A temperature-sensitive mutation in the acidic polymerase gene of an influenza A virus alters the regulation of viral protein synthesis

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The temperature-sensitive defect of mutant ts 263 of fowl plague virus (FPV) is located in the acidic polymerase (PA) gene and is due to a single base substitution (C2036T), which leads to an amino acid replacement (Ala671 to Val) in a highly conserved region of the protein. During passage at 33 °C ts 263 stably carries over a ninth RNA segment, which consists of a truncated PA gene. Although the deletion is in-frame and it is transcribed into mRNA, no corresponding protein is detected in vivo. After reversion to wild-type this extra RNA segment is immediately lost. At the non-permissive temperature of 40 °C no significant viral products of ts 263 are synthesized. Under semi-permissive conditions there is a relative, but very significant over-production of the M1 protein, which is not accompanied by a corresponding elevated M1 mRNA synthesis. These results are in agreement with the idea that the PA protein is involved in the regulation of viral protein synthesis at the level of expression of mRNA. Preinfection of chicken embryo cells with ts 263 at a semi-permissive temperature interferes with the replication of FPV wild-type indicating that premature availability of M1 might be detrimental for influenza virus replication.

Introduction

Studying the phenotype of temperature-sensitive (ts) mutants with a defect in a specific gene at the non- or semi-permissive temperature might provide us with information about the function of the corresponding gene product (for review, see Mahy, 1983). Although some knowledge already exists about the function of two of the components of the influenza virus polymerase complex, namely the two basic proteins (Braam et al., 1983), the function of the third component, the acidic polymerase (PA) protein, is not yet known.

Influenza viral protein synthesis is highly regulated. Some viral proteins are synthesized in small quantities and some in large quantities early in the infection cycle, such as the polymerase proteins or the nucleoprotein (NP) and non-structural NS1 protein, respectively. Some others are synthesized relatively late in small or large quantities, such as the neuraminidase (NA) or the haemagglutinin (HA) and matrix (M) proteins, respectively. The major regulation seems to occur at the level of the availability of the corresponding mRNA (Hay et al., 1977; Smith & Hay, 1982; Enami et al., 1985; Shapiro et al., 1987). However, there are some indications that additional regulation occurs at the level of translation (Hatada et al., 1989; Fischer et al., 1990). So far, little is known concerning the mechanism of regulation and the viral components involved. There are some indications that the NS1 protein plays a role in regulation (Wolstenholme et al., 1980; Koennecke et al., 1981; Ludwig et al., 1991; Hatada et al., 1990) and that there is some cooperation between the NS1 protein and the polymerase complex (Scholtissek & Spring, 1982; Ludwig et al., 1991). Since nothing is known about the possible role of the PA protein of the polymerase complex, we have investigated whether this protein might be involved in such regulation processes by studying the phenotype of a mutant with a ts defect in that gene in semi-permissive conditions.

The ts mutant ts 263 of fowl plague virus (FPV) carries a ts lesion in segment 3 (PA gene) (Scholtissek et al., 1976). After infection of chicken embryo cells (CEC) with ts 263 and incubation at the non-permissive temperature of 40 °C neither infectious virus, nor viral proteins, nor viral RNA (vRNA) are synthesized in significant quantities (Scholtissek & Bowles, 1975). Therefore we used intermediate temperatures in order to see how viral replication is influenced under these semi-permissive conditions. It was found that at elevated temperatures synthesis of the matrix protein M1 increased relative to the NP and NS1, whereas very little HA was detected at any temperature. In addition, at the permissive temperature a ninth RNA segment accumulated in virus particles, which turned out to be the product of a highly truncated PA gene.

Methods

Viruses and tissue culture. The FPV A/fowl plague/Rostock/34 (H7N1, FPV, Giessen isolate) and a ts mutant (ts 263) with a defect in
the PA of the polymerase complex (Scholtisseg et al., 1976) were used. The mutant was obtained after mutagenesis by growth in the presence of 5-fluorouracil (Scholtisseg & Bowles, 1975). The viruses were propagated either in primary CEC in culture or in embryonated chicken eggs.

Purification of ts 263 by back-crossing with wild-type FPV. In order to exclude the possibility that the ts mutant carries an additional mutation in another gene that does not contribute to the ts phenotype, ts 263 was purified twice by double infection of CEC with a surplus of FPV wild-type and ts 263, and plaques were picked at random after a plaque test at 33 °C, the permissive temperature. In parallel, a plaque enlargement test was performed (3 days at 33 °C, after marking the plaque size, shift to 40 °C for 24 h; Scholtisseg & Spring, 1982). In one experiment eight of 96, and in another 20 of 230, plaques after double infection did not grow further at 40 °C. However, when these plaques were picked and injected into embryonated chicken eggs, no virus yield was obtained at 33 °C. Fewer than 10% of the plaques isolated at random at 33 °C turned out to be ts. With one of the ts isolates the procedure was repeated with the same ratio of ts to wild-type plaques (1:10). Thus the chance of retaining a particular segment with an additional mutation was about 1:100. The location of the defect of the final ts isolate was checked by double infection with a set of standard mutants with defects in the various genes at 40 °C (Scholtisseg & Bowles, 1975). As a control, the same procedure was applied to ts 482, which contains a ts defect in the HA gene, and a second defect in the NA gene (about 1% NA activity when compared with FPV wild-type) that does not contribute to the ts character (C. Scholtisseg, unpublished). Under conditions of double infections with FPV, when about half of the plaques did not grow further after shift-up to 40 °C, about half of the isolated ts mutants with a defect in the HA gene, carrying the normal NA gene of the wild-type virus, had segregated. On this basis it was assumed that the purified ts 263 isolate had replaced most genes by those of FPV wild-type, except that carrying the ts defect.

Preparation of vRNA, cloning, PCR and sequencing procedures. The viruses were propagated in embryonated chicken eggs either at 33 °C (ts 263) or 37 °C (FPV). They were purified and concentrated by adsorption to and desorption from glutaraldehyde-fixed erythrocytes (Becth & Malole, 1975) and centrifugation. The RNA was isolated by the guanidinium thiocyanate–hot phenol method as described by Maniatis et al. (1982). The RNA was analysed by polyacrylamide gel electrophoresis (PAGE) and silver staining (silver stain kit, Bio-Rad) (Goldman & Merrill, 1982).

Two independent clones of the PA gene of FPV wild-type in a pBluescript SK + vector were obtained from Dr Ursula Schultz of our institute. They were subcloned using the restriction enzymes EcoRI, HindIII, or Stul. Using vector-specific or specific internal primers the sequences were obtained by the method of Sanger et al. (1977), as modified by Taber & Richardson (1987).

When the PCR was investigated to obtain corresponding clones of the ts 263 mutant, PA-specific DNA samples with a size of only about 500 bases were obtained. As shown below, this was because ts 263 particles contain an additional highly truncated PA gene that is preferentially amplified by the PCR. Therefore two pairs of primers were used to give rise to two subgenomic DNAs of the PA gene (from positions 1 to 1200 and from 917 to 2233 nucleotides) which were inserted into the pBluescript SK vector. Several independent subclones were used for sequencing according to Sanger et al. (1977). More than 90% of the wild-type and ts 263 PA gene was also sequenced directly on vRNA using Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL) (Brownlee & Cartwright, 1977).

Analysis of viral proteins. Viral proteins were labelled by incubating infected CEC at various temperatures in the presence of [35S]methionine for 1 h, starting at 4 h after infection. The labelled proteins were analysed by PAGE as described (Bosch et al., 1979). The X-ray films were scanned at a wavelength of 585 nm, and the peaks were cut out from the recording paper. The weights of the paper are given in milligrams to measure the relative amounts of the various viral proteins.

Analysis of viral mRNA. RNA was isolated at 4 h after infection of CEC as described by Chomczynski & Sacchi (1987). Either total RNA or mRNA as obtained by adsorption onto and desorption from oligo(dT)-coated Dynabeads (Dynal) were used. The RNA segments were separated by agarose-formamide gel electrophoresis and blotted onto a nylon membrane. For hybridization the [32P]UTP-labelled probes were synthesized on vectors derived from the pBluescript II-SK vector (Stratagene) using the T3 or T7 promoters. pAN1 contained nucleotides 341 to 1265 of the HA gene, pJU1 nucleotides 703 to 1565 of the NP gene, the pAN3 vector nucleotides 196 to 855 of the NS protein gene, pAN4 nucleotides 246 to 1024 of the M1 protein gene (Fischer et al., 1990), and pPA contained the truncated PA gene.

The mRNA-specific probes were synthesized after linearization by digestion with appropriate restriction enzymes prior to labelled RNA synthesis. The hybridization was carried out according to the procedure of Wahl et al. (1979) as modified by Fischer et al. (1990). For the Northern blot analysis of ts 263 viral RNA, a corresponding PA probe of positive polarity was prepared. The RNA was quantified by scanning of the X-ray films as described above.

Quantification of viral activities. Infectivity was determined by the usual plaque test on CEC (Klenk et al., 1972). HA tests were performed using chicken erythrocytes. NA activity was determined according to Aminoff (1961).

Results

Analysis of the RNA of ts 263 particles and localization of the ts defect

The RNA of purified ts 263 particles grown at 33 °C in embryonated eggs exhibited an abundant extra RNA segment with an estimated length of about 500 bases (Fig. 1). This extra RNA segment was found in the original ts 263 isolate as well as after back-crossing but it disappeared when the mutant was propagated at 37 °C. This extra RNA could be stained specifically with a PA plus-sense probe in Northern blot analysis (data not shown).

A comparison of the sequence of the wild-type (Giessen isolate) PA gene (plus-sense) with that of the published PA sequence (Robertson et al., 1984) revealed the following differences: A1325 to C, A306 to T, C394 to T, T1801 to C, G1891 to A, A1897 to G, A1573 to G and T2010 to G.

When the wild-type (Giessen) PA gene was compared with that of the ts 263 isolate a single base replacement from C to T at position 2036 (positive-sense strand) was observed, which leads to an exchange of alanine to valine at position 671. No further difference was found. Two revertants were isolated after independent plaque tests of
ts PA gene mutation in influenza A

Fig. 1. Polyacrylamide gel electrophoresis and silver staining of Is 263 vRNA. The over-representation of NP vRNA is not a common finding and has been seen only occasionally. The extra RNA hybridizes in a Northern blot to a PA plus-sense probe (not shown).

Fig. 2. Proteins of ts 263- or FPV-infected CEC were labelled with [35S]methionine from 4 to 5 h after infection at various temperatures, the results shown in Fig. 2 were obtained. In general, in ts 263-infected cells, even at the permissive temperature of 33 °C, the HA was scarcely or not visible after labelling with [35S]methionine. At semi-permissive temperatures the M1 protein became the most prominent component. Under these conditions a protein band at the position of the PA protein became visible, which was not observed at this intensity in wild-type-infected cells at any temperature. These observations were quantified by scanning (Table 1). The over-production of the M1 protein occurred as early at 2 h after infection (data not shown). Totally different results were obtained with wild-type-infected cells. In Fig. 2 no ts 263-specific protein of the size expected from the extra segment (147 amino acids) could be detected, nor when the pulse length was reduced to 10 min, in spite of the fact that the deletion is in-frame and the expected protein should contain five methionines.

Table 1. Quantification of viral proteins synthesized in CEC at different temperatures after infection with either FPV or ts 263*

<table>
<thead>
<tr>
<th>Incubation temperature (°C)</th>
<th>Strain</th>
<th>NP</th>
<th>M1</th>
<th>NS1</th>
<th>NP/M1</th>
</tr>
</thead>
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<tr>
<td></td>
<td>FPV</td>
<td>33</td>
<td>109.2</td>
<td>39.2</td>
<td>38.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>111.8</td>
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<td>44.8</td>
</tr>
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<td>39</td>
<td>144.9</td>
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<tr>
<td></td>
<td></td>
<td>40</td>
<td>118.4</td>
<td>104.6</td>
<td>74.8</td>
</tr>
<tr>
<td></td>
<td>ts 263</td>
<td>33</td>
<td>55.2</td>
<td>15.8</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>55.1</td>
<td>96.3</td>
<td>16.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39</td>
<td>30.7</td>
<td>56.2</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>12.3</td>
<td>14.7</td>
<td>ND†</td>
</tr>
</tbody>
</table>

* The X-ray films of Fig. 2 were scanned. The numbers in the table represent the mass of paper corresponding to the peaks.  
† ND, Not determined because signal too weak.

When CEC infected with either ts 263 or FPV wild-type were labelled with [35S]methionine from 4 to 5 h post-infection after incubation at different temperatures and separated by PAGE. Lanes 1, 33 °C; lanes 2, 37 °C; lanes 3, 39 °C; lanes 4, 40 °C; m.i., mock-infected. M1 became the most prominent product of ts 263 with increasing temperatures.

ts 263 at 40 °C. Both revertants again had C at position 2036. This indicates that the replacement of Ala271 with Val is responsible for the ts phenotype of ts 263. When the RNA of the revertants after multiplication in chicken eggs at 33 °C was analysed, the extra segment (about 500 bases) was no longer visible after silver staining.

The extra segment of ts 263 was sequenced using several independent PCR products subcloned in different orientations into the Bluescript vector. It was found that this segment contains 290 nucleotides from the 5' end and 233 nucleotides from the 3' end of the PA gene (vRNA). The point mutation at position 2036 (C changed to T) of the PA gene of ts 263 was also found in the highly truncated PA segment. Thus, this extra segment contains an in-frame deletion of 1710 nucleotides.

Viral protein synthesis in ts 263-infected cells at semi-permissive temperatures

When CEC infected with either ts 263 or FPV wild-type were labelled with [35S]methionine 4 to 5 h post-infection after incubation at different temperatures and separated by PAGE. Lanes 1, 33 °C; lanes 2, 37 °C; lanes 3, 39 °C; lanes 4, 40 °C; m.i., mock-infected. M1 became the most prominent product of ts 263 with increasing temperatures.
incubation at different temperatures does not correlate with the in vivo protein synthesis as shown in Fig. 2 and Table 1.

By using a PA-specific hybridization probe an mRNA of the size of the truncated PA gene could be detected (Fig. 3). Sometimes this mRNA was heterogeneous in size, which might be a sign of instability in vivo.

**Sequence of the M gene (segment 7)**

Because the synthesis of the M1 protein in ts 263-infected cells is extremely over-represented at 37 °C we sequenced the M genes of the mutant before and after plaque purification, and those of the revertants, and compared these sequences with that of the wild-type FPV. There were three base substitutions found in the ts 263 M genes when compared with FPV wild-type: T → C, T → C and T → G. Since the same replacements were found in the M genes of the two revertants, which phenotypically behave identically to FPV wild-type, we are confident that these differences in the RNA sequence do not contribute to the specific phenotype of ts 263.

**Trans-effect of the over-production of the M1 protein of ts 263 on the replication of wild-type FPV**

It is known that the M1 protein interferes with the transcription of influenza A virus nucleocapsids (Zvonarjev & Ghendon, 1980; Ye et al., 1989; Hankins et al., 1989). Since M1 is a late protein, its involvement has been suggested in the down-regulation of viral mRNA synthesis late in the infectious cycle, at a time when vRNA synthesis is still optimal. Therefore it was of interest to see whether preinfection with ts 263 at a semipermissive temperature interferes with the replication of FPV wild-type. A premature accumulation of M1 of ts 263 might interfere with transcription of the incoming nucleocapsid of FPV (Martin & Helenius, 1991). As can be seen in Table 3, a preinfection of CEC by ts 263 at 38 °C for at least 2 h reduced the yield of FPV wild-type by more than 90%. However, there was also a strong reduction in virus yield at 33 °C when the superinfection by FPV was delayed by 1 h. This did not occur when the superinfection was later. A plaque test at 33 °C and 40 °C of the virus yields (not shown) revealed that when superinfection by FPV was delayed by 1 h at 33 °C as well as 38 °C, the plaque yield at 40 °C was on average only 10% of that at 33 °C. This indicates that under these conditions ts 263 was produced preferentially and had interfered with FPV replication by an as yet unknown mechanism. At all later stages of superinfection with FPV the plaque yield at 33 °C and 40 °C was not significantly different, indicating that in these cases FPV prevailed over ts 263.
Table 3. Inhibition of FPV wild-type by preinfection of CEC by ts 263 at semi-permissive temperature*

<table>
<thead>
<tr>
<th>Infection with</th>
<th>Time FPV added after ts 263 (h)</th>
<th>HA yield after incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>33 °C</td>
</tr>
<tr>
<td>ts 263 FPV</td>
<td>-</td>
<td>1536</td>
</tr>
<tr>
<td>ts 263 FPV</td>
<td>0</td>
<td>512</td>
</tr>
<tr>
<td>ts 263 FPV</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>ts 263 FPV</td>
<td>2</td>
<td>ND†</td>
</tr>
<tr>
<td>ts 263 FPV</td>
<td>2.5</td>
<td>128</td>
</tr>
<tr>
<td>ts 263 FPV</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>ts 263 FPV</td>
<td>4</td>
<td>256</td>
</tr>
</tbody>
</table>

* CEC were either singly or doubly infected with ts 263 and/or FPV wild-type. In several cases the addition of FPV was postponed as indicated. HA units were determined either 9 h (33 °C) or 7 h (38 °C) after the secondary infection.
† ND, Not determined.

Discussion

Mutant ts 263 of FPV carries a mutation in the PA gene (Scholtissek et al., 1976). Comparison of sequences of the PA gene of ts 263 with the wild-type FPV and revertants showed that a single amino acid replacement (Ala, to Val), located in a completely conserved region of the PA protein (Okazaki et al., 1989; Schultz et al., 1991), was responsible for the ts phenotype.

In order to study the phenotype of a ts mutant obtained by mutagenesis it must be confirmed that a mutation in another gene is not responsible for the observations. Therefore ts 263 was ‘purified’ by two back-crosses with wild-type FPV so that all genes except that carrying the ts defect were replaced by the wild-type segments. Later analysis of the sequences of the M protein gene revealed that before and after back-crossing this ts 263 gene contained three substitutions compared with wild-type FPV. Therefore, segment 7 was the original one from the ts 263 isolate with two mutations introduced by 5-fluorouracil. However, since the two revertants carry the same replacements they do not play any role in the specific phenotype of ts 263.

In ts 263-infected CEC at semi-permissive temperatures there is a significant over-production of the M1 protein compared with wild-type-infected cells or after infection with ts 263 at the permissive temperature. HA synthesis is impeded at any temperature. Thus there is a clear deregulation of viral protein synthesis caused by a mutation in the PA protein. In ts 263-infected cells at elevated temperatures the over-production of M1 protein does not correlate with the amount of the corresponding mRNA. Thus, the deregulation is not likely to be at the level of mRNA synthesis. Although the RNA sequence of the ts 263 M gene is different to wild-type FPV at three positions, these base substitutions cannot be responsible for the increased translation because the same sequence differences were found in two independent revertants, which behave like wild-type FPV.

The important conclusion to draw from these results is that PA is somehow involved in the regulation of expression of viral mRNA. An explanation in molecular terms cannot be offered yet.

After double infection, concomitant with an accumulation of the M1 protein of ts 263 at a semi-permissive temperature, wild-type replication is severely impeded. This might be due to an inhibition of the transcription of the incoming nucleocapsid of FPV by the prematurely accumulated ts 263 M1 protein. Such premature synthesis of M1 could be one reason why ts 263 does not multiply at 40 °C.

An interesting observation is that a highly truncated PA gene is stably carried over as long as ts 263 is passaged at 33 °C. Although the deletion is in-frame, and mRNA is synthesized, a protein of a corresponding size was not discovered. It is not clear what role the extra segment plays in the viability of ts 263. Normally, extra RNA segments are detrimental for a virus but this segment accumulates at the permissive temperature and its production is impeded at elevated temperatures. The observation that during the plaque enlargement test the ts 263 plaques obtained after a shift for 24 h at 40 °C do not produce viable virus after injection into embryonated eggs at 33 °C can be explained by assuming that the extra segment is essential for replication of ts 263. However, a plausible mechanism for this cannot be provided at present. The PC Fold program (Jacobson et al., 1984) for calculating energies of secondary RNA structures did not reveal any significant change by the C2023 to T substitution (D. Wurkner, personal communication). No changes in secondary structure at the deletion site were expected to be caused by the mutation.

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References


