A POSSIBLE FUNCTION OF DNA POLYMERASE IN CHROMOSOME REPLICATION

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Received October 13, 1970

Summary: The Escherichia coli DNA polymerase mutant isolated by de Lucia and Cairns is impaired in its ability to convert low molecular weight, newly synthesized DNA into high molecular weight material. The mutation affects the joining of the low molecular weight strands and not their synthesis, but the strands are eventually incorporated into high molecular weight DNA. The in vitro DNA ligase activity of the mutant is normal. It is proposed both DNA polymerase and DNA ligase are required to close the breaks in newly synthesized DNA in vivo, and either the low levels of polymerase activity or interference with DNA ligase by an altered DNA polymerase reduce the rate of closing in the mutant.

INTRODUCTION

The in vivo function of the DNA polymerase isolated by Kornberg and co-workers has not been determined. The enzyme faithfully copies template DNA, and it could be the catalyst of chromosome replication (1,2). Other properties suggest DNA polymerase functions in repair (3,4), although most of these properties are not incompatible with models of chromosome replication (5).

A mutant of Escherichia coli that lacks virtually all DNA polymerase activity in vitro has been isolated by de Lucia and Cairns (6). The mutant grows at the same rate as its parent but is sensitive to ultraviolet light, which supports the suggestion DNA polymerase functions in repair and not in replication. As stressed by de Lucia and Cairns, conclusions based on such evidence must be drawn with caution. The lack of activity in vitro does not exclude activity in vivo.

DNA polymerase might function in chromosome replication, regardless of whether it is involved in replication directly at the replication fork. The newly synthesized strands of DNA are of low molecular weight (7), and there are gaps between these strands (8). The strands are joined by DNA ligase (9, 10, 11), and before this can occur, the gaps have to be closed. Both DNA polymerase and DNA ligase are required in vitro for the incorporation of newly synthesized DNA into high molecular weight material (12). The experi-

ments reported here test whether DNA polymerase functions similarly *in vivo*, using the DNA polymerase mutant isolated by de Lucia and Cairns.

MATERIALS AND METHODS

Bacterial strains. *E. coli* W3110 thy$^-$ pol$^+$ and W3110 thy$^-$ pol A1 were obtained from Dr. J. Cairns. The pol A1 cells had increased sensitivity to ultraviolet light and were deficient in DNA polymerase activity *in vitro*.

Growth medium. A low phosphate, Tris medium (13) supplemented with 4 mg/ml glucose and 2 μg/ml thymine was used.

Preparation of samples. Experiments were initiated when cultures had an optical density of 0.4 at 450 nm. Either ^{3}H-thymidine or ^{3}H-thymine was added to label DNA; the specific activities are given in the figure legends. At the designated times 1 ml samples were removed and immediately mixed with 10 ml of ice-cold "stop mix" containing 2 gm ice. The "stop mix" contained 0.01 M NaCN, 0.01 M EDTA, 0.01 M Tris adjusted to pH 8.1 with HCl, and either 200μg/ml thymidine or 100 μg/ml thymine. The samples were centrifuged 10 min at 0 C at 17,000 x g. The cells were resuspended in 0.4 ml of "stop mix", and 0.1 ml of "stop mix" containing 50 μg lysozyme and 15 mg sucrose was added. After 15 min incubation at 5 C, 0.5 ml 0.34 N NaOH was added. Phage T4 ^{14}C-DNA was added at this time. The preparations were gently mixed, allowed to stand at 5 C for at least 30 min, and poured onto the alkaline sucrose gradients.

Alkaline sucrose gradients. The low-salt gradients consisted of a 2 ml cushion of 80% sucrose (w/v) in 0.17 N NaOH and 31 ml of a linear 5 to 20% (w/v) sucrose gradient containing 0.17 N NaOH and 10^{-3} M EDTA. The high-salt gradients consisted of a 2 ml cushion, 80% sucrose in 0.1 N NaOH, and 31 ml of a linear 5 to 20% sucrose gradient containing 0.1 N NaOH, 0.9 M NaCl, and 10-3 M EDTA. Low-salt gradients were used for analyses of high molecular weight DNA, since some aggregation occurred in the high-salt gradients. The gradients were centrifuged at 20 C in a Spinco SW 27 rotor with 1 x 3½ inch buckets. Fractions were collected through a capillary pipet inserted to the bottom of the tube. Fractions were chilled to 5 C, and 50 μg calf thymus DNA, 20 μg lysozyme, and 5% trichloroacetic acid were added. The precipitates were collected on glass filters, washed extensively, dried at 100 C, and placed in vials containing 10 ml of scintillation fluid consisting of 8 gm butyl-PBD (Packard) in 1 liter toluene. The samples were counted in a Packard Tri-Carb Scintillation Counter.

DNA ligase assays. The procedure of Gellert was used (14), with the following modifications: hydrogen bonded circles of phage lambda DNA were prepared as described by Hershey *et al.* (15); cell extracts were obtained by 3 min sonication in a MSE sonicator; the incubations were conducted at 23 C; the assays contained 10^{-3} M NAD and either 30 or 300 μg/ml cell extract protein.

RESULTS

Exponentially growing W3110 pol$^+$ cells initially incorporated ^{3}H-thymidine into low molecular weight DNA, which was then rapidly incorporated into higher molecular weight material. After 15, 30 and 60 seconds incorporation at 37 C, 22, 31 and 39%, respectively, of the radioactivity was in DNA that sedimented as fast or faster than the strands of phage T4 DNA, which have a molecular weight of 65 x 10^6 (Fig. 1A).

The radioactivity did not appear as rapidly in high molecular weight DNA when similar experiments were conducted with W3110 pol A_1. After 60 seconds

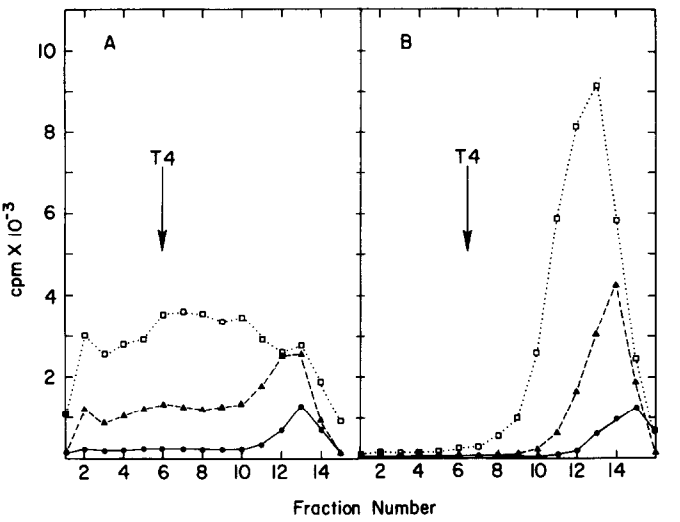

Figure 1. Sedimentation profiles of ^{3}H-thymidine (24.6 c/mmole, 5 μc/ml) incorporated during 15, 30, and 60 sec incubation at 37 C. A. W3110 pol^{+}. B. W3110 pol A_1. The low salt alkaline sucrose gradients were centrifuged for 5 hr at 24,000 rpm at 20 C. Sedimentation was from right to left. ●——●, 15 sec; ▲--▲, 30 sec; □···□, 60 sec.

incorporation, less than 3% of the radioactivity sedimented as fast or faster than T4 strands (Fig. 1B). This was not caused by large differences in the growth rates or rates of incorporation of ^{3}H-thymidine by the two strains. The generation times of W3110 pol^{+} and the pol A_1 mutant at 37 C were 82 and 92 minutes, respectively, in the minimal medium used, and although the rate of incorporation of ^{3}H-thymidine was sometimes less in W3110 pol A_1, it was never less than 75% of the rate in the parent.

The mutation in W3110 pol A_1 did not affect the rate of synthesis of very low molecular weight DNA. To demonstrate this, cells were grown at 23 C, which increased the generation time approximately fourfold. The ^{3}H-thymidine incorporated by both the mutant and its parent during 11 and 22 seconds at 23 C sedimented in a peak with an $S_{20,w}$ of 8-11 s (Fig. 2). This value was determined by comparison with the sedimentation under identical conditions of ΦX174 DNA, for which $S_{20,w}$ is 18.4 s (16). The low sedimentation rate indicates the newly synthesized DNA is in strands 1000 to 2000 nucleotides long, which is the same as the estimate originally given by Okazaki et al. (7). The mutant did not rapidly incorporate the newly synthesized DNA into higher molecular weight material, and this readily accounts for the differences between Figs. 2A and 2B. These differences are especially apparent between the samples taken after 60 seconds incubation.

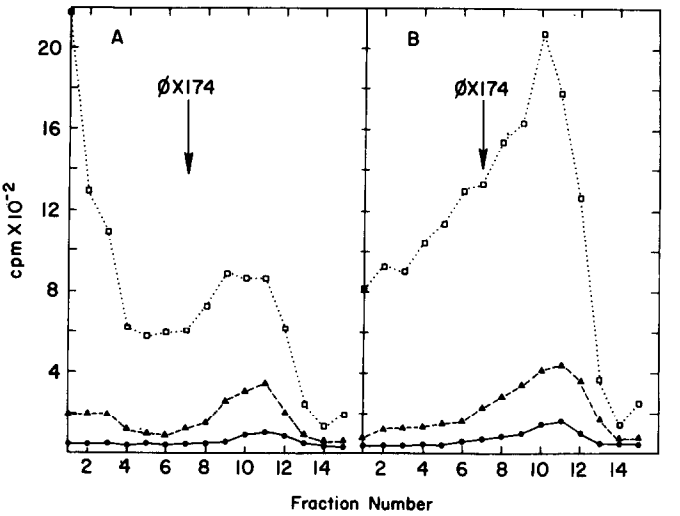

Figure 2. Sedimentation profiles of ^{3}H-thymidine (24.6 c/mmole, 5 μc/ml) incorporated during 11, 22, and 60 sec incubation at 23 C. A. W3110 pol^{+}. B. W3110 pol A$_{1}$. The low-salt alkaline sucrose gradients were centrifuged for 15.5 hr at 22,000 rpm at 20 C. ●——●, 11 sec; ▲--▲, 22 sec; □···□, 60 sec.

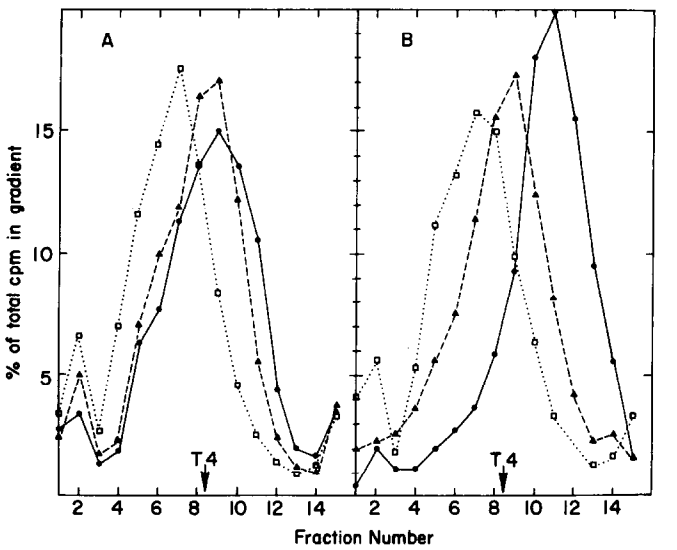

Figure 3. Sedimentation profiles of ^{3}H-thymidine (4.9 c/mmole, 1 μc/ml) incorporated during 30 sec incubation at 37 C followed by 2, 5, or 15 min growth in medium containing 50 μg/ml non-radioactive thymidine. A. W3110 pol^{+}. The total cpm in the gradients were 8743, 8580, and 10736 for 2, 5, and 15 min, respectively. B. W3110 pol A$_{1}$. The total cpm in the gradients were 6266, 5887, and 7678 for 2, 5, and 15 min, respectively. The high-salt alkaline sucrose gradients were centrifuged for 3.5 hr at 24,000 rpm at 20 C. ●——●, 2 min; ▲--▲, 5 min; □···□, 15 min.

The low molecular weight DNA is eventually incorporated into high molecular weight material. Exponentially growing cells of the mutant and its parent were incubated with ^{3}H-thymidine at 37 C for 30 seconds, a 1000-fold excess (50 μg/ml) of non-radioactive thymidine was then added, and samples were taken after 2, 5, and 15 minutes further incubation. The trichloroacetic acid precipitable radioactivity in the cultures increased approximately 15% between 2 and 5 minutes, and there was no further increase between 5 and 15 minutes. After 2, 5, and 15 minutes incubation, 20, 51, and 72%, respectively, of the radioactivity incorporated by the polymerase mutant sedimented as fast or faster than T4 strands (Fig. 3B). At the same times, 63, 73, and 77%, respectively, of the radioactivity incorporated by the parent sedimented as fast or faster thant T4 strands (Fig. 3A).

A few of the above experiments have also been conducted using ^{3}H-thymine to label the newly synthesized DNA. This was done to test whether the results reported above were unique to thymidine pulses. Fig. 4 shows the sedimentation profiles of the ^{3}H-thymine incorporated by the mutant and its parent during 30 and 60 seconds incubation at 37 C. The results are similar to those obtained with ^{3}H-thymidine (Fig. 1). After 60 seconds incorporation, less than 4% of the radioactivity incorporated by the mutant sediments as fast or faster than T4 strands (Fig. 4B). After 30 and 60 seconds incorporation, 26 and 31%, respectively, of the radioactivity incorporated by the parent sedimented as fast or faster than T4 strands (Fig. 4A).

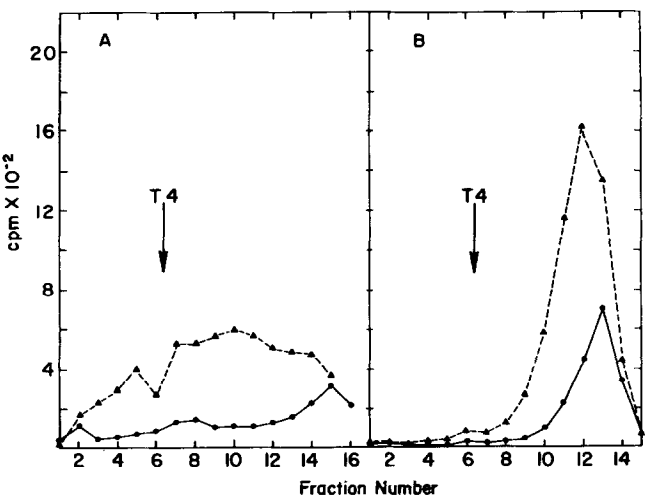

Figure 4. Sedimentation profiles of ^{3}H-thymine (2.56 c/mmole, 50 μc/ml) incorporated during 30 and 60 sec incubation at 37 C. A. W3110 pol^{+}. B. W3110 pol A_1. The low-salt alkaline sucrose gradients were centrifuged for 6 hr at 22,000 rpm at 20 C. ●——●, 30 sec; ▲--▲, 60 sec.

The difference between W3110 pol A_1 and its parent could be caused by a deficiency in DNA ligase activity, and the assay procedure of Gellert (14) was used to test this. Table I shows the rates at which extracts of the mutant and its parent converted hydrogen bonded circles of phage lambda DNA to covalently closed circles were similar. DNA polymerase was also assayed using the same extracts, and as reported by de Lucia and Cairns (6), the mutant had approximately 1% as much activity *in vitro* as the parent.

DISCUSSION

The *E. coli* mutant isolated by de Lucia and Cairns, which lacks virtually all DNA polymerase activity *in vitro* (6), is impaired in its ability to convert low molecular weight, newly synthesized DNA into high molecular weight material (Fig. 1B). The mutation affects the joining of the low molecular weight strands and not their synthesis, since the strands were of similar size and were synthesized at similar rates in both the mutant and its parent (Fig. 2). The low molecular weight strands were eventually incorporated into high molecular weight DNA, however (Fig. 3).

The results suggested the mutant might be defective in DNA ligase as well as DNA polymerase activity, since DNA ligase is involved in the conversion of low molecular weight DNA into higher molecular weight material (9, 10, 11). It is possible, for example, that the structural genes for both DNA polymerase and DNA ligase are in the same operon, and the amber mutation in W3110 pol A_1 (17) affects the synthesis of both enzymes. Assays for DNA ligase demonstrated that extracts of both mutant and parent had similar amounts of activity (Table I).

The simplest explanation for the results is the strands of low molecular weight DNA synthesized at the replication fork have gaps between their ends, and DNA polymerase normally elongates the strands until the 3' and 5' ends are contiguous. DNA ligase then joins the strands covalently. The process is slow in the mutant, presumably because of the low amount of DNA polymerase

TABLE 1. DNA LIGASE ASSAYS

Conversion of Hydrogen Bonded to Covalently Closed Circles of Lambda DNA

	Time		
	0 min	2 min	5.5 min
W3110 pol^+	0%	1.6%	6.7%
W3110 pol A_1	0%	2.3%	7.9%

Assays conducted as described by Gellert (14), with modifications given in Materials and Methods, with 30 μg/ml cell extract protein.

present in the cells. De Lucia and Cairns estimated the mutant cells contain 5 to 10 polymerase molecules (6), and this low number could be sufficient to facilitate a slow joining of the newly synthesized DNA. It is also possible, of course, that the mutant contains no "Kornberg" polymerase activity in vitro or in vivo, and the residual synthesis that occurs is catalyzed by some other enzyme.

An alternate explanation for the slow rate of conversion of newly synthesized DNA from low to high molecular weight is the mutant synthesizes an altered polymerase that inhibits some stage of the conversion process. The altered polymerase could (A) elongate the strands until the 3' and 5' ends are contiguous but remain bound at the nick and inhibit DNA ligase, or (B) bind at nicks between strands of newly synthesized DNA and inhibit DNA ligase, or (C) bind at nicks and remove some nucleotides by means of the polymerase associated exonuclease activities. Kanner and Hanawalt have suggested proposal (A) to explain the slow rate at which W3110 pol A_1 completes the final stages in the repair of damage induced by ultraviolet light (18). These proposals lead to definite preductions and they are being tested by more sophisticated assays of DNA ligase activity.

A final alternative that cannot yet be excluded is the amber mutation in W3110 pol A_1 is polar and causes the loss of some other enzyme normally required for the conversion from low to high molecular weight. There would consequently be no correlation between the lack of polymerase activity and the results presented here. Other DNA polymerase mutants will have to be isolated and tested to determine the validity of this hypothesis.

ACKNOWLEDGEMENTS

This research was supported by grants GM 15905 and GM 38077 (predoctoral fellowship awarded to G.E.V.) from the National Institutes of Health. We are especially thankful to Dr. J. Cairns for the use of his mutants.

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