The Relation of the DNA Polymerase to an Early Temperature-sensitive Event in the Replication of Variola Virus

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SUMMARY

An early temperature-sensitive event which prevents the replication of variola-virus DNA in HeLa cells at 40° was studied in 'temperature-shift' experiments using 5-fluorodeoxyuridine or 5-bromodeoxyuridine. 'Shift-up' from 35° to 40° suppressed the replication of virus DNA, rapidly halting it even after it had begun at 35°. 'Shift-down' from 40° to 35° permitted the replication of virus DNA after a delay of not more than 2 hr. These results were confirmed in studies of the incorporation of [3H]thymidine into DNA in the cytoplasmic fraction. It was also shown that the incorporation which followed 'shift-down' from 40° could be inhibited by adding p-fluorophenylalanine at the time of shift. When cytoplasmic fractions from cells incubated at different temperatures were examined for enzyme activities, there was a marked increase in thymidine kinase activity in infected cells both at 35° and at 40°. DNA polymerase activity was increased five- to sixfold in infected cells at 35° but was not increased at 40° or in the presence of p-fluorophenylalanine. The increased polymerase activity in infected cells at 35° was unstable at 40° both in vivo and in vitro, in contrast to its greater stability at 35°. The behaviour of this enzyme was thought to explain the observed temperature-sensitivity of the replication of variola virus DNA.

INTRODUCTION

The growth of variola virus in HeLa cells is temperature-sensitive and completely inhibited at 40° (Cruickshank & Bedson, 1968). The inhibition is due to temperature-sensitive events located both early and late in the growth cycle, the early event being probably concerned with the replication of virus DNA. We now report a more detailed investigation of this early event and present evidence that one of the ‘early’ enzymes appearing in virus-infected cells, the DNA polymerase (EC 2.7.7.7), is lacking at the high temperature.

METHODS

Virus, cells, virus assay and virus growth curves. These were described in detail elsewhere (Cruickshank & Bedson, 1968). In brief, one-step growth conditions were obtained in monolayer cultures of 'Bristol' HeLa cells grown to confluence in Petri dishes and infected with the HINDEN strain of variola virus at an input multiplicity of 10 to 20 p.f.u. per cell. Cultures were incubated at 35° or 40° (± 0.2°) in a humidified atmosphere containing about 5% CO₂. Anti-metabolites and radioactive chemicals were added pre-warmed and in small volume so as not to disturb temperature control. Harvests of intracellular virus were made at various intervals after infection and titrated by pock counts in 12-day chick embryos. Growth curves of 'functional' virus
DNA were made by titrating 24 hr yields of infectivity following the addition of either 5-fluorodeoxyuridine (FUdR) or 5-bromodeoxyuridine (BUdR) at various times early in the growth cycle.

Experiments were also made with cells infected in suspension. Cells were detached from the glass with 0.5 mM-EDTA in phosphate-buffered saline (PBS) and stored overnight at 4 °C in medium. They were then resuspended at a concentration of 10^6 per ml. in Eagle’s medium containing 10% calf serum, 0.02M-MgCl_2 and virus at 60 to 80 pk.f.u./cell. After adsorption for 40 min. at 37 °C the cells were washed, resuspended at a concentration of 2.5 x 10^6 per ml. in Eagle’s medium containing 2% calf serum and distributed to 6 cm. diam. Petri dishes, 2 ml. per dish. Subsequent incubation was at 35 °C or 40 °C as already described.

Studies of [3H]thymidine incorporation. At various times after infection or mock infection [3H]thymidine (30 c/m-mole) was added at a concentration of 1 µc/ml. The pulse was ended 30 min. later by adding ice-cold PBS. The cells were detached from the glass by gentle pipetting or with 0.5 mM-EDTA in PBS, washed and fractionated as described below to give whole-cell and cytoplasmic samples. Samples of these (0.1 ml.) were processed on discs of Whatman no. 3 filter paper as described by Regan & Chu (1956) to determine the radioactivity in material insoluble in 5% trichloracetic acid at 4 °C. Counts were made in a Packard ‘Tricarb’ liquid scintillation counter with an efficiency of c. 45%. In presenting data from these experiments the time of the pulse was taken as the mid-point of the 30 min. period.

Cell fractionation. We are grateful to Dr H. G. Klemperer for suggesting the following procedure. The cells, usually 1.5 to 2 x 10^7 per specimen, were washed with PBS, with sucrose buffer (0.25M-sucrose, 0.01M-tris, pH 7.4, 0.004M-2-mercaptoethanol) and with hypotonic buffer (0.01M-tris, pH 7.4, 0.004M-2-mercaptoethanol) and then resuspended in 3 ml. of the latter. After swelling for 10 min. at 4 °C they were broken in a Dounce homogenizer precalibrated for the required number of strokes. One ml. of sucrose was then added and a 1 ml. sample withdrawn for counts of nuclei and unbroken cells and for other determinations (whole-cell fraction). Nuclei were deposited from the remaining 3 ml. by centrifugation at 200 g for 5 min. and the supernatant fluid (cytoplasmic fraction) removed. Cell disruption always exceeded 98% and, in control experiments with either labelled uninfected cells or prelabelled and infected cells, only 2% to 3% of whole-cell radioactivity was recovered in the cytoplasmic fraction.

Enzyme assays. The cytoplasmic fractions of both infected and uninfected cells were stored at −20 °C and enzymes assayed usually within 1 or 2 days of their preparation. No appreciable loss of activity was observed with specimens stored for much longer periods at this temperature. Protein concentrations were determined by the method of Lowry et al. (1951). DNA polymerase activity was assayed by a standard method (Keir & Shepherd, 1965) with modifications suggested by Keir (personal communication). Each assay contained in final volume 0.25 ml.: 5 µmoles of tris+HCl buffer pH 7.5, 15 µmole of KCl, 2 µmole of MgCl_2, 0.1 µmole of EDTA, 1 µmole of 2-mercaptoethanol, 20 µg. of thermally denatured calf thymus DNA, 50 µmole each of dCTP, dGTP, dTTP and [3H]dATP (0.01 µc/µmole) and cytoplasmic fraction containing c. 40 to 60 µg. of protein. Incubation was for 1 hr at 35 °C. The reaction mixtures were then cooled to 0 °C and acidified with perchloric acid—final concentration 0.2N—after adding 400 µg carrier DNA and 4 µmole ATP. The resulting precipitates
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were collected on Teflon-coated glass-fibre filter papers (Fiberfilm, T1200–12R, Joymar Scientific Inc., 401 Broadway, New York, U.S.A.), washed with 0.2N-perchloric acid and then with alcohol, dried, and transferred to vials containing 0.5 ml of m-hyamine hydroxide and 10 ml of scintillation fluid (6 g. of 2,5-diphenyloxazole, 0.12 g. of bis-(4-methyl-5-phenyloxazolyl)-benzene/l. of toluene). The vials were heated at 57° for 10 min., cooled and counted in the liquid scintillation counter with an efficiency of c. 32%. Blank values, obtained by adding enzyme immediately before acidification, were subtracted and specific activities were calculated as μmole of dATP incorporated/mg. protein/hr of incubation at 35°C. Incorporation was linear with respect to time up to 90 min. and to protein concentration over the range 20 to 100 μg./assay. Both uninfected control and virus-induced activities appeared saturated at the primer concentration employed. Thymidine kinase (EC 2.7.1.21) activity was assayed as described by Klemperer et al. (1967), both infected and uninfected preparations being assayed at pH 7.9. Each reaction mixture of final volume 0.25 ml contained 2.1 μmole of [14C]thymidine (0.125 μC), 1.2 μmole ATP, 0.625 μmole MgCl2, 12.5 μmole tris+HCl buffer pH 7.9 and protein from the cytoplasmic fraction—usually from 3 to 30 μg, with infected cells and from 20 to 100 μg. with uninfected cells. Incubation was for 10 min. at 35°C and was terminated by heating in a boiling water bath for 4 min. Blank values were obtained by adding cell extract immediately before boiling. After centrifugation at 2000 g for 10 min. to sediment denatured protein, 0.05 ml volumes were applied to strips of DEAE-cellulose paper (Whatman Chromedia DE 81) and residual labelled thymidine separated from the phosphorylated product by descending chromatography for 2 hr in mM-ammonium formate. When dry, portions corresponding to the region of the origin were cut from the strips and placed in vials containing 12 ml of scintillation fluid. Counts were made in the liquid scintillation counter with an efficiency of c. 75% and specific activities calculated after subtraction of blanks and expressed as μmole of thymidine phosphorylated/mg. protein/10 min. incubation at 35°C. The reaction was linear with respect to protein concentration over the range 0 to 40 μg./assay with an infected cell extract of high specific activity.

Materials. FUDR, a gift from Roche Products Ltd, Welwyn Garden City, Herts., was used at a final concentration 10−5M. BUdR, used at a final concentration 10−4M, and p-fluorophenylalanine (FPA), used at a concentration of 100 μg./ml. in medium containing 6 μg./ml. phenylalanine, were both obtained from Koch–Light Laboratories Ltd, Colnbrook, Buckinghamshire. Puromycin dihydrochloride, obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, was used at a concentration of 15 μg./ml. [3H]dATP (3.8 c/m-mole) and initial supplies of dTTP, dGTP and dCTP were a gift from Dr H. M. Keir. Calf thymus DNA (Type I grade) and additional quantities of the non-radioactive triphosphates were obtained from Sigma (London) Chemical Co. Ltd. [14C]thymidine (59 mc/m-mole) and [3H]thymidine (3 c/m-mole) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

The effects of ‘temperature-shift’ on the growth of ‘functional’ virus DNA

We have shown previously that, when the growth of variola virus DNA is followed in ‘functional’ terms using either BUdR or FUDR (Salzman, 1960), production at 35°C increases steeply from 4 hr to give values equivalent to about 30 pk.f.u./cell at 8 hr,
whereas at 40° there is virtually no increase above the base line (Cruickshank & Bedson, 1968). It was therefore possible to examine the timing of the relevant temperature-sensitive events by determining the effects of 'temperature-shift' on the

Fig. 1. The growth of variola virus 'DNA' (24 hr titres after adding BUdR at the times plotted) in cultures at 35° (○—○) and at 40° (●—●), and in cultures 'shifted-up' from 35° to 40° at 4 hr (○—■) and at 6 hr (○—△).

Fig. 2. The growth of variola virus 'DNA' in experiments using BUdR (24 hr titres after adding inhibitor at the times plotted) in cultures at 35° (○—○) and in cultures 'shifted-down' from 40° to 35° at 2 hr (▲—△), at 4 hr (■—■) and at 6 hr (▼—▼).

Fig. 3. The growth of variola virus 'DNA' in experiments using FUdR (24 hr titres after adding inhibitor at the times plotted) in cultures at 35° (○—○) and in cultures 'shifted-down' from 40° to 35° at 4 hr (■—■), at 5 hr (▼—▼) and at 8 hr (▲—△).
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growth of ‘functional’ virus DNA. The basic premise of these experiments was that the amount of ‘functional’ DNA present in cultures at any point could be determined by adding BUdR or FUdR and incubating subsequently at 35°C.

In experiments in which cultures were ‘shifted-up’ from 35°C to 40°C at various times after infection, ‘DNA’ growth could be completely suppressed by shifts as late as 4 hr after infection, and even when ‘DNA’ growth was in progress at 35°C the process was rapidly halted by shift to 40°C (Fig. 1). In the latter situation, control cultures to which inhibitor was added at the time of ‘shift-up’ showed that the period at 40°C did not affect the expression of ‘DNA’ already synthesized at 35°C before the shift. Similar results were obtained with both FUdR and BUdR.

By contrast, the results of the ‘shift-down’ type of experiment were found to depend upon which of the inhibitors was used. With BUdR (Fig. 2) it appeared that only the first 2 hr after infection was insensitive to 40°C and that ‘shift-down’ at 4 or 6 hr was followed by a delay of 2 hr before the beginning of ‘DNA’ growth. With FUdR (Fig. 3) the first sensitive event appeared to be much closer to the start of virus ‘DNA’ growth and ‘shift-down’ at 4, 5 or 6 hr was followed almost at once by apparent ‘DNA’ growth. The difference in the pattern of results shown in Fig. 2, 3 was consistent and repeated in several further experiments.

**Studies of [3H]thymidine incorporation**

Because of the conflict in results and the necessarily indirect information given by experiments with BUdR and FUdR, a more direct approach to the question of virus DNA synthesis was required. Observations were therefore made of the cytoplasmic incorporation of [3H]thymidine in infected cells pulsed for 30 min. at various times after infection and under different conditions of incubation. These experiments were made with cells infected either in monolayers or in suspension. Although the over-all pattern of result was similar under the two conditions, our data are chiefly taken from infections made in suspension for these gave reduced background rates of cell DNA synthesis and a more synchronized synthesis of virus DNA. However, in relation to our other data, it should be noted that infection in suspension proceeded more rapidly and virus DNA synthesis was usually found as early as 3 hr after infection.

The results of straightforward incubation at 35°C and 40°C (Fig. 4) showed substantial incorporation at 35°C maximal at 6 hr, and the absence of any detectable activity at 40°C. Although not the point of primary interest in these experiments, and although no special measures were taken to reduce cytoplasmic contamination of nuclei, a clear indication of the effect of infection on host cell DNA synthesis emerged. Both at 35°C and at 40°C rates of nuclear incorporation, obtained by subtracting cytoplasmic counts from whole cell counts, decreased progressively after infection (Table 1). The slight stimulation of DNA synthesis seen in control cultures at 40°C in this experiment was a common but not invariable finding.

When ‘temperature-shifts’ were applied 5 hr after infection (Fig. 5), ‘shift-up’ from 35°C to 40°C resulted in a rapid halt in cytoplasmic incorporation as expected from the findings using BUdR or FUdR. ‘Shift-down’ from 40°C to 35°C was followed by the onset of activity but the rate of incorporation was not greatly increased until 2 hr later. This finding is more in keeping with the results obtained in the experiments with BUdR (Fig. 2) than those with FUdR (Fig. 3). The shorter delay period seen in Fig. 5 was probably due to the changed method of infection, for a longer delay was observed.
when the experiment was repeated using monolayer cultures. Figure 5 also shows the requirement for synthesis of an FPA-sensitive protein at 35 °. Cultures which received FPA when 'shifted-down' from 40 ° at 5 hr showed no increase in the rate of cytoplasmic incorporation in the period up to 8 hr.

![Graph 4](image)

**Fig. 4.** Rates of [³H]thymidine incorporation in the cytoplasm of infected cells: ○—○, at 35 °; ●—●, at 40 °.

**Fig. 5.** Rates of [³H]thymidine incorporation in the cytoplasm following 'temperature-shifts' at 5 hr: ○—△, 'shift-up' from 35 ° to 40 °; ●—△, 'shift-down' from 40 ° to 35 °; ●—□, 'shift-down' from 40 ° to 35 ° with addition of FPA.

**Table 1.** Rates of nuclear incorporation of [³H]thymidine in monolayer cultures of HeLa cells incubated at 35 ° or 40 ° following infection with variola virus

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Time (hr)</th>
<th>35 °</th>
<th>40 °</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>8</td>
<td>1925 (100)*</td>
<td>3149 (100)*</td>
</tr>
<tr>
<td>Infected</td>
<td>3</td>
<td>1485 (79)</td>
<td>1946 (62)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>439 (23)</td>
<td>428 (14)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>87 (5)</td>
<td>109 (35)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>116 (5)</td>
<td>71 (2)</td>
</tr>
</tbody>
</table>

* The figure in parenthesis is the percentage of the uninfected control value.

'Early' enzymes in virus-infected cells at 35 ° and 40 °

The preceding results focused attention on the 'early' enzymes which appear to be concerned with the replication of pox-virus DNA (Joklik, 1966). The activities of two of these, the DNA polymerase and the thymidine kinase, were examined in cytoplasmic extracts of cells from the different conditions of incubation.

**DNA polymerase.** In infected cells maintained at 35 ° there was a five- to sixfold increase in DNA polymerase activity, much of the increase taking place between 2 and 5 hr after infection (Fig. 6). In other experiments this increase was found to require new protein synthesis, being prevented by the addition of either puromycin or FPA 1 hr after infection. In infected cells at 40 °, activity was not increased above control
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Fig. 6. DNA polymerase activity in cytoplasmic extracts of infected cells: O—O, at 35°; •—•, at 40°; O—A, 'shift-up' from 35° to 40° at 4½ hr; •—A, 'shift-down' from 40° to 35° at 4½ hr; □, uninfected cells at 35°; ■, uninfected cells at 40°.

Fig. 7. DNA polymerase activity in cytoplasmic extracts of infected cells: •—•, at 35° after adding puromycin (15 μg./ml.) at 4½ hr; O—O, at 40° following 'shift-up' from 35° at 4½ hr; ----, level of activity expected from uninfected cells. The results are expressed as a percentage of the specific activity present at 4½ hr.

Fig. 8. The thermostability of virus-induced DNA polymerase in vitro: O—O, at 35°; •—•, at 40°; ----, level of activity expected from uninfected cells.

Fig. 9. Thymidine kinase activity in cytoplasmic extracts of infected cells: O—O, at 35°; •—•, at 40°; □, uninfected cells at 35°; ■, uninfected cells at 40°.
levels in uninfected cells at 35° or 40°, although these all rose slightly over the course of the experiment. When cultures which had been incubated for 4½ hr at 35° were 'shifted-up' to 40°, the increased activity which had been produced at 35° was lost. On the other hand, cultures 'shifted-down' from 40° to 35° at 4½ hr rapidly developed the increased activity associated with infection at 35°.

A comparison was made of the stability of the virus-induced enzyme in vivo at 35° and 40° (Fig. 7). Its stability at 35° was determined in cultures to which puromycin was added at 4½ hr, and at 40° in cultures 'shifted-up' to 40° at 4½ hr. Although the enzyme was not completely stable in vivo at 35°, there was a much more rapid loss of activity at 40°. When an infected-cell extract (protein concentration 2.2 mg./ml.) was incubated at 40° in vitro a similar rate of inactivation was obtained (Fig. 8), whereas incubation at 35° for up to 3 hr produced no significant change in activity.

Table 2. DNA polymerase activity of infected cell extracts from 35° and 40° assayed separately and mixed together in equal parts

<table>
<thead>
<tr>
<th>Specimen (protein concentration)</th>
<th>Volume</th>
<th>Counts*</th>
<th>Expected count</th>
</tr>
</thead>
<tbody>
<tr>
<td>35° 6 hr after infection (2.2 mg./ml.)</td>
<td>0.05 ml.</td>
<td>26,093</td>
<td>—</td>
</tr>
<tr>
<td>40° 6 hr after infection (1.7 mg./ml.)</td>
<td>0.05 ml.</td>
<td>4,052</td>
<td>—</td>
</tr>
<tr>
<td>35° 6 hr + 40° 6 hr</td>
<td>0.025 ml. of each</td>
<td>15,264</td>
<td>15,072</td>
</tr>
</tbody>
</table>

* Ten min. counts after blank subtraction.

The failure to find increased enzyme activity in infected cells at 40° could have been due either to the presence of an inhibitor or the failure to produce an activator of some pre-existing enzyme. In either case bizarre results would be expected in an assay made with a mixture of infected-cell extracts from 35° and 40°. However, when this was tested (Table 2) the activity of the mixture was almost exactly that expected from the separate activities of its constituents.

Thymidine kinase. In infected cells at 35° thymidine kinase activity increased 50- to 100-fold, maximum activities being reached by about 5 hr after infection (Fig. 9). At 40° the increase in activity over the first 3 hr was the same as at 35°, but thereafter it was slower and by 7 hr the activity was only 50% of that at 35°.

DISCUSSION

The experiments with FUdR and BUdR gave only indirect information about the replication of virus DNA and it was essential to verify the conclusions by other means. We relied principally on the incorporation of [3H]thymidine into DNA in the cytoplasm of fractionated cells, a method first introduced for vaccinia virus by Joklik & Becker (1964), but observations of the effect of 'temperature-shift' on the development of cytoplasmic DNA inclusions stained by acridine orange gave closely similar results. The only discrepancy in all these findings was that observed between 'shift-down' experiments with FUdR and those with BUdR. This can probably be explained by the action of FUdR as an inhibitor of thymidylate synthetase (Cohen et al. 1958), whereas BUdR competes for incorporation into abnormal DNA (Easterbrook & Davern, 1963). Our findings suggest, therefore, that at 40° there is neither DNA synthesis nor accumulation of thymidine nucleotides and that return to 35° is followed first by an increase in the pools of these precursors and only later by their incorporation
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into DNA. Be this as it may, the point does not affect the main conclusions of this section of the work, which are: (1) the replication of variola-virus DNA is temperature-sensitive and does not take place at 40°; (2) an FPA-sensitive protein must be synthesized at the permissive temperature before DNA replication starts; (3) DNA replication which has begun at 35° remains immediately sensitive to the imposition of high temperature; and (4) infection with variola virus, whether at 35° or 40°, produces a marked depression in the rates of host-cell DNA synthesis.

The depression of host-cell DNA synthesis is similar to that observed with vaccinia virus, and the situation at 40°, where this can be studied without the intervention of virus DNA synthesis, clearly resembles that produced by ultraviolet-inactivated virus (Joklik & Becker, 1964; Jungwirth & Launer, 1968).

The sensitivity to FPA of the temperature-sensitive step is of interest for we have argued elsewhere (Bedson & Cruickshank, 1968) that two protein functions known to be required for synthesis of virus DNA (Kates & McAuslan, 1967) differ in this respect and that sensitivity to FPA in the context of our experiments indicates involvement of an enzyme function. Unfortunately we have not been able to devise experiments to determine whether or not the stoichiometric function is also temperature-sensitive.

Our results suggest that thymidine kinase activity, which does not in any case appear necessary for virus replication (Dubbs & Kit, 1964), is not involved in temperature-sensitivity. The virus-induced DNA polymerase, on the other hand, could well account for the observed failure in replication of virus DNA, although the participation of other temperature-sensitive functions is not excluded. The importance of the DNA polymerase for virus replication has been most clearly established for the bacteriophages T4 and T5 in work with conditional lethal mutants (de Waard, Paul & Lehman, 1965).

Our evidence as to the virus-specificity of the variola-induced enzyme is reinforced by the observations of Kirn & Guir (1968, personal communication), who have found vaccinia virus to induce DNA polymerase activity at 41°, even though the 'cold' mutant employed was unable to replicate its DNA at this temperature. These findings coupled with earlier indications of the chemical and immunological specificity of the enzyme induced by vaccinia virus (Jungwirth & Joklik, 1965; Magee & Miller, 1967) make it highly probable that the DNA polymerase of pox viruses is specified by the virus genome. Alternative explanations involving modification of a pre-existing host enzyme, such as has been demonstrated by Chrispeels et al. (1968), seem unlikely but cannot at present be excluded.

The replication of the DNA of the larger animal viruses is clearly a complex process and the defect found at 42° with the herpes virus of infectious bovine rhinotracheitis appears to differ from those so far encountered with pox viruses. In this instance an initial temperature-sensitive step, not apparently involving the DNA polymerase, must take place at 37° but thereafter DNA synthesis can proceed at 42° (Stevens, 1966; Stevens & Jackson, 1967). The relevance of conditional lethal mutants (Fenner & Sambrook, 1964) to the study of these problems is obvious.

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