Studies of Fowl Plague Virus Temperature-sensitive Mutants with Defects in Transcription

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SUMMARY

Two fowl plague virus temperature-sensitive (ts) mutants belonging to different complementation groups were studied. Both were defective in the syntheses of unpolyadenylated complementary RNA [A(−)cRNA] and virus RNA (vRNA) at non-permissive temperature whereas primary transcription was unaffected. In addition, ts 29, in which the ts mutation is in gene 1 coding for polypeptide P3, has a defect in 'secondary' synthesis of mRNA at non-permissive temperature whereas inhibition of mRNA synthesis by ts 131, in which the ts mutation is in gene 2 coding for polypeptide P1, appeared to result from a defect in vRNA synthesis. These results indicate, therefore, that different enzymes are responsible for the syntheses of virus mRNAs and A(−)cRNAs, which is consistent with the apparent differences in initiation and termination of transcription in the two reactions. The patterns of synthesis of the various types of virus RNA during infection are discussed.

INTRODUCTION

The results of previous investigations (Ghendon et al., 1975) showed that among the ts mutants of fowl plague virus (FPV) isolated in this laboratory two mutants belonging to different complementation groups, ts 29 (complementation group C) and ts 131 (complementation group D), are defective in both RNA transcription and RNA replication at non-permissive temperature. Recently, our understanding of virus RNA transcription has increased and it is evident that two classes of complementary RNAs (cRNAs) which differ in structure, function and mechanism of synthesis, are involved in influenza virus replication (Hay et al., 1980). One of these, the virus mRNAs, are incomplete transcripts of the genome RNAs, lack sequences complementary to the 16 5’-terminal nucleotides of virus RNAs, but have additional 3’-terminal polyadenylated sequences and heterogeneous capped extensions of 10 to 15 nucleotides at their 5’ termini. These caps appear to be derived from the termini of cell mRNAs or their precursors during priming of transcription by these molecules (Skehel & Hay, 1978; Krug et al., 1979; Robertson et al., 1980). The other cRNAs [A(−)cRNAs] are complete genome transcripts, and have 5’-terminal pppA (Hay et al., 1982). Their synthesis does not require 'priming', and they are considered to function as intermediates in the replication of the virus genome.

This paper presents the results of experiments which have examined the syntheses of these two classes of cRNAs in cells infected with either mutant under non-permissive conditions and identifies the genes in which the mutations responsible for the transcriptional defects are located.

METHODS

Viruses and cell culture. Fowl plague virus (FPV; Weybridge strain, H7N7) and its ts mutants, ts 29 and ts 131, the origins and properties of which have been described elsewhere (Ghendon et al., 1973, 1975; Markushin & Ghendon, 1973), were used. Recombinants of FPV ts mutants and A/Krasnodar/101/59 (H2N2) having a ts+ phenotype were isolated as described by Ghenkina & Ghendon (1979). Viruses were grown in the allantoic cavity of chick embryos, concentrated by differential centrifugation and purified by sucrose density gradient centrifugation. Primary monolayer cultures of chick embryo fibroblasts (CEF) were used in all experiments.
cRNA synthesis. This was analysed as described by Hay et al. (1977a). CEF cultures in glass vials (3 x 10^6 cells/culture) were infected with viruses, at multiplicities of approximately 100, at 36 °C or 42 °C for 30 min, then washed and incubated in Gey's medium at the appropriate temperature. [3H]uridine (100 μCi/ml, 20 μCi/culture, sp. act. 46 Ci/mmol) was added for the appropriate time interval. RNA was extracted and annealed in 63% dimethyl sulphoxide with excess unlabelled vRNA (10 μg). The polyadenylated and unpolyadenylated hybrids were separated by precipitation of the former in 2 M-LiCl at 4 °C and residual precipitable radioactivity was removed from the supernatant following addition of unlabelled cell RNA (300 μg/ml). The LiCl precipitate was digested with nuclease S1 (2500 units/mg RNA in 100 mM-NaCl, 10 mM-sodium acetate pH 4.5, 1 mM-ZnSO₄) and then double-stranded RNAs of both fractions were analysed by electrophoresis in 4.5% polyacrylamide gels and fluorography.

Synthesis of virus polypeptides. CEF cultures were infected as described above and incubated in Medium 199 lacking methionine. [35S]methionine (20 μCi/culture, sp. act. 530 Ci/mmol; Amersham International) was added and after 15 min the cells were solubilized in a solution containing 5 M-urea, 1% SDS and 0.1% 2-mercaptoethanol and boiled for 3 min. Electrophoresis was performed in 25% or 15% polyacrylamide gels using the discontinuous buffer system described by Laemmli (1970) and autoradiography according to Russell & Skehel (1972).

Analyses of the genomes of recombinant viruses. These were performed as described previously (Hay et al., 1977b; Ghendon et al., 1979). CEF cultures were incubated at 36 °C for 30 min in medium containing cycloheximide (100 μg/ml), infected with viruses (100 to 300 p.f.u./cell) in the presence of cycloheximide and then incubated at 36 °C in medium containing cycloheximide. After 60 min, [3H]uridine (100 μCi/ml) was added to the infected cells and at 4 h RNA was extracted. Aliquots of labelled cell RNA were annealed in 63% dimethyl sulphoxide with unlabelled vRNA (5 to 10 μg) prepared from purified virus of each parental strain and, after treatment with nuclease S1 (500 units/100 μg RNA in 0.2 ml 10 mM-sodium acetate pH 4.5, 1 mM-ZnSO₄), the double-stranded RNAs were analysed by electrophoresis in 4% polyacrylamide gels.

RESULTS

Synthesis of virus cRNAs

The synthesis of cRNA in virus-infected cells was analysed as described by Hay et al. (1977a). Briefly, [3H]-labelled RNA extracted from infected cells was annealed with an excess of unlabelled virion RNA, and the polyadenylated and unpolyadenylated hybrid molecules were separated by LiCl fractionation. The former RNAs were incubated with nuclease S1 and the double-stranded RNAs analysed by electrophoresis in polyacrylamide gels. Fig. 1 shows the results of an analysis of virus mRNAs synthesized in chick cells between 2-25 and 3 h after infection at permissive (36 °C) and non-permissive (42 °C) temperatures with wild-type FPV or the mutants ts 29 or ts 131. Whereas synthesis in wild-type virus-infected cells was similar at 36 °C or 42 °C, the level of mRNA synthesis in cells infected with either mutant at 42 °C was less than 5% of that in cells infected at 36 °C, and approximated the level of primary transcription observed in cells infected in the presence of cycloheximide. Analyses of mRNAs synthesized at each temperature in cells infected with either mutant in the presence of cycloheximide showed that primary transcription occurred at the non-permissive temperature (Fig. 2). The synthesis of A(−)cRNAs was also drastically reduced in cells infected with either mutant at 42 °C as compared to that at 36 °C (Fig. 3): synthesis was barely detectable in cells infected at 42 °C with ts 131, and in cells infected at 42 °C with ts 29 it was less than 5% of that at 36 °C.

Following co-infection of cells with ts 29 and ts 131, synthesis of both mRNAs and A(−)cRNAs occurred normally at 42 °C, confirming that the defects of the two mutants complement. Since inhibition of cRNA synthesis at non-permissive temperature may be a secondary consequence of the inhibition of virus RNA (vRNA) synthesis, three different experiments were carried out in an attempt to identify the primary temperature-sensitive lesions.

Temperature shift-up experiments.

Cells infected with either mutant or wild-type virus at 36 °C were transferred to 42 °C after 1.75 h and incubated with [3H]uridine between 30 and 75 min following temperature shift-up. From the results shown in Fig. 1 and 3 it is apparent that raising the temperature reduces drastically the synthesis of both mRNAs and A(−)cRNAs in mutant-infected cells, to levels similar to those in cells incubated throughout at the non-permissive temperature. The reduction (approximately 50%) observed in the synthesis of A(−)cRNAs in wild-type virus-infected cells
Fig. 1. Synthesis of virus mRNAs in cells infected with wild-type FPV or mutants ts29 or ts131. CEF cultures were infected with wild-type FPV (lanes 1, 5 and 9), ts29 (lanes 2, 6 and 10), ts131 (lanes 3, 7 and 11) or ts29 plus ts131 (lanes 4 and 8) and incubated at 36 °C (lanes 1 to 4) or 42 °C (lanes 5 to 8). One set of cultures (lanes 9 to 11) infected at 36 °C was shifted to 42 °C at 1.75 h after infection. All cultures were incubated with [3H]uridine between 2.25 and 3 h, and 3H-labelled cRNAs were analysed as described in Methods.

Fig. 2. Primary transcription in cells infected with wild-type FPV or mutants ts29 or ts131. CEF cultures were preincubated at 36 °C for 30 min in medium containing cycloheximide (100 μg/ml), infected in the presence of cycloheximide (100 μg/ml) at 36 °C (lanes 1, 4 and 7) or 42 °C (lanes 2, 5 and 8) with FPV (lanes 1 to 3), ts29 (lanes 4 to 6) or ts131 (lanes 7 to 9), washed and subsequently incubated in cycloheximide medium. After 1 h one set of infected cultures was transferred from 36 °C to 42 °C (lanes 3, 6 and 9) and all cultures were incubated in medium containing [3H]uridine between 1.25 and 2.25 h.

following shift-up was not reproduced in other similar experiments. Analysis of vRNA synthesis following temperature shift-up, using the method described by Smith & Hay (1982), confirmed previous observations (Ghendon et al., 1975) that virion RNA synthesis is also inhibited under these conditions. The results of these and similar experiments indicate, therefore, that the enzymes responsible for synthesis of the three types of RNA are inhibited at non-permissive temperature. The results, though, do not distinguish between the direct inactivation of the
Fig. 3. Synthesis of unpolyadenylated cRNAs in cells infected with wild-type FPV or mutants ts 29 or ts 131. The results were obtained in the same experiment as that described in Fig. 1. Samples 1 to 4 are from cultures infected at 36 °C with FPV (lane 1), ts 29 (lane 2), ts 131 (lane 3) or ts 29 plus ts 131 (lane 4); samples 5 to 8 are from cultures infected at 42 °C with FPV (lane 5), ts 29 (lane 6), ts 131 (lane 7) or ts 29 plus ts 131 (lane 8); samples 9 to 11 are from cultures infected at 36 °C with FPV (lane 9), ts 29 (lane 10) or ts 131 (lane 11) and shifted to 42 °C at 1.75 h after infection.

Fig. 4. Synthesis of virus mRNA in the presence of cycloheximide in cells infected with wild-type FPV or mutants ts 29 or ts 131 in shift-up experiments. Cycloheximide (100 μg/ml) was added to cultures 2 h after infection at 36 °C with wild-type FPV (lanes 1 and 2), ts 29 (lanes 3 and 4) or ts 131 (lanes 5 and 6); at 2.5 h half the cultures were transferred to 42 °C (lanes 2, 4 and 6) and all were incubated with [3H]aridine (20 μCi/culture) between 2.75 and 3.5 h.
RNA synthesis in FPV mutants

Virus mRNA synthesis can be analysed independently of the syntheses of both virion RNA or unpolyadenylated cRNA since synthesis of the latter is inhibited by cycloheximide whereas synthesis of mRNA continues at a constant level following addition of the drug (Hay et al., 1980). The results of experiments as described in Fig. 4 showed that whereas the synthesis of
Table 1. Origin of genes of recombinants obtained by crossing FPV ts mutants (F) and A/Krasnodar/101/59 (K) virus

<table>
<thead>
<tr>
<th>Parental mutant</th>
<th>Recombinant</th>
<th>Gene number</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1 2 3 4 5 6 7 8</td>
</tr>
<tr>
<td>ts 29</td>
<td>R8</td>
<td>K F F F F F F</td>
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<tr>
<td></td>
<td>R10</td>
<td>K F F F F F F</td>
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<td></td>
<td>R12</td>
<td>K F F F F F F</td>
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<td></td>
<td>R14</td>
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<td></td>
<td>R20</td>
<td>K F F F F F F</td>
</tr>
<tr>
<td>ts 131</td>
<td>R40</td>
<td>K K F F F F F</td>
</tr>
<tr>
<td></td>
<td>R46</td>
<td>K K F F F F F</td>
</tr>
<tr>
<td></td>
<td>R48</td>
<td>K K F F F F F</td>
</tr>
<tr>
<td></td>
<td>R51</td>
<td>K K K F F F F</td>
</tr>
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</table>

mRNA following addition of cycloheximide at 2 h after infection with either wild-type FPV or ts 131 was increased somewhat following shift to 42 °C, the synthesis of mRNA in ts 29-infected cells was reduced by more than 90% (estimated from microdensitometer scans), indicating that 'secondary' mRNA synthesis is inhibited directly in these cells. The contrasting effects of temperature shift-up on 'secondary' mRNA synthesis in ts 131-infected cells in the presence or absence of cycloheximide suggest that the inhibition observed under the latter condition is secondary to the inhibition of vRNA synthesis at non-permissive temperature and that mRNA synthesis may normally be dependent on concurrent vRNA synthesis.

Unpolyadenylated cRNA synthesis after reversal of a cycloheximide block

Although unpolyadenylated cRNA may be transcribed mainly from the infecting virus genome (Hay et al., 1977a), the dependence of its synthesis on progeny vRNA is not known. Conditions which stimulate the synthesis of unpolyadenylated cRNA early in the replicative cycle were therefore used to investigate more specifically its sensitivity to non-permissive temperature in mutant-infected cells. As described in the legend to Fig. 5, these conditions involve incubating virus-infected cells in the presence of cycloheximide for 4 h and analysing RNA synthesized immediately following removal of the drug. The results obtained with wild-type FPV (Fig. 5) show that there is a stimulation in synthesis following prolonged infection in the presence of cycloheximide to a level equivalent to maximum synthesis between 1-25 and 1-75 h after normal infection. Synthesis is not affected by addition of actinomycin D, which inhibits mRNA synthesis, prior to removal of cycloheximide, indicating that the stimulation is independent of further mRNA synthesis. Although interpretation of the results is not unequivocal it is probable that synthesis of unpolyadenylated cRNA under these conditions is due largely to the enhanced transcription of the input virus genome by the increased synthesis of virus polypeptides translated from primary transcripts accumulated in the presence of cycloheximide (Skehel, 1973). Results similar to those shown for wild-type FPV were obtained with either ts mutant at 36 °C; however, following shifts to 42 °C before removal of cycloheximide, synthesis was barely detectable, indicating that the synthesis of unpolyadenylated cRNA in mutant-infected cells is inhibited directly by non-permissive temperature.

Location of the ts mutations

Recombinants with a ts⁺ phenotype were isolated by crosses between each ts mutant and the human influenza virus A/Krasnodar/101/59 and the compositions of their genomes were determined as previously described by Hay et al. (1977b). Altogether, six recombinants of ts 29 and A/Krasnodar were analysed and the results are shown in Table 1. Four recombinants (R8, R14, R16 and R20) inherited gene 1 from A/Krasnodar and all other genes from ts 29. Two other recombinants (R10 and R12) inherited two genes, 1 and 8, from A/Krasnodar and genes 2 to 7 from ts 29. Thus, substitution of gene 1 of ts 29 by the analogous gene of A/Krasnodar consistently resulted in recombinants with a ts⁺ phenotype, indicating that the temperature
RNA synthesis in FPV mutants

Table 2. Analysis of the electrophoretic mobilities of polypeptides of recombinants of FPV ts mutants (F) and A/Krasnodar/101/59 (K) virus

<table>
<thead>
<tr>
<th>Parental mutant</th>
<th>Recombinant</th>
<th>Polypeptide</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>P₁  P₂  P₃  HA  NP  M  NS</td>
</tr>
<tr>
<td>ts 29</td>
<td>R8</td>
<td>F  F  K  F  F  F  F</td>
</tr>
<tr>
<td></td>
<td>R14</td>
<td>F  F  K  F  F  F  F</td>
</tr>
<tr>
<td></td>
<td>R16</td>
<td>F  F  K  F  F  F  F</td>
</tr>
<tr>
<td>ts 131</td>
<td>R40</td>
<td>K  F  K  F  F  F  F</td>
</tr>
<tr>
<td></td>
<td>R46</td>
<td>K  F  K  F  F  F  F</td>
</tr>
<tr>
<td></td>
<td>R48</td>
<td>K  F  K  F  F  F  F</td>
</tr>
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sensitivity of ts 29 is due to a mutation in gene 1. Analyses of ts⁺ recombinants of ts 131 and A/Krasnodar showed that the genomes of three of them (R40, R46 and R48) were composed of RNAs 1 and 2 of A/Krasnodar and RNAs 3 to 8 of ts 131 (see Table 1) and a fourth had RNAs 1, 2 and 3 of A/Krasnodar and RNAs 4 to 8 of ts 131. Since ts 131 and ts 29 mutants complement and recombine with each other (Fig. 1 and 3; Markushin & Ghendon, 1973), it is unlikely that the temperature-sensitive mutations are in the same gene and, since the temperature-sensitive lesion of ts 29 is in gene 1, a mutation in gene 2 would appear to be responsible for the temperature sensitivity of ts 131.

In addition, examinations of the electrophoretic mobilities of virus-specified polypeptides synthesized at 36 °C in cells infected with recombinant or parent virus strains showed that only polypeptide P₃ of recombinants R8, R14 and R16 and polypeptides P₁ and P₃ of recombinants R40, R46 and R48 correspond to polypeptides of A/Krasnodar (Table 2). In view of the genome compositions of these recombinants it is evident that gene 1 codes for polypeptide P₃ and gene 2 for polypeptide P₁.

DISCUSSION

The results of these investigations have shown that in cells infected with mutants ts 29 or ts 131 the synthesis of A(-)cRNA and vRNA is inhibited at non-permissive temperature whereas primary transcription is unaffected. The two mutants are distinguished only by 'secondary' synthesis of mRNA, measured in the presence of cycloheximide. The sensitivity to non-permissive temperature in cells infected with ts 29, in which the ts mutation is in gene 1, indicates that the polypeptide product, P₃, of this gene is involved in secondary mRNA synthesis. It therefore appears from these results that the enzymes responsible for primary and secondary synthesis of mRNA differ in certain respects although it cannot be concluded that polypeptide P₃ is not also involved in primary mRNA synthesis. It has recently been shown directly that P₃ of influenza virus strain WSN functions as a cap-recognizing protein in the initiation of transcription in vitro (Ulmanen et al., 1981). The available information does not demonstrate that the ts mutation in gene 2 of ts 131 is responsible for the in vitro instability of the virion-associated transcriptase (Ghendon et al., 1975). This appears likely, however, in view of the similar properties of a mutant of WSN (Mowshowitz, 1978) and the involvement of P₁ in initiating transcription in vitro (Ulmanen et al., 1981). Results of investigations of a number of ts mutants of WSN have also indicated that viruses with defects in P₃ and P₁, resulting from mutations in RNAs 1 and 2 respectively, fail to direct the synthesis of both vRNA and cRNA in cells at non-permissive temperature (Sugiura et al., 1975; Krug et al., 1975; Palese et al., 1977).

One explanation for the differing sensitivities to non-permissive temperature of mRNA synthesis in the presence or absence of cycloheximide in ts 131-infected cells is that inhibition under normal conditions in these cells is secondary to a defect in vRNA synthesis. This would imply not only that the majority of mRNA is transcribed from vRNA synthesized de novo but also that under normal conditions continuing synthesis of vRNA is required and that newly synthesized vRNA may only be transcribed for a limited period of time. The correlation between the patterns of syntheses of mRNAs and vRNAs during infection (Smith & Hay, 1981, 1982) is consistent with such a mechanism. The continuing synthesis of mRNA in the absence of
concurrent vRNA synthesis, which occurs in virus-infected cells incubated in the presence of cycloheximide or p-fluorophenylalanine or at non-permissive temperature in cells infected with ts mutants having a vRNA⁻ cRNA⁺ phenotype (Ghendon et al., 1975; Scholtissek & Bowles, 1975; Krug et al., 1975; Wolstenholme et al., 1980), may result from the absence of functional proteins required in the 'processing' of vRNA thereby allowing its continued transcription.

The results of 'cycloheximide-reversal' experiments have suggested that, in cells infected by either mutant, synthesis of A(−)cRNA is directly affected at non-permissive temperature and, therefore, that both polypeptides P₁ and P₃ are components of the enzyme responsible for synthesis of this class of cRNA (although the cap-recognizing function of P₃ does not appear to be required), and furthermore that this enzyme is distinct from that involved in mRNA synthesis. The stimulation in synthesis immediately following cycloheximide reversal to a level similar to maximum synthesis under normal infection conditions suggests that unpolyadenylated cRNA is transcribed predominantly from the input genome of infecting virus particles, a conclusion also indicated by the phenotypes of two mutants with ts mutations in gene 8 (Wolstenholme et al., 1980). Although it is evident from these results that synthesis of virus polypeptides is required for synthesis of unpolyadenylated cRNA, it cannot be deduced from the available data whether de novo synthesis of the two polypeptides P₁ and P₃ is required or whether they are already present as components of the input virus ribonucleoprotein.

It is not possible at this stage to distinguish between direct inhibition of vRNA synthesis at non-permissive temperature and inhibition resulting from the cessation of synthesis of unpolyadenylated cRNA, its putative template. However, since the latter is produced in much lower amounts during infection and since maximum synthesis precedes that of vRNA it is probable that vRNA synthesis does not depend on continued synthesis of its template and, therefore, that cessation of vRNA synthesis following shift to non-permissive temperature is a consequence of the temperature sensitivity of the RNA replicase.

Finally, of some interest is the observation that, of four ts⁺ recombinants of ts 131 and A/Krasnodar/101/59, three inherited genes 1 and 2 and a fourth genes 1, 2 and 3 of the latter virus. This may reflect a partial 'linkage' of these genes during recombination, as observed by Lubeck et al. (1979), and indicates their functional relatedness although the apparent association is unilateral in that ts⁻ recombinants of ts 29 inherit only gene 1 of the wild-type virus.

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REFERENCES


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