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# Accumulation of ColE1 Early Replicative Intermediates Catalyzed by Extracts of *Escherichia coli dnaG* Mutant Strains

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To investigate the events occurring at the replication forks during DNA synthesis, we studied the replication of plasmid ColE1 DNA in vivo and in vitro, using strains of *Escherichia coli* carrying either the *dnaG3*(Ts) or *dnaG308*(Ts) mutation. Extracts of both mutant strains supported in vitro DNA synthesis, but the amount of [$^3$H]TMP incorporated into DNA was always less for mutant extracts than for extracts of revertant strains, which were able to grow at 42°C. Sucrose gradient analysis, Southern blot analysis, and electron microscopy showed that mutant extracts synthesize a large number of early replicative intermediates containing one or two (one on each template strand) fragments at the origin of replication and some completed molecules, either open circles or covalently closed circles. The revertant extracts synthesized more completed molecules although the fraction of templates used was about the same, 0.27 for mutant extracts and 0.21 for revertant extracts. Our results show that a mutation in *dnaG* causes a block in the synthesis of both leading and lagging strands after initiation, which results in the accumulation of early replicative intermediates. The average size of the newly replicated region in the early replicative intermediates is 730 bases as measured from electron micrographs of early replicative intermediates. We conclude that the DnaG protein functions in lagging strand synthesis and may be necessary for the continuation of leading strand synthesis as well.

When plasmid ColE1 DNA replication is initiated at the origin of replication, the first DNA fragment synthesized is primed from an RNA synthesized by *Escherichia coli* DNA-dependent RNA polymerase (13, 30). After initiation, RNA polymerase is no longer required for replication (30). A different priming activity is required for the discontinuous synthesis of the lagging strand and possibly for the continuation of the leading strand. (The lagging strand has 3′ to 5′ polarity and grows by the discontinuous synthesis of short DNA fragments, which are elongated in a 5′ to 3′ direction, opposite that of the replication fork. The leading strand is the strand with 5′ to 3′ polarity which is elongated by DNA polymerases in the direction of replication fork movement.) The most likely source of this activity is the *E. coli dnaG* gene product (27).

The *dnaG* gene product primase is a polymerase which has been shown to synthesize primers containing either ribonucleotides or deoxyribonucleotides for DNA synthesis on single-stranded DNA templates (23, 33). DnaG protein synthesizes a primer of 14 to 29 nucleotides on the bacteriophage G4 template coated with *E. coli* single-stranded DNA binding protein. This primer, which is initiated at a specific site (4), is used by DNA polymerase III holoenzyme to synthesize the complementary DNA strand (23, 33). Proteins DnaB, DnaC, i, n, n′, n″, and DnaG must be present to synthesize primers on the φX174 template (2). These proteins form a complex called a primosome, which moves along the template and synthesizes primers (2). The assembly of a primosome requires a site on the template which is recognized by protein n′. This protein binds to the DNA to initiate the formation of the primosome (2). *E. coli* DnaG protein is thought to prime discontinuous DNA synthesis since the initiation of short-fragment synthesis is blocked at the nonpermissive temperature in *dnaG*(Ts) mutants (15).

Plasmid ColE1 is a supercoiled DNA molecule with a molecular weight of $4.2 \times 10^6$ (3). Since ColE1 requires only *E. coli* proteins for its replication (10, 14), it is a good choice for

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studying the function of individual *E. coli* DNA replication proteins. In vivo and in vitro studies have demonstrated that semiconservative unidirectional replication of the plasmid occurs from a fixed origin (12, 17, 32). The in vitro initiation of ColEl DNA replication requires the purified *E. coli* proteins RNA polymerase, ribonuclease H, DNA polymerase I, and DNA gyrase (13). The product of initiation, the early replicative intermediate, has a replicating eye which contains a newly synthesized leading strand (6S L) fragment (24). This fragment of approximately 500 bases is annealed to the heavy (H) strand of the ColE1 plasmid. It has been shown that *dnaB* and *dnaC* are required for the syntheses of the leading strand and lagging strand after initiation (26, 27).

ColE1 DNA replication is arrested at 42°C in cells carrying the *dnaG3*(Ts) mutation, suggesting that *dnaG* must be required for ColE1 DNA replication (8). Staudenbauer et al. has studied the in vitro replication of ColE1 DNA in extracts of cells carrying the *dnaG1011*(Ts) mutation (27). They found that *dnaG* was required for the discontinuous synthesis of the H or lagging strand, but was not required for the continued synthesis of the leading strand. Initiation occurs, and covalently closed circles (CCCs) are made. However, the CCCs synthesized by these extracts contained only a nascent L-strand, which is the leading strand with respect to the unidirectional replication of ColE1 DNA.

Two binding sites for the *E. coli* replication protein n′ have been mapped just downstream from the ColE1 origin of replication, one on the lagging and one on the leading strand (19, 36). Nomura et al. have shown that fragments containing these sites can be used as origins of replication when cloned into the bacteriophage M13 (20, 21). In addition, a primosome is assembled from purified proteins on these single-stranded templates (20, 21). These authors speculate that the n′ site on the L-strand is used to assemble a primosome for the priming of the discontinuous synthesis of the ColE1 lagging strand and that the site on the H-strand may be used for transfer replication during conjugation (21).

In this report, we present our studies of the replication of ColE1 DNA in extracts of strains containing the *dnaG3*(Ts) or *dnaG308*(Ts) mutations and their respective revertants. We found an accumulation of replicative intermediates in the mutant extracts, which suggests a role for *dnaG* and possibly the primosome in the synthesis of both leading and lagging strands of ColE1 DNA. Since we found an accumulation of replication intermediates in strains containing the *dnaG3*(Ts) or *dnaG308*(Ts) mutations, although Staudenbauer et al. (27) found that leading strands are synthesized in a strain containing the *dnaG1011*(Ts) mutation, we propose that the various *dnaG* mutant alleles affect the two functions differentially.

TABLE 1. *E. coli* strains used

| Strain | Genotype/phenotype | Source/ reference |
|---|---|---|
| RB52[a] | *leu thi* Rif$^r$ *endA dnaG308*(Ts) | This study |
| RB52REV[a] | *leu thi* Rif$^r$ *endA* | This study |
| RB53[a] | *leu thi argG* Rif$^r$ *endA dnaG3*(Ts) | This study |
| RB53REV[a] | *leu thi argG* Rif$^r$ *endA* | This study |
| LF52[a] | RB52 *thy*(pBR322) | This study |
| LF52REV[a] | RB52REV *thy*(pBR322) | This study |
| LF53[a] | RB53 *thy*(pBR322) | This study |
| LF53REV[a] | RB53REV *thy*(pBR322) | This study |
| NY73 | *polA thy leu metE* Rif$^r$ Str$^r$ *dnaG3*(Ts) | 34 |
| NT446 | *thi thyA met*(ColE1) | 24 |

[a] The original *dnaG* recombinants of W1485 were provided by J.-M. Louarn.

## MATERIALS AND METHODS

**Bacterial strains and plasmid purification.** The *E. coli* K-12 strains used for this investigation are listed in Table 1. The *dnaG* parent strains of RB52 [*dnaG308*(Ts)] and RB53 [*dnaG3*(Ts)] were obtained from Jean-Michel Louarn. The *endA* mutation was introduced into the *dnaG* strains by conjugation with Hfr strain NT214. Spontaneous revertants of the *dnaG* mutations were isolated by plating liquid cultures at 42°C. Revertants were obtained at a frequency of approximately $10^{-8}$. The DnaG protein in the revertants has been shown to be insensitive to temperature by testing the ability of a revertant extract to complement a ϕX174 DNA replication assay, which required only DnaG protein to restore activity (34). For the in vivo experiments, *thy* derivatives of RB52, RB53 and revertants were selected in a medium containing trimethoprim and thymine (18). These were transformed (29) with pBR322, which renders transformants resistant to ampicillin and tetracycline.

Cleared lysates (7) were prepared from chloramphenicol-treated cultures (6). The plasmid DNA was purified from the cleared lysates by two centrifugations in cesium chloride-ethidium bromide gradients. The CCC band was extracted with *n*-butanol and dialyzed against 20 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA (TE). The resulting DNA was precipitated with ethanol and suspended in a smaller volume of TE. Radioactive marker DNA was further purified by centrifugation in a neutral sucrose gradient.

**Cell growth and radiolabeling for in vivo temperature shift experiments.** To uniformly label DNA, we diluted cells from an overnight culture in M9 minimal medium (1) supplemented with thiamine (10 μg/ml), Difco vitamin assay Casamino Acids (0.4% [wt/vol]), $CaCl_2$ (0.1 mM), $MgSO_4$ (1 mM), glucose (0.25% [wt/vol]), and [*methyl*-$^{14}$C]thymine (4 μg/ml; 1.7 μCi at 52.9 mCi/mmol). The cells were grown at 30°C to an absorbance of 0.5 at 600 nm (measured in a Zeiss

PMQII spectrophotometer). This is about $1.5 \times 10^8$ to $2.0 \times 10^8$ cells per ml for our strains. The cells were collected, washed twice with warmed unsupplemented M9 medium, suspended in an equal volume of warm supplemented M9 medium containing unlabeled thymine (4 μg/ml), and then incubated at 30°C for 1 h before they were shifted to 42°C. The cells were pulse labeled at various times before or after the temperature shift by pipetting a 2-ml sample of the culture into a tube containing 75 μl of [*methyl*-$^3$H]thymidine (37.5 μCi at 48 Ci/mmol). The 4-min pulses were stopped by adding 2 ml of a chilled ethanol stop solution (71% ethanol [vol/vol], 1.5% phenol [vol/vol], 2 mM EDTA, 16 mM sodium acetate [pH 5.7]). Samples were collected by centrifugation and washed twice with cold nonsupplemented M9 medium. The final pellets were stored at −20°C.

**DNA-DNA hybridization.** Filters which contained either salmon sperm, *E. coli* chromosomal, or *Eco*RI-cleaved pBR322 DNA were prepared for DNA-DNA hybridization. The DNAs were denatured in 0.15 N NaOH (10 min on ice), neutralized, and diluted to 0.1 μg/ml in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). DNA (2 μg) was added to each filter (16). The filters were washed with 6× SSC, air dried, heated at 80°C under vacuum, and stored in a desiccator.

To prepare the labeled sample DNAs for hybridization, we suspended the frozen cell samples in 0.5 ml of TE and 0.5 ml of 1 N NaOH (11). These mixtures were heated at 100°C for 10 min, cooled on ice, and neutralized with 0.1 ml of 1 M Tris-hydrochloride (pH 7.5) and 1 N HCl to pH 7.5. The radioactive DNA was incubated with filters in 2× SSC–50% formamide (pH 7.5) for 36 h at 42°C in glass scintillation vials. The filters were washed twice in 2× SSC–50% formamide for 15 min, once in 2× SSC, and once in 0.1× SSC. They were then dried and counted.

**Preparation of cell extracts.** The cells were grown in H broth (8 g of nutrient broth per liter, 5 g of Bacto-Peptone per liter, 1 g of glucose per liter) at 30°C to an absorbance of 1.0, at which time chloramphenicol was added (150 mg/liter). The cells were incubated with shaking at 42°C for an additional 4 h. Cell-free extracts were prepared by freeze-thaw lysis of lysozyme-treated cells. Nucleic acid was removed by streptomycin sulfate precipitation, and the extracts were concentrated by ammonium sulfate precipitation (9). The DNA-synthesizing capacity of the extracts did not decline significantly during storage at −70°C for up to 3 months.

**In vitro ColE1 DNA synthesis.** Assays were performed by the method of Conrad and Campbell (9). A 50-μl assay contained 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; ph 8.0); 100 mM KCl; 12 mM magnesium acetate; 2 mM ATP; 0.5 mM each of GTP, CTP, and UTP; 0.05 mM each of dATP, dGTP, dCTP, and [$^3$H]TTP (500 cpm/pmol); 0.1 mM dithiothreitol (DTT); 0.5 μg of ColE1 DNA (1.5 μg where indicated); and 20 μl of cell extract. The reactions were incubated at 30°C. Incubation at higher temperatures inactivated all extracts. To inactivate the temperature-sensitive DnaG activity, we had to incubate the chloramphenicol-treated cultures at 42°C for approximately 4 h immediately before the extracts were prepared. Extracts from *dnaG* strains grown at 30°C contained significant DnaG activity which was sensitive to heating; however, since heating an extract also inactivates DnaB activity (Bird, unpublished data), this method of inactivating DnaG activity could not be used.

**Product analysis.** The reactions to measure the incorporation of [$^3$H]TMP were stopped by the addition of 5% (wt/vol) trichloroacetic acid–0.05 M sodium pyrophosphate and incubated on ice for 5 min. The resulting precipitates were collected on glass filters. The filters were washed with 3% (wt/vol) trichloroacetic acid containing 0.03 M sodium pyrophosphate, washed with 95% ethanol, dried, and counted. The reactions to be analyzed by sedimentation were terminated by the addition of TE containing 0.25% sodium dodecyl sulfate and pronase at 1 mg/ml, followed by incubation at 37°C for 30 min. Product DNA and unincorporated nucleotides were separated by gel filtration on Sephadex G-50. Alkaline sucrose gradients (5 to 20% sucrose in 0.3 N NaOH–1 M NaCl–5 mM EDTA) and neutral sucrose gradients (5 to 20% sucrose in 1× SSC–50 mM Tris, pH 7.5) were run with $^3$H-labeled product DNA and $^{14}$C-labeled reference DNA in a Beckman SW60 Ti rotor at 45,000 rpm, 4°C, for 2.5 and 3.5 h, respectively. Fractions were collected from the bottom of the tube and counted in formula 963 scintillation cocktail (New England Nuclear Corp.).

**Southern blot analysis of radioactive DNA synthesized by RB52 and RB52REV extracts.** ColE1 DNA was digested with *Hae*III restriction endonuclease in a solution containing 20 mM Tris-hydrochloride (pH 7.9), 5 mM $MgCl_2$, 1 mM DTT, and 200 μg of bovine serum albumin per ml. Samples of the digests were run on a 1.5% agarose gel (agarose type II; Sigma Chemical Co.) in a horizontal apparatus. The gel was stained with ethidium bromide and photographed under UV light. The DNA in the gel was denatured in 1.5 M NaCl–0.5 N NaOH for 45 min with gentle agitation and neutralized in 3 M NaCl–0.5 M Tris-hydrochloride (pH 7.5) for another 45 min at room temperature. The denatured fragments were then transferred to HA sheets (Millipore Corp.) as described by Southern (25). The sheets were baked in a vacuum oven and then hybridized with either $^{32}$P-labeled ColE1 DNA fragments synthesized by a RB52 extract or total $^{32}$P-labeled ColE1 DNA synthesized by a RB52REV extract. The short fragments which were synthesized by an RB52 extract were pooled from an alkaline sucrose gradient (e.g., Fig. 3A, fractions 27 to 30). Hybridization was performed as described above, and the blots were autoradiographed with Kodak X-Omat AR film.

**Electron microscopic analysis of replicative intermediates.** The radiolabeled DNA synthesized by an RB52 extract was sedimented in a neutral sucrose gradient. The radiolabel, which sedimented faster than the CCC marker DNA, was pooled and dialyzed against TE containing 100 mM NaCl. DNA strands were cross-linked with trioxalen to prevent fork migration and the release of the nascent DNA fragments after cleavage with *Eco*RI (A. Luder, Ph.D. thesis, Vanderbilt University, Nashville, Tenn., 1981). DNA (50 μl) and 2 μl of trioxalen (8 μg/ml in methanol) were mixed and incubated at 4°C in the dark for 5 min. This mixture was exposed to long-wavelength UV light for 5 min at a distance of 10 cm. The DNA was precipitated in ethanol, collected by centrifugation, dried, suspended in 100 mM Tris-hydrochloride (pH 7.5)–50 mM NaCl–

10 mM $MgCl_2$–1 mM DTT, and cleaved with *Eco*RI. The DNA was diluted in a hyperphase (100 mM Tris-hydrochloride [pH 8.5], 10 mM EDTA, 40% formamide, 0.08 mg of cytochrome per ml), spread on a hypophase (10 mM Tris-hydrochloride [pH 8.5], 1 mM EDTA, 10% formamide), picked up on a Parlodion film, and shadowed with platinum-paladium by the method of Chow and Broker (5).

**ϕX174 *dnaG*-dependent in vitro replication assay.** RB52 and RB52REV extracts were used to complement a ϕX174 *dnaG*-dependent in vitro replication assay. Assays (25 μl) contained 50 mM Tris-hydrochloride (pH 7.5); 15 mM $MgCl_2$; 1 mM DTT; 4.5 mM ATP; 0.2 mM dGTP, dCTP, and dATP; [$^3$H]TTP (500 cpm/pmol); 4 μg of rifampin per ml; 0.2 μg of ϕX174 DNA (Bethesda Research Laboratories); 5 μl of a heated *dnaG* temperature-sensitive receptor extract [NY73 *dnaG3*(Ts)]; and a source of DnaG protein (34). The *dnaG* receptor extract was prepared as previously described (34). DnaG activity in various RB52 and RB52REV extracts was determined by a comparison of the stimulation of the *dnaG*-dependent assay by our extracts with known amounts of DnaG protein. Preparations of purified DnaG protein were provided by Sue Wickner and Lee Rowen.

## RESULTS

**Plasmid and chromosomal DNA replication in *dnaG* mutant and revertant strains.** DNA synthesis in *dnaG*(Ts) mutant and revertant strains containing pBR322 was measured before and after a shift from 30 to 42°C. Plasmid pBR322, an *E. coli* plasmid which carries genes for ampicillin and tetracycline resistance, was used in these in vivo studies since cells carrying this plasmid are easily selected. ColE1 and pBR322 have the same origin of replication (28). The results of these experiments demonstrate that both pBR322 and *E. coli* DNA replications are inhibited in the mutant strains at 42°C but not in the revertant strains (Fig. 1). In strain LF52 [*dnaG308*(Ts)], *E. coli* DNA replicated at 35% of the 30°C rate, and pBR322 DNA replicated at 40% of the 30°C rate 2 min after the shift to 42°C. In contrast, the rate of *E. coli* DNA replication in strain LF53 [*dnaG3*(Ts)] was still 75% of the 30°C rate 2 min after the temperature shift, and pBR322 DNA replication was 85% of the 30°C rate. In strains LF52 and LF53, pBR322 DNA replication ceased approximately 8 min after the temperature shift, whereas *E. coli* DNA replication stopped about 6 min after the shift. In the revertant strains, the initial increase and transient depression of the rate of DNA replication after the temperature shift were reproducible. After this time, the replication of both plasmid and chromosome DNA continued in the revertant strains. This observation has also been reported for plasmid ColE1 (11).

**Analysis of ColE1 DNA synthesized by cell extracts.** Cell-free extracts of *dnaG* mutant and revertant strains have been used for in vitro synthesis of ColE1 DNA (9). Figure 2 shows the time course of [$^3$H]TMP incorporation into ColE1 DNA by extracts of both *dnaG*(Ts) mutant and revertant cells. Even though some

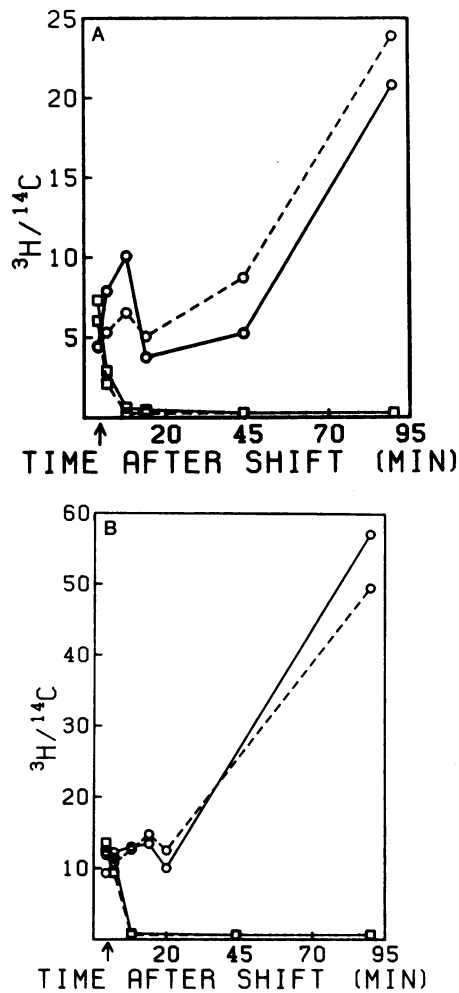


FIG. 1. Plasmid and chromosomal DNA synthesis at 42°C in LF52 and LF53 temperature-sensitive *E. coli* strains and their temperature-resistant revertants. Cultures of strains LF52, LF52REV, LF53, and LF53REV were grown at 30°C in supplemented M9 medium with [$^{14}$C]thymine. One hour before the shift to 42°C, the cultures were centrifuged, washed, and suspended in a nonradioactive medium. Samples were withdrawn before and after the temperature shift and exposed to [$^3$H]thymidine for 4 min at the temperature of the culture. DNA was extracted and hybridized to filters containing either pBR322, *E. coli*, or salmon sperm DNA. The points are plotted at 2 min into the pulse, and the arrow indicates 0 min after the shift in temperature. (A) LF52 and LF52REV. (B) LF53 and LF53REV. The dashed line represents *E. coli* chromosomal DNA; the unbroken line represents plasmid pBR322 DNA. Symbols: □, *dnaG* mutant strains; ○, *dnaG* revertant strains.

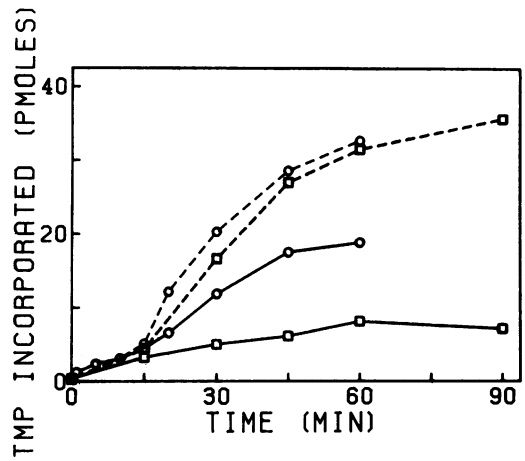


FIG. 2. Incorporation of dTMP into ColE1 DNA by *dnaG* mutant and revertant extracts. Extracts of strains RB52, RB52REV, RB53, and RB53REV were used to catalyze ColE1 DNA synthesis. Assays (50 μl) were prepared and incubated at 30°C. Incorporation was stopped at the indicated times by adding 5% (wt/vol) trichloroacetic acid–0.05 M sodium pyrophosphate. The precipitates were collected on glass filters (Whatman GFC) and counted in a scintillation counter. The unbroken line represents *dnaG* mutant strains; the dashed line represents *dnaG* revertant strains. Symbols: ○, RB53 and RB53REV extracts; □, RB52 and RB52REV extracts.

incorporation of [$^3$H]TMP was catalyzed by mutant extracts, the amount was always less than that observed with revertant extracts. In assays containing RB52REV extracts, the [$^3$H]TMP incorporation was up to four times that detected in assays containing RB52 [*dnaG308*(Ts)] extracts (Table 2). The difference in incorporation was less when RB53 [*dnaG3*(Ts)] and RB53REV extracts were compared (Fig. 2).

The products of in vitro reactions were analyzed by alkaline sucrose gradient sedimentation (Fig. 3). Most of the DNA synthesized by the RB52 [*dnaG308*(Ts)] extract sedimented as short fragments of about one-ninth the length of ColE1 DNA (Fig. 3A), whereas most of the DNA synthesized by the RB52REV extract sedimented as completed molecules, either CCCs or open circles (Fig. 3B). Less than 40% of the radiolabeled DNA synthesized by the mutant extracts and more than 90% of the radiolabeled DNA synthesized by the revertant extracts sedimented as completed molecules. Alkaline sucrose gradient analysis of the products synthesized by RB53 [*dnaG3*(Ts)] and RB53REV extracts gave similar results, but a larger fraction of the product in the RB53 mutant extract was in completed molecules (data not shown). Since RB52 extracts synthesized fewer completed molecules, this strain and its revertant were used for the remainder of the experiments. Plasmid pBR322 was also used as a template for in vitro replication assays. As with ColE1 DNA, the major product seen by alkaline sucrose gradient analysis of assays containing mutant extracts was short fragments (data not shown).

Neutral sucrose gradient profiles of ColE1 DNA synthesized by an RB52 [*dnaG308*(Ts)] and an RB52REV extract are shown in Fig. 4. The product of the mutant extract sedimented slightly faster than did the CCC reference DNA, similar to the sedimentation properties previously reported for ColE1 DNA early replicative intermediates (24). Most of the label in the product DNA synthesized by the revertant ex-

TABLE 2. ColE1 DNA synthesis and template utilization by RB52 and RB52REV extracts

| Prep | Strain | Total [$^3$H]TMP incorporated (pmol/60 min) | % Radioactivity in short fragments[a] | % of initiations leading to the synthesis of:[b] | | Relative[c] *dnaG* units |
|---|---|---|---|---|---|---|
| | | | | ERI | CCC and OC | |
| 1 | RB52 | 8 | 63 | 94 | 6 | 0.11 |
| | RB52REV | 32 | 6 | 47 | 53 | 4.25 |
| 2 | RB52 | 17 | 47 | 93 | 7 | 0.22 |
| | RB52REV | 38 | 13 | 68 | 32 | 2.35 |
| 3 | RB52 | 14 | 52 | 93 | 7 | 0.09 |
| | RB52REV | 22 | 15 | 69 | 31 | 4.05 |
| 4 | RB52 | 15 | 59 | 94 | 6 | ND[d] |
| | RB52REV | 32 | 19 | 75 | 25 | ND |
| Avg | RB52 | 13 | 55 | 93 | 7 | 0.14 |
| | RB52REV | 31 | 13 | 67 | 33 | 3.55 |

[a] The radioactivity in short fragments was measured by alkaline sucrose gradient sedimentation.
[b] ERI, Early replicative intermediates; CCC, covalently, closed circles. OC, open circles.
[c] DnaG protein activity was measured by the ability of these extracts to complement a ϕX174 DNA replication assay which required DnaG protein.
[d] ND, Not determined.

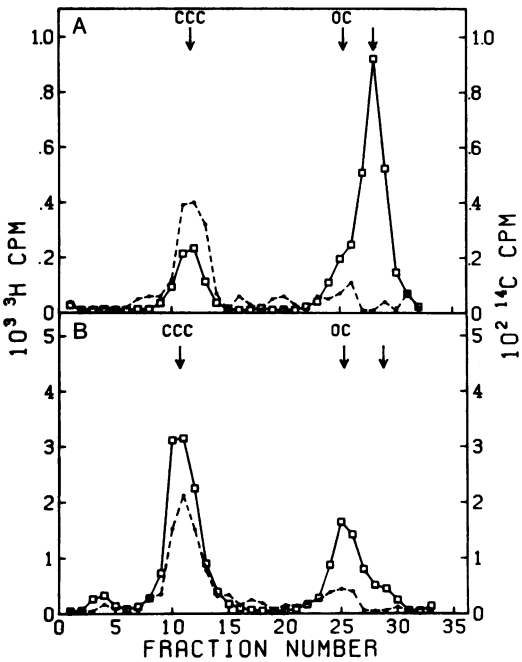


FIG. 3. Alkaline sucrose sedimentation analysis of ColE1 DNA synthesized in RB52 and RB52REV extracts. Extracts of strains RB52 and RB52REV (preparation 1 in Table 2) were used to catalyze ColE1 DNA synthesis in 200-μl reaction mixtures. After 60 min at 30°C, the reactions were stopped by the addition of sodium dodecyl sulfate, EDTA, and pronase. The unincorporated radioactivity was removed by gel filtration, and a portion of the product was layered on a 5 to 20% alkaline sucrose gradient and centrifuged in a Beckman SW60 Ti rotor for 2.5 h at 45,000 rpm. The peaks are labeled covalently closed circular (CCC) DNA and OC (open circular) DNA; the fragments are labeled by an arrow alone. Sedimentation in these gradients is from right to left. The unbroken line represents $^3$H-labeled sample DNA; the dashed line represents $^{14}$C-labeled reference DNA. (A) RB52; (B) RB52REV.

tract sedimented with the marker supercoiled DNA (Fig. 4B). The faster-sedimenting DNA synthesized by the mutant extract was isolated from neutral sucrose gradients and analyzed in the electron microscope. Examples of the two types of replicating molecules, which were cleaved with *Eco*RI, are seen in Fig. 5. Both types of molecules have a small replicating eye. The molecule in Fig. 5A is single stranded on one side of the eye and double stranded on the other. Such a molecule yields one newly replicated short fragment when denatured. The molecule in Fig. 5B is double stranded on both sides of the eye. Two-thirds of 100 molecules with eyes were of the first type, and one-third were of the second type. Forty-three molecules were measured to determine the location of the forks relative to the *Eco*RI-generated ends and to determine the size of the replicating eyes. The length of segment (a) in Fig. 5A and B from the end to the first fork encountered is 17% of the ColE1 length. This is the site where the ColE1 origin of replication has been mapped (12, 17, 32). The length of segment (b) was variable, depending upon the lengths of the arms of the replicating eyes. The length of the arms varied from 5 to 14% of the ColE1 length with an average length of 11%. This would indicate that the newly replicated fragments would be about 730 bases in length. The hybridization of fragments isolated by alkaline sucrose sedimentation to filters containing the separated strands of ColE1 DNA demonstrated that more L fragments (leading strand) than H fragments were synthesized by a mutant extract (data not shown). This result suggests that the first type of molecule (Fig. 5A) contains only a newly synthesized L fragment.

Since the major products synthesized by mu-

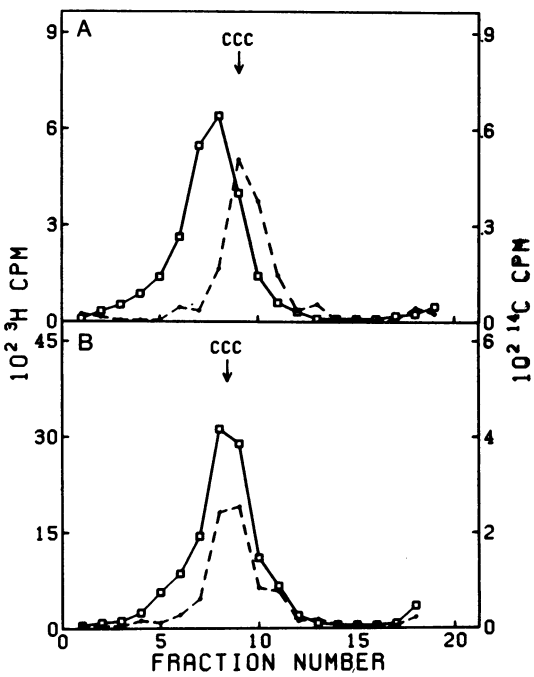


FIG. 4. Neutral sucrose sedimentation analysis of ColE1 DNA synthesized in RB52 and RB52REV extracts. Extracts of strains RB52 and RB52REV were used to catalyze ColE1 DNA synthesis. After 60 min at 30°C, the reactions were stopped as described in the legend to Fig. 3. Portions of the product were layered on neutral sucrose gradients and centrifuged for 3.5 h at 45,000 rpm. CCC designates the sedimentation of CCC marker DNA. Sedimentation in these gradients is from right to left. The unbroken line represents $^3$H-labeled sample DNA; the dashed line represents $^{14}$C-labeled reference DNA. (A) RB52; (B) RB52REV.

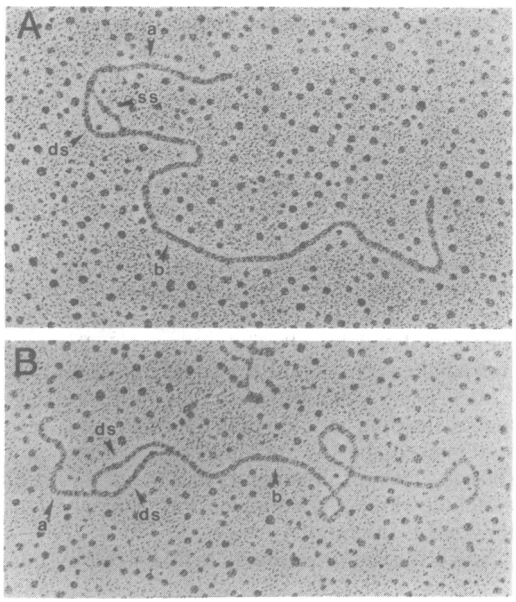


FIG. 5. Electron micrographs of *Eco*RI-cleaved replicative intermediates synthesized by an RB52 [*dnaG308*(Ts)] extract. (A) A molecule containing a single nascent DNA fragment; (B) a molecule containing two nascent DNA fragments. Abbreviations: ss, single stranded; ds, double stranded. For size data on segments (a) and (b), see the text.

tant extracts contain one or two newly synthesized short fragments and resemble early replicative intermediates, the short fragments should hybridize to ColE1 DNA immediately downstream from the origin of replication. Figure 6A shows the results of an experiment in which $^{32}$P-labeled short fragments synthesized by a mutant extract or total $^{32}$P-labeled product synthesized by a revertant extract were hybridized to Southern blots of *Hae*III-digested ColE1 DNA: lanes 1 and 3 show the stained digests, and lane 2 shows the hybridization of $^{32}$P-labeled short fragments synthesized by an RB52 [*dnaG308*(Ts)] extract to the blot of the fragments in lane 1. $^{32}$P-labeled fragments, synthesized by a mutant extract, hybridized only to fragments E and I, which are the two largest *Hae*III fragments immediately downstream from the origin. Fragments N and L are not seen since they are too small to be transferred efficiently. $^{32}$P-labeled product DNA synthesized by the revertant extract hybridized to all of the *Hae*III fragments which transferred to the nitrocellulose filter (Fig. 6, lane 4). Figure 6B shows the *Hae*III restriction map of *Eco*RI-digested ColE1 DNA (22, 31). Since electron microscopic analysis demonstrated that the arms of the replicating eyes were approximately 11% of unit length, the fragments should hybridize to ColE1 DNA from 0.17 to approximately 0.28 of the unit-length map of ColE1 (Fig. 6B). This includes fragment E and a small portion of I. The short fragments cannot be hybridizing to the other end of ColE1 since there are no fragments corresponding to E and I from that region.

Sucrose gradient analysis of the product DNA demonstrated that mutant extracts synthesize some CCC and open circular ColE1 DNA. Figure 7 shows a time course for the synthesis of completely replicated molecules by an RB52 [*dnaG308*(Ts)] and RB52REV extract. This experiment shows a major difference in the course of the synthesis of completely replicated molecules by these extracts. In contrast to RB52REV, a lag period of approximately 15 min occurs before CCC and open circular molecules are synthesized by the RB52 [*dnaG308*(Ts)] extract. By 60 min, the mutant extract incorporated only 9 pmol of [$^3$H]TMP into completely replicated molecules, whereas the RB52REV

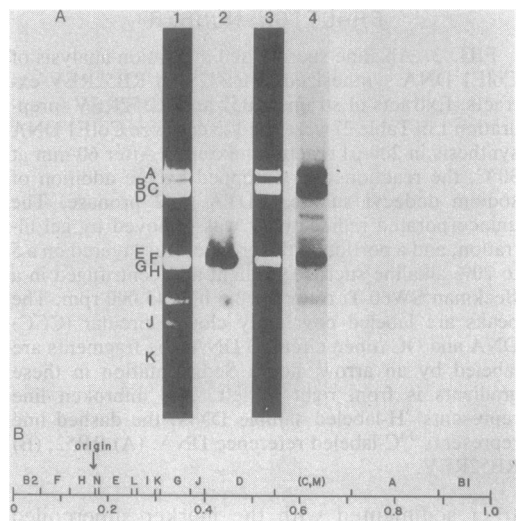


FIG. 6. Hybridization of ColE1 DNA synthesized in RB52 and RB52REV extracts to Southern blots of a *Hae*III digest of ColE1 DNA. (A) *Hae*III digests of ColE1 DNA were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and photographed under UV light (lanes 1 and 3). The DNA in the gel was transferred to a nitrocellulose sheet, and the blot of lane 1 was hybridized to $^{32}$P-labeled fragments synthesized by an RB52 [*dnaG308*(Ts)] extract (lane 2). The blot of lane 3 was hybridized to total $^{32}$P-labeled DNA synthesized by an RB52REV extract (lane 4). The autoradiogram shown in lane 2 was exposed for 5 days with an intensifying screen (Cronex lightning-plus; Du Pont Co.). The autoradiogram shown in lane 4 was exposed for 5 days without a screen. (B) *Hae*III restriction map of *Eco*RI-cut ColE1 DNA (22, 31). The letters in (A) correspond to the restriction fragments in (B).

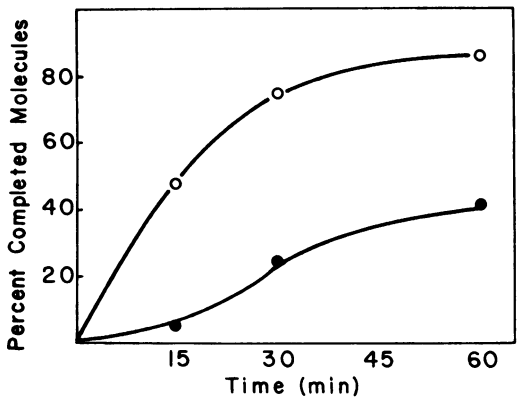


FIG. 7. Synthesis of completely replicated ColE1 molecules by RB52 and RB52REV extracts. Extracts of strains RB52 [*dnaG308*(Ts)] and RB52REV were used to catalyze ColE1 DNA synthesis (1.5 μg of DNA/50-μl assay) at 30°C. At the indicated times, samples were removed and used for alkaline sucrose gradient analysis and for the determination of incorporated [$^3$H]TMP. The percent completed molecules is the percentage of incorporated $^3$H label which appears in the open circular and CCC DNA peaks of alkaline sucrose gradient profiles. The [$^3$H]TMP incorporated into completely replicated molecules at the indicated times was (in picomoles) 0.4 (15 min), 3.3 (30 min), and 8.8 (60 min) for RB52 and 5.3 (15 min), 25.5 (30 min), and 74.0 (60 min) for RB52REV. Symbols: ●, RB52; ○, RB52REV.

extract incorporated a similar amount into completely replicated molecules by 15 min (Fig. 7).

**Product of four sets of extracts and the DnaG activity therein.** A summary of the results obtained with four mutant and four revertant extracts is shown in Table 2. The extracts were prepared and used to synthesize ColE1 DNA in vitro. The amount of incorporation of [$^3$H]TMP was measured, and a portion of the product was analyzed by sedimentation in alkaline sucrose gradients to measure the amount of radiolabel in short fragments and completed molecules. Individual extracts catalyzed the incorporation of [$^3$H]TMP to different extents. An RB52REV extract preparation incorporated, on the average, 2.5 times more [$^3$H]TMP than did an RB52 extract.

The data from these experiments can be used to calculate the number of initiations leading to replicative intermediates or completed molecules. It should be noted that two completed molecules are produced from a single initiation and that these two daughter molecules contain approximately 9 times more radiolabel than an intermediate that contains two fragments and 18 times more radiolabel than an intermediate that contains a single short fragment. All of these possibilities require a single initiation event. Therefore, simple analysis of the distribution of radiolabel in a sucrose gradient may not give a true measure of the number of initiations which lead to a particular product.

Extracts of RB52 [*dnaG308*(Ts)] produced about 13 times more replicative intermediates than did completely replicated molecules (Table 2). In contrast, extracts of RB52REV produced approximately two times more replicative intermediates. Although the product synthesized by mutant and revertant extracts was different, RB52 [*dnaG308*(Ts)] and RB52REV extracts initiated the replication of similar amounts of ColE1 DNA templates (27 and 21%, respectively). These results indicate that there is a block after the synthesis of early replicative intermediates in the *dnaG* mutant extracts. The reversion of the temperature-sensitive mutation removes this block in ColE1 DNA replication.

The relative DnaG activity of the extracts used in the experiments in Table 2 was determined by the complementation of a φX174 *dnaG*-dependent in vitro replication assay (34). Samples of extracts prepared from strains RB52 or RB52REV were added to reaction mixtures requiring an external source of DnaG protein. The results in Table 2 show that mutant extracts contained relatively little DnaG activity compared with the revertant extracts. The revertant extracts contained, on the average, 25 times more DnaG activity. The residual DnaG activity in the mutant extracts was temperature sensitive, whereas the DnaG activity in revertant extracts was not (data not shown). The amount of DnaG activity in RB53 [*dnaG3*(Ts)] and RB53REV extracts was similar to the activities obtained with RB52 and revertant extracts (data not shown). A strong correlation was found between the type of product synthesized by an extract in the ColE1 in vitro assay and the DnaG activity in the extract.

## DISCUSSION

**Accumulation of replicative intermediates.** To examine the events occurring at replication forks, we studied the effects of two strains carrying either the *dnaG3*(Ts) or *dnaG308*(Ts) mutations on the replication of ColE1 plasmids in vivo and in vitro. We have shown that replicative intermediates accumulate when extracts of cells carrying the *dnaG308*(Ts) or *dnaG3*(Ts) mutations are used for the in vitro synthesis of ColE1 DNA. This effect is reversed in strains selected at a frequency of $10^{-8}$ for their ability to grow at 42°C. This is about the frequency expected for a single-site reversion. Extracts of this revertant contain DnaG activity which is not sensitive to incubation at 40°C, whereas extracts of the mutant strain contain DnaG activity which

is sensitive to heating. From these results we conclude that the accumulation of replicative intermediates is the result of the *dnaG* mutation. The synthesis of a few completed molecules by the mutant extracts may be due to some residual DnaG activity or to an additional priming activity of *E. coli*.

**Effects of different *dnaG* mutations.** Staudenbauer et al. have reported that only lagging strand synthesis was blocked in extracts of a strain carrying the *dnaG1011*(Ts) mutation (27). Since we have shown that the *dnaG308*(Ts) mutation has an effect on leading strand, as well as lagging strand, synthesis, we propose an alternative model for the role of DnaG protein in ColE1 DNA replication. We propose that the *dnaG* gene product is required for the synthesis of both ColE1 DNA strands. The function of the DnaG protein in lagging strand synthesis would be to prime discontinuous DNA synthesis, using a primosome assembled at the protein n1 binding site on the lagging strand template that has been described by others (20, 21). In leading strand synthesis, DnaG protein may be involved in a function other than priming. This would not rule out the possibility of an additional priming role for DnaG protein in the synthesis of leading strands. The different *dnaG* mutations seem to affect these roles differentially. It seems likely that the *dnaG1011*(Ts) gene product lacks the activity required for lagging strand synthesis but not that required for leading strand synthesis, whereas the *dnaG308*(Ts) gene product lacks both activities.

Previously, it was shown that replicative intermediates accumulate in extracts of strains carrying a *dnaC*(Ts) mutation (26) or a *dnaB*(Ts) mutation (26) or in extracts of *dnaG1011*(Ts) strains treated with anti-DnaB protein antibody (27). DnaB protein and DnaC proteins are components of a primosome (2). These two proteins are required for the assembly of a primosome, and the loss of their activities blocks leading strand synthesis. Since the leading strand template contains a primosome assembly site (21), it is possible that the primosome may be required for leading strand synthesis.

**Complementation by DnaG protein.** We obtained complementation of *dnaG* mutant extracts by extracts of a strain carrying a plasmid which overproduces DnaG protein (35). We found a net increase in the incorporation of [$^3$H]TMP and a change in the product synthesized when small amounts of extract from the DnaG protein-overproducing strain were added to reactions containing a mutant extract (data not shown). As yet we have not been able to complement mutant extracts with purified DnaG protein obtained from others, but we are presently attempting to purify it under buffer and salt conditions compatible with the ColE1 replication system.


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