Replication of Bacteriophage ϕ X174 DNA in a Temperature-Sensitive *dnaC* Mutant of *Escherichia coli* C

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Received for publication 13 June 1973

Bacteriophage ϕ X174 cannot grow in a temperature-sensitive *dnaC* mutant of *Escherichia coli* C at the nonpermissive temperature. The inability to grow is the result of inhibition of virus DNA synthesis. Parental replicative form synthesis is not temperature sensitive. Single-stranded virus DNA continues to be synthesized for at least 45 min after shifting to the nonpermissive temperature late in infection. In contrast, the replication of the replicative form terminates within 5 min after shifting to the nonpermissive temperature.

Many temperature-sensitive mutants of *Escherichia coli* have recently been isolated that are defective in DNA replication. Several of these mutations specifically affect the initiation of DNA replication (2, 13). These initiation mutants map at two different loci on the *E. coli* chromosome, *dnaA* and *dnaC* (9, 26).

When examining the roles of the products of these genetic loci in the initiation of DNA replication, it is desirable to determine the effects of these mutations on the replication of a small DNA molecule whose replicating intermediates can be isolated intact. The bacteriophage ϕ X174 DNA molecule, and its replicating intermediates, can easily be isolated and characterized. This virus carries a limited amount of genetic information, and must therefore use the host cell DNA synthesis system to replicate its DNA. The ϕ X174 chromosome should be a useful probe in this examination.

We have isolated a temperature-sensitive *dnaC* mutant of *E. coli* C, the normal ϕ X174 host strain. We report here the effects of this mutation on the replication of the DNA of this virus in vivo.

MATERIALS AND METHODS

Bacteria and phage strains. LD331 (*uvrA*⁻, *thyA*⁻, *endI*⁻, *dnaC*^{ts}) is a nitrosguanidine-induced, temperature-sensitive mutant of H502 (*uvrA*⁻, *thyA*⁻, *endI*⁻) isolated in our laboratory. The temperature sensitive locus co-transduces with *dnaA* at a frequency of about 30%, thus identifying this locus as *dnaC* (26). The mutant bacteria grow well at 30°C but cannot form visible colonies on agar medium at 41°C. The DNA synthesis in this mutant continues in liquid culture at a decreasing rate for approximately 50 min after shifting from 30 to 41°C. The rate of protein synthesis is greater at 41°C than at 30°C. This strain

does not suppress amber mutants of bacteriophage ϕ X174. A spontaneous nontemperature-sensitive revertant of LD331, LD331R, was isolated and served as a control in the experiments described here. The efficiency of plating of ϕ X174 on LD331, LD331R, and H502 is the same. The details of the isolation of mutant LD331 and its characterization will be published elsewhere.

Strain C is the standard ϕ X174 host used here to prepare ϕ X174am3 stocks. HF4714 is an amber suppressor ϕ X174 host strain used to measure plaque-forming units and to prepare ϕ X174am9 stocks. ϕ X174am3 is an amber mutant in gene E (lysis defective). ϕ X174am9 is an amber mutant in gene G (spike protein). *E. coli* C, HF4714, and H502, and phages ϕ X174am3 and ϕ X174am9 were obtained from R. L. Sinsheimer.

Media and buffers. TPGA medium is TPG medium (23) with 1.0 g of KH₂PO₄ and 10 g of Casamino Acids per liter. Starvation buffer (3) has been described.

Growth and purification of ϕ X174. Nonradioactive phage were prepared as previously described (6). ³²P-labeled phage were grown as described by Sinsheimer, et al. (23) and were purified as described by Francke and Ray (7). The specific activity of the purified phage was approximately 10⁻⁵ counts per min per PFU.

Cell lysis and Pronase digestion. The methods used were those of Dumas and Miller (6a). Infected cells were lysed with lysozyme, EDTA, and Sarkosyl at 0°C. The lysates were then treated with Pronase at 37°C.

Sucrose density gradient analysis. Lysed, Pronase-digested samples of phage infected cells were layered onto 36-ml linear gradients of 5 to 20% sucrose in 0.05 M potassium phosphate, 2 mM EDTA, 1 M NaCl, pH 7.5. These gradients were centrifuged for 16 h at 25,000 rpm at 4°C in a Spinco SW27 rotor. Fractions of approximately 0.7 ml were collected from the bottoms of the centrifuge tubes. Portions of each fraction were diluted with water, and mixed with 3 ml

of scintillation fluid in 1-dram glass shell vials. The radioactivity in each vial was determined in a liquid scintillation spectrometer. ^3H -radioactivity was corrected for ^{32}P -overlap. The scintillation fluid consisted of 4 g of 2,5-diphenyloxazole (PPO) per liter in toluene: Triton X-100 (2:1).

Other methods. Measurements of acid-insoluble radioactivity and intracellular PFU were carried out as previously described (6a).

Chemicals. Thymine-methyl- ^3H , 23 Ci/mmol, and thymidine-methyl- ^3H , 18 to 25 Ci/mmol, were purchased from Amersham/Searle. ^{32}P -phosphoric acid, carrier free, was purchased from New England Nuclear Corp. Mitomycin C, chloramphenicol, egg white lysozyme, and Pronase (protease type VI) were purchased from Sigma Chemical Co.

RESULTS

Phage growth at 41 C. The effect of the *dnaC* mutation on the growth of ϕ X174 was tested in liquid cultures of host mutant LD331 incubated at 30 and 41 C. Figure 1 shows the effect of the temperature-sensitive mutation on the yield of mature phage. Phage growth is normal at 30 C, the yield being 500 PFU per cell after 3 h. After the 15-min adsorption period at 30 C in starvation buffer (zero time) a portion of the infected culture was shifted to 41 C. Phage synthesis is slower at this temperature and stops after 1 h. The yield is only 3 PFU per cell. When in a separate experiment a culture was shifted to 41 C at the time of addition of the phage, the yield was only about 0.05 PFU per cell (data not shown).

Portions of the infected culture were also shifted to 41 C at 15 and 45 min. Figure 1 shows that phage synthesis terminates approximately 40 min after the shifts.

These results indicate that the *dnaC* gene product of the host bacterium is required for the synthesis of progeny phage.

ϕ X174 DNA synthesis at 41 C. The product of the *dnaC* gene is required for the initiation of the replication of the host bacterial chromosome (2, 21). The effect of the *dnaC*^{ts} mutation on ϕ X174 DNA replication was examined in phage infected LD331. ^3H -thymine incorporation into newly synthesized ϕ X174 DNA was measured at 30 and 41 C. The host cells had been treated with mitomycin C prior to infection to specifically inhibit host DNA synthesis (19).

Figure 2 shows the results of such an experiment. The amount of phage DNA synthesized in host cells infected at 41 C is no higher than that in uninfected cells at 30 and 41 C. No detectable phage DNA synthesis occurs at 41 C.

The amount of phage DNA synthesis in a portion of the culture shifted to 41 C at 15 min

FIG. 1. Growth of ϕ X174am3 in mutant host LD331 at 30 and 41 C. The bacteria were grown to a cell density of 2×10^8 cells per ml on TPGA medium supplemented with $10 \mu\text{g}$ of thymine per ml. These cells were collected by centrifugation and resuspended in one-tenth volume of starvation buffer. ϕ X174am3 was added at a multiplicity of infection of 5. One volume of TPGA medium was added (zero time) after 15 min at 30 C without aeration. Portions of the infected culture were incubated at 30 and 41 C. At 15 and 45 min portions of the 30 C culture were shifted to 41 C. Samples (0.1 ml) were transferred into 1.2 ml of lysis buffer at the indicated times. The total PFU were then determined. Symbols: ●, PFU per ml from a culture incubated at 30 C; ○, PFU per ml from a culture infected at 30 C and shifted to 41 C at zero time; □, PFU per ml from a culture shifted to 41 C at 15 min; Δ, PFU per ml from a culture shifted to 41 C at 45 min. Arrows indicate the times of the temperature shifts.

is no higher than the background level of synthesis in the uninfected cultures. However, when shifted to 41 C at 45 min, phage DNA synthesis continues at a decreasing rate for approximately 1 h. Uninfected host bacteria, free of mitomycin C, cease net DNA synthesis at approximately 50 min after a shift from 30 to

FIG. 2. ^3H -thymine incorporation into phage DNA in $\phi\text{X174am3}$ -infected cells. A culture of LD331 was grown to a cell density of 3×10^8 cells per ml at 30 C on TPGA medium supplemented with 2 μg of thymine per ml. The cells were collected by centrifugation, washed in starvation buffer, and resuspended in one-tenth volume of the same. Mitomycin C was added to 0.1 mg per ml. After 20 min in the dark without aeration, the cells were collected and resuspended in one-tenth volume of starvation buffer. Phage were added to three-quarters of this suspension at a multiplicity of 3. Both the infected and uninfected suspensions were incubated at 30 C for 15 min without aeration. At zero time one volume of TPGA medium supplemented with 1 μg of thymine and 10 μCi of ^3H -thymine per ml was added. Portions of each culture were incubated at 30 and 41 C. At 15 and 45 min portions of the 30 C infected culture were shifted to 41 C. Samples (0.1 ml) were removed from each culture at regular intervals and assayed for acid-insoluble radioactivity. Symbols: $\bullet$, 30 C, infected; $\circ$, 30 C, uninfected; $\blacktriangle$, 41 C, infected; $\triangle$, 41 C, uninfected; $\square$, shifted to 41 C at 15 min; $\blacksquare$, shifted to 41 C at 45 min. Arrows indicate times of shifts.

41 C under similar experimental conditions (unpublished observations).

These data indicate that the host *dnaC* gene product is required for ϕX174 DNA synthesis.

Parental replicative form synthesis at 41 C. The replication of the single-stranded circular ϕX174 DNA occurs in three stages (22): parental replicative form (RF) synthesis—the synthesis of the complementary strand on the infecting virus strand template; the replication of the replicative form yielding approximately 20 progeny RF molecules per cell; the synthesis of the single-stranded virus DNA using the progeny RF molecules as template. The effect of the *dnaC* mutation in host strain LD331 on each of the three stages of ϕX174 DNA replication is described below.

To test the effect of the *dnaC* mutation on parental RF synthesis, cultures of LD331 were grown at 30 C and preincubated at 30 and 41 C prior to infection with ^{32}P -labeled $\phi\text{X174am3}$. The viral DNA extracted from these infected cells was then examined by zone sedimentation analysis to determine if the infecting virus DNA strand could be converted to RF at 41 C. The results of these analyses are shown in Fig. 3. Three distinct viral DNA species are seen in each gradient: single-stranded DNA and the two double-stranded forms RFI and RFII (closed, circular supercoils and open, nicked circles, respectively). Under the 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. 3A single-stranded ^{32}P -labeled ϕX174 DNA is seen in a band centered about fraction number 20, RFI in a band centered about fraction 29, and RFII in a band centered about fraction 33. ^3H -labeled viral DNA is also seen representing progeny RF molecules and single-stranded virus DNA synthesized during the 15 min infection period.

Comparison of Fig. 3A to 3B, 3C to 3D, and 3E to 3F shows that ^{32}P -labeled parental strand ϕX174 DNA is converted to RF at least as well at 41 C as at 30 C. The amount of ^{32}P -label found in ϕX174 RF DNA extracted from cells infected at 41 C is equal to or greater than that in RF DNA extracted from cells infected at 30 C. When parental RF synthesis at 30 C in mutant strain LD331 is compared to that in the nontemperature-sensitive revertant strain LD331R (Fig. 4A and B), it is seen that the amount of parental RF formed in the mutant is 80% of that in the revertant. Comparison of Fig. 4C to D shows that amount of parental RF

FIG. 3. Zone sedimentation of parental phage DNA from infected LD331. A culture (60 ml) of bacteria was grown at 30 C to a cell density of 3×10^8 cells per ml on TPG medium supplemented with 2 μ g of thymine and 2 mg of Casamino Acids per ml. One-half of the culture was shifted to 41 C. Incubation was continued at these two temperatures for 20 min (A and B, 30 and 41 C, respectively), 60 min (C and D), and 120 min (E and F) prior to infection. (In each case 0.1 mg of mitomycin C per ml was added to 10-ml portions at 20 min prior to infection.) 32 P- ϕ X174am3 was added at a multiplicity of 8. The specific activity of the phage was approximately 1.5×10^{-5} counts per min per PFU. 3 H-thymidine (20 μ Ci/ml) was added at the time of infection. Each culture was incubated 5 min without aeration, 15 min with aeration, and rapidly chilled. The infected cells were collected by centrifugation, washed, and lysed. The lysates were digested with Pronase and sedimented through linear sucrose gradients as described in Materials and Methods. The total radioactivity was determined in each fraction diluted with 0.3 ml water. Symbols: —, 3 H; ---, 32 P.

synthesized at 30 C in the mutant host is about half that in the parent host strain H502. In this and similar experiments the amounts of parental RF synthesized in the mutant host and its revertant are approximately the same, whereas that synthesized in the parent strain is one to two times that in the mutant.

The lysates of the mutant strain reproducibly contain more free single-stranded 32 P-labeled ϕ X174 DNA than those of the revertant and parent host strains. This might reflect less effective removal during the borate-EDTA washes of phage particles which have attached to the surface of the mutant cells and have failed to inject their DNA.

We conclude from these data that parental RF synthesis is not temperature sensitive in host strain LD331. This synthesis is partially defective at 30 C in LD331 and the revertant strain LD331R when compared to the parent host strain H502, but this is not a defect due to the *dnaC*^{ts} mutation. The host *dnaC* gene

product is not essential for ϕ X174 parental RF synthesis.

ϕ X174 RF replication at 41 C. Figure 3 shows that RF replication is inhibited during the first 15 min of infection at 41 C in host mutant LD331. The amount of 3 H-labeled RF synthesized at 41 C is less than that synthesized at 30 C. This inhibition was examined further in infected cultures containing 30 μ g of chloramphenicol per ml. This concentration of chloramphenicol inhibits ϕ X174 single-stranded DNA synthesis and allows RF replication to continue for several hours in wild-type host cells (23). At 30 min after infection with ϕ X174am3, one-half of the culture was shifted to 41 C. Portions of these cultures were then pulse labeled with 3 H-thymidine to measure the relative rates of RF replication at these two temperatures.

Figure 5 shows the results of zone sedimentation analysis of the lysates of portions of these cultures pulse labeled for 1 min at 5, 20, and 45 min after the time of the temperature shift. The

FIG. 4. Zone sedimentation of parental phage DNA from infected mutant host LD331, the nontemperature sensitive revertant host LD331R, and the parent host strain H502. Cultures of bacteria were grown at 30 C to a cell density of 3×10^8 cells per ml as described in Fig. 3. Incubation was continued at 30 C for 60 min. In each case 70 μ g of mitomycin C per ml was added to 15-ml portions at 20 min prior to infection. In one experiment LD331 (A) and LD331 R (B) were each infected with ^{32}P - $\phi\text{X174am3}$ (specific activity 10^{-5} counts per min per PFU) at a multiplicity of 10. ^3H -thymidine (13.3 μCi per ml) was added at the time of infection. In a second experiment LD331 (C) and H502 (D) were each infected at a multiplicity of 10 with ^{32}P - $\phi\text{X174am3}$ with a specific activity of 5×10^{-6} counts per min per PFU. ^3H -thymidine (10 μCi per ml) was added at the time of infection. At 20 min after infection 15-ml portions of each culture were rapidly chilled. The infected cells were collected by centrifugation, washed two times in 0.05 M sodium tetraborate, 0.006 M EDTA, pH 9, and once in 0.05 M Tris, 0.003 M EDTA, pH 8.1. The washed cells were lysed, digested with Pronase, and heated at 56 C for 20 min. The lysates were sedimented through linear sucrose gradients as described in Materials and Methods. Symbols: —, ^3H ; ---, ^{32}P .

amount of radioactive label in the RF region in Fig. 5B is only about 40% of that in the RF region in Fig. 5A. Thus the rate of RF synthesis at 41 C at 5 min after the shift is maximally 40% that at 30 C. At 20 and 45 min after the shift the rates of RF synthesis at 41 C are maximally 30 and 20% the rates at 30 C, respectively. Note however, that the amount of radioactive DNA that cosediments with the single-stranded ϕX174 DNA marker is greater in the lysates of the 41 C culture than in the lysates of the 30 C culture. It is possible that most of the RF labeled at 41 C functions as template for a low level of single-stranded DNA synthesis rather than RF replication.

To test this possibility host mutant LD331 was infected with $\phi\text{X174am9}$. This mutant phage is defective in single-strand ϕX174 DNA synthesis (10, C. A. Hutchison III, Ph.D. thesis, Cal. Inst. Tech., Pasadena, 1969). The results

of the zone sedimentation analyses of viral DNA extracted from $\phi\text{X174am9}$ infected LD331 are shown in Fig. 6. All conditions of this experiment are the same as described in Fig. 5. There is no detectable radioactive label in RF DNA in the extracts of the culture pulse-labeled at 41 C (Fig. 6B, D, and F). This indicates that little or no RF replication occurs at 41 C. There is some radioactively labeled material that sediments in a broad band at the same rate as the virus DNA marker. The amount of radioactivity in this material is no greater in the lysates of the culture pulse labeled at 41 C than in the lysates of the culture pulse labeled at 30 C. In contrast, the lysates of the $\phi\text{X174am3}$ infected culture pulse labeled at 41 C contained more radioactivity in this material than the lysates of the same culture pulse labeled at 30 C (Fig. 5). We conclude therefore that the radioactive label incorporated into RF DNA at 41 C in the

FIG. 5. Zone sedimentation of phage DNA from ϕ X174am3 infected LD331 pulse labeled during RF replication. A liquid culture (120 ml) of LD331 was grown at 30 C to a cell density of 3×10^8 cells per ml on TPGA medium supplemented with 2 μ g of thymine per ml. The cells were collected by centrifugation and resuspended in one-tenth volume of starvation buffer. Mitomycin C was added to 0.1 mg per ml. After 20 min in the dark without aeration the cells were collected and resuspended in one volume of TPGA medium supplemented with 1 μ g of thymine and 30 μ g of chloramphenicol per ml. Phage were added at a multiplicity of 3. The culture was incubated at 30 C for 10 min without aeration and 30 min with aeration. Half of the culture was then shifted to 41 C. At 5, 20, and 45 min after the shift 20-ml portions of the 30 and 41 C cultures were pulse labeled for 1 min with 200 μ Ci of 3 H-thymidine. The labeled cultures were rapidly chilled by the addition of an equal volume of acetone chilled in a methanol-dry ice bath. The cells were then collected, washed, lysed, digested with Pronase, and sedimented through sucrose gradients as described in Materials and Methods. Arrows indicate the peak positions of added 32 P-labeled ϕ X174 virus DNA marker. Total radioactivity was determined in each fraction diluted to 1 ml with water. A, 5 min, 30 C; B, 5 min, 41 C; C, 20 min, 30 C; D, 20 min, 41 C; E, 45 min, 30 C; F, 45 min, 41 C.

FIG. 6. Zone sedimentation of phage DNA from ϕ X174am9 infected LD331 pulse labeled during RF replication. The conditions were identical to those described in Fig. 4.

ϕ X174am3 infected cells (Fig. 5) was incorporated into RF templates for single-stranded DNA synthesis. ϕ X174 RF replication in LD331 ceases within 5 min after the shift to 41 C.

The relative rates of RF replication were measured at 30 and 41 C in the nontemperature-sensitive revertant LD331R. The rate of RF replication at 30 C in this revertant was approximately 15 times that at 30 C in LD331 (unpublished observations). The rate of ϕ X174 RF replication in LD331 is also much slower than in

parent host strain H502 (unpublished observations).

These data indicate that the host cell *dnaC* gene product is required for ϕ X174 RF replication, and that this gene product is partially defective in LD331 even at the permissive temperature.

ϕ X174 single-stranded DNA synthesis at 41 C. The effect of the *dnaC*^{ts} mutation on single-stranded ϕ X174 DNA synthesis was examined in cultures of LD331 shifted to 41 C late

in infection (45 min). By this time single-stranded virus DNA is being synthesized and encapsulated into mature phage particles (see Fig. 1). Radioactive label incorporated into RF molecules during short pulses at this stage of infection is found predominantly in the viral strand, and chases into virus particles (14).

Half of a ϕ X174am3 infected culture was shifted from 30 to 41 C at 45 min after infection. After 5, 30, and 45 min, portions of the 30 and 41 C cultures were pulse labeled with 3 H-thymidine for 1 min. The labeled phage DNA from these samples was examined by zone sedimentation. The data are shown in Fig. 7. In all of these gradients the RFII band is centered near fraction 35, and the free single-stranded virus DNA band, marked by added 32 P-DNA, is indicated by the arrow. Phage DNA not liberated from phage particles during the Pronase digestion is found on the dense cushion at the bottom of each gradient, while phage DNA still attached to partially disrupted particles sediments faster than the free DNA marker. If the Sarkosyl-Pronase lysates are incubated at 55 C for 20 min prior to sedimentation these aggregates are completely disrupted, and the radioactive DNA sediments as free 27S molecules.

The data in Fig. 7 show that the rate of single-stranded ϕ X174 DNA synthesis increases at 30 C during the time period observed. In contrast, the rate of single-stranded ϕ X174 DNA synthesis at 41 C is maximal at 5 min after the temperature shift, that rate being sixfold greater than the 30 C rate at the same time (compare Fig. 7B to 7A). The rate of single-stranded DNA synthesis at 41 C remains reasonably constant over the next 40 min (Fig. 7D and F), but the number of RFII molecules labeled during the 1-min pulses decreases with time. Essentially all of the pulse label incorporated into RFII molecules at 41 C can be chased into single-stranded virus DNA and mature phage particles at 41 C (data not shown).

These data show that the *dnaC*^{ts} mutation does not adversely affect the synthesis of single-stranded ϕ X174 DNA late in infection, but apparently causes a decrease in the number of RFII molecules participating in the reaction at 41 C with time after the shift. We conclude that the product of the host *dnaC* gene is not required for the continued synthesis of single-stranded ϕ X174 DNA once it has begun.

DISCUSSION

Of the three stages in ϕ X174 DNA replication, RF replication most closely resembles *E. coli* semiconservative DNA synthesis. RF replication is semiconservative (4), occurs on the cell

membrane (12), and requires the gene products of the host cell *dnaE* (6a), *dnaB* (L.B.D., unpublished observations), and *dnaC* (Fig. 5) loci. The *dnaC* gene product is required for the initiation of replication of the *E. coli* chromosome (2, 21), as is the product of the *dnaA* gene (9). Protein and RNA synthesis are also required (15, 16, 24). Lark has presented evidence suggesting a requirement for the synthesis of two different proteins for the initiation of *E. coli* chromosome replication. The synthesis of one of these two proteins is sensitive to chloramphenicol at 25 μ g per ml, whereas the synthesis of the other seems relatively resistant to this concentration of the drug (16). The data presented in Fig. 5 suggest that the synthesis of the *dnaC* gene product is relatively resistant to the presence of 30 μ g of chloramphenicol per ml as the replication of ϕ X174 RF continues for several hours in its presence under conditions (30 C) where the *dnaC* gene product is absolutely required. This gene product might correspond to the more resistant of the two proteins suggested by Lark.

The synthesis of the *dnaA* gene product is sensitive to the presence of 25 μ g of chloramphenicol per ml (1). The replication of ϕ X174 RF DNA is not (23, and Fig. 5). The product of ϕ X174 gene A, which is required for ϕ X174 RF replication, is synthesized at this chloramphenicol concentration (17, 18). Francke and Ray suggest that the ϕ X174 gene A product is required to initiate RF replication by promoting the formation of a nick in the viral strand of the RF template (8). The phage gene A product might substitute for the host *dnaA* gene product in initiation of replication of ϕ X174 RF molecules. This idea is supported by the recent finding that the host *dnaA* gene product is not required for ϕ X174 growth (25).

The activity of the host *dnaC* gene product is not required for parental RF synthesis (Fig. 3) or for continuation of the asymmetric single-stranded virus DNA synthesis *in vivo* once it has begun (Fig. 7). Both of these processes are different from normal semiconservative replication. The synthesis of the parental RF involves the polymerization of a complementary strand on the infecting strand template. This step is initiated by an RNA primer (20). Although parental RF synthesis is not temperature sensitive *in vivo* in our *dnaC*^{ts} mutant, cell-free extracts of temperature-sensitive *dnaC* mutants are defective in the conversion of added ϕ X174 single-stranded DNA to RF (27). This *in vitro* reaction may be different from the synthesis of parental RF *in vivo*.

The synthesis of single-stranded virus DNA molecules occurs in the cell cytoplasm, and seems to be initiated by a nick in the viral

FIG. 7. Zone sedimentation of phage DNA from ϕ X174am3 infected LD331 pulse labeled during single-stranded DNA synthesis. A liquid culture of bacteria was grown to a cell density of 4×10^8 cells per ml in TPGA medium supplemented with $2 \mu\text{g}$ of thymine per ml. The cells were collected by centrifugation, washed in starvation buffer, and resuspended in one-tenth volume of the same. ϕ X174am3 was added at a multiplicity of 3. After 15 min without aeration one volume of TPGA supplemented with $1 \mu\text{g}$ of thymine per ml was added (zero time). After 45 min at 30 C half of the culture was shifted to 41 C. Portions (10 ml) of each culture were pulse-labeled with 0.2 mCi of ^3H -thymidine for 1 min at 5, 30, and 45 min after the temperature shift. Each sample was rapidly chilled, lysed, digested with Pronase, and subjected to zone sedimentation analysis as described in Materials and Methods. The sucrose gradients in this experiment consisted of 33 ml of 5 to 20% sucrose over a 3-ml cushion of saturated CsCl in 60% sucrose. Total radioactivity was determined in each fraction diluted to 1 ml with water. A, 5 min, 30 C; B, 5 min, 41 C; C, 30 min, 30 C; D, 30 min, 41 C; E, 45 min, 30 C; F, 45 min, 41 C. Arrows indicate the peak positions of added ^{32}P -labeled ϕ X174 virus DNA marker.

strand of cytoplasmic RF DNA molecules (5, 11). Our data indicate that the *dnaC* gene product plays no direct role in the continued synthesis of single-stranded virus DNA molecules once this synthesis has begun. Single-stranded ϕ X174 DNA synthesis continues for at least 45 min after a shift of the culture to 41 C (see Fig. 7). Although the rate of single-stranded DNA synthesis is reasonably constant during this time period, the number of RFII molecules participating in the reaction as template decreases with time. The data in Fig. 2 show that after prolonged incubation of infected cells at 41 C during single-stranded DNA synthesis, the synthesis does cease. We have shown that RF replication, which does require the host *dnaC* gene product, is completely inhibited after 5 min at 41 C. One possible explanation of these results is that the host *dnaC* gene product plays no direct role in single-stranded ϕ X174 DNA synthesis. This synthesis ceases after prolonged incubation at 41 C only because RF replication must continue during single-stranded DNA synthesis in this mutant host in order that sufficient template be available for the prolonged synthesis of single strands in ϕ X174am3 (lysis defective) infection. Komano, Knippers, and Sinsheimer have shown that RF replication does continue at a slow rate during this period (14). Alternatively, the host *dnaC*

gene product could be involved directly in the first cycle of single-stranded DNA synthesis, but not required for any further cycles of synthesis. Our data do not distinguish between these possibilities.

The *dnaC* gene product may be involved in initiating each round of ϕ X174 RF replication in a manner similar to its role in the initiation of replication of the *E. coli* chromosome. We are examining the role of this gene product in more detail.

ACKNOWLEDGMENTS

We thank Christine Miller for assistance in several of these experiments. This investigation was supported in part by Public Health Service research grant number AI-9882 from the National Institute of Allergy and Infectious Diseases, research grant number GB-22867 from the National Science Foundation, and research grant number 70-21 from the American Cancer Society, Illinois Division, Inc.

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