Identification of an amino acid change that affects N protein function in vesicular stomatitis virus

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TsW16B is a temperature-sensitive mutant of vesicular stomatitis virus. Others have shown that it is temperature-sensitive for replication in vivo and for transcription in vitro and that these phenotypes are probably due to mutation of the N (nucleocapsid) gene. Five independent revertants were isolated from tsW16B based on their ability to grow at 39 °C. The thermosensitivity of in vitro transcription by these revertants was similar to that of the wild-type virus [wt(HR)] from which tsW16B was derived. Fractionation–reconstitution studies of two revertants indicated that the reversion was in the N or P (phosphoprotein) gene. The N and P genes of wt(HR), tsW16B, and these two revertants were sequenced. There were no differences between the P genes. Comparison of the predicted N protein sequences of wt(HR), tsW16B and the two revertants indicated that the growth and in vitro transcription phenotypes of tsW16B were due to a change of amino acid residue 238 from threonine to isoleucine. The amino acid at position 238 in the other three revertants also showed an exact reversion to threonine. Amino acid residue 238 lies in a domain of the N protein which is highly conserved among vesiculoviruses.

Vesicular stomatitis virus (VSV), a member of the Rhabdoviridae family, has a negative-stranded RNA genome that codes for at least five proteins. The P (phosphoprotein) and L (large) proteins are packaged in the virion and form a polymerase complex which is responsible for the synthesis of five monocistronic mRNAs (transcription) and full-length RNA (replication). The template for RNA synthesis consists of viral RNA tightly complexed with the N (nucleocapsid) protein which renders the RNA nuclease-resistant (Banerjee & Barik, 1992). Nucleocapsid-bound N protein can modulate the properties of the transcriptase (Ngan et al., 1974; Perrault & McLean, 1984; Beckes et al., 1987). Nucleocapsid-bound N protein is not only bound to RNA but also binds other molecules of N protein and probably the polymerase complex. It has been proposed that the concentration of soluble N protein modulates the switch from transcription to replication (Leppert et al., 1979).

Transcription of viral mRNA predominates early in infection; the viral polymerase complex binds to the 3′ end of the genome and synthesizes a 47 nucleotide ‘leader’ RNA before sequentially transcribing the five mRNAs. As viral protein levels rise, soluble N protein binds to an encapsidation signal on the leader RNA and this is followed by co-operative binding of further N protein monomers. It is postulated that binding of N protein to the nascent RNA suppresses polymerase termination, resulting in the production of genome-length RNA. One important function of the P protein appears to be its ability to bind to soluble N protein and prevent N protein aggregation into complexes which fail to support replication (Peluso & Moyer, 1984, 1988; Davis et al., 1986; Wertz et al., 1987; Masters & Banerjee, 1988; Howard & Wertz, 1989). Comparison of the N protein sequences of various rhabdoviruses suggests that there is a domain structure in which the central domain is highly conserved but the N- and C-terminal domains are more variable (Crysler et al., 1990). The function(s) of the domains of the N protein have not been defined, although the C-terminal five amino acids may be critical for the association with the P protein (Takacs et al., 1993).

To investigate the N protein further, we examined a temperature-sensitive (ts) mutant of VSV(Indiana), tsW16B (Holloway et al., 1970). TsW16B can transcribe mRNA upon infection of cells at the non-permissive temperature of 38 °C, but is defective in replication (Wong et al., 1972). However, transcription in vitro is more temperature-sensitive at 38 °C than transcription.
by the parental wild-type (wt)HR virus (Cairns et al., 1972). Although the N protein of tsW16B has been implicated in conferring the temperature-sensitive phenotype (Ngan et al., 1974; Cormack et al., 1975) the exact location of the genetic lesion was unknown. Evidence is presented here to suggest that an amino acid change at position 238 of the N protein can confer both the inability to grow at 39 °C and the in vitro temperature-sensitive transcription phenotypes. The crucial role of this amino acid residue is indicated by the fact that it reverted to the wild-type amino acid in all five revertant viruses examined.

To investigate the altered amino acid(s) that cause the phenotypic differences in tsW16B, we isolated spontaneous revertants. This was done with minimal selection pressure and for as few passages as possible so that revertants had few nucleotide changes compared to tsW16B. TsW16B was obtained from Dr R. R. Wagner who received it from Drs D. V. Cormack and A. F. Holloway; the parental wt(HR) strain was obtained from Dr R. A. Lazzarini. Plaque purified tsW16B was titrated on L-929 cells at 31 °C and 39 °C and potential revertants were isolated by picking plaques from plates incubated at 39 °C. Only one revertant from each plaque-purified stock of tsW16B was used to ensure that all revertants were independent. Putative revertant plaques were grown on BHK-21 cells at 31 °C for 24 h and titrated at 31 °C and 39 °C on L-929 cells (Hutchinson et al., 1990). Revertants were recloned by growing a plaque from a 31 °C plate on BHK-21 cells at 31 °C, followed by titration at 31 °C and 39 °C. Revertants were isolated from plaque-purified stocks of tsW16B at a frequency of $10^{-5}$ to $10^{-6}$, consistent with their being single-site revertants (Pringle, 1987). Revertants are designated by the letter R and plaque numbers (pXX) corresponding to the arbitrary number of the plaque isolate of tsW16B from which they were derived.

Five revertants were isolated. Their plating efficiency at 39 °C compared to 31 °C was much higher than that of tsW16B and was comparable to that of the parental wt(HR) strain (Table 1). The temperature sensitivity of in vitro transcription was examined as previously described (Hutchinson et al., 1990). RNA synthesis at 39 °C compared to 31 °C was greater for the revertants than for tsW16B and was comparable to wt(HR) virus (Table 1). Thus, all five revertants had at least partially regained the wt(HR) phenotype with respect to both growth and the temperature-sensitivity of in vitro transcription. This agrees with the data of Cairns et al. (1972) and Ngan et al. (1974) who also found that all the revertants of tsW16B that they examined had co-reverted for both phenotypes.

It has been reported that tsW16B has a temperature-sensitive defect in a template-associated protein, probably the N protein (Ngan et al., 1974; Cormack et al., 1975). Fractionation–reconstitution experiments were performed to ascertain whether the altered protein in a particular revertant was template-associated or if extracistronic suppression had occurred. Wt(HR), tsW16B, RW16Bp2 or RW16Bp33 virions were fractionated into pellet (template-containing) and supernatant (polymerase-containing) fractions and subjected to homologous or heterologous transcription reconstitution reactions (Hunt & Wagner, 1974). The published method was modified in that the pellet fraction was obtained after dilution with seven volumes of a modified Triton-high salt solubilizer [which contained a higher concentration of NaCl (2 M), no MgSO₄, and 2 mM-EDTA] and the diluted solubilized virus was incubated at 0 °C for 1 h prior to ultracentrifugation. In homologous reconstitution reactions, although both revertants were rather more thermosensitive after fractionation–reconstitution, they were clearly more temperature-resistant than tsW16B (Table 2). In heterologous reconstitutions, when the pellet fraction from tsW16B was combined with the supernatant fraction from either of the two revertants, the temperature-sensitive phenotype was observed, suggesting that the revertant protein was not a component of the supernatant fraction. However, if the pellet fraction from either of the revertants was mixed with the supernatant fraction of tsW16B, the revertant phenotype was obtained (Table 2), indicating that the altered protein responsible for the revertant phenotype in RW16Bp2 and RW16Bp33 was a component of the pellet fraction. SDS–PAGE showed that the pellet fraction contained a substantial amount of the P protein as well as the N protein (data not shown). Thus, since the altered protein might be N or P, the N and P genes from wt(HR), tsW16B, RW16Bp2 and RW16Bp33 were sequenced.

### Table 1. Efficiency of plaque formation and temperature sensitivity of in vitro transcription at 39 °C compared to 31 °C

<table>
<thead>
<tr>
<th>Virus</th>
<th>P.f.u. (39 °C/31 °C × 100)</th>
<th>RNA synthesis (39 °C/31 °C × 100)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt(HR)</td>
<td>22</td>
<td>35 (20 to 52; 10)</td>
</tr>
<tr>
<td>tsW16B</td>
<td>0.0001; 10</td>
<td>8 (4 to 12; 11)</td>
</tr>
<tr>
<td>RW16Bp2</td>
<td>21</td>
<td>41 (26 to 60; 8)</td>
</tr>
<tr>
<td>RW16Bp33</td>
<td>26</td>
<td>40 (26 to 61; 8)</td>
</tr>
<tr>
<td>RW16Bp35</td>
<td>15</td>
<td>45 (34 to 73; 5)</td>
</tr>
<tr>
<td>RW16Bp36</td>
<td>6</td>
<td>31 (23 to 36; 4)</td>
</tr>
<tr>
<td>RW16Bp37</td>
<td>24</td>
<td>33 (26 to 36; 3)</td>
</tr>
</tbody>
</table>

* [3H]UTP incorporation determined after a 2 h incubation. The range of values and number of independent experiments are shown in parentheses.
† Range for the plaque purified stocks of tsW16B from which revertants were isolated. Values have not been corrected for revertants. All plaques seen at 39 °C that were tested arose from revertants.
RNA was reverse transcribed into cDNA and the cDNA was amplified using the PCR. Single-stranded DNA generated by asymmetric PCR was directly sequenced in order to obtain a consensus sequence for both strands (Hunt & Hutchinson, 1993). Data were analysed with the IBI-Pustell programs (Kodak-IBI). The N gene of wt(HR) differed at 10 nucleotide positions from that of the San Juan strain of VSV(Indiana) and the P gene differed at 22 nucleotide positions (our data, GenBank U12967; Rose & Schubert, 1987). Comparison of the predicted amino acid sequence of wt(HR) with that of wt(San Juan) revealed two amino acid differences in the N protein (out of 422 amino acids) and nine amino acid differences in the P protein (out of 265 amino acids). This is in agreement with reports that the N protein is more conserved than the P protein (Rose & Schubert, 1987).

The P genes of tsW16B and wt(HR) were identical. This confirms the conclusion of Ngan et al. (1974) that the altered protein in tsW16B was probably not the P protein but was more likely to be the N protein. The N gene of tsW16B differed from that of the wt(HR) virus at four nucleotide positions (Table 3). Only the mutations at nucleotides 776 and 906 changed the predicted amino acid sequence. Thus these were potentially involved in conferring temperature sensitivity on tsW16B.

The P gene of RW16Bp2 and RW16Bp33 was identical to the P gene of tsW16B. The N genes differed from tsW16B only at VSV nucleotide residue 776. In both revertants this nucleotide had changed to the C residue found in wt(HR). The regions containing nucleotides 776 and 906 of RW16Bp35, RW16Bp36, and RW16Bp37 were sequenced. These revertants were all identical to tsW16B at residue 906 but had all reverted to the C observed in wt(HR) at residue 776. Thus, it appears that a mutation in nucleotide 776, which results in amino acid 238 being isoleucine rather than threonine, confers both the temperature-sensitive growth phenotype and the in vitro temperature-sensitive transcription phenotype on tsW16B. However, we cannot tell from these results whether amino acid 281 [Phe in wt(HR) and Leu in tsW16B and the revertants] is important in conferring an environment in which the effects of amino acid 238 Thr → Ile can be detected.

Since all five revertants had the same nucleotide change, the nature of the amino acid residue 238 may play some key role in N protein function. This residue is located in a central domain of 34 amino acids that is highly conserved in various vesiculoviruses (Crysler et al., 1990). This conserved domain is also seen in the lyssavirus genus of the rhabdovirus family (Bourhy et al., 1993) as well as some other rhabdoviruses (Gilmore & Leong, 1988; Bras et al., 1994). A limited similarity at the amino acid sequence level is also detected when rhabdoviruses and members of other non-segmented negative-strand virus families such as the paramyxoviruses and filoviruses are compared (Barr et al., 1991; Sanchez et al., 1992). Interestingly, when the alignments are examined, the position equivalent to amino acid 238 (Thr → Ile in tsW16B) is threonine, serine or isoleucine in all these viruses with the exception of the pneumovirus, respiratory syncytial virus.

The function of this conserved domain (domain II) is not known, although it may play a particularly important role in the elongation reaction during RNA synthesis (Moyer, 1989). Nucleocapsid proteins bind to RNA, as
as well as to other viral proteins, including the N protein, the P protein and possibly the M protein during virus maturation. Since the P protein, the M protein, and domains I and III of the N protein are far less conserved than domain II of the N protein, it has been suggested that the most likely ligand for this region would be the RNA (Masters & Banerjee, 1987; Crytsler et al., 1990; Bernard et al., 1990). The presence of positively charged amino acids in a relatively hydrophobic domain might be consistent with this (Bernard et al., 1992). In addition, electron microscopy suggests that the central domain of the N molecule may interact with the RNA (Thomas et al., 1985), although the correlation between linear sequence data and three-dimensional structure is not clear. Others have speculated that since the carboxyl terminus of the N proteins of rhabdoviruses contains a high percentage of basic amino acids it may interact with the phosphate backbone of the RNA (Gilmore & Leong, 1988). If this were the case, the central conserved domain of the protein may be involved in protein–protein interactions and the change to isoleucine at amino acid 238 in tsW16B may disrupt or destabilize P:N or N:N binding, especially at elevated temperatures. We are currently investigating the properties of the tsW16B and RW16B N proteins in an attempt to elucidate the role of the central domain of the N protein.

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References


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