Dependence of RNA Replication on Continuous Protein Synthesis in a Temperature-sensitive Mutant of Foot-and-Mouth Disease Virus

(Accepted 4 July 1974)

SUMMARY

A temperature-sensitive mutant of foot-and-mouth disease virus (FMDV) of the RNA− phenotype induces active RNA dependent RNA polymerase only at the permissive temperature. Once induced this enzyme is active when assayed under cell-free conditions, even at the non-permissive temperature. However, when the enzyme is induced at the permissive temperature and the cultures are shifted to the non-permissive temperature, a sharp decline in activity is observed. The RNA− character can be phenocopied by blocking protein synthesis. The evidence obtained suggests that virus RNA replication is dependent on continuous production of the enzyme.

In a previous communication (Manor & Goldblum, 1973) we described a temperature-sensitive mutant ts-8 of FMDV, its origin and its growth under various thermal conditions. This mutant, of an RNA− phenotype, grows almost normally at 30 °C while at 38 °C over 90 % inhibition of its RNA synthesis takes place. Moreover, thermal shift-up from 30 to 38 °C at various times during active RNA synthesis results in a rapid arrest of this process. This effect may be caused by various types of lesions: (a) the virus-directed RNA polymerase (replicase) is inactivated by the elevated temperature, as for some mutants of vesicular stomatitis virus (Szilagyi & Pringle, 1972); (b) there is an in vivo degradation of the RNA template or progeny, as in poliovirus mutants (Fiszman et al., 1970; Adler et al., 1973); (c) the synthesis of the virus RNA polymerase is inhibited at the restrictive temperature, as suspected by Delagneau (1970).

This report presents evidence that the production of active replicase by the ts-8 mutant is impaired at the restrictive temperature. In addition, evidence is presented that the synthesis of FMDV RNA may be closely dependent on a continuous production of the polymerase.

The BHK 21 line of hamster cells was used for all experiments, as well as for the production of various FMDV stocks. Monolayer cultures of these cells were grown in Eagle's modified medium supplemented with 5 % foetal calf serum, in air with 5 % CO₂. Serum was omitted during virus infection. The growth, harvest and concentration of the SAT-1 (wild type) and ts-8 (thermosensitive mutant) viruses have been described (Manor & Goldblum, 1973).

The activity of virus directed RNA polymerase (replicase) was determined essentially according to Lazarus et al. (1972). At various times after infection, FMDV-infected cells were washed with phosphate-buffered saline and lysed in the cold using a solution containing the following: 0.25 M-sucrose, 10 mM-tris-HCl buffer, pH 7.8, 0.1 mM-EDTA, 1 mM-DDT and 0.5 % NP-40 (Nonidet, P40, Shell, Israel). Nuclei and unaffected cells were sedimented at 3000 rev/min for 5 min. The pellets were discarded, and the cell-free cytoplasmic extracts used for determination of enzymatic activity. Reaction mixtures contained the following in 100 μl final volume: 10 mM-tris-HCl, pH 8.1, 0.2 mM-KCl,
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Fig. 1. Induction of FMDV RNA-polymerase. BHK cells were infected with FMDV in the presence of 1.5 μg actinomycin D/ml and incubated at the appropriate temperature. Infected cultures were withdrawn at hourly intervals; the cells were lysed by 0.5% NP-40, the nuclei spun out and cytoplasmic extracts were assayed for RNA-polymerase induction by the incorporation of [3H]-UTP into acid-insoluble material, during 30 min of incubation. (a) Cells infected with mutant ts-8 virus. •—•, induction of RNA-polymerase at 30 °C; ○—○, induction of RNA-polymerase at 38 °C. (b) Induction of RNA polymerase in cells infected with SAT-1 virus. •—•, infected cells incubated at 38 °C; ○—○, uninfected cells incubated at 38 °C. The in vitro assay was performed at 38 °C.

20 mM-MgCl₂, 2 mM-2-mercaptoethanol, 2 mM-phosphocreatine, 5 μg phosphokinase, 80 μM each of ATP, GTP and CTP, 1.8 μM-[3H]-UTP (sp. act. 11 Ci/mmol, The Radiochemical Centre, Amersham, England) and 50 μl of cytoplasmic extracts containing replicase and endogenous virus RNA template. Standard reaction mixtures were incubated for 30 min, and the reaction terminated by the sequential addition of pyrophosphate to 0.01 M and TCA to 10%. Acid precipitated material was collected on Whatman GF/C filters, washed with 5% TCA and acetone, air dried and the radioactivity determined in a liquid scintillation spectrometer using a toluene based fluid.

Confluent cultures of BHK cells on 60 mm plates were infected with mutant virus ts-8 at 100 to 500 p.f.u./cell in the presence of 1.5 μg/ml of actinomycin D (Merck, Sharp & Dohm). After 30 min adsorption at 30 or 38 °C, fresh medium was added and incubation continued at the permissive or restrictive temperatures. The actinomycin D level was maintained through all stages in all experiments. At hourly intervals duplicate cultures were withdrawn, rinsed and lysed (final vol. 0.5 ml per plate). Cytoplasmic extracts were then assayed for replicase activity. As shown in Fig. 1(a), polymerase activity was evident only in extracts from cells incubated at the permissive temperature. Activity appeared at the third hour after infection and was maximal 3 to 4 h later. In contrast, no activity was detected in extracts from cultures incubated at 38 °C. A parallel set of cultures was similarly infected with the SAT-1 strain of virus and incubated at 38 °C. Here enzymic activity appeared 1 h after infection and reached a maximal level 2 to 3 h later. Uninfected control cultures showed no activity (Fig. 1b).

A possible cause for the thermosensitivity of the ts-8 mutant could have been the formation of a thermolabile polymerase. To test this hypothesis we measured the enzymic activity...
under cell-free conditions both at the permissive and non-permissive temperatures. Cultures were infected with ts-8 at 30 °C. Samples were withdrawn at various times, lysed and the cytoplasmic extracts assayed for polymerase activity at 30 and 38 °C. We found that the level of [aH]-UTP incorporation was not reduced when the reaction took place at 38 °C (Fig. 2). In fact, the elevated temperature caused some increase in the amount of [aH]-UMP incorporated. Thus, once induced at 30 °C, the polymerase activity was virtually unaffected by the non-permissive temperature.

These results are in conflict with the observed arrest of RNA synthesis when shift-up experiments are carried out with intact cells (Manor & Goldblum, 1973). Apparently, although the enzymic activity is insensitive to incubation at 38 °C under cell-free conditions, it is affected by the restrictive temperature while ‘contained’ in the intact cells. To test this hypothesis, we infected cells with the ts-8 mutant at 30 °C. After the induction of the enzymic activity, the infected cells were shifted up to the non-permissive temperature. At various times thereafter, samples were lysed and the cytoplasmic extracts assayed for polymerase activity at 30 °C. In this experiment we observed a sharp decline in enzymic activity (Fig. 3), and no activity could be detected at 1 h after the shift-up.

These results indicate the following: (a) ts-8 induced replicase, once formed, is active at the non-permissive temperature; (b) the ts-8 induced replicase activity falls-off upon a shift-up of the infected cells to the non-permissive temperature; (c) if incubated from the onset of infection at the non-permissive temperature, no replicase induction is apparent in the ts-8 infected cells; (d) overall ts-8 RNA synthesis is inhibited upon shift-up of infected cells to
the non-permissive temperature (Manor & Goldblum, 1973). These findings suggest that the thermosensitivity of ts-8 is not due to a thermolabile replicase, but is due to the inability to form active enzyme at the non-permissive temperature. If this assumption is correct, then the observed inhibition of RNA synthesis at 38 °C, as well as the decay of enzyme activity at that temperature, may suggest that the virus-induced replicase must be synthesized continuously during infection, so as to allow production of normal levels of virus RNA. This conclusion can be tested since, if true, the synthesis of ts-8 RNA would be inhibited even at the permissive temperature, in a manner similar to a thermal shift-up, as a consequence of protein synthesis inhibition. This prediction was confirmed in the following experiment.

Cultures of BHK cells were infected with ts-8 at 100 p.f.u./cell in the presence of actinomycin D. After 30 min adsorption, the cultures were labelled with 2 μCi/ml of [3H]-uridine, (27 Ci/mmol) and incubation continued at 30 °C. Cycloheximide at 100 μg/ml was added to the cultures at 0, 5 or 6 h. Duplicate cultures from sets receiving the various treatments, as well as sets without cycloheximide, were withdrawn, lysed and precipitated with TCA. As seen in Fig. 4, the incorporation of [3H]-uridine was inhibited whenever cycloheximide was added. Similar results were obtained in cells infected with SAT-1 virus (data not shown).

Our observations seem to indicate that the synthesis of FMDV-RNA is dependent upon continuous synthesis of an active replicase. The RNA− character of the ts-8 mutant may be due to the inability to produce active replicase at the non-permissive temperature. These results imply that the active enzyme has a short half-life, and that inactive molecules are the waste-product of virus RNA synthesis.
Fig. 4. Effect of cycloheximide on RNA synthesis at 30 °C. Cells were infected with ts-8 in the presence of 1.5 μl/ml actinomycin D and incubated at the permissive temperature. RNA synthesis was measured by incorporation of [3H]-uridine (2 μCi/ml) into TCA precipitable material.

-•-, untreated cultures; ○---○, cultures with 100 μg/ml cycloheximide at zero time; △-△, cycloheximide (100 μg/ml) added at the 5th or 6th hour.

This research was aided generously by the Chanock Foundation and a Research Grant No. A 10-ADP-13 (FG-Is-258) from the United States Department of Agriculture. One of us (R. B.) is indebted to Messrs Omry and Nimrod for stimulating discussion.

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(Received 15 February 1974)