Cold-Sensitive Mutants of Bacteriophage ϕ X174.

II. Comparison of Two Cold-Sensitive Mutants

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Cold-sensitive bacteriophage ϕ X174 mutants, another class of conditional lethals, were examined with regard to growth parameters, DNA synthesis, and particle properties. Two mutants, *cs70* and *cs82*, were examined. Mutant *cs70* was eclipse defective, showing altered eclipse kinetics at permissive temperature (40 C) and failing entirely to eclipse at restrictive temperature (25 C). Mutant *cs70* replicated well at 25 C if allowed prior eclipse at 40 C. Mutant *cs82* had wild-type eclipse at both temperatures but was defective in single-strand synthesis at 25 C, which led to delayed progeny phage appearance, decreased progeny phage synthesis rate, and greatly reduced burst size. The *cs82* block could not be bypassed by temperature shift. Since complementation analysis of *cs70* and *cs82* was not feasible due to the unique properties of these mutants, those ϕ X174 properties affected by the virus coat were examined as an index of a mutation in a coat protein gene. Mutant *cs70* had aberrant attachment kinetics at both 25 C and 40 C, evidence of a coat protein alteration. Mutant *cs70* also exhibited significantly decreased thermal stability, further evidence of an altered virus structure. Mutant *cs82* had increased thermal stability, but the difference was not sufficient to allow unequivocal assignment of this mutant to a coat protein gene. Both mutants had wild-type antiserum inactivation and host range, although *cs70* was subject to less of (low-level) plating restriction by endogenous F⁺ factors.

Bacteriophage ϕ X174, because of its limited genome size of about 5,500 nucleotides, has been examined by many laboratories with the objective of elucidating all of the virus functions. To this end, nine (and perhaps ten) genes have been defined (4, 5) through the use of conditional lethal mutants of the temperature-sensitive and nonsense types. In 1967, Dowell (15) isolated a class of ϕ X174 mutants which failed to grow at low temperature; these mutants were called cold-sensitive (*cs*) since they were phenotypically opposite to temperature-sensitive mutants. It was anticipated that such mutants might define previously uncharacterized ϕ X174 functions. In that preliminary report, two classes of cold-sensitive mutants were described: those defective in eclipse, and those whose cold sensitivity was manifest in some later replication step(s). The present paper compares the growth properties of *cs70* (an eclipse mutant) with *cs82* (a replication mutant) at both permissive (40 C) and restrictive

(25 C) temperatures. The results of single-step growth experiments and intracellular DNA analyses show that the *cs70* mutation prevented eclipse completely at 25 C and partially at 40 C, but had little effect on subsequent replication steps at either temperature. The *cs82* mutation, on the other hand, had no effect on eclipse at either temperature, but severely inhibited single-strand (SS) DNA synthesis at 25 C.

At least four ϕ X174 genes specify virus structural proteins, and the growth properties of the *cs70* mutant suggested that it would be located in a coat protein gene. Although ϕ X174 mutations have been mapped through both complementation analysis (8, 39; C. A. Hutchison, Ph. D. thesis, California Institute of Technology, Pasadena, 1969) and recombination (2, 4; C. A. Hutchison, Ph. D. thesis), several properties of *cs70* and *cs82* precluded the use of these conventional genetic techniques (see Discussion). Instead, evidence that *cs70* is a coat protein mutant was sought by examining those properties determined by the virus coat, such as particle stability, attachment kinetics, anti-

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serum inactivation, and host range. Other investigators have also used such nongenetic criteria to assign ϕ X174 mutations to coat protein genes (2, 7, 14, 16, 21, 28; C. A. Hutchison, Ph. D. thesis).

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MATERIALS AND METHODS

Bacterial strains. The strains used were: (i) *Escherichia coli* C (BTCC 122), with genotype *sup⁻, thy⁺, ϕ X^{*}*, the usual host for ϕ X174; (ii) *E. coli* HF4704, with genotype *sup⁻, thy⁻, ϕ X^{*}, hcr⁻* (27); (iii) *E. coli* HF4714, with genotype *sup⁺, thy⁺, ϕ X^{*}*, a C-K-12 hybrid (C. A. Hutchison, Ph.D. thesis); and (iv) *E. coli* C-2, (v) HF4704F⁺, and (vi) CRF⁺, which are male derivatives of *E. coli* C, HF4704, and CR (15).

Viral strains. Mutants *cs70* and *cs82* were isolated and described previously (15). Amber mutants were obtained from R. L. Sinsheimer; *am33*(A), *am14*(B), *am10*(D), *am3*(E), *am9*(G), and *am23*(H) were isolated by C. A. Hutchison (Ph. D. thesis), *am88*(F) by F. Funk (21), and *amN-1*(H) by M. Hayashi (34). Double mutants of *am3* and *cs70* or *cs82* were constructed according to C. A. Hutchison (Ph. D. thesis).

Media. Top agar, bottom agar, and KC broth were as previously described (16) except that KC broth contained 0.001 M CaCl₂. C-medium is TPG medium (22) containing 0.1% Casamino Acids and 0.001 M CaCl₂. Starvation buffer has been described (13). Sodium tetraborate solution is a solution of sodium tetraborate saturated at 4 C.

Chemicals. The chemicals [*methyl-³H*]thymine (23 Ci/mmol), [*methyl-³H*]thymidine (50 to 60 Ci/mmol), [*methyl-¹⁴C*]thymine (1 to 5 mCi/mmol), Triton X-100, and Aquasol were purchased from New England Nuclear (Boston, Mass.). Spectrafluor was from Amersham/Searle Corp. (Des Plains, Iowa). Pronase (Calbiochem, Los Angeles, Calif.), was self-digested for 2 h at 37 C and 2 min at 80 C as described by Iwaya and Denhardt (26). Sarkosyl (Geigy Chemical Corp. Ardsley, N.Y.) was 10% in 0.8% EDTA containing 0.1 M Tris buffer. Mitomycin C was obtained from Sigma Chemical Co. (St. Louis, Mo.), and lyophilized lysozyme from Mann Research Labs., Inc. (New York, N.Y.). Phenol was redistilled and stored at -20 C in a sealed container.

Biological assays. Assays of infective centers and intracellular phage were performed as described by Denhardt and Sinsheimer (13), using the soft-agar overlay method (1). Indicator bacteria were grown to 3×10^8 cells/ml and kept in an ice bath until required.

Starvation synchrony. A stationary culture was diluted at least 1:10,000 into growth medium and allowed to reach a density of about 2×10^8 cells/ml at 37 C with aeration. Cells were collected by centrifugation, washed twice with starvation buffer, and starved

for 90 min at 37 C (13). A sample of starved cells was placed at 40 C for 5 min and then infected at a multiplicity of infection (MOI) of about 5. After 15 min to allow eclipse, growth was initiated by diluting 1:100 in starvation buffer at 4 C and then 1:100 into growth medium at the desired temperature. For DNA-labeling experiments, after eclipse as previously described growth was initiated by collecting infected cells by centrifugation, washing once, and resuspending at input density in fresh growth medium at the proper temperature.

Burst size determination. Cultures of *E. coli* C or HF4704 were grown to 2×10^8 cells/ml in KC broth or C-medium, and synchronously infected at a MOI of about 5 as described above. Growth was initiated by 1:10,000 dilution into appropriate medium at 40 C or 25 C. Wild-type, *cs70*, and *cs82* burst sizes were calculated as the ratio of infectious centers at $t = 60$ (for 40 C) or $t = 240$ (for 25 C) to input infectious centers at $t = 0$. Burst sizes for *am3*, *cs70am3*, and *cs82am3* were calculated as the ratio of intracellular phage at $t = 120$ (for 40 C) or $t = 240$ (for 25 C) to the number of input-starved cells measured just prior to phage addition.

Preparation of ¹⁴C-labeled SS DNA. *E. coli* HF4704 was grown to 3×10^8 cells/ml in 500 ml of C-medium containing 2 μ g/ml of thymine, and synchronously infected with *am3* (MOI = 5) in C-medium supplemented only with 2.4 μ g of [¹⁴C]thymine per ml. After 180 min of infection, the cells were collected by centrifugation, suspended in Tris-lysozyme-EDTA (13), and lysed by six cycles of freeze-thaw. Saturated borate-eluted intracellular phage particles were purified twice by isopycnic centrifugation and dialyzed against 0.05 M sodium tetraborate. DNA was extracted by the hot-phenol method (24), with removal of residual phenol by dialysis against 0.05 M borate.

Measurement of DNA synthesis. At 40 C, ϕ X174 DNA synthesis was measured by the method of Lindqvist and Sinsheimer (27), in mitomycin C-treated cells growing in C-medium supplemented with 2 μ g of nonradioactive thymine per ml and 10 μ Ci of [³H]thymine per ml. At 25 C, ϕ X174 DNA synthesis was measured using a method similar to that of Iwaya and Denhardt (26); cells were starved, mitomycin treated, and synchronously infected in the absence of exogenous thymidine or thymine, since at lower temperatures *E. coli* HF4704 no longer requires these supplements (12). Virus DNA synthesized at 25 C was labeled in cells growing in C-medium supplemented with 10 μ Ci of [³H]thymidine per ml plus 20 μ g of adenosine per ml to potentiate thymidine uptake (12).

Analysis of intracellular DNA. Infected 20-ml portions of *E. coli* HF4704 cultures were pulsed with [³H]thymidine from 60 to 240 min; the cultures were chilled by addition of an equal volume of 4 C starvation buffer; and the cells were collected and washed three times by centrifugation. The pellet was lysed in Tris-lysozyme-EDTA and treated with Sarkosyl and Pronase as described (26). DNA was deproteinized by room temperature phenol extraction, precipitated by isopropanol, and dissolved in 0.1 M NaCl as previ-

ously described (16); efficiency of recovery of phage-specific DNA was 67 to 77%. Samples containing equal volumes of [3 H]DNA and [14 C]SS DNA were layered over linear 5 to 20% sucrose gradients containing 0.5 M NaCl and 0.05 M sodium citrate (pH 7) and centrifuged at 37,000 rpm for 5.5 h in an SW50.1 rotor of a Beckman L2-65B ultracentrifuge. The gradient tube was punctured, and 3-drop fractions were collected in scintillation vials and counted using a scintillation cocktail consisting of 1,875 ml of toluene, 1,000 ml of Triton X-100, 333 ml of water, and 125 ml of Spectrafluor.

Eclipse studies. *E. coli* C cultures were starved and synchronously infected at 25 C or 40 C as described above. Periodic samples were diluted 1:100 into KC broth at 4 C, and the number of uneclipsed particles was determined by intracellular assay at permissive temperature. Starvation conditions, which permit ϕ X174 attachment and eclipse, but not DNA replication, were employed to prevent eclipse from being masked by subsequent virus replication.

Thermal stability studies. Virus stocks in 0.05 M sodium tetraborate were diluted to 1×10^{10} to 5×10^{10} PFU/ml in saturated borate solution and left overnight at 4 C. The experiment was begun by adding 0.1 ml of phage solution to 9.9 ml of saturated borate maintained at 56.5 C in a constant-temperature water bath. Samples were diluted 1:100 into KC broth at 4 C, and survivors were plated on *E. coli* HF4714 at 37 C. Wild-type survival was 1×10^{-5} to 3×10^{-5} after 180 min of exponential inactivation under these conditions. Inactivation rates and survival ratios were calculated as described (35, 39; C. A. Hutchison, Ph. D. thesis).

Attachment studies. Attachment kinetics were measured essentially as described by Newbold and Sinsheimer (28). *E. coli* C, grown to 2.5×10^8 cells/ml and starved in the usual fashion, was equilibrated at 25 or 40 C. Phage stocks were diluted in starvation buffer to 1.2×10^8 PFU/ml and temperature-equilibrated; equal volumes of *cs70* (or *cs82*) and *am3* were combined, and the experiment was begun by addition of 0.1 ml of phage mixture (1.2×10^7 phage) to 5 ml of cells (1.2×10^8 cells). The culture was aerated, and 0.1 ml samples were diluted into 9.9 ml of KC broth (at 4 C) containing 2×10^8 *E. coli* K-12 W-6 carrier cells, a strain to which ϕ X174 cannot attach (15, 35). Cells and adsorbed virus were collected by centrifugation, and the supernatant fluids were titrated for unattached *cs* phage by plating on *E. coli* C at 37 C and for *am3* phage by plating on *E. coli* HF4714 at 25 C. Wild-type attachment kinetics were measured in the same way, but not in the same tube as with *am3*. The design fulfilled the following criteria. (i) *am3* and *cs70* (or *cs82*) were in the same tube to normalize sampling errors; (ii) MOIs were low enough to ensure no cooperative effect of attachment on adsorption of subsequent particles (1). (iii) A dilution of 1:100 effectively stopped further attachment by reducing cell concentration to below 2×10^8 cells/ml (20). (iv) Calculations were based on only the first-order portion of attachment kinetics. Attachment rate constants were determined by the method of Adams (1).

Antiserum inactivation. Experiments were performed by the method of Rolfe and Sinsheimer (31). Virus stocks were diluted at least 100-fold into 10 ml of KC broth at 37 C, to a final virus concentration of 5×10^7 PFU/ml. An equal number of *am3* particles was added to each tube, and the phage mixtures were equilibrated at 37 C for 15 min. The ϕ X174 antiserum (attachment rate constant $k \cong 2 \times 10^4$ /min; kindly supplied by D. T. Denhardt) was diluted 1:500 into KC broth, and the experiment was begun by adding 0.1 ml of antiserum mixture to 10 ml of virus mixture (final $k \cong 4 \times 10^{-1}$ /min). Samples taken at 1-min intervals were diluted 1:100 into KC broth. Wild-type and *cs* survivors were assayed on *E. coli* C at 37 C, and *am3* survivors were assayed on *E. coli* HF4714 at 25 C. Phage mixtures provided an internal control against variation in sampling time and dilution. Under these conditions, it was possible to obtain 10 points on the inactivation curve and yet remain within the quantitative limits of the Adams equation for calculating antiserum k values (1).

RESULTS

Eclipse studies. Examining the efficiencies of plating of wild-type and *cs70* and *cs82* at 25 C and 40 C, it was found that wild type had an EOP of 0.55 at 25 C relative to 40 C, but that *cs70* and *cs82* had efficiencies of plating of 1.6×10^{-7} and 1.7×10^{-5} , respectively, over the same range. The initial report on these mutants (16) suggested that *cs70* cold sensitivity was due to a block in eclipse, although *cs82* was thought to be cold sensitive in subsequent replication. Eclipse kinetics experiments (Fig. 1) showed that at 25 C, no *cs70* PFUs were lost during the interval in which more than 98% of *cs82* and wild-type particles became eclipsed. In addition, *cs70* eclipsed at a slower rate at 40 C and had a higher level of noneclipsed particles than the other two viruses. These results showed conclusively that the *cs70* mutation inhibited eclipse completely at 25 C and partially at 40 C. The *cs82* mutation, on the other hand, had little effect on eclipse at either temperature. Experiments with *am3*, *cs70am3*, and *cs82am3* showed a similar relationship.

Growth experiments. Single-stop growth experiments were performed to see if the *cs70* mutation affected any post-eclipse function and also to define the *cs82* replication defect. At permissive temperature (40 C), *cs70*, *cs82*, and wild type had similar growth kinetics, with eclipse times of 12 min, latent periods of 14 min, and burst sizes of 148, 83, and 89 for *cs70*, *cs82*, and wild-type, respectively. The growth of *cs70* at 25 C (Fig. 2), after eclipse at 40 C, was very much like wild-type, which suggested that the *cs70* mutation had little effect on any replication steps subsequent to eclipse. The growth of

FIG. 1. Eclipse of wild type *cs70*, and *cs82* at 25 C and 40 C. Starved *E. coli* C was infected at 25 or 40 C with wild type, *cs70*, or *cs82* at MOI about 4. Samples were diluted 1:100 into KC broth at 4 C and assayed for intracellular phage. Uneclipsed phage is percentage (PFU per ml, after intracellular assay, of sample at $t = X$ divided by PFU per ml, after intracellular assay, of sample at $t = 0$).

cs82 at 25 C, on the other hand, showed delayed onset of intracellular phage production, retarded rate of intracellular synthesis, and greatly reduced burst size. This growth inhibition of *cs82* at 25 C, contrasted with the wild-type growth of *cs70* at both temperatures (after permissive temperature eclipse), was observed in a variety of growth conditions (Table 1).

The data in Fig. 2 suggested that *cs82* was defective not only in initiation of intracellular phage synthesis but also in some subsequent process(es). An infected culture was shifted from 40 C to 25 C after 20 min of infection, with the rationale that if the *cs82* mutation affected a function continually required for phage synthesis, production would cease after a down-shift. The data in Fig. 3 showed that wild type (*am3*) continued replication after such a shift, but that the *cs82am3* culture ceased replication. At the end of a 20-min period, replicative form (RF) formation, replication, transcription, and translation are essentially complete (32, 35), so that an RF defect was in itself not sufficient to explain *cs82* cold sensitivity. It was also not likely that a lysis defect was responsible for *cs82* cold sensitivity, since in the absence of lysis at 25 C the *cs82* mutation still delayed appearance of intracellular phage, retarded intracellular synthesis rate, and reduced burst size (experiment not shown).

DNA synthesis. The growth experiments showed that *cs82* growth inhibition at 25 C was

due to a block in some late replication process other than RF replication or lysis. To determine if the *cs82* mutation affected progeny SS DNA synthesis at 25 C, *cs82am3*-infected cultures were labeled with [^3H]thymidine from 60 to 240 min during the period of SS synthesis, and the intracellular phage DNA was analysed on a sucrose gradient. The results (Fig. 4) show that the *cs82* mutation severely inhibited SS synthesis at 25 C, but permitted normal synthesis of RF I and RF II, relative to both *am3* and *cs70am3*. The [^3H]SS DNA peak sedimented two to three fractions ahead of the ^{14}C -labeled SS DNA marker; it is doubtful that this reflected altered intracellular SS DNA in the infected cultures, but instead probably reflects the effects of different methods of DNA isolation (19).

Attachment kinetics. The effect of the *cs70* and *cs82* mutations on virus attachment rate at 25 C was examined by incubating virus and cells under conditions which permitted phage attachment but not replication, and then by assaying the amount of virus present in the supernatant fluids of samples taken at various times (Fig. 5). Wild type (not shown), *am3*, and *cs82* had similar linear kinetics, but *cs70* exhibited a distinctly biphasic pattern at 25 C. This suggested that two classes of *cs70* particles were present which attached at different rates (Table 2). These two classes may have been present in the lysate, or may have arisen through activa-

tion of *cs70* during the experiment (6, 9). Alternatively, *cs70* may have spontaneously detached more frequently than wild type, *am3*, or *cs82* (see *cs70* eclipse kinetics at 40 C, in Fig.

1). Regardless, the presence of a class of *cs70* particles with a significantly decreased attachment rate supported the hypothesis that the *cs70* mutation affects some component of the ϕ X174 coat. However, this attachment defect was not sufficient to account for the complete

FIG. 2. Growth of *cs70* and *cs82* at 25 C. Starved *E. coli* C was synchronously infected with *cs70* (MOI = 4.2) or *cs82* (MOI = 5.3) at 40 C, and growth was initiated by 1:10,000 dilution into KC broth at 25 C. Mutant *cs70* had an eclipse time of 38 min, a latent period of 50 min, and a burst size of 264; mutant *cs82* had an eclipse time of 65 min, a latent period of 70 min, and a burst size of 4.4; wild type (not shown) had an eclipse time of 40 min, a latent period of 50 min, and a burst size of 230. Relative titer is (infective centers or intracellular PFU per ml) divided by (input infectious centers per ml during the latent period).

FIG. 3. Growth of *am3* and *cs82am3* after temperature downshift late in infection. *E. coli* C was synchronously infected with *am3* (MOI = 5) or *cs82am3* (MOI = 5) at 40 C in the presence of 0.009 M KCN (16). Growth was initiated by 1:100 dilution into KC broth at 40 C. After 20 min of infection, portions were diluted 1:10 into KC broth at 25 C. Lysis defective *am3* derivatives were used to prevent lysis from prematurely terminating the experiment.

TABLE 1. Burst sizes of cold-sensitive mutants^a

Virus	Burst size ^b			
	40 C KC broth	40 C C-medium	25 C KC broth	25 C C-medium
Wild type	78 ± 17 (6)	241 ± 102 (6)	235 ± 26 (6)	534 ± 63 (4)
<i>cs70</i>	160 ± 34 (6)	234 (1)	322 ± 79 (4)	N.D. ^c
<i>cs82</i>	103 ± 16 (6)	192 ± 63 (6)	2.9 ± 1.2 (5)	15.3 ± 2.2 (3)
<i>am3</i>	1045 ± 45 (2)	415 ± 44 (3)	347 ± 21 (3)	176 ± 63 (3)
<i>cs70am3</i>	1940 (1)	2647 ± 400 (3)	401 ± 114 (2)	400 (1)
<i>cs82am3</i>	862 ± 87 (2)	611 ± 112 (3)	3.5 ± 0.5 (3)	9.0 ± 1.2 (3)

^a Starved *E. coli* C cultures were infected at MOI about 5, and growth initiated by 1:10,000 dilution into appropriate growth conditions.

^b Mean ± standard deviation, figure in parenthesis is the number of separate determinations.

^c ND, Not done.

FIG. 4. Analysis of intracellular DNA synthesized at 25 C. Starved *E. coli* HF4704 was treated with mitomycin C ($50 \mu\text{g}$ per 10^8 cells per ml) for 15 min at 40 C, and 25-ml portions were infected with *am3* (MOI = 4.7), and *cs70am3* (MOI = 5) or *cs82am3* (MOI = 4.8). Growth was initiated by suspension in C-medium (containing $20 \mu\text{g}$ of adenosine per ml) at 25 C. After 60 min [^3H]thymidine (specific activity 50 to 60 Ci/mmol) was added to a level of $10 \mu\text{Ci}/\text{ml}$, and at 240 min infected cells were subjected to DNA extraction and gradient analysis. Counts have been corrected for isotope spill and for background. RF I sedimented with a peak about fraction 35, and RF II about fraction 44.

FIG. 5. Attachment kinetics at 25 C. Starved *E. coli* C was incubated with virus at 25 C. Unattached phage is percentage (PFU/ml in supernatant fluid at $t = X$ divided by PFU/ml in supernatant fluid of sample at $t = 1$).

failure of *cs70* to eclipse at 25 C. Attachment kinetics were also examined at 40 C. Again, *cs70* attached more slowly than other ϕ X174 strains, although the decrease in rate was not as striking as at 25 C, and only one class of particles was apparent. Mutant *cs82* showed no significant attachment difference.

Table 2 also includes ϕ X174 attachment rate constants observed by other laboratories in other media. Our values were greater than those literature values, but this may have been due to increased adsorption that occurs when cells are aerated (1), or when temperature is increased (20, 37). Alternatively, the increased attachment rates may reflect increased ability of starved cells to adsorb ϕ X174.

Thermal stability. Heat inactivation studies were carried out on *cs70* and *cs82* to determine whether either cold-sensitive mutation affected virus particle stability. Various ϕ X174 mutants, whose particle stabilities (except for *am88* and *amN-1*) have been previously examined (35; C. A. Hutchison, Ph. D. thesis; D. Tseui, Ph. D. thesis, University of California, Davis, 1969),

were incubated in saturated sodium tetraborate at 56.5 C. The data in Fig. 6 showed that only those amber mutants with altered coat proteins (suppressed, but still missense mutations) exhibited altered inactivation kinetics, thus dem-

TABLE 2. Attachment rate constants of *cs* mutants

Virus	Temp (C)	Attachment rate constants (<i>k</i>) ^a
Wild type	25	3.02 ± 0.20 (2)
<i>am3</i>	25	3.51 ± 0.14 (4)
<i>cs82</i>	25	3.76 ± 0.40 (2)
<i>cs70</i>	25	3.45 ± 0.55 (2)
		0.80 ± 0.17 (2) ^b
Wild type	40	14.8
<i>am3</i>	40	15.7
<i>cs82</i>	40	14.3
<i>cs70</i>	40	12.9
Wild type	37	7.8 ± 1.8 (15) ^c ; 6.0 ^d ; 8.2 ^e
<i>am3</i>	37	8.6 ± 0.59 (4) ^e

^a Experiments were performed as described in Materials and Methods, and *k* values (1) expressed as milliliters per minute × 10⁹. Both *cs70* and *cs82* determinations were from a tube containing *am3* as well. Multiple values represent the results from more than one experiment.

^b The second set of values represent the biphasic component of the curves; the first set derives from the first 10 min of the experiments, the second set from the final 20 min.

^c In KC broth (3).

^d In 0.1 M CaCl₂ at 36 C (20).

^e In starvation buffer (28).

onstrating the ability of heat inactivation studies to distinguish coat from noncoat ϕ X174 amber mutants. The greatly increased heat sensitivity of *cs70* was therefore considered evidence of an altered *cs70* coat protein; *cs82* showed decreased heat sensitivity, but the difference was not sufficiently compelling to claim that *cs82* also is a coat protein mutant.

Although other laboratories have used particle stability to define coat protein mutations, the use of different temperatures and incubation media makes the comparison of heat stabilities difficult. To determine whether the differences observed for *cs70* and *cs82* were significant according to criteria adopted in other studies, we chose experimental times when the extent of wild-type inactivation was equivalent to that observed by others. For example, Sinsheimer (35) and Hutchison (Ph. D. thesis) observed 10⁻³ wild-type survival after 30 min in 0.025 M Tris buffer at 60 C, and the data in Fig. 6 showed about the same survival at 120 min. Therefore, by applying the same criterion to the *t* = 120 values in Fig. 6 as used by Hutchison and Sinsheimer at *t* = 30 to define a significant change, we can conclude from the resulting comparison (Table 3) that *cs70* and perhaps *cs82* had significantly altered particle stabilities. Similarly, by applying the criteria of Baker and Tessman (2) to the *t* = 30 value in Fig. 3 (which gave the wild-type inactivation equivalent to 21 min at 51.5 C in 0.1 M phosphate buffer), it was found that *cs70* again exhibited what other investigators would have considered

FIG. 6. Thermal stabilities of *am* and *cs* mutants at 56.5 C in saturated sodium tetraborate solution.

TABLE 3. Thermal stabilities of ϕ X174 mutants

Mutant	Cistron	Inactivation rate ^a (mutant/wild type)	Survival ratio ^b (mutant/wild type)	
			<i>t</i> = 30	<i>t</i> = 120
<i>am33</i>	A	1.07	0.89	0.63
<i>am14</i>	B	1.47*	0.49*	0.043*
<i>am10</i>	D	1.03	1.03	0.89
<i>am3</i>	E	1.04	1.00	0.85
<i>am88</i>	F	1.30*	0.56	0.119*
<i>am9</i>	G	0.62*	2.00*	15.8*
<i>am23</i>	H	1.33*	0.59	0.128*
<i>amN-1</i>	H	4.68*	0.0009*	<0.0009*
<i>cs70</i>	?	4.50*	0.0024*	<0.0024*
		4.98*	0.0006*	<0.0006*
<i>cs70am3</i>	?, E	3.12*	0.016*	<0.0005*
<i>cs82</i>	?	0.78	1.50	4.88*
		0.81	1.33	4.26*
<i>cs82am3</i>	?, E	0.78	1.42	4.38*

^a Calculated as described by Sinsheimer (35); values with asterisk (*) are considered significantly different, where significance is a value <0.77 or >1.25 (35).

^b Calculated as described by Tessman and Tessman (39) and Hutchison (Ph.D. thesis). Values with asterisk (*) are considered significantly different; significance at *t* = 30 is a value less than 0.5 or greater than 1.5 (39), and at *t* = 120 is a value less than 0.2 or greater than 4.0 (Hutchison, Ph.D. thesis).

to be an altered virus coat.

Serology and host range. The effects of the *cs70* and *cs82* mutations on other coat protein-determined ϕ X174 functions were also examined. ϕ X174-neutralizing antibody reacts with the virus coat (35; C. A. Hutchison, Ph.D. thesis), so that coat protein mutants may well exhibit altered antiserum inactivation kinetics. Furthermore, ϕ X174 host range mutants may also show altered antiserum inactivation (6, 7, 9). However, examination of the responses of *cs70* and *cs82* to inactivating antiserum showed no significant differences from wild-type inactivation, with *k* values of 2.01×10^3 to 2.33×10^3 /min for *cs70*, *cs82*, *am3*, and wild-type.

With regard to host range, many *E. coli* strains were examined including some sensitive only to ϕ X174 host range mutants, but the only strains showing any differences among wild-type, *cs70*, and *cs82* were host cells containing an F⁺ factor. The data in Table 4 showed that in these strains, *cs70* plated relatively better than wild type (which was similar to *cs82*), although all were restricted by the F⁺ factor. The degree of restriction was slight, but similar to a previous observation of ϕ X174 restriction by F⁺ factors (23).

DISCUSSION

Conditional lethal mutations, a powerful genetic technique developed by Campbell (11) for phage λ and by Epstein et al. (17) for phage T4, can define more genes than mutations affecting specific functions. Considerable progress in understanding ϕ X174 replication has been made with such mutants (4, 5, 35, C. A. Hutchison, Ph. D. thesis), but most investigators have concentrated on mutants of the temperature-sensitive and nonsense types. Our laboratory has sought additional types of ϕ X174 conditional lethal mutants, and we have isolated cold-sensitive mutants (15) as well as strains capable of growth at high temperatures (30) and in strains normally excluding ϕ X174 (7).

The present paper extends the initial description (15) of the cold-sensitive ϕ X174 mutants *cs70* and *cs82*. We show that the *cs70* mutant was blocked in eclipse at 25 C (Fig. 1) but was capable of wild-type replication at this temperature if allowed prior eclipse at 40 C (Fig. 2). The *cs82* mutant exhibited wild-type eclipse kinetics at both temperatures, but at 25 C was severely impaired in SS DNA synthesis (Fig. 4), with concomitant delayed initiation of intracellular phage production, decreased rate of progeny synthesis, and reduced burst size. The rapid cessation of intracellular *cs82am3* production following temperature downshift late in infection (Fig. 3) shows that the *cs82* gene function was continually required during replication, and that the decreased SS DNA synthesis observed at 25 C was not due simply to a failure to initiate SS DNA synthesis.

At permissive temperature (40 C) both *cs70* and *cs82* grew in a way similar to wild type. We were intrigued by the consistently higher burst sizes of *cs70* relative to wild type (Table 1), even though the rate and quantity of *cs70am3* DNA synthesis was the same as *am3* (Fig. 7). This implied that the *cs70* gene product, in

TABLE 4. Restriction of ϕ X174 by F⁺ factors

Virus	Efficiency of plating		
	Ratio of C-2 ^a to C	Ratio of CRF ^b to CR	Ratio of HF4704F ^{++c} to HF4704
Wild type	0.409	0.421	0.214
<i>cs70</i>	0.693	0.698	0.492

^a F⁺ Derivative of *E. coli* C, obtained from R. L. Sinsheimer.

^b F⁺ Derivative of *E. coli* CR (16), derived by our laboratory and carrying M13 *ts5*, *ts9*.

^c F⁺ Derivative of HF4704 previously derived by our laboratory (10).

FIG. 7. DNA synthesis at 40 C. Starved *E. coli* HF4704 was treated with mitomycin C as described (27) and infected with *am3* (MOI = 4.4), *cs70am3* (MOI = 4.4) or *cs82am3* (MOI = 4.4) at 40 C. Growth was initiated by suspension in C-medium containing 2 μ g of nonradioactive thymine per ml and 10 μ Ci of [3 H]thymidine per ml (specific activity 23 Ci/mmol). Samples (0.5 ml each) were assayed for [3 H] uptake as described (27). The burst sizes were 57 for *am3*, 88 for *cs82am3*, and 245 for *cs70am3*.

addition to controlling eclipse, may also have had a regulatory role in ϕ X174 replication. Further support for this hypothesis was that both *cs70* and *cs82* could replicate at 44 C, a temperature at which wild-type ϕ X174 would not plate. As such, these mutants may be useful in studies of viral replication in host strains temperature-sensitive for DNA synthesis (7, 9, 30; C. E. Dowell, unpublished data).

The ϕ X174 mutations have been assigned to specific genes by both complementation and recombination analysis, although complementation analysis is the more fruitful approach because of low recombination frequencies (29, 40; C. A. Hutchison, Ph. D. thesis) and site-specific effects (4) in ϕ X174. It was our intention to use complementation testing to determine whether *cs70* or *cs82* defined new ϕ X174

functions. Complementation mapping involves mixed-infection under restrictive conditions with the degree of intracellular complementation determined by plating at restrictive conditions. It can be seen that the very nature of the *cs70* defect (inability to eclipse at 25 C) precluded complementation testing of this mutant, since at the restrictive temperature the *cs70* genome cannot even enter the cell to participate in complementation. Since *cs70* was thus not amenable to complementation analysis, non-genetic methods of assigning *cs70* to a genetic function were investigated.

Virus attachment is obviously a property of the virus protein coat, and ϕ X174 mutations affecting attachment are found in coat protein genes (28, 36, 38). The aberrant attachment kinetics of *cs70* at both 25 and 40 C were therefore considered as evidence of an altered *cs70* coat protein. The most commonly used nongenetic criterion to identify coat protein mutants is particle thermal stability. Intact phage are diluted into a chemically defined solution maintained at constant temperature (about 60 C), and the rate of inactivation determined by plating survivors at specific intervals. This treatment has little effect on DNA primary structure or infectivity (6, 18); thus, the inactivating event is the breaking of the phage coat. Mutants with heat stability different from wild-type are thought to have an altered particle structure, and hence a mutated coat protein (33, 35, 39, C. A. Hutchison, Ph. D. thesis). The significantly decreased heat stability of *cs70* in saturated borate solution at 56.5 C was again interpreted as evidence of a coat protein alteration. A previous heat inactivation of *cs70* (15) showed increased heat stability, but that experiment was performed in a different buffer and at a lower temperature.

Benbow et al. (4) have placed *cs70* in gene H on the basis of three- and four-factor crosses, and some of the *cs70* properties support this hypothesis. For example, the only other mutant affecting eclipse is the gene H S13 mutant t1069 (14), and the only other mutant exhibiting such extreme heat sensitivity is the gene H mutant *amN-1*. Also, *cs70* seems to affect the efficiency of maturing progeny DNA into infectious particles (contrast the increased *cs70* burst sizes in Table 1 with equal *cs70* DNA synthesis in Fig. 7), and the three gene H mutants *amN-1*, *am80*, and *ts4* also affect the ability of SS DNA to be matured into infectious virus particles (16, 21, 33).

With regard to *cs82* and conventional genetic approaches, preliminary complementation tests

TABLE 5. Effect of *cs* mutations on co-infecting *am3* replication at 25 C

Virus	MOI	Total burst ^a	<i>am</i> (%)	<i>cs</i> (%)
<i>am3</i>	4.9	193	100	
<i>cs70am3</i>	5.2	386		100
<i>cs82am3</i>	5.6	1.4		100
<i>cs70am3</i> + <i>am3</i>	4.3	294	54 ^b	46 ^b
	4.0			
<i>cs82am3</i> + <i>am3</i>	4.0	38	86 ^c	14 ^c
	3.8			

^a *E. coli* C was infected at 40 C with the desired mutants in the presence of 0.009 M KCN (16). After 15 min at 40 C with mild aeration, growth was initiated by 1:10,000 dilution into KC broth at 25 C. Samples were assayed for intracellular phage at *t* = 180, and total burst size determined by plating on *E. coli* HF4714 at 37 C.

^b *am3* contribution determined by plating at 25 C, *cs70am3* as the difference between 25 C and 37 C values. The calculation considered the efficiency of plating of 0.7 for *am3* at 25 C relative to 40 C.

^c *cs82am3* contribution determined at 44 C (since *am3* will not plate at this temperature), and *am3* contribution as the difference between 37 C and 44 C values. Similar percentages were determined from 37 C versus 25 C plating, as in the prior footnote.

suggested that the *cs82* mutation somehow interfered with coinfecting virus replication. This occurred even when the coinfecting virus was *am3* (Table 5). Although *am3* gave a burst of 193 at 25 C, the presence of co-infecting *cs82am3* reduced the burst size to 38, most of which (86%) was *am3* progeny. This *trans*-dominance effect suggests that the *cs82* defect at 25 C is not due to the *absence* of a necessary gene product, but is due instead to the *presence* of an aberrant gene product capable of inhibiting expression of a co-infecting wild-type genome. The possibility that the *cs82* gene product at 25 C is a protein present in an inactive configuration is supported by the previous downshift experiment (Fig. 3), which showed immediate cessation of intracellular growth following temperature change. These are precisely the effects predicted from changes in protein configuration at different temperatures.

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