Mapping Temperature-sensitive Mutants of Vesicular Stomatitis Virus by RNA Heteroduplex Formation

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(Accepted 1 July 1981)

SUMMARY

Duplex RNA molecules made by hybridization of virion and mRNA of vesicular stomatitis virus (VSV) were digested with ribonuclease and separated into five size classes, each containing the gene and the mRNA for one of the VSV proteins. Denaturation of the duplexes yielded full size mRNA lacking poly(A) tails. Utilizing duplex formation between the RNAs from VSV temperature-sensitive (ts) mutants and their revertants and subsequent RNase digestion under varying salt conditions, specific cleavages within a certain duplex were seen for representative mutants from complementation groups III, IV and V. Specific cleavages were not seen for a group II mutant. From these results gene assignments cannot be made for group II; equivocal assignments are made for group III and clear assignments made for group IV and V. The assignment for the group V mutants, however, does not conform to expectations. Nevertheless, from these studies and other published ones, there is the suggestion that interactions may exist between the gene products of complementation groups II and V during VSV transcription and morphogenesis. These results also support the lack of transcriptional splicing for VSV mRNAs.

INTRODUCTION

Vesicular stomatitis virus (VSV) of the Indiana serotype is a well-characterized, large RNA-containing virus (see Wagner, 1975). Because its genome has been mapped (Abraham & Banerjee, 1976; Ball & White, 1976) and all of its gene products identified, VSV offers a defined system for studying macromolecular controls involving protein–nucleic acid and protein–protein interactions. As a tool for such analyses, conditional-lethal temperature-sensitive (ts) mutants were obtained and genetically categorized into five complementation groups.

Four sets of ts mutants, designated by the initials G, O, W and M, are available. G stands for Glasgow (Pringle, 1970), O for Orsay (Flamand, 1970), W for Winnipeg (Holloway et al., 1970) and M for the Massachusetts Institute of Technology (Rettenmier et al., 1975).

Assignment of the five complementation groups to individual genes has been complicated by pleomorphic and overlapping phenotypes. However, enzymic analyses of the RNA-dependent RNA polymerase activity and in vitro reconstitution of polymerase activity with the ts mutants indicate unequivocally that complementation group I mutants have lesions in

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the polymerase gene, which codes for the largest (L) of the VSV proteins (Szilagyi & Pringle, 1972; Hunt et al., 1976).

The group II mutants have RNA+ and RNA− phenotypes (Pringle & Duncan, 1971; Unger & Reichmann, 1973). Those which synthesize RNA have a maturational defect which is sometimes observed as a migrational alteration of the surface (G) glycoprotein of VSV on polyacrylamide gels (Printz & Wagner, 1971). In contrast, Combard et al. (1977) showed that one of the Orsay group II mutants contained nucleocapsids that were more sensitive to ribonuclease and more heat-labile than wild-type nucleocapsids, thus implicating the N protein as the site of the lesion. On the other hand, more by default than by direct gene assignment, group II mutants are thought to have a primary lesion in the nucleocapsid-associated (NS) protein. Some support for this is found in partial protease digests of NS protein from group II mutants (Metzel & Reichmann, 1981). In summary, there is evidence for the lesion in group II being in the NS, N or G genes. However, analysis of the comparable complementation group E mutants of the related New Jersey serotype of VSV indicates that although other migrational alterations may occur, only those involving the NS protein are associated with temperature sensitivity (Evans et al., 1979; Huang & Pringle, 1980).

The group III mutants are all RNA+ (Pringle & Duncan, 1971) to the extent of increasing transcription by fivefold at the non-permissive temperature (Clinton et al., 1978 b). They synthesize a membrane-associated matrix (M) protein that is more thermosensitive at non-permissive temperatures and which is not incorporated into progeny virions (Printz & Wagner, 1971; Lafay, 1974; Knipe et al., 1977 a). Therefore, the group III mutants are assigned to the M gene.

The group IV mutants are RNA− for genome replication (Pringle & Duncan, 1971; Unger & Reichmann, 1973; Perlman & Huang, 1974) and synthesize a thermosensitive nucleocapsid (N) protein at non-permissive temperatures (Knipe et al., 1977 a; Little & Huang, 1977). By reconstitution experiments with purified nucleocapsids and soluble polymerase, Cormack et al. (1975) showed that the lesion in group IV mutants resides in the nucleocapsids. Since the N protein is the major protein in nucleocapsids, the ts lesion in group IV mutants is assigned to the N gene.

The group V mutants have a thermolabile G protein (Zavada, 1972; Lafay, 1974) and during maturation the G protein is under-glycosylated, resulting in a lack of incorporation into virions and a more rapid migration of the G protein on polyacrylamide gels (Knipe et al., 1977 b; Little & Huang, 1977). For these reasons, group V mutants are likely to map in the G gene.

We concluded that a more direct method of analysing ts defects would help to resolve some of the discrepancies in gene assignments, map the lesion in more detail and permit studies on control mechanisms to progress at a rapid rate. To this end, heteroduplex analysis of nucleic acids was adapted to this RNA virus system, where the genomic RNA is annealed to small complementary messenger RNAs and trimmed with ribonucleases. The resultant RNA duplexes are separated on polyacrylamide gels (Freeman et al., 1977; Rhodes et al., 1977). Should a base mismatch occur between the mutant gene and mRNA isolated from revertant-infected cells, the duplex containing the lesion would be expected to either migrate differently on the gels (Ito & Joklik, 1972), or be differentially sensitive to digestion by ribonucleases or the S1 single-stranded specific nuclease (Shenk et al., 1975). This mapping strategy was applied to heteroduplexes of mutant and revertant RNAs of ts G22 (II), ts G31 (III), ts G41 (IV) and ts O45 (V).

The results presented here assign complementation groups III and IV to the genes coding for the M and N proteins respectively. Although complementation groups II and V remain unresolved, there is the suggestion that interactions occur during transcription and virus
maturation between the NS and G proteins. These interactions may account for our inability to assign complementation groups II and V.

METHODS

Cells and viruses. The growth of Chinese hamster ovary (CHO) cells (Puck et al., 1964) and baby hamster kidney cells (BHK-21, clone 13; adapted to suspension culture) have been described in detail (Freeman et al., 1979). Wild-type stocks of VSV were all of the Indiana serotype: the San Juan strain described by Wagner et al. (1963), the Glasgow strain and the Orsay strain kindly supplied by Drs P. Printz and Anne Flamand. Mutants ts G22 (II), ts G31 (III) and ts G41 (IV) were kindly supplied by Dr Craig Pringle (Pringle, 1970). Mutant ts O45 (V), clone 201 and its ts+ revertants: clones 207, 277, 290 and 296 were kindly supplied by Dr Robin Weiss. The wild-type San Juan strain was cloned by seven successive plaque isolations (Stampfer et al., 1969, 1971). All other viruses were similarly plaque-purified three times. Only cloned and sucrose gradient-purified, standard infectious particles were used throughout these studies.

Selection of revertants from ts mutants. Spontaneous revertants of ts mutants were selected by infecting CHO cell monolayers at 38.5 °C with appropriate dilutions of cloned ts mutants. An effort was made to pick well isolated plaques of different size and morphology. Revertant clones were plaque-purified twice on CHO cells at 36.5 °C and then screened for their ability to efficiently form plaques at 38.5 °C.

Preparation of 32P-labelled VSV mRNA. 32P-labelled VSV mRNA was prepared by phenol–chloroform extraction of infected BHK cell cytoplasm as described in Freeman et al. (1979). Polyadenylated mRNA was purified by binding to and elution from oligo(dT)-cellulose as described in Rose & Knipe (1975).

Purification of VS virion RNA. Infected, 32P-labelled BHK cells were pelleted by centrifugation at 10 h post-incubation. Virus in the supernatant was concentrated by centrifugation, purified on a 5 to 40% sucrose gradient (Stampfer et al., 1969) and re-pelleted. The virus pellet was resuspended in 0.1 M-sodium acetate pH 6.9, 1% SDS and the RNA extracted with phenol, chloroform and isooamyl alcohol.

Electrophoresis of RNA in formamide–polyacrylamide gels. Electrophoresis of RNA in formamide–polyacrylamide gels was performed according to Duesberg & Vogt (1973), except that a 3.75% polyacrylamide slab gel was formed in a vertical slab gel apparatus.

Hybridization conditions. Large scale hybridizations were performed according to Rhodes et al. (1977). To ensure saturation hybridization of 32P-labelled RNA, a ratio of 10^5 ct/min of dT-selected VSV mRNA per 1 μg VS virion RNA or 10^3 ct/min of VS virion RNA per 20 μg of dT-selected VSV mRNA was employed. Typically, 10^6 ct/min of dT-selected VSV mRNA would be annealed with 10 μg of VS virion RNA in 0.5 ml.

Ribonuclease treatment of duplexes. After hybridization, the ethanol-precipitated RNA was dissolved in sterile water and adjusted to the appropriate concentration of SSC (1 × SSC = 0.15 M-NaCl, 0.015 M-sodium citrate pH 7). Standard digestion conditions were in 2 × SSC for 0.5 h at 37 °C with ribonucleases at the following concentrations: 1 μg/ml RNase A, 12.5 U/ml RNase T1 and 10 U/ml RNase T2. Ribonucleases were then inactivated by adding 2 vol. phenol and adjusting the SSC concentration to 1 × SSC. The aqueous layer was then collected and the RNA duplexes ethanol-precipitated.

Electrophoresis of double-stranded RNA in polyacrylamide slab gels. Double-stranded RNAs were separated by electrophoresis in a 5% polyacrylamide gel and radiolabelled RNA was recovered from the gel as described in Freeman et al. (1977).

Materials. The source of all materials has been described in detail (Freeman et al., 1979), except for the following: actinomycin D was a kind gift from Merck Sharp & Dohme,

**RESULTS**

Growth and plating efficiencies of mutants and revertants

Spontaneous $ts^+$ revertants, selected at 38.5 °C and their parental $ts$ mutants, as well as wild-type virus strains were assayed at three different temperatures in order to verify their respective phenotypes (Table 1). Most of the wild-type and revertants showed some temperature sensitivity at 37 to 38.5 °C (Table 1). They were, however, never reduced by more than 1 log$_{10}$ when compared to the lower temperature of 31 °C. In contrast, $ts$ mutants chosen for this study had a 3 log$_{10}$ or greater differential when grown between 31 and 37 or 38.5 °C. In addition, their ability to synthesize intracellular RNA and proteins, and release progeny virions at the permissive temperature of 31 °C as well as at the non-permissive temperature of 38 °C, have been checked by examining their protein products on SDS slab gels (Little & Huang, 1977, 1978; Clinton et al., 1978b; Rao & Huang, 1980, unpublished results).

**Identification of denatured RNA obtained from individual duplex RNA molecules**

Previously, formation of duplex RNA molecules between virion RNA and mRNA of VSV permitted the separation of all five individual mRNAs and each of the five coding regions (Freeman et al., 1977, 1979; Rhodes et al., 1977; Clewley & Bishop, 1979). To ensure that random internal strand scissions did not occur during the hybridization procedure and subsequent nuclease treatment, duplexes labelled in their mRNA were purified and then denatured. The resulting mRNAs were analysed on a polyacrylamide gel containing formamide along with poly(A)-containing mRNA molecules which had not undergone hybridization and nuclease treatment.

Fig. 1 shows that in comparison to the polyadenylated mRNA (lane a), the denatured RNAs from duplexes migrated slightly faster giving four distinct bands (lane b). L mRNA was in too small an amount to be seen clearly. The higher mobility of the other mRNAs after duplex formation, nuclease treatment and denaturation is consistent with the loss of an average poly(A) length of about 100 nucleotides. These results show that there were no internal strand scissions during ribonuclease digestion and that the loss of sequences was accounted for by the expected loss of unduplexed poly(A). The identification of these bands was further verified by eluting individual duplexes, denaturing the RNA and noting their migration rates (data not shown for the L, G and N duplexes). When purified NS and M duplexes were denatured and electrophoresed in a formamide–polyacrylamide gel, the NS mRNA (lane d) had a higher mobility than the M mRNA (lane c). This was a reversal of their mobility as double-stranded duplexes (Freeman et al., 1977). This lower mol. wt. for the NS mRNA compared to the M mRNA has recently been observed by separating $^3$H-labelled mRNAs and translating them individually in an *in vitro* system (Lynch et al., 1979) and by direct sequence analysis of the cloned M and NS genes (J. K. Rose, personal communication).

Thus, by examining denatured RNA after duplex formation the identity of each of the duplex bands was further verified (Freeman et al., 1977). In addition, the lack of internal strand scissions during duplex formation assured that analysis of $ts$ mutants by hybrid RNA formation would not be complicated by non-specific breaks in the RNA molecules.
Fig. 1. Autoradiogram of the migration of denatured VSV duplex RNAs on formamide-polyacrylamide gels. $^{32}$P-labelled VSV mRNAs were hybridized with VS virion RNA and single-stranded regions digested with ribonucleases. The ribonuclease-resistant duplexes were denatured and electrophoresed on a 3.75% formamide-polyacrylamide slab gel. (a) VSV mRNAs; (b) VSV mRNAs after duplex formation and denaturation; (c) purified M duplex after denaturation; (d) purified NS duplex after denaturation.

Table 1. Growth properties of VSV temperature-sensitive mutants and their revertants

<table>
<thead>
<tr>
<th>Mutant (complementation group)</th>
<th>Ratio of titres at 37 °C/31 °C</th>
<th>Ratio of titres at 38.5 °C/31 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (San Juan strain)</td>
<td>6.7 x $10^{-1}$</td>
<td>2.0 x $10^{-1}$</td>
</tr>
<tr>
<td>ts G22 (II)</td>
<td>5.6 x $10^{-2}$</td>
<td>2.5 x $10^{-5}$</td>
</tr>
<tr>
<td>ts+ G22-Rev 1</td>
<td>7.8 x $10^{-1}$</td>
<td>1.4 x $10^{-1}$</td>
</tr>
<tr>
<td>ts+ G22-Rev 2</td>
<td>7.4 x $10^{-1}$</td>
<td>1.6 x $10^{-1}$</td>
</tr>
<tr>
<td>ts+ G22-Rev 3</td>
<td>7.9 x $10^{-1}$</td>
<td>2.0 x $10^{-1}$</td>
</tr>
<tr>
<td>ts G31 (III)</td>
<td>1.0 x $10^{-3}$</td>
<td>2.7 x $10^{-5}$</td>
</tr>
<tr>
<td>ts+ G31-Rev 1</td>
<td></td>
<td>3.0 x $10^{-1}$</td>
</tr>
<tr>
<td>ts+ G31-Rev 4</td>
<td></td>
<td>3.8 x $10^{-1}$</td>
</tr>
<tr>
<td>ts G41 (IV)</td>
<td></td>
<td>1.2 x $10^{-4}$</td>
</tr>
<tr>
<td>ts+ G41-Rev 3</td>
<td>1.02</td>
<td>4.2 x $10^{-1}$</td>
</tr>
<tr>
<td>ts+ G41-Rev 5</td>
<td>1.23</td>
<td>6.5 x $10^{-1}$</td>
</tr>
<tr>
<td>ts O45 (V), clone 201</td>
<td>9.8 x $10^{-1}$</td>
<td>2.7 x $10^{-3}$</td>
</tr>
<tr>
<td>ts+ O45, rev clone 277</td>
<td>7.5 x $10^{-1}$</td>
<td>1.7 x $10^{-1}$</td>
</tr>
</tbody>
</table>
Determining conditions for digestion of heteroduplexes

In preliminary experiments on RNA heteroduplexes formed between mutant RNA and its revertant mRNA, specific cleavages by ribonucleases were made only when the salt concentration was lower or equal to 1 × SSC instead of the usual 2 × SSC. Digestions of homoduplexes were included as controls to exclude cleavages in regions of transient strand separation such as stretches rich in A–U base pairs. Several other nuclease digestion conditions were also examined. No specific cleavage of a heteroduplex was observed in 2 × SSC when the temperature was varied from 45 to 55 °C, when S1 nuclease was used instead of ribonuclease, when 10 to 30% dimethyl sulphoxide was included in the digestion buffer or when the time of digestion was extended from 0.5 to 3 h. In addition, cross-linking of duplexed RNA with 4'-hydroxymethyl-4,5',8-trimethylpsoralen (Isaacs et al., 1977) was examined as a way of reducing the non-specific cleavages in the duplexes. The cross-linking was successful since the duplexes could not be denatured; however, after ribonuclease digestion in 1 to 2 × SSC and electrophoresis of the duplexes, only indistinct wide bands were observed.

Rather than using mutants and their revertants, heteroduplexes were formed between RNA of the strain of origin of the mutants (wild-type VSV, Glasgow strain) and 32p-labelled mRNA from cells infected with tsG41, tsG31 or tsG22. A general result was that each of the five heteroduplexes formed with each pair of mutant and wild-type virus RNAs was specifically cleaved at no more than one site. Although the excess number of cleavages did not permit specific gene assignments, their number indicated that there have been very few base changes between the wild-type virus and temperature-sensitive mutants derived from it.

Group II ts G22 heteroduplexes

Because the group II VSV mutants are the least well-characterized and their gene assignments are the most controversial, the initial testing of heteroduplex formation was done with 32P-labelled mRNA from cells infected with tsG22 and unlabelled RNA extracted from virions of two of its ts+ revertants. As a control, homoduplexes were made between the mRNA and virion RNA of tsG22. Fig. 2 shows that there was no detectable difference between the digested homoduplexes (lanes a to g) and the heteroduplexes (lanes h to n and o to u). As the salt concentration was decreased during digestion with RNases all of the duplexes became more susceptible to digestion, with the larger ones being the most susceptible. Quantification of the N, NS and M bands showed that none of these heteroduplex RNA molecules was more susceptible to digestion by RNase than their respective homoduplex molecules. Similar results were obtained with heteroduplexes made between tsG22 and a third revertant (data not shown).

Although these results did not permit a gene assignment for tsG22, it showed that decreasing salt concentration increased hydrolysis of the duplexes by RNases and that the non-specific, increased susceptibility was randomly distributed among all the duplex molecules with the larger ones more susceptible than the smaller ones.

Mapping of ts G31 (III) by strand scission of the M heteroduplex

To map tsG31, homoduplexes were formed between 32P-labelled mRNA from tsG31-infected cells and unlabelled virion RNA of tsG31; heteroduplexes were made between labelled mRNA of tsG31 and virion RNA from two of its revertants. Two new duplexes, labelled Y1 and Y2, were produced by ribonuclease digestion of heteroduplexes (Fig. 3, lanes h to n and o to u) and were not seen after digestion of homoduplexes (Fig. 3, lanes a to g). Both revertants yielded identical results with the production of Y1 and Y2 duplexes of the same mobility. These duplexes had too high a mobility on these gels for an accurate molecular weight determination.
Fig. 2. Mapping of ts G22 (II). Duplexes were formed between 23P-labelled ts G22 mRNA and unlabelled virion RNA from ts G22 or ts + revertants. The duplexes were digested with ribonuclease for 30 min at 37°C in varying concentrations of SSC. The ribonucleases were inactivated by phenol extraction and the duplexes separated by electrophoresis in a 5% polyacrylamide gel. The lanes contain the following duplexes digested in the indicated concentrations of SSC. These were ts G22 homoduplex digested in: (a) 2, (b) 1, (c) 0.8, (d) 0.6, (e) 0.5, (f) 0.4, (g) 0.3 × SSC; ts G22-Rev 1 homoduplex digested in: (h) 2, (i) 1, (j) 0.8, (k) 0.6, (l) 0.5, (m) 0.4, (n) 0.3 × SSC.
Fig. 3. Mapping of ts G31 (III) by strand scission of the M heteroduplex. Duplexes were formed between 32P-labelled ts G31 mRNA and unlabelled virion RNA from ts G31 or ts+ revertant virions. The duplexes were digested with ribonucleases for 30 min at 37 °C in varying concentrations of SSC. The ribonucleases were inactivated by phenol extraction and the duplexes separated by electrophoresis in a 5% polyacrylamide gel. The lanes contain the following duplexes digested in the indicated concentrations of SSC. These were ts G31 homoduplex digested in: (a) 2, (b) 1, (c) 0·8, (d) 0·6, (e) 0·5, (f) 0·4, (g) 0·3 × SSC; ts G31–Rev 1 heteroduplex digested in: (h) 2, (i) 1, (j) 0·8, (k) 0·6, (l) 0·5, (m) 0·4, (n) 0·3 × SSC; ts G31–Rev 4 heteroduplex digested in: (o) 2, (p) 1, (q) 0·8, (r) 0·6, (s) 0·5, (t) 0·4, (u) 0·3 × SSC.

To answer the question of which heteroduplex gave rise to the Y1 and Y2 duplexes, the digestions of both homo- and heteroduplexes were compared (Fig. 3). Digestion of the L and G duplexes could not have produced the Y duplexes since digestion caused both to disappear similarly from homo- and heteroduplexes at salt concentrations less than 1 × SSC, whereas the Y duplexes were most clearly seen when digestion was in 0·5 × SSC. In addition, the Y duplexes were much too small to be two halves of the L or G duplex. Both the N and NS homo- and heteroduplexes were stable at the salt concentrations in which the Y duplexes were produced and were thus excluded. The amount of M heteroduplex diminished at the salt concentrations in which the Y duplexes were produced, while the M homoduplex appeared to
Mapping of ts mutants of VSV

Fig. 4. Mapping of ts G41 (IV) by strand scission of the N heteroduplex. Duplexes were formed between 32P-labelled ts G41 mRNA and unlabelled virion RNA from ts G41 or ts+ revertant 3 virions. The duplexes were digested with ribonucleases for 30 min at 37 °C in varying concentrations of SSC. The ribonucleases were inactivated by phenol extraction and the duplexes separated by electrophoresis in a 5% polyacrylamide gel. The lanes containing homoduplexes of ts G41 were digested in: (a) 1.5, (b) 2, (d) 1, (f) 0.8, (h) 0.6 x SSC; the lanes containing heteroduplexes of ts G41 mRNA and revertant 3 virion RNA were digested in: (c) 2, (e) 1, (g) 0.8, (i) 0.6 and (j) 1.5 × SSC.

Mapping of ts G41 (IV) by strand scission of the N heteroduplex

Heteroduplexes were formed with virion RNA of revertant 3 of ts G41 and 32P-labelled mRNA from ts G41-infected cells. To ensure that any cuts would be specific, a homoduplex

be more stable at these concentrations (compare lanes k and l with d and e). In addition, the small sizes of the Y duplexes were consistent with them being products of a scission of the small M duplex.

Although gene assignment of M to complementation group III from these results is somewhat equivocal, it agrees with previous studies on the more rapid turnover rate of the M protein in cells infected with ts G31 at the non-permissive temperature (Knipe et al., 1977a; Little & Huang, 1977). Further characterization of the Y1 and Y2 duplexes was not carried out because of the small amounts of available material.
Fig. 5. Specific strand scission of the N heteroduplex of ts G41 (IV). Duplexes were formed between $^{32}$P-labelled ts G41 mRNA and unlabelled virion RNA from ts G41 or ts + revertant 3 virions. The duplexes were digested with ribonucleases for 30 min at 37 °C in 2 x SSC. The ribonucleases were inactivated by phenol extraction and the duplexes purified by electrophoresis in a 5% polyacrylamide gel. The G and N duplexes were excised and eluted from the gel. The purified G and N duplexes were digested with ribonucleases for 30 min at 37 °C in varying concentrations of SSC. The ribonucleases were inactivated by phenol extraction and the duplexes separated by electrophoresis in a 5% polyacrylamide gel. The lanes contain purified duplexes digested in the indicated concentration of SSC. G homoduplex digested in: (a) 2, (b) 0.8, (c) 0.6 x SSC; G heteroduplex (d) undigested and digested in: (e) 2, (f) 1, (g) 0.8, (h) 0.6 x SSC. N heteroduplex (i) undigested and digested in: (j) 2, (k) 1, (l) 0.8, (m) 0.6, (n) 0.5 x SSC. N homoduplex (p) undigested and digested in: (q) 2, (r) 1, (s) 0.8, (t) 0.6, (u) 0.5 x SSC. Lane (o) is N heteroduplex digested in 0.6 x SSC and applied to the gel late so as to be electrophoresed for only 40% as long.
of virion RNA and $^{32}$P-labelled mRNA of $ts$ G41 was also formed. In Fig. 4, lanes (a), (b), (d), (f) and (h) contained homoduplexes, and lanes (c), (e), (g), (i) and (j) contained heteroduplexes which were obtained after digestion in different salt concentrations. The N heteroduplex began to disappear after digestion in 0.8 x SSC (lane g) and was absent after digestion in 0.6 x SSC (lane i). The digestion produced a new duplex (labelled X in Fig. 4) with a mobility between the N and NS duplexes, corresponding to about 1350 base pairs compared to 1525 base pairs for the N duplex. In contrast, the N homoduplex was stable in all the salt concentrations used, as were both the NS and M homo- and heteroduplexes. Digestion caused both the L and G homo- and heteroduplexes to disappear in a parallel fashion. These results suggest that a specific strand scission was made 175 base pairs from one end of the N heteroduplex, producing the X duplex. Similar results were obtained when heteroduplexes were formed between $ts$ G41 and another of its $ts^+$ revertants (Freeman et al., 1978).

The N heteroduplex made with RNA from $ts$ G41 and its revertant is the precursor to the X duplex

To establish that the X duplex was produced from the N heteroduplex, G and N heteroduplexes and homoduplexes (as shown in Fig. 4) were purified by preparative gel electrophoresis, individually excised and eluted. These purified duplexes which were first produced by digestion with RNases at 2 x SSC were then digested again at progressively lower salt concentrations to see which of the two duplexes gave rise to the X duplex. When purified N heteroduplex was digested with ribonucleases (Fig. 5, lanes i to n), the X heteroduplex was produced. This established that the X duplex was produced by strand scission of the N heteroduplex.

In an attempt to find the 175 base pair duplex which should also be produced by the cleavage that produced the X duplex, a sample was electrophoresed for a time short enough to retain this smaller duplex on the gel (lane o); however, this duplex was not seen. In control experiments, the X heteroduplex was not produced by digestion of the G heteroduplex (lanes d to h), the G homoduplex (lanes a to e) or the N homoduplex (lanes p to u). They were, in general, stable in the low salt digestion conditions.

Mapping of $ts$ O45 (V) by strand scission of the NS heteroduplex

The $ts$ O45 was also mapped by specific strand scission of heteroduplexes between mutant and revertant RNAs. Duplexes were formed between $^{32}$P-labelled mRNA of $ts$ O45 and unlabelled virion RNA of $ts$ O45, or revertants 207, 277, 290 and 296 of $ts$ O45 (Fig. 6). A new duplex, labelled Z, was produced by digestion of heteroduplexes (Fig. 6, lanes h to o, p to v, w to bb and cc to hh) but was not seen after digestion of homoduplexes (Fig. 6, lanes a to g). The production of the new Z duplex was paralleled by the gradual disappearance of the NS heteroduplex. The result was the same for each of the revertants, whereas the NS homoduplex remained stable. If the gel was electrophoresed for less time, another specific new duplex with a higher mobility was seen (data not shown). This probably represented the other portion of the cleaved NS heteroduplex. The amounts of the L, G, N and M homo- and heteroduplexes decreased in a parallel fashion as the salt concentration was lowered and thus digestion of these duplexes would not have produced the Z duplex.

In summary, when a heteroduplex of mutant and any of four revertant $ts$ O45 RNAs was digested with ribonucleases, a specific scission was made only in the NS heteroduplex, producing two new duplexes. This suggested that the temperature-sensitive lesion of complementation group V was located in the gene for the NS protein; however, the parentage of the revertants and a previous gene assignment (Lafay, 1974) qualify this conclusion. This somewhat surprising result will be considered further in Discussion.
Fig. 6. Mapping of ts O45 (V) by strand scission of the NS duplex. Duplexes were formed between 32P-labelled ts O45 mRNA and unlabelled virion RNA from ts O45 or ts+ revertant virions. The duplexes were digested with ribonucleases for 30 min at 37 °C in varying concentrations of SSC. The ribonucleases were inactivated by phenol extraction and the duplexes separated by electrophoresis in a 5% polyacrylamide gel. The lanes contained the following duplexes digested in the indicated concentration of SSC: ts O45 homoduplex digested in: (a) 2, (b) 1.5, (c) 1, (d) 0.8, (e) 0.6, (f) 0.5, (g) 0.4 × SSC; ts O45–Rev 207 heteroduplex digested in: (h) 2, (i) 1.5, (j) 1, (k) 0.8, (l) 0.6, (m) 0.5, (n) 0.4, (o) 0.3 × SSC; ts O45–Rev 277 heteroduplex digested in: (p) 2, (q) 1.5, (r) 1, (s) 0.8, (t) 0.6, (u) 0.5, (v) 0.4 × SSC; ts O45–Rev 290 heteroduplex digested in: (w) 2, (x) 1.5, (y) 1, (z) 0.8, (aa) 0.6, (bb) 0.5 × SSC; ts O45–Rev 296 heteroduplex digested in: (cc) 2, (dd) 1.5, (ee) 1, (ff) 0.8, (gg) 0.6, (hh) 0.5 × SSC.
These results on VSV RNA duplexes extend the methodology to a direct analysis of ts mutants by biochemical means. Genetic mapping of VSV mutants had not hitherto been done, since the single-stranded RNA genome of VSV undergoes neither recombination nor reassortment. By heteroduplex analysis we can assign complementation groups III and IV to genes coding for the M and N proteins respectively. Complementation group I was not examined because the protein results are clear-cut (Szilagyi & Pringle, 1972; Hunt et al., 1976). The inability to assign complementation groups II and V to a single gene by this method can be explained in several ways.

Revertants were used in the formation of the heteroduplex instead of the parental wild-type. This was because silent mutations may have accumulated in both the parental wild-type and ts genomes during passage. The use of revertants in this strategy of gene assignment has, however, the weakness that the reversion event may be in a gene other than the originally mutated one (extragenic suppression; Jarvik & Botstein, 1975). Ribonuclease cleavage at such a point of reversion would lead to an erroneous gene assignment.

Heteroduplex analyses of ts O45 (V) assigned group V mutants to the NS gene. However, thermolability and morphogenetic studies suggest that the group V mutants have lesions in the glycoprotein gene. Because of this result, proteins synthesized by this particular clone of ts O45 at the non-permissive temperature were examined by polyacrylamide gel electrophoresis and the phosphorylated forms of the NS protein appeared completely normal, whereas the G protein showed the expected migrational difference (G. M. Clinton, unpublished results). Similar changes in the migration of G protein have been reported for group II mutants (Printz & Wagner, 1971). Extragenic suppression of the G lesion by reversion in the NS gene would explain these varying results. Moreover, both NS and G proteins affect transcription and morphogenesis (Emerson & Yu, 1975; Clinton et al., 1978 a; Com bard et al., 1979).

Aside from extragenic suppression, there is another possible explanation for these observations made with ts O45. All of the revertants are ts+ for growth but only some have regained virion thermostability. Because of these two phenotypic changes, the mapping was done ‘blind’, i.e. the phenotype of the revertants was not known. After the mapping, from the information provided, it was apparent that the ts O45 stock used in the mapping was not the direct parent of the revertants but was instead derived after one further plaque purification from the parental stock of the ts mutant. Because the ts O45 used in the mapping was not the direct parent of the revertants, it is possible that a silent mutation in the NS gene occurred during the subcloning of ts O45, resulting in the cleavage in the NS heteroduplexes. Thus, no conclusive gene assignment can be made for ts O45 from this result.

When RNA heteroduplexes of ts G22 (II) and its revertants were digested with ribonucleases, no specific cleavage was observed (Fig. 2). For reversion to have occurred, there must have been a nucleotide change; however, ribonuclease may not necessarily cleave at this base mismatch because not all base mismatches destabilize the duplex. The larger size of the purine mismatches in G–A, A–A or G–G sterically push the duplex apart. Because of the smaller size of the pyrimidines, C–U, C–C or U–U, such mismatches are less destabilizing. The other possible mismatches will destabilize the duplex to an intermediate extent. The G–C content of the flanking sequences will also influence the tendency for the mismatch to make the region single-stranded. A combination of these influences may make the base mismatch in the ts G22 heteroduplex too stable to be recognized as single-stranded by ribonuclease.

Undoubtedly, duplex analysis could be made more sensitive if some way were found to stabilize regions of homologous base pairs under low salt conditions. Unfortunately, the attempt to cross-link base-paired regions with psoralen failed to yield decisive results. Without the ability to stabilize homologous regions, this approach is limited to gene assignments and
extensions of this methodology for fine mapping within a gene would be too cumbersome and inaccurate. Nevertheless, some limited conclusions on gene assignments can be made. Moreover, the finding that denatured duplexes resulted in the recovery of intact mRNA species lacking only their poly(A) tails further supports the suggestion that post-transcriptional processing, such as splicing, of VSV mRNA does not occur (Freeman et al., 1979).

This paper is based on studies submitted to the Faculty of Arts and Sciences in partial fulfillment of the requirements for the degree Doctor of Philosophy. A preliminary report of part of these results was made at the 1977 meeting on Negative Strand Viruses and the Host Cell, Cambridge, U.K.

This work was supported by research grants AI 16625 from the National Institutes of Health and MV 54 from the American Cancer Society. G.J.F. was a trainee supported by an institutional NRSA T32 CA-09031. We thank Trudy Lanman for excellent technical support and Suzanne Ress for the computer-aided preparation of this manuscript.

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(Received 24 April 1981)