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Process of Infection with Bacteriophage φX174

XXIV. New Type of Temperature-sensitive Mutant

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A group of temperature-sensitive mutants of φX174 has been isolated which can go through a single, normal one-step growth cycle at 40 C but fail to form plaques at this temperature. Such mutants fail to initiate a second cycle at 40 C; however they can gain the capacity to infect at 40 C, upon incubation for 10 min in broth at 30 C. In regaining the ability to infect, the phage appear to undergo a temperature-dependent conformational alteration. The inverse process, a reversible loss of ability to infect at 40 C, is observed when such phage produced at 30 C are incubated for 2 hr at 40 C. The defect in initiation of a second cycle of infection appears to be in the injection of viral deoxyribonucleic acid. A two-step complementation test has been used to identify the cistron coding for the affected function. Such mutants are also unusually sensitive to an irreversible thermal inactivation when incubated at 40 C.

The characteristic phenotypic expression of temperature-sensitive mutations in genes controlling the structure of a bacteriophage is the absence of infective progeny virus in cultures at the restrictive temperature (2). We have isolated a group of temperature-sensitive mutants of φX174 (6) which, in a one-step growth experiment under the restrictive conditions (40 C), produce a normal burst of viable progeny phage but do not form plaques at 40 C. The properties of a representative of this class of mutants are described and the affected function is partially characterized.

MATERIALS AND METHODS

Media. KC broth, bottom agar, and top agar have been described (4).

Escherichia coli strains. C is the usual host of φX used in this laboratory. C600.5 is a λ-sensitive strain of *E. coli* to which φX does not attach. X is a suppressor-containing strain (Su$^+$) of *E. coli*.

φX174 strains. *Wt* is the φX wild-type (6). Amber (*am*) mutants of φX were obtained from C. Hutchison; the designation of *am* mutants to particular cistrons is from Sinsheimer et al. (7).

Temperature-sensitive (*ts*) mutants of φX were selected by the following procedure. *Wt* φX was treated with nitrous acid (*p*H 4) for sufficient time to decrease the titer 10^3-fold. The mutagenized stock was used to infect *E. coli* at 30 C in KC broth; after 10 min at 30 C, the growing stock was transferred to 40 C. After lysis, phage were plated out at 30 C and shifted to 40 C when plaques appeared. Small, sharp-edged plaques were selected and spot-tested for temperature sensitivity. Mutants selected for further investigation formed plaques at 30 C but not at 40 C.

Biological assays. Synchronization of φX infection with cyanide was as described previously (1), except that 0.005 M cyanide (final concentration) was used.

Phage attachment was measured by diluting samples 10^3-fold in broth at timed intervals after addition of *E. coli* C; the diluted suspension was cooled to 0 to 4 C, and *E. coli* C600.5 (to which φX does not attach) was added. The suspension was centrifuged at 10,000 rev/min (8,000 × *g*) for 10 min, and the supernatant fluid was assayed for unattached phage.

Phage eclipse was assayed by diluting infected complexes 10-fold into chloroform-saturated broth at 0 C, before dilution for phage assay. This step inactivates φX-infected cells and releases adsorbed but uneclipsed phage. The extent of eclipse was calculated by comparison with a control in which phage were treated identically except that cells were absent.

Wt φX and *tss*6 produced at 30 and 40 C were assayed by plating on *E. coli* C at 30 C. If the plating efficiency of *tss*6 on *E. coli* C at 30 C is taken as 100%, the efficiencies at 37 and 25 C were 65 and 94%, respectively. Phage *tss*6 plated on *E. coli* X at 30 and 25 C with relative efficiencies of 85 and 82%, respectively. In mixed infections such as *tss*6 × *am*9 or *tss*6 × *am*33, *tss*6 progeny were assayed on C at 30 C and amber progeny on X at 40 C.

Growth of *am*16 and *am*10 is temperature-sensitive in the permissive host. Therefore, progeny of mixed infections with *tss*6 were assayed at 30 C on double-layer plates; approximately equal amounts of *E. coli* C in the lower layer and *E. coli* X in the upper layer were used. Plaques of *tss*6 were large and clear; *am* plaques were turbid and smaller.

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Preparation of ^{32}P-labeled phage. *E. coli* C was grown in TPG medium (8) to which was added 2.8 g of special L-amino acid mixture per liter (Nutritional Biochemicals Corp., Cleveland, Ohio). A culture (20 ml, 2 × 10^8 cells/ml) was infected with *tss*6 at 30 C [multiplicity of infection (MOI), approximately 3], and 4.5 mc of carrier-free ^{32}P-phosphate was added. The resultant lysate was centrifuged, and the debris (with which 90% of the phage was associated) was resuspended in 0.05 M sodium borate (*p*H 8.1); the suspension was dialyzed overnight and centrifuged at 10,000 × *g* for 10 min. The supernatant fluid, containing free phage, was centrifuged in a 5 to 20% sucrose gradient containing 0.05 M sodium borate for 3.5 hr at 24,000 rev/min in an SW25 rotor. The most radioactive fractions in the region of free phage were pooled and dialyzed against borate. CsCl was added to the solution (0.625 g/g of solution; Harshaw Chemical Co., Cleveland, Ohio), which was then centrifuged at 4 C for 24 hr at 37,000 rev/min in an SW39 rotor. The most radioactive fractions were again dialyzed against borate, and the centrifugation in CsCl was repeated. A typical ^{32}P-labeled phage preparation contained 4 × 10^{-6} counts per min per plaque-forming unit (PFU).

Preparation of phage stocks. Genetically pure phage stocks were prepared as described (4); these were used in most growth experiments. For heat-inactivation experiments, phage stocks purified as described above for ^{32}P-phage were used.

Preparation of E. coli cell walls. Cell walls were prepared according to the method of Wylie and Johnson (9). An assay of colony formers in the preparation showed 3 × 10^4 viable cells per 10^8 cell equivalents of cell wall preparation.

Formation of spheroplasts and membrane isolation. Approximately 1.5 × 10^8 *E. coli* C cells were washed three times by centrifugation in sodium borate (0.05 M, *p*H 8.1) and then resuspended in a solution of 30% sucrose, 0.15 M NaCl, 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-chloride (*p*H 7.4), and 0.005 M ethylenediaminetetraacetate (EDTA); 0.2 ml of a solution of lysozyme (2 mg/ml) in 0.01 M EDTA was added, and the preparation was incubated at 37 C for 30 min. Spheroplast formation was followed in a phase-contrast microscope. The preparation was dialyzed for 12 hr at 4 C against a solution of 0.15 M NaCl, 0.075 M Na_2HPO_4, and 0.005 M EDTA (*p*H 7.4); the membrane fraction was isolated by filtration on membranes (Millipore Corp., Bedford, Mass.) by means of a procedure recently developed in this laboratory by W. Salivar.

Distribution of total counts in cellular fractions after productive and abortive eclipse of ^{32}P-labeled tss6. ^{32}P-labeled *tss*6 (3.6 × 10^9 PFU/ml) was incubated in broth at 30 and 40 C for 2 hr (postincubation titers, 3.3 × 10^9 and 2.8 × 10^9 PFU/ml, respectively). Samples (0.4 ml) were added to 5.0 ml of *E. coli* C (5 × 10^7 cells/ml, MOI ~ 7) preincubated for 5 min at 40 C with 0.005 M cyanide. Eclipse was measured after 10 min (99%), a sample was diluted at 40 C to assay phage growth, and the remainder was cooled and centrifuged to separate cells from the supernatant fraction. The pellet was washed three times with 1.5 ml of sodium borate to remove divalent cations and attached phage (washes 1, 2, and 3, Table 2), resuspended as described in Materials and Methods, and converted to spheroplasts with lysozyme-EDTA. The spheroplast preparation was dialyzed, and the membrane fraction was isolated by filtration on membranes (*see above*). The washings from this filtration are represented as wash 4 (Table 2). Total counts per minute were as follows: *tss*6 (30 → 30), 11,135 (90% recovery); *tss*6 (30 → 40), 11,588 (93% recovery). Infective centers present 5 and 40 min after dilution at 40 C were: *tss*6 (30 → 30), 6 × 10^7 and 9.5 × 10^9 PFU/ml, respectively; *tss*6 (30 → 40), 7 × 10^6 and 9 × 10^8 PFU/ml, respectively.

Two-step complementation test. Cultures of *E. coli* C in KC broth at 30 C (4.5 × 10^7/ml) were infected with *tss*6 (MOI ~ 12), with *tss*6 and *am*9 (MOI ~ 6 + 6), with *tss*6 and *am*33 (MOI ~ 6 + 6), with *tss*6 and *am*10 (MOI ~ 6 + 6), with *tss*6 and *am*16 (MOI ~ 6 + 6), and with *wt* (MOI ~ 12). The infections were synchronized with cyanide (1). After 10 min, anti-φX serum was added ($K \approx 2$ min^{-1}, final titer) and incubation was continued for 6 min. The infected cultures were then diluted at 30 C in broth to 10^{-3}, 10^{-5}, 10^{-6}, and 10^{-7}; infective centers were plated directly from the appropriate dilutions at various times to assay phage growth. After lysis, cyanide (0.0075 M, final concentration) was added to each 10^3-fold dilution to prevent growth of resistant bacteria. These lysates were transferred to 40 C and incubated for 16 hr. [This procedure prevents successful initiation of a second-cycle of infection by phage of *tss*6 phenotype (*see* Results).]

Residual phage was then assayed, and the possibility of a second cycle of infection was tested by addition of 0.1 volume of *E. coli* X (grown to ~ 10^8 cells/ml at 40 C; MOI of 0.01 to 0.001). After 7 min at 40 C, the infected complexes (synchronized by the presence of the cyanide previously added for the 16-hr incubation period) were diluted to 10^{-3}, 10^{-4}, and 10^{-5} into broth at 30 C, and phage development was assayed by plating directly at the appropriate temperature. Phage growth in the second cycle was initiated at 40 C but was continued by dilution into broth at 30 C because of the temperature sensitivity of *am*16 and *am*10 growth in *E. coli* X.

RESULTS

Fifteen *ts* mutants of φX were isolated by the selection procedure described. All formed plaques on *E. coli* at 30 C but not at 40 C. Reversion frequencies were of the order of 5 × 10^{-6} to 1 × 10^{-5}. Approximately two-thirds of the *ts* mutants were of the conventional type; i.e., no infective progeny were formed in a one-step growth experiment in broth at 40 C. However, several (*tss*6, *tss*9, *tss*12, *tss*13) carried out an apparently normal one-step infection at 40 C (progeny were assayed on *E. coli* at 30 C) but initiated a second cycle with very low efficiency. One representative of this type of mutant (*tss*6) has been studied in detail.

Two-step growth experiment at 40 C. Figure 1

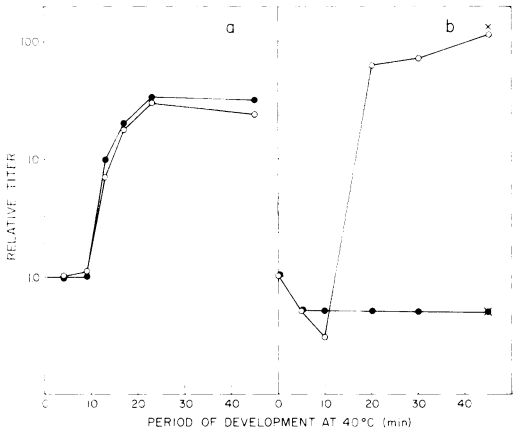

FIG. 1. *Two-step growth experiment for wt φX and tss6 at 40 C. (a) First cycle. Two cultures of E. coli C (10^8 cells/ml) grown in KC broth at 40 C were infected at 40 C with wt φX (MOI ~ 4) and tss6 (30) (MOI ~ 6); cyanide synchronization was employed. After an 8-min adsorption period, anti-φX serum (final K ~ 2 min^{-1}) was added and incubation was continued for 3 min. The cultures were diluted to 10^{-3}, 10^{-5}, 10^{-6}, and 10^{-7}, and were assayed for wt φX and tss6 by directly plating infective centers from the appropriate dilutions. The average burst sizes for wt φX and tss6 were 24 and 32, respectively [in other comparable experiments, the average burst was generally higher (150 to 200/cell) than observed in this experiment]. (b) Second cycle. After lysis (45 min), 2 ml of each of the 10^3 dilutions from the first cycle were added to 2 ml of E. coli C in broth (10^8 cells/ml) at 40 C, and the suspension was aerated. The MOI for wt φX and tss6 were 0.012 and 0.016, respectively. Samples were removed at various times from the wt culture and diluted in ice-cold borate for assay; tss6 infective centers were plated from the aerated undiluted culture. The average burst sizes for wt φX and tss6 were 117 and nil, respectively. Crosses indicate the titer after lysis of residual cells with lysozyme-EDTA. Symbols: ○, wt φX infective centers; ●, tss6 (40) infective centers.*

shows the result of a two-step growth experiment for *wt* φX and *tss*6 in broth at 40 C. Growth of *tss*6 is normal in the first cycle at 40 C and follows similar kinetics to *wt* φX (Fig. 1a). In the second cycle, the titer of *tss*6 actually decreases, whereas the average burst size for *wt* is 117 phage/cell (Fig. 1b). In some experiments, a very small burst of *tss*6 was observed in the second cycle. Lysis of the culture with lysozyme-EDTA (crosses in Fig. 1b) provided essentially identical titers, indicating that the lack of *tss*6 formation as assayed by infective centers is not merely a consequence of a failure to lyse the cells.

Other experiments indicated no marked difference between *tss*6 and *wt* φX in the dependence of average burst size upon input multiplicity. Hence, the lack of production of *tss*6 in the second cycle is not a result of the low multiplicity of infection (MOI) in this cycle as compared with the first cycle.

Reversal of the tss6 phenotype: acquisition by tss6 (40) of the ability to infect at 40 C. Since *tss*6 produced at 40 C [*tss*6 (40)] was assayed by plating at 30 C, the defect which prevents a second cycle of infection at 40 C must be reversible at the lower temperature. When *tss*6 (40) is plated out at 30 C, plaques appear 0.5 to 1 hr later than those of wild-type φX or of other representative *ts* mutants plated out under the same conditions.

Incubation of freshly produced *tss*6 (40) in broth at 30 C also permits the phage to regain the capacity to infect at 40 C (or 30 C). This process is relatively rapid since, after 10 min at 30 C, 62% of the mutant phage which are capable of forming plaques at 30 C are also able to initiate a single-step infection at 40 C (Fig. 2). The reversal under these conditions is not complete, however, and the ability to infect at 40 C subsequently declines slightly upon continued incubation at 30 C. The reason for this decline is not known.

It seemed possible that the reversal of phenotype at 30 C was either a result of a reattachment of a necessary component to an incomplete phage or of a conformational alteration of the complete, but defective, phage. To distinguish between these possibilities, freshly produced *tss*6 (40) was diluted through broth held at 40, 30, and 0 C prior to plating out at 30 C. The same titer was obtained in each instance. Thus, the restoration of infectivity which occurs at 30 C is probably a consequence of a conformational alteration in *tss*6 (40) and not the reattachment of a necessary component.

Loss (at 40 C) of the ability of tss6 (30) to infect at 40 C. Purified *wt* φX and *tss*6 (30) were incubated in broth at 40 C; samples were removed at intervals and assayed for plaque formers (at 30 C) and for capacity to initiate a single cycle of infection at 40 C (Fig. 3). Unlike *wt* φX, *tss*6, formed at 30 C and incubated at 40 C [*tss*6 (30 → 40)], almost completely loses the ability to initiate infection at 40 C after 90-min incubation at this temperature; such phage still form plaques at 30 C with only a slight decrease in titer.

The results in Fig. 3 demonstrate that the reversal of *tss*6 (30) phenotype (active → inactive) during incubation at 40 C is slower but more complete than the reversal of *tss*6 (40) phenotype (inactive → active) during incubation at 30 C (Fig. 2).

Thermal sensitivity of tss6. During prolonged incubation in broth at 40 C, purified *tss*6 loses

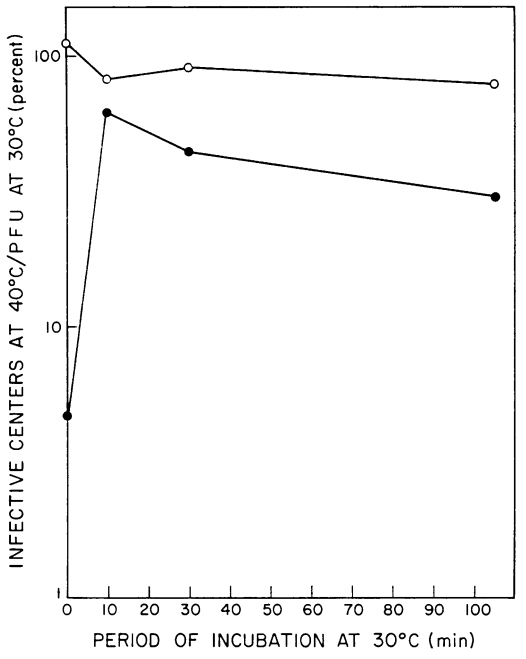

FIG. 2. *Recovery of ability of tss6 (40) to infect at 40 C upon incubation at 30 C. Two E. coli C cultures (5 × 10⁷ cells/ml) grown in KC broth at 40 C were infected at 40 C with wt φX and tss6 (30) at an MOI of ~ 5. After lysis, cyanide (final concentration, 0.005 M) was added to the 10³ dilutions of wt φX (7 × 10⁶ PFU/ml) and tss6 (40) (5.7 × 10⁶ PFU/ml); the lysates were then placed at 30 C. At zero-time and after 10, 30, and 105 min of incubation at 30 C, samples (2.0 ml) were added to 1.0 ml of E. coli at 40 C (~ 10⁸ cells/ml) previously preincubated for 5 min with 0.005 M cyanide; the MOI was approximately 0.1. After an 8-min adsorption period, anti-φX serum was added (final K≈ 2 min⁻¹), and incubation was continued at 40 C for 3 min. The suspension was then diluted at 40 C and assayed for growth of wt φX and tss6 (40–30) by directly plating infective centers at various times from the appropriate dilution. The ordinate represents infective centers present 5 min after dilution at 40 C as a percentage of the PFU (assayed at 30 C) in a corresponding virus sample diluted in 0.05 M sodium borate at room temperature. [The final titer in each growth experiment (at 50 min after dilution) was, in each case, nearly 200 times the infective centers present at 5 min; thus, infective centers present at 5 min are a good measure of the ability to initiate infection at 40 C.] The titers of wt φX and tss6 after incubation for 105 min at 30 C were 6.5 × 10⁶ and 6.4 × 10⁶ PFU/ml, respectively, equivalent to the initial titers. Symbols: ○, wt φX; ●, tss6.*

the ability to form plaques at 30 C (irreversible inactivation) more rapidly (Table 1) than *am3* (or *wt* which is inactivated at the same rate as *am3*). However, purified *tss6* and *am3* are inactivated at identical rates at 50 C or higher, presumably by a different process. Thus, the degree of thermal sensitivity of *tss6* is specifically anomalous at the lower temperatures.

This thermal sensitivity (at 40 C) of *tss6* as assayed by plaque-forming ability *at 30 C* is to be distinguished from the more rapid loss of ability to initiate infection *at 40 C*; it may, of course, be a secondary consequence of the conformational alteration presumed to be responsible for the latter effect.

Electron microscopic appearance of tss6. Freshly produced *tss6* (40) was fixed at 40 C and stained with uranyl acetate at the same temperature (we thank C. A. Hutchison for assistance in this experiment). No differences from wild-type φX particles were observed; in particular, the pro-

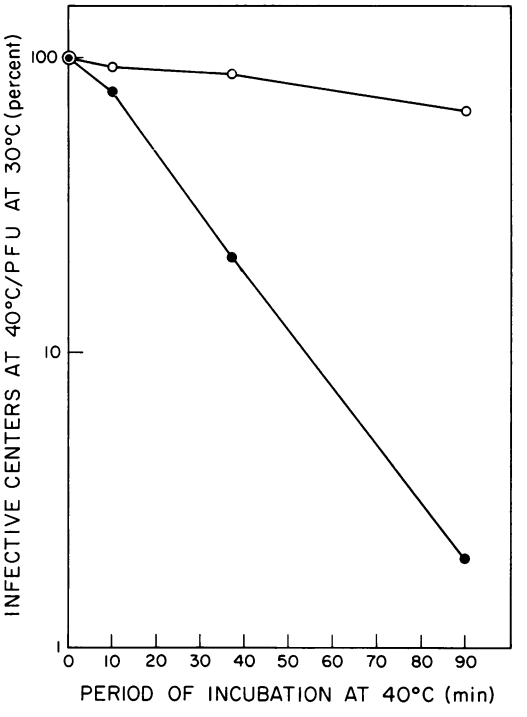

FIG. 3. *Loss of ability of tss6 (30) to infect E. coli C at 40 C, upon incubation at 40 C. Samples of purified wt φX (5.7 × 10⁸ PFU/ml) and of tss6 (30) (3.4 × 10⁸ PFU/ml) were incubated separately in broth at 40 C for 90 min (final titers, 6.1 × 10⁸ and 3.4 × 10⁸ PFU/ml, respectively). At zero-time and after 10, 37, and 90 min of incubation at 40 C, samples were assayed for PFU (on E. coli C at 30 C) and for ability to initiate a single cycle of infection in E. coli C at 40 C (~ 10⁸ cells/ml, with MOI 0.05 to 0.2). (The procedure was as described for Fig. 1.) The ordinate expresses infective centers 5 min after dilution of the infected cultures at 40 C as a percentage of the PFU (at 30 C) in the corresponding phage sample (diluted into 0.05 M sodium borate at room temperature). Symbols: ○, wt φX; ●, tss6.*

TABLE 1. *Thermal inactivation of φX am3 and tss6*

Temp (C)	Half-life[a] of	
	am3	*tss6*
40	19.0 hr	10.5 hr
46	3.8 hr	3.0 hr
50	1.0 hr	1.0 hr
60	2.1 min	2.1 min

[a] In KC broth.

jecting "spikes" seen on *wt* phage were found in preparations of *tss*6 (40).

Polyacrylamide gel electrophoresis of tss6. Certain amber and *ts* mutants of φX differ from *wt* in electrophoretic mobility (3). By use of the procedure of Hutchison et al. (3), *tss*6 (40) was electrophoresed at 40 C together with *am*3. No differences in the mobility of *tss*6 (40), other *tss* mutants, and *am*3 (*am*3 has the same mobility as *wt* φX) were found.

Nature of the block in the second cycle of infection. Although the kinetics of eclipse of *tss*6 (30) were normal at 40 C, preliminary results indicated that the abortive second cycle of infection was a result of a defective eclipse of *tss*6 (40). Eclipse of φX, as measured by loss of plaque formers upon dilution of infected complexes into chloroform broth, requires attachment of the phage (reversible) and then a structural alteration of the phage particle, which leads to irreversible loss of infectivity (J. E. Newbold, *unpublished data*). Eclipse, thus defined, may occur without (the normal) subsequent injection of deoxyribonucleic acid (DNA) into the cell. The experiments which follow are an attempt to define the nature of the second-cycle defect in terms of these three initial steps in the infective process.

Attachment of phage. Attachment of *tss*6 (40) and *wt* (40) to *E. coli* at 40 C was measured. Fresh lysates of wild-type and of mutant phage (produced at 40 C) were mixed with *E. coli* C cultures at 40 C (MOI ~ 0.2). After 5 and 10 min, respectively, 87 and 93% of *wt* φX had attached; the corresponding figures for *ts*6 (40) were both 60%. Attachment of *ts*6 (40) therefore occurs in the abortive second cycle, but to a reduced extent.

Irreversible loss of phage infectivity. Eclipse of freshly made *tss*6 (40) does occur to a limited but significant extent in a second cycle at 40 C (Fig. 4a); however, only about 3% of those phage which do eclipse give rise to infective progeny.

Eclipse of *tss*6 (30 → 40, 2 hr) differs from that of *tss*6 (40); *tss*6 (30 → 40) eclipses more rapidly at 40 C than do either *tss*6 (40) or *wt* (40) (Fig. 4b). However, eclipsed *tss*6 (30 → 40) also give rise to very few infective progeny.

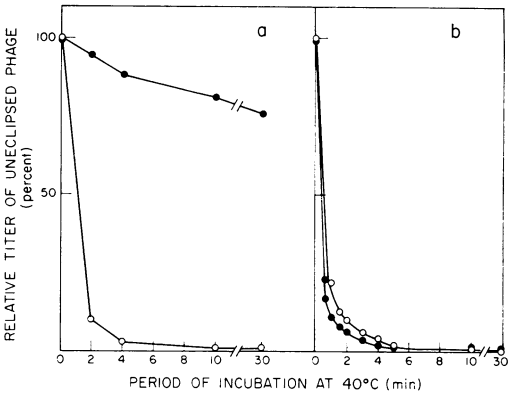

FIG. 4. *(a) Eclipse of wt (40) and tss6 (40) at 40 C. Mutants wt φX and tss6 were grown in separate single-cycle growth experiments at 40 C (see legend to Fig. 1). Soon after lysis, an equal volume of E. coli C (~ 10⁸ cells/ml) was added to an appropriate dilution of each lysate (the final MOI was 0.016 for wt φX and 0.01 for tss6). Samples were removed for assay of phage eclipse at timed intervals. After 8 min, a sample was removed from each culture, diluted into broth at 40 C, and assayed for phage development. A normal second-cycle growth curve was observed for wt φX; tss6 yielded an average burst of less than one phage per eclipsed input phage. (b) Eclipse of wt (30 → 40) and tss6 (30 → 40) at 40 C. Samples of purified wt (30) and of tss6 (30) were incubated separately in broth at 40 C for 2 hr, each at 5 × 10⁸ PFU/ml. Portions (40 μliters) of each were removed and added to separate tubes containing 2 ml of E. coli C at 40 C (5 × 10⁷ to 1 × 10⁸ cells/ml, MOI 0.1 to 0.2) in the presence of 0.005 M cyanide. Eclipse was followed for 30 min. After 8 min, a sample was removed and diluted into broth at 40 C; phage development was followed. Mutant wt φX gave rise to a normal infection (average burst, 130 phage per cell); tss6 (30 → 40) yielded an average burst of 3.4 phage per eclipsed input phage. Symbols: ○, wt φX infective centers; ●, tss6 infective centers.*

When incubation of nascent *tss*6 (40) was continued at 40 C, the extent of subsequent eclipse upon infection increased, although the virus preparation did not fully acquire the rapid-eclipse characteristics of *tss*6 (30 → 40). In one experiment, 52% of freshly produced *tss*6 (40) eclipsed within 10 min on addition of cells; after a prior 4-hr incubation (with cyanide) at 40 C, 78% of the phage eclipsed in the same period of time. However, the proportion of eclipsed phage which yielded progeny (assuming a normal burst from a few infected cells) decreased from 3% to 1% after the 4-hr incubation at 40 C.

To determine whether any reduction in cell viability occurred after these abortive eclipses, *wt* (40) and *tss*6 (40) were added to cells preincubated with cyanide at 40 C (final MOI ~ 15 to 20). Samples were removed after 10, 20, and 30

min, diluted at 40 C, and assayed for bacterial colony-forming ability. After the 30-min trial, *wt* (40) reduced colony-forming ability to 13% of the initial level; however, cell killing by *tss*6 (40) was below a detectable level over the same period.

"Eclipse" of *tss*6 (30) held at 30 C for 2 hr [*tss*6 (30 → 30)] and *tss*6 (30 → 40, 2 hr) onto *E. coli* cell-wall preparations was compared, at 40 C (MOI ~ two phage/cell equivalent of cell-wall preparation). In both cases, the rate of eclipse was lower than for whole cells but was the same (33% "eclipse" in 10 min).

Injection of DNA. To determine the fate of the viral DNA in these abortive infections, two portions of ^{32}P-labeled *tss*6 (30) were incubated for 2 hr in broth at 30 and 40 C, respectively. A portion of each was added to cells preincubated at 40 C with cyanide (MOI ~ 7). Eclipse was measured after 12 min (99% in both cases) and a sample was diluted at 40 C to assay phage development. As compared with *tss*6 (30 → 30), the *tss*6 (30 → 40) phage yielded 10% of the initial infective centers and 10% of the final titer.

After the 12-min eclipse period, the cell suspension was centrifuged and washed several times with borate to remove attached phage; the cells were converted to spheroplasts. Total radioactive counts were assayed in the initial supernatant fraction, in washings of the cell pellet, and in the cell membrane fraction isolated by filtration of the ruptured spheroplasts. The supernatant fluid contained 45 and 71% of the total counts after eclipse of *tss*6 (30 → 30) and *tss*6 (30 → 40), respectively (Table 2); the corresponding figures in the membrane fraction were 34 and 12%, respectively. (In other comparable experiments, less than 8% of the total counts were found in acid-soluble fractions.)

Complementation of the defective eclipse function. The complementation test measures the ability of progeny from a mixed infection of *tss*6 and various *am* mutants in *E. coli* C (su$^-$) to initiate a second cycle of infection at 40 C in *E. coli* X (su$^+$). Conditions were selected which would prevent phage of *tss*6 phenotype from initiating the second cycle of infection.

Preliminary experiments showed that complementation of *am*9 growth by *tss*6 in *E. coli* C was low at 40 C but three times more efficient at 30 C; the initial mixed infection was therefore performed at 30 C. Since *tss*6 produced at 30 C is capable of initiating a second cycle of infection, progeny from the first cycle were maintained at 40 C (for 16 hr) before initiating the second cycle. By this time, phage of *tss*6 phenotype are unable to infect cells successfully at 40 C (Fig. 3). The extent of complementation is expressed as the proportion of phage present after incubation at 40 C which are capable of initiating a cycle of infection at the same temperature.

TABLE 2. *Distribution of total counts in cellular fractions after productive and abortive eclipse of ^{32}P-labeled tss6*

Fraction	Preincubation temp	
	30 C	40 C
Supernatant fraction	45.0[a]	71.2
Wash 1	10.7	11.1
Wash 2	4.4	4.2
Wash 3	2.2	1.8
Wash 4	3.5	nil
Membrane fraction	34.1	11.7

[a] Percentage of total counts per minute.

In the first growth cycle (mixed infection in *E. coli* C), selective assays of the progeny indicate that complementation of *am*9 (cistron III), *am*16 (IV), and *am*10 (V) growth by *tss*6 occurs (Table 3, first and second columns). Only a slight complementation of *am*33 (VI) growth by *tss*6 is found; this is consistent with previous observations on cistron VI mutants (7).

After incubation of first-cycle progeny at 40 C, only 4% of the residual *tss*6 in the unmixed infection initiated infection at 40 C (Table 3, last column). The corresponding figure for *wt* φX is 37%. Progeny from the mixed infections of *tss*6 with *am*16, *am*10, and *am*33 all show an increased proportion capable of initiating a second cycle of infection in *E. coli* X at 40 C. Thus, defective protein of *tss*6 is not coded by cistrons IV, V, or VI.

Complementation by *am*16 is relatively symmetrical; i.e., progeny of both genotypes show an increase in ability to initiate a second cycle over that for *tss*6 alone. Complementation by *am*10 is less effective and is asymmetrical in this particular experiment, although in other experiments symmetric complementation by *am*10 was observed. As noted above, *am*33 is produced to only a low titer in the first cycle and does not initiate the second cycle to a significant degree; it is noteworthy, however, that although the parental *am*33 replicative form does not replicate under the restrictive conditions (5) sufficient protein is apparently formed to complement the defective *tss*6 function.

Mutant *am*9 (cistron III) is the only mutant which wholly fails to complement the defective *tss*6 function. These results indicate that the defective protein is probably coded by cistron III.

TABLE 3. *Two-step complementation test of tss6*

Mutant	First growth cycle[a] infective centers[c]		Titer after incubation of first-cycle lysate (10^{-3} dilution) at 40 C	Second growth cycle[b] infective centers[c] (final)	Proportion of first-cycle progeny which can initiate second infection (%)[d]
	Initial	Final			
wt	3×10^7	1×10^{10}	2.7×10^5	1×10^7	37
tss6	4.5×10^7	1.6×10^{10}	1.5×10^5	6.6×10^5	4
tss6 × *am9*	4×10^7	8×10^9	2.1×10^4	4.3×10^4	2
	3×10^7	2.8×10^9	9×10^3	3×10^4	3
tss6 × *am16*	3×10^7	3×10^9	2.7×10^4	5.4×10^4	20
	1×10^6	2×10^9	1.7×10^4	2×10^4	12
tss6 × *am33*	4.5×10^7	2×10^{10}	1×10^5	1.6×10^6	16
	1×10^7	3×10^7	1×10^3	nil	—
tss6 × *am10*	3×10^7	4×10^9	2.2×10^4	2×10^5	9
	1×10^6	8×10^8	8×10^3	3×10^4	4

[a] The first cycle of growth was in *E. coli* C (su^-) at 30 C (high MOI).

[b] The second cycle, initiated after incubation of first-cycle progeny at 40 C for 16 hr, was in *E. coli* X (su^+) at low MOI (0.01 to 0.001). After attachment (7 min) at 40 C, dilution was into broth at 30 C, since *am16* and *am10* are temperature-sensitive in *E. coli* X.

[c] Initial and final titers refer to infective centers 10 and 45 min (first cycle) and 60 min (second cycle) after dilution.

[d] The percentage of progeny phage which can initiate infection at 40 C is calculated by dividing column 4 by column 3; a burst of 100 phage per successful second-cycle infection is assumed.

DISCUSSION

These results show that, by means of an appropriate selection procedure, temperature-sensitive mutants of ϕX may be isolated which undergo one cycle of growth at the "restrictive" temperature but fail to initiate a second cycle at the same temperature. The selection procedure ensures that *ts* mutants produced at the "restrictive" temperature have, or can acquire, the ability to infect at the permissive temperature. A representative mutant of this class, *tss6*, may reproduce in a single-cycle infection in broth at 40 C [*tss6* (40)]. It cannot initiate a second cycle at 40 C, but can, at 30 C, acquire the ability to form plaques, probably as the result of a conformational change in the phage particle during incubation at this temperature; this alteration in capacity to infect is not a consequence of the reattachment of a necessary component to a defective particle at 30 C. During the first 10 min of incubation at 30 C (in broth), a large proportion of *tss6* (40) regain the capacity to infect at 40 C (in broth) as well.

Phage produced at 30 C [*tss6* (30)] almost completely lose their original capacity to infect at 40 C upon incubation for 2 hr in broth at 40 C; this change occurs with no marked decrease in plaque-forming ability at 30 C. There is also a slower, but relatively rapid, irreversible inactivation of *tss6* (assayed at 30 C) which occurs during prolonged incubation at 40 C. This unusual sensitivity of *tss6* to irreversible inactivation at 40 C may be related to the postulated conformational alteration at this temperature.

The phenotypic expression of the *tss6* mutation (inability to initiate infection) is thus reversible, and its expression depends on either the temperature at which the phage is produced or the temperature at which it is incubated prior to infection. However, the alteration of phenotypic expression of *tss6* (30) which occurs after 2 hr at 40 C apparently does not give rise to phage which are in all respects identical to those produced at that temperature; thus, although *tss6* (40) and *tss6* (30 → 40) both fail to infect successfully at 40 C, the kinetics of the abortive eclipse differ markedly (Fig. 4).

The primary defect in the abortive infection of *tss6* appears to be in the injection of the viral DNA. Attachment and subsequent irreversible loss of phage infectivity (eclipse) occur in the

abortive infection, although the kinetics of both processes differ from those of *wt* φX. However, experiments with ^{32}P-labeled *tss*6 show a decrease in viral DNA associated with the cell membrane fraction after eclipse in the abortive infection; there is a corresponding increase in labeled DNA in the supernatant fraction. Since parental φX DNA is known first to associate with the membrane fraction upon entering the cell (R. Knippers, *personal communication*), the DNA of abortively eclipsed *tss*6 possibly does not pass through the cell membrane.

The results of complementation studies indicate that the defective protein is likely coded for by cistron III. This protein, together with cistron II protein, appears to constitute the "spike" component of the φX virus particle (M. H. Edgell and C. A. Hutchison, *unpublished data*); cistron II protein is believed to be responsible for attachment specificity (host range). Our results indicate that cistron III protein probably plays a role in the injection of φX DNA. The alteration of attachment kinetics observed during abortive eclipse indicates that a conformational alteration in the cistron III protein may also include a change in the configuration or disposition of the cistron II protein(s) in the "spike" component.

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