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Replication of Bacteriophage M13

IX. Requirement of the *Escherichia coli dnaG* Function for M13 Duplex DNA Replication

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Temperature-shift experiments with an *Escherichia coli dnaG* strain indicate a requirement for the *dnaG* function for M13 phage production only at an early stage of infection. Mutant cells infected at nonpermissive temperature form the parental RF (SS → RF) but do not replicate further. A shift to nonpermissive temperature after infection inhibits RF → RF replication but not RF → SS synthesis. The synthesis of both strands of the duplex RF was inhibited equally after a temperature shift during RF → RF replication. We infer that the *dnaG* protein is required for M13 production only during RF replication and that it is required for the synthesis of both strands of the RF.

Bacteriophage M13 contains fewer than 10 genes and consequently utilizes many of its host's functions in its replication (10). With the availability of many bacterial mutants defective in their own DNA replication, it is now possible to determine which of the known *Escherichia coli* genes involved in bacterial DNA replication also function in M13 DNA replication. *E. coli polA* mutants defective for *dnaA*, *dnaB*, *dnaC(D)*, *dnaE*, or *dnaG* are all blocked in M13 production at nonpermissive temperature (11). A recently described mutant, *dnaZ*, also fails to support M13 replication at high temperature (18).

Studies with *dnaB* and *dnaE* mutants (12, 17) suggest that neither of these genes are absolutely essential for the in vivo conversion of the M13 viral DNA to its replicative form (SS → RF). However, both of these gene products are required for later stages of replication—the *dnaB* product being required for RF replication (RF → RF) but not for single-strand synthesis (RF → SS) and the *dnaE* product required for RF replication and possible for single-strand synthesis as well. The *dnaA* gene appears to be required for one of the final steps of replication, the closure of linear viral strands (4). At nonpermissive temperature this mutant accumulates linear rather than circular viral strands.

The *dnaG* gene has been implicated recently in the initiation of Okazaki fragments (8) and in the formation of an RNA primer for the complementary strand during parental RF formation in vitro by G4 (J. P. Bouché, K. Zechel, and A. Kornberg, J. Biol. Chem., in press; K. Zechel, J. P. Bouché, and A. Kornberg, J. Biol. Chem., in press), a ΦX-related bacteriophage. This stage of ΦX replication as well as RF replication and single-strand synthesis are all inhibited in vivo in a *dnaG* mutant at nonpermissive temperature (9).

Our studies indicate a requirement for the *dnaG* gene product in only a single stage of M13 replication—the replication of RF. Neither parental RF formation (SS → RF) nor single-strand synthesis (RF → SS) requires this host function.

MATERIALS AND METHODS

Phage and bacterial strains. M13 wild-type and M13 *5am*H3 are from the collection of David Pratt and have been described previously (13, 14). *E. coli* PC3 (F^-, *leu*$^-$, *thy*$^-$, *str*^{R}, *dnaG*^{ts}) was kindly provided by Randy Schekman. (We have confirmed the location of the temperature-sensitive locus by genetic mapping of the mutation.) *E. coli* PC3X8 is an F^+ derivative of PC3. PC3X8 rev. is a temperature-resistant revertant of PC3X8 which forms colonies at 41 C; however, the reversion of the *dnaG*^{ts} locus is only partial since PC3X8 rev. neither forms colonies nor synthesizes DNA at 42 C. *E. coli* K37 (*sup D*) was used as indicator for M13 in plating experiments. Phage were titered by the agar overlay method described by Adams (1).

Media. Bacteria were grown in glucose-Casamino Acids medium (16) containing thymidine at 2 μg/ml. For radioactive labeling of DNA the medium was supplemented with [^{3}H]thymidine at 10 μCi/ml.

Preparation of ^{32}P-labeled phage. For the preparation of ^{32}P-labeled M13, *E. coli* K37 was grown in 50

ml of glucose-Casamino Acids medium in which the Casamino Acids were replaced by an equal mixture of the 20 common amino acids. The cells were grown on a rotatory shaker at 37 C to approximately 2×10^8 cells/ml. Wild-type M13 was added to 10^{10} PFU/ml and carrier-free $^{32}PO_4$ was added to 100 μCi/ml. After 2 h of growth the culture was chilled in ice and sedimented for 15 min at 15,000 rpm in a Sorvall SS-34 rotor. Phage were precipitated from the supernatant by adding NaCl to 0.5 M and polyethylene glycol to 5% (22). After 30 min in ice, the precipitated phage were collected by centrifugation for 5 min at 15,000 rpm in a Sorval SS-34 rotor. The phage were resuspended in 0.01 M Tris (pH 8.0) containing 1 mM EDTA and 0.5% Sarkosyl and precipitated a second time with polyethylene glycol and NaCl. This precipitate was finally resuspended in 1 ml of 0.01 M Tris (pH 8) containing 1 mM EDTA and sedimented for 16 h through a 5 to 20% sucrose gradient at 24,000 rpm and 5 C in a Beckman Spinco SW27 rotor. Fractions were collected from the centrifuge tube at the end of the run and assayed for both ^{32}P and PFU.

Harvest and lysis technique. Isotope incorporation was terminated by pouring infected cultures into an equal volume of ice cold TEN-CN buffer (0.01 M Tris, pH 8.0, 1 mM EDTA, 50 mM NaCl, and 10 mM NaCN). The cells were harvested and washed by centrifugation for 1 min at 15,000 rpm in a Sorvall SS-34 rotor at 4 C and resuspension in 1 ml of cold TEN-CN buffer. After washing and resuspending the cells a second time, the cells were lysed by adding 100 μl of lysozyme (4 mg/ml in water), incubating for 10 min at 37 C, adding 100 μl of 10% Sarkosyl, and incubating for an additional 10 min at 37 C. Care was taken not to shear the viscous lysate during the addition and mixing of the Sarkosyl. This was accomplished by gently pipetting the detergent into the lysate and slowly rolling the tube by hand until well mixed.

Neutral velocity sedimentation of lysates. The viscous lysate can be gently poured onto the top of a 5 to 20% sucrose gradient for sedimentation analysis. Pipetting such lysates should by avoided since this leads to breakage of the bacterial DNA and consequently an increased background of bacterial DNA sedimenting in the region of the small phage DNA species. Neutral gradients contained 0.01 M Tris(pH 8), 1 mM EDTA, and 1.0 M NaCl. Sedimentation in an SW27 ultracentrifuge rotor was for 17 h at 23,000 rpm and 5 C. Fractions were collected from the top by pumping 50% sucrose into the bottom of the gradient and collecting the fractions through a specially devised adaptor placed on top of the centrifuge tube.

Alakline centrifugation techniques. Alkaline equilibrium centrifugation was performed as previously described (5, 15).

Radioactive counting. Aliquots from neutral sucrose gradient fractions were spotted directly onto Whatman no. 3 filter paper circles, dried, and counted by liquid scintillation counting in a Beckman LS 230 two-channel counter. Fractions from alkaline CsCl gradients were collected directly onto Whatman no. 3 filter paper circles, dried, and counted as above.

RESULTS

Requirement for the dnaG function early in infection. Infection of *E. coli* PC3X8 *dnaG* at 40 C leads to a reduction of the phage yield by almost 100-fold at 2 h after infection relative to that at 32 C (Fig. 1a). In contrast, the phage yield from the revertant strain at 40 C is increased by more than threefold relative to that at 32 C. If, however, the infection of the *dnaG* strain is initiated at 32 C and then shifted to 40 C after 40 min the final phage yield approaches that of the culture held at 32 C for the entire time (Fig. 1b). We infer from this observation that the *dnaG* function is required for SS → RF and/or RF → RF replication but not for RF → SS synthesis, the final stage of viral DNA synthesis.

Parental RF formation (SS → RF) in PC3X8 dnaG cells. The first stage of replication of M13 DNA, the conversion of the infecting viral strand to the parental RF, does not require the synthesis of viral proteins. This set of reactions is readily carried out in crude extracts of uninfected cells and can be reconstituted in vitro using purified bacterial proteins (6). To investigate the possible role of the *dnaG* protein in this stage of replication in vivo we have analyzed RF molecules formed in *E. coli* PC3X8 *dnaG* at different temperatures. Figure 2 shows the neutral velocity sedimentation profiles of DNA from lysates of cells infected at 32, 39, and 41 C. The cells were infected with ^{32}P-labeled M13 in growth medium containing [^3H]thymidine, harvested at 5 min after infection, lysed, and sedimented through neutral sucrose gradients. Since the parental RF contains ^{32}P label from the infecting viral strand, the amount of ^{32}P found in replicative forms is a direct measure of the amount of parental RF formed. More parental RF is formed at both 39 and 41 C than at 32 C (Fig. 2). Similar amounts of parental RF were formed (data not shown) in a revertant of the *dnaG* mutant strain and in two different wild-type strains, indicating a lack of a requirement for the *dnaG* function in parental RF formation.

Replication beyond this initial stage of replication was inhibited at 39 and 41 C as shown by the incorporation of ^3H label into only the complementary strand of RF II molecules (Fig. 3). Extensive RF replication would have resulted in the labeling of both strands of the RF. Even a single round of duplex DNA replication would have led to an easily detectable amount of ^3H in the viral strand of progeny RF molecules, unless the progeny RF molecule having

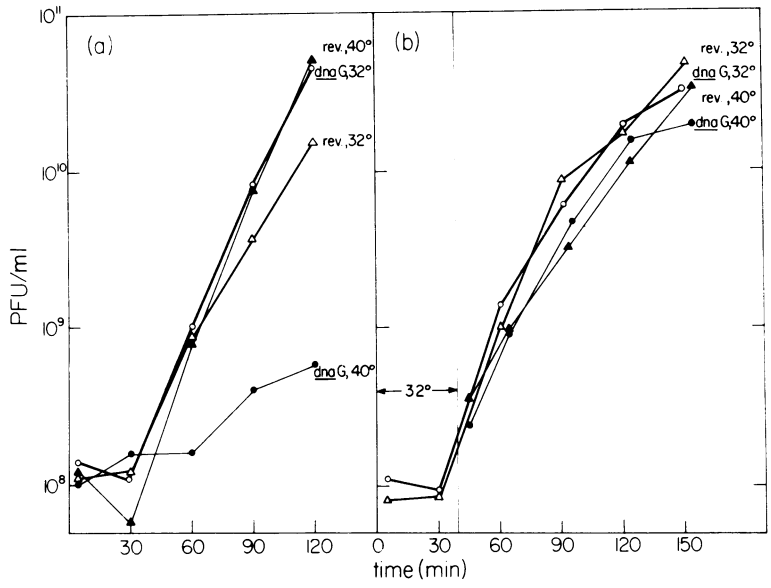

FIG. 1. *Requirement for the dnaG function early in M13 replication. Cultures of E. coli PC3X8 dnaG and E. coli PC3X8 rev. were each grown in glucose-Casamino Acids medium at 32 C to a cell density of 2 × 10⁸/ml and infected with M13 at a multiplicity of infection of 1 to 2. (a) Phage production in cultures either held at 32 C or shifted to 40 C at 10 min prior to infection; (b) phage production in cultures either held at 32 C or shifted to 40 C at 40 min after infection. Symbols: circles, PC3X8 dnaG; triangles, PC3X8 rev.; open symbols, 32 C; closed symbols, shifted to 40 C.*

the parental viral strand is selectively retained in the RF II form while the other daughter RF is closed to form an RF I. Should such a preferential closure occur, it would be possible for a single round of RF → RF synthesis, but no more, to go undetected by this method of analysis. Whether or not preferential closure of RF II molecules might occur, extensive RF replication does not take place in the *dnaG* mutant at nonpermissive temperature. The observed block to RF → RF synthesis but not to SS → RF synthesis indicated that the *dnaG* function is required only after the initial formation of the parental RF.

Since parental RF formation might conceivably involve residual activity of the *dnaG* protein it was of interest to investigate this reaction in cells preincubated for a longer time at nonpermissive temperature. Figure 4 shows the sedimentation profile of DNA from a lysate of PC3X8 cells infected with unlabeled M13 at 40 min after transfer from 32 to 41 C. The amount of ^{3}H-labeled RF observed here is similar to that found in cells infected at only 8 min after transfer (Fig. 2c). These results strongly support the lack of a requirement for the *dnaG* protein in the SS → RF conversion in vivo.

Requirement of the dnaG protein for RF → RF replication. To explore further the involvement of the *dnaG* protein in RF → RF replication we have investigated the temperature sensitivity of RF → RF synthesis in mutant and revertant cells infected with M13 mutants defective in gene 5. RF → RF synthesis takes place in the absence of the gene 5 DNA-binding protein but not that of RF → SS (13). Figure 5 shows the neutral velocity sedimentation profiles of lysates of PC3X8 and a partially temperature-resistant revertant of the same strain. Both strains were infected with M13 5*am*H3, and portions were transferred to separate culture tubes at 32, 39, and 41 C at 15 min after infection. Each culture was pulse labeled with [^{3}H]thymidine from 25 to 30 min after infection, harvested, lysed, and sedimented through neutral sucrose gradients. Labeling of replicative forms was inhibited at 39 and 41 C in the *dnaG* mutant strain but less so in the revertant strain. These results directly implicate the *dnaG* protein in M13 RF → RF replication.

To investigate the possible selective inhibition of the synthesis of the M13 complementary strand we have analyzed the distribution of label between the two strands of RF II molecules formed in the *dnaG* mutant at 32 and 39 C. Separation of the two strands is achieved by equilibrium centrifugation in alkaline CsCl. Figure 6 shows the ^{3}H label contained in the

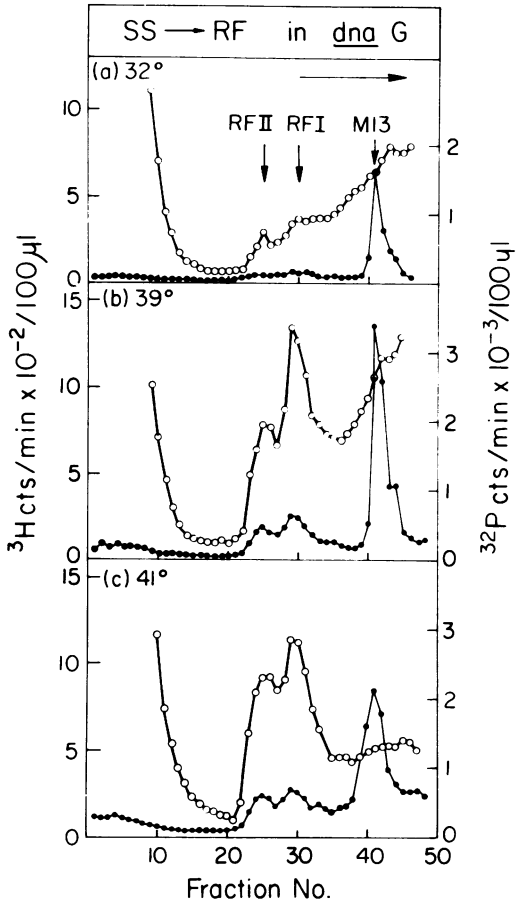

FIG. 2. *Parental RF formation (SS → RF) in PC3X8 dnaG. Three 20-ml cultures of PC3X8 dnaG were grown in glucose-Casamino Acids medium at 32 C to a cell density of approximately 2 × 10^8 cells/ml. Two of the cultures were transferred to 39 and 41 C, and after 7 min [^{3}H]thymidine was added to all three cultures. At 8 min after the temperature shift each culture was infected with ^{32}P-labeled M13 at 10^{10}/ml, and after an additional 5 min all cultures were harvested, lysed, and sedimented through neutral sucrose gradients as described. The direction of sedimentation is indicated by the horizontal arrow. The sedimentation positions of RF I, RF II, and phage are indicated by vertical arrows. Symbols: ●, ^{32}P; ○, ^{3}H.*

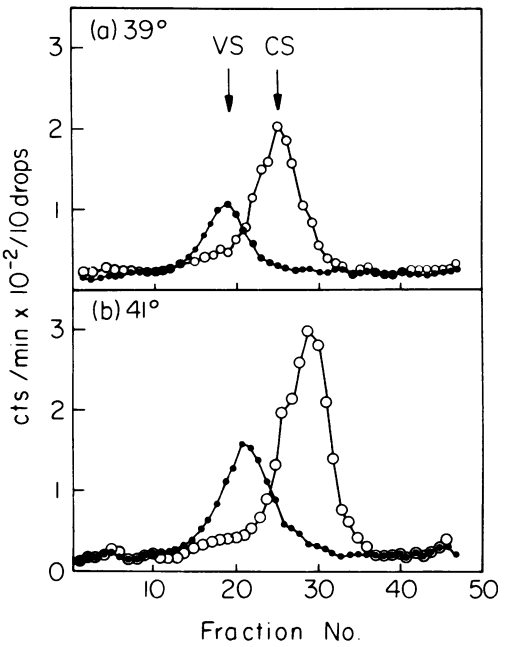

FIG. 3. *Distribution of ^{3}H label between the viral and complementary strands of RF II formed in PC3X8 dnaG at 39 and 41 C. Pooled fractions from the RF II regions of Fig. 2b and c were dialyzed against 0.01 M Tris (pH 8) containing 1 mM EDTA and then sedimented to equilibrium in alkaline CsCl gradients. Density increases from right to left in these gradients. The positions in the density gradient of viral strands (VS) and complementary strands (CS) are indicated by vertical arrows. Symbols: ●, ^{32}P; ○, ^{3}H.*

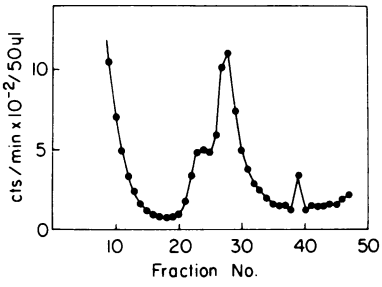

FIG. 4. *Parental RF formation in PC3X8 dnaG after extended incubation at 41 C. A 40-ml culture of PC3X8 dnaG was grown at 2 × 10^8 cells/ml as in the legend to Fig. 2. The culture was shifted to 41 C for 40 min before infection by unlabeled wild-type M13. One minute prior to infection [^{3}H]thymidine was added to the culture to 10 µCi/ml. At 5 min after infection the culture was harvested, lysed, and sedimented through a neutral sucrose gradient as described. The direction of sedimentation is from left to right.*

separated strands of the RF II's. The extent of labeling of the complementary strand relative to that of the viral strand is the same at 39 C as at 32 C. A difference in the relative labeling of the two strands would have been expected if the viral strand had been synthesized preferentially at the nonpermissive temperature. This evidence suggests that the *dnaG* protein is not required exclusively for the synthesis of the complementary strand of the RF during RF → RF replication.

Single-strand synthesis (RF → SS) in PC3X8 dnaG. The final stage of M13 DNA

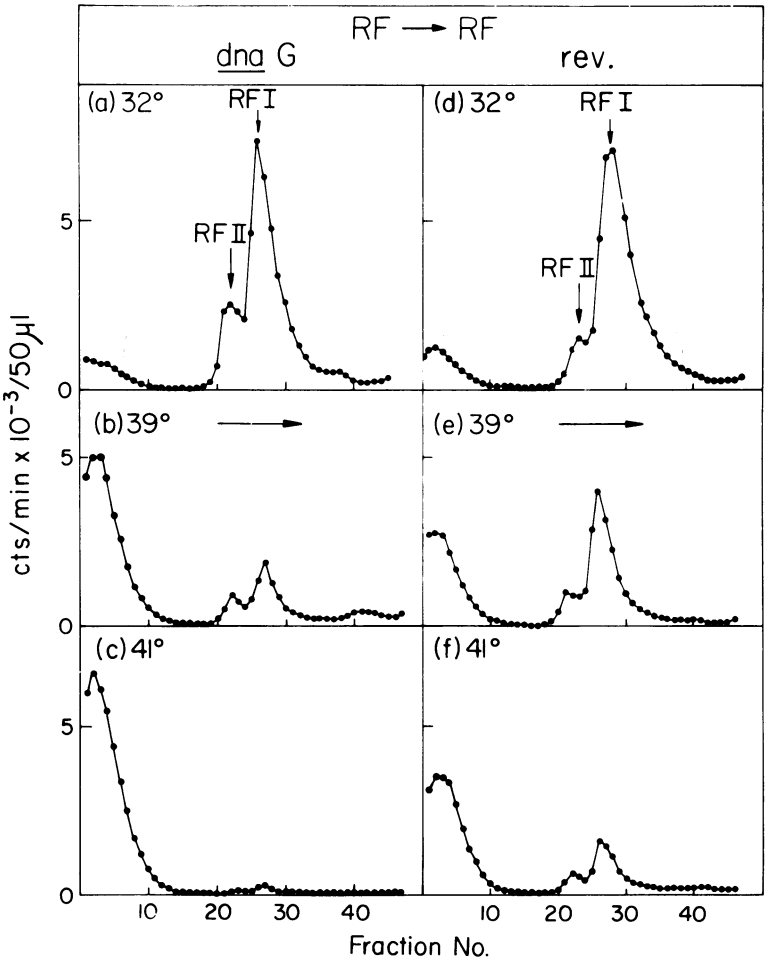

FIG. 5. *RF replication (RF → RF) in PC3X8 dnaG and PC3X8 rev. Three 30-ml cultures of PC3X8 dnaG and three 30-ml cultures of PC3X8 rev. were each grown at 32 C in glucose-Casamino Acids medium to cell densities of approximately 2 × 10⁸/ml and then infected with M13 5amH3 at 10¹⁰/ml. At 15 min after infection two cultures of each set were transferred to 39 and 41 C, and after 10 min each culture was pulse labeled with [³H]thymidine for 5 min. All cultures were harvested, lysed, and sedimented through neutral sucrose gradients as described. The direction of sedimentation is indicated by the horizontal arrows.*

replication, the asymmetric synthesis of viral strands, requires two viral gene functions in addition to host functions (13). To investigate the potential role of *dnaG* in this stage of replication we have pulse labeled both the *dnaG* mutant and its revertant after shifting to 39 and 41 C at 45 min after infection. The cells were pulse labeled with [³H]thymidine for 5 min at 10 min after the temperature shift. Figure 7 shows the velocity sedimentation pattern of lysates from each of the infected cultures. Substantial amounts of viral single strands were synthesized even at 41 C in the *dnaG* mutant (Fig. 7c). Comparison with the amount of synthesis observed in the revertant strain shows no significant difference in single-strand synthesis. We infer from this that asymmetric viral strand synthesis does not require a functional *dnaG* protein for continued synthesis.

DISCUSSION

The observations presented here indicate that the *E. coli dnaG* function is required for M13 RF replication but not for parental RF formation (SS → RF) or single-strand synthesis (RF → SS). A point of concern with regard to the significance of these results for the SS → RF reaction is the need for only a single round of

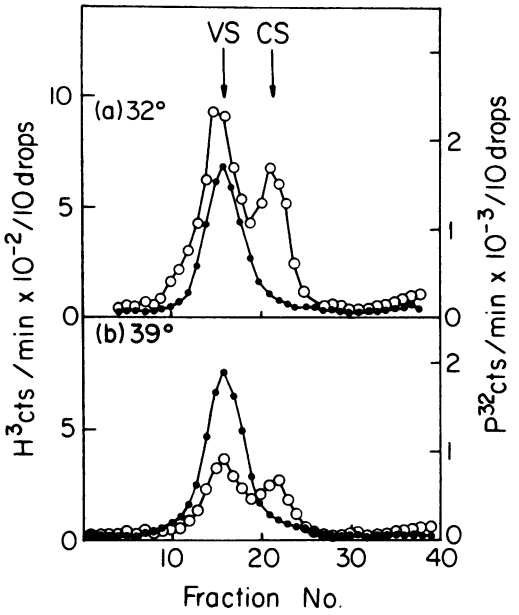

FIG. 6. *Distribution of ^3H label between the viral and complementary strands of M13 5amH3 RF II formed at 32 and 39 C in PC3X8 dnaG. Pooled fractions from the RF II regions of Fig. 5a and b were dialyzed and sedimented to equilibrium in alkaline CsCl as in the legend to Fig. 3. ^{32}P-labeled viral DNA was added as a density marker. Density increases from right to left. The positions of viral strands (VS) and complementary strands (CS) are indicated by vertical arrows. Symbols: ●, ^{32}P; ○, ^3H.*

replication for parental RF formation to occur. This small amount of synthesis might readily take place due to either residual enzyme activity at the nonpermissive temperature or renaturation of the enzyme during some stage of processing of the infected cells prior to lysis. The strongest argument against such an artifact is the clean inhibition of RF → RF replication in cells infected at nonpermissive temperature. Had there been a similar requirement for the *dnaG* function for the SS → RF reaction then there should have been a significant decrease in the amount of parental RF formed at high temperature. Yet, just the opposite was observed. There was an actual stimulation of parental RF formation at temperatures of 39 and 41 C. This lack of a requirement for the *dnaG* protein for the conversion of M13 SS to RF agrees with the observations made on in vitro systems (6, 21). This reaction has now been reconstituted from purified proteins and requires only RNA polymerase, a DNA polymerase III holoenzyme, and DNA unwinding protein (7).

Our finding that the *dnaG* function is required only at an early stage of infection when RF replication is occurring is in contrast with the observation by Mitra and Stallions (12) who found that phage production in a *pol A dnaG* double mutant was temperature sensitive even late in infection. This difference may reflect a characteristic of the double mutant that differs from our *dnaG* mutant.

Experiments designed to investigate a preferential or exclusive involvement of the *dnaG* protein with the synthesis of only one of the two strands of the RF indicated a requirement of the *dnaG* function for the synthesis of both strands. Residual labeling of RF molecules in the *dnaG* mutant at high temperature labeled both strands equally. In parallel experiments with a *dnaB* mutant (unpublished data) we also found equal inhibition of synthesis of both strands of M13 RF at nonpermissive temperature. We therefore infer that neither the *dnaB* nor the *dnaG* proteins are required solely for complementary strand synthesis during RF replication.

The *dnaG* protein has been shown recently to be a rifampicin-resistant RNA polymerase capable of forming an oligoribonucleotide primer on single-stranded G4 DNA coated with the *E. coli* unwinding protein (Bouché et al., J. Biol. Chem., in press). A transcript of a unique region of the G4 DNA can serve as a primer for the synthesis of the G4 complementary strand during SS → RF synthesis. This function is similar to that of RNA polymerase in initiating the M13 complementary strand (4, 6, 20). It therefore is likely that the *dnaG* protein might prime the synthesis of one of the strands of the M13 RF. However, should the *dnaG*, protein be involved in the initiation of either strand of the RF during RF → RF replication, the mechanism of initiation would need to be unique to that stage of replication since neither complementary strand initiation during SS → RF synthesis nor viral strand initiation during RF → SS synthesis require the *dnaG* function. It is unlikely that the *dnaG* protein is directly involved in complementary strand initiation since rifampin selectively inhibits M13 complementary strand synthesis during RF → RF synthesis (4). This observation suggests that, as for M13 SS → RF synthesis, the complementary strand primer is formed by RNA polymerase.

We are thus left with the possibility that the *dnaG* protein initiates viral strand synthesis. Inhibition of viral strand synthesis might also prevent complementary strand initiation since RF → RF replication appears to occur by a rolling-circle mechanism (7) in which the viral

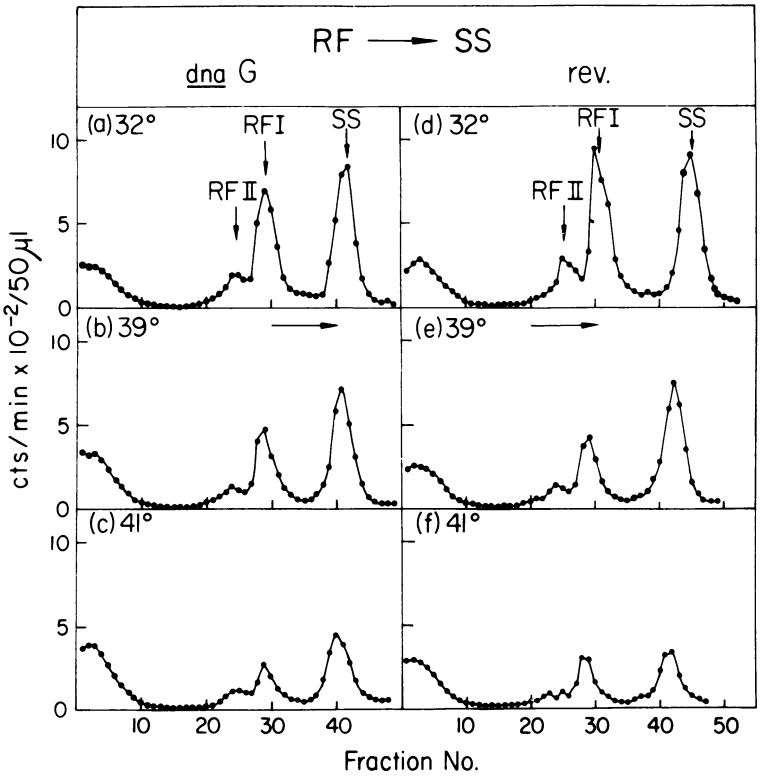

FIG. 7. *Single-strand synthesis (RF → SS) in PC3X8 dnaG and PC3X8 rev. Three 30-ml cultures of PC3X8 dnaG and three 30-ml cultures of PC3X8 rev. were each grown at 32 C in glucose-Casamino Acids medium to a cell density of approximately 2 × 10⁸/ml. At 45 min after infection two cultures of each set were transferred to 39 and 41 C, and after 10 min each culture was pulse labeled with [³H]thymidine for 5 min. All cultures were harvested, lysed, and sedimented through neutral sucrose gradients as described. The direction of sedimentation is indicated by the horizontal arrows. The sedimentation positions of RF I, RF II, and SS are indicated by vertical arrows.*

strand is the elongated strand (19). In this case, the template for complementary strand synthesis, the elongated tail of the viral strand, would not be formed and the synthesis of both strands would be inhibited equally. The experiments presented here would therefore not discriminate between a direct requirement of the *dnaG* protein for the synthesis of both strands of the RF or of only the viral strand. Should the *dnaG* protein prove to be directly involved only in viral strand synthesis during RF → RF synthesis, it will be of interest to compare the mechanism of initiation of viral strands during both RF → RF and RF → SS synthesis. From results presented here, we infer that the *dnaG* protein is not required for viral strand synthesis during RF → SS synthesis.

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