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Analysis of DNA Polymerases II and III in Mutants of *Escherichia coli* Thermosensitive for DNA Synthesis

(*pol*A1 mutants/phosphocellulose chromatography/*dna*E locus)

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ABSTRACT **A series of double mutants carrying one of the thermosensitive mutations for DNA synthesis (*dnaA*, B, C, D, E, F, and G) and the *pol*A1 mutation of DeLucia and Cairns, were constructed. Enzyme activities of DNA Polymerases II and III were measured in each mutant. DNA Polymerase II activity was normal in all strains tested. DNA Polymerase III activity is thermosensitive specifically in those strains having thermosensitive mutations at the *dna*E locus. From these results we conclude that DNA Polymerases II and III are independent enzymes and that DNA Polymerase III is an enzyme required for DNA replication in *Escherichia coli*.**

The isolation by DeLucia and Cairns (1) of an *Escherichia coli* mutant that lacks DNA Polymerase I activity (*polA*1) has prompted many investigations into the nature of the DNA synthetic capacity of such strains. The purification and characterization of DNA Polymerase II has been reported by ourselves (2) and others (3, 4). In addition, we have reported the existence of a third DNA polymerase in *E. coli* (DNA Polymerase III) (2). A physiological function for these enzymes has not been determined.

The viability of cells devoid of measurable DNA Polymerase I activity suggests that this enzyme is not an obligatory component of the DNA replication machinery of *E. coli*. To determine whether polymerases II and III are essential for replication, we examined the DNA polymerases of *E. coli* mutants that were temperature-sensitive for DNA replication in an attempt to correlate the genetic lesions with altered DNA polymerase activity *in vitro*. We will present evidence indicating that DNA Polymerase III is the product of an essential gene mapping at the *dna*E locus.

MATERIALS AND METHODS

The following bacterial strains were used†:

(*1*) CRT4637: F⁻ *thr*⁻ *leu*⁻ *his*⁻ *str*ʳ *mal*A *mtl*⁻ *thi*⁻ *dna*AT46
(*2*) CRT2667: F⁻ *his*⁻ *str*ʳ *mal*A *thi*⁻ *pol*A1 *sup*⁻ *dna*BT266
(*3*) BT1029: H560 *thy*⁻ *endo*I⁻ *pol*A1 *dna*B
(*4*) PC22: F⁻ *his*⁻ *str*ʳ *mal*A *xyl*⁻ *arg*⁻ *mtl*⁻ *thi*⁻ *pol*A1 *sup*⁻ *dna*C2
(*5*) PC79: F⁻ *his*⁻ *str*ʳ *mal*A *xyl*⁻ *mtl*⁻ *thi*⁻ *pol*A1 *sup*⁻ *dna*D7
(*6*) E5111: F⁻ *his*⁻ *str*ʳ *mal*A *xyl*⁻ *mtl*⁻ *arg*⁻ *thi*⁻ *sup*⁻ *pol*A1 *dna*E511
(*7*) E4860: F⁻ *his*⁻ *str*ʳ *mal*A *xyl*⁻ *mtl*⁻ *arg*⁻ *thi*⁻ *sup*⁻ *dna*E486
(*8*) E4868: F⁻ *his*⁻ *str*ʳ *mal*A *xyl*⁻ *mtl*⁻ *arg*⁻ *thi*⁻ *sup*⁻ *pol*A1 *dna*E486
(*9*) BT1026: H560 *thy*⁻ *endo*I⁻ *pol*A1 *dna*E
(*10*) BT1040: H560 *thy*⁻ *endo*I⁻ *pol*A1 *dna*E
(*11*) E1011: F⁻ *his*⁻ *str*ʳ *mal*A *xyl*⁻ *mtl*⁻ *arg*⁻ *thi*⁻ *sup*⁻ *pol*A1 *dna*F101
(*12*) JW207: *thy*⁻ *rha*⁻ *str*ʳ *pol*A1 *dna*F101
(*13*) NY73: *leu*⁻ *thy*⁻ *met*E *rif*ʳ *str*ʳ *pol*A1 *dna*G3
(*14*) CRT2668: F⁻ B1⁻ *his*⁻ *mal*A *str*ʳ *sup*⁻ *pol*A1 *dna*⁺
(*15*) JG112: W3110 *thy*⁻ *rha*⁻ *lac*⁻ *sup*⁻ *pol*A1 *dna*⁺

The isolation of the double mutant *dnaB polA1*, was described (5). A further series of *dna–pol*A1 double mutants, *dna*C, D, E, and F, with *pol*A1, were constructed through two successive steps. (*i*) Each thermosensitive mutation was introduced into an Hfr strain (HfrP4x8: an Hfr that injects its chromosome in the order, O-*pro*A-*leu*—*lac*-F, or Hfr-Cavalli: an Hfr that injects its chromosome in the order, O-*lac*-*leu*—*gal*-F) by crossing the Hfr with an F-strain carrying a thermosensitive mutation affecting DNA synthesis. *lacy* (The site of F integration of HfrP4x8 is near *lacy*) (6) and *gal* (the site of F integration of Hfr Cavalli is near *gal*) (6) are used for selection. (*ii*) Each thermosensitive Hfr strain isolated was then crossed with an F⁻ strain, PA33612; F⁻ *arg*⁻ *his*⁻ *thi*⁻ *leu*⁻ *mal*A⁻ *xyl*⁻ *mtl*⁻ *pol*A1⁻ *sup*⁻, using a closely linked marker (*leu* for *dna*E, C, and D and *his* for *dna*F) for selection. Recombinants were then tested for both *pol*A1, *dna*, and *sup*.

As controls, thermoresistant *dna*⁺*pol*A1⁻ were constructed by selection at a high temperature, either after P1-transduction of the thermosensitive allele, *dna*⁺, or spontaneous occurrence of revertants from the double mutants. Strain JW 207 was isolated after bacteriophage P1 transduction of *dna*F101 from strain E101 (7) into W3110 *thy*⁻ *rha*⁻ *pol*A1 *pur*F, by selection for *pur*F⁺. Strain NY73 was isolated by introduction of *pol*A1 into PC3 with JG78 (Hfr *R1*, *metE rha*⁺ *pol*A1 Rifʳ) (Peacey, M., and J. D. Gross, unpublished data).

† In the text, these strains will be referred to by their number in the above list, followed by the *dna* mutation designation in parenthesis.

The isolation and mapping of thermosensitive mutants have been reported by others (5, 7–14). Strains PC2:*dna*C2 and PCF *dna*D7 were a gift from Dr. P. Carl. Strains 1026, 1040 (*dna*E), and 1029 (*dna*B) were a gift from Dr. F. Bonhoeffer (classification of *dna* lesion was by co-transduction, ref. 11).

The materials used for purification and assay of DNA Polymerases II and III were described. [^{3}H]TTP (2×10^5 cpm/nmol) was used throughout to assay DNA polymerase activity.

Cells were grown in three-times concentrated L.B. broth (Difco) (15) at 25°C with aeration, and harvested in mid-log phase at 3–4 $\times$ 10^9 cells/ml. Cell-free extracts (10 g of cells) and the S100 fraction were prepared as described (2). Separation of DNA Polymerases II and III (see Fig. 1*A*) was also described, except that all volumes and column dimensions were scaled down 10-fold and preliminary dialysis and batch elution from phosphocellulose (step II) were omitted. For the addition of large amounts of DNA Polymerase III to reaction mixtures, the enzyme activity that eluted from phosphocellulose was concentrated 10-fold by precipitation with ammonium sulfate.

Assays of rates of reaction at 30 and 45°C were done by first equilibrating the assay mixture (0.9 ml) at the appropriate temperature. The reaction was begun by the addition of enzyme. 0.2-ml aliquots were withdrawn at various times and pipetted into 1.0 ml of 5% trichloroacetic acid. The acid-insoluble material was collected on a Millipore filter and the radioactivity was determined in a liquid scintillation counter.

RESULTS

The results of a typical isolation of DNA Polymerase II and III are shown in Fig. 1*A*. Polymerases II and III are distinguished on the basis of chromatographic behavior; Polymerase III elutes at 0.1 M PO_4^{3-} (fraction 17) and Polymerase II at 0.2 M PO_4^{3-} (fraction 37). The two enzymes can further be distinguished on the basis of their response to ionic strength (2). In two instances (strains 1 and 7), Polymerases II and III were isolated from cells containing the normal amount of DNA Polymerase I. The result of phosphocellulose chromatography of extracts from such cells is shown in Fig. 1*B*. Although DNA Polymerase III is not completely resolved from Polymerase I activity (fraction 20), the activity of Polymerase III can be uniquely determined by assay of column fractions in the presence of either *N*-ethylmaleimide (dotted line, Fig. 1*B*) or antiserum to DNA Polymerase I. Since Polymerase III activity is completely abolished in the presence of *N*-ethylmaleimide, and is unaffected by antiserum directed against DNA Polymerase I (2), it is possible to obtain preparations active only due to Polymerase III despite the presence of Polymerase I. DNA Polymerase II is obtained in normal yield from *pol*$^+$ strains; it is completely resolved from Polymerase I by phosphocellulose chromatography.

The peak fraction of each polymerase activity was used directly for measurements of the rate of synthesis at 30 and 45°C. The rate of reaction catalyzed by Polymerase II was 1.8-times faster at 45°C than at 30°C; the rate of the Polymerase III reaction was 1.5-times faster at 45°C than at 30°C. DNA Polymerase III activity is not linear with time after 5 min at 45°C and, therefore, relative rates were calculated only from the initial slopes.

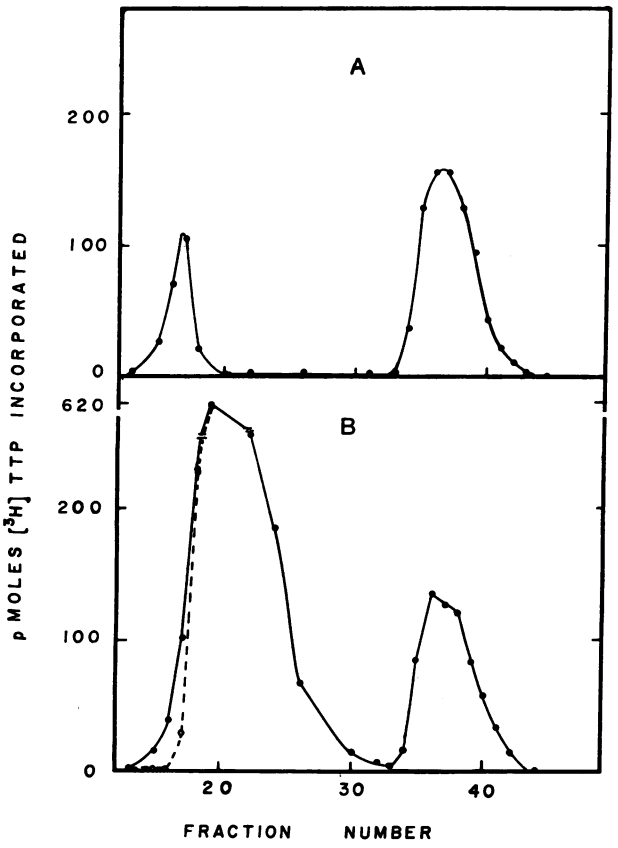

FIG. 1. Separation of DNA Polymerases II and III by phosphocellulose chromatography. Cell-free extracts from *pol*A1 strains (*A*) and *pol*$^+$ strains (*B*) were prepared and subjected to phosphocellulose chromatography. Each fraction was assayed for DNA polymerase activity. Results obtained by assay of fractions (12–19) in the presence of *N*-ethylmaleimide are shown by the *dotted line*. Polymerase II and III activities elute at fractions 35–45 and 12–20, respectively.

The results of a typical analysis of DNA Polymerase II activity at 30 and 45°C are shown in Fig. 2. Fig. 2*A* represents the Polymerase II activity isolated from strain 13 (*dna*G) and 2*B* the results from strain 9 (*dna*E). On the basis of these analyses, DNA Polymerase II activity appeared normal in all strains tested. The results of these relative rate measurements are summarized in Table 1.

The results of a typical analysis of wild-type DNA Polymerase III activity are shown in Fig. 3*A*. These results were obtained with DNA Polymerase III isolated from strain 13 (*dna*G) and are representative of all enzyme preparations tested except for those isolated from strains 6, 9, and 10 (carrying *dna*E mutations). These results are also summarized in Table 1.

The results obtained for DNA Polymerase III activity isolated from strain 9 (*dna*E) are shown in Fig. 3*B*. (Polymerase III activity isolated from strain 10 (*dna*E) behaves essentially the same way.) In contrast to the rate observed with a normal enzyme, the rate of synthesis at 45°C with enzyme preparations from the *dna*E mutants 9 and 10 was undetectable. To rule out the possibility of a temperature-dependent inhibitor present in these preparations, concentrated Polymerase III preparations (see *Methods*) from strains 9 (*dna*E) and 13 (*dna*G) were mixed and the rates at

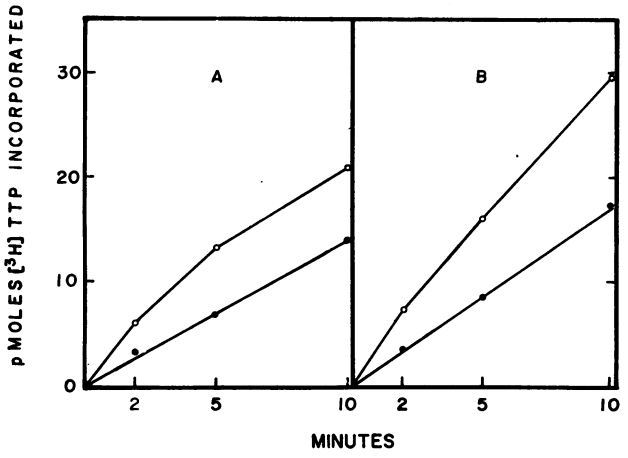

FIG. 2. Effect of temperature on Polymerase II-catalyzed synthesis. The rate of polymerization at 30°C (—●—) and 45°C (—○—) was measured. The results obtained for enzymes isolated from strain 13 (*dna*G) and strain 9 (*dna*E) are shown in parts *A* and *B*, respectively.

30 and 45°C were determined. The presence of Polymerase III from strain 9 does not render the wild-type enzyme temperature sensitive (Fig. 3*C*).

In order to obtain further evidence that the temperature-sensitive character of Polymerase III, derived from strains 9 or 10, was due to a specific alteration of the enzyme, Polymerase III from strain 9 was further purified. The procedures used (T. K. and M. G., manuscript in preparation) are sufficient to purify the wild-type enzyme 2000-fold with respect to the S100 fraction. The properties of such an enzyme preparation are identical to those described for the enzyme activity eluted from the phosphocellulose column.

The specific activity (assayed at 30°C) of preparations from strains 9 and 10 are 10% that of the wild-type enzyme throughout the purification procedure. In all cases "mutant" Polymerase III is totally inactive when assayed at 45°C.

Polymerase III activity could not be detected in extracts from strains 7 and 8 (*dna*E486). The DNA Polymerase III isolated from strain 6 (*dna*E511) was only marginally temperature sensitive.

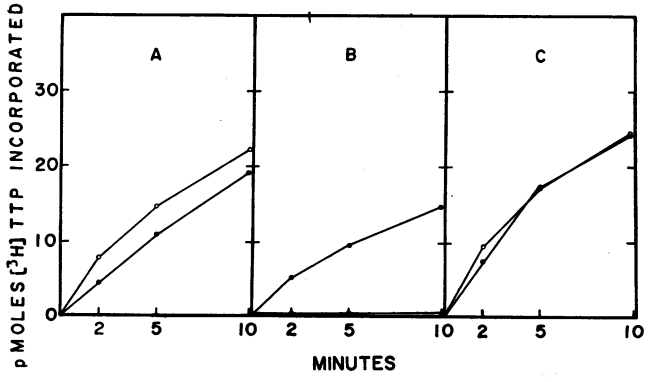

FIG. 3. Effect of temperature on Polymerase III-catalyzed synthesis. The rate of polymerization at 30°C (—●—) and 45°C (—○—) was measured. The results obtained for enzymes isolated from strain 13 (*dna*G) and strain 9 (*dna*E) are shown in Parts *A* and *B*, respectively. The results obtained with a mixture of equal amounts of enzyme from strain 13 and strain 9 are shown n part *C*.

TABLE 1. *Effect of temperature on the rate of reaction*

Strain	Reaction rate $\frac{45°C}{30°C}$ DNA Polymerase II	DNA Polymerase III
1 (*dna*A)	1.85	1.45
2 (*dna*B)	1.73	1.74
3 (*dna*B)	1.95	1.55
4 (*dna*C)	1.86	1.20
5 (*dna*D)	1.99	1.47
6 (*dna*E)	1.87	1.0
7 (*dna*E)	1.70	—*
8 (*dna*E)	2.00	—*
9 (*dna*E)	1.95	<0.1
10 (*dna*E)	1.70	<0.1
11 (*dna*F)	1.55	1.33
12 (*dna*F)	1.77	1.43
13 (*dna*G)	1.75	1.57
14 (*dna*+)	1.92	1.70
15 (*dna*+)	1.90	1.55

* A dash indicates that the enzyme activity was not detectable at 30°C.

DISCUSSION

The enzymes DNA Polymerase II and III have been analyzed in several mutants thermosensitive for DNA synthesis. DNA Polymerase II activity appears to be normal in all strains tested, including a strain carrying a thermosensitive *rec*A gene (data not presented). The failure to associate Polymerase II activity with any *dna* locus tested does not prove that this enzyme is dispensable; isolation of a mutant defective in Polymerase II will be required to resolve this question. That Polymerase II is not thermolabile in *dna*E mutants indicates that this enzyme is not related to Polymerase III, as was previously suggested (2).

DNA Polymerase III activity appears to be normal in strains carrying mutations at the *dna*A, B, C, D, F, and G loci. Of four independently isolated *dna*E mutants, all had altered DNA Polymerase III activity *in vitro*. DNA Polymerase III activity could not be detected in extracts of *dna*E486 mutants, either in the presence or absence of DNA Polymerase I. We believe that this result is due to instability *in vitro* of the Polymerase III in these strains. DNA Polymerase III in the *dna*E511 mutant is only slightly temperature sensitive, which perhaps reflects the slow cessation of *in vivo* DNA synthesis that this mutant displays at the restrictive temperature (11). Polymerase III activity in strains 9 and 10 (*dna*E) is thermolabile.

Our results suggest that the structural gene for DNA Polymerase III is located at the *dna*E locus. Furthermore, since *dna*E mutants fail to replicate their DNA at 42°C *in vivo* (10) and contain a thermolabile Polymerase III *in vitro*, we conclude that this enzyme is required for DNA replication in *E. coli*.

NOTE ADDED IN PROOF

Drs. H. Schaller, B. Otto, V. Nüsslein, J. Huf, R. Herrmann, and F. Bonhoeffer have isolated 50 independent

dna^{ts} mutants of *E. coli*. Of these, 20 were shown to be temperature-sensitive in highly concentrated lysates designed to measure DNA replication *in vitro*. Four such mutants that include strains 9 and 10, were shown not to complement each other but were complemented by a soluble factor isolated from *dna*$^{+}$ cells. Drs. B. Otto and V. Nüsslein have purified the soluble factor (E-protein) 1,000-fold using a complementation assay, and have independently shown that its properties (polymerase activity, stability, response to ionic conditions, and molecular weight) are in keeping with those determined for DNA polymerase 111. According to the above criteria, DNA polymerase 111 and the E-protein appear to be the same.

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1. DeLucia, P., and J. Cairns, *Nature*, **224**, 1164 (1969).
2. Kornberg, T., and M. L. Gefter, *Proc. Nat. Acad. Sci. USA*, **68**, 761 (1971).
3. Knippers, R., *Nature*, **228**, 1050 (1970).
4. Moses, R., and C. C. Richardson, *Biochem. Biophys. Res. Commun.*, **41**, 1565 (1970).
5. Mordoh, J., Y. Hirota, and F. Jacob, *Proc. Nat. Acad. Sci. USA*, **67**, 773 (1970).
6. Jacob, F., and E. Wollman, *Sexuality and Genetics of Bacteria* (Academic Press, Inc., New York, 1961).
7. Carl, P., *Mol. Gen. Genet.*, **109**, 107 (1970).
8. Ricard, M., and Y. Hirota, *C.R.-Acad. Sci.*, **268**, 1335, (1969).
9. Bonhoeffer, F., *Z. Vererbungslehre.*, **98**, 141 (1966).
10. Gross, J. D., *Current Topics in Microbiology and Immunology* (Berlin, Springer-Verlag, 1971).
11. Wechsler, J. A., and J. D. Gross, *Mol. Gen. Genet.*, in press.
12. Kohiyama, M., D. Cousin, A. Ryter, and F. Jacob, *Ann. Inst. Pasteur*, **110**, 465 (1966).
13. Hirota, Y., A. Ryter, and F. Jacob, *Cold Spring Harbor Symp. Quant. Biol*, **33**, 677 (1968).
14. Hirota, Y., J. Mordoh, and F. Jacob, *J. Mol. Biol*, **53**, 369 (1970).
15. Bertani, G., *J. Bacteriol.* **62**, 293 (1951).