Genotypic and Phenotypic Characterization of a Mammalian Cell-adapted Mutant of Fowl Plague Virus (FPV)

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SUMMARY

A mammalian cell-adapted mutant of the Dobson strain of fowl plague virus (FPV-B) was characterized. Genetic analyses of recombinants between a ts mutant of this virus and either the non-adapted Dobson strain or the Rostock strain of FPV showed that the gene coding for the P3 protein of the adapted Dobson strain was sufficient to enable any recombinant to grow in L cells.

The abortive cycle of wild-type Dobson strain (FPV+) was compared to the productive cycle of the mutant. By using 100 p.f.u./cell, no quantitative difference could be detected in infected L cells between polypeptides and cRNAs induced by FPV+ and FPV-B. However, the maturation of virions at the plasma membrane did not proceed correctly. At a lower m.o.i. the amounts of virus polypeptides decreased with the m.o.i. This decrease was not the same for all polypeptides and cRNA segments: HA, M and NA and their mRNAs decreased to a greater extent than the others. These results are discussed in relation to a possible biological activity of polypeptide P3.

INTRODUCTION

Influenza A viruses are characterized by a narrow host-range, they form plaques on a very limited number of tissue culture cells and in many cells undergo an abortive cycle. The Dobson strain of fowl plague virus (FPV+) is able to replicate productively in chick embryo cells (CEC), while L cells have been shown to be non-permissive for this virus (Israël et al. 1975) as for a number of other influenza A viruses (Franklin & Breitenfeld, 1959; Fraser, 1967; Gandhi et al. 1971; Avery, 1975; Bosch et al. 1978). Our previous results have shown that the abortive cycle of FPV+ in L cells is characterized by the production of mainly non-infectious particles. However, these particles possess all polypeptides and RNA segments usually detected in infectious virions (Israël, 1979). Zavada (1969) has isolated, by repeated passage on L cells, an adapted mutant (FPV-B). We have analysed this mutant and describe here the localization of the mutation. We also present the results of investigations comparing the productive and abortive replication cycles of FPV in L cells.

METHODS

Acrylamide, N,N'-methylen bisacrylamide, N,N,N',N'-tetramethylethylenediamine and diallyltartardiamide were purchased from Eastman Kodak (Rochester, N.Y., U.S.A.). S1 nuclease and 5-fluorouracil were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and all other chemicals were purest grade from Merck (Darmstadt, Germany).
Radiochemicals, 5-³H-uridine (25 Ci/mmol) and L-³⁵S-methionine (300 to 600 Ci/mmol), were obtained from the CEA (Saclay, France).

**Cells and viruses.** Secondary chick embryo cells (CEC) and mouse L929 cells were grown in minimum essential medium (MEM) supplemented with 10% calf serum and 10% tryptose phosphate broth (TPB). Two viruses were compared in this study: the wild-type Dobson strain of FPV (FPV+ : Hav1Neq1) which grows only on CEC and a mammalian cell-adapted mutant of this strain (FPV-B) isolated by Dr J. Zavada (Bratislava, Czechoslovakia) by selection and cloning in L cells (Zavada, 1969). This virus grows in several types of mammalian cells and is genetically stable (Israël *et al.* 1975). The Rostock strain of FPV (HavlN1) was kindly provided by Dr A. J. Hay (Mill Hill, London, U.K.). The three viruses were plaque-purified three times before growing stocks in embryonated eggs.

**Peptide maps.** One-dimensional peptide maps were obtained by the method of Cleveland *et al.* (1977). *Staphylococcus aureus* V8 protease was used for partial digestion of the polypeptides.

**Infectious centre assay.** L cells were infected at various m.o.i. and after 1 h at 4 °C the inoculum was removed by successive washings with PBS including one with PBS adjusted to pH 3. The cells were covered with prewarmed medium and incubated at 37 °C. After 30 min the medium was replaced for 15 min by a 1/100 dilution of anti-FPV antiserum. These treatments were found to reduce the titre of unadsorbed virus by a factor of 10⁶. The cells were then washed with PBS, trypsinized and various dilutions of this suspension were plated on to secondary CEC. After 30 min at 37 °C the CEC monolayers were overlaid with medium containing 1% agar. Plaques were counted on the third day.

**Other methods.** Isolation of ts mutants, preparation of recombinants, analysis of labelled virion RNA (vRNA) and complementary RNA (cRNA), determination of haemagglutinin and neuraminidase activities, pulse-labelling of infected cells with ³⁵S-methionine or ³H-uridine, and analysis of virus polypeptides and RNAs by polyacrylamide gel electrophoresis (PAGE) have been described previously (Israël, 1980a, b).

**RESULTS**

**Genetic characterization of FPV-B**

The strategy used to identify the gene(s) responsible for the adapted character of FPV-B has been described by Almond (1977). It involves the genetic analysis of recombinants between a ts mutant of FPV-B and the Rostock strain of FPV. The ts mutant used in this study was td4 which we isolated and characterized previously (Israël, 1980b). It has a lesion in the P1 gene and is temperature-sensitive in L cells but not in CEC. L cells were infected at 39.5 °C simultaneously with the td4 and Rostock viruses. In addition, td4 was u.v.-irradiated before infection in order to reduce the number of recombinants to be screened.

All recombinants growing in L cells must contain from Rostock at least the gene corresponding to the defective gene of td4 (the P1 gene in this case) and from td4 the gene(s) responsible for the ability to multiply in L cells. The genetic analysis of the recombinants obtained has been described in detail elsewhere (Israël, 1980b). Briefly, we used the fact that P2, P3, HA, NP, M and NS polypeptides of td4 and Rostock have different migration rates in PAGE using various gel systems. The assignment of the two other genes was based on the analysis of virus RNAs by the technique previously described by Hay *et al.* (1977a). By these techniques 30 recombinants have been analysed. Their genetic composition is shown in Table 1. P3 and HA are the only td4 genes common to all recombinants.
Table 1. Genome composition of recombinants between u.v.-irradiated td4 and Rostock

<table>
<thead>
<tr>
<th>Recombinant*</th>
<th>Gene</th>
<th>6, 11, 23, 26</th>
<th>7, 15, 27</th>
<th>4, 12, 14, 28</th>
<th>8, 17, 25</th>
<th>1, 13, 16, 24</th>
<th>5, 19, 28</th>
<th>2, 20</th>
<th>9, 22, 29</th>
<th>10, 21, 30</th>
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<tbody>
<tr>
<td>P1</td>
<td>Ro†</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
</tr>
<tr>
<td>P2</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>P3</td>
<td>B†</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>HA</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>NP</td>
<td>Ro</td>
<td>Ro</td>
<td>B</td>
<td>B</td>
<td>Ro</td>
<td>Ro</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>Ro</td>
</tr>
<tr>
<td>NA</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>B</td>
<td>Ro</td>
<td>B</td>
<td>Ro</td>
<td>B</td>
</tr>
<tr>
<td>M</td>
<td>Ro</td>
<td>B</td>
<td>B</td>
<td>Ro</td>
<td>Ro</td>
<td>B</td>
<td>Ro</td>
<td>B</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>NS</td>
<td>Ro</td>
<td>B</td>
<td>Ro</td>
<td>B</td>
<td>Ro</td>
<td>B</td>
<td>Ro</td>
<td>B</td>
<td>B</td>
<td>B</td>
</tr>
</tbody>
</table>

* The recombinants have been numbered in the order of isolation.
† Ro, Rostock; B, td4.

Table 2. Genome composition of recombinants between u.v.-irradiated FPV-B and Rostock

<table>
<thead>
<tr>
<th>Recombinant</th>
<th>Gene</th>
<th>1, 3</th>
<th>2</th>
<th>7</th>
<th>4, 6</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Ro</td>
<td>Ro</td>
<td>B</td>
<td>Ro</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>Ro</td>
<td>B</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>HA</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>B</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>NP</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td>B</td>
<td>Ro</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>Ro</td>
<td>B</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>Ro</td>
<td>B</td>
<td>Ro</td>
<td>Ro</td>
<td>Ro</td>
<td></td>
</tr>
<tr>
<td>NS</td>
<td>Ro</td>
<td>Ro</td>
<td>B</td>
<td>Ro</td>
<td>Ro</td>
<td></td>
</tr>
</tbody>
</table>

In another experiment L cells were mixedly infected at 37 °C with u.v.-irradiated FPV-B (Dobson) and the Rostock strain. Plaques were picked at random. Some were due to true recombinants. The genetic composition of seven such recombinants is shown in Table 2: it can be seen that all of these have the P3 gene of FPV-B and four of them also contain the HA gene of Rostock. Therefore, it can be concluded that the P3 gene of Dobson is sufficient to enable a recombinant to grow in L cells. However, it is necessary to demonstrate that this gene would also be sufficient to allow the non-adapted Dobson strain (FPV+) to grow in L cells. The use of the techniques described above was difficult since the proteins and RNAs of FPV+ and FPV-B have similar migration rates except for the HA polypeptide (Israel, 1979). The use of the RNA technique of Hay et al. (1977a) allowed us to detect mutations in genes 2 and 3 of FPV-B as compared to FPV+ (Fig. 1).

The mutation in gene 3 which codes for P2 is apparently not relevant to the adaptation phenomenon. The slight displacement of the heterologous HA double-strand probably correlates with the previously detected mutation. The presence of three detectable mutations in FPV-B allowed us to analyse recombinants between u.v.-irradiated FPV-B and FPV+. Only a few recombinants could be recovered because of the higher interfering activity of FPV+ (A. Israel, unpublished results).

The genetic composition of the five recombinants isolated is shown in Table 3: they all have HA from FPV+ (which means that they are true recombinants since FPV+ does not multiply in L cells) and P3 from FPV-B. The origin of P2 is variable. The five remaining genes are indistinguishable in FPV+ and FPV-B by all techniques used and most likely did not undergo mutation during the selection of FPV-B (see Discussion). This finding strongly suggests that substitution of the P3 gene is sufficient to enable FPV+ to grow in L cells.

An intriguing result was the observation that the P2 and P3 polypeptides of Dobson-B are functionally equivalent to P2 and P3 respectively of Rostock in contrast to the results
Fig. 1. Detection of mutations in the P genes of FPV-B compared to FPV+. "H-labelled cRNA from FPV+ was annealed with an excess of unlabelled vRNA of either (1) FPV+ or (2) FPV-B, treated with S1 nuclease and analysed on a 4% acrylamide 0.2% diallyltartardiamide gel containing 4 M-urea. Electrophoresis was for 40 h at 100 V. The gel was treated for fluorography as described by Laskey & Mills (1975).

Table 3. Origin of the P2, P3 and HA genes of five recombinants between u.v.-irradiated FPV-B and FPV+

<table>
<thead>
<tr>
<th>Recombinant</th>
<th>Gene</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P2</td>
<td>P3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>1, 3, 5</td>
<td>2, 4</td>
</tr>
<tr>
<td>HA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>*</td>
<td>+, FPV+; B, FPV-B</td>
<td></td>
</tr>
</tbody>
</table>

of Almond & Barry (1979). This result was confirmed by peptide mapping of the proteins by the partial proteolytic digest fingerprint method of Cleveland et al. (1977). Bands corresponding to the three P polypeptides were excised from the gel shown in Fig. 2(a), which contained diallyltartardiamide instead of bisacrylamide; this gel system allows an unambiguous separation of the three P polypeptides. Peptide maps shown in Fig. 2(b) indicate that P1, P2 and P3 of Rostock have digestion patterns similar to those of P1, P2 and P3 respectively, of Dobson-B.

Phenotypic expression of FPV+ and FPV-B in L cells

Previous results (Israël, 1979) have shown that the abortive cycle of FPV+ in L cells is characterized by the production of mainly non-infectious particles (called FPV+L): the yield after 12 h is 0.1 to 0.5 p.f.u./cell. However, analysis of polypeptides and cRNAs induced by FPV+ and FPV-B in L cells infected with a high multiplicity shows only minor differences (Israël, 1980a).

Moreover, the non-infectious particles produced by FPV+ apparently contain the same polypeptides and RNA segments as the infectious particles produced by FPV-B (Israël, 1979).
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Fig. 2. (a) Analysis of the polypeptides of td4-infected and Rostock-infected CEC labelled with \textsuperscript{35}S-methionine on an 8\% acrylamide 0.2\% diallyl tartardiamide gel containing 4 M-urea. Electrophoresis was for 15 h at 100 V. (b) Analysis of the three P proteins of td4 and Rostock by limited digestion using \textit{S. aureus} V8 protease as described by Cleveland et al. (1977). The virus polypeptides were excised from the gel shown in (a) and placed directly into the slots of a 15\% polyacrylamide gel. A 500 ng amount of \textit{S. aureus} V8 protease was added on to each slice. Electrophoresis was for 18 h at 70 V. R, Rostock; 4, td4.

Table 4. Comparison between virus production of FPV+ and FPV-B in L cells and direct infectious centre assay of the infected cells*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Total production(\dagger)</th>
<th>Infectious centres(\ddagger)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPV+</td>
<td>(0.8 \times 10^8)§</td>
<td>(5 \times 10^6)</td>
</tr>
<tr>
<td>FPV-B</td>
<td>(7.2 \times 10^6)</td>
<td>(7 \times 10^6)</td>
</tr>
</tbody>
</table>

* L cells were infected with FPV+ or FPV-B at 50 p.f.u./cell. After 1 h at 4°C the inoculum was neutralized as described in Methods. Cells were either trypsinized and various dilutions plated on to CEC plates, or further incubated for 12 h at 37°C. At the end of this period the total production (supernatant plus intracellular virus obtained by three cycles of freezing and thawing) was titrated on CEC. The residual infectivity after inactivation of the inoculum (5 \(\times\) 10\textsuperscript{7} p.f.u. per 10\textsuperscript{8} cells) as described in Methods was usually 50 to 100 p.f.u.
\(\dagger\) Amount of virus produced by 10\textsuperscript{6} L cells.
\(\ddagger\) Number of infectious centres obtained from 10\textsuperscript{6} L cells.
§ Each value is the mean of four different determinations.

An indication that the assembly of FPV+\(_L\) could be deficient has been given by experiments involving the use of latex spheres coupled with fetuin (Israel et al. 1979). These spheres are able to attach to the plasma membrane of FPV-B-infected L cells due to the interaction between fetuin and viral neuraminidase exposed at the cell surface. L cells infected with FPV+ are unable to bind these spheres which may indicate that neuraminidase could be incorrectly inserted into the plasma membrane. A similar indication was obtained by an infectious centre assay: L cells infected with FPV+ or FPV-B were either incubated overnight at 37°C or trypsinized after the adsorption period and plated on CEC. Plaque titration was performed on the supernatant plus intracellular virus in the first case and directly on the infected CEC in the second case. Results in Table 4 indicate that with FPV-B, as expected, the titre by infectious centre assay is 5 to 10 times lower.
Fig. 3. Polypeptides synthesized in L cells infected with different multiplicities of FPV+ or FPV-B. Cells were labelled with 35S-methionine from 4 to 4.25 h and then analysed on a 15% polyacrylamide gel. Electrophoresis was for 19 h at 100 V. +, FPV+; B, FPV-B; u, uninfected cells. The numbers indicate the m.o.i. used.

than by direct titration of the product. However, in the case of FPV+ the titre by infectious centre assay is 5 to 10 times higher than by titration of the product.

We have also analysed the effect of varying the m.o.i. on the synthesis of polypeptides in FPV+- and FPV-B-infected L cells. The results are shown in Fig. 3. At 4 h p.i., the synthesis of virus polypeptides decreased with the m.o.i. in FPV+-infected L cells but not in FPV-B-infected cells. Densitometer tracings of the gel shown in Fig. 3 followed by quantitative analysis of the profiles show (Table 5) that proteins M and HA decrease exactly in proportion to the m.o.i. while proteins P1, 2, 3, NP and NS decrease at a slower rate. This decrease has been found at all times tested for FPV+-infected L cells. Enzymic assay of the viral neuraminidase showed that in FPV+-infected cells this activity decreased at the same rate as M and HA. For FPV-B this phenomenon was tested only until 4 h p.i. since at later times a second cycle of infection is initiated at low multiplicities.

It could be argued that such a decrease was caused by a lower affinity of the haemagglutinin of FPV+ for the receptors at the surface of L cells, compared with the haemagglutinin of FPV-B; this would correlate with the mutation observed. In that case the number of L cells infected with FPV+ would be much less than indicated by the m.o.i. This hypothesis has been eliminated by the following observation: a recombinant carrying the HA gene of FPV+ and the P3 gene of FPV-B behaves exactly as FPV-B when the
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Table 5. Quantitative analysis of the amounts of virus polypeptides synthesized by FPV+ and FPV-B in L cells in relation to the m.o.i.

<table>
<thead>
<tr>
<th>M.o.i.</th>
<th>P1 +</th>
<th>B</th>
<th>P2, 3 +</th>
<th>B</th>
<th>HA +</th>
<th>B</th>
<th>NP +</th>
<th>B</th>
<th>M +</th>
<th>B</th>
<th>NS +</th>
<th>B</th>
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<tbody>
<tr>
<td>200</td>
<td>15†</td>
<td>10</td>
<td>14</td>
<td>9</td>
<td>280</td>
<td>220</td>
<td>250</td>
<td>150</td>
<td>260</td>
<td>470</td>
<td>240</td>
<td>260</td>
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<tr>
<td>100</td>
<td>27</td>
<td>19</td>
<td>34</td>
<td>19</td>
<td>220</td>
<td>390</td>
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<td>480</td>
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<td>50</td>
<td>36</td>
<td>37</td>
<td>47</td>
<td>51</td>
<td>240</td>
<td>705</td>
<td>740</td>
<td>605</td>
<td>290</td>
<td>1140</td>
<td>1130</td>
<td>1330</td>
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<td>20</td>
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<td>5</td>
<td>ND†</td>
<td>230</td>
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<td>380</td>
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<td>7450</td>
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<td>8840</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>590</td>
<td>ND</td>
<td>1080</td>
<td>ND</td>
<td>15300</td>
<td>2850</td>
<td>14160</td>
<td>310</td>
<td>20400</td>
<td>1840</td>
<td>15110</td>
</tr>
</tbody>
</table>

* Each number represents the ratio between the peak surface (arbitrary units) of the corresponding polypeptide as measured from the densitometer tracings of three different gels such as that shown on Fig. 3, and the m.o.i. Each value is the mean of three determinations.
† ND, Not detected.

Fig. 4. Virus mRNAs synthesized by FPV+ and FPV-B in L cells at different m.o.i. Cells were labelled from 2 to 3 h p.i. with 100 μCi/ml of 5-3H-uridine and extracted. Labelled RNA was annealed with an excess of homologous unlabelled vRNA and polyadenylated double-strands were isolated by LiCl precipitation and analysed on a 7.5% polyacrylamide gel as described by Hay et al. (1977b). Electrophoresis was for 40 h at 100 V. †, FPV+; B, FPV-B. The numbers indicate the m.o.i. used.

m.o.i. is decreased. This indicates that only the P3 protein is involved in the multiplicity phenomenon. The amount of virus mRNA synthesized was also multiplicity dependent in the abortive system (Fig. 4): messengers 4 and 7 coding for HA and M were the first to disappear when the m.o.i. was lowered (decrease of the messenger coding for NA could not be detected since it is synthesized in small amounts even in a productive cycle).
Table 6. Synthesis of vRNA by FPV+ and FPV-B in L cells at different m.o.i.*

<table>
<thead>
<tr>
<th>Virus</th>
<th>200</th>
<th>50</th>
<th>10</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPV+</td>
<td>11.7</td>
<td>4.9</td>
<td>1.1</td>
<td>ND2</td>
</tr>
<tr>
<td>FPV-B</td>
<td>10.5</td>
<td>9.8</td>
<td>10.1</td>
<td>8.2</td>
</tr>
</tbody>
</table>

* Infected or mock-infected L cells were labelled with 100 μCi/ml of 5,6-3H-uridine from 2 to 3 h p.i. RNA was extracted at this time and annealed with increasing amounts of unlabelled in vitro synthesized cRNA (Scholtissek & Rott, 1970) until a plateau was reached.

† Amount of vRNA synthesized was expressed as the percentage of total labelled RNA rendered RNase-resistant by saturating amounts of unlabelled cRNA after subtraction of the values obtained in the same annealing conditions with labelled RNA from mock-infected cells. Each value is the mean of four different determinations.

‡ ND, Not detected.

Since amplification of mRNA synthesis depends on synthesis of vRNA, we have also estimated the amount of vRNA produced by the method described by Scholtissek & Rott (1970). Data in Table 6 indicate that the amount of vRNA synthesized by FPV+ in L cells was also multiplicity-dependent, but this was not the case with FPV-B.

The decrease in synthesis of FPV+-specific components with the m.o.i. correlated well with the amount of virus particles released by L cells and estimated by plaque titration of the supernatant or by labelling with 35S-methionine. For 1 p.f.u./cell or less, no virus particle can be detected in the supernatant by any technique and at any time after infection. It may be noted that at all multiplicities the amount of virus detected was 10 to 20 times higher by using the infectious centre assay than by direct titration.

DISCUSSION

The data presented here indicate that the adaptation of the Dobson strain of fowl plague virus to mammalian cells is most probably due to a mutation in the gene coding for the P3 protein. This gene has been unambiguously shown to confer to the Rostock strain (by replacement of the corresponding P3 gene) the ability to multiply in L cells. However, the result is less clear in the case of FPV+ since the parental origin of five genes cannot be attributed to FPV+ or FPV-B by all techniques used. As such it is impossible to rule out the role of one or several of these genes in conferring on a given recombinant the ability to multiply in L cells. However, the RNA annealing technique used (Hay et al. 1977a) is probably able to detect a point mutation in a virus gene (Hay et al. 1979), but in this case it did not permit us to detect any additional mutation in these five genes. This probably indicates that these five genes did not undergo mutation during the selection of FPV-B and that substitution of the P3 gene is sufficient to enable FPV+ to grow in L cells. This is consistent with the results of Almond (1977). However, we found that the P3 protein of Dobson is functionally equivalent to the P3 protein of Rostock and not to P2 as found by Almond (1977). Several reasons could explain such a discrepancy. The numbering of the P proteins solely according to their electrophoretic mobility is confusing. P2 and P3 have very similar mol. wt. and therefore could have inverted migration rates depending on the polyacrylamide concentration. We used diallyltartardiamide instead of bisacrylamide (Fig. 2a) which allows an unambiguous separation of the three P proteins.

It is intriguing that all recombinants between td4 and Rostock able to grow in L cells have the HA gene from the td4 parent, while recombinants between FPV-B and either Rostock or FPV+ can receive the HA gene from one or the other parent. A possible explanation could be that td4 carries a mutation in the HA gene in addition to the one in
the P1 gene. This mutation would give a selective advantage to the recombinants carrying HA from td4 over those carrying HA from Rostock, e.g. by increasing the rate of virus release by infected cells, or the infectivity of the released virions. In favour of such an interpretation involving an additional mutation in the genome of td4 is the fact that the recombinants isolated by Almond (1977) using another ts mutant of FPV-B can receive the HA gene from one or the other parent.

FPV-B, when compared to FPV+, appears as a triple mutant but only the mutation in the P3 gene is relevant to the adapted character. The mutation in the P2 gene detected on Fig. 1 and the mutation in HA previously described (Israël et al. 1975) and confirmed by peptide mapping (not shown) are apparently chance co-mutations which appeared during the adaptation of FPV-B to L cells.

The abortive infection of L cells by FPV+ is followed at high multiplicities (more than 20 to 50 p.f.u./cell) by death of the cells and by release of mainly non-infectious particles (Israël, 1979). When the m.o.i. is decreased fewer cells show a c.p.e. and also the amount of infectious particles released decreases. When the m.o.i. is less than 1 p.f.u./cell, neither c.p.e. nor infectious particles can be detected.

The analysis of intracellular events during the replication cycles of FPV+ and FPV-B in L cells shows that virus mRNA and polypeptide synthesis is multiplicity-dependent in the case of FPV+ but not in that of FPV-B. Polypeptides M, HA and NA of FPV+ decrease proportionately to the m.o.i. while the other virus polypeptides decrease at a slower rate. When the m.o.i. is less than 1 no virus polypeptide can be detected in FPV+-infected cells, correlating with the lack of c.p.e. and of detectable released particles.

In the replication cycle of influenza A viruses, M, HA and NA have been characterized as late proteins (Skehel, 1972, 1973; Meier-Ewert & Compans, 1974; Inglis et al. 1976; Hay et al. 1977b) and the transition from early to late protein synthesis has been attributed to newly made and presumably virus-specified protein (Inglis & Mahy, 1979). In addition, vRNA synthesis has been shown to be also multiplicity-dependent in the abortive cycle but not in the productive one.

These data suggest that P3 is involved in the amplification of vRNA and mRNA synthesis and perhaps in the switch from early to late protein synthesis. The m.o.i. effect in the abortive system could be explained as follows: if the P3 protein has to interact with a cellular factor in order to be functional (or to be protected from degradation), it is possible to assume that the affinity of the P3 protein of FPV+ for the cellular components of L cells is much lower than that of P3 of FPV-B. Therefore, formation of a functional complex would require larger amounts of P3 in an abortive than in a productive cycle. Increasing the amount of input P3 would increase the chance to initiate a replication cycle. The phenomenon would also apply to the newly synthesized P3 proteins, thus making the final amount of all virus components dependent on the initial m.o.i. If this were so, the adapted character of FPV-B would result from an increased affinity of the P3 protein for the L cell component.

However, it cannot be decided from the available data whether the initial defect in the abortive system is at the level of transcription (thus allowing only limited amounts of poly A+ cRNA and of replicase molecules to be synthesized and consequently restricting vRNA synthesis) or at the level of replication (thus preventing the amplification of cRNA). Alternatively, P3 could be involved in both transcription and replication. On the other hand, infection of L cells with high multiplicities (over 100 p.f.u./cell) of FPV+ or FPV-B leads to the synthesis of equivalent amounts of virus proteins and RNAs (Israël, 1980a). Under such conditions, productive and abortive cycles differ only by the amount of infectious particles produced: 0.1 to 0.5 p.f.u./cell for FPV+ and 5 to 10 p.f.u./cell for FPV-B, although apparently equivalent amounts of physical particles are released in both
systems (Israël, 1979). This rather low production in a permissive infection is due to a Von Magnus phenomenon; infection of L cells at very low multiplicity with FPV-B yields after multiple cycles more than 100 p.f.u./cell. Therefore, the mechanism of abortive infection at high m.o.i. may be different from that prevalent at low multiplicities.

At high multiplicities, we have found some indications of a maturation defect at the plasma membrane of abortively infected cells, where the viral neuraminidase is abnormally integrated (Israël et al. 1979). Furthermore, the amount of infectious centres detectable in the abortive system is much larger when L cells are directly plated on to CEC 1 h after the adsorption period than those measured when the supernatant plus intracellular virus of such FPV+ infected L cells are titrated on CEC at the time when there is maximum virus production (12 h). This increase is not due to residual FPV+ which remained adsorbed on to the plasma membrane of L cells since these infectious particles would also have been detected by titrating the supernatant plus intracellular virus (obtained by several cycles of freezing and thawing). This effect cannot be explained by the action of trypsin on the haemagglutinin precursor since evidence has been provided that cleavage of this molecule occurs normally in the abortive system and that trypsin has no effect on the infectivity of FPV+ and FPV+L for CEC or L cells (Israël, 1979). A possible explanation would be that a spontaneous fusion can occur between the plasma membranes of L cells and CEC in the infectious centre assay, which either allows the particles produced by L cells to mature correctly, or provides factors derived from CEC which are necessary for the correct replication of FPV+. Alternatively, in the infectious centre assay the virus particles released by FPV+-infected L cells could be protected from physical inactivation which may take place in the plaque assay due to their prolonged stay in the culture medium. However, it has not been possible to detect a particular sensitivity of the FPV+L infectivity to heat or pH inactivation.

A maturation defect has been invoked to explain some influenza virus abortive infections (Caliguiri & Holmes, 1979). However, the relationship existing between the P3 protein and virus maturation remains obscure.

The abortive cycle examined here is quite distinct from that described for the Rostock strain in L cells. In this latter case the abortive infection is characterized by an apparent retention of the RNP in the nucleus (Franklin & Breitenfeld, 1959) and by a dramatic decrease in the synthesis of the M and NA polypeptides (Bosch et al. 1978), with no release of virus particles. In the present case, the abortive cycle is characterized by an apparent lack of amplification of virus polypeptide synthesis, resulting in an absence of spread of the virus at low m.o.i. On the other hand, at high multiplicities similar amounts of virus constituents are synthesized in both productive and abortive systems and non-infectious particles are released in the abortive system, possibly as a consequence of a maturation defect at the plasma membrane. It is interesting to note that, although the abortive cycles undergone by FPV+ and Rostock in L cells seem to be due to different causes, the replacement of the P3 gene of each of these viruses by the corresponding gene of FPV-B is enough to enable a productive replication cycle in L cells to occur. This may indicate that the abortive cycle undergone by these two strains of influenza virus may have a unique cause, the expression of which differs from one system to another. The involvement of the P3 gene in the control of the host-range is to be put in the context of the observation of Scholtissek & Murphy (1978) who showed that a ts mutant of the Rostock strain carrying a lesion in the P3 gene is no longer able to multiply in MDCK cells even at the permissive temperature. These authors suggest that the spread of the ts mutant is inhibited in MDCK cells. Mutants carrying a lesion in the Pt gene exhibit the same growth restriction.
These data suggest that the correct functioning of the P3 protein requires interaction with some particular cellular component(s). In the case of the Dobson strain FPV+ (and also probably in the case of the Rostock strain) this component would be present in CEC but either missing or different in L cells. The adapted character of FPV-B would result also probably in the case of the Rostock strain) this component would be present in CEC and L cells.

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REFERENCES


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