polA6, a mutation affecting the DNA binding capacity of DNA polymerase I.

William S. Kelly and Nigel D. F. Grindley*

Department of Biological Sciences, Mellon Institute of Science, Carnegie-Mellon University, Pittsburgh, PA 15213, USA

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ABSTRACT

The polA6 mutation is an allele of the polA gene of Escherichia coli which produces a DNA polymerase I species readily distinguishable from that produced by the wild type allele. Experiments described here show that this enzyme has an altered pH optimum for polymerization and a lower binding affinity for DNA. The defect clearly lies within the carboxyl-terminal large fragment of the enzyme produced by in vivo or in vitro proteolysis since the fragment has the same pH optimum for polymerization as the intact enzyme. The polA6 enzyme and its fragment are more sensitive to phosphate ions than the wild type polymerase, and the large fragment is less efficient at binding poly d(AT) in in vitro binding assays. Although the specific nucleolytic activity of the polA6 enzyme is higher than that of the wild type, there is no apparent alteration in pH optimum for the hydrolysis of either double or single stranded DNA.

INTRODUCTION

In 1971 we reported the isolation of an altered species of DNA polymerase I from an E. coli K12 strain bearing the mutation polA6 (Kelley and Whitfield, 1971). Since that time we have demonstrated that strains carrying this allele are defective in the replication of certain non-conjugative plasmids (Grindley and Kelley, 1976) and that the polA6 mutation lies toward the carboxyl-terminus of the polA cistron in recombinational mapping experiments (Kelley and Grindley, 1976). We are reporting here evidence which demonstrates that the polA6 mutation results in a defect in the enzyme's polymerization function, localized in the carboxyl-terminal portion of the polypeptide. The result of this defect is an altered pH optimum for polymerization and a sensitivity to phosphate which reflects a diminished affinity of the enzyme for its DNA primer/template.

MATERIALS AND METHODS

Nucleic Acids

DNA from E. coli was prepared from unlabelled or $^{32}$P-labelled cells
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by the method of Murray (1970). $^3H$-d(AT) alternating copolymer was prepared using E. coli DNA polymerase I by the method of Modrich and Lehman (1970) and nicked with micrococcal nuclease as described by Deutscher and Kornberg (1969). Unlabelled d(AT) alternating copolymer was purchased from Miles Chemicals. Calf thymus DNA was purchased from Calbiochem or Sigma Chemicals. Maximally activated calf thymus DNA was prepared by the method of Fansler and Loeb (1974).

Enzymes

Micrococcal nuclease and deoxyribonuclease I were purchased from Worthington Biochemicals. The preparation of DNA polymerase I is described in the text below.

Assays

DNA polymerase I was assayed by the procedure of Setlow (1974) using the DNA primer/template indicated and units calculated on the basis of nucleotides incorporated based on known composition and the amount of $^3H$-dATP or $^3H$-dTTP incorporated, assuming an efficiency for liquid scintillation counting of ten percent. Single and double stranded exonucleolytic activity was estimated using the assay system of Lehman and Richardson (1964) with either double stranded DNA or heat-denatured DNA from E. coli. To enhance 5' to 3' exonuclease activity an equimolar mixture of dATP, dCTP, dGTP and dTTP was added to the double stranded DNA substrate at the same concentration levels used for the polymerization assays.

DNA binding assays were carried out using S and S B-6 membrane filters prepared by the method of Yuan and Meselson (1970) in the buffer systems described by Riggs, Suzuki and Bourgeois (1970). Each sample contained 3 nanomoles of micrococcal nuclease-nicked dAT copolymer (equal to 25,600 cpm) in 1 ml of BB buffer to which was added 50 μl of DNA polymerase I large fragment diluted in BB buffer. Addition and mixing took place at zero degrees and samples were filtered after ten minutes of incubation in an ice-water bath.

SDS-polyacrylamide slab gels were used to analyze column fractions and purified enzyme preparations as previously described (Kelley and Grindley, 1976). Protein was estimated by the Lowry method, final concentrations of whole DNA polymerase I and large fragment of both wild type and the polA6 mutant were determined by amino acid analysis for greater accuracy in specific activity calculations.
Bacterial strains
The E. coli K12 strains carrying the wild type and polA6 mutant alleles are described in Kelley and Grindley (1976).

Chromatography media
Phosphocellulose was obtained from Whatman and washed and equilibrated as described by Jovin, Englund and Bertsch (1969). Bio-Gel P-150 and hydroxylapatite, DNA grade, were obtained from Bio-Rad Laboratories. Agarose was obtained from Sigma Chemicals and DNA-agarose was prepared by the method described by Schaller, Nusslein, Bonhoeffer, Kurz and Nietzschmann (1972).

Other reagents
Unless specifically noted, other reagents were purchased from standard laboratory supply house stocks and used without further purification. Liquid scintillation counting systems were as described in Grindley and Kelley (1976).

RESULTS
Purification of DNA Polymerase I
In our initial experiments (Kelley and Whitfield, 1971) we experienced substantial difficulties in purifying the polA6 enzyme due to our inability to assay the enzymatic activity during the latter stages of the standard preparative protocol of Jovin, et al. (1969). Much of this difficulty may be attributed to the assay conditions, as explained more fully below. Although polymerase may be obtained in pure form by this protocol, we have devised an alternative procedure utilizing DNA-agarose affinity chromatography for initial separation of all DNA binding proteins. Subsequent column chromatography on phosphocellulose, hydroxylapatite and Bio-Gel P-150 yields enzyme preparations of purity and specific activity which are comparable to those produced by the method of Jovin, et al. (1969). More important, our procedure does not involve a prolonged autolysis step which could result in inactivation of a thermolabile mutant enzyme.

The overall purification scheme for polA6 DNA polymerase I is summarized in Table I. Yields at the various steps are calculated on the basis of the enzyme's response to heavily nicked calf thymus DNA (Fasman and Loeb, 1974) in the assay system of Setlow (1974). Activities of the crude and DNA agarose fractions are calculated on the basis of enzymatic activities at pH 9.2 in sodium glycinate buffer. Activities of the fractions including and following the phosphocellulose chromatographic
Table I

Summary of DNA Polymerase I and Large Fragment Purification Steps:

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude DNA Agarose</td>
<td>48,720 mg</td>
<td>16.3 unit/mg</td>
<td>100%</td>
</tr>
<tr>
<td>DNA Agarose</td>
<td>1,472</td>
<td>124</td>
<td>23</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>44</td>
<td>2,520</td>
<td>14</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>3.6</td>
<td>22,000</td>
<td>10</td>
</tr>
<tr>
<td>Bio-Gel P-150</td>
<td>2.6</td>
<td>38,125</td>
<td>12</td>
</tr>
<tr>
<td>a) polA6 Enzyme</td>
<td>67,808 mg</td>
<td>6.2 unit/mg</td>
<td>100%</td>
</tr>
<tr>
<td>Crude DNA Agarose</td>
<td>1,787</td>
<td>139</td>
<td>62</td>
</tr>
<tr>
<td>DNA Agarose</td>
<td>58</td>
<td>3,261</td>
<td>43</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>20</td>
<td>11,028</td>
<td>52</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>16</td>
<td>9,411</td>
<td>28</td>
</tr>
<tr>
<td>b) polA(^+) Enzyme</td>
<td>67,808 mg</td>
<td>6.2 unit/mg</td>
<td>100%</td>
</tr>
<tr>
<td>Crude DNA Agarose</td>
<td>1,787</td>
<td>139</td>
<td>62</td>
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<td>Hydroxylapatite</td>
<td>16</td>
<td>9,411</td>
<td>28</td>
</tr>
</tbody>
</table>

All assays are as described in the text. Yield is calculated on the basis of units recovered.

The steps are based on measurements at pH 7.4 (polA\(^+\)) and pH 8.7 (polA6) in tris buffers. No further attempts were made here to maximize incorporations by altering substrate, pH, primer/template or ionic strength conditions. The enzymes were homogeneous as judged by SDS slab gel electrophoresis after the final chromatography step.

(1) DNA-Agarose Affinity Chromatography

A 19.6 cm\(^2\) x 25 cm DNA-agarose affinity column was prepared according to the method of Schaller et al., (1972). Cells were broken by grinding with glass beads and the crude lysate clarified by high speed centrifugation, fractionated with polyethylene glycol 6000, dialyzed and adsorbed to the column as described by Schaller et al., (1972) and Heijneker, Ellens, Tjeerde, Glickman, van Dorp and Pouwels (1973), except that sodium rather than potassium chloride was used in the buffer systems. Elution of the column with a wash of 1.0 M sodium chloride buffer yielded a fraction containing the DNA-binding proteins. A single passage through this column adsorbed approximately eighty to ninety percent of the binding proteins from one kilogram of cells as judged from the recycling of the unadsorbed material. The DNA-agarose was not adversely affected by this procedure and has been reused repeatedly.

(2) Phosphocellulose Chromatography

After concentrating the resulting fraction by ammonium sulfate pre-
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cipitation (eighty percent saturation at zero degrees), the material was
dialyzed against 50 mM potassium phosphate, pH 6.5, 10 mM mercaptoethanol,
onight. The dialyzed material was diluted with 10 mM mercaptoethanol
until its conductivity corresponded to that of 25 mM potassium phosphate
and it was then adsorbed to a column of phosphocellulose 4.9 cm² x 25 cm.
This column was developed with a one liter linear gradient of 20 to 300 mM
potassium phosphate, pH 6.5, 10 mM mercaptoethanol, as described in Jovin
et al., (1969). Fractions were collected and analyzed for DNA polymerase
activity and tested by SDS polyacrylamide gel electrophoresis. Fractions
containing polymerase activity also include substantial contamination with
two nucleases of lower subunit molecular weight, one of which is presumed
to be exonuclease III/endonuclease II (Weiss, 1976) on the basis of its
size and known chromatographic behavior (Jovin et al., 1969; Setlow,
Brutlag and Kornberg, 1972). No attempt has been made to identify the
second nuclease which has an apparent subunit size of 50-60,000 dalton.
Fragments of DNA polymerase I corresponding to the amino-terminal portion
of the whole enzyme containing the 5' to 3' exonuclease have not been
detected. The phosphate gradient used to develop this column does not
resolve the large fragment of DNA polymerase I from the whole enzyme.

(3) Hydroxylapatite Chromatography

The fractions of the phosphocellulose chromatogram containing the
polymerase were concentrated by ammonium sulfate precipitation and dialyzed
against 25 mM potassium phosphate, pH 6.5, 10 mM mercaptoethanol. This
material was then applied to a column of hydroxylapatite (0.64 cm² x 8 cm)
which had been equilibrated in 20 mM potassium phosphate, pH 6.5, 10 mM
mercaptoethanol. The column was then developed with a 300 ml linear
gradient of 20 to 300 mM potassium phosphate, pH 6.5, 10 mM mercaptoethanol.
This procedure closely parallels that of Richardson (1963) and Jacobsen,
Klenow and Overgaard-Hansen (1974). The resulting fraction containing
polymerase activity is essentially free of nuclease contamination but con-
tains a mixture of whole DNA polymerase I and large fragment.

(4) Bio-Gel P-150 Chromatography

After concentration of the hydroxylapatite fractions by ammonium
sulfate precipitation, final traces of impurities were removed and the
whole polymerase was separated from the large fragment by chromatography
on a 4.9 cm² x 180 cm column of Bio-Gel P-150. This column was equilibrated
and eluted with a buffer of 0.1 M potassium phosphate, pH 7.0, 0.1 M amm-
nium sulfate, 1 mM mercaptoethanol. Elution was in the downward direction
using a 50 cm pressure head. Calibration of the column with excluded and included protein standards indicated that essentially all of the protein applied chromatographed as included material. Most of the material eluted as a peak containing DNA polymerase activity and the capacity to degrade $^3$H-d(AT) copolymer which is characteristic of the 5' to 3' nucleolytic activity of the intact enzyme. A satellite follows the main polymerase peak which has polymerase activity but little, if any, 5' to 3' nucleolytic activity (see Figure 1). SDS-polyacrylamide gel electrophoresis of the column fractions indicated that bands of 110,000 and 75,000 dalton subunit size appeared in fractions from the first and second peaks, respectively. These bands were identical in mobility to those of whole polymerase produced from previous preparations and its subtilisin proteolytic fragment. Following the precedent of Setlow et al., (1972) we have treated the naturally occurring and the artificially produced fragments as equivalent in our subsequent experiments.

Enzymatic characterization

In experiments with the polA6 enzyme previously reported (Kelley and Whitfield, 1971) we noted that this species of DNA polymerase I did not

![Figure 1](image-url)
utilize d(AT) copolymer as a primer/template. A similar phenomenon was noted in the case of the DNA polymerase from the polA5 and polA12 mutants (Lehman and Chien, 1973) and reported to be pH dependent. In testing the pH dependence of the polA6 whole enzyme and large fragment we were able to demonstrate clearly that, in fact, this enzyme does have a substantially altered pH optimum for polymerization when compared to the whole wild type enzyme or its fragment (see Figure 2). This dramatic difference in pH optimum partly explains our earlier finding that the enzyme is inactive when assayed in the standard assay described by Jovin et al., (1969) with d(AT) copolymer as a primer/template at pH 7.4. The observation that the polymerization pH optima are the same for both the whole enzyme and its large fragment demonstrates that the mutational lesion in polA6 must be located within the polypeptide comprising the carboxyl-terminal end of the molecule.

Inefficiency of the polA6 enzyme in the assay system of Jovin et al., (1969) is also due to the presence of phosphate buffer in that assay. This has become apparent when comparing the efficacies of different buffer systems in the neutral pH range. Assays conducted with tris buffer gave substantially greater polymerase activity than assays conducted in potassium phosphate buffer of the same molarity and pH. This effect did not appear

Fig. 2. pH Optima of the Whole DNA Polymerase I and Large Fragment of polA6 and polA+. Assays were conducted in triplicate in 33 mM tris buffers with nicked calf thymus DNA primer/template at the indicated pH's as described in the text.

\[
\begin{array}{c}
\text{Whole Enzyme} \\
\text{Polymerase Activity (unit/mg) x 10^3} \\
\text{pH} \\
\text{polA6} \\
\text{polA+} \\
\end{array}
\]

\[
\begin{array}{c}
\text{Large Fragment} \\
\text{Polymerase Activity (unit/mg) x 10^3} \\
\text{pH} \\
\text{polA6} \\
\text{polA+} \\
\end{array}
\]

\[\text{o-o-o = polA+} \quad \text{- - - - = polA6}\]
to be dependent on potassium ion concentration or on ionic strength since
the addition of potassium chloride at equivalent ionic strength levels did
not inhibit the enzyme when assayed in tris buffer. The polA6 and polA+
enzymes were assayed under identical conditions using E. coli DNA as primer/
template in pH 8.7 tris buffer with varying amounts of added potassium
phosphate (see Figure 3). Here it is clear that the addition of phosphate
inhibits the polA6 polymerase activity at concentrations which actually
stimulate the activity of the polA+ enzyme.

The large fragment liberated from the polA6 enzyme by subtilisin
cleavage is inhibited by phosphate (Kelley and Grindley, 1976) under assay
conditions which stimulate the polymerizing capacity of the large fragment
of the polA+ enzyme. Previous studies by Englund, Kelly and Kornberg (1969)
indicate that the binding of DNA to DNA polymerase I is inhibited by
phosphate and can be eliminated by phosphate levels greater than 100 mM.
This suggested that polA6 DNA polymerase I and its large fragment might be
deficient in the capacity to bind DNA, particularly d(AT) copolymer. Since

![Figure 3](image-url)
the enzyme does catalyze some polymerization in the presence of phosphate, binding should be altered but not necessarily abolished. We sought to demonstrate this by assaying the enzyme's capacity to bind $^3\text{H}$-d(AT) copolymer.

The experiments of Englund et al. (1969) demonstrated that whole DNA polymerase I binds tightly enough to d(AT) copolymer that the complex can be sedimented into a sucrose gradient and recovered. Since the enzyme's 5' to 3' exonuclease function hydrolyzes the double stranded poly d(AT), their experiments were conducted in the absence of added Mg$^{2+}$, a condition which inhibited the 5' to 3' exonuclease action. Such assays require large amounts of polymerase and poly d(AT) and recovery is not necessarily quantitative, particularly if the sedimentation is extended. Conversely, the nitrocellulose filter binding techniques utilized by Riggs et al., (1960) to study binding of lac repressor to lac operator DNA and by Yuan and Meselson (1970) to study binding of E. coli RI restriction endonuclease to DNA require less enzyme, and are rapid and quantitative. However, such binding assays depend on the effects of Mg$^{2+}$ to stabilize the complex and assure its retention on the filter.

We have examined the effects of phosphate on the binding of DNA polymerase I large fragment to 3'-phosphoryl terminated d(AT) copolymer. Since the large fragment of the enzyme lacks 5' to 3' exonucleolytic activity and 3' to 5' exonucleolytic activity is blocked by 3'-phosphoryl groups, Mg$^{2+}$ can be added to binding assay incubations to stabilize complexes and assure their binding to filters without fear of nucleolytic digestion of the DNA. Our results are presented in Figure 4.

Here the binding of d(AT) copolymer was examined in 17 and 64 mM phosphate at pH 7.0. At this pH the specific activity of the wild type large fragment is approximately twice that of the polA6 fragment (see Figure 2) when measured in the absence of phosphate. At both of the phosphate concentrations tested, the binding curves for the polA6 enzyme are substantially lower than those for the polA$^+$ enzyme. At 17 mM phosphate polA6 appears to be about half as effective as polA$^+$ in its poly d(AT) binding capacity and at 64 mM phosphate it is about one-third as effective. Thus, the polA6 enzyme binds poly d(AT) less effectively than the polA$^+$ enzyme. This behavior could explain the low activity of polA6 polymerase when assayed in phosphate buffers.

Nucleolytic activities of the polA6 and polA$^+$ enzymes do not appear significantly different although the polA6 enzyme has a slightly higher specific activity when measured in tris and glycine buffers in the pH 7 to
Fig. 4. Binding Activity of DNA Polymerase I Large Fragment. Assay conditions are described in the text. Each assay sample contained 3 nanomoles of \(^{3}H\)-poly d(AT) at a specific activity of 8500 cpm per nanomole.

The higher specific 5' to 3' exonuclease activity may reflect the elevated specific polymerizing activity of the intact polA6 enzyme in the high pH range (see Figure 2). The 5' to 3' exonuclease activities were measured during polymerization. Experiments of Setlow and Kornberg (1972) have indicated that the levels of 5' to 3' exonuclease activities are closely coordinated with polymerization rates.

DISCUSSION

The results presented above provide evidence to support the argument that the mutation in the polA6 allele results in a structural alteration of the DNA polymerase I molecule in the carboxyl-terminal end of the polypeptide. This is consistent with genetic data presented elsewhere (Kelley and Grindley, 1976) which have allowed us to orient the polA cistron in the E. coli genetic map with respect to external markers. A result of the structural alteration is a reduced affinity of the enzyme for poly d(AT), especially at the low pH at which polA\(^{+}\) DNA polymerase I exhibits optimal activity.

The polA6 mutation is one of a collection originally isolated by Cairns (personal communication) by the techniques used to isolate the
initial DNA polymerase I mutant polA6 (de Lucia and Cairns, 1969; Gross and Gross, 1969). Earlier studies indicated that the mutant enzyme is virtually identical to wild type DNA polymerase I in size (Kelley and Whitfield, 1971) and amino acid analysis of homogeneous polA6 enzyme indicate no significant alterations in overall composition from that of wild type polymerase (W. E. Brown, personal communication). The mutation is not suppressed by supD, supE or supP (Kelley and Reehl, unpublished) and is thus not likely to be a nonsense mutation. The most reasonable rationalization of these observations is that polA6 is a missense mutation. Since the enzyme functions well at elevated pH and appears defective in its capacity to bind DNA, especially at pH's near neutrality, one reasonable hypothesis is that the mutation has resulted in substitution of a basic amino acid into a region of the polypeptide at or near the DNA binding site. Since basic amino acids are particularly abundant in the enzyme, a single amino acid substitution of this sort would be difficult to detect, particularly in an enzyme as large as DNA polymerase I. We are presently attempting to identify the particular polypeptide region in which substitution has occurred by fingerprinting techniques.

Our binding studies indicate differences in the affinity of wild type
and polA6 DNA polymerase I for d(AT) copolymer. Other data also allow us to draw the same conclusion, although less quantitatively. Kinetic measurements of the enzymes' requirements for DNA primer/template in the polymerization reaction imply that the apparent \( K_m \) of the enzymes for DNA differ. However, calculation of precise \( K_m \) values is difficult using the data obtained with heavily nicked DNA molecules of ill-defined size and conformation. Since DNA polymerase I is a processive enzyme (Uyemura, Bambara and Lehman, 1975) the interpretation of kinetic data of this sort may not be particularly relevant in any case, especially when considering the complexity of the overall mechanism of polymerase-exonuclease action. In addition to our kinetic analysis, initial experiments with the DNA agarose technique clearly demonstrated that polA6 DNA polymerase I elutes from the single stranded DNA of the affinity column at a lower ionic strength than does the polA+ enzyme, also supporting the hypothesis of weaker binding to DNA. Despite careful attempts to find differences, both enzymes eluted from phosphocellulose and hydroxylapatite columns at or about the same phosphate concentrations and in the same position with respect to contaminating nuclease.

The data presented here do not permit us to calculate actual dissociation constants for the two enzymes with respect to the poly d(AT) used in our study. In obtaining these initial data we did not take particular care in preparing a poly d(AT) substrate of uniquely defined size or conformation. More precise determinations of DNA binding constants may be obtained by using more carefully defined molecules of the type used by Englund et al., (1969). We are currently pursuing such experiments.

Recent experiments of Uyemura and Lehman (1976) and Uyemura, Eichler and Lehman (1976) have described the purification and properties of the mutant polymerases from E. coli K12 strains with the allelic mutations polAexl and polA12. Previously Heijneker et al., (1973) and Heijneker and Klenow (1975) have reported isolation and partial characterization of the polA+107 polymerase. Thus, data for enzymatic activities of four different mutant species of DNA polymerase I are now available. The polAexl and polA+107 polymerases are both deficient in 5' to 3' exonuclease activity with the former being abnormally labile at elevated temperatures. The polA12 polymerase is unstable in low salt buffers and lacks the capacity for concerted 5' to 3' exonuclease/polymerase activity (nick translation) even at 30°. Its pH optimum for polymerization is essentially the same as that of wild type enzyme although it is unable to utilize poly dA-oligo dT
as a primer/template. The enzymatic defect in polA12 is clearly different from that in polA6. The latter enzyme can withstand low ionic strength conditions during chromatography and can polymerize and degrade d(AT) copolymer at its pH optimum. We have not examined the capacity of polA6 polymerase for nick translation under a variety of conditions. However, experiments such as those shown above (Figure 5) and elsewhere (Kelley and Grindley, 1976) indicate that at 37° 5' to 3' exonuclease is at least equally stimulated by concomitant polymerization in both polA6 and polA enzymes. Hopefully, further study of the defects of these and other mutant forms of DNA polymerase I will help us elucidate the mechanism of action of the enzyme.

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*Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06510, USA.

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