Growth of Bacteriophage \( \phi X-174 \) at Elevated Temperatures

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SUMMARY

The replication of bacteriophage \( \phi X-174 \) is impaired at temperatures above 40 °C. Mutants (ht) that replicate at high temperature were isolated and partially characterized. Wild-type \( \phi X \) fails to grow at high temperature because, unlike the mutants, it does not make appreciable amounts of single-stranded (ss)DNA. An unusual form of ssDNA, not found in complete virions, is described.

INTRODUCTION

Bacteriophage \( \phi X-174 \) is a small virus whose biological and genetic properties have been extensively described (Denhardt, 1977). This virus exhibits reduced growth at temperatures above 37 °C (Hutchison, 1969). In attempting to ascertain the \textit{in vivo} host requirements for virus DNA synthesis (Dumas & Miller, 1974; Taketo & Kodaira, 1978) synthesis is usually assessed in a \textit{dna} temperature-sensitive (ts) bacterial host at 40 to 42 °C. The results can sometimes be difficult to interpret due to the lability of virus growth at these temperatures. To overcome this problem we have isolated mutants of \( \phi X \) that are capable of growth at temperatures up to 44 °C. This paper describes some characteristics of the mutants and compares mutant and wild-type growth at high temperatures.

METHODS

\textbf{Bacterial and phage strains.} \( \phi X-174 \) wild-type and \( \phi X \ am-3 \) (lysis-defective, gene E) were originally obtained from Dr R. L. Sinsheimer. Stocks were stored at 4 °C. All bacterial strains were obtained from R. L. Sinsheimer and were stored at 4 °C on KC agar slants. \textit{E. coli} C is the standard host for \( \phi X \) wild-type. HF4704 \textit{uvrA}, \textit{T1}, \textit{thy} was used for radioactive labelling experiments. HF4714 \textit{arg}, \textit{his}, \textit{leu}, \textit{thr}, \textit{pro}, Su + was utilized in the plaque assay of \( \phi X \ am-3 \). \textit{E. coli} C-406 \textit{pur}, \textit{arg}, \textit{trp} was used in the preparation of \(^{14}\text{C} \phi X \ am-3 \) DNA.

\textbf{Media and chemicals.} KC broth, top and bottom agar have been previously described (Dowell & Sinsheimer, 1966). KC slants have the same composition as KC bottom agar except that they contain 2 % instead of 1 % agar. The medium TPG-2A has been described by Lindquist & Sinsheimer (1967). When a \textit{thy} strain was employed, 2 \( \mu \)g/ml thymine was added to the medium. \(^{14}\text{C}\)-adenine and \(^3\text{H}\)-thymine were purchased from New England Nuclear, Boston, Mass., U.S.A.

\textbf{Isolation and propagation of high temperature (ht) mutants.} Unmutagenized wild-type \( \phi X \) or \( \phi X \ am-3 \) was plated on \textit{E. coli} C or HF4714 at 42 to 43 °C. At these temperatures,
wild-type phage makes pin-point plaques but an occasional large plaque can be observed. The large plaques were picked and cloned through three separate plaque isolations. I have termed them ht or high temperature mutants and have chosen to study two of them: φX 5 ht, derived from wild-type φX and φX 6 ht am-3 derived from φX am-3.

Lysates of the mutants were usually made by picking a plaque into growing cells (1 × 10⁶ to 2 × 10⁶/ml) in KC broth at 37 °C. After lysis occurred, the debris was collected by centrifugation, resuspended in 0.05 m-sodium tetraborate and stored overnight at 4 °C to allow the phage to elute. The mixture was then recenterfuged (12000 g for 10 min) and the supernate which contained most of the phage was decanted and stored at 4 °C. Lysates made in this manner usually had a titre of 1 × 10¹⁰ to 1 × 10¹¹ p.f.u./ml.

Growth curves and burst size determinations. E. coli C was grown to 1 × 10⁸ cells/ml in KC broth and used for a starvation-synchronized growth curve as described by Denhardt & Sinsheimer (1965). The number of phage released per infected cell (burst size) was calculated from such experiments.

Heat inactivation experiments. Phage, suspended in cold (4 °C) saturated sodium tetraborate were added to the same solution at 56 °C in a Haake circulating water bath. At selected times samples were removed and assayed for surviving virus.

Preparation of ¹⁴C φX am-3 single-stranded marker DNA. E. coli C-406 was grown to 3 × 10⁸ cells/ml in TPG-2A medium containing 6 μg/ml adenine. The cells were starved as described by Denhardt & Sinsheimer (1965) and 60 ml were infected at 37 °C with φX am-3, at a m.o.i. of 3. After 25 min, 49 ml of the infected culture were centrifuged and resuspended in 3 ml of 0.033 m-tris buffer (pH 8.1). A 0.5 ml amount of a solution of lysozyme (2 mg/ml in distilled water) and 0.5 ml of 4% (w/v) EDTA in 0.033 m-tris buffer (pH 8.1) were added and the mixture was shaken for 2 min and then frozen and thawed 6 to 7 times in a dry ice-acetone bath. To this preparation of lysed cells 4 ml of 4% (w/v) EDTA in 0.033 m-tris, pH 8.1 was added with shaking. The debris was pelleted by centrifugation. (12000 g, 5 min) and the supernatant fluid decanted. A 2 ml amount of 2 m-NaCl was added to the supernate. The mixture was shaken, and 2 ml of a 30% (w/v) solution of carbowax 6000 was added. The mixture was stored at 4 °C for at least 1 h and then centrifuged at 17400 g for 10 min.

The supernate was discarded and the pelleted phage was resuspended in 1 ml of cold (4 °C) saturated sodium tetraborate. The phage was purified by two cycles of CsCl density gradient centrifugation and the DNA extracted by the hot phenol method of Guthrie & Sinsheimer (1963).

Measurement of phage-specific DNA synthesis at elevated temperature. A 30 ml amount of E. coli HF4704 was grown in TPG-2A to 1 × 10⁸ cells/ml at 37 °C. The cells were centrifuged and resuspended in starvation buffer (Denhardt & Sinsheimer, 1965) plus 2 μg/ml thymine and aerated at 37 °C for 75 min. The cells were then treated with mitomycin C (50 μg/ml) for 10 min at 37 °C with no aeration. The cells were subsequently washed twice with starvation buffer and finally resuspended in starvation buffer plus 2 μg/ml thymine. A portion of cells (usually 10 ml) was infected with phage (m.o.i. 3 to 4) at 40 °C and 30 min was allowed for eclipse. The infected cells were centrifuged and resuspended in TPG-2A plus 2 μg/ml thymine and 0.2 μCi ³²H-thymine at 42 or 44 °C. At intervals, 0.1 ml samples were removed, added to 1 ml ice-cold 10% (w/v) trichloroacetic acid (TCA) containing 50 μg/ml thymine and 0.1 ml carrier DNA (denatured salmon sperm DNA at 800 μg/ml). After 30 min the precipitate was collected by filtration on Gelman filters (Metricel-GA6 presoaked in H₂O containing 50 μg/ml thymine) and washed twice with 10 ml of cold H₂O containing thymine (50 μg/ml). The filter was then transferred to a scintillation vial and dried at 40 °C for 30
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Table 1. Burst size of $\phi X$ wild-type and $\phi X$ 5ht at various temperatures*

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Phage 37</th>
<th>40</th>
<th>42</th>
<th>44</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\phi X$ wild-type</td>
<td>234.5</td>
<td>49.7</td>
<td>6.12</td>
<td>0.43</td>
</tr>
<tr>
<td>$\phi X$ 5ht</td>
<td>274.6</td>
<td>189.1</td>
<td>106.6</td>
<td>10.36</td>
</tr>
</tbody>
</table>

* The burst size was determined after starvation synchronization as described in Methods.

RESULTS

Burst size and growth kinetics of $\phi X$ wild-type and $\phi X$ 5ht at various temperatures

The burst size at various temperatures of the mutant 5ht and wild-type $\phi X$ are compared in Table 1. The mutant is capable of larger bursts especially at temperatures above 37 °C. The growth kinetics in a starvation-synchronized infection at 42 °C are shown in Fig. 1. The development of 5ht is accelerated relative to wild-type; both the appearance of mature intracellular phage and the subsequent lysis and release of phage occur much earlier than in a wild-type infection. The growth kinetics of 6ht am-3 are similar to that of 5ht (data not shown).

Heat inactivation of 5ht and 6ht am-3 particles

Both 5ht and 6ht am-3 were subjected to heat inactivation at 56 °C and the results compared with those obtained by heating $\phi X$ wild-type. The results in Fig. 2 affirm the reproducibility of the technique as shown in separate wild-type heat inactivation experiments (Fig. 2a) and illustrate the 5ht and 6ht am-3 are considerably more heat resistant than wild-type $\phi X$ (Fig. 2b).
Fig. 1. The growth of φX sht and φX wild-type at 42 °C. A starvation-synchronized growth curve was carried out as described in Methods. ●—●, sht intracellular phage; ○—○, sht infective centres; ▲—▲, wild-type intracellular phage; △—△, wild-type infective centres.

Fig. 2. Heat inactivation of φX ht mutants and φX wild-type. Experiments were carried out as described in Methods. (a) Separate experiments with wild-type φX: ●—●, expt. 1; ○—○, expt. 2. (b) ht mutants and wild-type φX: ●—●, sht; ▲—▲, sht am-3; ○—○, wild-type.
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Fig. 3. Incorporation of 3H-thymine into infected cells at elevated temperatures. Experiments were carried out as described in Methods. (a) Incorporation at 42 °C; (b) incorporation at 44 °C.

Virus DNA synthesis at 42 and 44 °C

Cells were synchronously infected at 42 and 44 °C with either ϕX am-3 or 6ht am-3 and the uptake of 3H-thymine into TCA-precipitable material measured. The results are presented in Fig. 3. For 10 to 20 min, the incorporation curves at both temperatures are similar for both mutant and wild-type; after that time the amount of acid-precipitable counts increases greatly for 6ht and am-3 with a far less dramatic increase for ϕX am-3-infected cells. The difference between mutant and wild-type is more pronounced at 44 °C. The rate change at 10 to 12 min coincides with the approximate time of onset of single-stranded synthesis. It is known that the synthesis of single-stranded (ss)DNA is an integrated process involving virus structural proteins (Dowell & Sinsheimer, 1966; Iwaya & Denhardt, 1971; Fujisawa & Hayashi, 1977), so the synthesis at 44 °C of the different species of virus DNA was determined.

Analysis of virus DNA synthesized at 44 °C

Cells were infected with either 6ht am-3 or ϕX am-3 at 44 °C and the intracellular virus DNA labelled and analysed on sucrose gradients as described in Methods. The results presented in Fig. 4 typify the sedimentation profiles obtained at early times (5 to 15 min) after infection. The ϕX am-3 profile shows that both RFI (fraction 45) and RFII (fraction 51) are present in infected cells at 44 °C early in infection. The 6ht am-3 early profile reveals the presence of RFI and RFII but also contains a peak fraction 32) sedimenting in advance of the marker for ssDNA.

At later times (50 to 70 min) the differences between the 6ht am-3 and wild-type am-3
Fig. 4. Sucrose gradient analysis of intracellular DNA synthesized from 5 to 15 min after infection at 44 °C. Experiments were carried out as described in Methods. (a) φX wild-type am-3; (b) φX 6ht am-3. ●—●, 3H intracellular DNA; ○—○, 14C φX am-3 single-stranded marker DNA.

Fig. 5. Sucrose gradient analysis of intracellular DNA synthesized from 50 to 70 min after infection at 44 °C. Experiments were carried out as described in Methods. (a) φX wild-type am-3; (b) φX 6ht am-3. ●—●, 3H intracellular DNA; ○—○, 14C φX am-3 single-stranded marker DNA.

Profiles are even more marked, as shown in Fig. 5. The wild-type am-3 gradient contains RFI and RFII and a small amount of material sedimenting in advance of the marker for ssDNA. There is a notable absence of a large amount of ssDNA. In contrast, 6ht am-3 gradients contain large amounts of ssDNA sedimenting faster than the marker DNA. This fast sedimenting material is infective in the spheroplast assay system (data not shown).

Properties of the ssDNA made at 44 °C

6ht am-3 infected cells contain ssDNA of normal sedimentation characteristics when the infection and labelling are carried out at 37 °C (Fig. 6).

Proteolytic enzyme (Pronase) treatment of the fast sedimenting ssDNA failed to change its sedimentation characteristics (data not shown). However, denaturation with alkali or heating and quick-cooling altered the sedimentation properties of the material so that it then co-sedimented with the marker DNA (Fig. 7 and 8).
**Fig. 6.** Sucrose gradient analysis of intracellular DNA synthesized from 50 to 70 min p.i. at 37 °C with \( \phi X 6ht am-3 \). The experiment was carried out as described in Methods except labelling was performed at 37 °C. ●—●, \(^3\)H \( 6ht am-3 \) DNA; ○—○, \(^{14}\)C \( \phi X am-3 \) single-stranded marker DNA.

**Fig. 7.** Alkaline sucrose gradient analysis of \( 6ht am-3 \) DNA. A 0.4 ml amount of the \( 6ht am-3 \) late DNA (Fig. 5b) was combined with 0.04 ml of \(^{14}\)C marker DNA and treated with 0.07 ml 1 M NaOH for 15 min at room temperature. A 0.15 ml amount of the treated sample was then layered on a 5 to 20% sucrose gradient containing 0.05 M Na3PO4 (pH of gradient, 12.4), centrifuged and collected as described in Methods. ●—●, \(^3\)H \( 6ht am-3 \) DNA; ○—○, \(^{14}\)C \( \phi X am-3 \) single-stranded marker DNA.

**Fig. 8.** Sucrose gradient analysis of heat-denatured \( 6ht am-3 \) DNA. \( 6ht am-3 \) late DNA (Fig. 5b) and \(^{14}\)C marker DNA were heated separately at 100 °C for 10 min and then quickly cooled by immersing the tubes in ice water. The samples were combined and 0.15 ml was layered on a sucrose gradient and analysed as described in Methods. ●—●, \(^3\)H \( 6ht am-3 \) DNA; ○—○, \(^{14}\)C \( \phi X am-3 \) single-stranded marker DNA.
Fig. 9. Sucrose gradient analysis of phenolated and unphenolated late 6ht am-3 DNA. HF4704 cells (40 ml) were infected with 6ht am-3 at 44 °C as described in Methods. After 30 min 0.8 mCi 3H-thymine was added to the infected culture. At 50 min the culture was split into 2 to 20 ml portions and washed twice in cold starvation buffer. The cells were collected by centrifugation and both portions were lysed as described by Dowell & Sinsheimer (1966). The DNA from one portion was phenol-extracted and precipitated as described by Dowell & Sinsheimer (1966). Then 10 μl of 14C marker DNA and 0.15 ml of the 6ht am-3 DNA were mixed, layered on sucrose gradients, centrifuged and the radioactivity counted as described in Methods. (a) Phenol-extracted DNA; (b) unphenolated DNA. ●—●, 3H 6ht am-3 intracellular DNA; ○—○, 14C φX am-3 single-stranded marker DNA.

The fast-sedimenting material is not contained within mature phage particles

Although 6ht am-3 cells synthesized a large amount of fast-sedimenting virus DNA at 44 °C, the yield of infective virus was small (less than 20 p.f.u. in a lysis-inhibited Su- cell). To determine whether the fast-sedimenting material was contained in complete virions, a sample of infected cells was lysed and layered directly on a sucrose gradient without prior phenol treatment to remove virus proteins. The results of such an experiment, which includes a phenol-treated control, are shown in Fig. 9. The sedimentation profiles are essentially identical although the cells which were not phenol-treated contain a large amount of unincorporated 3H-thymine which is found at the top of the gradient. It seems evident that the majority of the radioactivity is not contained in complete phage particles which would have sedimented much further down the gradient.

DISCUSSION

The synthesis of ssDNA in φX-infected cells is integrated with the assembly of the virion and requires the function of at least six phage-coded genes including several virus structural proteins (Dowell & Sinsheimer, 1966; Iwaya & Denhardt, 1971; Fujisawa & Hayashi, 1977). The results presented here indicate that at temperatures above 40 °C this
process is disrupted, with a resulting impairment in the synthesis of ssDNA, but show that it is possible to isolate mutants which overcome this block. The mutations which allow DNA synthesis at elevated temperatures are probably in a gene that determines a capsid structural protein. Alternatively, the mutations could be in genes that function in the assembly of the virus but which are not components of the final virus structure. Pollock et al. (1978) have shown that there are multiple products of certain phage genes, in particular the G-protein, which is a part of the virus capsid. It may be that the ht mutation selects for the inclusion of certain of these forms in the capsid, resulting in a more heat-stable virus. At present the location of the ht mutation is not known nor whether the mutations in 5ht and 6ht am-3 are in the same gene. Electrophoretic analysis of the virus-coded proteins might be useful in resolving these questions.

Although the high temperature mutation permits the synthesis of ssDNA, most of this DNA is found in a form which is not packaged into mature virions (Fig. 9). The small amount of wild-type ssDNA synthesized at 44 °C also has a similar form, as judged by sedimentation (Fig. 5). The most likely explanation for the sedimentation behaviour of the single-stranded material made by 6ht am-3 infected cells at 44 °C is that the virus DNA synthesized at 44 °C has a more compact structure, probably due to a more extensive secondary structure than is found in such DNA synthesized at lower temperatures. While the virus DNA sediments identically, regardless of phenol treatment (Fig. 9), there is a possibility that it was associated with a subviral complex (Fujisawa & Hayashi, 1977) which was disrupted by the artificial lysis of the infected cells or with a protein that is not removed by the phenol treatment. It is most unusual for intracellular φX virus DNA to be found apparently unassociated with a subviral complex.

Espejo & Sinsheimer (1976) have shown that φX infection is abortive at 15 °C due to the production of heterogeneous size fragments of ssDNA. Clearly, infection of cells with φX at both high and low temperatures disturbs the integration between virus assembly and single-stranded synthesis.

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REFERENCES


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