Temperature-Sensitive Mutants of Complementation Group E of Vesicular Stomatitis Virus New Jersey Serotype Possess Altered NS Polypeptides

D. EVANS, C. R. PRINGLE, AND J. F. SZILÁGYI*

Medical Research Council Virology Unit, Institute of Virology, University of Glasgow, Glasgow G11 5JR, Scotland

Received for publication 29 March 1979

In vesicular stomatitis virus New Jersey serotype polyacrylamide gel electrophoresis was unable to distinguish the polypeptides of the temperature-sensitive (*ts*) mutants of complementation groups A, B, C, and F from those of the wild-type virus. However, the NS polypeptide of the representative mutant of group E, *ts* E1, had a significantly greater electrophoretic mobility than that of the wild-type virus NS polypeptide. The electrophoretic mobilities of the NS polypeptides of the three mutants of complementation group E varied, being greatest in the case of *ts* E1, slightly less for *ts* E2, and only a little greater than that of wild-type virus NS polypeptide in the case of *ts* E3. Since the NS polypeptides of the revertant clones *ts* E1/R1 and *ts* E3/R1 have mobilities identical to that of wild-type NS polypeptide, the observed altered mobilities of the group E mutants are almost certainly the direct result of the *ts* mutations in the E locus. The electrophoretic mobilities of the intracellular NS polypeptides of the group E mutants were indistinguishable from those of their virion NS polypeptides. The electrophoretic mobilities of the NS polypeptides of the group E mutants synthesized in vitro using mRNA synthesized in vitro by TNP were identical to those of the NS polypeptides of their purified virions. The NS polypeptides of all three mutants were labeled with ^{32}P to approximately the same extent as wild-type virus NS polypeptide, indicating that gross differences in phosphorylation of this polypeptide are unlikely to account for the altered mobilities. We propose a model in which the NS polypeptide consists of at least three loops held in this configuration by hydrophobic or ionic forces or both and stabilized by phosphodiester bridges. If a mutation affects one of the amino acids to which the phosphate is covalently linked, the phosphodiester bridge cannot be formed, and, as a result, in the presence of sodium dodecyl sulfate the affected loop opens and thus the NS polypeptide migrates further into the gel. Such a configuration may also explain the multifunctional nature of the NS polypeptide.

Vesicular stomatitis virus (VSV) New Jersey serotype consists of a single-stranded RNA molecule and five polypeptides, L, glycosylated G, N, phosphorylated NS, and M (12, 13, 15, 22, 28-30). Polypeptides N, NS, and M, as well as the unglycosylated precursor to G, have been synthesized in the rabbit reticulocyte lysate system by using RNA synthesized in vitro by the transcribing nucleoprotein (TNP) complex (15).

The temperature-sensitive (*ts*) mutants of VSV New Jersey fall into six nonoverlapping complementation groups; the majority isolated belong to groups A and B, whereas group C contains only four mutants, group E contains three, group F contains two, and group D contains only one (16, 17).

At the restrictive temperature viral RNA syn-

thesis was defective in BHK-21 cells infected with mutants of groups A, B, and F, but not in those infected with mutants of groups C and D (17). Mutants of group E were heterogeneous; *ts* E1 and *ts* E3 did not synthesize viral RNA at 39°C, whereas *ts* E2 did (17). Viral protein synthesis at 39°C in infected BHK-21 cells exhibited a similar pattern (30). Subsequent analysis showed that primary transcription was inhibited at 39.5°C in cells infected with *ts* B1 and *ts* F1 but not in cells infected with *ts* A1, *ts* C1, *ts* D1, and *ts* E1 (9).

Recently, Szilágyi and Pringle (26) showed that in vitro RNA synthesis at 39°C by the RNA-negative *ts* A1 and the RNA-positive *ts* C1 and *ts* D1 was similar to RNA synthesis in wild-type virus, whereas the RNA-negative *ts* B1

synthesized only small amounts of RNA in vitro at 39°C. Mutant *ts* E1 synthesized very little RNA, *ts* E2 synthesized moderate amounts, and *ts* E3 was not inhibited. The group F mutants were also dissimilar; *ts* F1 synthesized only very little RNA in vitro at 39°C, whereas *ts* F2 synthesized as much RNA as did wild-type virus.

We have analyzed the polypeptides of some of these *ts* mutants of VSV New Jersey to determine whether differences in the electrophoretic mobilities of any of the structural polypeptides are associated with any of the complementation groups. Initially, we examined one representative mutant from each complementation group and found that the mobility of the NS polypeptide of *ts* E1 differed from that of wild-type NS polypeptide. The present paper describes our consequent study of the NS polypeptide of all three complementation group E mutants.

MATERIALS AND METHODS

Mutants. The *ts* mutants of VSV New Jersey described previously by Pringle et al. (17) were used. The revertant clone *ts* E1/R1 has been described previously by Szilágyi and Pringle (26), and *ts* E3/R1 was isolated from BS-C-1 monolayers incubated at 39°C.

Growth and purification of virus. Virus was grown in BHK-21 clone 13 monolayers and purified by the method of Szilágyi and Pringle (24). Purified virus was suspended in 20 mM Tris-hydrochloride buffer, pH 8.0, and the protein was determined by the method of Lowry et al. (10).

Preparation of radioactively labeled virus. Virus was grown in 80-ounce (2,400-ml) Burrell bottles as described above except that (i) the medium contained only 20% of its normal amino acid composition when the virus was to be labeled with [³⁵S]methionine, or (ii) the medium was phosphate-free when the virus was to be labeled with ³²P_i. [³⁵S]methionine or ³²P_i (200 μCi in 35 ml of medium per bottle) was added 6 h postinfection. After harvesting, the medium containing the virus was clarified by centrifugation at 2,500 rpm for 30 min to remove cellular debris, and then the virus was pelleted by centrifugation at 21,000 rpm for 90 min through a 30% glycerol cushion containing 20 mM Tris-hydrochloride, pH 8.0, 1 mM EDTA, and 0.1 M NaCl. The pellets were then resuspended in a buffer consisting of 20 mM Tris-hydrochloride buffer, pH 8.0, 1 mM EDTA, and 100 mM NaCl.

Preparation of intracellular [³⁵S]methionine-labeled polypeptides. Throughout the experiment the medium contained only 20% of its normal amino acid composition. Each BHK-21 cell monolayer in a Burrell bottle was infected with a low multiplicity of purified virus (24) and incubated at 31°C for 22 h in 35 ml of medium. After this incubation the medium was removed, and the surface of the cell monolayer was washed twice with fresh medium. Then 20 ml of medium containing 300 μCi of [³⁵S]methionine was added, and incubation was continued at 31°C. After 90 min of incubation, the medium was removed, and the cell surface was washed twice with fresh medium.

Finally, 15 ml of 20 mM Tris-hydrochloride buffer, pH 8.0, was added, the cell monolayer was removed by sterile glass beads, and a homogeneous suspension was obtained by extensive sonication.

A mock-infected cell suspension was obtained in the same way except that the virus was omitted.

Preparation of TNP. TNP complexes were prepared by the method of Szilágyi and Uryvayev (27), incorporating the modifications of Szilágyi and Pringle (25).

In vitro RNA and protein synthesis. Complementary RNA was synthesized in vitro at 31°C for 90 min in a 0.2-ml incubation mixture containing 0.02 ml of TNP, 0.05 ml of reagent mixture (containing 400 mM Tris-hydrochloride buffer, pH 8.0, 400 mM NaCl, 3.2 μg of actinomycin D per ml, 2.56 mM ATP, 2.56 mM CTP, 2.56 mM GTP, 0.256 mM UTP, and 12 μCi of [5,6-³H]UTP), 3.5 mM dithiothreitol, 2 mM additional ATP, 25 μM S-adenosylmethionine, and 1 U of rat liver RNase inhibitor (Amersham/Searle) per ml.

An unfractionated rabbit reticulocyte lysate was prepared as described by Preston and Szilágyi (15). The endogenous RNA from the lysate was removed by treating it with micrococcal nuclease as described by Pelham and Jackson (14).

The cell-free translation system (25 μl) contained 100 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.7), 40 mM KCl, 0.8 mM spermidine trihydrochloride, 1 mM ATP, 0.1 mM GTP, 4 mg of creatine phosphate per ml, 200 μg of creatine phosphokinase per ml, each of the 19 common amino acids except methionine at a concentration of 50 μM, 500 μCi of L-[³⁵S]methionine, 12.5% lysate, and 200 μM hemin. Finally, 2.5 μl of the incubation mixture was added, and it was incubated at 31°C for 60 min. Protein synthesis was terminated by the addition of 25 μl of a solution containing 100 mM EDTA, 2% methionine and 300 μg of RNase A per ml, followed by a further incubation at 31°C for 15 min.

Polyacrylamide gel electrophoresis. Samples of purified virus or cell-free translation system were analyzed by discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gradient gel electrophoresis as described by Marsden et al. (11). To obtain greater separation of the N and NS polypeptides, the electrophoresis was continued for an additional 2 h after the bromophenol blue dye front had reached the bottom of the gel. Kodirex film was used for the autoradiography.

RESULTS

Polypeptide composition of the virions of the *ts* mutants of VSV New Jersey. We chose a representative mutant from each of the complementation groups A, B, C, E, and F and analyzed their polypeptide compositions by discontinuous polyacrylamide gradient gel electrophoresis. Group D was excluded for reasons outlined below.

Figure 1 shows that wild-type VSV New Jersey contains five polypeptides, L, G, N, NS, and M, although at least two minor bands (MB1, MB2) of unknown origin were also detected. The

polypeptides of the mutants *ts* A1, *ts* B1, *ts* C1, and *ts* F1 had electrophoretic mobilities identical to those of the wild-type virus polypeptides.

However, the NS polypeptide of *ts* E1 has a significantly greater electrophoretic mobility than that of the wild-type virus NS polypeptide, whereas the mobilities of the other polypeptides were not different (Fig. 1). This indicated that the *ts* E1 mutation might have affected the electrophoretic mobility of the NS polypeptide.

Polypeptide composition of the virions of the group E mutants. Since the NS polypeptide of *ts* E1 had an altered electrophoretic mobility in a polyacrylamide gel, we decided to examine all three mutants of complementation group E, as well as the revertant clones *ts* E1/R1 and *ts* E3/R1.

FIG. 1. Virion polypeptides of the *ts* mutants of VSV New Jersey. [35 S]methionine-labeled purified virions of wild-type virus and *ts* mutants were analyzed on a discontinuous SDS-polyacrylamide gradient gel as described in the text. Each track contained approximately 60,000 cpm of [35 S]methionine. The polypeptides L, G, N, NS, and M, as well as two minor bands of unknown origins (MB1, MB2), are indicated. To emphasize the position of the NS polypeptides, they are marked by open triangles.

Figure 2 shows that the mobilities of polypeptides L, G, N, and M of each mutant and the two revertants were indistinguishable from those of wild-type virus polypeptides although the NS polypeptides of the three *ts* mutants had different mobilities.

Because the electrophoretic mobility of N was identical in each case, it was convenient to express the relative mobilities of the NS polypeptides of the mutants by comparing the extent of the separation of their N and NS polypeptides with the separation observed in wild-type virus. The separation of polypeptides N and NS was approximately 1.6 times greater for *ts* E1 than for wild-type virus (Fig. 2, tracks 1 through 3), it was slightly less in the case of *ts* E2 (approximately 1.4 times greater; tracks 5 through 7), and in *ts* E3 the separation was only 1.3 times greater than in wild-type virus (tracks 8 through 10).

The differences in the electrophoretic mobilities of the NS polypeptides of the three *ts* mutants were clearly resolved in those tracks where mutants and wild-type virus were mixed in various combinations (Fig. 2, tracks 13 through 15).

Thus, the order of the mutants, according to the extent of migration of their NS polypeptide, is *ts* E1, *ts* E2, *ts* E3.

The electrophoretic mobilities of the NS polypeptides of the revertant clones *ts* E1/R1 and *ts* E3/R1 appear to be identical to that of the NS polypeptide of the wild-type virus (Fig. 2, tracks 4, 5, 11 and 12). The simultaneous reversion of the *ts* phenotype and the electrophoretic mobility of the NS polypeptide strongly suggests that the *ts* mutation is responsible for the altered mobility of the NS polypeptides of the mutants *ts* E1 and *ts* E3, and probably of *ts* E2 as well.

Polypeptide synthesis in cells infected with the group E mutants. BHK-21 cells were infected with the three group E mutants and the two revertants, and the intracellular polypeptides labeled during a 90-min pulse of [35 S]methionine were analyzed by polyacrylamide gel electrophoresis (Fig. 3).

Actin was the predominant labeled polypeptide in mock-infected cells (Fig. 3, tracks 2 and 13). In cells infected with the wild-type (tracks 3, 7, 8, and 11), mutant (tracks 4 through 6), and revertant (tracks 10 and 11) viruses there were, besides the actin, six major labeled polypeptides. These were the viral polypeptides L, G, N, NS, and M, as well as a further polypeptide, pG, which is presumably a precursor of G. In each case polypeptides L, G, pG, N, and M were indistinguishable from the polypeptides of the wild-type virus (tracks 1 and 14). However, the electrophoretic mobilities of polypeptide NS in cells infected with the three mutants (tracks

FIG. 2. Virion polypeptides of the *ts* mutants of complementation group E. [^{35}S]methionine-labeled purified virions of wild-type VSV New Jersey (wt), the three *ts* mutants (*ts* E1, *ts* E2, *ts* E3) of complementation group E, and the revertant clones (*ts* E1/R1, *ts* E3/R1) were analyzed on a discontinuous SDS-polyacrylamide gradient gel individually or in mixed tracks, as indicated. Each track contained approximately 60,000 cpm of [^{35}S]methionine. Therefore, 60,000, 30,000, or 20,000 cpm of [^{35}S]methionine for each virus was placed on the tracks, depending on whether the viruses were alone or in combination. The positions of the NS polypeptides are indicated by the open triangles.

4 through 6) were different from that of NS polypeptide synthesized in cells infected with wild-type virus (tracks 3, 7, 9, and 12) and were also different from each other. The separations of the N and NS polypeptides for *ts* E1, *ts* E2, and *ts* E3 were 1.6, 1.4, and 1.3 times greater, respectively, than the separation for wild-type virus. Thus, the mobilities of the intracellular NS polypeptides of the group E mutants were indistinguishable from those of the purified virion NS polypeptides.

The intracellular NS polypeptides synthesized in cells infected with the revertant clones (Fig. 3, tracks 10 and 11) were indistinguishable from the NS polypeptide of the wild-type virus (tracks 9 and 12), which strengthens the suggestion that the altered electrophoretic mobilities of the NS

polypeptides of the mutants are the result of the *ts* mutations.

In vitro-synthesized polypeptides. As the aberrant electrophoretic mobilities of the NS polypeptides in group E appear to be the result of mutation, these differences should also be observed in polypeptides synthesized in vitro. We tested this by synthesizing mRNA's in vitro, using TNP complexes and translating them in a rabbit reticulocyte lysate cell-free system.

Figure 4 shows that four major polypeptides were translated when the RNA synthesized by wild-type TNP was used. Three of these correspond to the N, NS, and M polypeptides of the virion, whereas the fourth polypeptide (pG) is presumably the unglycosylated G polypeptide (Fig. 4, tracks 1, 2, 11, and 12). In vitro-synthe-

FIG. 3. Intracellular polypeptides of cells infected with the group E mutants. [^{35}S]methionine-labeled infected cell extracts were obtained and analyzed by SDS-polyacrylamide gradient gel electrophoresis as described in the text. Each tract contained approximately 5,000 cpm of labeled cell extract. For comparison, [^{35}S]methionine-labeled purified wild-type VSV New Jersey virions (*wt virus*) or a mock-infected cell preparation was used. The NS polypeptides are indicated by the open triangles.

sized polypeptides pG, N, and M of all three mutants were identical to those of the wild-type virus (tracks 3, 5, and 9).

The electrophoretic mobilities of the in vitro-synthesized NS polypeptides of *ts* E1 and *ts* E2 were significantly greater than the mobility of wild-type virus NS polypeptide (tracks 3 through 6); the mobility of the NS polypeptide of *ts* E3, although lower, was still greater than the mobility of the wild-type NS polypeptide (tracks 9 and 10).

The differences in the electrophoretic mobilities of the NS polypeptides of the mutants are easier seen here than in the virion analysis, since the NS polypeptide is a major product of the in vitro translation. The in vitro results fully confirm the differences in the electrophoretic mobilities of the NS polypeptides of the group E mutants.

Since the mobilities of the NS polypeptides of the virions and infected cells, as well as of the in

vitro-synthesized NS polypeptides of all of the mutants, were identical, it seems unlikely that the altered mobilities of the NS polypeptides are due to a posttranslational cleavage. The lack of low-molecular-weight cleavage products observed in gels where electrophoresis was stopped before the dye front ran off the gel is further evidence that cleavage does not take place.

Phosphoprotein composition of the group E mutants. The mutants were grown in the presence of $^{32}\text{P}_i$ to determine whether the alterations in the electrophoretic mobilities of the NS polypeptides of the group E mutants were due to differences in the degree of phosphorylation. Equal amounts of protein were placed on each track to determine whether there were any differences in the degree of phosphorylation of the mutants.

Figure 5 shows that only NS was phosphorylated (tracks 1, 2, 8, 12, and 16). One of the minor polypeptides of unknown origin (MB2) was also phosphorylated. The ^{32}P -labeled NS polypeptides of the three mutants showed differences in mobilities similar to those obtained previously with [^{35}S]methionine-labeled virus.

The chief conclusion from Fig. 5 is that the NS polypeptides of all three mutants and the two revertants are phosphorylated. The degree of phosphorylation of the NS polypeptide of the individual mutants is approximately of the same order of magnitude as that of the wild-type virus NS polypeptide. This is clearly illustrated in mixed tracks where equal amounts of the mutant and wild-type virus were present (tracks 4, 7, and 10).

DISCUSSION

Polyacrylamide gel electrophoresis was unable to distinguish differences between the polypeptides of the *ts* mutants of complementation groups A, B, C, and F and the polypeptides of the wild-type virus. Previously Wunner and Pringle (31) reported that the G and N polypeptides of *ts* D1, the only mutant of complementation group D, exhibited aberrant electrophoretic mobilities and that the N polypeptide defect was associated with the *ts* lesion. Subsequent work suggests that neither polypeptide defect may be responsible for temperature sensitivity of *ts* D1. This work will be the subject of a separate report and is therefore excluded from this discussion.

The experiments described in this paper show that the NS polypeptides of all three mutants have altered electrophoretic mobilities in relation to one another and to wild-type virus. Since the electrophoretic mobilities of the NS polypeptides of the revertant clones *ts* E1/R1 and *ts*

FIG. 4. Polypeptides synthesized *in vitro* using RNA produced *in vitro* by TNP complexes of the group E mutants. The preparation of the TNP complexes and the *in vitro* RNA and protein syntheses are described in the text. Tracks 1, 7, and 12 contained [^{35}S]methionine-labeled purified virions of wild-type VSV New Jersey (35S virus). Track 8 contained cell-free translation system from which the TNP was omitted. The other tracks contained cell-free translation systems of the various mutants and wild-type virus, as indicated. Single tracks contained 20 μl . Mixed tracks contained 10 μl from each of the cell-free translation systems. The NS polypeptides are indicated by the open triangles.

E3/R1 were identical to that of wild-type virus NS polypeptide, the altered mobilities of the NS polypeptides of the mutants *ts* E1 and *ts* E3 are almost certainly the result of the *ts* mutation. Presumably this is also true for *ts* E2, although as yet we have been unable to obtain a stable spontaneous revertant from this mutant. Thus, the inhibition of virus development of the group E mutants at the restrictive temperature is almost certainly due to inability of their mutated NS polypeptides to function normally at this temperature.

Recently, Szilágyi and Pringle (26) found that the virion-associated transcriptase activity of *ts*

E1 is temperature sensitive, although *in vitro* RNA synthesis is not inhibited at 39°C in the case of *ts* E2 and *ts* E3. *In vivo* studies showed that *ts* E3 is defective in the replication of the virion RNA (17), whereas in the case of *ts* E2 replication did not appear to be inhibited (17). Therefore, Szilágyi and Pringle suggested that the polypeptide affected by the mutation in group E is a multifunctional polypeptide involved in transcription, replication, and possibly some late stage of virus development (26). Since the results presented in this paper show that the mutated polypeptide is almost certainly NS, we conclude that NS is the multifunctional poly-

FIG. 5. Phosphoprotein composition of complementation group E mutants. ^{32}P -labeled purified virions of the group E mutants and their revertants were analyzed by discontinuous SDS-polyacrylamide gradient gel electrophoresis as described in the text. Each track contained $9\text{ }\mu\text{g}$ of virus protein. In tracks which contained more than one virus, the amounts of each virus were proportionately reduced in order to maintain the total protein concentration at a constant level. The NS polypeptides are indicated by the open triangles. wt, wild-type virus.

peptide. That the NS polypeptide of VSV Indiana is involved in transcription has already been shown by dissociation and reconstitution experiments (4, 7). Our evidence suggests that the NS polypeptide is also involved in the replication of virion RNA, although we cannot say whether it is the replicase itself or part of a replicase complex. Since viral RNA synthesis takes place in *ts* E2-infected cells at the restrictive temperature, the involvement of NS polypeptide in a late stage of virus development is suggested. Previously, Szilágyi and Pringle (26) showed that the heat stability of the transcriptase of *ts* E1 was dependent on an interaction between the viral coat and the core.

At present it is not certain what causes the marked alteration in the electrophoretic mobilities of the NS polypeptides of the three group

E mutants. Two possible explanations arise: (i) either the NS polypeptides of the group E mutants are shorter than the NS polypeptide of the wild-type virus, or (ii) the phosphorylation of mutant NS differs from that of the wild-type NS.

The occurrence of a shortened polypeptide can be explained in several ways, although there are objections to all of them. (i) A section of the NS cistron may have been deleted by the mutation. However, we consider this unlikely since the base analog 5-fluorouracil, which favors point mutation, was used as the mutagen and since revertants were obtained from *ts* E1 and *ts* E3 at high frequency.

(ii) The mRNA for NS may have been prematurely terminated. However, all of the evidence available suggests that transcription is

initiated only at the 3' end of the genome (1, 2). Thus, premature termination of the mRNA synthesis would produce polar effects whereby the subsequent messengers for the M, G, and L polypeptides would not be transcribed. To exclude this possibility, the mRNA's of the mutants will have to be compared with those of the wild-type virus by gel electrophoresis and oligonucleotide fingerprinting.

(iii) The mutant mRNA may contain an early termination or late initiation codon resulting in a shorter NS polypeptide. To determine whether this is the case, the sequence of the mRNA's of the various group E mutants will have to be determined.

However, it is difficult to reconcile early termination or late initiation of transcription or translation with the loss of functions of the various group E mutants.

(iv) Another possibility is that mutation altered the NS polypeptide in such a way as to render it more susceptible to cleavage by proteolytic enzymes. This is also unlikely since the in vitro-synthesized NS polypeptides had similarly altered mobilities. Also, it is generally held that in the rabbit reticulocyte lysate system the in vitro-synthesized polypeptides are not cleaved (3, 18, 21), and there was no evidence for the presence of a low-molecular-weight cleavage product. Furthermore, the mobilities of the intracellular NS polypeptides of the group E mutants were indistinguishable from those of the purified virion NS polypeptides, making proteolytic cleavage even more unlikely.

Since NS is a phosphorylated polypeptide, the second possibility is that the aberrant mobilities of this polypeptide in the various group E mutants are a consequence of the differences in the extent of phosphorylation. However, the experiment with ^{32}P -labeled virions indicated that, in the case of all three mutants, polypeptide NS is phosphorylated to approximately the same extent as it is in wild-type virus. Therefore, gross differences in the level of phosphorylation in the various mutants are unlikely to be the cause of the differences in the mobilities of the NS polypeptide.

We propose a model which could explain both the altered mobility of the NS polypeptide and the nature of the group E mutation. According to this model the NS polypeptide chain is folded into at least three loops. Each loop is held together by ionic or hydrophobic bonds or both and stabilized in this configuration by a phosphodiester bridge. Phosphate diesters cannot be hydrolyzed by alkaline phosphatase (6, 19), which explains why polypeptide NS was not dephosphorylated by this enzyme (12, 22; unpublished observations on VSV New Jersey).

Therefore, the structure of polypeptide NS cannot be denatured by boiling in SDS and 2-mercaptoethanol during preparation for polyacrylamide gel electrophoresis, and hence it would not migrate as far into the gel as a fully denatured polypeptide. This would also explain why the NS appears as a high-molecular-weight polypeptide in polyacrylamide gels despite the relatively small coding capacity of its mRNA and also would account for the differences in the mobilities of the NS polypeptide in different gel systems (5, 8, 20, 23).

If mutation results in the substitution of one of the amino acids to which the phosphate is covalently bonded, the phosphodiester bridge cannot be formed and the stabilization of the loop does not take place. Therefore, under denaturing conditions this loop opens, and the mobility of the NS polypeptide in the gel increases. The extent of this increase presumably depends on the size of the loop affected by the mutation, which would explain the differing mobilities of the NS polypeptides of the group E mutants.

Our model can also explain the *ts* nature of the mutation. At the permissive temperature the ionic and hydrophobic bonds alone are sufficient, but at the restrictive temperature the phosphodiester bond is required to hold the configuration of the loop. Thus, a mutation which results in the breaking of the phosphodiester bridge will be temperature sensitive for the function of that loop. Mutations affecting any other amino acid not linked by the phosphodiester bond will presumably be either a silent or a lethal mutation.

According to this model each loop corresponds to a different function of the NS polypeptide. One of the loops (loop *ts* E1) is involved in the transcription of mRNA's, the second (loop *ts* E3) is involved in the replication of virus RNA, and the third (loop *ts* E2) is involved in the late function in which the mutant *ts* E2 is defective.

The model would explain why there is no correspondence between the loss of functions by the mutants and the differences in the electrophoretic mobilities of the NS polypeptide.

Although at present we have no experimental evidence to substantiate our model, we are actively engaged in the determination of the structure of the NS polypeptide. Also, we will have to isolate more revertant clones, especially from *ts* E2, since attempts to isolate stable revertants from this mutant have so far been unsuccessful.

ACKNOWLEDGMENTS

We thank J. H. Subak-Sharpe for a critical reading of the manuscript. We also thank C. Cunningham for his excellent technical assistance, C. M. Preston for helping with the in

vitro translation, and J. T. Poyner for helping with the preparation of the manuscript.

D. E. was a recipient of a Science Research Council scholarship for training in research methods.

LITERATURE CITED

1. Abraham, G., and A. K. Banerjee. 1976. Sequential transcription of the genes of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. U.S.A.* 73:1504-1508.
2. Ball, L. A., and C. N. White. 1976. Order of transcription of genes of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. U.S.A.* 73:442-446.
3. Cancedda, R., and M. J. Schlesinger. 1974. Formation of Sindbis virus capsid protein in mammalian cell-free extracts programmed with viral messenger RNA. *Proc. Natl. Acad. Sci. U.S.A.* 71:1843-1847.
4. Emerson, S. U., and Y.-H. Yu. 1975. Both NS and L proteins are required for in vitro RNA synthesis by vesicular stomatitis virus. *J. Virol.* 15:1348-1356.
5. Freeman, G. J., J. K. Rose, G. M. Clinton, and A. S. Huang. 1977. RNA synthesis of vesicular stomatitis virus. VII. Complete separation of the mRNA's of vesicular stomatitis virus by duplex formation. *J. Virol.* 21:1094-1104.
6. Heppel, L. A., D. R. Harkness, and R. J. Hilme. 1962. A study of the substrate specificity and other properties of the alkaline phosphatase of *Escherichia coli*. *J. Biol. Chem.* 237:841-846.
7. Imblum, R. D., and R. R. Wagner. 1975. Inhibition of viral transcriptase by immunoglobulin directed against the nucleocapsid NS protein of vesicular stomatitis virus. *J. Virol.* 15:1357-1366.
8. Knipe, D., J. K. Rose, and H. F. Lodish. 1975. Translation of individual species of vesicular stomatitis viral mRNA. *J. Virol.* 15:1004-1011.
9. Lesnaw, J. A., and M. E. Reichmann. 1975. RNA synthesis by temperature-sensitive mutants of vesicular stomatitis virus, New Jersey serotype. *Virology* 63:492-504.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
11. Marsden, H. S., T. K. Crombie, and J. H. Subak-Sharpe. 1976. Control of protein synthesis in herpes virus-infected cells: analysis of the polypeptides induced by wild type and sixteen temperature-sensitive mutants of HSV strain 17. *J. Gen. Virol.* 31:347-372.
12. Moyer, S. A., and D. F. Summers. 1974. Phosphorylation of vesicular stomatitis virus in vivo and in vitro. *J. Virol.* 13:455-465.
13. Obijeski, J. F., A. T. Marchenko, D. H. L. Bishop, B. W. Cann, and F. A. Murphy. 1974. Comparative electrophoretic analysis of the virus proteins of four rhabdoviruses. *J. Gen. Virol.* 22:21-33.
14. Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247-256.
15. Preston, C. M., and J. F. Szilágyi. 1977. Cell-free translation of RNA synthesized in vitro by a transcribing nucleoprotein complex prepared from purified vesicular stomatitis virus. *J. Virol.* 21:1002-1009.
16. Pringle, C. R. 1977. Genetics of rhabdoviruses, p. 239-289. In H. Fraenkel-Conrat and R. R. Wagner (ed.), *Comprehensive virology*, vol. 9. Plenum Press, New York.
17. Pringle, C. R., I. B. Duncan, and M. Stevenson. 1971. Isolation and characterization of temperature-sensitive mutants of vesicular stomatitis virus, New Jersey serotype. *J. Virol.* 8:836-841.
18. Purchio, A. F., E. Erikson, and R. L. Erikson. 1977. Translation of 35S and of subgenomic regions of avian sarcoma virus RNA. *Proc. Natl. Acad. Sci. U.S.A.* 74:4661-4664.
19. Reid, T. W., and T. B. Wilson. 1971. *E. coli* alkaline phosphatase, p. 373-415. In P. D. Boyer (ed.), *The enzymes*, vol. 4, 3rd ed. Academic Press Inc., New York.
20. Rhodes, D. P., G. Abraham, R. J. Colonno, W. Jelinek, and A. K. Banerjee. 1977. Characterization of vesicular stomatitis virus mRNA species synthesized in vitro. *J. Virol.* 21:1105-1112.
21. Simmons, D. T., and J. H. Strauss. 1974. Translation of Sindbis virus 26S RNA and 49S RNA in lysates of rabbit reticulocytes. *J. Mol. Biol.* 86:397-409.
22. Sokol, F., and H. F. Clark. 1973. Phosphoproteins, structural components of rhabdoviruses. *Virology* 52:246-263.
23. Stampfer, M., and D. Baltimore. 1973. Identification of the vesicular stomatitis virus large protein as a unique viral protein. *J. Virol.* 11:520-526.
24. Szilágyi, J. F., and C. R. Pringle. 1972. Effect of temperature-sensitive mutations on the virion-associated RNA transcriptase of vesicular stomatitis virus. *J. Mol. Biol.* 71:281-291.
25. Szilágyi, J. F., and C. R. Pringle. 1975. Virion transcriptase activity differences in host range mutants of vesicular stomatitis virus. *J. Virol.* 16:927-936.
26. Szilágyi, J. F., and C. R. Pringle. 1979. Effect of temperature-sensitive mutation on activity of the RNA transcriptase of vesicular stomatitis virus New Jersey. *J. Virol.* 30:692-700.
27. Szilágyi, J. F., and L. Uryvayev. 1973. Isolation of an infectious ribonucleoprotein from vesicular stomatitis virus containing an active RNA transcriptase. *J. Virol.* 11:279-286.
28. Wagner, R. R. 1975. Reproduction of rhabdoviruses, p. 1-93. In H. Fraenkel-Conrat and R. R. Wagner (ed.), *Comprehensive virology*, vol. 4. Plenum Press, New York.
29. Wagner, R. R., L. Prevec, F. Brown, D. F. Summers, F. Sokol, and R. MacLeod. 1972. Classification of rhabdovirus proteins: a proposal. *J. Virol.* 10:1228-1230.
30. Wunner, W. H., and C. R. Pringle. 1972. Protein synthesis in BHK cells infected with vesicular stomatitis virus. II. *ts* mutants of the New Jersey serotype. *Virology* 50:250-253.
31. Wunner, W. H., and C. R. Pringle. 1974. A temperature-sensitive mutant of vesicular stomatitis virus with two abnormal virus proteins. *J. Gen. Virol.* 23:97-106.