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Studies on a Temperature-sensitive Mutant of Fowl Plague Virus Having a Mutation in Gene 7 Coding for the M Protein

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SUMMARY

A fowl plague virus (FPV) temperature-sensitive mutant, ts 303/1 having a ts mutation in gene 7 coding for the matrix (M) protein has been obtained. The mutant induced synthesis of virus-specific RNA and polypeptides as well as ribonuclear protein (RNP) formation in cells under non-permissive conditions; however, haemagglutinin cleavage was reduced, functionally active haemagglutinin and neuraminidase were absent and virions were not formed. In mutant-infected cells at 36 °C haemagglutinin cleavage was also reduced and virions formed had an altered NP:M ratio as well as a decreased haemagglutinin content. A population of virions formed under these conditions was heterogeneous both in morphology and in buoyant density. The data obtained suggest that a mutation in the M proteins of orthomyxoviruses can affect processing of the haemagglutinin and impair final stages of virion morphogenesis.

INTRODUCTION

It is common knowledge that the matrix (M) protein of orthomyxoviruses plays an important role in formation of infectious virions and in maintenance of the structural integrity of virions (Kendal et al., 1977; Bukrinskaya et al., 1978). It has been supposed that the M protein is a key factor influencing the rate of virion formation (Klenk & Rott, 1973; Lazarowitz et al., 1971). One cannot rule out the possibility that it can influence synthesis of virus-specific RNA by the virion transcriptase (Zvonarjov & Ghendon, 1980). However, there have been few investigations to date concerning the exact role of the M protein in orthomyxovirus reproduction.

There are data on an influenza virus ts mutant having a mutation in gene 7 coding for M protein (Sugiura et al., 1975; Ritchey & Palese, 1977); however, the physiological defects of this mutant were not studied in detail.

The present paper describes an FPV ts mutant having a mutational lesion in gene 7 coding for the M protein. The data show that a ts mutation in M polypeptides can influence both the processing of the haemagglutinin (HA) and the final stages of virion morphogenesis.

METHODS

Virus and cells. Fowl plague virus (FPV) Weybridge strain (H7N7) and a ts mutant of this strain, ts 303/1, obtained by treatment of this virus with N-methyl-N-nitroso-N-nitroguanidine were used in the experiments. FPV Rostock strain (H7N1) and a ts mutant of this strain, ts 46, having a mutation in the gene coding for the haemagglutinin (Almond et al., 1979) and kindly provided by Dr B. W. J. Mahy, were used in a number of experiments. All the experiments were carried out using primary chick embryo fibroblast (CEF) cultures.

Recombination and complementation of FPV ts mutants were carried out as described earlier (Markushin & Ghendon, 1973).

A temperature-sensitive stage in the reproduction cycle of the ts 303/1 mutant was determined as described previously (Ghendon et al., 1975).

Analysis of virus-specific RNA and protein synthesis. Synthesis of virus-specific complementary RNA (cRNA) was studied using the method of Hay et al. (1977 a). Synthesis of virion RNA (vRNA) was studied as described by Smith & Hay (1982).
Synthesis of virus-specific proteins was studied as described by Ghendon et al. (1981). Synthesis of functionally active haemagglutinin and neuraminidase was studied as described by Ghendon et al. (1973).

Analysis of the genome composition of ts+ recombinants. Recombinants possessing a ts+ phenotype were obtained by crossing the ts 303/1 mutant with FPV Rostock strain (H7N1). The genome composition of ts+ recombinants was analysed as described earlier (Hay et al., 1977b; Ghendon et al., 1979).

Derivation of recombinants by crossing FPV ts 303/1 (H7N7) with human influenza virus A/England/42/72 (H3N2). Fertile eggs were co-infected with ts 303/1 and A/England/42/72 at $10^4$ EID$_{50}$ of each virus. After 48 h of incubation at 36 °C, virus-containing allantoic fluid was harvested and a series of dilutions prepared which were treated with monospecific antiserum against FPV haemagglutinin; chick embryos were infected with the material obtained. After 48 h of incubation, the virus was harvested and titrated in chick embryos. The materials of each of the last two final dilutions were inoculated into 10 chick embryos into which monospecific serum against the neuraminidase of A/England/42/72 virus was added. After 48 h of incubation, the allantoic fluid was harvested, the presence of the virus was determined using a haemagglutination test, and virus-containing material studied in a haemagglutination-inhibition test with antisera against FPV and A/England/42/72 haemagglutinins, as well as in a neuraminidase-inhibition test with antisera against N2 and N7 neuraminidases. Recombinants having the haemagglutinin of A/England/42/72 (H3) and the neuraminidase of FPV (N7) were passed once in chick embryos and used for further studies.

Isopycnic centrifugation. CEF cultures were infected with viruses at an m.o.i. of 5 to 10 and incubated in medium containing [3H]uridine (20 μCi/ml, specific activity 10 Ci/mmol) for 10 h at 36 °C. Infected cell medium was then harvested, clarified, and the virus was pelleted (MSE Superspeed-65 centrifuge, rotor 6 × 250 ml, 19000 rev/min, 60 min, 6 °C). The pellet was resuspended in 1 ml of STE buffer (0.1 M-NaCl, 0.01 M-Tris-HCl, 10 mM-EDTA pH 7.4). Virus was purified using a 20 to 60% sucrose density gradient prepared in STE buffer (rotor 3 × 6 ml, 48000 rev/min, 30 min, 6 °C). A virus-containing band was collected, diluted with buffer to 6 ml and pelleted in the same rotor (35000 rev/min, 40 min). The virus pellet resuspended in STE buffer was subjected to isopycnic centrifugation in a sucrose gradient at a density of 1.06 to 1.33 g/ml (20 to 60% sucrose in STE buffer, rotor 3 × 25 ml, 27000 rev/min, 20 h, 6 °C). The gradient was fractionated, and radioactivity, infectivity and haemagglutinating activity were determined in the fractions obtained. Sucrose density was determined by its refractive index.

Electron microscopy. This was carried out using viruses grown in CEF cultures during one cycle, pelleted from virus-containing fluid and purified as described in 'Isopycnic centrifugation'. Virus particles were examined by the negative staining technique with 2% phosphotungstic acid. The preparations were examined in a JEM-100 B/JEOL electron microscope at 80 kV and instrumental magnification of 60000 to 100000.

Calculation of the number of virus particles contained in 1 p.f.u. This was carried out by the method of Miller et al. (1973), based on calculation of the amount of virus particles contained in a given volume of fluid with known virus titre. For this purpose, virions of wild-type FPV and ts 303/1 were sedimented by centrifugation for 90 min at 40000 rev/min (MSE, rotor 3 × 6 ml) on to a Millipore filter membrane. After fixation with glutaraldehyde and OsO$_4$ solution, dehydration with isopropanol and clarification with toluene, the membrane with sedimented virions was set in Epon 812. Ultrathin sections, 90 to 100 nm thick (golden interference colour), of FPV virions were prepared in a ultramicrotome (Ultratome III). Virions were counted from not less than 24 meshes. The total number, $V_m$, of particles present on the surface of the filter was calculated using the formula:

$$V_m = n(D^2) Vf/LT,$$

where D is the diameter of the Millipore filter (4 mm), Vf the average number of particles in LT area, L the effective length of a section and T the thickness of a section. Calculation of the average number of particles contained in 1 p.f.u. of virus were determined in the material before sedimentation, and in the supernatant after sedimentation.

RESULTS

Studies of ts 303/1 mutant in a complementation test, and biological properties of the mutant

FPV mutants we obtained earlier were classified into the complementation groups A, B, C, D and E (Markushin & Ghendon, 1973). As can be seen from the data shown in Table 1, ts 303/1 belonged to none of these complementation groups and was assigned to a sixth complementation group.

The efficiency of plating (42 °C/36 °C) of ts 303/1 (Table 2) was 3.9 × 10³. It possessed a reduced ability to reproduce in chick embryos during incubation at 36 °C. Incubation of infected chick embryos for longer periods of time (to 48 or 72 h) did not increase virus titres (not shown).
Table 1. Complementation of FPV ts mutants

<table>
<thead>
<tr>
<th>Complementation group</th>
<th>Mutant</th>
<th>ts 43</th>
<th>ts 166</th>
<th>ts 29</th>
<th>ts 131</th>
<th>ts 5</th>
<th>ts 303/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ts 43</td>
<td>2.9*</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>ts 166</td>
<td>&lt;1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>ts 29</td>
<td>3.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>ts 131</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>ts 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>F</td>
<td>ts 303/1</td>
<td>5.8</td>
<td>4.8</td>
<td>6.1</td>
<td>5.4</td>
<td>4.5</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

* Titres are expressed as \( \log_{10} \) p.f.u./ml. CEF cultures were infected with two mutants at m.o.i. of 2 for each mutant, or (in the controls) with one of the mutants under study (4 p.f.u./cell) and incubated at 42 °C for 10 h. The virus was titrated using a plaque technique in CEF cultures at 36 °C.

Table 2. Biological properties of ts 303/1 mutant

<table>
<thead>
<tr>
<th>Virus</th>
<th>Reproduction (EID(_{50})/0.1 ml)*</th>
<th>Pathogenicity (LD(_{50})/p.f.u.)†</th>
<th>Plaque size (mm)‡</th>
<th>P.f.u./HAU ratio§</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts 303/1</td>
<td>( 3.9 \times 10^{-3} )</td>
<td>0.31</td>
<td>2.0 ± 0.27</td>
<td>2 \times 10^{7}</td>
</tr>
<tr>
<td>Wild-type FPV</td>
<td>1.0</td>
<td>10³</td>
<td>2.8 ± 0.57</td>
<td>1 \times 10^{6}</td>
</tr>
</tbody>
</table>

* A chick embryo was inoculated with \( 10^3 \) EID\(_{50}\) of the virus and incubated for 30 h at 36 °C. Average data of 12 experiments.
† One-day-old chickens were intramuscularly inoculated with various virus doses (in p.f.u.) and observed for 7 days. The LD\(_{50}\) was then calculated, and LD\(_{50}\) in 1 p.f.u. determined.
‡ Not less than 500 plaques were measured in three different experiments at day 3.
§ Haemagglutinating, plaque-forming activities and p.f.u./HAU ratio were determined in the same virus preparations grown in chick embryos at 36 °C. Average data of 18 experiments.

Yield of infectious virions both from CEF cultures infected with ts 303/1 and incubated at 36 °C, and from infected chick embryos was reduced by 1 to 2 \( \log_{10} \) compared to the yield of wild-type FPV (not shown). Plaques formed by ts 303/1 in CEF cultures were significantly smaller than those formed by wild-type virus. The mutant was significantly less pathogenic for 1-day-old chickens. Virions of ts 303/1 formed at 36 °C in fertile eggs had low haemagglutinating activity: approximately 20 times less than that of wild-type virus. Studies of the thermostability of infectious and haemagglutinating activities of ts 303/1 virions by heating \textit{in vitro} showed that the haemagglutinin of the virions did not possess increased thermostability while titres of infectivity decreased more rapidly on heating than those of wild-type virus (data not shown).

Determination of a thermosensitive stage of reproduction

Fig. 1 shows that when infected CEF cultures were incubated for the first 4-5 h under non-permissive conditions (42 °C) and then at 36 °C, virus yields were not decreased during the reproductive cycle. At the same time, when infected cells were incubated at the optimal temperature and then under non-permissive conditions, virus yields were reduced drastically, even if incubation at 36 °C was continued for 6 h. These results suggest that a thermosensitive stage of the reproduction of ts 303/1 occurs during the late stages of the cycle.

Identification of the ts 303/1 gene having a ts lesion

To determine the gene carrying a ts mutation in the ts 303/1 mutant, we studied the genome composition of recombinants possessing a ts\(^+\) phenotype obtained by crossing of ts 303/1 and wild-type FPV Rostock strain. Weybridge and Rostock strains of FPV were shown previously to differ in all eight segments of their genomes (Markushin \textit{et al.}, 1981) and it allowed us to determine the origin of each RNA segment of the recombinants of these viruses. As can be seen from the data of Table 3, ts\(^+\) recombinants R 112, R 114, R 117 and R 119 inherited genes 1, 2, 3, 4, 5, 6 and 8 from the Weybridge strain, and gene 7 from the Rostock strain; recombinants R 115, R 126 and R 128 inherited genes 6 and 7 from Rostock strain, and recombinant R 123...
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Fig. 1. Studies on a temperature-sensitive stage in reproduction of ts 303/1 in CEF cultures. Cells were infected with virus at a m.o.i. of 2 to 5 and incubated at the indicated temperatures. (a) Transfer from permissive to non-permissive temperature. 1, 36 °C for 9 h; 2, 36 °C for 3 h then 42 °C for 6 h; 3, 36 °C for 4-5 h then 42 °C for 4-5 h; 4, 36 °C for 6 h then 42 °C for 3 h. (b) Transfer from non-permissive to permissive temperature. 1, 42 °C for 9 h; 2, 42 °C for 3 h then 36 °C for 6 h; 3, 42 °C for 4-5 h then 36 °C for 4-5 h; 4, 42 °C for 6 h then 36 °C for 3 h.

Table 3. Analysis of the genome composition of ts+ recombinants obtained by crossing of ts 303/1 mutant of FPV Weybridge strain with wild-type FPV Rostock strain*

<table>
<thead>
<tr>
<th>Recombinant studied</th>
<th>Origin of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>R 112</td>
<td>W†</td>
</tr>
<tr>
<td>R 114</td>
<td>W</td>
</tr>
<tr>
<td>R 115</td>
<td>W</td>
</tr>
<tr>
<td>R 117</td>
<td>W</td>
</tr>
<tr>
<td>R 119</td>
<td>W</td>
</tr>
<tr>
<td>R 123</td>
<td>R</td>
</tr>
<tr>
<td>R 126</td>
<td>W</td>
</tr>
<tr>
<td>R 128</td>
<td>W</td>
</tr>
</tbody>
</table>

* CEF cultures were infected with recombinants and parent strains (100 p.f.u./cell) and incubated in the presence of cycloheximide (100 µg/ml) for 1 h at 36 °C; 3H-uridine was then added (100 µCi/ml) and incubation continued for 4 h. The cRNA was isolated and hybridized with unlabelled vRNA extracted from purified virions of every parent strain. After hybridization, the material was treated with nuclease S1 and analysed by electrophoresis in a 4% polyacrylamide gel.

† W, gene inherited from Weybridge strain; R, gene inherited from Rostock strain.

inherited genes 1, 6 and 7 from this strain, while all other genes were inherited from Weybridge strain. Thus, these data indicate that substitution of segment 7 of RNA of ts 303/1 mutant by a corresponding segment of RNA of the wild-type FPV Rostock strain results in restoration of a ts+ phenotype which implies that the ts phenotype of ts 303/1 is due to the mutation in gene 7 coding for the M proteins. Since extragenic suppression of the gene carrying a ts mutation (Ghendon et al., 1982) can occur by crossing ts mutants of the FPV Weybridge and Rostock strains, we studied the possibility of such a phenomenon in crossing of ts 303/1 (Weybridge) with the FPV Rostock strain. For this purpose, a ts+ recombinant, R 119, containing seven genes inherited from FPV Weybridge strain, and gene 7 from FPV Rostock strain (Table 3) was crossed at 36 °C with wild-type FPV Weybridge strain. Then we analysed the temperaturesensitivity of clones isolated from plaques formed at 36 °C in CEF culture infected with parent
FPV temperature-sensitive mutant

Fig. 2. Synthesis of virus-specific poly(A⁺) cRNA and vRNA of the ts 303/1 mutant. (a) Densitometer traces of the fluorographs obtained in studies of synthesis of poly(A⁺) cRNA. CEF cultures were infected with the viruses (100 p.f.u./cell) and incubated for 1.5 h or 4.0 h at 36 °C or 42 °C. [3H]Uridine was then added, and incubation continued for 30 min. RNA–RNA hybridization was carried out as described in Methods. After treatment of the hybrids obtained with nuclease S1, RNA was analysed by electrophoresis in a 4% polyacrylamide gel. Cells were infected with wild-type FPV and labelled for 1.5 h (A) or 4.0 h (B) after infection at 36 °C, and 1.5 h (C) or 4.0 h (D) after infection at 42 °C. Cells were infected with ts 303/1 and labelled for 1.5 h (E) or 4.0 h (F) after infection at 36 °C, and 1.5 h (G) or 4.0 h (H) after infection at 42 °C. The numbers, 1 to 8, indicate the locations of the corresponding double-stranded complexes of poly(A) cRNA–vRNA. (b) Densitometer traces of fluorographs obtained in studies of vRNA synthesis. Conditions of the experiment and designations are the same as in (a).

strains (R 119 or FPV Weybridge) or with the material resulting from crossing R 119 and FPV Weybridge. All the clones studied (18 clones of R 119, 19 clones of FPV and 31 clones of R 119 × FPV) turned out to possess a distinct ts⁺ phenotype. Our earlier experiments (Ghendon et al., 1982), which gave evidence of extragenic suppression in crosses of ts mutants of FPV Weybridge and Rostock strains having a ts mutation in gene 1, revealed four clones possessing a ts phenotype, by the analysis of 30 clones obtained by crossing of a ts⁺ recombinant with wild-type FPV strain. Thus the data obtained suggest that the ts⁺ phenotype of the R 119 recombinant is due to substitution of the mutant gene 7 by a corresponding gene of Rostock strain, rather than to extragenic suppression of some gene of ts 303/1 mutant having a ts mutation.
Fig. 3. Synthesis of virus-specific polypeptides and cleavage of the haemagglutinin in ts 303/1-infected cells. CEF cultures were infected at a m.o.i. of 100 and incubated at 36 °C or 42 °C for 4 h. [35S]Methionine was then added (20 μCi/sample) and incubation continued for 15 min. Extracts were then prepared from a portion of samples, (a), and other samples were supplemented with an excess of unlabelled methionine (b), and incubation was continued for 45 min at the appropriate temperature. The solubilized extract was analysed in a 25% acrylamide gel (120 V, 18 h). The autoradiographs obtained were subjected to densitometry.

*Synthesis of virus-specific RNAs*

Fig. 2 shows that in cells infected with *ts* 303/1 a normal level of synthesis of poly(A⁺) cRNA and vRNA is observed at 42 °C. We observed no impairment in regulation of the processes of secondary transcription or replication. We failed to reveal any impairment in the synthesis of poly(A⁻) cRNA under these conditions (not shown).
**Table 4. Effect of trypsin on formation of infectious virus of ts 303/1 mutant and A/Krasnodar/101/59 influenza virus in CEF cultures***

<table>
<thead>
<tr>
<th>Virus</th>
<th>Temperature of incubation of CEF cultures (°C)</th>
<th>Titre (log₁₀ EID₅₀/ml)</th>
<th>without trypsin</th>
<th>with trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Krasnodar/101/59</td>
<td>36</td>
<td>5.00</td>
<td>5.75</td>
<td>6.75</td>
</tr>
<tr>
<td>ts 303/1</td>
<td>36</td>
<td>7.00</td>
<td>6.75</td>
<td>6.75</td>
</tr>
<tr>
<td>ts 303/1</td>
<td>42</td>
<td>4.25</td>
<td>4.50</td>
<td>4.50</td>
</tr>
</tbody>
</table>

*CEF cultures were infected with the viruses at a multiplicity of 1 to 3 EID₅₀/cell, incubated for 24 h at 36 °C or 42 °C, and yields of infectious virus were determined by titration in chick embryos.

**Table 5. Studies of ts 303/1 and ts 46 in a recombination test***

<table>
<thead>
<tr>
<th>Ts mutants being crossed</th>
<th>Number of plaques (p.f.u./ml) formed</th>
<th>ts⁺ clones (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts 303/1 × ts 303/1</td>
<td>4·3 × 10⁶</td>
<td>&lt;1 × 10¹ (ts⁺)</td>
</tr>
<tr>
<td>ts 46 × ts 46</td>
<td>8·1 × 10⁶</td>
<td>&lt;1 × 10¹ (ts⁺)</td>
</tr>
<tr>
<td>ts 303/1 × ts 46</td>
<td>7·2 × 10⁶</td>
<td>5·1 × 10⁵ (ts⁺)</td>
</tr>
</tbody>
</table>

*Cells were infected with ts mutants (2 p.f.u./cell), incubated for 18 h at 36 °C; titres were determined using a plaque technique at 36 °C and 41 °C.

**Synthesis of virus-specific polypeptides**

It can be seen from Fig. 3 that in mutant-infected cells virus-specific proteins P1, P2, P3, HA, NP, M and NSI are synthesized under both permissive and non-permissive conditions. We have revealed that cleavage of the precursor of the haemagglutinin of ts 303 is reduced at both 42 °C and 36 °C. In ts 303/1-infected cells, synthesis of functionally active haemagglutinin and neuraminidase was reduced at 36 °C, while under non-permissive conditions it was not observed at all (data not shown). The presence of trypsin (5 μg/ml) in CEF cultures infected with ts 303/1 did not increase the yields of infectious virus at either 42 °C or 36 °C (Table 4). Approximately equal amounts of ribonuclear protein (RNP) were formed in ts 303/l-infected cells incubated at 36 °C or 42 °C (data not shown).

**Studies on the haemagglutinin of ts 303/1**

The genome analysis of ts⁺ recombinants showed that ts 303/1 had a mutation in the gene coding for the M proteins. Nevertheless, certain impairments in the haemagglutinin of this mutant were observed: in particular, its cleavage was affected. In this connection we decided to study (i) whether this protein contained an unidentified mutation, and (ii) whether it is able to function normally in virions having no ts mutation in the M protein. To investigate the first possibility we studied ts 303/1 in a recombination test with ts 46 of FPV Rostock, obtained by Almond et al. (1979), which had a mutation in the gene coding for the haemagglutinin. Table 5 shows that ts 303/1 recombined well with ts 46, yielding ts⁺ recombinants. To exclude a possible intracistronic complementation, 18 clones were isolated from plaques formed at 41 °C; the ts phenotype of these clones was studied after two passages at 41 °C. All the clones retained their ts⁺ phenotype, which allowed us to exclude the possibility of intracistronic complementation. Thus, all these data indicate that the gene coding for the haemagglutinin of ts 303/1 has no ts mutation.

To clear up the second point, we studied yields of infectious virions and haemagglutinin titres of the ts⁺ recombinants obtained in which the gene coding for the haemagglutinin of ts 46 might have been substituted by the corresponding gene of ts 303/1. We showed earlier (Markushin et al., 1981) that the haemagglutinins of FPV Weybridge and Rostock strains differ slightly in their
antigenic specificity and can be differentiated. So, we studied the recombinants in a haemagglutination-inhibition test with sera against the haemagglutinins of FPV Weybridge and Rostock strains and found that ts\(^{+}\) recombinants obtained by crossing of ts 303/1 (Weybridge) and ts 46 (Rostock) contained the haemagglutinin of FPV Weybridge, but not that of Rostock strain (not shown). In these experiments chick embryos were infected with 10\(^3\) EID\(_{50}\) of the virus and incubated for 30 h at 36 °C; haemagglutinin titres were determined in a haemagglutination test, and titres of infectious virus were determined in CEF cultures using a plaque technique. The results showed that ts 303/1 and ts 46 as well as ts\(^{+}\) recombinants (R 14, R 18 and R 21) obtained, gave similar virus yields in chick embryos at 36 °C (1 \times 10^7 to 5 \times 10^7 p.f.u./0.1 ml). At the same time, these recombinants were close to ts 46 in haemagglutinin production; their p.f.u.: HA ratio was 7 \times 10^5 to 1 \times 10^6, while the synthesis of the haemagglutinin of ts 303/1 was reduced significantly, and the p.f.u.: HA ratio was nearly 10-fold higher (8 \times 10^6 to 2 \times 10^7). Thus, these data indicate that impairment in the processing of the haemagglutinin of ts 303/1 is apparently due to a mutation in the M protein rather than to the mutation in the haemagglutinin itself.

Studies on the neuraminidase of ts 303/1

According to the published data, cleavage of the haemagglutinin may be associated with neuraminidase function (Schulman & Palese, 1977; Ghendon et al., 1979). Since cleavage of the haemagglutinin of ts 303/1 was impaired, and synthesis of functionally active neuraminidase was not detectable in mutant-infected cells at the non-permissive temperature, we decided to study whether there was a non-ts mutation in the neuraminidase. We crossed the ts 303/1 mutant of FPV with human influenza virus A/England/42/72 (H3N2) and obtained recombinants RA/303/1 and RA/303/2 containing the haemagglutinin of A/England/42/72 (H3) and neuraminidase of FPV Weybridge strain (N7). Table 6 shows that neuraminidase activity of the recombinants possessing the haemagglutinin of human influenza virus (H3) and the neuraminidase of ts 303/1 (N7) is rather high, including that of the viruses which reproduced at high temperature (39 °C). In addition, the recombinants possessing the neuraminidase N7 gave high yields of infectious virions with normal haemagglutinating activity; they did not differ from those of the parent A/England/42/72 strain. These results indicate that the gene of ts 303/1 coding for the neuraminidase has no mutation, and that inhibition of cleavage of the haemagglutinin of ts 303/1 is not due to any mutational changes in the neuraminidase.

Studies on the formation of non-infectious virions under non-permissive conditions

Since we observed synthesis of virus-specific proteins and RNA in ts 303/1-infected cells under non-permissive conditions, as well as formation of RNPs but not of infectious virions, we decided to study whether or not non-infectious virions are formed under these conditions. CEF cultures were infected with ts 303/1 and incubated at 42 °C in the presence of hydrolysate of \(^{14}\)C-labelled proteins from \textit{Chlorella} (0.5 ~tCi/ml). Virus-containing fluid was then harvested, clarified and centrifuged to pellet the presumed virions. The pellet was solubilized and analysed by polyacrylamide gel electrophoresis, but this revealed no bands corresponding to virus-specific polypeptides (not shown). It should be noted that electron microscope studies of CEF cultures infected with ts 303/1 and incubated at the non-permissive temperature also failed to reveal formation of virions under these conditions (Anisimova et al., 1980).

Studies on the virions formed at 36 °C

Polypeptide analysis of ts 303/1 virions formed in infected CEF cultures at 36 °C (Fig. 4) showed that they contain small amounts of haemagglutinin and that the amount of the M polypeptide was reduced significantly with respect to the NP polypeptide as compared to the amount of these polypeptides in virions of wild-type FPV formed under similar conditions. The M:NP ratio of wild-type FPV was 2:26, while in the ts 303/1 mutant it was 0:52.

One could suppose that a change in molar ratios of the polypeptides of ts 303/1 virions formed at 36 °C could influence density characteristics of the mutant virions. We carried out sucrose gradient analysis of a population of virions of ts 303/1 formed in CEF cultures at 36 °C. The data
Fig. 4. Comparative studies of polypeptides of wild-type FPV (●) and ts 303/1 (○) virions formed at 36 °C. CEF cultures were infected at a m.o.i. of 50 and incubated for 8 h at 36 °C in the presence of a hydrolysate of labelled *Chlorella* proteins. Virions were pelleted by centrifugation from culture fluid and analysed by polyacrylamide gel electrophoresis.

Table 6. Properties of recombinants obtained by crossing of ts 303/1 mutant and A/England/72 influenza virus (H3N2)*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Reproduction in chick embryos</th>
<th>36 °C, 48 h</th>
<th>39 °C, 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Titre (log_{10} EID_{50}/ml)</td>
<td>Neuraminidase activity (E_{549}/0.1 ml)</td>
<td>Titre (log_{10} EID_{50}/ml)</td>
</tr>
<tr>
<td>ts 303/1 (H7N7)</td>
<td>7.3</td>
<td>32</td>
<td>undetectable</td>
</tr>
<tr>
<td>A/England/72 (H3N2)</td>
<td>9.2</td>
<td>1024</td>
<td>0.375</td>
</tr>
<tr>
<td>RA/303/1 (H3N7)</td>
<td>9.2</td>
<td>2048</td>
<td>0.480</td>
</tr>
<tr>
<td>RA/303/2 (H3N7)</td>
<td>9.0</td>
<td>1024</td>
<td>0.350</td>
</tr>
</tbody>
</table>

* The technique of obtaining recombinants is described in Methods.
Fig. 5. Isopycnic centrifugation of wild-type FPV and ts 303/1 virions formed in CEF cultures during one cycle or reproduction at 36 °C. CEF cultures were infected at a m.o.i. of 5 to 10 and incubated at 36 °C for 10 h in the presence of [3H]uridine. The virus was pelleted from culture fluid, purified and analysed in a sucrose gradient with a density of 1.06 to 1.33 g/ml. (a) Wild-type FPV virions and (b) ts 303/1 virions were analysed for radioactivity (●), haemagglutinating activity (○) and infectivity (□).

presented in Fig. 5(a) show that sucrose gradients of FPV virions resulted in a homogeneous peak at a density of 1.18 g/ml and sometimes a small fraction in the area of 1.15 g/ml (not shown in Fig. 5a). Infectious virions are concentrated in the area with a density of 1.18 g/ml. Studies of the virions of ts 303/1 revealed heterogeneity of the population. The buoyant density of these virions was distributed over a wide range, having a small peak in the area of 1.18 g/ml in a number of experiments (as in Fig. 5b), and a much larger peak in the area of 1.15 g/ml. In other experiments this distribution did not have such definite peaks, but a clearly marked decrease of the density was observed. Both infectivity and haemagglutinating activity were associated with the virions detected in the region of 1.18 g/ml, the PFU:HA ratio in this peak being close to that of wild-type FPV (2.3 × 10^5 in FPV and 1.7 × 10^5 in ts 303/1). No infectious or haemagglutinating material was observed in the region of 1.15 g/ml.

Electron microscope studies of preparations of purified virions stained with phosphotungstic acid revealed significant differences in structure between virions of FPV and ts 303/1 formed at 36 °C. Wild-type FPV virions had a typical structure. They were spherical, with a diameter of 100 to 130 nm, and contained haemagglutinin spikes about 10 nm long on their surface (Fig. 6a). Studies of ts 303/1 showed that the virus population was polymorphous. In this population typical virions (which did not differ from those of wild-type FPV) constituted not more than 1 to 5%, while the majority of virions were represented by atypical particles (Fig. 6b). These had a spherical, ring or irregular form. Their diameters varied from 70 to 400 nm, and the length of individual filamentous particles was 1200 nm. The most characteristic morphological feature of
Fig. 6. Electron micrographs of FPV and *ts* 303/1 virions formed in CEF cultures at 36 °C. Bar markers represent 5 nm. (a) Population of FPV virions characterized by stability of sizes, form, electron optical density and by constant presence of haemagglutinins. (b) Group of *ts* 303/1 virions of irregular shape and varying sizes containing morphologically defective haemagglutinins. Arrows indicate large virions nearly 200 nm in diameter, with their surfaces partially covered with, or without, haemagglutinins.

ts 303/1 virions involved the haemagglutinins. In a high proportion of virions in the population, haemagglutinins could not be detected at all or covered unevenly only a part of the virion surface. The haemagglutinins were morphologically defective and of varying length (6 to 20 nm), and irregular with respect to the virion surface orientation.

In further experiments, we determined the number of physical particles corresponding to 1 p.f.u., in a population of *ts* 303/1 and wild-type virus. Our calculations showed that 1 ml of a purified preparation of wild-type FPV contained 1·078 × 10¹⁰ physical particles and the infectious titre to be 2·5 × 10⁷ p.f.u./ml. Thus, 1 p.f.u. of wild-type FPV corresponded to 430 physical virus particles. Studies of *ts* 303/1 have shown that 1 ml of the purified preparation contained 1·480 × 10¹⁰ to 1·505 × 10¹⁰ particles. With virus titres of 4·75 × 10⁶ p.f.u./ml, it corresponded to the content of 3·029 to 3·120 physical particles per p.f.u., i.e. eightfold higher than in the wild-type FPV population.
According to published data, 1 infectious unit of influenza virus corresponds to 10 to 80 physical virus particles (Donald & Isaacs, 1954). However, in those experiments, infectivity was determined by inoculation of chick embryos (EID$_{50}$). In our experiments, infectivity was determined by counts of plaques formed in CEF cultures under agar. Comparative studies of the titres of wild-type virus and $ts$ 303/1 in p.f.u. and EID$_{50}$ showed that 1 p.f.u. corresponded to 10 to 20 EID$_{50}$. So, our data on the ratio of infectious and physical units in wild-type FPV are in good agreement with the published data.

In addition, we determined virion sizes of the populations of the viruses studied. The data were obtained from analysis of ultrathin sections of sediments of the virus-containing material on Millipore membrane filters. The mutant population was more heterogeneous with respect to the sizes of the particles than was wild-type FPV, and the average particle size of the mutant was larger than those of FPV (200 nm for $ts$ 303/1 and 130 nm for wild-type FPV).

**DISCUSSION**

To date, $ts$ mutants of orthomyxoviruses having a $ts$ mutation in gene 7 coding for the M proteins, have been obtained only by one group of investigators (Sugiura et al., 1975). According to their data, these mutants induced normal synthesis of virus-specific RNA and RNP, as well as synthesis of functionally active neuraminidase in infected cells under non-permissive conditions; however, synthesis of functionally active haemagglutinin was decreased (to 10%). At the same time these mutants induced normal synthesis of all virus-specific polypeptides at the non-permissive temperature (Ritchey & Palese, 1977).

Studies of the $ts$ 303/1 mutant of FPV by genome analysis of recombinants possessing a $ts^+$ phenotype obtained by crossing $ts$ 303/1 of FPV Weybridge with FPV Rostock showed that $ts$ 303/1 had a $ts$ mutation in gene 7 coding for the M polypeptides. Our previous studies on the physiology of this mutant, in which cleavage of the haemagglutinin was inhibited, suggested that it might have a mutation in the haemagglutinin (Ghendon & Markushin, 1980). However, further experiments showed that the gene coding for the haemagglutinin of $ts$ 303/1 had no mutations and was able to function normally in the genome of other recombinants having no mutations in the gene coding for the M protein.

Phenotypic expression of the mutation in $ts$ 303/1 was dependent on temperature conditions. In cells infected with $ts$ 303/1 and incubated under non-permissive conditions (42 °C) virus-specific RNAs and polypeptides were synthesized and RNPs formed; whereas cleavage of the haemagglutinin was affected and virions, including non-infectious virions, were not formed. Synthesis of functionally active haemagglutinin and neuraminidase was not observed in $ts$ 303/1-infected cells under non-permissive conditions.

Cleavage of the haemagglutinin in $ts$ 303/1-infected cells was affected even at 36 °C, and synthesis of functionally active haemagglutinin and neuraminidase was decreased. The virions formed under these conditions had a decreased content of the M protein and a decreased content of the haemagglutinin which had a defective conformation. All these data testify to the fact that the mutation in the M protein may impair maturation of virus-specific glycoproteins and their incorporation into the virion envelope in a functionally active form.

There are published data (Hay, 1974) that the M protein of orthomyxoviruses is incorporated directly into the sites of cell plasma membranes containing molecules of virus glycoproteins. A reduced content of the M protein in virions of $ts$ 303/1 suggests that incorporation of the mutant M polypeptide into the inner surface of plasma membrane is affected.

The fact that a reduced number of haemagglutinin spikes is present on the surface of the mutant virions formed at 36 °C as morphologically defective formations, indicates that the mutant polypeptide is incapable of correcting formation of the virus envelope by fixation of virus-specific glycoproteins in definite sites of the plasma membrane. Presumably the mechanism of fixation is closely associated with an increase in rigidity of the lipid bilayer at definite sites of the plasma membranes of infected cells. According to the data of Gregoriades & Frangione (1981), molecules of the M proteins are in close contact with the plasma membrane and some sites on the M protein are embedded into the inner lipid bilayer of the membrane. An increase in the rigidity of the lipid bilayer resulting from interaction of the M protein with the...
inner surface of the plasma membrane appears to be one of the conditions for maintenance of native conformation of virus-specific glycoproteins.

Cleavage of the haemagglutinin was impaired in ts 303/1-infected cells. Experiments have shown that this impairment is associated neither with a mutation in the neuraminidase, which may be involved in cleavage of the haemagglutinin-precursor (Schulman & Palese, 1977; Ghendon et al., 1979), nor with a mutation in the haemagglutinin itself. Since cleavage of the haemagglutinin is known to occur on plasma membranes (Klenk et al., 1975; Lazarowitz & Choppin, 1975), impairment in cleavage of the haemagglutinin might be due to alterations in its functional conformation, or, probably, in the functional conformation of the neuraminidase resulting from the incorporation of the defective M protein into the inner surface of plasma membranes. The latter circumstance may also be associated with the absence of neuraminidase and haemagglutinating activities in ts 303/1-infected cells in spite of adequate synthesis of these polypeptides.

It should be noted in conclusion that RNA segment 7 of orthomyxoviruses codes for at least two (Lamb & Choppin, 1981), and possibly three (Lamb et al., 1981) virus-specific proteins. The results obtained by us so far do not allow us to determine the exact location of the ts mutation in segment 7 of ts 303/1 with respect to the M or M2 protein. Exact localization of a ts mutation in segment 7 of ts 303/1 demands further studies.

REFERENCES


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