A Mutant of Fowl Plague Virus (Influenza A) with an Enhanced Electrophoretic Mobility of RNA Segment 8

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SUMMARY

A temperature-sensitive mutant (ts 1/9) obtained by undiluted passage of fowl plague virus (FPV) at 33 °C carried a strong ts defect in RNA segment 6 [neuraminidase (NA) gene] and a weak ts defect in RNA segment 8 [non-structural (NS) protein gene]. Although the viral proteins have normal migration rates, the NS gene migrated during polyacrylamide gel electrophoresis (PAGE) significantly faster than the NS gene of wild-type FPV, even after denaturation by glyoxal. Despite this observation, the NS gene of ts 1/9 did not carry a deletion as shown by sequence determination. There were only five base replacements which resulted in three changes in amino acids. Three of the base replacements led to a more compact secondary structure of RNA segment 8, which seems to be responsible for the faster migration rate during PAGE and which seems to resist, at least partially, the treatment with glyoxal.

INTRODUCTION

Mutations caused by deletions are expected to be genetically stable, in contrast to point mutations which can revert relatively easily. Therefore, we have screened temperature-sensitive (ts) mutants of fowl plague virus (FPV) obtained by undiluted passage at low temperature (Scholtissek & Müller, 1983) for deletions by determining the electrophoretic mobility of the proteins and/or vRNA segments. One of these mutants with a ts defect in the haemagglutinin (HA) gene was found to carry an HA2 molecule with an enhanced electrophoretic mobility. However, it was concluded that this enhanced mobility was due to a loss of one of the oligosaccharide side chains (Scholtissek et al., 1984). We now describe another ts mutant, obtained by the same technique, which carries a non-structural (NS) protein gene (RNA segment 8) with an enhanced electrophoretic mobility. This enhanced mobility is partially retained even after treatment with glyoxal, which is thought to destroy the secondary structure of RNA. We have shown by direct sequencing that the mutant does not carry a deletion in RNA segment 8, but that the difference in mobility is due exclusively to differences in secondary structure of the RNA caused by point mutations.

METHODS

Virus and cell cultures. A/FPV/Rostock/34 (H7N1) and a ts mutant (1/9) thereof have been used throughout. The ts mutant was obtained by undiluted passage at 33 °C and carries a strong ts defect in RNA segment 6 [neuraminidase (NA) gene] as shown by rescue with standard ts mutants at the non-permissive temperature (40 °C) (Scholtissek & Müller, 1983). In addition, it carries a weak ts defect in RNA segment 8 (NS gene), since after double infection with ts 412 (NS gene mutant; Koennecke et al., 1981) the plaques formed at 40 °C are significantly smaller than wild-type plaques.

Primary chick embryo monolayers prepared from 11-day-old chickens were used 48 h after seeding.

Biological tests. Plaque and haemagglutination tests were performed according to established procedures (Klenk et al., 1972). The NA activity was determined by the method of Aminoff (1961).

Polyacrylamide gel electrophoresis (PAGE). The viral proteins were labelled by incubating infected cells (multiplicity of infection of 5 to 20 p.f.u./cell) either with [35S]methionine (800 Ci/mmol) or [2-3H]mannose
(10 Ci/mmol, Amersham). The cells were processed and the proteins separated by PAGE as described by Bosch et al. (1979), except that 25% polyacrylamide gels were used.

Viral RNA was isolated from virus particles labelled with carrier-free $^{32}$P orthophosphate (Amersham) (Scholtissek et al., 1976). The RNA was extracted from the viral particles by phenol and was analysed by PAGE in 6 M-urea directly (Floyd et al., 1974), or after treatment with glyoxal to destroy the secondary structure of RNA (McMaster & Carmichael, 1977; Desselberger & Palese, 1978).

Reverse transcription, cloning into pBR322 and sequencing of the NS gene of ts 1/9. Mutant ts 1/9 was propagated in 11-day-old chick embryos and purified by centrifugation through a 40% sucrose cushion (for details, see Steuler et al., 1984). Viral RNA was extracted in the presence of 2 M-guanidine isothiocyanate by treatment with phenol/chloroform/isooamyl alcohol at 60°C. The RNA segments were separated by PAGE and the NS gene (segment 8) was extracted from the gel. It was reverse-transcribed in the presence of the deoxycytodinenucleotide complementary to the 3' end of the viral RNA segment (forward primer, kindly provided by Dr E.-L. Winnacker, München, F.R.G.). The RNA–DNA hybrid molecule was used directly for homopolymer tailing and cloning into the PstI site of the pBR322 vector (Lobban & Kaiser, 1973; Peacock et al., 1981; Land et al., 1981).

The insert DNA of two clones (37 and 74) was subcloned after cleavage with the restriction endonuclease Sau3A into the BamHI cut of the M13mp8 or M13mp9 vectors, respectively (Messing & Vieira, 1982). From the recombinant M13 clones single-stranded phage DNA was prepared and used for sequence determination by the chain-termination method (Sanger et al., 1977). Since clone 74 was missing 91 bases corresponding to the 5' end of the vRNA, the residual bases were sequenced directly on the viral RNA template by using the deoxyoligonucleotide GAAGATAACAGAGAA complementary to position 759 to 773 of RNA segment 8 (Porter et al., 1980) labelled at the 5' end with $^{32}$P. The deoxyoligonucleotide primer was synthesized by Dr B. Müller-Hill (Köln, F.R.G.) and was purified by PAGE by Dr W. Garten at our Institute.

RESULTS

Biological characterization of ts 1/9

When chick embryo cells were infected with ts 1/9 and incubated either at 33°C or 40°C, the yield of infectious virus was only about 1% or 1%, respectively, when compared to cells infected with wild-type FPV at these temperatures (data not shown). Thus, even at the so-called permissive temperature of 33°C, when plaques are still formed, virus yield from mutant-infected cells is relatively low. Although the HA and NA titres were comparable at 33°C in mutant virus- and wild-type virus-infected cells, the HA/NA ratios were consistently somewhat higher in FPV-infected cells (Table 1). At 40°C, both the absolute titres as well as the HA/NA ratios were very low in mutant virus-infected cells when compared with those of FPV-infected cells (Table 1). Thus, ts 1/9 seems to have a defect in HA synthesis despite the fact that the ts mutation is located in the NA and not in the HA gene as determined by rescue with standard ts mutants (Scholtissek & Müller, 1983).

When infected cells were labelled from 6 to 7 h after infection at 33°C with $^{35}$S-methionine,

<table>
<thead>
<tr>
<th>Virus</th>
<th>Incubation temperature (°C)</th>
<th>Time after infection (h)</th>
<th>HA units</th>
<th>NA activity/ml†</th>
<th>Ratio HA/NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPV</td>
<td>33</td>
<td>8</td>
<td>32</td>
<td>29</td>
<td>1-1</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>24</td>
<td>128</td>
<td>74</td>
<td>1-7</td>
</tr>
<tr>
<td>ts 1/9</td>
<td>33</td>
<td>8</td>
<td>32</td>
<td>51</td>
<td>0-6</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>24</td>
<td>32</td>
<td>57</td>
<td>0-6</td>
</tr>
</tbody>
</table>

* Cells in the supernatant medium were frozen and thawed three times, the debris was removed by centrifugation, and the activities were determined in the supernatant.
† Sialic acid (µg) released from fetuin after 30 min incubation at 37°C.
Fig. 1. Labelling with \[^{35}\text{S}]\text{methionine of chick embryo cells infected with ts 1/9 or FPV wild-type and incubated at 33 °C. The infected cells were labelled from 6 to 7 h after infection with the following quantities per ml: (a) ts 1/9, 20 μCi; (b) mock-infected, 30 μCi; (c) FPV, 5 μCi; (d) ts 1/9, 40 μCi; (e) FPV, 10 μCi. NP, Nucleoprotein; HA, haemagglutinin; HA1 and HA2, corresponding cleavage products of HA; M, membrane protein; NS1, non-structural protein 1. The labelled proteins were separated by electrophoresis on a 25% polyacrylamide gel.}

the results presented in Fig. 1 were obtained. The shut-off of cellular protein synthesis by ts 1/9 was less efficient than that caused by the wild-type virus. However, this depended somewhat on the multiplicity of infection. Therefore, it was difficult to recognize the P proteins and HA1. No differences in the migration rates of nucleoprotein (NP), HA2, membrane (M) and NS1 proteins could be seen. There was a relatively enhanced labelling of the NS1 protein in ts 1/9-infected cells compared to labelling of HA2 and M. However, this may be due to a slower rate of multiplication of the mutant when compared with FPV wild-type: NS1 is an early protein, while M and HA are late proteins (for review, see Skehel & Hay, 1978). After longer exposure, NS2 could also be recognized (not shown), but there seemed to be no significant difference in migration rates of NS2 between the mutant and FPV either.

When infected cells were labelled with \[^{3}H\text{mannose they were incubated with the isotope}
at 33 °C from 4 to 7·5 h, and at 40 °C from 2·5 to 5 h after infection. As can be seen in Fig. 2, at 33 °C the NA in mutant-infected cells was relatively highly labelled when compared with cells infected with FPV. At 40 °C, the absolute incorporation into \( ts 1/9 \) glycoproteins was drastically reduced when compared with FPV-infected cells.

**Characterization of viral RNA by PAGE**

When viral RNA segments labelled *in vivo* with \( {^{32}}P \)orthophosphate were separated by PAGE without prior denaturation by glyoxal, all RNA segments of the mutant co-migrated with the corresponding RNA segments of the wild-type FPV, except for RNA segment 8 (NS gene), which migrated ahead of the FPV gene (Fig. 3a). When the RNA was treated with glyoxal under conditions which should theoretically completely abolish its secondary structure (McMaster & Carmichael, 1977; Desselberger & Palese, 1978), the NS gene of \( ts 1/9 \) still migrated significantly ahead of that of wild-type FPV (Fig. 3b), even though the difference in migration between mutant and wild-type genes was reduced. These results have been obtained in three independent experiments.
Ribosomal RNA——

(a) ts 1/9 FPV

(b) FPV ts 1/9

Fig. 3. PAGE of ts 1/9 and FPV wild-type RNA labelled in vivo with $^{32}$Porthophosphate. After labelling and phenol extraction the viral RNA was separated without prior treatment with glyoxal (a) or after treatment with 1 M purified glyoxal in 50% DMSO at 50 °C for 1 h (b). The middle lane of (a) contains another ts mutant which grew to such low titres that only the contaminating 18S ribosomal RNA became visible. PB2, PB1 and PA are the three polymerase genes. The bar at the top indicates gel origin.

Sequencing of RNA segment 8 of ts 1/9

Since the faster migration of the NS gene of ts 1/9 under denaturing conditions could indicate that this gene carried a deletion, the gene was sequenced after partially cloning RNA segment 8 into pBR322 and subcloning into M13 vectors. The rest of the sequence was determined by direct sequencing of viral RNA using an appropriate deoxyoligonucleotide primer as described in Methods. Fig. 4 depicts the relative length of clones 37 and 74 and the strategy of sequencing using corresponding subclones. No differences in base sequence between the two individual clones were found. The residual sequence on the right was determined using a synthetic primer complementary to the viral RNA sequence between position 759 and 773, and vRNA segment 8.

In Fig. 5, the total sequence of the cDNA of the wild-type FPV NS gene and the deduced amino acid sequence as determined by Porter et al. (1980) are compared with the corresponding sequence of ts 1/9. There were five base replacements, one of which was silent. The four other replacements altogether led to three amino acid changes. No deletion was discovered.
DISCUSSION

Recently, we have developed a new technique for producing \textit{ts} mutants of influenza viruses by undiluted passage at low temperature. Since many of the mutants obtained in this way had properties different from mutants generated by chemical mutagens, we thought that the former mutants could arise by an unknown mechanism, possibly causing deletions (Scholtissek & Müller, 1983). Therefore, we have analysed the RNA of several mutants by PAGE. One of these mutants which carried a strong \textit{ts} defect in the NA gene is described here. It contains an NS gene (RNA segment 8) with a faster mobility in PAGE, although the \textit{ts} defect in this gene is rather weak. It is not yet clear whether the decreased HA activity found in mutant-infected cells is caused by the latter mutation or is due to the mutation in RNA segment 6 (NA gene).

The enhanced migration rate of RNA segment 8 without denaturation by glyoxal could be due either to changes in the secondary structure of the RNA by point mutations or to deletion of bases. However, the two NS proteins of \textit{ts} 1/9 have the same mobilities on PAGE as those of the wild-type FPV. Thus, if there is a deletion, it should be either very small or it should be located beyond the coding region of the two NS proteins.

The secondary structure of RNA can be destroyed by treatment with glyoxal (McMaster & Carmichael, 1977; Desselberger & Palese, 1978) and thus differences in mobilities caused by secondary structure should be abolished. When glyoxal-treated RNA segment 8 of \textit{ts} 1/9 and wild-type FPV were examined by PAGE the difference in mobility was reduced but not totally abolished. Therefore, RNA segment 8 of \textit{ts} 1/9 was sequenced and compared to the wild-type FPV sequence, determined by Porter \textit{et al.} (1980). As can be seen in Fig. 5, there were no deletions, and only five base replacements with four of them leading to three changes in amino acids. As a result of these mutations, more-stable hairpin structures can be constructed in two regions of the gene (Fig. 6) when compared with the wild-type sequence. The construction of the more-stable secondary structures over longer distances has not been tried. Thus, even a few point mutations can change the secondary structure of an RNA to such an extent that the mobility in PAGE is significantly changed, and this change in secondary structure cannot be completely abolished by treatment with glyoxal under the most stringent conditions. Thus, comparative analysis of single-stranded RNAs after treatment with glyoxal by PAGE does not give an unequivocal answer to whether an RNA contains a deletion or not.
Fig. 5. Total base sequence of the NS gene of FPV wild-type and the deduced amino acid sequence of NS1 and NS2, showing also the mutations in the corresponding gene of ts 1/9. The replacements of bases in ts 1/9 are shown above the base sequence of the wild-type, and the amino acid replacements below the wild-type sequence. The corresponding sites are boxed in.

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Mutation | FPV (vRNA) | ts 1/9 (vRNA)
---|---|---
1 | \(-CUAGUCUUCAGG^A\_C\ \_C\) | \(-CUAGUCUUCAGG^A\_C\ \_C\)
 at position 150 |

2/3 | \(-UAACUCCCCUUCUG\_C\) | \(-UAACUCCCCUUCUG\_C\)
 at positions 400/401 |

Fig. 6. Changes in secondary structure induced by mutation. The presence of theoretically more-stable hairpin configurations in the immediate neighbourhood of the mutations has been deduced.

REFERENCES


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