Temperature-sensitive Mutant of Newcastle Disease Virus which has an Altered Nucleocapsid-associated Protein

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SUMMARY

Analysis of six temperature-sensitive (ts) mutants of Newcastle disease virus (NDV) representing each of six complementation groups by both SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis revealed that in only one mutant was there an alteration in the isoelectric point of a protein. This altered protein was the nucleocapsid-associated protein, NAP. In addition, the mobility of the haemagglutinin–neuraminidase protein, HN, was decreased on non-reduced SDS-PAGE in this mutant.

All independent ts+ clones derived from this mutant had normal NAP but HN protein migrated at the decreased rate. Haemagglutinating activity of wild-type (ts+) and ts virions was equally thermostable. Wild-type and ts+ clones derived from this ts mutant were RNA (+) at both permissive and non-permissive temperatures, whereas the ts mutant was RNA (−) at the non-permissive temperature. This ts mutant appears to be a double mutant in both HN and NAP genes, the latter only being a temperature-sensitive lesion which affects virus-directed RNA synthesis.

Temperature-sensitive (ts) mutants of animal viruses have proved invaluable aids in the elucidation of virus gene functions. Mutations to temperature sensitivity are missense rather than nonsense and therefore are not necessarily associated with changes in polypeptide chain length, which can be readily detected by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Approximately one-third of all missense mutations are expected to result in a net charge change for the polypeptide if these occur at random within the confines of the genetic code. High resolution two-dimensional PAGE is capable of distinguishing single charge changes in polypeptides (O'Farrell, 1975) and electrofocusing has already been successfully used to identify charge changes in missense mutants of foot-and-mouth disease virus (King & Newman, 1980). In this study ts mutants of Newcastle disease virus (NDV) strain Beaudette C representing each of six complementation groups were examined by SDS–PAGE and by two-dimensional PAGE to see if there were any changes in the size and/or the isoelectric point of any of the virus-induced proteins synthesized in chick embryo fibroblast (CEF) cells. Demonstration of a polypeptide difference between wild-type virus and mutant is of importance in assignment of virus proteins to virus genes.

Stocks of ts mutants (derived by nitrous acid mutagenesis of plaque-purified wild-type ts+ stock) were plaque purified at the permissive temperature (34 °C) three times on CEF monolayers. Dilutions of independent clones of ts mutants were plated at the non-permissive temperature (41 °C) and several spontaneously arising normal-sized plaques derived from each ts clone were picked as putative revertants. These were grown on CEF monolayers under liquid medium for 4 days at the permissive temperature, harvested and plated at permissive and non-permissive temperatures. Clones which gave only normal-sized plaques at the non-permissive temperature were termed ts+ ‘revertants’. High titre stocks of wild-type virus, ts mutants and ts+ ‘revertants’ were prepared by inoculating 10-day-old embryonated eggs which were then incubated at the permissive temperature for 3 days. Chorioallantoic fluid was harvested, clarified by low-speed centrifugation and used undiluted and without further
purification to infect CEF monolayers. Polypeptides synthesized 5 to 7 h post-infection at the permissive temperature were radioactively labelled with $^3$H-leucine or $^{14}$C-leucine and analysed by SDS-PAGE (Laemmli, 1970) and by two-dimensional PAGE as previously described (Chambers & Samson, 1980).

Fig. 1(a to d) shows common areas from four two-dimensional PAGE fluorograms of $^{14}$C-leucine-labelled proteins from (a) uninfected and (b), (c) and (d), CEF cells infected with wild-type, ts mutant (ts172) and the corresponding 'revertant' (CR172) respectively. The figure includes the region of the gel corresponding to the following NDV-induced proteins: HN, F₀, NAP and F₁ (identified in Fig. 1b).

The focusing pattern obtained here using the detergent Nonidet P40 (NP40) and ampholines 3.5 to 10 and 5 to 7, differed slightly from that used earlier (Tween 80 and ampholines 3.5 to 10; Chambers & Samson, 1980). This is because NP is not soluble in NP40 but glycoproteins F₁ and F₀ reach their equilibrium focus positions within 2000 V h in this detergent (P. Chambers, unpublished results).

NAP in Fig. 1(b to d) exhibits charge heterogeneity (Chambers & Samson, 1980) which is associated with the level of phosphorylation of this protein; charge heterogeneity of the glycoprotein HN is also found with other virus glycoproteins (Raghow et al., 1978; Privalsky & Penhoet, 1978). Comparison between the wild-type virus-infected cells (Fig. 1b) and the ts mutant-infected cells (Fig. 1c) reveals that the ts NAP was shifted to the left (towards the cathode) with respect to the wild-type virus and that the NAP position for the 'revertant' ts$^+$ clones derived from the ts mutant (Fig. 1d) was the same as that for the wild-type virus. This shift is consistent with the addition of one net positive charge to the native polypeptide and was found for all independent ts$^+$ revertants analysed (12/12).

Samples of $^3$H-leucine labelled polypeptides from uninfected and NDV-infected CEF were analysed on two separate 7.5% (w/v) acrylamide SDS-PAGE slab gels (Laemmli, 1970) after boiling extracts in either the presence or absence of 2-mercaptoethanol as reducing agent. A small but significant increase in the apparent mol. wt. of HN protein was seen in the ts mutant-infected cells (Fig. 1e, lane 3) compared to wild-type virus-infected cells (Fig. 1e, lane 2). This apparent mol. wt. change was seen on non-reduced SDS gels (Fig. 1e; mol. wt. approx. 2000) but was barely noticeable on reduced SDS gels (mol. wt. approx. 500 to 1000; not shown). Subsequently, when ts$^+$ 'revertants' were analysed, all these had retained the altered (ts) HN protein.

The effect on the activity of two biological properties of the ts$^+$ and ts viruses was tested. First, the thermostabilities of the haemagglutinin activities of wild-type and ts virions were indistinguishable (not shown). Second, the capacity of wild-type virus, ts mutants and ts$^+$ 'revertants' to direct RNA synthesis at a non-permissive temperature (42.5 °C) and permissive temperature (34 °C) was determined.

Triplicate CEF monolayers were infected with the same high m.o.i. of wild-type, ts and ts$^+$ 'revertant' virus or mock-infected and incubated at 34 °C for 12 h before 60 min incubation with or without actinomycin D (Aldrich) at 34 or 42.5 °C. RNA synthesis was measured as the amount of $^3$H-uridine incorporation into TCA-precipitable material at either temperature over a 60 min period with or without actinomycin D.

Table 1 shows that host (DNA)-directed RNA synthesis was reduced to less than 1% by actinomycin D treatment and that virus-directed RNA synthesis considerably exceeded this level. Monolayers infected with any of the three virus preparations synthesized RNA at similar rates at the permissive temperature (34 °C), but only wild-type virus and ts$^+$ 'revertant' synthesized RNA at a much higher rate at a non-permissive temperature (42.5 °C). The rate of RNA synthesis by the ts mutant was only slightly higher than the rate at 34 °C but was clearly not abolished at the higher temperature. A similar pattern has been found for other clones of the ts mutant and other ts$^+$ revertants from other ts clones.
Fig. 1. Common areas from fluorograms of two-dimensional gel electrophoresis of $^{14}$C-labelled proteins from (a) mock-infected and (b to d) NDV-infected CEF cells; (e) fluorogram of SDS–PAGE of non-reduced $^3$H-labelled proteins from mock-infected (lane 1) and NDV-infected (lanes 2, 3 and 4) CEF cells. (a) and (e, lane 1) mock-infected; (b) and (e, lane 2) wild-type infected; (c) and (e, lane 3) $ts$ mutant ($ts172$)-infected; (d) and (e, lane 4) $ts^+$ 'revertant' (CR172)-infected. NDV proteins indicated are: haemagglutinin-neuraminidase (HN), uncleaved fusion ($F_0$), cleaved fusion ($F_{1,2}$), larger cleaved fusion ($F_{1,3}$), nucleocapsid protein (NP), nucleocapsid-associated protein (NAP) and matrix protein (M). Vertical arrows point to host protein reference points. SDS–PAGE from top (−) to bottom (+). Isoelectric focusing was from right (+) to left (−) according to Chambers & Samson (1980) mode B but with NP40 (not Tween 80) and LKB ampholines, 1% 3·5 to 10 and 1% 5 to 7. The pH gradient is linear over the region shown.

On the basis of the data mentioned above we believe that this $ts$ mutant is a double mutant affected in both the gene coding for the NAP and the gene coding for the HN protein. The former but not the latter appears to be the temperature-sensitive lesion since 'reversion' to $ts^+$ invariably (12/12) resulted in a change from mutant NAP to an NAP which we cannot distinguish from wild-type virus, but HN remains mutant. A single temperature-sensitive
### Table 1. Incorporation of $^3$H-uridine into TCA-precipitable material following infection of CEF with wild-type, $ts$ mutant and $ts^+$ 'revertant' NDV strain Beaudette C*

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Actinomycin D</th>
<th>$3^4$ C °C</th>
<th>$42.5$ C °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock-infected</td>
<td>-</td>
<td>43,311</td>
<td>66,624</td>
</tr>
<tr>
<td>Mock-infected</td>
<td>+</td>
<td>346</td>
<td>370</td>
</tr>
<tr>
<td>Wild-type</td>
<td>+</td>
<td>1620</td>
<td>4269</td>
</tr>
<tr>
<td>$ts$ mutant</td>
<td>+</td>
<td>1438</td>
<td>1917</td>
</tr>
<tr>
<td>$ts^+$ 'revertant'</td>
<td>+</td>
<td>1726</td>
<td>8086</td>
</tr>
</tbody>
</table>

* Confluent monolayers of CEF cells were infected with each virus at an m.o.i. of 85 and incubated for 12 h at 34 °C. Actinomycin D was added as indicated to a final concentration of 5 μg/ml and monolayers were re-incubated at 34 or 42.5 °C for 1 h prior to a 1 h labelling period with $^3$H-uridine (4 μCi/dish; 29.7 Ci/mmol) with or without actinomycin D at 34 or 42.5 °C. Monolayers were rinsed once with tris–saline buffer pH 7.4, washed three times with ice-cold 5% trichloroacetic acid before being taken up in 0.7 ml of a 0.1 M NaOH solution and radioactivity measured by liquid scintillation spectrometry.

† Mean of triplicate monolayers minus background.

lesion is consistent with the complementation behaviour of this mutant in that (i) it only occupies one complementation group and (ii) it complements another $ts$ mutant which has thermolabile haemagglutination and neuraminidase activities. The finding that the $ts$ mutant with the altered NAP protein is RNA (−) at the non-permissive temperature but that $ts^+$ 'revertants' are RNA (+) lends support to the suggestion (Chambers & Samson, 1980) that NAP protein is the NDV analogue of the Sendai virus P protein believed to be involved in virus RNA polymerase activity (Marx et al., 1974; Lamb & Choppin, 1978; Lamb et al., 1978).

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### References


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