Genetic evidence for multiple functions of the matrix protein of vesicular stomatitis virus

P. Coulon,* V. Deutsch, F. Lafay, C. Martinet-Edelist, F. Wyers, R. C. Herman and A. Flamand

Laboratoire de Génétique des Virus, CNRS, 91198 Gif-sur-Yvette Cedex, France and Syntex Research, 3401 Hillview Avenue, Palo Alto, California 94304, U.S.A.

TsO82, a spontaneous temperature-sensitive (ts) mutant of vesicular stomatitis virus (VSV) isolated in chick embryo fibroblasts (CEFs), complements the five prototype ts mutants of the virus. The data presented here indicate that the defect in tsO82 is localized in the M gene. The mutation changed a methionine to an arginine at position 51 of the M protein. Only true revertants could be isolated, and their frequency was low, perhaps due to the type of substitution required to return to the wild-type phenotype. TsO82 does not exhibit hypertranscription, in contrast to the data reported for all of the other ts mutants affected in the M protein. Moreover, tsO82 is conditionally ts, since it grows normally in BHK-21 cells at all temperatures. It exhibits no c.p.e. at the non-permissive temperature in CEFs. Our data argue for multiple functions of the M protein of VSV, the domain affected by the tsO82 mutation possibly being implicated both in the shut-off of cellular RNA synthesis, and for the recognition of a cellular factor required for efficient viral RNA synthesis.

Vesicular stomatitis virus (VSV) is an enveloped negative strand RNA virus that belongs to the rhabdovirus family (Wagner, 1987). Five proteins are encoded by the viral genome, three of which (N, NS and L) are associated with the RNA to constitute the ribonucleoprotein core (RNP). The other two are membrane-associated: the G protein which is the only external protein, and the M protein which is not exposed at the surface of the virion (Zakowski & Wagner, 1980). In addition, the M protein also interacts with the nucleocapsid and causes it to form a coiled structure (Newcomb & Brown, 1981; Newcomb et al., 1982).

Studies of temperature-sensitive (ts) mutants of VSV defined complementation groups (Flamand, 1970; Pringle, 1970) and the genes encoding the five proteins have each been assigned to a complementation group (for a review see Pringle, 1987). Nevertheless, some ts mutants complement representatives of all five complementation groups. Most of these have proved to belong to complementation group I (mutated in the L gene), and exhibit intracistronic complementation (Flamand, 1980). A few others are clearly distinct from these group I mutants and up to now have been unclassified. The mutant tsO82 belongs to this category.

TsO82 is a spontaneous ts mutant that was isolated in chick embryo fibroblasts (CEFs) (Flamand, 1973). It grows slowly at the permissive temperature, and small plaques devoid of infectious particles appear when it is titrated at the non-permissive temperature (Martinet, 1977). The tsO82 mutant has been analysed by its rescue at the non-permissive temperature by u.v.-irradiated virus. The results suggest that rescue is mediated through two complementation processes: gene survival and structural protein supply by the u.v.-irradiated virus. These properties are shared with the ts mutants of complementation group II, suggesting that tsO82 may also belong to group II (Deutsch et al., 1979). Group II ts mutants have been identified as NS protein mutants by peptide mapping experiments (Lafay & Bénéjéan, 1981). Moreover, by in vitro translation of NS mRNA it has been shown that this mRNA directs the synthesis of a second product, in addition to the NS protein, by initiating translation at an internal AUG codon within the same open reading frame (ORF) (Herman, 1986). By analysis of the nucleotide sequence, it has been suggested that another protein could also be encoded by the NS mRNA in a second ORF, but that hypothetical product has not been detected (Hudson et al., 1986). The possible polycistronic nature of the NS gene of VSV would make it similar to the phosphoprotein gene of the non-segmented negative strand paramyxoviruses which has been shown to encode multiple proteins (Morrison, 1988; Curran & Kolakofsky, 1989). Thus an attractive hypothesis is that tsO82 is mutated in the NS gene.

Other published characteristics of tsO82 are that it does not induce inhibition of cellular RNA synthesis in
CEFs, in contrast to the wild-type and all other ts mutants which have been tested, and that infection of CEFs by VSV induces a modification of the plasma membrane which leads to an inhibition of uptake of extracellular uridine; no such modification is observed at the non-permissive temperature with group III and tsO82 mutants (Genty, 1978). Group III has been assigned to the M protein (Lafay, 1974). Therefore the possibility that tsO82 is mutated in the M protein could not be ruled out. To elucidate the origin of the ts mutation of tsO82, we examined some of its biological properties and have attempted to locate its molecular defect by nucleotide sequencing.

The ts phenotype of tsO82 is exhibited in CEFs and in HeLa cells, but it grows as efficiently as the wild-type in BHK-21 cells at all temperatures (data not shown). This conditional phenotype has been described for temperature-dependent (td) mutants of VSV serotypes Indiana and New Jersey (e.g. tdCE) (Pringle, 1978). Only the New Jersey tdCE mutants have been studied and they were shown to be affected in polymerase function. At the non-permissive temperature tsO82 had no c.p.e. on CEFs, whereas this c.p.e. is conserved at all temperatures on BHK-21 cells (data not shown). This indicates that the ts defect and the lack of c.p.e. are related.

Experiments were undertaken to isolate independent revertants from tsO82. Three successive passages in CEFs at low temperature followed by two passages at the non-permissive temperature were necessary to isolate large plaques in the Petri dishes. The migration profile of tsO82 proteins was examined by SDS-PAGE and compared to that of the Orsay wild-type virus. The migration profile presented in Fig. 1 (lane 2) shows that M protein from tsO82 migrated faster than that from wild-type virus (lane 1). On the other hand, M protein of two temperature-resistant revertants of tsO82 co-migrated with M protein of wild-type virus (lanes 3 and 4). This suggested that an alteration of the M protein could be responsible for the tsO82 defect.

One of the criteria for the classification of ts mutants of VSV is the synthesis of mRNA at the non-permissive temperature. Viral RNA species from HeLa cells infected with tsO23 or tsO82 were analysed in sucrose gradients as described previously (Martinet et al., 1979). At the non-permissive temperature only tsO23 was found to exhibit hypertranscription, whereas tsO82 made all RNA species but in lower amounts than at the permissive temperature (Table 1), or than the wild-type virus (data not shown).

More precise quantification of viral mRNA was performed at 3 and 6 h post-infection of CEFs by tsO82 or by one temperature-resistant revertant (tsO82-RII). Total intracellular nucleic acids were extracted, denatured with glyoxal, and then serial dilutions of the

![Fig. 1. Comparison of electrophoretic mobilities of proteins from tsO82 and its revertants. Proteins were synthesized in BHK cells infected at the permissive temperature with the wild-type virus (lane 1), tsO82 (lane 2), or either of two temperature-resistant revertants (lanes 3 and 4). Cells infected with the different viruses were incubated in presence of 20 μCi of [35S]methionine from 4 to 4.5 h post-infection, and the proteins of the cell extracts were resolved in a 10% discontinuous SDS-polyacrylamide gel.](image)

<table>
<thead>
<tr>
<th>Virus</th>
<th>38S</th>
<th>28S</th>
<th>13S–17S</th>
</tr>
</thead>
<tbody>
<tr>
<td>TsO23</td>
<td>0.188†</td>
<td>0.364</td>
<td>0.760</td>
</tr>
<tr>
<td>NPT</td>
<td>0.084 (45%)</td>
<td>0.183 (50%)</td>
<td>0.952 (125%)</td>
</tr>
<tr>
<td>TsO82</td>
<td>0.267</td>
<td>0.493</td>
<td>0.648</td>
</tr>
<tr>
<td>NPT</td>
<td>0.096 (36%)</td>
<td>0.107 (22%)</td>
<td>0.314 (48%)</td>
</tr>
</tbody>
</table>

* Permissive (PT) and non-permissive (NPT) temperatures are 31 °C and 39.6 °C, respectively. HeLa cells were infected at an m.o.i. of 200, treated with actinomycin D (10 μg/ml), and labelled with [3H]uridine (20 μCi/ml) from 2 to 5 h p.i. Cytoplasmic RNAs were analysed by sucrose velocity gradients as previously described (Martinet et al., 1979).
† Quantity of RNA, expressed in arbitrary units.
‡ Percentage of RNA syntheses at NPT with respect to PT.
samples were spotted onto nitrocellulose membrane as described previously (Blondel et al., 1988). Viral mRNAs were detected by hybridization with \( ^{32}P \)-labelled genomic RNA. Viral antigenome is also detected with this probe, but this RNA species is present in very low amounts and does not contribute much to the hybridizations. The data presented in Fig. 2 show that transcription of tsO82 was reduced by approximately threefold at the non-permissive temperature. Thus tsO82 can be considered as an RNA +/− mutant.

The NS and M genes of tsO82 and of one temperature-resistant revertant (tsO82-RII) were sequenced by the dideoxynucleotide chain-terminating technique in an attempt to locate the defect. Four positive sense oligonucleotide primers were used to sequence each gene on genomic RNA (Table 2).

With respect to the NS gene, the sequences of both tsO82 and revertant viruses were identical. However, as the first oligonucleotide primer covered the beginning of the NS coding region, the codons for the first seven amino acids were missing from our sequences. The NS sequence of the Orsay strain was compared to previously published NS sequences (Table 3). The Orsay strain differed from the Mudd-Summers strain by three nucleotides (two amino acids), from the Glasgow strain by five nucleotides (four amino acids) and from the San Juan strain by 20 nucleotides (eight amino acids). The Glasgow and Mudd-Summers strains differed from each other by six nucleotides (four amino acids). The San Juan strain differed from the Mudd-Summers and Glasgow strains by 23 nucleotides (10 amino acids), indicating a larger divergence between this strain and the three others.

The M sequence of the revertant tsO82-RII was identical to the sequence published by Gopalakrishna & Lenard (1985) for the M of wild-type virus, Orsay. The comparison between the tsO82 and tsO82-RII M sequences indicated only a single nucleotide substitution in the tsO82 mutant. At position 193, a thymine in the wild-type (and revertant) sequence was changed to a guanine in tsO82, which led to the replacement of a methionine by an arginine at position 51 in the M protein. Since the tsO82-RII revertant had the same M sequence as the Orsay wild-type strain it was a true revertant. Another independent revertant clone was subsequently sequenced in the amino-terminal region of the M gene. The sequence obtained was identical to the wild-type sequence, confirming the selection of a second true revertant.

Thus a single amino acid substitution seems to be responsible for the different migration profile of the tsO82 M protein in SDS–polyacrylamide gels when compared to the M protein of the Orsay wild-type virus and of the revertants (Fig. 1).

**Table 2.** Nucleotide sequence of the oligonucleotides used to determine the positive sense sequence of the coding region of the NS and M genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Location</th>
</tr>
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<tbody>
<tr>
<td>NS</td>
<td>5'-ATATCATGGATAATCTCAC-3' (6-24)</td>
<td>(6–24)</td>
</tr>
<tr>
<td></td>
<td>5'-CTGAACAGAAAATGGAAGA-3' (201-219)</td>
<td>(201–219)</td>
</tr>
<tr>
<td></td>
<td>5'-GAGAGGAGAAATCCCAGTG-3' (405-423)</td>
<td>(405–423)</td>
</tr>
<tr>
<td></td>
<td>5'-GGTCTCTCTCAAGACATC-3' (603-621)</td>
<td>(603–621)</td>
</tr>
<tr>
<td>M</td>
<td>5'-TTATCATGAGTCCCTAATC-3' (36–54)</td>
<td>(36–54)</td>
</tr>
<tr>
<td></td>
<td>5'-ATGATCGCCATGAATTAAGG-3' (202-221)</td>
<td>(202–221)</td>
</tr>
<tr>
<td></td>
<td>5'-GTCTTTGCTCTAATGAGGC-3' (376–394)</td>
<td>(376–394)</td>
</tr>
<tr>
<td></td>
<td>5'-CGGTTCGCTCAATGAC-3' (550-568)</td>
<td>(550–568)</td>
</tr>
</tbody>
</table>

It is difficult to isolate temperature-resistant revertants from tsO82. If tsO82 were a double mutant, the frequency of revertants would be so low that it would be nearly impossible to select them. Moreover, tsO82 is not
ts in BHK-21 cells, and, again, it is very improbable that two hypothetical mutations would be host range-restricted. However, we have sequenced only the NS and the M genes, and we cannot exclude the possibility that tsO82 has a second mutation elsewhere in the genome. One possible explanation for the low frequency of reversion could be the presence of the ATG codon in the wild-type cDNA sequence. Indeed, methionine is encoded by one codon only and the mutation in tsO82 is a transversion. Only a second transversion (G to T) would reestablish the initial wild-type codon. Interestingly, in each attempt it was only possible to select true revertants. This result could be an indication that second site suppressors of the tsO82 phenotype do not exist, and it implies that the methionine at position 51 has a critical role.

The mutation in the M gene does not impair the complementation between tsO82 and tsO23. This is the first example of intracistronic complementation between VSV mutants affected in a gene other than the L gene. This result implies that M protein has more than one functional domain. Many data argue in favour of multiple functions for this protein. First, it acts structurally through its interactions with both the lipid bilayer and with the nucleocapsid. Cross-linking experiments on intact virions lead to the conclusion that the M protein is located on the inner side of the membrane (Zakowski & Wagner, 1980). Interactions with the nucleocapsid have been reported by Newcomb et al. (1982) who observed reassociation of nucleocapsids in the presence of M protein after disruption by detergent plus salt, and subsequent dialysis. A third role for M protein is functional, because it has been demonstrated that M acts as an inhibitor of transcription (Combard & Printz-Ané, 1979; Carroll & Wagner, 1979). These three functions are impaired at the non-permissive temperature for all complementation group III mutants studied so far. Cells infected with ts group III mutants, and especially by tsO23, exhibit an overproduction of viral mRNAs at the non-permissive temperature (Clinton et al., 1978; Marti-net et al., 1979), and there is no maturation of virions at the permissive temperature if the tsO23 proteins are synthesized at the non-permissive temperature (Lafay, 1974). In contrast, shift-down experiments showed that the structural proteins of tsO82 synthesized at the non-permissive temperature are incorporated into the virions at the permissive temperature (data not shown).

Using monoclonal antibodies specific for the M
protein (Pal et al., 1985a), it has been shown that the amino-terminal region of M regulates transcription (Pal et al., 1985b; Ogden et al., 1986). More precisely, the RNP-binding site has been localized to region 17 to 31 of the M protein (Shipley et al., 1988). However, most of the group III mutants are not mutated in this region, suggesting that many amino acids outside of the binding region can affect the RNA transcription inhibition property (Morita et al., 1987).

Quantification of viral mRNA synthesis by tsO82 in CEFs showed that transcription is less efficient at the non-permissive temperature than at the permissive temperature (Table 1, Fig. 2). Thus, tsO82 did not exhibit the hypertranscription typical of group III mutants. That is, amino acid 51 is not involved in this function.

TsO82 does not inhibit cellular RNA synthesis at the non-permissive temperature (Genty, 1978). Since M protein has been observed in the nuclear fraction from VSV-infected cells (Lyles et al., 1988), an attractive hypothesis would be that the M protein domain modified by the tsO82 mutation could play a role in the shut-off of cellular RNA synthesis. Whether the tsO82 M protein is found in the nucleus at the non-permissive temperature is not known at present.

TsO82 is host range temperature-sensitive. This implies that the structural role of the M protein is not affected at high temperature, and secondly that the domain modified by this mutation may interact with a cellular product. Like all the host range tdCE mutants isolated by Pringle (1978), tsO82 is restricted in CEFs but not in BHK cells. It has been hypothesized that a host cell factor must interact directly with the L protein for maximum RNA polymerase activity. TsO82 is affected in the M protein, leading to the possibility that the M protein may play a role in the interaction between L protein and the cellular factor(s); this function is impaired in the tsO82 mutant and this could explain the RNA +/− phenotype of the mutant. This function can be taken over by the structural proteins supplied by the u.v.-irradiated virus, and explains the results obtained by Deutsch et al. (1979).

In conclusion the M protein seems to have at least two separate domains. The first is responsible for the structural interactions between the RNP and the lipid bilayer, as well as for the regulation of viral transcription; both of these functions are affected in complementation group III ts mutants. The second domain may be responsible for the shut-off of cellular RNA synthesis and perhaps also for the recognition of a cellular factor required for efficient viral RNA synthesis; these functions are affected in the tsO82 mutant.

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