Evidence for the Involvement of Influenza A (Fowl Plague Rostock) Virus Protein P2 in ApG and mRNA Primed in vitro RNA Synthesis

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SUMMARY

Eleven temperature-sensitive (ts) mutants of influenza A (fowl plague. Rostock) virus were analysed for in vitro RNA transcriptase activity in reactions primed by ApG or globin mRNA at 31 °C or at 40.5 °C, the restrictive temperature for ts mutant growth. Only those ts mutants studied which were defective in RNA segment 1, coding for the virion P2 protein, were defective in RNA transcriptase activity when compared to wild-type virus. Mutants having a defect in the P2 protein had no significant RNA transcriptase activity in reactions at 40.5 °C primed by globin mRNA. However, one mutant showed RNA transcriptase activity similar to wild-type virus at 40.5 °C when ApG (0.3 mM) was used as primer. The results suggest that influenza (fowl plague. Rostock) P2 protein is directly involved in the mRNA priming reaction, as well as in the RNA transcription reaction in vitro.

The eight distinct negative-strand RNA segments of fowl plague virus, an avian influenza A virus, can be transcribed in vitro by the virion-bound RNA-dependent RNA polymerase (for reviews, see Barry & Mahy, 1979; Hay & Skehel, 1979). Under optimal conditions the products of the influenza virion RNA-dependent RNA polymerase reaction have many of the properties of virus mRNA synthesized in infected cells, in that they are approx. full-length, polyadenylated transcripts of each genome segment (Plotch & Krug, 1977, 1978; Hay et al., 1977). However, the structures of in vivo and in vitro synthesized mRNA do differ in that the in vitro mRNAs are shorter and lack a 5' methylated cap structure (Hay et al., 1977; Plotch et al., 1978). Furthermore, primary transcription in vivo is sensitive to the drugs actinomycin D and alpha-amanitin (inhibitors of host cell DNA-dependent RNA polymerase II), whereas transcription in vitro is insensitive to these drugs (Scholtissek & Rott, 1970; Penhoet et al., 1971; Mahy et al., 1972; Lamb & Choppin, 1977; Spooner & Barry, 1977). In addition, the influenza virion transcriptase was shown to possess another unusual property in that a number of guanosine related compounds, particularly the dinucleoside adenyl (3' to 5') guanosine (ApG), stimulate RNA synthesis in vitro by acting as primers for RNA synthesis (McGeoch & Kitron, 1975; Plotch & Krug, 1977).

Recently, an explanation for these findings became apparent when it was reported that various eukaryotic mRNAs stimulate the in vitro transcription reaction (Bouloy et al., 1978) and that the 5'-terminal cap structure plus 9 to 15 nucleotides is transferred to the 5' end of the transcript in vitro (Plotch et al., 1979; Bouloy et al., 1980; Robertson et al., 1980). The authors suggested that capped host cell RNA molecules are used as ‘primers’ for synthesis of influenza mRNAs in vivo and that the continued synthesis of these primer molecules is the alpha-amanitin-sensitive step in influenza virus replication (Krug et al., 1979). In an attempt to determine which virion proteins participate in this priming reaction, we have studied the ApG and globin mRNA-primed in vitro transcriptase activities of a number of genetically characterized temperature-sensitive mutants of fowl plague virus (FPV) (Ghendon et al., 1973; Almond et al., 1979). The results presented here suggest that a function of FPV (Rostock) P2 protein is essential for virion transcriptase activity and that this protein is involved in the utilization of the mRNA primer.
Temperature-sensitive mutants of FPV Weybridge (A/FPV/Dutch/27, H7N7) (Ghendon et al., 1973), FPV Rostock (A/FPV/Rostock/34, H7NI) (Almond et al., 1979) and the parent wild-type strains for these mutants were used. Virus stocks were grown in the allantoic cavity of 11-day-old fertile hens' eggs and plaqued on primary chick embryo fibroblast cells (CEF) as described by Almond et al. (1977). Virus was purified as described previously (Inglis et al., 1976) and the final virus pellet was resuspended in NT buffer (0.1 M-NaCl, 0.01 M-tris–HCl pH 7.4) and stored at 0 °C. Protein concentrations of purified virus suspensions were determined by the method of Lowry et al. (1951). The procedure used for recombination analysis of ts mutants was that described by Almond et al. (1979).

Purified rabbit globin messenger RNA was extracted from rabbit reticulocyte lysate prepared as described by Hunt & Jackson (1974). Polysomes were pelleted from the lysate by centrifugation at 140 000 g for 2.5 h at 4 °C. The pellet was resuspended in NET buffer (100 mM-NaCl, 1 mM-EDTA, 10 mM-tris–HCl pH 7.5) and the RNA extracted by Pronase digestion followed by phenol-chloroform extraction and ethanol precipitation (Barrett et al., 1979). RNA pellets were resuspended in water, made 1% SDS, 5 mM-EDTA, 0.1 M-NaCl and 10 mM-tris–HCl pH 7.5, before loading on to linear 10 to 40% sucrose gradients formed in NET. Gradients were centrifuged at 100 000 g for 18 h at 15 °C. then fractionated using an automatic Isco gradient fractionator and fractions monitored for absorbance at 254 nm. Fractions containing the peak of RNA sedimenting in the 9S to 10S region of the gradient were pooled and ethanol-precipitated. The globin mRNA was further purified by two rounds of oligo(dT)-cellulose chromatography (Glass et al., 1975; Inglis & Mahy, 1979).

The procedure for assay of influenza virion-associated RNA polymerase was based on that described by McGeoch & Kitron (1975). Purified virus (in a small vol. of NT buffer) was disrupted with 0.5% Nonidet P40 for 10 min at the incubation temperature. The disruption mix was then diluted sixfold by addition of incubation buffer (prewarmed to incubation temperature for 1 min) to 150 mM-KCl, 50 mM-tris–HCl pH 8.2, 5 mM-dithiothreitol, 0.3 mM-ApG or 15 µg/150 µl reaction volume of rabbit globin mRNA, 2 mM-ATP, 0.4 mM-CTP and either (i) 0.4 mM-UTP, 20 µM-GTP and 10 µCi [α-32P]GTP per 150 µl reaction volume or (ii) 0.2 mM-GTP, 40 mM-UTP and 15 µCi 3H-labelled UTP per 150 µl reaction volume. Duplicate samples were immediately removed for acid-insoluble radioactivity assay (regarded as time zero), then MgCl2 was added to a concentration of 8 mM and the reaction was incubated at the temperature indicated for each experiment. Assays were performed using purified virus at a concentration of 50 µg virus protein per 150 µl reaction volume. The incubation temperature for FPV (Rostock) wild-type and ts mutants was either 31 °C or 40.5 °C and for FPV (Weybridge) wild-type and ts mutants was either 31 °C or 42 °C. Reactions were sampled at timed intervals for determination of acid-insoluble radioactivity.

The virion transcriptase activities of FPV wild-type and ts mutants were measured at the optimum in vitro temperature of 31 °C (Skehek 1971; Bishop et al., 1971; Plotch & Krug, 1977; Nichol, 1980) and at the non-permissive temperature used in the initial selection of the mutants (40.5 °C for FPV Rostock and 42 °C for FPV Weybridge) in the presence of ApG. In each case, a kinetic analysis of the reaction was performed with six time-points taken over a 90 min incubation period.

As expected, the transcriptase activity of the wild-type viruses was greatly reduced at the upper temperature, possessing only approx. 10 to 13% of the activity expressed at 31 °C. The temperature sensitivity of the virion transcriptase of the Weybridge ts mutants, ts 29 and ts 166, was similar to that of the wild-type enzyme. However, the transcriptase activity of ts 131 at the upper temperature was only 2% of that at 31 °C (data not shown). Previous analysis of this mutant under different reaction conditions had also shown it to be negative for transcriptase activity at 42 °C (Ghendon et al., 1975). Thus, for the assay conditions used here, mutants expressing transcriptase activity at the upper temperature of approx. 2% or less
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Fig. 1. (a) Rostock wild-type and ts mutants were assayed for in vitro transcriptase activity at 31 °C (●●●●) and 40.5 °C (○○○○) with 0.3 mM-ApG as primer as described in the text using [32P]GTP as the labelled nucleotide. Activity is expressed as a percentage of acid-precipitable counts after 90 min at 31 °C. The 90 min values (ct/min incorporated per 10 μl sample × 10⁻⁴) were as follows: wild-type, 16.2; ts 17, 4.32; ts 44, 4.82. (b) Rostock wild-type and ts mutants were assayed for in vitro transcriptase activity at 31 °C (●●●●) and 40.5 °C (○○○○) in the presence of globin mRNA as described in the text using [32P]GTP as the labelled nucleotide. Activity is expressed as a percentage of acid precipitable counts after 120 min at 31 °C. The 120 min values (ct/min incorporated per 10 μl sample × 10⁻⁴) were as follows: wild-type, 19.5; ts 17, 5.34; ts 44, 6.65.

of that expressed at the lower temperature, were regarded as negative. Using this criterion, a number of Rostock ts mutants were screened (Fig. 1a and Table 1) and the only mutant which was negative for ApG-primed transcriptase activity at 40.5 °C was ts 17, which has a defect in RNA segment 1 coding for protein P2 (Almond et al., 1979).

In an attempt to identify the virus-specific proteins involved in priming of influenza RNA transcription by capped mRNA, which is apparently essential for mRNA synthesis in vivo (Krug et al., 1979; Caton & Robertson, 1980; Dhar et al., 1980), the same Rostock ts mutants were screened using a globin mRNA-stimulated transcriptase reaction. Again a kinetic analysis was carried out for each mutant; however, only the kinetics of more interesting mutants are shown (Fig. 1b), the transcriptase activities of other mutants being summarized in Table 1. Two mutants, ts 17 and ts 44, possessing ts lesions in gene 1 (coding for the P2 protein) were negative for mRNA-stimulated transcriptase activity at 40.5 °C. The result obtained with ts 17 is consistent with this mutant also being negative for transcriptase activity at 40.5 °C in the non-mRNA-primed reaction (Fig. 1a). The inactivity of ts 44 in the mRNA-stimulated transcriptase reaction at 40.5 °C is interesting; this mutant was positive in
Table 1. In vitro transcriptase activity of Rostock wild-type and ts mutants at the non-permissive temperature (40.5 °C)

<table>
<thead>
<tr>
<th>Recombination group</th>
<th>Virus</th>
<th>Defective protein</th>
<th>ApG primed transcriptase activity†</th>
<th>Globin mRNA primed transcriptase activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>Wild type</td>
<td>−</td>
<td>9.4 (+)</td>
<td>11.6 (+)</td>
</tr>
<tr>
<td>I</td>
<td>ts US1 NP</td>
<td>HA</td>
<td>22.4 (+)</td>
<td>19.1 (+)</td>
</tr>
<tr>
<td>VI</td>
<td>ts 46 HA</td>
<td>P1</td>
<td>6.1 (+)</td>
<td>7.4 (+)</td>
</tr>
<tr>
<td>II</td>
<td>ts US4 HA</td>
<td>P1</td>
<td>7.4 (+)</td>
<td>7.7 (+)</td>
</tr>
<tr>
<td>III</td>
<td>ts 15 P1</td>
<td>P1</td>
<td>5.6 (+)</td>
<td>5.0 (+)</td>
</tr>
<tr>
<td>III</td>
<td>ts 17 P2</td>
<td>P2</td>
<td>4.8 (+)</td>
<td>10.2 (+)</td>
</tr>
<tr>
<td>V</td>
<td>ts mN4 P3</td>
<td>P3</td>
<td>1.4 (+)</td>
<td>1.3 (+)</td>
</tr>
<tr>
<td>V</td>
<td>ts 44 P2</td>
<td>P3</td>
<td>6.2 (+)</td>
<td>2.2 (+)</td>
</tr>
<tr>
<td>IV</td>
<td>ts mN3 NS1 &amp;/or NS2</td>
<td>P3</td>
<td>20.2 (+)</td>
<td>26.6 (+)</td>
</tr>
<tr>
<td>IV</td>
<td>ts 45 P3</td>
<td>P3</td>
<td>9.7 (+)</td>
<td>6.7 (+)</td>
</tr>
<tr>
<td>IV</td>
<td>ts 47 NS1 &amp;/or NS2</td>
<td>P3</td>
<td>7.3 (+)</td>
<td>7.2 (+)</td>
</tr>
</tbody>
</table>

† Virion transcriptase activity expressed as percentage activity at 40.5 °C after 90 min (ApG primed reactions) or 120 min (mRNA primed reactions).

ApG-primed reactions at 40.5 °C, suggesting that it has a specific defect in the utilization of mRNAs as primers for initiation of transcription.

Several groups of investigators have attempted to identify the virus-specific proteins involved in the transcription of influenza virus RNA. Both fractionation studies of virus ribonucleoprotein complexes (Caliguiru & Compans, 1974; Caliguiru & Gerstein, 1978; Inglis et al., 1976) and the study of RNA synthesis in cells infected with influenza virus ts mutants (Sugiura et al., 1975; Krug et al., 1975; Scholtissek et al., 1974; Scholtissek & Bowles, 1975; Scholtissek, 1978, 1979; Ghendon et al., 1973, 1975; Barry & Mahy, 1979) have indicated a requirement for the P proteins and the nucleocapsid protein in transcription of influenza virus RNA, although the precise function of these proteins remained unclear.

The results obtained with FPV Weybridge mutant ts 131 (containing a ts lesion in RNA segment 2 coding for P1 protein) indicated that a functional P1 protein was required for virion transcriptase activity of the Weybridge virus strain. These results are in agreement with earlier findings by Ghendon et al. (1975). A more extensive study of FPV Rostock ts mutants revealed that out of eleven mutants tested only one, ts 17, was negative for ApG-primed transcriptase activity at 40.5 °C, indicating that for this strain of FPV, a functional P2 protein (coded for by RNA segment 1) was required for virion transcriptase activity. When mutants were tested for mRNA-stimulated transcriptase activity at 40.5 °C, one other RNA segment 1 mutant, ts 44, also appeared to be negative, suggesting that the P2 protein of this influenza virus strain is directly involved in the mRNA priming event, as well as in the transcription reaction itself. Recently, analysis of seven other FPV Rostock P2 mutants in our laboratory has shown that all are defective in the mRNA-stimulated transcriptase reaction at 40.5 °C (C. R. Penn & B. W. J. Mahy, unpublished results). The mRNA priming mechanism apparently involves recognition of the 5' methyl cap structure (Bouloy et al., 1980) and a specific endonucleolytic cleavage of primer molecules (Robertson et al., 1980; Plotch et al., 1981). The exact role of the P2 protein in the priming mechanism is as yet unclear, but is currently under investigation.

The comparison of data obtained with different influenza virus strains is complicated by the difference in electrophoretic mobility of equivalent genes and proteins (Palese, 1977). However, as it has been demonstrated that RNA segment 1 of fowl plague virus Rostock is functionally equivalent to RNA segment 2 of the WSN strain of influenza virus (Scholtissek,
1978, 1979) there is a clear correlation between the results presented here and those obtained in previous studies with influenza WSN (Mowshowitz & Ueda, 1976: Mowshowitz, 1978), influenza A/Ann Arbor/6/60 (Kendal et al., 1978, 1979) and fowl plague virus Weybridge (Ghendon et al., 1975). In all cases, a protein encoded in the genome RNA segment functionally equivalent to influenza WSN RNA segment 2 is essential for the function of the virion transcriptase.

The fact that ts mutants from one recombination group can display more than one phenotype, as shown here with group III mutants and elsewhere with other ts mutants (Scholitissek & Bowles, 1975; Scholitissek, 1978; Szilagyi & Pringle 1972, 1979) demonstrates the importance of analysing a series of ts mutants from each recombination group before assigning minimum functions to the defective protein of that group. Thus, this type of analysis does not rule out the possible role of other virus-specific proteins in the transcription process or other roles for the P2 protein. Indeed, we now know that purified influenza virions are capable of performing several activities, including cap recognition and endonucleolytic cleavage of primer RNA molecules (Bouloy et al., 1980; Robertson et al., 1980; Nichol, 1980; Plotch et al., 1981), transcription of template RNA and poly(A) synthesis (for review, see Barry & Mahy, 1979; Hay & Skehel, 1979) and phosphorylation of virion proteins (Kamata & Watanabe, 1977; Nichol, 1980). The exact role of each of the P proteins and the NP protein in these processes will no doubt become clear from further genetic and phenotypic analysis of ts mutants.

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Division of Virology, Department of Pathology  
University of Cambridge, Laboratories Block  
Addenbrooke's Hospital, Cambridge, U.K.

Stuart T. Nichol†  
Charles R. Penn  
Brian W. J. Mahy*

† Present address: Department of Biology, University of California, San Diego, California 92093, U.S.A.

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