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Persistence of Deoxyribonucleic Acid Polymerase I and Its 5′ → 3′ Exonuclease Activity in *PolA* Mutants of *Escherichia coli* K12*

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SUMMARY

Partially purified enzyme preparations derived from a strain of *Escherichia coli* K12 with an *amber* mutation (*pol*A1) in the DNA polymerase I gene contain approximately 1% as much polymerase I activity as the preparations from wild type strain purified by the same procedure. Two other polymerase I mutants, one temperature-sensitive (*pol*A12) and the other nonsuppressible (*pol*A5), also contain significant levels of polymerase I (12% and 35% of wild type, respectively). In the case of *pol*A12, the polymerase I activity is abnormally thermolabile. The level of polymerase I observed in a given mutant is dependent, in part, on the pH and the template-primer used.

Despite their low DNA polymerase I levels, these mutants possess nearly normal amounts of the 5′ → 3′ exonuclease activity that is known to be part of DNA polymerase I and is separable as a 2.8 S fragment by proteolytic cleavage. In the temperature-sensitive and nonsuppressible mutants, the 5′ → 3′ exonuclease and polymerase I activities cosediment at approximately 5.4 S just as in the wild type. In contrast, the polymerase I activity in the *amber* mutant sediments at 5.4 S, but the 5′ → 3′ exonuclease activity sediments at 2.8 S. These findings suggest that the residual DNA polymerase I activity sedimenting like the wild type enzyme in the *pol*A1 mutants is the result of a low level read-through of the *amber* mutation, and that the 5′ → 3′ exonuclease represents the *amber* peptide, or a proteolytic cleavage product of it.

In their initial description of the *pol*A1 mutation in *Escherichia coli* strain P3478, deLucia and Cairns reported that extracts of the mutant contained 0.5 to 1% as much DNA polymerase activity as the parent strain, W3110 (1). Whether the residual activity was the result of some "leakiness" of the *amber* mutation or represented a novel polymerase was not determined. Subsequent examination of *pol*A1 extracts in several laboratories (2–6) led to the discovery of two additional DNA polymerases (II and III) and the general impression that DNA polymerase I was totally absent in *pol*A1 mutants.

We have examined a series of DNA polymerase I (*pol*A) mutants to determine whether they do, in fact, contain any detectable DNA polymerase I activity. We have also inquired whether these mutants retain the 5′ → 3′ exonuclease function associated with the wild type enzyme (7). All of the *pol*A mutants that we have examined contain low but significant amounts of DNA polymerase I activity. On the other hand, the levels of 5′ → 3′ exonuclease activity that they possess are nearly normal. In the case of *pol*A1 *amber* mutants, the 5′ → 3′ exonuclease is associated with a protein of substantially lower molecular weight than the native enzyme, probably the prematurely terminated polypeptide chain generated by the *amber* mutation or, even more likely, its proteolytic cleavage product.

EXPERIMENTAL PROCEDURE

Materials

Strains and Media—*E. coli* strains W3110 (*pol*A$^+$) and P3478 (*pol*A1) were provided by Dr. John Cairns (Cold Spring Harbor Laboratory); strain JG112 (*pol*A1) was obtained from T. Kornberg (MIT); strain MM383 (*pol*A12) was obtained from Dr. Sidney Kushner (Stanford University), and strain JG110 (*pol*A5) was from Dr. Douglas Berg (Stanford University). Cell cultures were grown in H-broth (8) at 37°, except for MM383 which was grown at 30°. Fresh tryptone-yeast extract plates (9) supplemented with 0.04% methyl methane sulfonate were used to determine sensitivity to this alkylating agent.

Nucleotides and Polynucleotides—Unlabeled deoxynucleoside triphosphates were purchased from P-L Biochemicals, and [^{3}H]dTTP was purchased from Schwarz BioResearch. [α-^{32}P]-dTTP was synthesized according to Symons (10). d(pA)$_{800}$·d(pT)$_{10}$, [^{3}H]d(pT)$_{275}$, and [5′-OH] [^{3}H]d(pT)$_{275}$[1] were synthesized with calf thymus terminal deoxynucleotidyltransferase as described by Kelly *et al.* (11). d(pA)$_{5000}$ was synthesized and purified by the method of Riley *et al.* (12). "Nicked" calf thymus DNA was prepared by limited digestion with pancreatic deoxyribonuclease (13). [5′-^{32}P] [^{3}H]d(pT)$_{275}$ was prepared by treating

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[1] The abbreviations used are: [5′-OH]d(pT)$_{275}$, a polymer composed of 275 deoxythymidylate residues with a 5′-hydroxyl terminus; [5′-^{32}P] [^{3}H]d(pT)$_{275}$, polymer composed of 275 deoxythymidylate residues labeled in the thymine with ^{3}H and in its 5′-phosphoryl terminus with ^{32}P.

[5′-OH] [^{3}H] $(pT)_{275}$ with [γ-^{32}P]ATP and polynucleotide kinase as described by Richardson (14). Polynucleotide concentrations are expressed as the molarity of nucleotide residues.

Enzymes—*E. coli* DNA polymerase I (Fraction VII) (15) was provided by Dr. A. Kornberg. Polynucleotide kinase was isolated by the method of Richardson (14). Calf thymus terminal deoxynucleotidyltransferase (16) was a gift from Dr. R. Ratliff (Los Alamos Scientific Laboratory, Los Alamos, New Mexico).

Other Reagents—Antiserum against *E. coli* DNA polymerase I (Fraction VII) and normal rabbit serum were provided by Dr. A. Kornberg. They were diluted 1:10 with 0.15 M NaCl and heated for 30 min at 70°, then cooled before use. Human hemoglobin was a gift from Dr. Philip Laipis. The protease inhibitor, phenyl methyl sulfonyl chloride, was purchased from the Pierce Chemical Company (Rockford, Ill.) and *N*-ethylmaleimide was purchased from the Mann Research Laboratories.

Methods

Enzyme Assays—DNA polymerase activity was assayed in reaction mixtures (0.1 ml) that contained 0.06 M Tris-HCl (pH 8.6); 8 mM $MgCl_2$; 0.06 mM each of dATP, dCTP, and dGTP; 0.015 mM [^{3}H]- or [α-^{32}P]dTTP, (300 to 1000 cpm per pmole); 0.14 mM $d(pA)_{800}$, and 0.009 mM $d(pT)_{10}$, or 0.04 mM nicked calf thymus DNA; and enzyme fraction as specified in the table and figure legends. *N*-Ethylmaleimide (10 mM) was routinely included in assays of sucrose density gradient fractions. Where indicated, 15 μl of diluted antiserum to *E. coli* DNA polymerase I were added. After incubation for 30 min at 37°, the reaction was terminated, and acid-insoluble ^{32}P was determined by the method of Richardson *et al.* (13).

5′ → 3′ exonuclease activity was assayed in a reaction mixture (0.08 ml) that contained 0.067 M Tris-HCl (pH 7.5); 20 mM potassium phosphate (pH 7.5); 6.7 mM $MgCl_2$; 0.0145 mM $d(pA)_{5000}$; 0.0145 mM [5′-^{32}P] [^{3}H]$d(pT)_{275}$, (1000 to 5000 cpm per pmole of ^{32}P, 10 cpm per pmole of ^{3}H); 10 mM *N*-ethylmaleimide; 50 μg per ml of bovine serum albumin; 0.5 A_{260} unit of *E. coli* tRNA; and 5 μl of sucrose density gradient fraction. Where indicated, 10 μl of diluted *E. coli* polymerase I antiserum (or 5 μl of diluted normal rabbit serum) were added. Incubation was at 30°. Samples (20 μl) were removed at zero time (immediately after addition of enzyme) and at 5 and 10 min, and pipetted into 0.2 ml of an ice-cold solution containing 3 mM calf thymus DNA and 10 mM NaCl. Three-tenths milliliter of cold 10% trichloroacetic acid was added, and, after mixing, the precipitate was removed by centrifugation at 20,000 × *g* for 15 min. An aliquot of the supernatant fluid (0.3 ml) was added to 10 ml of toluene-Triton X-100 scintillator, and ^{32}P and ^{3}H were determined in a Beckman LS-230 spectrometer. Zero time samples contained less than 1% acid-soluble ^{32}P.

Partial Purification of DNA Polymerase I—Mid-log phase cultures grown in H-broth were harvested, and the pellet (2 g) was suspended in 8 ml of a solution composed of 20 mM Tris-acetate (pH 8.2), 10 mM magnesium acetate, 5 mM EDTA, 0.2 mM dithiothreitol, and 1 mM phenyl methyl sulfonyl chloride. The suspension was treated twice in a French pressure cell at 9000 p.s.i. according to Kornberg and Gefter (2). Nine milliliters of extract (approximately 20 mg of protein per ml) were made 0.06 M in $MgCl_2$ and kept at 0° for 2 to 4 hours. The precipitate was removed by centrifugation at 20,000 × *g* for 30 min. The supernatant fluid (8 ml) was treated with 2.35 ml of a freshly prepared 5% solution of streptomycin sulfate per 1000 A_{260} units. After 20 min at 0°, the suspension was centrifuged at 20,000 × *g* for 15 min. The Tris-HCl was adjusted to 0.05 M by the addition of 1 M Tris-HCl (pH 7.5). Solid ammonium sulfate (0.282 g per ml) was added with stirring, and, after 20 min at 0°, the suspension was centrifuged at 20,000 × *g* for 30 min. The precipitate was dissolved in 1 ml of 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM dithiothreitol, and 0.1 mM phenyl methyl sulfonyl chloride (ammonium sulfate Fraction I; 0 to 40% saturation). Solid ammonium sulfate (0.141 g per ml) was added to the supernatant fluid. After 20 min at 0°, the suspension was centrifuged and the resulting precipitate was dissolved as described above (ammonium sulfate Fraction II; 40 to 60% saturation). Ammonium sulfate Fractions I and II both contained 15 to 20 mg of protein per ml. Greater than 80% of the DNA polymerase I recovered from the wild type and mutant extracts appeared in ammonium sulfate Fraction II.

Sucrose Density Gradient Centrifugation—The ammonium sulfate Fraction II was dialyzed immediately after preparation against three changes of 500 ml of a solution composed of 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 0.1 mM phenyl methyl sulfonyl chloride for 2 hours at 0°. A 0.1-ml sample of the dialyzed preparation was centrifuged in a 5 to 20% sucrose density gradient containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.05 M ammonium sulfate, and 1 mM dithiothreitol, using the SW 56 rotor in a Beckman L265 B ultracentrifuge at 50,000 rpm for 16 hours at 2–3°. Crystalline human hemoglobin (5 μl, containing 0.5 mg of protein) was included as sedimentation standard ($s_{20,w}$ = 4.2 S). At the end of the run, 0.19-ml fractions were collected by puncturing the bottom of the tube. They were immediately assayed for DNA polymerase I and 5′ → 3′ exonuclease activities.

RESULTS

Detection of DNA Polymerase I in Partially Purified Extracts of P3478—Two reagents were used to identify DNA polymerase I, antiserum prepared against the pure enzyme and *N*-ethylmaleimide. Each of these distinguishes labeled deoxynucleoside triphosphate incorporation into acid-insoluble material due to polymerase I from that due to polymerases II and III, even in relatively crude fractions. Thus, activity due to polymerase I is defined as that which is inhibited by polymerase I antiserum and unaffected by concentrations of *N*-ethylmaleimide up to 0.01 M. On the other hand, polymerases II and III are not affected by polymerase I antiserum, but are completely inhibited by 0.01 M *N*-ethylmaleimide (2–6).

Assay of crude, French pressure cell extracts of strain P3478 suggested that they contained very low levels of polymerase I activity as defined by these criteria. The extracts were then fractionated with streptomycin and ammonium sulfate as described under "Methods." As shown in Table I, DNA polymerase I can be easily detected and effectively separated from polymerases II and III by ammonium sulfate fractionation as judged by its response to *N*-ethylmaleimide and to antibody directed against DNA polymerase I. The inhibition of DNA polymerase I activity by polymerase I antiserum is specific; no such inhibition (<10%) was observed upon addition of normal rabbit serum. In a series of such preparations, the level of DNA polymerase I ranged from 0.5 to 2% of that found in the parent wild type strain, W3110, fractionated in the same way. The DNA polymerase I activity observed in P3478 was not the result of 0.5 to 2% *pol*A$^+$ revertants, since each of the cultures from which the ammonium sulfate fraction was prepared plated with an efficiency of only about 10^{-6} in the presence of 0.04% methyl methane sulfonate, as compared with an efficiency of 0.66 for W3110 (1).

TABLE I

Separation of DNA polymerase I from DNA polymerases II and III in polA1 strain P3478

Ammonium sulfate Fractions I and II were prepared and assayed as described under "Experimental Procedure," with additions as indicated. One microliter of enzyme fraction (approximately 15 μg of protein) was used for each assay.

Template-primer	Additions	[^{32}P]dTMP incorporated
		pmoles
Ammonium sulfate I		
$d(pA)_{800}\cdot d(pT)_{10}$	None	1.02
$d(pA)_{800}\cdot d(pT)_{10}$	Polymerase I antiserum	0.1
Nicked calf thymus DNA	None	12.9
Nicked calf thymus DNA	Polymerase I antiserum	14.2
Nicked calf thymus DNA	0.01 M *N*-ethylmaleimide	0.8
Ammonium sulfate II		
$d(pA)_{800}\cdot d(pT)_{10}$	None	10.3
$d(pA)_{800}\cdot d(pT)_{10}$	Polymerase I antiserum	0.5
$d(pA)_{800}\cdot d(pT)_{10}$	0.01 M *N*-ethylmaleimide	10.7
Nicked calf thymus DNA	None	2.2
Nicked calf thymus DNA	Polymerase I antiserum	0.2

Polymerase I can also be distinguished from polymerases II and III on the basis of its template-primer requirement. Thus DNA polymerase I from P3478 (and W3110) shows a strong preference for the $d(pA)_{800}\cdot d(pT)_{10}$ homopolymer pair over nicked calf thymus DNA. The homopolymer pair which contains a ratio of 20-deoxyadenylate residues to 1 thymidylate residue has relatively large gaps, and such a structure has been shown to be an excellent template-primer for polymerase I but a poor one for polymerases II and III (5, 6). We presume that the capacity of nicked calf thymus DNA to serve polymerases II and III at all is due to the conversion of nicks to small gaps by the exonuclease III activity present in the ammonium sulfate fractions.

Sucrose Density Centrifugation of PolA1 (Amber) Mutants—The low level of DNA polymerase I in P3478 could be the result of some "leakiness" or misreading of the *amber* mutation. Alternatively, it might represent an intrinsically low activity of the *amber* fragment itself. To distinguish between these two possibilities, the ammonium sulfate II fraction derived from P3478 was centrifuged in a sucrose density gradient, and its sedimentation coefficient was compared with the same fraction derived from the *pol*A$^+$ strain, W3110. As shown in Figs. 1 and 2, the sedimentation coefficient (5.4 S) of DNA polymerase I activity in ammonium sulfate Fraction II of P3478 is identical with that in the wild type strain and with that of the homogeneous DNA polymerase I (15), suggesting that the mutant polymerase does not differ greatly from the wild type enzyme in its molecular weight. An analysis of this kind, however, would not distinguish an *amber* fragment, for example, of molecular weight 100,000, from a missense protein with a molecular weight equal to that of the wild type enzyme (109,000), resulting from readthrough of the *amber* codon.

JG112, a methyl methane sulfonate-sensitive strain of W3110 into which the *pol*A1 *amber* mutation had been transferred from the heavily mutagenized background of P3478 (17), was also examined by sucrose density gradient centrifugation. The sedimentation coefficient of the polymerase I activity in this mutant strain was indistinguishable from that of the wild type enzyme (Fig. 3). On the other hand, the total amount of activity sedimenting at 5.4 S in JG112 was substantially below that found in P3478 (Table II). We have also noted that the DNA polymerase I activity in JG112 is considerably more labile than the activity in P3478, and the lower value for JG112 may reflect its instability.

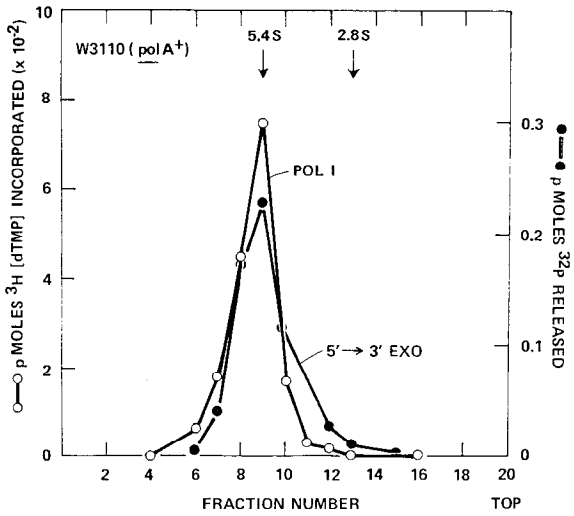


FIG. 1. Sucrose density gradient centrifugation of ammonium sulfate Fraction II of strain W3110 (*pol*A$^+$). Polymerase I and 5′ → 3′ exonuclease assays were performed as described under "Methods," with 1-μl samples for polymerase and 5-μl samples for exonuclease assays. $d(pA)_{800}\cdot d(pT)_{10}$ was used as template-primer for the polymerase assays. The polymerase and exonuclease activities of the peak fraction were >90% inhibited by the addition of 15 μl of polymerase I antibody. Polymerase and exonuclease activities are expressed per microliter of enzyme fraction.

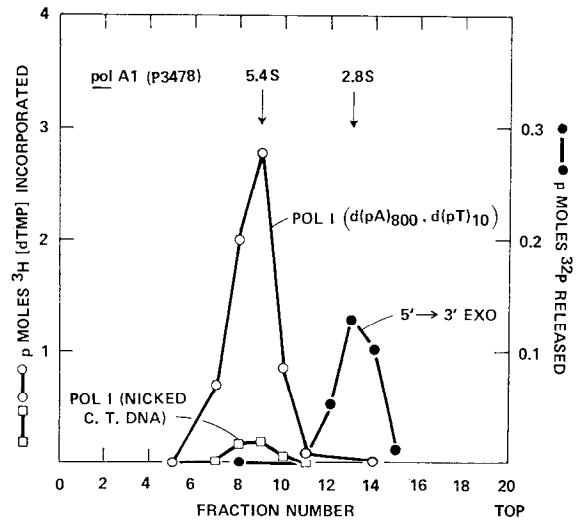


FIG. 2. Sucrose density gradient centrifugation of ammonium sulfate Fraction II of P3478 (*pol*A1). Samples of 5 μl were used for polymerase and for exonuclease assays as described under "Methods." *Nicked C. T. DNA* refers to nicked calf thymus DNA. The polymerase activity of the 5.4 S peak fraction and the exonuclease activity of the 2.8 S peak fraction were >90% inhibited by the addition of 15 μl of polymerase I antibody. Polymerase and exonuclease activities are expressed per microliter of enzyme fraction.

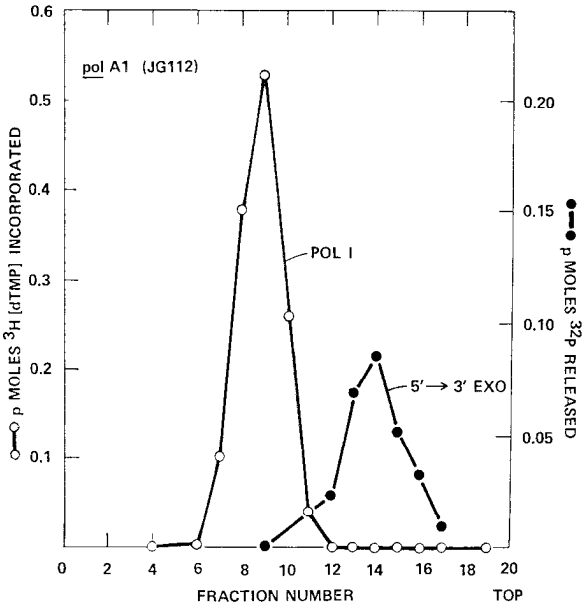


FIG. 3. Sucrose density gradient centrifugation of ammonium sulfate Fraction II of JG112 (*pol*A1). Samples of 10 μl were used for polymerase, and 5-μl samples were used for exonuclease assays as described under "Methods." $d(pA)_{800}\cdot d(pT)_{10}$ was the template-primer in the polymerase assays. The sedimentation coefficient of the polymerase activity was 5.3 S, and the 5′ → 3′ exonuclease was 2.6 S. The polymerase and exonuclease activities of the peak fractions were >90% inhibited by the addition of 15 μl of polymerase I antibody. Polymerase and exonuclease activities are expressed per microliter of enzyme fraction.

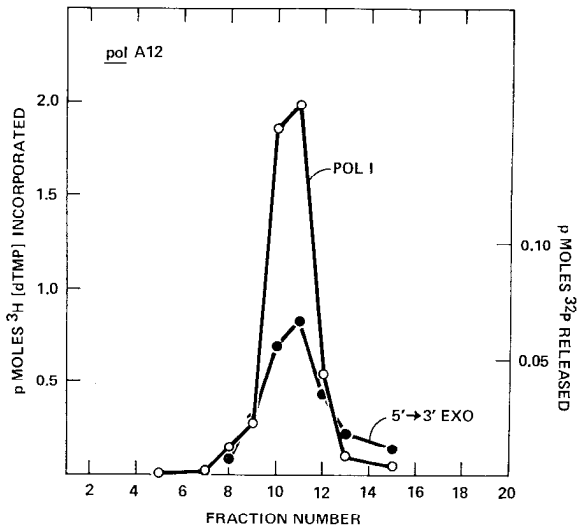


FIG. 4. Sucrose density gradient centrifugation of ammonium sulfate Fraction II of MM383 (*pol*A12). Samples of 10 μl were used for polymerase, and 5-μl samples were used for exonuclease assays. The 5′ → 3′ exonuclease assays were carried out as described under "Methods." The DNA polymerase activity was assayed using nicked calf thymus DNA as template-primer as described under "Methods," except that Tris-HCl buffer at pH 7.5 was used and the incubation temperature was 30°. The sedimentation coefficients of the polymerase I and 5′→ 3′ exonuclease were both 5.0 S. Both activities in the peak fraction were >90% inhibited by the addition of 15 μl of polymerase I antibody. Polymerase and exonuclease activities are expressed per microliter of enzyme fraction.

TABLE II

DNA polymerase I activity in sucrose gradient fractions of polA mutants of Escherichia coli

The values shown represent the total DNA polymerase I activity sedimenting at 5.0 to 5.4 S in Figs. 1 to 5.

Strain	Total activity in peak		
	$d(pA)_{800}\cdot d(pT)_{10}$	Nicked calf thymus DNA	Percentage of wild type[a]
	pmoles dTMP incorporated		
W3110 (*pol*A^{+})	185,000	23,000	(100)
P3478 (*pol*A1)	1,170	99	0.6
JG112 (*pol*A1)	140	n.d.[b]	0.08
MM383 (*pol*A12)	96	935[c]	12.2
JG110 (*pol*A5)	<10	7,950	35

[a] Calculated using the template-primer optimal for the particular mutant.

[b] n.d., not determined.

[c] This assay was at 30° and pH 7.5. When W3110 was assayed under these conditions, 7700 pmoles were incorporated.

Sucrose Density Gradient Centrifugation of PolA12 and PolA5 Mutants—Two additional *pol*A mutants were examined: MM383 (*pol*A12), a temperature-sensitive mutant which is abnormally sensitive to methyl methane sulfonate at 42° but not at 25° (18); and JG110 (*pol*A5), which bears a nonsuppressible mutation in the *pol*A gene.[2] In both of these mutants, DNA polymerase I activity showed a sedimentation coefficient close to 5.4 S (Figs. 4 and 5).

Initial experiments employing conditions that are optimal for DNA polymerase I in the wild type (W3110) and *amber* mutants (P3478 and JG112), *i.e.* $d(pA)_{800}\cdot d(pT)_{10}$ as primer-template, Tris-HCl buffer (pH 8.6) at 37°, were suboptimal for the polymerase I activity in the *pol*A12[3] and *pol*A5 mutants. In fact, polymerase I activity in *pol*A5 was undetectable under these conditions (Fig. 5). Assay of the *pol*A12 polymerase I using nicked calf thymus DNA as a template-primer, at pH 7.5 and 30°, gave a level of activity approximately 12% of that found for the wild type enzyme assayed under the same conditions (Table II). Similarly, assay of the *pol*A5 polymerase I with nicked calf thymus DNA in place of the $d(pA)_{800}\cdot d(pT)_{10}$ homopolymer pair gave a value for this enzyme of 35% of the wild type (Table II).

Properties of PolA1 and PolA12 DNA Polymerases—The DNA polymerase I activity in ammonium sulfate Fraction II of P3478 (*pol*A1) was not significantly different from the activity in the same fraction of the wild type strain in terms of its temperature sensitivity, its preference of $d(pA)_{800}\cdot d(pT)_{10}$ over nicked calf thymus DNA as a template-primer, and its activity at pH 8.6 relative to pH 7.5 (Table III). It was also indistinguishable in its K_m for deoxynucleoside triphosphates, DNA, and Mg^{++} (not shown). A similar result was obtained with strain JG112. On the other hand, the polymerase I activity in *pol*A12 was markedly different from the wild type. Thus, it was very thermosensitive, utilized $d(pA)_{800}\cdot d(pT)_{10}$ very poorly, and showed greater activity at pH 7.5 than at pH 8.5 (Table III).

5′ → 3′ *Exonuclease Activity in PolA Mutants*—Although the level of polymerase I activity displayed in sucrose density gradients of the ammonium sulfate II fractions derived from the *pol*A1 mutants, P3478 and JG112, was very low compared to

[2] B. Konrad, unpublished experiments.

[3] The initial observation that DNA polymerase I activity in partially purified preparations derived from *pol*A12 showed a strong preference for nicked calf thymus DNA over $d(pA)_{800}\cdot d(pT)_{10}$ as a template-primer was made by Dr. Sidney Kushner.

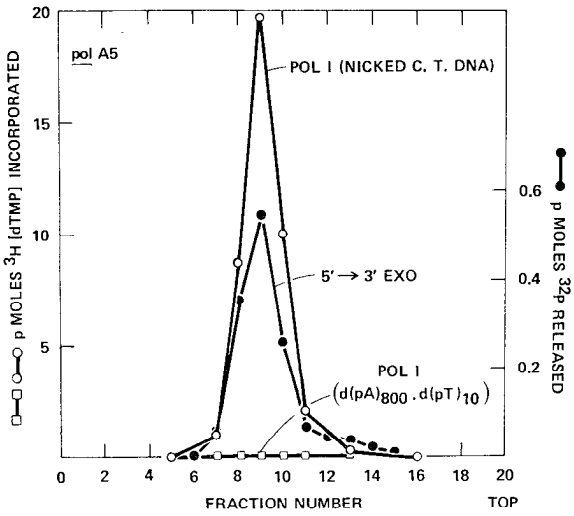


FIG. 5. Sucrose density gradient centrifugation of ammonium sulfate Fraction II of JG110 (*pol*A5). Samples of 10 μl were used for polymerase, and 5-μl samples were used for exonuclease assays as described under "Methods." *Nicked C. T. DNA* refers to nicked calf thymus DNA. The sedimentation coefficients of the polymerase I and the 5′ → 3′ exonuclease were both 5.4 S. The polymerase and exonuclease activities of the peak fraction were >90% inhibited by the addition of 15 μl of polymerase I antibody. Polymerase and exonuclease activities are expressed per ml of enzyme fraction.

TABLE III

Comparison of DNA polymerase I activity in polA12, polA1, and polA⁺

DNA polymerase I assays were performed using ammonium sulfate II fractions as described under "Methods" with variations as indicated. For assays at pH 7.5, Tris-HCl (pH 7.5) replaced the Tris buffer at pH 8.6.

Parameter	MM383 (*pol*A12)	P3478 (*pol*A1)	W3110 (*pol*A⁺)
Activity: 42°/30°	0.13	2.2	2.6
Activity: $d(pA)_{800}\cdot d(pT)_{10}$/Nicked DNA	0.04	5.0	6.0
Activity: pH 8.6/pH 7.5	0.43	1.1	1.2

the wild type (0.6 and 0.1%, respectively), there was a nearly normal level of 5′ → 3′ exonuclease activity in these mutants. Unlike the wild type enzyme, in which the 5′ → 3′ exonuclease and polymerase I co-sedimented at 5.4 S, the P3478 and JG112 polymerase peaks showed no detectable 5′ → 3′ exonuclease activity.[4] Instead, the 5′ → 3′ exonuclease activity sedimented more slowly than the polymerase, in a discrete peak at 2.8 S. The 5′ → 3′ exonuclease activities sedimenting at 5.4 S in W3110 and at 2.8 S in P3478 were specifically inhibited by antiserum to polymerase I (Fig. 6). A similar result was obtained with JG112.

An analysis of sucrose density gradients of *pol*A12 and *pol*A5 showed that like the wild type, but in contrast to the *pol*A1 *amber* mutants, the 5′ → 3′ exonuclease activity co-sedimented with the polymerase I activity at or near 5.4 S (Figs. 4 and 5, Table IV). In *pol*A12 and *pol*A5, as in the *pol*A1 mutants, the

[4] Although some 5′ → 3′ exonuclease activity (approximately 1% of wild type for P3478) should co-sediment with the polymerase I activity at 5.4 S, the relatively low sensitivity of the assay for 5′ → 3′ exonuclease as compared to polymerase I, prevented its detection in the 5.4 S peak.

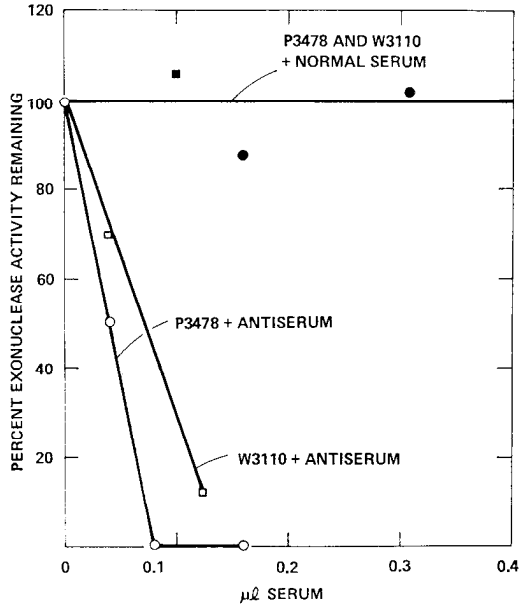


FIG. 6. Inhibition of 5′ → 3′ exonuclease activity by antiserum against purified DNA polymerase I. 5′ → 3′ exonuclease assays were performed with 5 μl of the peak sucrose density gradient fractions from W3110 (Fig. 1) and P3478 (Fig. 2). Polymerase I antiserum or normal rabbit serum were added as indicated.

TABLE IV

5′ → 3′ exonuclease in sucrose gradient fractions of polA mutants of Escherichia coli

The values shown represent the total 5′ → 3′ exonuclease activity in the peaks sedimenting at 2.6 or 2.8 S and at 5.0 or 5.4 S in Figs. 1 to 5. The peak fractions were also assayed at 30° and 42° to determine the 42°/30° ratio.

Strain	Total activity in peak	$s_{20,w}$	Activity 42°/30°
	pmoles 32P released		
W3110 (*pol*A⁺)	82	5.4	1.6
P3478 (*pol*A1)	56	2.8	2.1
JG112 (*pol* A1)	48	2.6	1.7
MM383 (*pol*A12)	38	5.0	1.7
JG110 (*pol* A5)	231	5.4	

total amount of 5′ → 3′ exonuclease activity was near that of wild type levels. In both cases the 5′ → 3′ exonuclease was quantitatively inhibited by polymerase I antibody. The *pol*A5 mutant appeared to possess more than twice the normal 5′ → 3′ exonuclease activity (Table IV); however, the significance of this increase is not known. Although the polymerase I activity of *pol*A12 is abnormally thermolabile, the 5′ → 3′ exonuclease is not (compare Tables III and IV).

DISCUSSION

DNA polymerase I activity can be recognized by the following properties: sedimentation at 5.4 S, insensitivity to 0.01 M *N*-ethylmaleimide, and complete inhibition by antibody to homogeneous DNA polymerase I. As judged by these criteria, partially purified extracts of the *pol*A mutants that we have investigated contain from 0.1 to 35% as much polymerase activity as extracts of wild type strains that have been subjected to the same fractionation procedures. None of the mutants that we have examined, including two additional derivatives of P3478,

strains H560 (19) and D110 (3) obtained by further nitrosoguanidine mutagenesis of P3478, are totally devoid of DNA polymerase I. Obviously, then, no conclusions can be drawn as to the essentiality of DNA polymerase I for cell viability in these strains. When the *pol*A1 mutation is introduced into a strain with particularly low nonsense suppression (due to a streptomycin resistance mutation (20)) such strains form colonies less efficiently on rich than on minimal media.[5] This finding suggests that under certain conditions at least, DNA polymerase I may be required for cell viability.

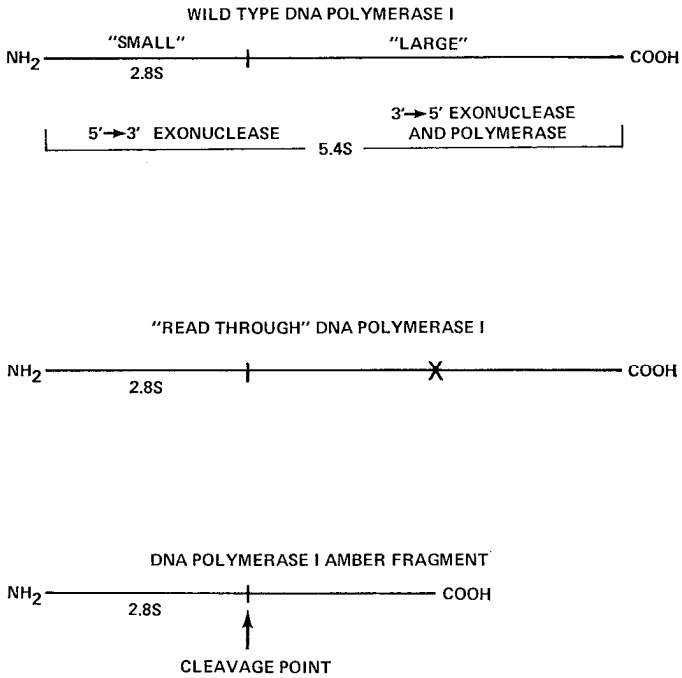
Monk and Kinross were unable to detect polymerase I activity in extracts of the temperature-sensitive mutant *pol*A12 (18). We have found a level of approximately 12% of wild type when the enzyme was assayed at pH 7.5 at 30° with the appropriately nicked calf thymus DNA as template-primer, rather than under conditions that we have found to be optimal for the wild type enzyme (pH 8.6, 37°, $d(pA)_{800}\cdot d(pT)_{10}$). Recent experiments have further shown that the *pol*A12 enzyme is very rapidly inactivated in solutions of ionic strength less than 0.1 M. When care was taken to maintain ionic strength at this or a higher level during isolation and assay of the enzyme, levels of polymerase I up to 60% of wild type were found.[6] Similarly, the *pol*A5 enzyme was undetectable when the $d(pA)_{800}\cdot d(pT)_{10}$ homopolymer pair was used as template-primer, yet a level of 35% of normal activity was observed using nicked calf thymus DNA. Other conditions may reveal an even higher level of activity. Thus, mutations in the *pol*A gene can have a profound influence on polymerase activity, and assays of crude extracts under a single set of conditions can easily lead to erroneous conclusions regarding the presence or absence of the enzyme. On the other hand, partially purified preparations of polymerase I derived from strain JG112 possess a significantly lower level of DNA polymerase I activity than P3478 under all conditions that we have examined. We do not know the reason for this difference, but it may have to do with the efficiency with which the *amber* mutation is suppressed in the two strains (see below). Alternatively, we have found the polymerase I activity in JG112 to be considerably more labile than in P3478; the difference may, therefore, reflect an intrinsic instability of the JG112 enzyme in extracts.

In spite of the low level of DNA polymerase I activity in the *pol*A1 *amber* mutants, P3478 and JG112, these strains possess nearly normal amounts of a 5′ → 3′ exonuclease activity that is inhibitable by polymerase I antibody. In these two mutants, and in contrast to the wild type, the temperature-sensitive, and the nonsuppressible *pol*A strains, the 5′ → 3′ exonuclease is separate from the polymerase and sediments at 2.8 S. A reasonable interpretation of these findings is that the polymerase activity in the *pol*A1 mutants is due to a low level misreading or read-through of the *amber* mutation and the 5′ → 3′ exonuclease activity with a sedimentation coefficient of 2.8 S is the result of rapid proteolytic cleavage of the *amber* peptide to generate a polypeptide similar to or identical with the polymerase I "small fragment" (whose sedimentation coefficient is also 2.8 S) (21). Thus, the site at which the wild type polymerase I is particularly susceptible to proteolysis may be even more vulnerable in the case of the incomplete polypeptide (22). An alternative but, in our view, less likely interpretation is that the 5′ → 3′ exonuclease represents an intrinsic activity of the *amber* peptide whose sedimentation coefficient happens to be the same as the polymerase I "small fragment."

[5] I. Herskowitz, personal communication.

[6] D. Uyemura and I. R. Lehman, unpublished results.

The association of the 5′ → 3′ exonuclease component of DNA polymerase I with the *amber* fragment (or its proteolytic cleavage product) in *pol*A1 permits positioning of this function within DNA polymerase I (23); it is very probably located in the NH_2-terminal portion of the molecule (24) (Scheme 1).



SCHEME 1. Hypothetical scheme to account for the 2.8 S 5′ → 3′ exonuclease activity in *pol*A1 *amber* mutants. The position in the read-through DNA polymerase I at which an amino acid has been inserted in response to the UAG *amber* codon (*X*) is presumed to be distal to the point in the polypeptide chain vulnerable to cleavage by subtilisin.

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