2

Phenotypic Revertants of Temperature-Sensitive M Protein Mutants of Vesicular Stomatitis Virus: Sequence Analysis and Functional Characterization

KOUICHI MORITA, ROGER VANDEROEF, AND JOHN LENARD*

Department of Physiology and Biophysics, Robert Wood Johnson Medical School,† University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854-5635

Received 20 June 1986/Accepted 8 October 1986

Twenty-five spontaneous temperature-stable revertants of four different temperature-sensitive (*ts*) M protein mutants (complementation group III: *ts*G31, *ts*G33, *ts*O23, and *ts*O89) were sequenced and tested for their ability to inhibit vesicular stomatitis virus RNA polymerase activity in vitro. Consensus sequences of the coding region of each M protein gene were determined, using total viral RNA as template. Fifteen different sequences were found among the 25 revertants; 14 differed from their *ts* parent by a single amino acid (one nucleotide), and 1 differed by two amino acids (two nucleotides). Amino acids were altered in various positions between residues 64 and 215, representing over 60% of the polypeptide chain. Resequencing of the Glasgow and Orsay wild types and the four *ts* mutants confirmed previously published differences (Y. Gopalakrishna and J. Lenard, J. Virol., 56:655–659, 1985), and one or two additional differences were found in each. The relative charges of the revertant M proteins, as determined by nonequilibrium pH gradient electrophoresis, were consistent with the deduced sequences in every case. The ability of each revertant M protein to inhibit the RNA polymerase activity of nucleocapsids prepared from its parent *ts* mutant was also tested. Only 13 of the 25 revertants had M protein with high (wild type-like) polymerase-inhibiting activity, while 5 had low (*ts*-like) activity, and 7 had intermediate activity, demonstrating that this property is not an essential concomitant of the temperature-stable phenotype. It is concluded that the high reversion frequency observed for these mutants arises from a very high incidence of pseudoreversion, i.e., many different molecular changes can repair the *ts* phenotype.

The M protein of vesicular stomatitis virus (VSV) is one of the three major proteins in purified virions, making up nearly one-third of the total viral protein (29). M protein is thought to coordinate viral budding, implying interactions with both viral nucleocapsids and membranes, but little is yet known about the molecular basis for these interactions (27, 28, 31). One approach to this problem is through the study of temperature-sensitive (*ts*) mutants, i.e., mutants that fail to produce virions at some restrictive temperature and in which the relevant mutations occur in the M protein gene (*ts*M mutants, complementation group III [8, 21]). In general, the characterization of *ts* mutants and their revertants offer the possibility of identifying different gene products, and even specific segments of individual polypeptide chains, that interact to form specific functional complexes. The simple composition of this system, only five genes of which three code for structural proteins, makes it especially suitable for detailed analysis by this genetic approach.

All the *ts*M mutants studied so far behave very similarly. Cells infected with them at the restrictive temperature overproduce viral RNA (2, 10) and synthesize normal or elevated amounts of all the viral proteins (8, 9). Lack of viral budding at the restrictive temperature is thought to be due to the inability of the mutant M protein to function at that temperature, although accelerated intracellular degradation of the mutant protein has also been described (8) and may either contribute to or arise from the phenotype.

The study of the mechanism of action of M protein has been hindered by its relative insolubility and also by its lack of any measurable enzymatic activity. Electron microscopic studies have suggested that M protein can interact with isolated or intracellular nucleocapsids to produce new structures that closely resemble the cores of purified virions (12–14). The ability of M protein to inhibit viral RNA polymerase activity in a cell-free assay has provided one convenient measure of M protein function (1, 34). This property has been thought to reflect an essential element of the budding function of the M protein, since *ts*M proteins all possess much lower polymerase-inhibiting activity than wild-type (wt) M proteins (1, 34). This difference is not itself a *ts* function, however (T. Wilson and J. Lenard, unpublished observations), and so the relationship between the polymerase-inhibiting activity of M protein and its budding function has remained speculative. Purified M protein has also been shown to interact with acidic phospholipid bilayers (16, 33, 35), but again, it is not clear how this property relates to viral budding.

*ts*M mutants have a remarkably high rate of reversion, around 10^{-3} (21). Much lower frequencies are generally associated with highly specific requirements, e.g., when only one amino acid will function properly in the active center of an enzyme. High frequencies, on the other hand, suggest that many different amino acid substitutions, i.e., many intragenic pseudoreversions, can produce a functional protein. Results reported in this paper support this idea for M protein.

In this paper we report the deduced amino acid sequences and polymerase-inhibiting activities of 25 spontaneous revertants isolated from four *ts*M mutants. Single amino acid changes occur in 14 of the 15 different sequences found and span over 60% of the polypeptide chain. Polymerase-

* Corresponding author.
† Formerly Rutgers Medical School.

inhibiting activity remains below wt levels in nearly half the revertants, suggesting that this convenient assay is not a necessary concomitant of wt M protein function.

MATERIALS AND METHODS

Cells and viruses. The Orsay and Glasgow variants of VSV Indiana and the group III mutants were originally obtained from A. Huang and A. Flamand. All stocks were plaque purified. Frozen aliquots (−80°C) from the identical stocks used in the previous sequencing paper (7) were grown to prepare viral RNA as template for resequencing the M protein regions of Glasgow and Orsay wt and the four *ts* mutants in the present paper. The San Juan wt was obtained from J. Rose. Viruses were grown in BHK-21 cells and isolated and purified as described previously (11). *ts* mutants were grown at 31°C. wt and revertants were grown at 37°C. Temperature-stable revertants designated with the suffix TW were originally described by Wilson and Lenard (34); all others were isolated subsequently, using identical procedures. The temperature stability of the revertants used in this study is expressed as the ratio of plaques formed on Vero cells at 39°C and at 31°C (Table 1).

Sequence analysis. Extracted viral RNA was used as a template for reverse transcription. Three primers were used in this and a previous paper (7), corresponding to nucleotides 36 to 54, 201 to 219, and 376 to 394 of M mRNA as numbered by Rose and Gallione (24). In addition, a primer corresponding to nucleotides −40 to −22 of the NS protein mRNA (as numbered by Rose [23]) was prepared for sequencing the nucleotides corresponding to the first few amino acids of the M protein.

TABLE 1. Temperature stability of plaque formation by wt, *ts*M mutants, and phenotypic revertants

Strain	Plaques at 39°C/plaques at 31°C
Glasgow wt	0.60
*ts*G31	0.0002
*ts*G31r1	0.52
*ts*G31r2	0.48
*ts*G31r3	0.48
*ts*G31r4	0.66
*ts*G31r5	0.57
*ts*G31r4TW	0.57
*ts*G33	0.0002
*ts*G33r1	0.41
*ts*G33r3	0.93
*ts*G33r4	0.72
*ts*G33r6	0.67
*ts*G33r2TW	0.81
*ts*G33r3TW	0.86
*ts*G33r5TW	0.58
Orsay wt	0.44
*ts*O23	0.00003
*ts*O23r1	0.66
*ts*O23r2	0.50
*ts*O23r3	0.58
*ts*O23r4	0.38
*ts*O23r5	0.90
*ts*O23r5TW	0.13
*ts*O23r7TW	0.29
*ts*O89	0.0003
*ts*O89r1	0.53
*ts*O89r3	0.44
*ts*O89r4	0.37
*ts*O89r5	0.25
*ts*O89r8TW	0.58

The entire M protein-coding region of genomic RNA was sequenced by the dideoxy (dd) method (26). Viral RNA was denatured at 95°C for 1 min in 8 μl of annealing solution (4 μg of viral RNA, 50 ng of primer, 10 mM Tris, 1 mM EDTA, pH 7.5), 1 μl of 750 mM KCl was added, and the mixture was cooled to 42°C over a period of 30 min (30). To this mixture was then added 3 μl of [^{32}P]dCTP (30 μCi, 600 Ci/mmol), 2 μl of avian myeloblastosis virus reverse transcriptase (10 U), and 1.5 μl of buffer (500 mM Tris, 3 mM dithiothreitol, 80 mM $MgCl_2$, pH 8.3). Samples of this mixture (3.3 μl) were incubated with 2 μl of each deoxy-dd nucleoside triphosphate mixture (micromolar concentrations: A lane, 36 dATP, 71 dGTP, 71 dTTP, 20 ddATP; C lane, 63 dATP, 63 dGTP, 63 dTTP, 1 ddCTP; G lane, 71 dATP, 36 dGTP, 71 dTTP, 10 ddGTP; T lane, 71 dATP, 71 dGTP, 36 dTTP, 15 ddTTP) at 42°C for 20 min and electrophoresed on sequencing gels.

NEPHGE. Nonequilibrium pH gradient electrophoresis (NEPHGE) was performed by a modification of the O'Farrell technique as previously described (7, 15, 25).

Polymerase assay. Reactions were carried out in 100 μl of total volume containing: 10 μl of buffer (500 mM Tris, 50 mM $MgCl_2$, 40 mM dithiothreitol, pH 7.8); 18 μl of nucleocapsid fraction (containing 0.3 to 0.5 mg of N protein per ml); 12 μl of high-salt extract (containing 0.16 to 0.46 mg of M protein per ml) or high-salt blank (250 mM NaCl, 10% glycerol, 10 mM Tris, 1.5 mM dithiothreitol, pH 8.0); 15 μl of nucleoside triphosphates (6.7 mM each ATP, CTP, GTP, 0.67 mM UTP, 5 μCi of [^{3}H]UTP); 45 μl of water. Reactions were incubated at 30°C for 90 min, and the reaction was terminated by the addition of 100 μl of 67 mM sodium PP_i containing 200 μg of yeast RNA. RNA was precipitated by the addition of 5% trichloroacetic acid and counted after filtration on Whatman GF/C filters.

RESULTS

Sequence analysis. M protein-coding regions of Glasgow and Orsay wt VSV were resequenced by the dd procedure as described in Materials and Methods. The sequence of the Orsay wt M protein published previously was confirmed and completed (Fig. 1). A difference of two nucleotides from the previously published sequence was found in the Glasgow wt (Fig. 1): T to C at position 724, causing a Phe to Ser change at amino acid position 228; and A to G at position 731, causing a change in the termination codon, to Trp at position 230 (Fig. 1 and 2). This makes the Glasgow wt M protein eight amino acids longer than the Orsay wt (Fig. 1 and 2).

The sequence of a cDNA clone prepared from the M protein mRNA of the San Juan strain of VSV has previously been reported by Rose and Gallione (24). Since the procedure used in this paper determines the consensus sequence of the entire population of viral RNA rather than that of a single molecule from which the clone was derived, it was of interest to sequence this wt M gene as well. Our sequence differed in only one nucleotide from that reported by Rose and Gallione (24): A to G at position 438, resulting in a Thr to Ala change in the previously published sequence (24) at amino acid position 133, in agreement with the sequences of both Glasgow and Orsay wt proteins at this position (7) (Fig. 1).

We attempted to confirm the predicted size difference between Orsay and Glasgow M proteins by sodium dodecyl

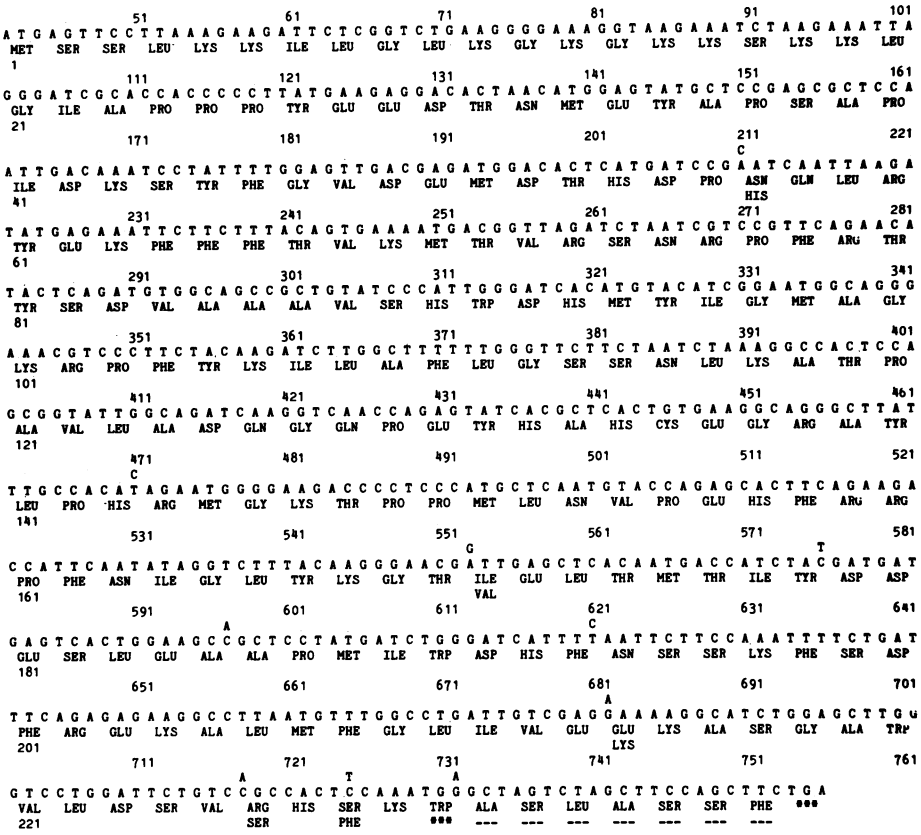

FIG. 1. Sequence of cDNA from coding regions of Glasgow and Orsay wt M protein genes. Where differences exist, the Orsay nucleotide sequence is shown above, and the protein sequence below, the Glasgow sequence. Numbering according to Rose and Gallione (24).

sulfate-gel electrophoresis. Although the predicted difference in mobility (corresponding to about a 2.5% difference in molecular weight) was observed when sodium dodecyl sulfate-gel electrophoresis was performed at pH 9.5, differences of similar magnitude were also found between different M proteins predicted to be of identical size, e.g., between Orsay wt, *ts*O23, and certain of its revertants. It appears, therefore, that sequence differences of only two or three amino acids in M proteins of the same size can impart small mobility differences in sodium dodecyl sulfate gels. This occurs in a way that is not readily predictable from the sequence change, or from the charge. Accordingly, the predicted size difference between Glasgow and Orsay wt M proteins could not be unambiguously confirmed by this method.

The deduced amino acid sequences of the four *ts* mutants and the 25 revertants are shown in Fig. 3. The previously described point mutations of the mutants (7) were all confirmed. In addition, two nucleotide changes were found in *ts*O23, *ts*G31, and *ts*G33, and one additional change was seen in *ts*O89, in the region not previously sequenced. The nucleotide and amino acid sequences of each mutant are represented by the top lines of each panel of Fig. 3.

Several of the revertants isolated as separate plaques possessed identical sequences (Fig. 3). While all the revertants in each group might not represent independent muta-

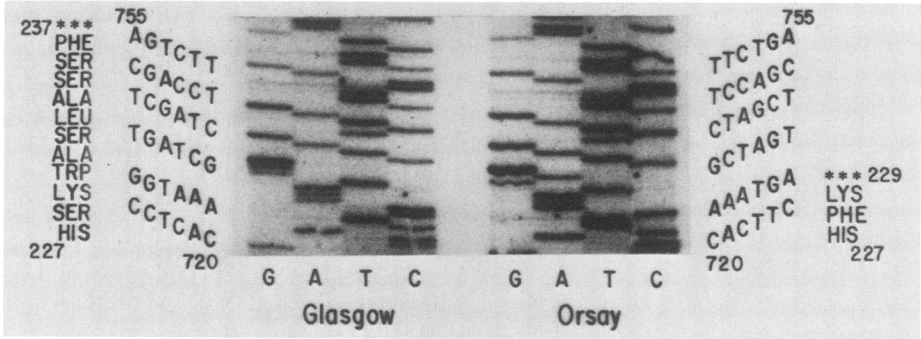

FIG. 2. dd sequencing gels of nucleotides 720 to 755 of cDNAs from the Glasgow and Orsay wt M protein genes, showing corresponding predicted amino acids.

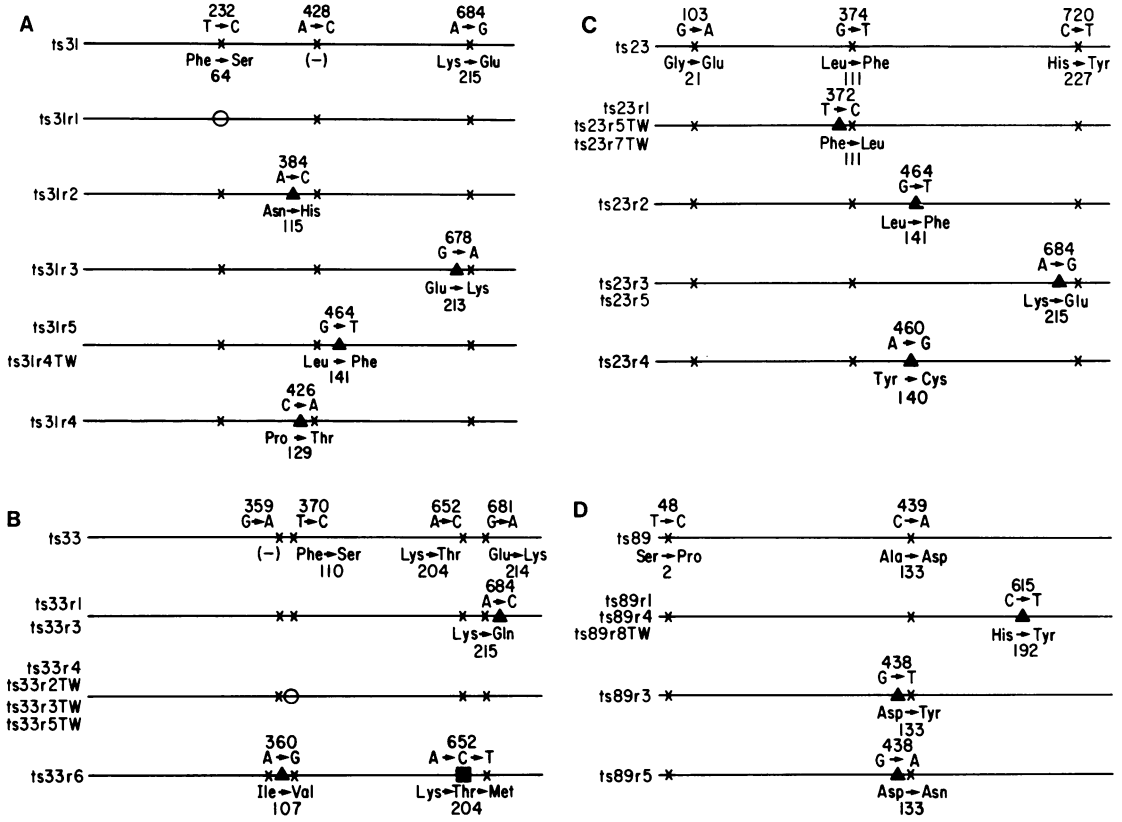

FIG. 3. Sequences of cDNAs from coding regions of M protein genes of *ts* mutants and revertants. Symbols: ×, *ts* and revertant nucleotides that differ from corresponding wt; ○, revertant nucleotides that have changed back from *ts* to corresponding wt; ▲, ■, revertant nucleotides that differ from both corresponding wt and *ts*. Nucleotides not identified by these symbols are identical to corresponding wt (Fig. 1) and are indicated by the solid line. (A) *ts*G31 family. (B) *ts*G33 family. (C) *ts*O23 family. (D) *ts*O89 family. *ts*G31 and *ts*G33 are derived from Glasgow wt; *ts*O23 and *ts*O89 are derived from Orsay wt.

tional events, there are four groups (one from each family) that contain representatives of both the TW series (34) and of the series isolated several years later from recloned mutant stock in connection with the present study. Thus, each of these revertants was independently isolated at least twice.

NEPHGE gels. The charge on each revertant M protein was compared, using NEPHGE gels, with the *ts*M and wt M proteins from which it was derived (Fig. 4). As predicted from the sequences, there was considerable variation in charge among the different revertant M proteins. Of the 25, only 5 migrated identically to their wt grandparents, while 15 migrated identically to their *ts* parents, and 5 migrated differently from either. A small but consistent difference was observed between *ts*O89 and its parent, Orsay wt (Fig. 4D), in contrast to a previous report (7). The mutant was more acidic, as predicted from its amino acid sequence (7) (Fig. 3D). All 25 revertants migrated in a manner consistent with their deduced amino acid sequences (Fig. 3).

The reason for the double bands formed by *ts*O23 and most of its revertants (Fig. 4C) is not known. They are unlikely to represent phosphorylation, since this would make the protein more acidic, while most of the minor bands are in the more basic direction. It also seems unlikely that these bands arise from genetic variants present in the plaque-purified stocks, since they are most prominent in members of the *ts*O23 family and are absent from the families derived from the Glasgow wt.

Polymerase-inhibiting activity. Figure 5 shows the ability of high-salt extracts prepared from each of the two wt and four *ts* virions to inhibit the RNA polymerase activity of nucleocapsids prepared from the appropriate *ts* mutant. In each case, the wt extract inhibited more effectively than the *ts* extract, in agreement with conclusions from previous studies (1, 34). The ability of each revertant M protein to inhibit viral RNA polymerase activity was tested by adding high-salt extract prepared from revertant virions to nucleocapsids from the appropriate *ts* parent (Fig. 5). The inhibitory activities of the revertant extracts differed considerably from one another. Each revertant was classified according to whether its inhibitory activity most closely resembled its wt grandparent, or its *ts* parent, or whether it was intermediate between the two. Of the 25 revertant high-salt extracts, 13 were judged to have wt activity (Fig. 5, open circles), 5 to have *ts* activity (Fig. 5, open triangles), and 7 to have intermediate activity (Fig. 5, open squares). A variability of polymerase-inhibiting activity between two different revertants of *ts*O23 was previously suggested by the data of Pal et al. (17).

DISCUSSION

The finding that each *ts* mutant differs in two or three amino acids (two to four nucleotides) from its parent wt necessitated analysis of revertants to assess the relative importance of each point change. The revertants proved highly informative, since 14 of the 15 unique sequences differed from their parent *ts*M by only one amino acid (one nucleotide), while one, *ts*G33r6, differed by two amino acids

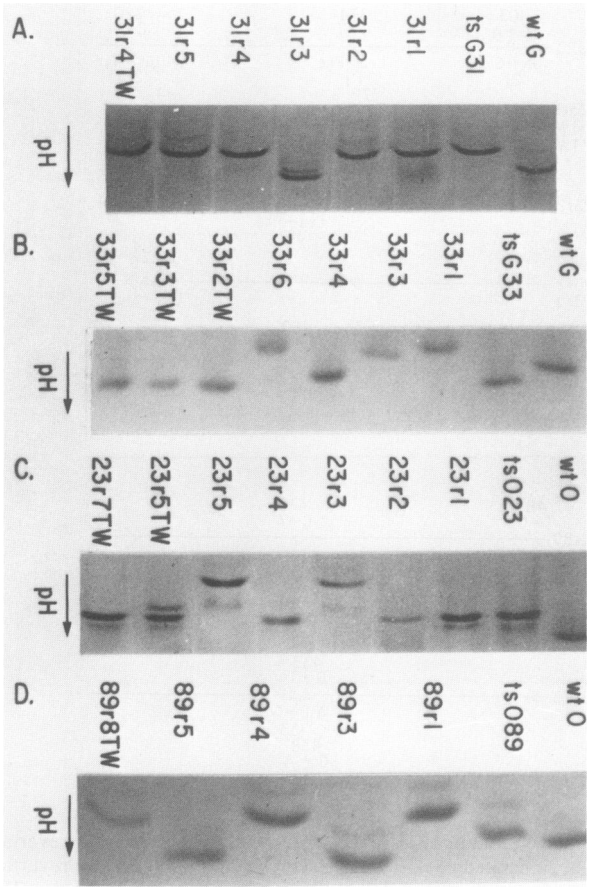

FIG. 4. NEPHGE gels of M proteins of wt VSV, *ts* mutants, and revertants. pH increases toward the bottom of each gel. (A) *ts*G31 family. (B) *ts*G33 family. (C) *ts*O23 family. (D) *ts*O89 family.

(two nucleotides; Fig. 3B). The larger number of changes between wt and *ts*M than between *ts*M and revertants may reflect genetic drift of both wt and *ts*M strains occurring during passage since the mutants were isolated over 17 years ago (4, 5, 19, 20). The revertants, in contrast, were isolated relatively recently in this laboratory from our *ts*M strains, and random divergence should therefore be less extensive.

The well-known hypermutability of RNA viral genomes raises the question of whether the sequence changes found in the M proteins of the revertants represent the actual sites of reversion. This question becomes especially pressing in view of the finding (Fig. 5) that nearly half the revertants have polymerase-inhibiting activities that are significantly lower than that of their ancestral wt. Polymerase-inhibiting activity has been tacitly assumed to be obligatorily linked to the *ts* phenotype in *ts*M mutants (1, 34). The possibility to be considered is whether some of the revertant sequence changes might be drift or noise. In that case, since all except one differ from *ts*M at a single point, the mutation actually controlling reversion would have to reside in a different gene, coding for a protein (perhaps N) that interacts with M during budding. While we cannot definitively exclude this possibility, several observations argue against it. (i) All 25 revertants isolated have M proteins that are altered from the *ts* parent, generally in a single position (Fig. 3). (ii) Over 99% of the N proteins of *ts*O23 and two of its revertants possessing *ts*-like polymerase-inhibiting activity, *ts*O23r2 and *ts*O23r4, have been sequenced (418 of 422 amino acids; see reference 6). While these differed from the published sequence (6) in several places (seven nucleotides and two amino acids; data not shown), no differences were found between *ts*O23 and its revertants. (iii) The polymerase-inhibiting activities of the M proteins of four revertants, *ts*O23r2, *ts*O23r4, *ts*G33rl, and *ts*G33r3, were tested against those of the homologous nucleocapsids instead of against those of *ts*O23 as shown in Fig. 5. Polymerase-inhibiting activity was *ts*-like in this homologous system (data not shown) as it was against the parent *ts* nucleocapsids (Fig. 5), suggesting that no compensatory changes had occurred in any of the nucleocapsid proteins. The discussion below will therefore assume that the sequence changes of M protein shown in Fig. 3 are those responsible for the *ts* or revertant phenotype. If this assumption is correct, then it follows that wt-like polymerase-inhibiting activity in disrupted virus preparations is not a necessary concomitant of the temperature-stable phenotype.

In a previous paper the NaCl dependence of M protein inhibition of in vitro polymerase activity was shown to differ between wt M and *ts*M proteins (34). NaCl dependence was much more pronounced for inhibition by wt M than by *ts*M. Two revertants derived from each of the four *ts*M mutants were tested, and all eight showed wt-like NaCl dependence (34). Four of these revertant strains have survived and were tested in this study, *ts*O23r7TW, *ts*O23r5TW, *ts*G33r5TW, and *ts*G31r4TW. It is noteworthy that the first three of these all exhibited wt-like polymerase-inhibiting activity in the quite different in vitro assay used in the present paper (Fig. 5). *ts*G31r4TW was judged to have intermediate polymerase-inhibiting activity (Fig. 5A), but its activity was also very close to the line defined by wt M protein. The two different assays of M protein function in vitro thus appear to be entirely consistent with each other.

Point mutations leading to reversion of *ts*M occur throughout the region from residues 64 to 215, a region making up over 60% of the polypeptide chain. This provides evidence in support of the suggestion (7) that the protein functions in a highly folded, comformationally stabilized form and that residues along most of the chain contribute to the formation of its functional form, or to its site of interaction with other viral structures, or both.

In all four families, revertants were found involving single residues that were altered (relative to wt) in the *ts* parent: residues 64 (*ts*G31), 110 (*ts*G33), 111 (*ts*O23), and 133 (*ts*O89). The first three, at positions 64, 110, and 111, were simple reversions to the wt residue. Two changes were seen in different revertants of the *ts*O89 family, both of which replaced the charged residue in *ts* with an uncharged residue different from that found in wt. It is noteworthy that none of these residues were altered in heterologous families, i.e., in families that possessed wt residues at these positions, suggesting that separate interactions involving specific residues were disrupted by each *ts* mutation. Only residues 141 and 215 were involved in reversions in two different families.

The remaining single-residue reversions may be imagined to compensate for the disruptions caused by the *ts* substitution, most simply by direct interactions with the substituted residue. We may then assign the following pairs of residues as possibly interacting, or at least in close proximity, in the wt protein: 64 with 115, 129, 141, and 213; 110 with 215; 111 with 140, 141, and 215; and 133 with 192. For these interactions to occur simultaneously, the structure of the residue 64 to 215 region of M protein would evidently have to be very compactly folded. Further, the stabilizing interactions in this

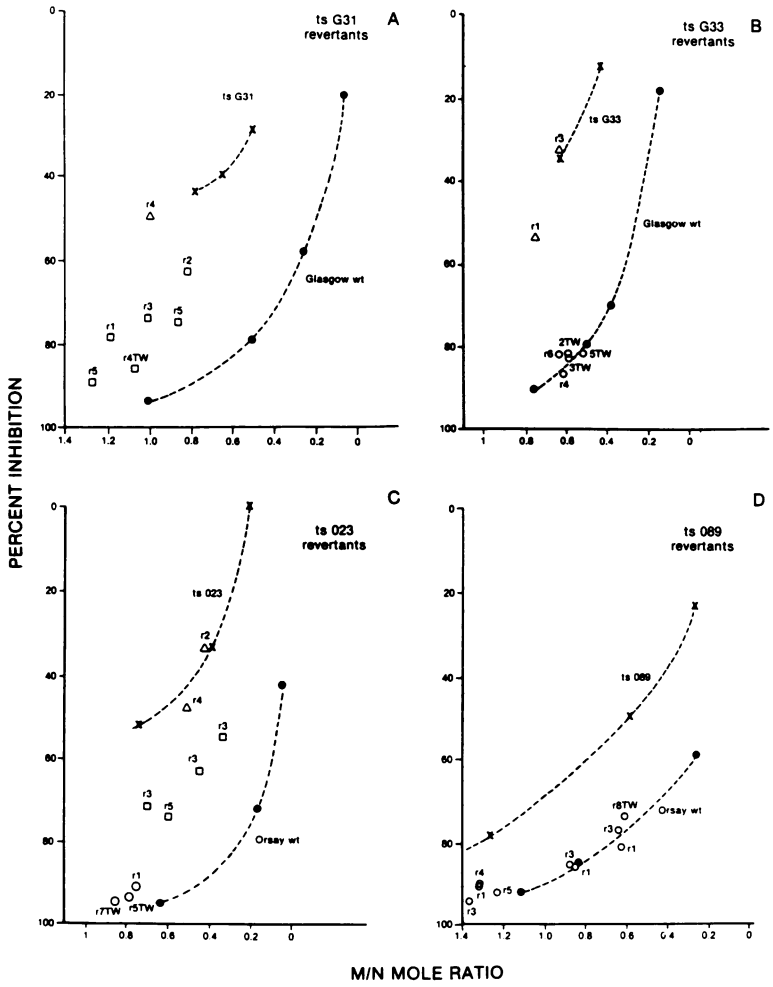

FIG. 5. Polymerase inhibition activity of high-salt extracts from wt and *ts* mutants. Activity was tested against nucleocapsids prepared from the *ts* mutant indicated in each panel. High-salt extract was prepared from: Glasgow (A and B) or Orsay (C and D) wt (●); *ts* mutant, as indicated (×); homologous revertants exhibiting wt-like activity (○), *ts*-like activity (△), or intermediate activity (□). (A) *ts*G31 family. (B) *ts*G33 family. (C) *ts*O23 family. (D) *ts*O89 family.

TABLE 2. Sequences of amino acid residues 213 to 215 in wt, *ts*, and revertant strains of VSV

Strain	Sequence	Net charge	Polymerase-inhibiting activity[a]
	213 214 215		
wt Glasgow	Glu-Glu-Lys	−1	W
wt Orsay	Glu-Lys-Lys	+1	W
wt San Juan			W
*ts*G33			M
*ts*G33r1	Glu-Lys-Gln	0	M
*ts*G33r3			M
*ts*G31	Glu-Glu-Glu	−3	M
*ts*G31r3	Lys-Glu-Glu	−1	I
*ts*O23r3	Glu-Lys-Glu	−1	I
*ts*O23r5			I

[a] As classified in Fig. 5. W, wt; M, mutant; I, intermediate.

region appear to be quite precise: substitution of Leu at residue 111 with another hydrophobic amino acid, Phe, is sufficient to create the *ts* phenotype in *ts*O23, while reversion of this residue to Leu returns the wt phenotype (Fig. 3C).

The charged amino acids at positions 213 to 215 appear to be highly mutable, but the acceptable amino acids are limited. The six different sequences found in this region are shown in Table 2: either Glu or Lys appear at positions 213 and 214, while Glu, Lys, or Gln appear at position 215. The net charge on positions 213 to 215 is −3, −1, 0, and +1 in different sequences, and there is no obvious correlation of charge or residue with either temperature stability or polymerase-inhibiting activity. On the other hand, residues 213 and 215 were the only amino acids changed in three different revertants, so this region, while not informative by itself, is presumably involved in phenotype-determining interactions with other parts of the molecule.

The sequences of *ts*O23 and its revertants presented here provide an interesting perspective in which to reconsider the recent findings of Ogden et al. (16). These workers showed that proteolytic removal of the N-terminal 43 amino acid residues of M protein yielded a fragment whose polymerase-

inhibiting activity was diminished to about 40% of intact protein levels. Further, one specific monoclonal antibody epitope, previously found to be absent from *ts*O23, was also absent from this trypsin fragment; further analysis restricted the site of antibody binding to residues 18 to 43 of the M protein. In only one of four *ts*O23 revertants examined by Paul et al. (17) was this epitope regained. While one of the three differences between Orsay wt and *ts*O23 is in the residue 18 to 43 region, at residue 21, neither this residue nor any other in this region was altered in any of the revertants examined by us; indeed, reversion of amino acid 111 alone was sufficient for recovery of wt polymerase-inhibiting activity, and amino acid substitutions elsewhere allowed recovery of partial activity. It seems most probable, therefore, that the residue 18 to 43 region pinpointed by Ogden et al. (16), while important, does not by itself determine either polymerase-inhibiting activity or specific antibody binding. Rather, it seems more likely that this region is involved either in stabilizing the native conformation of the protein or in interacting with other regions of the molecule to create the necessary specific site(s). Both from the sequence data presented here and from the complicated picture of overlapping epitopes defined by monoclonal antibodies (18), the M protein appears to be a tightly interacting structure whose functional domains are likely to be determined by the conformation of the folded protein rather than by individual small regions of linear amino acid sequence.

ACKNOWLEDGMENTS

J.L. acknowledges valuable discussions with D. Lyles.

This work was supported by Public Health Service grant AI-13002 from the National Institutes of Health.

ADDENDUM IN PROOF

Several additional sequences have been obtained. Orsay wt, stored by A. Flamand since 1970, was identical with ours. *ts*O23 from A. Flamand, stored after its first multiplication in 1969 (4), lacked the mutation at base 720 but was otherwise identical to ours. *ts*O23 from R. R. Wagner, maintained separately for over 10 years, was identical to ours. *ts*O23 revertants from Pal et al. (17) showed single-base changes: R12 and R14, T-373→G(Phe-111→Cys); R13, C-615→T(His-192→Tyr).

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