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Novel *Escherichia coli dnaB* Mutant: Direct Involvement of the *dnaB252* Gene Product in the Synthesis of an Origin-Ribonucleic Acid Species During Initiation of a Round of Deoxyribonucleic Acid Replication

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The initiation process of deoxyribonucleic acid (DNA) replication in *Escherichia coli* has been studied using the thermoreversible *dna* initiation mutant *E. coli* HfrH165/120/6 *dna-252*. This *dna* mutation was incorrectly classed as a *dnaA* mutation. Biochemical and genetic evidence suggests that the *dna-252* mutant is a novel *dnaB* mutant, possessing phenotypic properties which distinguish it from other *dnaB* mutants. Sensitivity of reinitiation in the *dna-252* mutant to specific inhibitors of protein, ribonucleic acid (RNA), and DNA synthesis was studied. Reinitiation is shown to be sensitive to rifampin and streptolydigin but not to chloramphenicol. Thus, the *dna-252* gene product appears to be required during the initiation process for a step occurring either before or during synthesis of an RNA species (origin-RNA). Using reversible inhibition of RNA synthesis by streptolydigin of a streptolydigin-sensitive derivative of the *dna-252* mutant, the *dna-252* gene product is shown to be directly involved in the synthesis of an origin-RNA species. These results are included in a schematic model presented in the accompanying paper of the temporal sequence of events occurring during the initiation process.

The initiation of a new round of deoxyribonucleic acid (DNA) replication at the origin of the bacterial chromosome requires the synthesis of ribonucleic acid (RNA). Evidence for this includes the following: (i) both rifampin and streptolydigin inhibit initiation after the time when chloramphenicol is no longer inhibitory in *Escherichia coli* (20, 26); (ii) Laurent (22) has found that reinitiation in a *Bacillus subtilis* thermoreversible DNA initiation mutant is sensitive to rifampin and streptolydigin but not to chloramphenicol; (iii) Messer and co-workers (27, 45) have isolated a putative origin-RNA from *E. coli*, the synthesis of which is independent of the *dnaC* gene product but is influenced by the product of the *dnaA* gene; and (iv) reinitiation in a thermoreversible *dnaA* mutant is sensitive to rifampin but not to chloramphenicol, and the sensitivity to rifampin is due specifically to RNA polymerase inhibition by rifampin (47).

We have studied reinitiation in *E. coli* HfrH165/120/6 *dna-252* (2-4, 12, 32), one of the first *dna* initiation mutants isolated. This mutant has been incorrectly classed as a *dnaA* mutant (13). Reinitiation in this mutant was found to be sensitive to rifampin and streptolydigin but not to chloramphenicol. Results of experiments with this mutant indicate that the *dna-252* gene product is directly involved in the synthesis of an origin-RNA. The temperature-sensitive mutation, *dna-252*, maps in the same region as the *dnaB* locus, between *uvrA* and *ampA*, and *dna-252* cell extracts are complemented by purified *dnaB* protein in the bacteriophage ϕX174 viral DNA to replicative form in vitro DNA synthesis system. However, the phenotype of the *dna-252* mutant is that of a mutant blocked in the initiation process of DNA replication rather than in the elongation process.

MATERIALS AND METHODS

Strains. The *E. coli* K-12 strains used in this study are listed in Table 1. Strain ZS15, the streptolydigin-sensitive derivative of strain HfrH165/120/6, was isolated as described by Clewell and Evenchik (8).

Media and growth conditions. Fresh overnight cultures grown in TG_0 medium (29) containing 0.1% Casamino Acids (Difco), 4 μg of thymine per ml, and 40 μg of L-proline per ml (TG+), were inoculated into prewarmed TG+ medium. Experiments were performed only on cultures which had undergone at least three generations from the time of inoculation. Transfer of media was accomplished by rapid filtration (25) in a room kept at 43°C. L broth contained

TABLE 1. *Escherichia coli K-12 strains used*

Strain	Genotype and phenotype	Source
HfrH165/120/6	*dna-252 thi thy pro*	W. Messer
ZS15	HfrH165/120/6 *stl*^{s}	This lab, see Materials and Methods
ZS16	HfrH165/120/6 *ampA*	This lab, spontaneous penicillin-resistant mutant
AB2500	*uvrA thy thr leu arg his thi pro ara lac gal mtl xyl str*^{r} T6^r	D. H. Helinski
H882	*purA thr leu thi arg pro str*^{r} *tonA tsx supE44*	D. H. Helinski
KL209 (HfrJ4)	*malB16 supE44*, λ^r, λ^-	B. J. Bachmann
KLF4/AB2463	F104/*thi-1 thr-1 leu-6 argE3 strA31 his-4 proA2 recA13 mtl-1 supE44 xyl-5 ara-14 galK2 lacY1 tsx-33*, λ^-	B. J. Bachmann
KLF16/KL110	F116/*argG6 metB1 his-1 thyA23 leu-6 recA1 mtl-2 xyl-7 malA1 fal-6 lacY1 strA204 tonA2 tsx-1*, λ^r, λ^-	B. J. Bachmann
KLF43/KL259	F143/*thi-1 tyrA2 pyrD34 his-68 trp-45 thyA33 recA1 mtl-2 xyl-7 malA1 galK35 strA118*, λ^r, λ^-	B. J. Bachmann
NY100	F118 *ampA1 pyrB*$^+$/*ampA*$^+$ *pyrB thr leu thi his lac recA56 val*^{r} *strA*	J. A. Wechsler
P801	Hfr, *thi mtl xyl lacY*, λ^{ind}	B. J. Bachmann
Ra-2	Hfr, *mal-28 sfa-4 supE42*, λ^r, λ^-	B. J. Bachmann

1.0% tryptone (Difco), 1.0% yeast extract (Difco), 0.5% NaCl, and 0.2% glucose.

Inhibitors. Preparation of inhibitors was as described in the accompanying paper (47). Streptolydigin, lot no. 2677dev11710799, was a gift of Upjohn.

Radioactive labeling of DNA and RNA. Radioactive labeling was as described in the accompanying paper (47).

Preparation of phage P1(*kc*) and transduction. The protocols described in reference 23 were followed for preparation of phage P1(*kc*) and transduction.

RESULTS

Reinitiation of strain HfrH165/120/6 in the presence and absence of chloramphenicol. When cultures of strain HfrH165/120/6 were transferred to the restrictive temperature of 42°C, DNA synthesis gradually ceased (Fig. 1A and Fig. 7, ●; see also reference 2). If, however, such cultures were returned again to 42°C after only 10 min at 30°C (reinitiation control, ×), DNA synthesis ceased after the amount of DNA in the culture had approximately doubled (Fig. 1A and 7). This doubling in DNA content is the predicted value if each chromosome initiated one new round of DNA replication during the 10-min exposure to 30°C. Occasionally, less than a complete doubling in the amount of DNA synthesized in this reinitiation control was observed (e.g., see Fig. 1A), probably because some cells failed to recover an active *dna-252* gene product.

The effect of chloramphenicol on reinitiation in these strains was tested by adding chloramphenicol to cultures exposed to the restrictive temperature for about 1.5 generations. Ten minutes later the cultures were returned to the permissive temperature and DNA synthesis was measured (Fig. 1A, □, and see Fig. 7, ◇). The amount of DNA synthesized was approximately equal to the amount of DNA synthesized in the reinitiation control, demonstrating that protein synthesis is not required for reinitiation in this mutant, and therefore that this mutant is thermoreversible, as was observed by others (2).

Effect of rifampin on reinitiation. The effects of rifampin (38, 39), a specific inhibitor of bacterial DNA-dependent RNA polymerase (EC 2.7.7.6), on reinitiation in this strain were also examined. Rifampin (100 μg/ml) was added to cultures exposed to the restrictive temperature for 1.5 generations, the cultures were returned to the permissive temperature 10 min later, and DNA synthesis was measured. Essentially no DNA was synthesized in the presence of rifampin in the *dna-252* mutant (Fig. 1A, △, and see Fig. 7, ■). Thus, rifampin added 10 min before the return to the permissive temperature prevents reinitiation in this mutant, indicating that the thermosensitive block occurs either before or during a rifampin-sensitive step of the initiation process.

Effect of time of addition of rifampin on reinitiation. Previous reports showed that rifampin added to cultures of *dnaA* mutants 5 min prior to return to the permissive temperature did not completely block initiation of new rounds of DNA replication (14). However, Reid and Speyer (28), noting that *E. coli* is relatively impermeable to rifampin (see also 15 and 31), estimated the time required for 100 μg of rifampin per ml to inhibit the rate of RNA synthesis 99% in an *E. coli* K-12 strain to be between 5.1

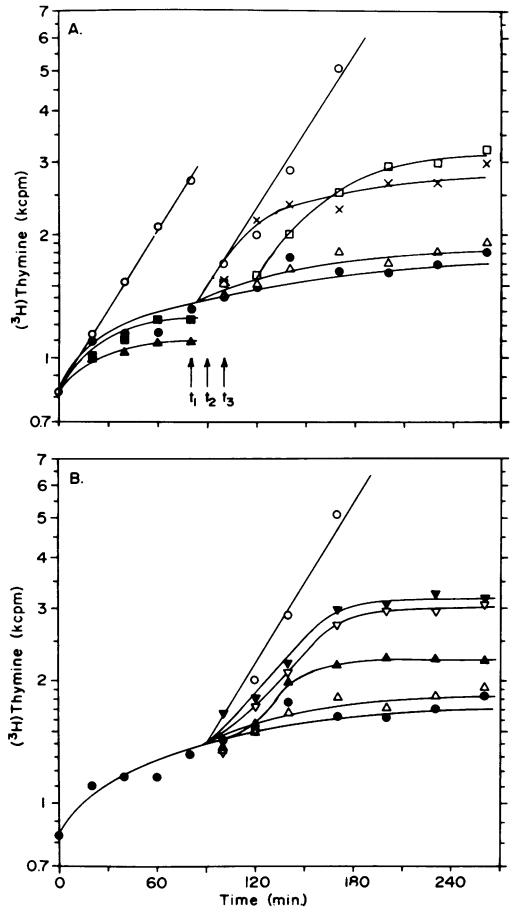

FIG. 1. *(A) Effect of rifampin or chloramphenicol on the reinitiation of DNA replication in strain HfrH165/120/6 dna-252. Strain HfrH165/120/6 was grown in 80 ml of TG+ medium plus [^{3}H]thymine (1 μCi/4 μg per ml) for three generations at 30°C. At 0 min (about 3 × 10^7 cells/ml), 50 ml was shifted to 42°C (●). The remaining 30 ml was split into three aliquots and incubated at 30°C. One aliquot received rifampin (100 μg/ml; ▲), one received chloramphenicol (100 μg/ml; ■), and the third received no additions (○). At time t_1 (80 min), rifampin (100 μg/ml; △) or chloramphenicol (100 μg/ml; □) was added to each of two 10-ml aliquots of the 42°C culture, and 10 min later (time t_2) both cultures were returned to 30°C and incubated further. Two additional 10-ml aliquots of the 42°C culture were returned to 30°C at time t_2. One was incubated further at 30°C (○), and the second was returned to 42°C 10 min later at time t_3 (×, reinitiation control). (B) Effect of time of addition of rifampin on the reinitiation of DNA replication in strain HfrH165/120/6 dna-252. Strain HfrH165/120/6 grown as described in (A) above was incubated at 42°C for 90 min (●), at which time (0 min) five 10-ml aliquots were returned to 30°C and incubated further. Four of the five aliquots received rifampin (100 μg/ml) 10 min before (△), 5 min before (▲), 0 min before (▽), and 10 min after (▼) the return to 30°C; the fifth aliquot received no additions (○). DNA synthesis was assayed as described in the text.*

and 6.1 min. The kinetics of inhibition of RNA synthesis by rifampin were determined for strain HfrH165/120/6. As seen in Fig. 2, the incorporation of [^{3}H]uridine was inhibited approximately 95% after 5 min and 98% after 10 min of exposure to rifampin. Thus, the rifampin inhibition of RNA synthesis is not immediate, and 10 min of exposure to rifampin is necessary to reduce the rate of RNA synthesis to a few percent of the initial rate. The specific effects of rifampin on reinitiation when added to a culture of strain HfrH165/120/6 at varying times before and after return of the culture to 30°C are shown in Fig. 1B. The data shown in Fig. 1A and B are from the same experiment. When added at the same time or 10 min after return of the culture to the permissive temperature, rifampin had no effect on reinitiation: the amount of DNA synthesized was the same as in the reinitiation control (see Fig. 1A). In contrast, when added 10 min prior to return of the culture to the permissive temperature, rifampin blocked reinitiation nearly 100%. When added 5 min prior to the return of the culture to the permissive temperature, reinitiation was

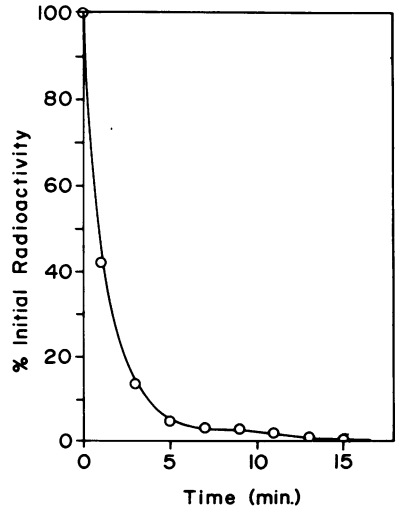

FIG. 2. *Effect of rifampin on the rate of RNA synthesis in strain HfrH165/120/6 dna-252. Rifampin (100 μg/ml) was added to a culture growing exponentially (about 1.5 × 10^8 cells/ml) at 30°C in TG+ medium. At the times indicated, duplicate 0.2-ml samples received [^{3}H]uridine (50 μCi/2.4 μg per ml). After incubation at 30°C for 3.0 min, incorporation was terminated and RNA synthesis was assayed as described in the text. Incorporation in the untreated culture (100%) was 35,700 counts/min.*

blocked approximately 50%, as was observed by others (14) for a *dnaA* mutant. Thus, complete inhibition of reinitiation in the *dna-252* mutant requires 5 to 10 min of exposure to rifampin, a time necessary for greater than 95% inhibition of the rate of RNA synthesis.

Effects of streptolydigin on reinitiation. The data of Fig. 1 show that the thermosensitive block in the *dna-252* mutant occurs either before or during a rifampin-sensitive step in the initiation process. An experiment was designed to differentiate between these two possibilities (see Fig. 6). This experiment requires that RNA synthesis be reversibly inhibited. Rifampin inhibits the initiation of transcription by forming a very stable complex with the β subunit of RNA polymerase (38, 39, 46). Consequently, inhibition of RNA synthesis, as well as inhibition of reinitiation of DNA replication (unpublished observations), by rifampin is essentially irreversible. Streptolydigin, another specific inhibitor of RNA polymerase (6), binds much less tightly to the β subunit of RNA polymerase and the inhibitory effect is reversed simply by removing streptolydigin from the culture medium or by diluting the cells into streptolydigin-free medium (6). Since most *E. coli* strains are impermeable to streptolydigin, a derivative of *E. coli* HfrH165/120/6, called ZS15, was isolated whose growth on tryptone agar plates is completely inhibited by 50 μg of streptolydigin per ml at 30°C.

In contrast to this growth inhibition, no inhibition of DNA replication by 50 μg of streptolydigin per ml was observed at 30°C (Fig. 3). A complete inhibition of DNA synthesis, after an initial residual synthesis consistent with completion of rounds of DNA replication in progress and no initiation of new rounds, was observed only for concentrations of 400 μg of streptolydigin per ml or higher (Fig. 3). The effects of two concentrations of streptolydigin on the rate of RNA synthesis are shown in Fig. 4. In the presence of 50 μg of streptolydigin per ml (Fig. 4A), a residual rate of RNA synthesis 12 to 15% of the initial rate was observed, whereas 500 μg of streptolydigin per ml (Fig. 4B) reduced the rate of RNA synthesis to less than 5% of the initial rate. Such inhibition is apparently required to inhibit the RNA synthesis event which occurs during the initiation process (synthesis of origin-RNA).

The kinetics of the reversal of RNA synthesis inhibition by 500 μg of streptolydigin per ml were studied using a temperature protocol identical to that of the experiment outlined in Fig. 6. The rate of RNA synthesis was inhibited about 98% within 10 min after addition of 500 μg of streptolydigin per ml to a culture of *E. coli* ZS15 exposed to the restrictive temperature for 1.5 generations, and this inhibition was further increased during the subsequent 10-min exposure to the permissive temperature (Fig. 5). When the streptolydigin was removed by rapid filtration, the rate of RNA synthesis immediately increased, attaining a rate of 50% of

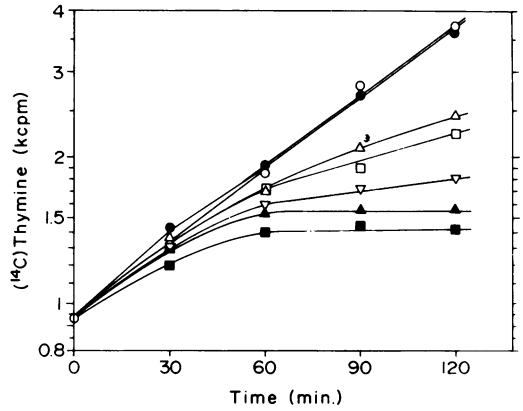

FIG. 3. *Effect of different concentrations of streptolydigin on DNA replication in strain ZS15 dna-252 stl^s. Strain ZS15 was grown in 80 ml of TG+ medium plus [^{14}C]thymine (0.5 μCi/4 μg per ml) for three generations at 30°C. At 0 min (about 3 × 10^7 cells/ml), 10-ml aliquots received 0 (○), 50 (●), 100 (△), 200 (□), 300 (▽), 400 (▲), or 500 (■) μg of streptolydigin per ml, and were incubated further at 30°C. DNA synthesis was assayed as described in Materials and Methods.*

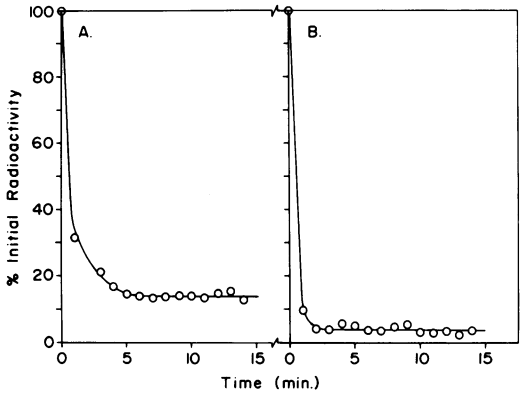

FIG. 4. *Effect of streptolydigin on the rate of RNA synthesis in strain ZS15 dna-252 stl^s. Streptolydigin was added at 0 min to two cultures of ZS15 growing exponentially (about 0.5 × 10^8 cells/ml) at 30°C in TG+ medium. At the times indicated, duplicate 0.2-ml samples received [^{3}H]uridine (40 μCi/2 μg per ml). After incubation at the permissive temperature for 2.5 min, incorporation was terminated and RNA synthesis was assayed as described in the text. Incorporation in the untreated culture (100%) was 18,360 counts/min. (A) 50 μg of streptolydigin per ml; (B) 500 μg of streptolydigin per ml.*

the initial rate within 10 min after filtration (Fig. 5). Thus, RNA synthesis can be reversibly inhibited in this strain using 500 μg of streptolydigin per ml.

To demonstrate whether the thermosensitive block in the *dna-252* mutant occurs before or during the origin-RNA synthesis event in the initiation process, the experiment outlined in Fig. 6 was performed. Using a culture of ZS15 thermally blocked at the step controlled by the *dna-252* gene product, RNA synthesis was inhibited by the addition of 500 μg of streptolydigin per ml, and 10 min later the culture was returned to the permissive temperature. After allowing 10 min for the *dna-252* gene product to act functionally in the initiation process, the culture was returned to the restrictive temperature and incubated for 10 min to inactivate the *dna-252* gene product. Streptolydigin was then removed by rapid filtration, permitting RNA synthesis to resume (see Fig. 5). If the RNA synthesis step occurs only after the step controlled by the *dna-252* gene product, then reinitiation should be observed. If, however, synthesis of the origin-RNA directly requires a functional *dna-252* gene product, then no reinitiation should be observed.

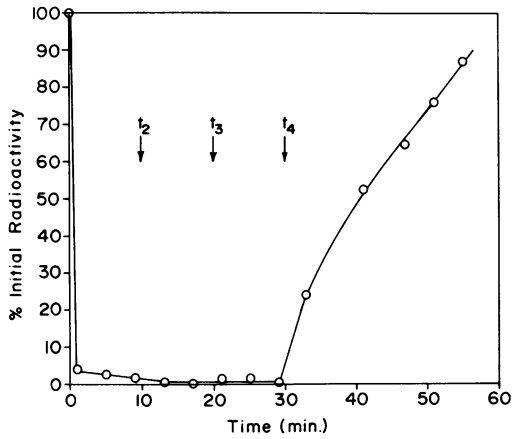

FIG. 5. *Reversal of inhibition of RNA synthesis by streptolydigin in strain ZS15 dna-252 stl^s. Strain ZS15 was grown in 10 ml of TG+ medium for three generations at 30°C (to about 3 × 10^7 cells/ml) and then incubated at 41°C for 80 min. At this time (0 min), streptolydigin (500 μg/ml) was added to the culture. The culture was incubated at 41°C for 10 min, shifted to 30°C (at time t_2) for 10 min, and then returned to 41°C (at time t_3) for 10 min. The streptolydigin was then removed (at time t_4) by rapid filtration at 41°C, and the culture was incubated further at 41°C. At the times indicated, duplicate 0.2-ml samples received [^{3}H]uridine (40 μCi/2 μg per ml). After incubation for 3.0 min at the temperature indicated, incorporation was terminated and RNA synthesis was assayed as described in the text. Incorporation in the untreated culture (100%) was 15,570 counts/min.*

The results of this experiment are shown in Fig. 7. When chloramphenicol (100 μg/ml, ◇) was added to a culture of strain ZS15 incubated at the restrictive temperature for 1.5 generations 10 min prior to return to the permissive temperature, no inhibition of reinitiation was observed, whereas similar addition of streptolydigin (500 μg/ml, □) or rifampin (100 μg/ml, ■) completely inhibited reinitiation, consistent with the data of Fig. 1. When streptolydigin (500 μg/ml, ▽) was added 10 min after the return of the cells to the permissive temperature, a 100% increase in the DNA content was observed. This control experiment demonstrates that a new round of DNA replication will occur in the presence of streptolydigin (500 μg/ml) when the conditions for the initiation process have already been met. When streptolydigin was removed by rapid filtration at the restrictive temperature after the culture had been exposed to the permissive temperature for 10 min in the presence of the streptolydigin, DNA synthesis resumed immediately if the culture was then returned to the permissive tem-

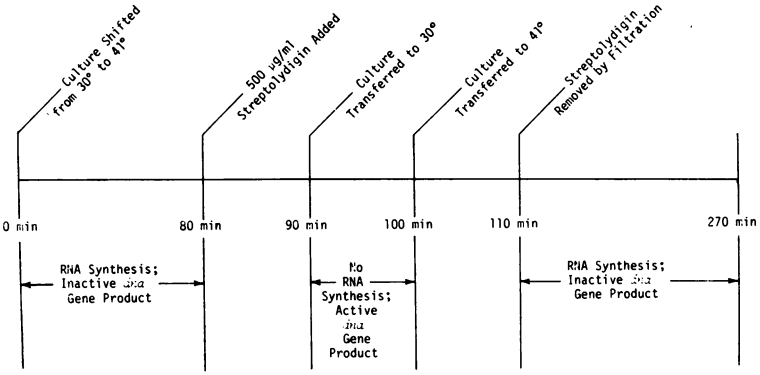

FIG. 6. *Experimental protocol to demonstrate whether dna-252 gene product is directly involved in the synthesis of origin-RNA.*

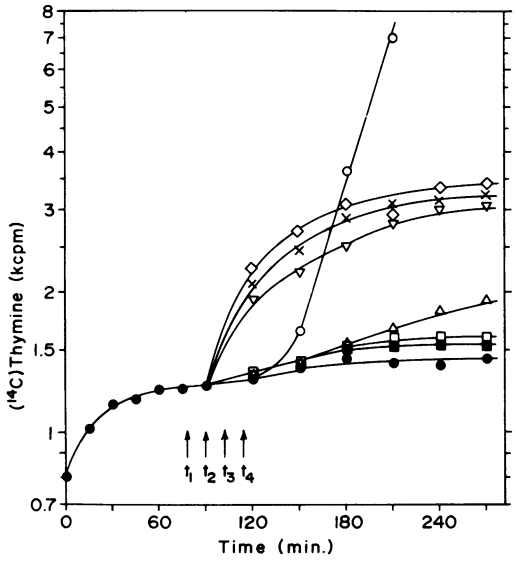

FIG. 7. *Effect of streptolydigin, rifampin, or chloramphenicol on the reinitiation of DNA replication in strain ZS15 dna-252 stl^s. Strain ZS15 was grown in 50 ml of TG+ medium plus [^{14}C]thymine (0.5 μCi/4 μg per ml) for three generations at 30°C to about 3 × 10^7 cells/ml. The culture was then shifted at 0 min to 41°C and incubated further (●). At time t_1 (80 min after 0 min), rifampin (100 μg/ml; ■) and chloramphenicol (100 μg/ml; ◇) were added to each of two 5-ml aliquots, and streptolydigin (500 μg/ml) was added to a 15-ml aliquot of the 50-ml culture. Ten minutes later (time t_2), these cultures and two untreated 5-ml aliquots were returned to 30°C. A 5-ml aliquot of the 15-ml streptolydigin-treated culture (□) and the rifampin- and chloramphenicol-treated cultures were incubated further. Ten minutes later (time t_3), one untreated 5-ml aliquot received streptolydigin (500 μg/ml) and was incubated further at 30°C (▽). The other untreated 5-ml aliquot was returned to 41°C at time t_3 and incubated further (×, reinitiation control). The remaining 10 ml of streptolydigin-treated culture was returned to 41°C at time t_3, incubated for 10 min (to time t_4), rapidly filtered at 41°C to remove the streptolydigin, suspended in 10 ml of prewarmed (41°C) TG+ medium containing [^{14}C]thymine (0.5 μCi/4 μg per ml), and split into two 5-ml aliquots. One of these was incubated further at 41°C (△) and the other was incubated at 30°C (○). DNA synthesis was assayed as described in the text.*

perature (Fig. 7, ○); therefore, the cells were fully capable of DNA replication after the experimental manipulations. However, when a culture treated in this manner was maintained at the restrictive temperature, no reinitiation occurred (Fig. 7, △). Thus, reinitiation in strain ZS15 requires that both an active *dna-252* gene product and an RNA synthetic activity must be present simultaneously.

Genetic analysis of the *dna-252* mutation. The reversion rate for strain HfrH165/120/6 of temperature sensitivity to temperature resistance as determined by the ability to form colonies at 43°C was between 10^{-5} and 10^{-6}, indicating that the *dna-252* mutation is a single mutation. In initial P1 transduction studies, no cotransduction between *dna-252* and *ilv* (linked to *dnaA*) or *serB* (linked to *dnaC*) could be detected. The rapid mapping method of Low (24) using the Hfr kit from the *E. coli* Genetic Stock Center showed that the *dna-252* locus mapped in region 17 between 90 and 98 min on the modified *E. coli* genetic map (1). This result was confirmed via time of transfer conjugation experiments using phenotypic F$^-$ cells of strain HfrH165/120/6 *dna-252* as recipients and either *E. coli* P801 or *E. coli* Ra-2 as donor cells. Additional genetic information was obtained with conjugation experiments using four different F-prime strains as donors and phenotypic F$^-$ cells of strain HfrH165/120/6 *dna-252 thyA ampA$^+$ pro* as the recipient (Table 2). Only F118, which covers the region of the chromosome between 90 and 96 min, and also contains the *dnaB* locus (34), gave temperature-resistant colonies when mated with the *dna-252* mutant. When a ColE1-*E. coli* DNA chimera plasmid (7) known to carry the *dnaB* locus (R. McMacken, personal communication) was transferred into strain HfrH165/120/6 *dna-252*, all HfrH165/120/6 colonies resistant to colicin E1 were temperature resistant, demonstrating that the *dna-252* locus maps at least within about 0.5 min of the *dnaB* locus on the *E. coli* genetic map. These complementation experiments also show that the *dna-252* mutation is

TABLE 2. *Complementation experiments between F' plasmids and the dna-252 locus*

Plasmid	Marker selected[a]	Position of marker (min)	No. of temperature-sensitive F-ductants	No. of temperature-resistant F-ductants	Total F-ductants tested[b]	% Temperature resistant
F104	*pro$^+$*	6	28	3	31	10
F116	*thyA$^+$*	60	31	0	31	0
F118	*ampA*	93	1	29	30	97
F143	*thyA$^+$*	60	31	0	31	0

[a] Cultures of the F' donor strains growing exponentially in L broth were mixed with the same volume of a stationary-phase culture of recipient cells (strain HfrH165/120/6 *dna-252 pro thyA ampA$^+$*) grown in TG+ medium. The mating mixture was held at 30°C for 1 h. It was then diluted and samples were spread on selective media plates and incubated at 30°C.

[b] A portion of the F-ductants that formed colonies on the selective media plates was tested further for temperature sensitivity by streaking on selective media plates and incubating the plates at 42°C.

recessive to wild type. Further mapping experiments were performed by transduction using bacteriophage P1, and the results obtained are summarized in Table 3. In another P1 cross using strain ZS16 *malB*$^+$ *dna-252* as the donor and strain KL209 (HfrJ4) *malB16 dna-252*$^+$ as the recipient, the cotransduction frequency for *malB*$^+$ and *dna-252* was 0.86. Different *dnaB* mutations have cotransduction frequencies with *malB* ranging from 0.05 to 0.80 (35, 36; P. Schendel, Ph.D. thesis, University of Wisconsin, Madison, Wis., 1974) even though they appear to represent a single cistron as defined by complementation (33). Based on the transduction data in Table 3 and other published data (5), the probable gene order in this region of the *E. coli* chromosome is *malB* - *uvrA* - *dna-252* - *ampA* - *purA*. This position of the *dna-252* gene is in the same region of the *E. coli* chromosome as the *dnaB* locus, and may be a *dnaB* mutation exhibiting novel phenotypic properties. Furthermore, biochemical evidence (S. Wickner, personal communication) suggesting that the *dna-252* gene product is identical with the *dnaB* gene product has been obtained in complementation experiments done by S. Wickner using the bacteriophage ϕX174 viral DNA to replicative form DNA in vitro DNA synthesis system. [Three-liter cultures of strain HfrH165/120/6 *dna-252* and a temperature-resistant revertant of this strain were grown at 30°C to an A_{650} of 1.0. Cells were collected and frozen (43), and crude extracts, streptomycin sulfate supernatants, and ammonium sulfate precipitates were prepared (43). Precipitates were suspended in 0.03 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5), 1 mM ethylenediaminetetraacetic acid, 2 mM dithiothreitol, 5% glycerol, and 2 mM $MgCl_2$ and dialyzed against the same buffer for 2 h. The final protein concentration of each preparation was about 20 mg/ml. Preparations from both the *dna-252* mutant and temperature-resistant revertant catalyzed bacteriophage fd DNA-dependent DNA synthesis in vitro, using previously described assay conditions (40). The preparation from the temperature-resistant strain also catalyzed ϕX174 DNA-dependent in vitro DNA synthesis. However, the preparation from the *dna-252* mutant was inactive with ϕX174 DNA, but could be stimulated 10-fold by the addition of purified *dnaB* protein (isolated as described in reference 44). As measured by the *dnaB* complementation assay (44) at 30°C, the preparation from the *dna-252* mutant contained less than 0.01 U of *dnaB* protein per mg and that from temperature-resistant revertant cells contained 1.30 U of *dnaB* protein per mg.]

TABLE 3. *Transduction mapping of the dna-252 mutation by phage P1*

Expt[a] and selected marker	No. of transductants examined[b]	Unselected markers[c]				Cotransduction frequency of *dna-252* with:	
		ampA$^+$ *dna-252*$^+$	*ampA*$^+$ *dna-252*	*ampA dna-252*$^+$	*ampA dna-252*	*purA*	*uvrA*
I. *purA*$^+$	90	62	0	28	0	<0.01	
II. *uvrA*$^+$	58	28	27	0	3		0.52

[a] For experiment I, the donor was ZS16 *dna-252 ampA purA*$^+$ and the recipient was H882 *dna-252*$^+$ *ampA*$^+$ *purA*. For experiment II, the donor was ZS16 *uvrA*$^+$ *dna-252 ampA* and the recipient was AB2500 *uvrA dna-252*$^+$ *ampA*$^+$.

[b] Number of transductants examined for unselected markers.

[c] Number of transductants scored.

DISCUSSION

E. coli strain HfrH165/120/6 *dna-252* is temperature-sensitive in the process of initiation of DNA replication by the following criteria. (i) The observed amount of residual synthesis and number of residual cell divisions are those expected if rounds of DNA replication in progress at the time of exposure to the restrictive temperature are completed, but new rounds are not initiated (2). (ii) After completion of the residual DNA synthesis observed during starvation of required amino acids (25) at the permissive temperature, no further DNA synthesis at the restrictive temperature occurs, indicating that both the temperature-sensitive block in DNA synthesis and that caused by amino acid starvation are at the same point in the DNA replication cycle (2). (iii) Reinitiation of DNA replication at the permissive temperature after completion of residual DNA synthesis at the restrictive temperature is not sensitive to chloramphenicol, indicating thermoreversibility (Fig. 1A, and reference 2), but is sensitive to rifampin and streptolydigin (Fig. 1A and 7). (iv) A doubling in the amount of DNA is observed in the presence of chloramphenicol when cells exposed to 42°C for 1.5 generations are returned to 30°C, as would be expected if most chromosomes initiate one new round of DNA replication at 30°C (Fig. 1A, and reference 2). (v) This approximate doubling in DNA content is also observed when cells exposed to 42°C for 1.5 generations are returned to the permissive temperature for 10 min and then returned to 42°C (Fig. 1A, and reference 2). (vi) Temperature-shift experiments with simultaneous density labeling show that the residual DNA synthesis observed at 42°C is not due to new rounds of DNA replication aberrantly initiated in a small percentage of the cell population, but

rather is due to the completion of already initiated replication rounds (2).

It is reasonable to assume that after 1.5 generations at the restrictive temperature DNA initiation mutants contain completed chromosomes with DNA synthesis blocked at the step involving the product of the mutated gene. If that step is prior to or involved in the obligatory transcriptional event (synthesis of origin-RNA), then RNA synthesis inhibitors would prevent DNA reinitiation on return to the permissive temperature. RNA synthesis inhibitors should have no effect on reinitiation if the step that was blocked followed the synthesis of origin-RNA. Results presented here show that reinitiation is inhibited in *dna-252* mutants by rifampin and streptolydigin but not by chloramphenicol (Fig. 1A and 7). These results demonstrate that the *dna-252* gene product is either involved in a step prior to or is directly involved in the synthesis of an origin-RNA.

Lark (20) observed that streptolydigin as well as rifampin inhibits replication from the origin at a late time in the DNA replication cycle when chloramphenicol is no longer inhibitory. We have observed that in a streptolydigin-sensitive mutant the amount of DNA synthesized in the presence of streptolydigin exceeds the 39% amount expected for the inhibition of initiation events unless a streptolydigin concentration of 400 μg/ml or greater is used. However, a streptolydigin concentration of 50 μg/ml is sufficient to prevent colony formation, as well as to inhibit RNA synthesis by 85 to 90%. The following two possibilities would explain the decreased sensitivity of the initiation process to streptolydigin inhibition compared with colony formation. First, initiation of nascent RNA chains must be inhibited in order to inhibit DNA synthesis at the origin. Cassani et al. (6), using an in vitro RNA synthesis system, observed that at sufficiently high concentrations of streptolydigin (300 μg/ml) initiation of nascent RNA chains as well as elongation was inhibited, whereas at lower concentrations only elongation of RNA chains was inhibited. Second, streptolydigin may inhibit transcription of the DNA segment coding for origin-RNA to a lesser extent than transcription of other regions of the chromosome. Cassani et al. (6) found that the concentration of streptolydigin necessary to produce 50% inhibition of transcription of poly(dA)·poly(dT) is 10 times higher than the dose required for a 50% inhibition of transcription of T4 DNA, suggesting that the degree of inhibition of the rate of transcription by streptolydigin may be a function of the composition and secondary structure of the DNA segment that is being transcribed. Experiments designed to distinguish between these two possibilities are currently being pursued in this laboratory.

Results from the experiment designed to test whether the block in DNA replication in the *dna-252* mutant grown at the restrictive temperature occurs at the origin-RNA transcription step or prior to it (Fig. 5 and 6) demonstrate that, in order for reinitiation to occur in the *dna-252* mutant, there must be an active *dna-252* gene product and an RNA synthetic activity present simultaneously. Therefore, the *dna-252* gene product appears to be directly involved in the transcription of an origin-RNA, rather than in a step prior to its synthesis. These results are included in a schematic model of the temporal sequence of events which occur in the initiation process presented in the accompanying paper (47).

The biochemical and genetic evidence presented here suggest that the *dna-252* mutation is in the *dnaB* gene. However, the *dna-252* mutant exhibits phenotypic properties which distinguish it from other *dnaB* mutants that have been studied. The *dna-252* mutant is temperature sensitive in the initiation process, as determined by the criteria given above, whereas *dnaB* mutants, with no residual DNA synthesis at the restrictive temperature, are temperature sensitive in the elongation process. The *dna-252* mutant differs from a *dnaB* mutant with residual synthesis at the restrictive temperature, strain FA21*dnaB21* (11), in that the temperature-sensitive block in the *dna-252* mutant is the same as the block in DNA synthesis caused by amino acid starvation (2); this is not true for the *dnaB21* mutant (11). Also, the *dnaB21* mutant is unable to support the synthesis of bacteriophage λDNA or support the growth of λ at the restrictive temperature (11); however, both the synthesis of a λ DNA and the production of λ phage occur in the *dna-252* mutant at the restrictive temperature (19, 32). Finally, several differences with regard to bacteriophage and plasmid DNA replication and propagation exist between *dnaB* mutants and the *dna-252* mutant; these differences are summarized in Table 4.

At least two distinct possibilities exist consistent with results presented here which would reconcile the above apparent phenotypic differences between the *dna-252* mutant and other *dnaB* mutants, both of which may be true. First, the primary function of the *dnaB* gene product may be a direct involvement in the synthesis of RNA species necessary for the initiation process. The *dnaB* protein also is required for bacteriophage lambda DNA replication and for synthesis of OOP RNA, an RNA

TABLE 4. *Plasmid ColE1 and bacteriophage P1 and λ propagation: differences between E. coli dna-252 and dnaB mutants*

Plasmid or phage	Response in the *dna-252* mutant at the restrictive temp	Response in the *dnaB* mutant at the restrictive temp	References
P1	No phage production but phage DNA still synthesized at 42°C	Replication and phage production accompanied by transient recovery of bacterial DNA synthesis[a]	4, 19
λ	DNA replication and phage production	No DNA replication and no phage production[a]	19, 32
ColE1	No DNA replication and no colicin production	DNA replication at a reduced rate and high colicin production[b]	12

[a] Strain HfrH165/70 *dnaB70* was examined.
[b] Strain CR34/43 *dnaB43* was examined.

species which may serve a primer role in the initiation of lambda DNA replication (16). If the role of the *dnaB* protein in DNA chain elongation is the synthesis of RNA primers, then the *dna-252* mutation may be in a region of the *dnaB* cistron such that the temperature-sensitive *dnaB* protein is active in vivo in nascent DNA short piece synthesis during elongation but is inactive in vivo in the synthesis of origin-RNA during initiation. This mutant protein may be unstable and hence inactivated during extract preparation for in vitro studies, accounting for the results in the φX174 viral DNA-dependent studies.

Second, the *dnaB* gene product may be a necessary component of a "replication complex" formed during the initiation process and subsequently required for the elongation process. If the *dnaB* protein is a component of a replication complex formed during initiation and subsequently active during elongation, an RNA synthesis event requiring the *dnaB* protein may be necessary either for formation of the complex or for conversion of the complex into a stable or mature form. Suggestive evidence for such a replication complex has been presented by Lark and Wechsler (21) in in vivo studies of several *dnaB* mutants and by Kogoma and Lark (18) in studies of "stable DNA replication." Further, in vivo studies of stable replication by Kogoma (17) suggest that the *dnaB* protein performs two functions during stable replication, functions that may be related to initiation and elongation processes of normal DNA replication. In vitro studies using the φX174 viral DNA-dependent system also provide evidence that the *dnaB* protein functionally enters a complex with other proteins. First, a complex involving five proteins (including the *dnaB* protein), adenosine 5'-triphosphate, and φX174 viral DNA is formed prior to *dnaG* protein participation in conversion of φX174 viral DNA to duplex replicative form DNA (37). Second, an ATP-dependent complex of *dnaB* and *dnaC* proteins can be isolated by gel filtration (41, 42). In this complex, *dnaC* protein inhibits the DNA-dependent ATPase activity associated with *dnaB* protein, and *dnaB* protein protects *dnaC* protein from *N*-ethylmaleimide inactivation (41, 42). This complex may be physiologically important for initiation and for elongation in *E. coli* DNA replication (42). Some *dnaC* mutants appear to be deficient in the elongation process (34), consistent with this hypothesis. By comparison, plasmid ColE1 DNA replication is reduced in *dnaB43* (12) and *dnaC* (9) mutants, whereas initiation of lambda DNA replication occurs in *dnaC1* and *dnaC2* mutants but not in strain FA22 *dnaB22* at the restrictive temperature (S. Hayes, personal communication). Thus, if the in vitro *dnaB-dnaC* protein complex is physiologically important for *E. coli* DNA replication, this complex may not be required for replication of all other DNA molecules in the *E. coli* cell.

The biochemical and genetic evidence presented here strongly suggests that the *dna-252* mutation is a mutation in the *dnaB* cistron. However, it is possible that the "*dnaB* locus" is composed of more than one cistron, coding for more than one protein involved in DNA initiation events, and that the native "*dnaB* protein" of molecular weight 250,000 (44) is a protein complex containing these polypeptide chains as subunits. The following biochemical and genetic observations support this concept. First, sodium dodecyl sulfate acrylamide gel electrophoresis showed that greater than 98% of *dnaB* protein migrates as a single band of molecular weight 48,000 (44). Second, analysis of *dnaB* heteroallelic diploids suggests that the products of the cistrons defined by the individual *dnaB* mutations studied aggregate to form a multimeric complex (21; P. Schendel, Ph.D. thesis). Third, phage P1 cotransduction frequencies of different *dnaB* mutants with *malB* vary from 0.05 to 0.80, suggesting that the "cistron" defined by these mutations is unusually large

(35, 36; P. Schendel, Ph.D. thesis). Fourth, gel filtration studies of purified *dnaB* protein suggests that high-molecular-weight aggregates possessing *dnaB* activity in the ϕX174 DNA-dependent DNA synthesis system are formed when ATP is removed from the *dnaB* protein (41, 42).

Thus, in summary, the *dna-252* mutation defines a gene whose product appears to be directly involved in the synthesis of an RNA species required for initiation of a round of DNA replication in *E. coli*. Genetic and biochemical evidence strongly suggest that the *dna-252* mutation is in the *dnaB* gene even though the mutation affects the initiation process rather than the elongation process in DNA replication.

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