Inhibition of Bacteriophage ϕ X174 DNA Replication in *dnaB* Mutants of *Escherichia coli* C

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Bacteriophage ϕ X174 DNA replication was examined in temperature-sensitive *dnaB* mutants of *Escherichia coli* C to determine which stages require the participation of the product of this host gene. The conversion of the infecting phage single-stranded DNA to the double-stranded replicative form (parental RF synthesis) is completely inhibited at the nonpermissive temperature (41 C) in two of the three *dnaB* mutants tested. The efficiency of phage eclipse and of phage DNA penetration of these mutant host cells at 41 C is the same as that of the parent host strain. The defect is most likely in the synthesis of the complementary strand DNA. The semiconservative replication of the double-stranded replicative form DNA (RF replication) is inhibited in all three host mutants after shifting from 30 to 41 C. Late in infection, the rate of progeny single-stranded phage DNA synthesis increases following shifts from 30 to 41 C. Approximately the same amounts of phage DNA and of infectious phage particles are made following the shift to 41 C as in the control left at 30 C. The simplest interpretation of our data is that the product of the host *dnaB* gene is required for ϕ X174 parental RF synthesis and RF replication, but is not directly involved in phage single-stranded DNA synthesis once it has begun. The possible significance of the synthesis of parental RF DNA at 41 C in one of the three mutants is discussed.

We have isolated several temperature-sensitive *dna* mutants of *Escherichia coli* C in order to examine the requirements for host DNA synthesis factors in bacteriophage ϕ X174 DNA replication. We have previously demonstrated that the host *dnaC* gene product is required for the semiconservative replication of ϕ X174 double-stranded replicative form DNA (RF replication) (8), and that the host *dnaE* gene product is required for both RF replication and the asymmetric semiconservative synthesis of single-stranded phage DNA (single strand synthesis) late in infection (4). Neither host gene product is apparently required for conversion of the infecting single-stranded phage DNA to the double-stranded replicative form (parental RF synthesis). The role that these essential host gene products play in DNA replication in general can perhaps be determined by a detailed examination of their participation in ϕ X174 RF replication, the stage of ϕ X174 DNA synthesis most closely resembling general semiconservative replication of double-stranded DNA.

Previous investigations have shown that host range mutants of ϕ X174 that productively infect *E. coli* K12 are unable to replicate in *dnaB*

mutants of this strain at the nonpermissive temperature (1, 21). Parental RF synthesis is inhibited, although the phage attach to the host cells and eclipse. RF replication and the synthesis of mature phage late in infection are also inhibited.

M13, a small phage carrying a single-stranded circular DNA molecule similar in size to that of ϕ X174, will not replicate in *dnaB* mutants of host strain K12 at the nonpermissive temperature (13). The host *dnaB* gene product is required for RF replication, but not for parental RF synthesis or single-stranded DNA synthesis.

Our *dnaB* mutants of *E. coli* C fall into two classes with respect to effects on ϕ X174 DNA replication. These effects are examined in this report and are compared to those previously described.

MATERIALS AND METHODS

Bacteria and phage strains. LD311, LD312, and LD313 (*uvrA*⁻, *thyA*⁻, *endI*⁻, *dnaB*^{ts}) are nitrosoguanidine-induced temperature-sensitive mutants of H502 (*uvrA*⁻, *thyA*⁻, *endI*⁻) isolated in our laboratory. The temperature-sensitive markers lie

within the region of the chromosome carried by F-prime factor 118, and co-transduce with *mal B101* at frequencies of 0.17, 0.18, and 0.08, respectively. These data indicate that the temperature-sensitive markers map at the *dnaB* locus (22). These mutant strains grow well at 30 C, but cannot form visible colonies on agar medium at 41 C. Cellular DNA synthesis ceases within 15 min after shifting from 30 to 41 C, whereas protein synthesis continues uninhibited for several hours. The efficiency of plating of bacteriophage ϕ X174 on these host strains at 30 C is the same as that on H502. These three mutant strains are used interchangeably in the experiments reported here, and show similar effects on ϕ X174 DNA replication at 41 C except where specifically noted.

E. coli C is the standard ϕ X174 host used here to prepare stocks of ϕ X174*am3* (gene E, lysis defective). HF4714 is an amber suppressor host strain used to measure ϕ X174*am3* plaque-forming units.

Media and buffers. TPGA medium is TPG medium (19) with 1.0 g of KH_2PO_4 and 10 g of Casamino acids per liter. Starvation buffer (SVB) has been described (2).

Phage stocks. Nonradioactive phage were prepared as previously described (3). ^{32}P -labeled phage were prepared as described by Sinsheimer et al. (19), and purified as described by Francke and Ray (5). The specific activity of the purified phage was approximately 10^5 counts/min/plaque-forming unit.

Cell lysis and protease digestion. [^{32}P]phage-infected cells were collected by centrifugation, and washed twice with a solution of 50 mM sodium tetraborate, 6 mM EDTA, pH 9, and once with a solution of 50 mM Tris, 3 mM EDTA, pH 8. When cells were infected with nonradioactive phage, they were washed twice with Tris-EDTA. The washed cells were resuspended in 0.5 ml of 50 mM Tris, 10 mM EDTA, pH 8. A 0.1-volume of 2 mg of lysozyme per ml was added, and the mixture was incubated for 1 h at 0 C. A 0.1-volume of 10% Sarkosyl was then added, and the mixture was incubated overnight at 0 C. A 0.2-ml amount of 5 mg of protease (type VI, Sigma Chemical Co.) per ml was then added, and the mixture was incubated 8 h at 37 C.

Sucrose density gradient analysis. The lysed, protease-digested samples were layered onto 36-ml linear gradients of 5 to 20% sucrose in 50 mM potassium phosphate, 2 mM EDTA, 1 M NaCl, pH 7. The gradients were centrifuged for 16 h at 4 C in a Beckman SW27 rotor at 25,000 rpm. Fractions (0.6 ml) were collected from the bottoms of the tubes into 1-dram glass shell vials. Water (0.4 ml) and scintillation fluid (3 ml) were added, and the radioactivity in each sample was measured in a liquid scintillation spectrometer. ^3H radioactivity was corrected for ^{32}P overlap. The scintillation fluid consisted of 4 g of 2,5-diphenyloxazole per liter in 2 parts toluene to 1 part Triton X100.

Phage DNA penetration of the host cell. The procedures are similar to those described by Newbold and Sinsheimer (12). Host bacterial cultures (50 ml) were grown at 30 C to cell densities of approximately 2×10^8 cells per ml on TPGA medium supplemented with 2 μg of thymine per ml. The cells were collected

by centrifugation and resuspended in 5 ml of SVB. Half of this suspension was immediately treated with mitomycin C as described below. The cells from the other half were again collected by centrifugation and resuspended in SVB. These washed cells were then incubated with aeration for 90 min at 30 C to deplete the intracellular thymine pools.

Mitomycin C (100 $\mu\text{g}/\text{ml}$) was added to each cell suspension. After 20 min at 30 C without aeration, the cells were again collected by centrifugation. Those cells that had been starved for thymine for 90 min were resuspended in 25 ml of SVB. The other cells were resuspended in 25 ml of TPGA supplemented with 2 μg of thymine per ml.

Portions (10 ml each) of these suspensions were preincubated for 30 min at 30 and 41 C. Each culture was then infected with [^{32}P] ϕ X174*am3* (3×10^5 counts/min per PFU) at an MOI of 1. After 30 min at the appropriate temperature, 40 ml of a prewarmed solution of 50 mM sodium tetraborate, 6 mM EDTA, pH 9, were added to prevent further attachment and eclipse. Five minutes later the cultures were chilled on ice. The cells were collected by centrifugation and washed five times with 2-ml portions of borate-EDTA solution. The final cell pellet was resuspended in 1 ml of the same. The radioactivity in 1-ml portions of washes and 0.1 ml of the resuspended cells was measured, and the total radioactivity was computed.

Other methods. The methods of measuring the amount of acid-insoluble radioactivity in intracellular phage DNA and the number of intracellular plaque-forming units have been described (4).

Chemicals [Methyl- ^3H]thymidine (15 Ci/mmol) and [methyl- ^3H]thymine (23 Ci/mmol) were purchased from Amersham/Searle. [^{32}P]phosphoric acid, carrier-free, was purchased from New England Nuclear Corp. Mitomycin C, chloramphenicol, and lysozyme were purchased from Sigma Chemical Co.

RESULTS

Phage production at 41 C. The effect of the host *dnaB* mutations on the synthesis of infective phage particles was tested in three mutant strains. Liquid cultures were treated with mitomycin C as required in other experiments described below to selectively inhibit host DNA synthesis (11). Cell cultures incubated at 30 and 41 C were infected with ϕ X174*am3* (lysis defective), and the number of intracellular infective phage particles was measured as a function of time after infection. The results of one such experiment, shown in Fig. 1, show that phage synthesis is normal at 30 C. Approximately 100 infective phage per cell were synthesized by 2 h after infection. The yield was two- to threefold higher when the host cells were not treated with mitomycin C (data not shown). When bacteria from the same culture were infected and incubated at 41 C, the yield of infectious phage was about 3% that at 30 C. When a portion of the culture infected and incubated at 30 C was

shifted to 41 C at 20 min, the number of infectious phage increased 10-fold in the first 40 min after the shift and remained constant thereafter. When a portion of 30 C culture was shifted to 41 C at 60 min, the number of infective phage increased more rapidly than at 30 C, but the final yield was approximately the same. Phage production at 41 C in temperature-insensitive revertants of these host mutants is the same as that in the parent strain H502 (data not shown).

These results indicate that the host *dnaB* gene product is required for replication of phage ϕ X174.

ϕ X174 parental replicative form synthesis at 41 C. The product of the host *dnaB* gene is required for the replication of the host cell chromosome (7, 22). We examined the effects of the temperature-sensitive *dnaB* mutations in our strains on each of the three stages of bacteriophage ϕ X174 DNA replication (18). The first of the three stages involves the conversion of the infecting single-stranded DNA molecule to its double-stranded replicative form, the parental RF molecule. To examine the effect of the *dnaB* mutations on this stage mitomycin C treated LD311, LD312, and LD313 were infected in three different experiments with 32 P-labeled ϕ X174am3 after preincubation of the cultures at 30 and 41 C for 30 min. [3 H]thymidine was included to label newly synthesized ϕ X174 DNA. Infection was terminated, and the labeled intracellular viral DNA was extracted and examined by zone sedimentation to determine if the infecting phage DNA was converted to the parental replicative form.

The results of the analyses of the extracts of samples of ϕ X174-infected LD311 and LD312 taken at 20 min after infection are shown in Fig. 2. Three distinct viral DNA species are seen in extracts from the 30 C infections: single-stranded DNA, and the two double-stranded forms RFI and RFII (closed, circular supercoils and open, nicked circles, respectively). Under conditions of these analyses these three species sediment at rates of approximately 27, 21, and 16S, respectively. In addition some of the single-stranded virus DNA is found in phage particles which sediment to the bottom of each gradient. In Fig. 2a 32 P-labeled single-stranded ϕ X174 DNA is seen in a band centered about fraction 21, RFI in a band centered about fraction 32, and RFII centered about fraction 38.

Figure 2a shows that infecting 32 P-labeled single-stranded DNA is converted to parental RF at 30 C. Tritium label is also incorporated into parental and progeny RF DNA molecules.

FIG. 1. Replication of ϕ X174am3 in mutant host LD311 at 30 and 41 C. The bacteria were grown at 30 C in 15 ml of TPGA medium supplemented with 2 μ g of thymine per ml to a cell density of approximately 2×10^8 cell per ml. The cells were collected by centrifugation and resuspended in 2 ml of SVB. Mitomycin C was added to 100 μ g per ml. After 20 min at 30 C the cells were again collected by centrifugation and resuspended in 1.6 ml of SVB. Portions of this suspension were incubated 15 min at 30 and 41 C. ϕ X174am3 was added at a multiplicity of infection of 3 (zero time). After 5 min, 9 volumes of prewarmed TPGA medium, supplemented with 2 μ g of thymine per ml, were added. At the indicated times 0.1-ml samples of each culture were transferred into 1.2 ml of lysis buffer. Total plaque-forming units were then determined. Symbols: $\bullet$, PFU per ml from the culture incubated at 30 C; $\circ$, 41 C; $\square$, shifted to 41 C at 20 min postinfection; Δ , shifted to 41 C at 60 min postinfection. The arrows indicate the times of the shifts from 30 to 41 C.

At 41 C (Fig. 2b) no 32 P-label or 3 H-label is seen at the positions of the RF DNA bands, indicating the failure to convert the infecting strand to parental RF in LD311. Comparison of Fig. 2c to d shows that parental RF is not synthesized at 41 C in LD312 either, although it is at 30 C.

Extracts of samples of ϕ X174am3 infected LD313 taken at 15 and 30 min after infection were also analyzed by zone sedimentation. The results are shown in Fig. 3. Parental RF synthesis occurs at 41 C (Fig. 3c and d). In this and similar experiments the efficiency of formation of parental RF DNA at 41 C was 50 to 100% of that at 30 C (Fig. 3a and b). The efficiency is

about the same as that at 30 C in the presence of 150 μg of chloramphenicol per ml (Fig. 3e and f), a drug concentration that allows parental RF synthesis, but markedly reduces the rate of RF replication (9, 16). The efficiency of parental RF synthesis is maintained even after 60 min preincubation of the culture prior to infection (data not shown). Little, if any, RF replication occurs at 41 C as judged by comparing the amount of ^3H -labeled RF DNA in Fig. 3d to that in Fig. 3b.

We conclude that the host *dnaB* gene product is essential for ϕX174 parental RF synthesis, but that not all *dnaB* mutations prevent this synthesis.

ϕX174 attachment, eclipse, and DNA penetration at 41 C. Mutations at the *dnaB* locus cause altered bacterial membrane protein aggregations at the nonpermissive temperature (17). We asked whether the inhibition of ϕX174 parental RF synthesis at 41 C in two of our

dnaB mutants might be explained by the inability to carry out one of the early events in infection occurring on the cell surface, or by a direct effect on the synthesis of the complementary DNA strand on the infecting phage DNA molecule.

Newbold and Sinsheimer (12) have shown that the early events in ϕX174 infection consist of three stages: attachment, eclipse, and DNA penetration. The three stages are distinguished by the kinds of phage particles that can be eluted from the surface of host cells by borate-EDTA washes. Attachment involves no detectable structural change in the phage particle; attached phage can be eluted as infectious particles. Eclipse involves a structural alteration of the phage resulting in partial extrusion of the DNA; eclipsed phage are eluted as noninfectious particles. After DNA penetration only DNA-free phage ghosts can be eluted.

FIG. 2. Zone sedimentation of intracellular phage DNA from $\phi\text{X174am3}$ infected LD311 and LD312. In two separate experiments bacteria were grown on TPGA medium supplemented with 2 μg of thymine per ml at 30 C to cell densities of approximately 3×10^8 cells per ml. The cells were collected by centrifugation, resuspended in starvation buffer, and treated with 100 μg of mitomycin C per ml for 20 min. The cells were again collected by centrifugation and resuspended in one volume of TPGA medium supplemented with 1 μg of thymine per ml. Portions (25 ml) of each culture were incubated at 30 and 41 C with aeration for 30 min. ^{32}P -labeled $\phi\text{X174am3}$ (10 PFU per cell) was then added to each culture. The specific activity of the phage added to the LD311 cultures was 10^{-5} count per min per PFU; that of the phage added to the LD312 cultures was 5×10^{-6} count per min per PFU. ^3H thymidine (0.1 mCi) was added at the same time. After 5 min without aeration and 15 min with aeration the cultures were chilled on ice. The cells were collected by centrifugation, washed, lysed, and the lysates were digested with protease. These lysates were sedimented through linear sucrose gradients and analyzed. (A) LD311 incubated and infected at 30 C; (B) LD311, 41 C; (C) LD312, 30 C; (D) LD312, 41 C. Symbols: $\bullet$, ^3H ; $\square$, ^{32}P .

FIG. 3. Zone sedimentation of intracellular phage DNA extracted from mutant host LD313. A liquid culture of bacteria was grown, treated with mitomycin C, and resuspended in medium as described in Fig. 2. Portions (20 ml each) of the culture were incubated at 30 and 41 C for 30 min. Chloramphenicol (150 μ g/ml) was added to two of the samples at 30 C halfway through the 30-min period. At the end of the 30-min preincubation [32 P] ϕ X174am3 (4×10^{-6} counts/min/PFU) was added at a multiplicity of 10. [3 H]thymidine (10 μ Ci/ml) was added at the same time. At 15 and 30 min after infection 10-ml portions of each culture were chilled rapidly. The cells were collected by centrifugation, washed, lysed, and the lysates were digested with protease. These lysates were sedimented through sucrose gradients and analyzed. (a) 15 min postinfection, 30 C; (b) 30 min, 30 C; (c) 15 min, 41 C; (d) 30 min, 41 C; (e) 15 min, 30 C, 150 μ g of chloramphenicol per ml; (f) 30 min, 30 C, 150 μ g of chloramphenicol per ml. Symbols: ●, 3 H; □, 32 P.

The effects of our *dnaB* mutations on ϕ X174 attachment were examined by measuring the reduction in extracellular phage titer at 30 and 41 C. ϕ X174am3 was mixed with cultures of parent host H502 and mutant host LD312 in 30 μ g of chloramphenicol per ml (to prevent phage synthesis). At various times after addition of the phage, samples were withdrawn into a solution of lysozyme in sodium borate and EDTA. The EDTA concentration was sufficient to cause detachment of both attached and eclipsed particles. The phage titer measured at these times indicates the number of phage that have failed to eclipse.

The results of such an experiment are shown in Fig. 4. The titer of the phage mixed with mutant host LD312 decreased at approximately the same rate as the titer of phage mixed with

parent host H502 at both 30 and 41 C, indicating that the *dnaB* mutant host can eclipse ϕ X174 phage particles as efficiently as the parent host strain at both the permissive and restrictive temperatures. Mitomycin C treatment has no adverse effect on attachment and eclipse. Figure 1 shows that these steps occur normally in mitomycin C treated cells at 41 C. We conclude that the *dnaB* mutation alters neither attachment nor eclipse.

The efficiency of ϕ X174 DNA penetration of the *dnaB* mutant hosts at 30 and 41 C was determined by measuring the amount of 32 P-labeled phage DNA that could not be washed off the surface of the cells. As a control, we measured the amount of 32 P-labeled DNA that could not be eluted from cells that had been starved for thymine and infected in starvation

FIG. 4. Ability of ϕ X174am3 to attach to H502 and LD312 and eclipse at 30 and 41°C. Liquid cultures of the host bacteria were grown at 30°C in TPGA medium supplemented with 10 μ g of thymine per ml to cell densities of approximately 3×10^8 cells per ml. Chloramphenicol was added to 30 μ g/ml. Halves of the culture were incubated 15 min at 30 and 41°C. ϕ X174am3 was added to each at a multiplicity of infection of 3 (zero time). At the indicated times 0.1-ml portions were transferred into 10 ml of 0.05 M sodium tetraborate, 0.01 M EDTA, 2 mg of lysozyme per ml, pH 8. The total PFU were then measured. Symbols: ●, H502, 30°C; ○, H502, 41°C; ▲, LD312, 30°C; △, LD312, 41°C; □, control, without bacteria, 30°C.

buffer. Such cells allow attachment and eclipse, but not DNA penetration. (12). The control numbers, which represent the background level of phage DNA in attached and eclipsed particles that was not eluted from the surface, were then subtracted from the values measured after infection in TPGA medium to give the actual amount of DNA penetration.

The summary of the results of such an experiment are shown in Table 1. The details of the experimental procedure are described in Materials and Methods. The data show that the net penetration of ϕ X174 DNA in mutants LD311 and LD312 is about the same as that in H502 and LD313 at 30 and 41°C. This indicates that ϕ X174 DNA penetrates these *dnaB* mutant cells at 41°C even though it cannot be converted to the parental replicative form.

We conclude that the defect is most likely in the synthesis of the complementary DNA strand on the penetrated virus DNA molecule.

ϕ X174 RF replication at 41°C. The data in Fig. 3 suggest that the second stage of ϕ X174 DNA synthesis, RF replication, is inhibited at 41°C in LD313. We examined the inhibition of this stage in more detail in all three *dnaB*

mutants. Chloramphenicol (30 μ g per ml) was included in the infected cultures to inhibit phage single-stranded DNA synthesis and allow prolonged RF replication (19).

A mitomycin C treated culture of LD311 was infected with ϕ X174am3 at 30°C. At 20 min after the addition of phage half of the culture was shifted to 41°C. At 22, 30, and 50 min after addition of phage portions of the infected cultures at 30 and 41°C were pulse-labeled for 2 min with [3 H]thymidine. The lysates of these pulse-labeled cultures were then examined by zone sedimentation, and the rates of RF replication at these two temperatures were compared.

Figure 5 shows the results of these zone sedimentation analyses. Comparison of Fig. 5a, c, and e shows that the rate of RF replication at 30°C increases with time. At 22, 30, and 50 min after infection, the relative rates are 1.0, 1.9, and 3.4, respectively. Comparison of Fig. 5b, d, and f shows that the rate of RF replication at 41°C decreases markedly by 10 min after the shift. The ratio of the rates of RF replication at 41°C to those at 30°C at 22, 30, and 50 min after infection are 1.7, 0.26, and 0.15, respectively.

A more complete analysis of the inhibition of ϕ X174 RF replication at 41°C is shown in Fig. 6. Host bacteria H502 (a), LD312 (b), and LD313 (c) were treated with mitomycin C and infected with ϕ X174am3 in the presence of 30 μ g of chloramphenicol per ml. At 20 and 40 min after

TABLE 1. ϕ X174am3 DNA penetration of host cells

Host cells, medium, temp	Counts per min		Fraction penetrated [B/(A+B)]	Net penetration ^a
	Total in washes (A)	Total in washed cells (B)		
H502				
TPGA, 30°C	70,970	8700	0.11	0.09
TPGA, 41°C	66,780	11,300	0.14	0.12
SVB, 30°C	89,650	1670	0.018	
SVB, 41°C	85,670	2000	0.023	
LD311				
TPGA, 30°C	65,020	8220	0.11	0.08
TPGA, 41°C	82,220	12,500	0.13	0.08
SVB, 30°C	84,780	2500	0.029	
SVB, 41°C	94,340	4740	0.048	
LD312				
TPGA, 30°C	59,440	5750	0.088	0.05
TPGA, 41°C	109,200	21,200	0.16	0.11
SVB, 30°C	106,800	4310	0.039	
SVB, 41°C	96,280	4880	0.048	
LD313				
TPGA, 30°C	48,360	3460	0.067	0.02
TPGA, 41°C	70,190	8080	0.10	0.07
SVB, 30°C	69,470	3530	0.048	
SVB, 41°C	94,840	3260	0.033	

^a Net penetration equals fraction penetrated in TPGA minus that in SVB.

FIG. 5. Zone sedimentation of intracellular phage DNA from ϕ X174am3 infected LD311 pulse-labeled during RF replication. A 60-ml culture of bacteria was grown at 30 C to a cell density of about 4×10^8 cells per ml and treated with mitomycin C as described in Fig. 2. The cells were resuspended in 60 ml of TPGA medium containing 1 μ g of thymine and 30 μ g of chloramphenicol per ml. ϕ X174am3 (3 PFU per cell) was added. The culture was incubated 5 min at 30 C without aeration and aeration was then resumed. One half of the culture was shifted to 41 C 15 min later. At 22, 30, and 50 min after addition of the phage 0.1 mCi of [3 H]thymidine was added to 10-ml portions of each of the two cultures. Two minutes later 10 ml of cold acetone were added to terminate the pulse. The cells were collected by centrifugation, washed, lysed, and digested with protease. The lysates were sedimented through linear sucrose gradients and analyzed. (a) Incubated at 30 C, pulse-labeled at 22 min postinfection; (b) 41 C, 22 min postinfection; (c) 30 C, 30 min postinfection; (d) 41 C, 30 min postinfection; (e) 30 C, 50 min postinfection; (f) 41 C, 50 min postinfection. Arrows indicate the peak positions of added 32 P-labeled ϕ X174 virus DNA marker.

infection portions of each culture at 30 C were shifted to 41 C. At regular intervals the rate of ϕ X174 RF replication was measured in 2-ml portions of each culture by pulse-labeling with [3 H]thymidine for 2 min. The amounts of alkali-stable, acid-insoluble radioactivity incorporated during the pulses were measured. These values are plotted against time after addition of phage.

In the parent host strain H502 the rate of ϕ X174 RF replication increases with time after infection, reaching a maximum at approximately 50 min. This maximum rate is achieved as early as 40 min and as late as 60 min after infection in similar experiments. Parental RF synthesis is complete by 30 to 40 min after

infection at 30 C in all hosts as judged by the accumulation of 32 P-label in ϕ X174 RF DNA upon infection with 32 P-labeled phage (data not shown). Thus, part of the increase in rate is probably due to continued synthesis of parental RF DNA, the template for RF replication, during the first 30 to 40 min. The remainder of the rate increase could be due to participation of daughter RF molecules in RF replication. In the time period after 50 min postinfection the rate of RF replication decreases continuously. The rate of RF replication at 41 C, following the temperature shift from 30 C, achieves its maximum earlier than at 30 C.

Comparison of Fig. 6b and c to a shows that the rate of RF replication in *dnaB* mutant hosts

FIG. 6. Rate of ϕ X174am3 RF DNA replication in host bacteria treated with chloramphenicol. Host bacterial strains H502, LD312, and LD313 were grown at 30 C on TPGA medium (80 ml) supplemented with 2 μ g of thymine per ml to cell densities of approximately 3×10^8 cells per ml. These cultures were treated with mitomycin C as described in Fig. 2, and resuspended in 5 ml of TPGA medium. ϕ X174am3 was added at a multiplicity of 3 (zero time). After 5 min at 30 C 75 ml of TPGA medium supplemented with 1 μ g of thymine per ml were added. Chloramphenicol was added to 30 μ g/ml. At 20 and 40 min after infection portions of the culture were shifted to 41 C. At 10-min intervals 2 ml of each culture were transferred into separate vessels containing 10 μ Ci of [3 H]thymidine in 0.5 ml of TPGA medium and incubated for 2 min at either 30 or 41 C. Cold acetone (2.5 ml) was added to terminate the pulse. The cells were collected by centrifugation and resuspended in 1 ml of 0.05 M sodium tetraborate, 0.01 M EDTA, pH 8. Lysozyme (200 μ g) was added, and the mixtures were incubated 15 min at room temperature. A 0.1-ml amount of 3 N KOH was then added, and the lysates were incubated 14 h at 30 C. Calf thymus DNA carrier (200 μ g) and 2 ml of cold 10% trichloroacetic acid were added. The amount of acid-insoluble radioactivity was then determined. (a) H502; (b) LD312; (c) LD313. Symbols: ●, 30 C; ○, shifted to 41 C at 20 min; Δ, shifted to 41 C at 40 min.

rapidly decreases upon shifting from 30 to 41 C. DNA synthesis continues at a reduced rate for at least 1 h. It is not clear whether this slow synthesis at 41 C represents RF replication, or some abnormal DNA synthesis.

We conclude that the host *dnaB* gene product is required for the semiconservative replication of ϕ X174 RF DNA.

ϕ X174 single-stranded DNA synthesis at 41 C. The effect of the temperature-sensitive *dnaB* mutations on ϕ X174 single-stranded DNA synthesis, the third stage, was examined at times later than 45 min after phage infection. By this time at 30 C single-stranded ϕ X174 DNA and infectious phage particles are being synthesized (see Fig. 1). The onset of phage single-stranded DNA synthesis coincides with the cessation of net phage and host cell double-stranded DNA replication (10). Portions of the infected cultures were shifted from 30 to 41 C at 45 min after infection. The rates of phage DNA synthesis at these two temperatures were measured. Figure 7 shows the rates of ϕ X174 DNA synthesis in LD311 and the temperature-insensitive revertant strain LD311 R. In both hosts the rate of ϕ X174 single-stranded DNA synthesis increases markedly after the shift to 41 C. After approximately 20 to 30 min at this temperature the rate begins to decrease. Similar experiments using parental host strain H502 and the other *dnaB* mutants (data not shown)

indicate the same pattern of change in rate of ϕ X174 single-stranded DNA synthesis with time after infection. The initial increase in rate at 41 C is followed by a rapid decrease in rate. The rate of synthesis at 41 C sometimes declines more rapidly in the *dnaB* mutants, as seen in Fig. 7.

Figure 8 shows zone sedimentation analyses of the ϕ X174 intracellular DNA pulse-labeled in similar experiments. These data show the same increase in rate of synthesis after shifting 41 C, and the decrease in rate that follows. These data also show that the products labeled at 41 C are single-stranded phage DNA and the RF DNA templates, the same species synthesized at 30 C. This is consistent with the data in Fig. 1 which show that mature phage continue to be synthesized after shifts to 41 C.

The data from Fig. 7 and 8 indicate that ϕ X174 single-stranded DNA is made faster following shifts to 41 C late in infection than at 30 C. The data in Fig. 1 show that the rate of mature phage synthesis following such a late shift is also increased, and that the final yield is the same as that in the culture kept at 30 C. The decline in rate of ϕ X174 single-stranded DNA synthesis after 20 to 30 min at 41 C is seen in the *dnaB* mutant hosts, the revertant hosts, and the parent strain H502. ϕ X174 single-stranded DNA synthesis is not significantly more temperature sensitive in the *dnaB* mutant

hosts than in the revertants and the parent strain, in contrast to the temperature sensitivity of RF replication. We conclude, therefore, that the host *dnaB* gene product is not required for this synthesis.

DISCUSSION

Bacteriophage ϕ X174 cannot replicate in our temperature-sensitive *dnaB* mutants of *E. coli* C at 41 C (Fig. 1). The phage attaches to the host cell and eclipses normally at 41 C (Fig. 4). The phage DNA penetrates the mutant host cells as efficiently as 41 C as at 30 C (Table 1). The inability to produce phage is due to inhibition of DNA synthesis. The synthesis of ϕ X174 parental RF DNA molecules is inhibited at 41 C in mutant strains LD311 and LD312 (Fig. 2), but not in LD313 (Fig. 3). The replication of the replicative form is inhibited at 41 C in all three host mutants (Fig. 5 and 6). The rate of single-stranded phage DNA synthesis late in infection increases after a shift from 30 to 41 C. After 20 to 30 min at 41 C the rate decreases. The same pattern is seen in temperature-insensitive revertants and in the parent host strain H502 (Fig. 7 and 8; unpublished data). The simplest interpretation of these observations is that the host *dnaB* gene product is required for ϕ X174 parental RF DNA synthesis and RF replication, but not for the synthesis of progeny phage single-stranded DNA once it has begun.

The three host *dnaB* mutants can be divided into two classes on the basis of their effects on ϕ X174 parental RF synthesis; one class prevents this synthesis at 41 C, the other does not. There are also two classes of *dnaB* mutants which differ in their ability to support bacteriophage λ DNA synthesis (6, 23). Wechsler has shown that 17 *dnaB* alleles fail to complement one another (23). This suggests, but does not prove, that the *dnaB* locus as defined by these alleles is a single gene. Assuming that the *dnaB* mutations in our three strains are all in the same gene, our observations would suggest that different alterations in the same gene product cause different effects on ϕ X174 parental RF synthesis, but have the same effect on RF replication. The *dnaB* protein is perhaps part of an aggregate of proteins that coordinately catalyzes ϕ X174 RF replication. Mutations that alter the *dnaB* protein structure would have unusually large effects on the ability to replicate ϕ X174 DNA if the conformation of the protein aggregate were critically dependent on the structure of this protein. The *dnaB* protein might act more independently in the catalysis of ϕ X174 parental RF synthesis such that minor

changes in its structure would not result in inhibition (as in LD313).

The possibility that the genes that code for the temperature-sensitive proteins in mutant host strains LD311 and LD312 versus LD313 are actually different cannot be excluded. If this were the case, we would conclude that only one of these gene products is required for ϕ X174 parental RF synthesis.

Our observations are consistent in part with those of Steinberg and Denhardt (21) who showed that reproduction of a host range mutant of ϕ X174 was temperature sensitive in a mutant of *E. coli* K12. The temperature-sensitive mutation has since been mapped at the *dnaB* locus (22). They observed that ϕ X174 parental RF synthesis was prevented at the nonpermissive temperature, and that little or no RF DNA replication occurred. Phage production was abruptly terminated upon shifts to the nonpermis-

FIG. 7. Rate of ϕ X174 DNA synthesis at 30 and 41 C. Liquid cultures (50 ml) of LD311 and LD311R, a spontaneous temperature-insensitive revertant of LD311, were incubated at 30 C until the cell density reached approximately 3×10^8 cells per ml. The cells were collected by centrifugation, resuspended in 5 ml of starvation buffer, and treated with mitomycin C as described in Fig. 2. The cells were again collected by centrifugation and resuspended in 5 ml of TPGA supplemented with 1 μ g of thymine per ml. ϕ X174am3 was added at a multiplicity of infection of 3. After 5 min without aeration, 45 ml of the same medium was added. The cultures were aerated at 30 C. At 45 min after infection half of each culture was shifted from 30 to 41 C. At 10-min intervals the rate of ϕ X174 DNA synthesis was measured as described in Fig. 6. (a) LD311R; (b) LD311. Symbols: $\bullet$, 30 C; $\circ$, shifted to 41 C at 45 min postinfection.

FIG. 8. Zone sedimentation of intracellular phage DNA extracted from ϕ X174am3 infected LD311 pulse labeled during single-stranded DNA synthesis. Bacteria were grown at 30 C on TPGA medium (40 ml) supplemented with 2 μ g of thymine per ml to a cell density of approximately 4×10^8 cells per ml. The cells were collected by centrifugation and treated with mitomycin C in starvation buffer as described in Fig. 2. The cells were again collected by centrifugation and resuspended in 4 ml of TPGA. ϕ X174am3 was added at a multiplicity of infection of 3 (zero time). After 5 min at 30 C 36 ml of TPGA supplemented with 2 μ g of thymine per ml were added. At 45 min postinfection 30 ml of the culture were shifted to 41 C. At 55, 65, and 85 min postinfection 50 μ Ci of [3 H]thymidine were added to 10-ml portions of the culture at 41 C. At 85 min postinfection the same was added to 10 ml of the culture at 30 C. One minute after the addition of the radioactive label, 10 ml of cold acetone was added to terminate the pulse. The cells were collected by centrifugation, washed, lysed, and digested with protease. The lysates were sedimented through linear sucrose gradients and analyzed (1.2-ml fractions were collected). (a) Incubated at 30 C, pulse labeled at 85 min postinfection; (b) 41 C, 55 min postinfection; (c) 41 C, 65 min postinfection; (d) 41 C, 85 min postinfection. Arrows indicate the peak positions of added 32 P-labeled ϕ X174 virus DNA marker.

sive temperature late in infection, which they interpreted as indicating inhibition of phage single-stranded DNA synthesis. Our data suggest no requirement for the host *dnaB* gene product in this late single-stranded DNA synthesis. Direct evidence indicates that single-stranded DNA synthesis is not inhibited at the nonpermissive temperature when a host strain carrying the same *dnaB* mutation as that used by Steinberg and Denhardt is infected with a different host range mutant of ϕ X174 (C. Dowell, personal communication). The host *dnaB*

gene product probably plays no direct role in phage single-stranded DNA synthesis.

ϕ X174 parental RF DNA synthesis is defective at the nonpermissive temperature in an unmapped mutant of *E. coli* 15 (18). Progeny phage single-stranded DNA synthesis is apparently not dependent upon the activity of the temperature-sensitive gene product (14). These effects on ϕ X174 DNA replication are similar to those in our *dnaB* mutants LD311 and LD312.

The replication of bacteriophage M13 DNA has been examined in a *dnaB* mutant of *E. coli*

K12 (13, 20). The synthesis of M13 parental RF DNA molecules occurs at the nonpermissive temperature, as does progeny phage single-stranded DNA synthesis. M13 RF DNA replication is markedly inhibited. Under certain conditions conversion of ϕ X174 single-stranded DNA to a double-stranded molecule in cell-free extracts of *E. coli* requires the *dnaB* gene product, whereas the conversion of M13 single-stranded DNA does not (15, 24). These data suggest that M13 parental RF synthesis *in vivo* occurs via a different pathway from that of ϕ X174.

The apparent protein product of the *dnaB* gene has recently been isolated (25). Its actual role in DNA replication has not yet been determined.

The host *dnaB*, *dnaC* (8), *dnaE* (4), and *dnaG* (G. McFadden and D. Denhardt, personal communication) gene products are all required for ϕ X174 RF DNA replication. Further examination of the mechanism of ϕ X174 RF replication and the roles of these host gene products in it should provide information useful to the explanation of the general mechanism of the semi-conservative replication of double-stranded DNA.

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