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Isolation and Partial Characterization of a Mutant of *Escherichia coli* Deficient in DNA Polymerase II

(mutagenesis/*polA1* mutants/*polB1* mutants/DNA replication)

JUDITH L. CAMPBELL*, LARRY SOLL†, AND CHARLES C. RICHARDSON*

*Department of Biological Chemistry and † Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT **A mutant of *Escherichia coli* deficient in DNA polymerase II has been isolated from *E. coli polA1* by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and assay of polymerase activity in extracts of survivors. The *polA1* mutation was suppressed during mutagenesis by introduction of the suppressor, su7$^+$, into the parental strain. The mutant, HMS83 *polA1 polB1*, contains less than 0.5% of the normal levels of DNA polymerase II. The only polymerase activity detected in the mutant is DNA polymerase III. *E. coli* HMS83 grows normally at both 25 and 42°, and supports the growth of bacteriophages T4, T7, lambda, φX174, 186, P2, and fd. The *polB* mutation does not affect sensitivity to ultraviolet irradiation or recombination frequencies.**

Three distinct DNA polymerases have been found in extracts of *Escherichia coli*. The major activity, polymerase I, has been purified to homogeneity and studied extensively (1). In order to determine the role of polymerase I *in vivo*, DeLucia and Cairns (2) isolated a mutant of *E. coli*, *E. coli polA1*, that has greatly reduced levels of polymerase I activity in extracts. The mutant grows normally, but has definite physiological defects in repair mechanisms (2, 3).

The residual polymerase activity detectable in extracts of *E. coli polA1* has been resolved into two enzymes: polymerase II (4–11) and polymerase III (5). In a survey of mutants temperature-sensitive for DNA synthesis, dna_{ts}, Gefter *et al.* (12) found that strains with a mutation at the *dnaE* locus (13) contained a thermolabile polymerase III. Nüsslein *et al.* (14) have partially purified the *dnaE* gene product using an *in vitro* complementation assay for replication, and have shown that the purified fraction contains an activity that has all of the properties of polymerase III. Therefore, it seems that polymerase III is essential for DNA replication.

In contrast to polymerase III, polymerase II activity is normal in all the known classes of dna_{ts} mutants (7, 12). Thus, in order to investigate the function of polymerase II *in vivo*, we decided to isolate a mutant lacking the enzyme. We tried to minimize the bias inherent in any selection by screening a highly mutagenized stock of *E. coli* for polymerase II in individual extracts. Because DNA polymerase I activity masks polymerase II activity in extracts, it was necessary to use *E. coli polA1* as the parental strain. This communication describes the isolation and partial characterization of a mutant lacking DNA polymerase II in extracts.

Abbreviations: NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MMS, methylmethanesulfonate.

MATERIALS AND METHODS

Bacterial and Phage Strains. The mutants were isolated from strains derived from *E. coli* W3110 *thy*$^-$. The parental strain was JG138 *thy*$^-$ *polA1 rha*$^-$ *lacZ*$_{am}$. Bacteriophage P1KC was grown on LS448F'14 and used for the transduction to *su7*$^+$.

E. coli EC-0 is (*proB* lac)$_{X111}$ S_m^S carrying $F_{TS\ 114}$ *lac*$^+$.

Media. Minimal medium was that of Vogel and Bonner (15), supplemented with 0.5% of the desired sugar and 40 μg/ml of any required amino acids. Thymine was present at 10 μg/ml where necessary. L-Broth contained 1% Difco Bacto-Tryptone, 1.0% NaCl, 0.5% Bacto-Yeast Extract, and 0.1% glucose. MacConkey Agar was prepared with Difco Bacto-MacConkey Agar Base, and contained 1% of the desired sugar. Methylmethanesulfonate (MMS) (Eastman Kodak Co.) plates were prepared by addition of 0.04% MMS to T-Broth agar, and were used immediately after pouring and cooling.

Transductions. Transductions were performed according to the method of Lennox (16).

Mutagenesis. The parental strain was *E. coli* JG138, which had been made *su7*$^+$ by transduction with P1KC (LS448). Mutagenesis was performed according to the method of Adelberg *et al.* (17) with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Aldrich Chemical Co., Milwaukee, Wis.) in Tris·maleate buffer (pH 6.0) for 30 min at 37°. Mutant segregation and phenotypic expression were performed at 25° for 17 hr in L-Broth on aliquots of the NTG-treated cells that had been washed free of mutagen and diluted 1:10. The extent of mutagenesis of the surviving cells was determined by the frequency of appearance of clones unable to ferment maltose (see *Results*). Segregants were diluted 1:20, and were stored in 20% glycerol at −60°.

Preparation of Extracts. Bacteria were grown at 25° on T-Broth plates. Single colonies were picked and grown in L-Broth overnight at 25°. An aliquot containing 5×10^9 cells was centrifuged at 5900 × *g* for 10 min. The cell pellet was resuspended in 0.1 ml of a 1% emulsion of toluene in 0.05 M Tris buffer (pH 7.6)–5 mM EDTA. The emulsion was prepared by sonic irradiation. After 5 min at room temperature, 50 μg of lysozyme (Calbiochem, 10,000 units/mg) was added. The tubes were incubated for 5 min at 37°, then chilled to 0° after the addition of 0.1 ml of 25% Triton X-100. The

lysate was passed rapidly through a 27-gauge needle. After 20 min at 0°, 0.15 ml of a 50% suspension of microgranular DEAE-cellulose in 0.6 M potassium phosphate buffer (pH 7.4) was added to adsorb DNA. The suspension was centrifuged at 5900 × *g* for 10 min, after which 0.02–0.05 ml of the supernatant fluid was assayed for polymerase II.

Assay for Polymerase Activity in Extracts of Mutagenized Cells. Each reaction mixture contained 66 mM potassium phosphate buffer (pH 7.4), 6.6 mM $MgCl_2$, 0.033 mM (each) dCTP, dATP, dGTP, and [^{3}H]TTP (1.2×10^8 cpm/μmol), 0.25 mM "activated" salmon-sperm DNA (18), and cell extract in a final volume of 0.3 ml. The tubes were incubated at 37° for 30 min. The reaction was stopped by the addition of 2 ml of 1 N trichloroacetic acid–20 mM sodium pyrophosphate. After 5 min, the pellet was collected by centrifugation at 9000 × *g* for 10 min. The pellet was redissolved in 0.5 ml NaOH (0.1 M at room temperature). The DNA was precipitated by the addition of 3 ml of the above solution of trichloroacetic acid, and the precipitate was collected on a GF/C glass filter, washed, and dried; the radioactivity was determined as described (6).

Purification of Polymerase Activity. Extracts of strains lacking polymerase I were prepared as described (6). Subsequent steps were modified as follows: All buffers contained 1 mM 2-mercaptoethanol–1 mM EDTA–10% (v/v) glycerol. The extract was diluted 9-fold with 0.015 M KPO_4 buffer (pH 7.0), and loaded onto a microgranular DEAE-cellulose column (2 cm² × 25 cm). The column was washed with 100 ml of the above buffer, and the protein was eluted with 0.2 M KPO_4 buffer (pH 7.0) and collected in 10-ml fractions. The two fractions that contained 90% of the polymerase activity were dialyzed against 100 volumes of 0.02 M KPO_4 buffer (pH 6.5) at 0° for 12 hr and were applied to a phosphocellulose column (0.79 cm² × 25 cm) previously equilibrated with the 0.02 M KPO_4 buffer (pH 6.5). The column was then washed with 40 ml of the same buffer; a linear gradient from 0.02 M to 0.4 M KPO_4 buffer (pH 6.5) was pumped through the column, and 2-ml fractions were collected.

Other Methods. Sensitivity to ultraviolet irradiation was determined according to the method of Howard-Flanders and Therriot (19). Bacteria in logarithmic phase of growth were harvested by centrifugation and resuspended in 0.85% NaCl at 1×10^8 cells/ml. An aliquot, 6.5 ml, that gave a depth of liquid of 1 mm was placed in a flat-bottomed glass petri dish, 50 cm from a Westinghouse Sterilamp, and then irradiated with continuous gentle swirling for various lengths of time. Aliquots were diluted at 0° in subdued light, plated, and incubated in the dark for 18 hr at 37° to determine the number of survivors. Recombination frequencies were determined by the method of Adelberg and Burns (20). Spheroplast assays of φX174 DNA were performed according to the method of Guthrie and Sinsheimer (21). Protein was measured according to the method of Lowry *et al.* (22); bovine-serum albumin was used as a standard. HMS83 $F_{TS114}lac$ was prepared by mixing *E. coli EC-0* $F_{TS114}lac^+$ with HMS83 in a ratio of 1:10 and selecting pro^+lac^+ phenotype at 30°.

RESULTS

Mutagenesis of *E. coli* JG138 *polA1 su7*$^+$

When *E. coli* JG183 *polA1*, lacking DNA polymerase I, was mutagenized with nitrosoguanidine, the number of survivors was much less than that observed with JG139, the parental $polA^+$ strain (Table 1). In addition, as shown in Table 1, the frequency of appearance of mutants among the survivors was at least 10-fold lower for JG138 than for JG139, an observation also made by Smirnov *et al.* (23).

Because the *polA1* mutation is an *amber* mutation (3), it was possible to obtain a highly mutagenized stock of JG138 by introduction of a suppressor during mutagenesis. The suppressor used, $su7^+$ (24, 25), is a recessive lethal mutation, and as such can exist only in strains that are diploid for the suppressor locus. When $su7^+$ is introduced by P1 transduction into a haploid bacterium, a tandem duplication is created containing one $su7^+$ allele and one $su7^-$. Such duplications are genetically unstable and in rec^+ strains segregate su^- clones at a high frequency, thus making it possible to easily remove the suppressor after mutagenesis. The $su7^+$ allele was introduced by transduction of JG138 with P1 grown on LS448 F'14. Since JG138 also contains the $lacZ_{am}$ gene, selection was first for lac^+ phenotype. From the lac^+ colonies a clone with the $polA^+$ phenotype was obtained by screening for sensitivity to ultraviolet light and MMS. The lac^+ $polA^+$ phenotype was shown to be due to suppression by $su7^+$ by the high frequency of simultaneous segregation to lac^- *polA1*. This clone, JG138$su7^+$, was used as the parental strain in the subsequent mutagenesis. When JG138-$su7^+$ was treated with nitrosoguanidine (1 mg/ml), survival was 10^{-3}, thus establishing the effectiveness of the $polA^+$ phenotype during NTG treatment. Of the survivors, 3.7% were unable to grow on maltose.

Identification of polymerase II deficient mutants

The mutagenized bacteria were plated out from storage and grown at 25°. Colonies were streaked onto Lactose-MacConkey indicator plates, and lac^- segregants (which had lost $su7^+$) were picked and grown. Extracts of the mutant cells were prepared and assayed for DNA-polymerase activity. All cells were grown at 25°, and assays were done at 43° in order to detect a thermolabile enzyme in a conditional lethal mutant. Polymerase III is not active under these con-

TABLE 1. *Effect of nitrosoguanidine on E. coli JG138 polA*$^-$

NTG added (μg/ml)	Fraction surviving		*mal*$^-$	
	JG139 *polA*$^+$	JG138 *polA*$^-$	JG139 *polA*$^+$ (%)	JG138 *polA*$^-$ (%)
10	2.4×10^{-1}	1.6×10^{-3}	1.5	<0.01
50	1.3×10^{-2}	3.1×10^{-5}	3.3	<0.01
100	7.8×10^{-3}	1×10^{-5}	3.3	<0.01
400	7.9×10^{-3}	$<10^{-6}$	3.8	<0.01

Cultures of JG138 and JG139 in the logarithmic phase of growth were mutagenized with the concentrations of nitrosoguanidine indicated, at 37° for 30 min. Cells were collected by centrifugation at 0°, and were washed twice to remove the nitrosoguanidine. They were then plated for determination of survival. An aliquot was diluted and allowed to grow for 17 hr at 25° to express phenotypic properties. Frequency of appearance of *mal*$^-$ phenotype among the survivors was determined by plating on MacConkey Agar with maltose. Mutagenesis is expressed as the percentage of the survivors that are *mal*$^-$.

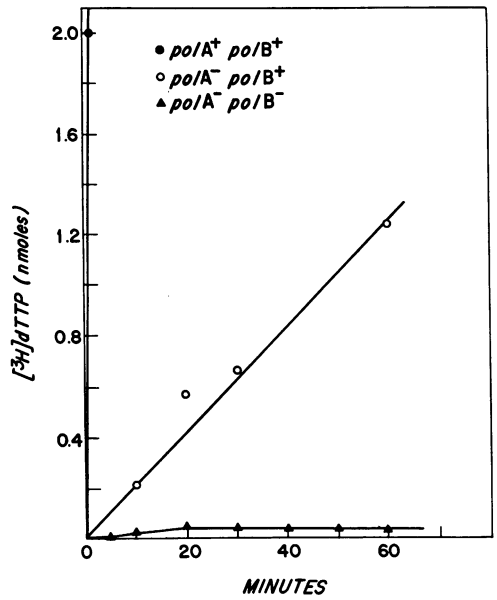

FIG. 1. Polymerase activity in extracts of *E. coli* JG138 and HMS83. Bacteria were grown at 25° to 2–3 × 10^9 cells per ml in L-Broth. Extracts were prepared, and polymerase activity was measured (6) as incorporation of [^{3}H]dTTP into acid-insoluble form. ●——● JG139; ○——○ JG138; ▲——▲ HMS83.

ditions of extraction and assay, and only appears during further fractionation (see below).

The thirty-fifth strain assayed, designated as *E. coli* HMS83, had no detectable polymerase activity in extracts prepared for the screening assay (Table 2). As shown in Fig. 1, extracts of *E. coli* HMS83, prepared as described by Moses and Richardson (6), contained less than 5% of the polymerase activity present in *E. coli* JG138, and less than 0.2% of that present in wild-type *E. coli*. Extracts of HMS83 did not inhibit the polymerase activity observed in extracts of *E. coli* JG138 (less than 10%) when added in equal amounts.

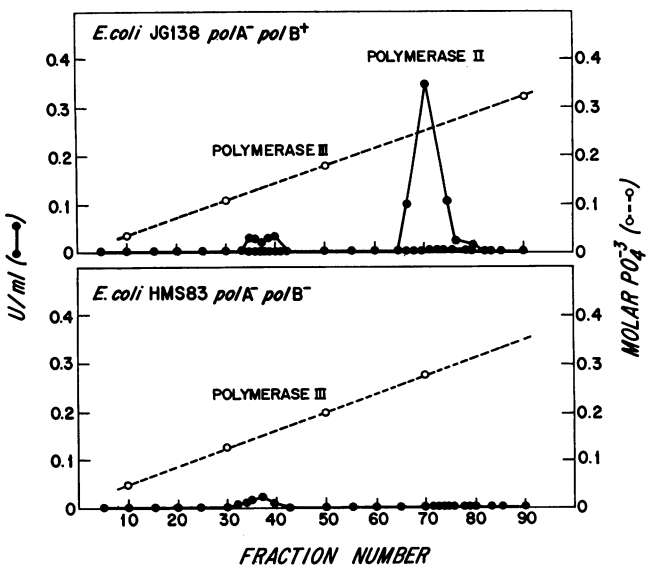

FIG. 2. Phosphocellulose chromatography of extracts of JG138 *polA1 polB*$^+$ and HMS83 *polA1 polB1*. Extracts were fractionated as described in *Methods*. Polymerase activity was measured (6), and fractions 45–55 and 75–85 were also assayed with 6 mM *N*-ethylmaleimide. Polymerase III eluted at 0.17 M KPO_4 in fractions 45–55, and polymerase II eluted at fractions 75–85 (see text).

TABLE 2. *DNA-polymerase activity in extracts of mutagenized E. coli JG138*

	nmol per ml of extract per 30 min	
	25°	42°
JG138 *polA1 polB*$^+$	0.21*	0.21
HMS83 *polA1 polB1*	<0.01	<0.01

* Incubation of the extracts in a screening assay did not give linear rates of reaction with time. Therefore, the values recorded for activities only represent the extent of reaction during the 30-min incubation.

Polymerase III—the residual activity in HMS83

In order to characterize the residual polymerase activity present in the mutant, an extract of HMS83 was prepared, and the polymerase activity was partially purified. As a control, JG138 was treated in a similar manner. As shown in Fig. 2, when extracts of HMS83 were purified, no polymerase II activity (less than 0.5% of that found in JG138) was present in the phosphocellulose fractions where the enzyme normally elutes. When extracts of HMS83 were mixed with equal amounts of extracts of JG138 and purified in an identical manner to that shown in Fig. 2, polymerase II activity was present in normal amounts. This result indicates that the reduction in polymerase activity in HMS83 is not due to an inhibitor in extracts of HMS83.

However, extracts of both JG138 and HMS83 contained a polymerase activity that eluted from phosphocellulose in 0.17 M KPO_4 (pH 6.5) (Fig. 2). The activity was completely inhibited by 6 mM *N*-ethylmaleimide and is stimulated up to 3-fold by the addition of 10% ethanol in the reaction. The addition of 10 mM ammonium sulfate to the reaction mixture results in a 60% reduction in activity. Finally, the enzyme is absent from *E. coli* carrying the *dnaE*486 mutation. Since these properties correspond to those described by Gefter *et al.* (refs. 5 and 12 for polymerase III, and are clearly different from the properties of polymerase I, we conclude that the residual polymerase activity in HMS83 is DNA polymerase III. The polymerase III activity observed in the phosphocellulose fractions shown in Fig. 2 does not reflect the actual level of polymerase III in HMS83, since extracts were prepared and assays were done under conditions optimal for polymerase II. Polymerase III has been partially purified from this strain and, under optimal conditions, the activity exceeds the polymerase II activity in *polB*$^+$ strains (D. C. Hinkle, D. M. Livingston, and C. C. Richardson, unpublished results).

Biological characteristics of HMS83

During mutagenesis, the HMS83 strain acquired the auxotrophic marker, *lys*$^-$. The genotype of the strain is, therefore, HMS83 *polA1 polB1 thy*$^-$ *lys*$^-$ *lacZ*$_{am}$ *rha*$^-$. *E. coli* HMS83 is viable at 25° and 42° in L-Broth. The generation time of both

the mutant and the parental strain (JG138) was 45 min at 37°.

As shown in Fig. 3, *E. coli* HMS83 *polA1 polB1* is no more sensitive to ultraviolet light than JG138 *polA1 polB*$^+$. Similarly, when a *polA*$^+$ *polB1* strain, HMS85, was prepared (see legend to Fig. 3), it was found to have the same sensitivity to ultraviolet light as JG139 *polA*$^+$ *polB*$^+$ (Fig. 3).

Bacteriophages T4, T7, and lambda grow with normal efficiency on HMS83 and HMS85. Spheroplasts of HMS83 allow normal growth of ϕX174. Phage 186 grows on HMS83 and HMS85. P2 does not grow on *polA1* strains, but does grow on the *polB1* mutant, HMS85. Therefore, the *rep*$^-$ locus is probably not the gene for polymerase II (26). The male-specific phage fd gives a normal burst size when grown on HMS83 F_{TS114}*lac*$^+$, although the plaques are more turbid than those obtained on JG138 F_{TS114}*lac*$^+$.

Recombination mechanisms in HMS83 were sufficient to allow the preparation of the *polA*$^+$ *polB1* strain by transduction of HMS83 by the phage P1 (see legend to Fig. 3). In addition, HMS83 can act as a recipient in bacterial conjugation. A mating experiment was performed with *E. coli* AT982 *dap4 thi-1 rel-1 HfrKL16* as donor. This strain transfers *thy* with good efficiency. Frequency of appearance of *thy*$^+$ recombinants was the same for both JG138 *polA*$^-$ *polB*$^+$ and HMS83 recipients. When this particular *Hfr* was used as donor, the frequency of appearance of *lac*$^+$ and *rha*$^+$ recombinants, more remote markers, were also equal in HMS83 and JG138. Similar matings were performed with HfrH and HfrC. The frequency of appearance of *lac*$^+$ recombinants was equal in HMS83 and JG138.

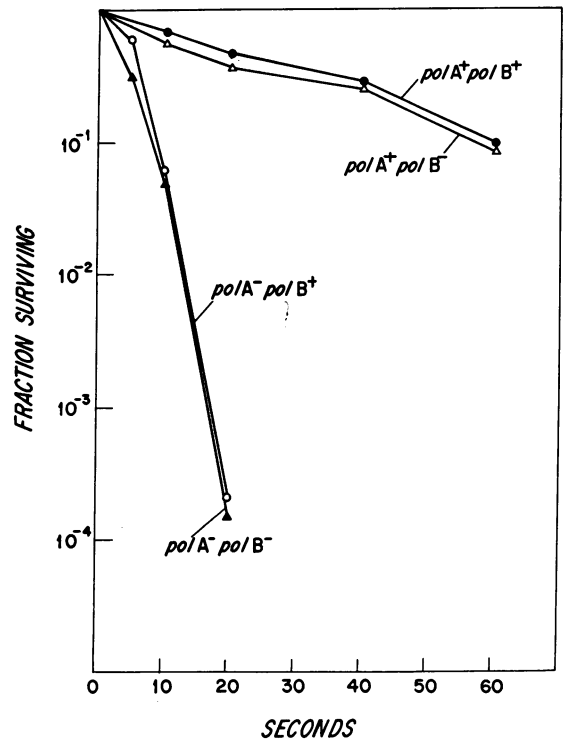

FIG. 3. Survival of *E. coli* at various doses of ultraviolet light. Suspensions were irradiated at 1×10^8/ml, diluted, plated, and incubated in the dark at 37°. *E. coli* HMS85 *polA*$^+$ *polB1* was prepared by transduction of HMS83 with P1KC grown on *E. coli* W3110 *polA*$^+$ *rha*$^+$. Selection was for ability to grow on rhamnose. Among the *rha*$^+$ transductants 11% were *polA*$^+$ as determined by assay of polymerase I activity. Transductants were shown not to be P1 lysogens by plating of bacteriophages lambda and T7, which are restricted by strains that carry P1. Extracts of HMS85 were fractionated as described in Fig. 2, and were shown to be lacking polymerase II activity. O——O JG138 *polA1 polB*$^+$; ▲——▲ HMS83 *polA1 polB1*; ●——● JG139 polA$^+$polB$^+$; △——△ HMS85 polA$^+$polB1.

DISCUSSION

Extracts of *E. coli* HMS83 are deficient in both polymerases I and II, yet the mutant does not appear to differ from its polymerase I-deficient parent in growth or properties. One must be cautious, however, in interpreting the results that might occur *in vivo* obtained with mutants isolated by an *in vitro* assay. A strain lacking all or most of the assayable enzyme in extracts might retain sufficient residual activity for the cell to function normally *in vivo*. For example, *E. coli* mutants that have detectable (but less than 1%) ligase activity in extracts grow normally (28), but conditional lethal mutants have been isolated that have no detectable activity (29). Similarly, while several studies that used *polA*$^-$ strains of *E. coli* have established that polymerase I is important in repair processes (27), it now appears that extracts of these strains contain low, but detectable, amounts of polymerase I activity (I. R. Lehman, personal communication). Furthermore, even if there is no detectable enzyme activity in extracts, the mutant protein may be stable and may function normally *in vivo*. Therefore, we cannot define the complete functions of polymerases I and II until deletion mutants are available, or until polymerase II mutants are isolated by selection for specific physiological defects.

However, the availability of a strain with significantly reduced levels of polymerases I and II greatly aids enzymatic studies on DNA replication. The specific roles of the three known DNA polymerases are not known. The finding that the only residual polymerase activity observed in HMS83 is DNA polymerase III lends support to the studies of Gefter *et al.* (12) and Nüsslein *et al.* (14). Clearly polymerase III is essential for DNA replication, but its role in initiation and elongation of polynucleotides remains to be established, as well as its interaction with other proteins of DNA metabolism. The absence of polymerase I and II activities in extracts has facilitated the purification of polymerase III, particularly in the early fractionation steps where the specific identification of polymerase III is difficult (D. Hinkle and C. C. Richardson, unpublished results).

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