DNA Polymerase-Primase from Embryos of Drosophila melanogaster

THE DNA POLYMERASE SUBUNIT*

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The DNA polymerase-primase from Drosophila melanogaster has been separated into its constituent polymerase and primase subunits by sedimentation in glycerol gradients containing 50% ethylene glycol. Both activities have been obtained in good yield. The properties of the 182-kDa polymerase subunit are similar to those of the intact four-subunit enzyme. However, there are three significant differences. (i) The polymerase activity of the 182-kDa subunit shows an increased thermolability; (ii) the pause sites during replication of singly primed, single-stranded circular DNA by the 182-kDa subunit are altered; and (iii) unlike the intact enzyme, the 182-kDa subunit is highly processive in the presence of the single-stranded DNA-binding protein of Escherichia coli.

EXPERIMENTAL PROCEDURES

Materials

Nucleotides and Nucleic Acids—Unlabeled deoxy- and ribonucleotides were purchased from Pharmacia Biotechnology, Inc. [32P]dATP and [32P]dCTP (20,000 cpm/pmol, 3,000 Ci/mmol) were purchased from Amersham Corp. Poly(dT) and poly(dC-T) were purchased from Pharmacia Biotechnology, Inc. Activated calf thymus DNA was prepared as described (4). M13 and dXI74 ssDNAs were prepared as described (5, 6). All DNA concentrations are expressed as moles of nucleotide unless otherwise indicated. Synthetic 15-mer primers for M13 (map position 1376–1361) and dXI74 (map positions 2808–2793, 887–872, 4792–4777, and 4046–4031) DNAs were kindly provided by M. O’Donnell (of this department) (7). The primer was annealed to the DNA using a 10:1 molar ratio of primer to template. The components were heated to 65 °C in a buffer consisting of 50 mM Tris (pH 8.0) and 25 mM MgCl2 for 10 min. They were then allowed to cool slowly to room temperature.

Enzymes—D. melanogaster polymerase-primase was purified as described (1). A complex of the 182- and 73-kDa polypeptides generated from the polymerase-primase by dissociation of the 60- and 50-kDa subunits from the intact enzyme in the course of purification was a kind gift of L. Kaguni (Michigan State University). Escherichia coli DNA polymerase I and E. coli SSB were kindly provided by J. Kelly and D. Julin, respectively (of this department).

Methods

DNA Polymerase Assay—Standard reaction mixtures (50 µl) contained 25 mM Tris-HCl (pH 8.5), 10 mM MgCl2, 50 µg/ml bovine serum albumin, 2 mM diethiothreitol, 200 µg/ml activated DNA, 100 µM unlabelled dNTPs, 10 µM [32P]dNTPs (either dATP or dCTP), and 0.25–0.5 units of enzyme. Singly or multi-primed ssDNAs were added at the concentrations given in the figure legends. Incubation was at 25 °C. All rates were determined from a time course of the appearance of acid-insoluble radioactivity (1).

DNA Primase Assay—Primase activity was assayed by coupling oligoribonucleotide synthesis to polymerization of dNTPs by E. coli DNA polymerase I (1). The rate of the reaction was determined by monitoring the incorporation of labeled dNTPs into acid-insoluble material. Reaction mixtures (50 µl) contained 10 mM Tris-HCl (pH 8.5), 5 mM MgCl2, 1 mM diethiothreitol, 10 µg/ml poly(dT) or poly(dC-T), 0.25–0.5 units of primase, and 0.6 unit of DNA polymerase I. ATP (poly(dT) template) or ATP and GTP (poly(dC-T) template) were present at 250 µM. [32P]dATP was added at 100 µM with the poly(dC-T) template; unlabeled dGTP was also added at the same concentration. Incubations were for 20 or 40 min at 25 °C.

Analysis by Gel Electrophoresis of Products of DNA Synthesis—The products to be analyzed were precipitated with ethanol (10 µg of salmon sperm DNA added as carrier) to remove unincorporated nucleotides, and the precipitates were resuspended in deionized formamide containing 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue. The samples were heated to 100 °C for 2–5 min and then loaded onto the appropriate gel (see figure legends).

Sequencing Gels—6% gradient gels (200 × 500 × 0.38 mm) containing 7 µmol urea were prepared as described (8). The gels were run at 1500 V for 2–3 h and then washed in 10% acetic acid, 10% methanol for 20 min to remove urea and fix the samples. The samples were then dried under vacuum. Products were visualized by exposing the gel to Kodak X-AR5 film at −70 °C using a Du Pont-New England Nuclear Cronex Lightning Plus intensifying screen for an appropriate length of time.

Polyacrylamide Slab Gels—160 × 270 × 1.5-mm slab gels (8%) contained 90 mM Tris-HCl (pH 8.3), 2.5 mM EDTA, and 7 µg urea. The gels were run at 300 mV for 3 h. Autoradiography was performed

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1 The abbreviations used are: ssDNA, single-stranded DNA; SSB, E. coli single-stranded DNA-binding protein; dNTPs, deoxyribonucleoside triphosphates; NTPs, ribonucleoside triphosphates.
as described above, without prior drying of the gel. The procedures for polyacrylamide gel electrophoresis and staining of polymerase-primase subunits were as published (9).

**Alkaline Agarose Gel Electrophoresis**—2% agarose gels containing 50 mM NaOH, 2 mM EDTA were run at 75 mA for 16 h. After electrophoresis, the gels were washed in 90 mM Tris borate (pH 8.3), 2.5 mM EDTA for 30 min to remove the alkali and then dried and autoradiographed as described above.

**Subunit Separation**—DNA polymerase-primase (1200 units of polymerase in 0.05 ml) was added to a 0–10% glycerol gradient containing 50% ethylene glycol, 20 mM potassium phosphate (pH 7.5), 2 mM dithiothreitol, 1 mM EDTA, and 20 mM ammonium sulfate. Centrifugation was performed at 4 °C for 44 h at 60,000 rpm in a Beckman SW 60 rotor, 64 h at 50,000 rpm in a Beckman SW 56 rotor, or 78 h at 40,000 rpm in a Beckman SW 41 rotor. Gradient fractions were collected in siliconized Eppendorf tubes.

**RESULTS**

**Dissociation of Polymerase-Primase Subunits**

Sedimentation of the *Drosophila* polymerase-primase through a 0–10% glycerol gradient containing 50% ethylene glycol resulted in a clean separation of polymerase and primase activities (Fig. 1A). Although several peptides were visible in the DNA polymerase peak, only the 182-kDa polypeptide cosedimented with polymerase activity. The primase activity sedimented more slowly than the polymerase at a position corresponding to a protein with molecular size of 100–120 kDa; it consisted principally of the 50- and 60-kDa subunits, which were not separated (Fig. 1B). The 73-kDa subunit sedimented at a position consistent with its molecular size. Further separation of the 60/50-kDa polypeptides from the 73-kDa subunit could be achieved by longer periods of centrifugation; however, this resulted in pelleting of the 182-kDa subunit. These findings confirm earlier results in which the subunits were separated with urea as the dissociating agent (2). The use of ethylene glycol, however, consistently resulted in a high yield of both polymerase and primase activity (>40%) in contrast to the urea treatment, which gave only a 4% recovery of the polymerase and a 10% recovery of primase.

**Characterization of Isolated Polymerase Subunit**

With activated DNA as primer-template, the basic properties of the polymerase activity associated with the isolated 182-kDa subunit were not significantly different from those of the intact enzyme (Table I). The sensitivity of the two forms of the enzyme to ionic strength was the same, as were the *Km* values for both dNTPs and DNA. Neither form could use an RNA template or NTPs in place of dNTPs; neither was significantly stimulated by ATP. In addition, both the 182-kDa subunit and the intact enzyme showed the same pattern of inhibition by aphidicolin. The only significant difference observed was the increased sensitivity of the isolated 182-kDa subunit to heat inactivation. After preincuba-

**TABLE I**

Comparison of properties of intact *Drosophila* polymerase-primase with the isolated polymerase (182 kDa) subunit

| Parameter | Intact enzyme | Isolated 182-kDa subunit |
|-----------|---------------|------------------------|
| NaCl dependence (mM) | 0–60 | 0–60 |
| *km*<sub>ATP</sub> (µM) | 4–10 | 4–10 |
| *km*<sub>DNA</sub> (µM) | 1.6 | 4 |
| ATP stimulation | No | No |
| NTP utilization | No | No |
| Aphidicolin sensitivity | | |
| % activity remaining with |  | |
| 10 µg/ml | 30 | 30 |
| 50 µg/ml | 8 | 6 |
| % activity after 30 min at 37 °C | 100 | 18 |

*Activated DNA.*
tion for 30 min at 42 °C, the intact enzyme remained fully active; however, preincubation of the isolated 182-kDa subunit at 37 °C for the same period of time resulted in the loss of 82% of polymerase activity. Both forms of the enzyme were stable to prolonged incubation at 30 °C.

Replication of Singly Primed Templates

The isolated 182-kDa subunit could use both singly and multiprimed φX174 ssDNA templates. The rate of synthesis on the singly primed template was, however, 10-fold less than that observed with activated DNA. A similar difference in rate between the singly primed template and activated DNA was observed with the intact enzyme.

Analysis of Products Synthesized with Singly Primed Templates

Analysis of Pause Sites—Earlier experiments (10) had shown that when Drosophila α-polymerase replicates a singly primed template, a number of discrete bands are observed on a nucleotide sequencing gel. These bands have been interpreted as preferential sites of pausing by the polymerase; and some, but not all, of these can be identified as sites of potential secondary structure (10). Analysis of the pause sites for three different forms of the Drosophila polymerase (the intact enzyme, the combined 182- and 73-kDa subunits, and the 182-kDa subunit) is shown in Fig. 2. Whereas all the pause sites observed for the combined 182- and 73-kDa subunits were the same as those for the intact enzyme, the pattern observed with the 182-kDa subunit alone was different. Some of the pause sites were similar; however, others were absent, and bands appeared at new positions. The appearance of different pause sites for the 182-kDa subunit was observed with several different preparations of singly primed φX174 ssDNA. Computer analysis of DNA secondary structure failed to reveal any significant correlation between structure and pause sites for either form of the enzyme.

Processivity—To determine whether the 73-kDa subunit influences the processivity of the polymerase, the lengths of the products synthesized by the intact enzyme, the 182-kDa subunit, and the combined 182- and 73-kDa subunits on a singly primed M13mp8 ssDNA were determined. Since the template was in large excess (moles of template:moles of enzyme > 20:1), a large proportion (>95%) of the template remained unused at early time points. The lengths of the products should therefore correspond to a single elongation event and reflect the processivity of the enzyme. The results of such an experiment are shown in Fig. 3. The average length of the product in each case was calculated by scanning the gels. Comparison of the results from such an analysis showed that the processivities of all three forms of the enzyme were similar. At the earliest time points, the average length of the products was 20–30 nucleotides, in agreement with the value for the processivity of the α-polymerase reported earlier (11).

Effect of E. coli SSB on Replication of Singly Primed ssDNA—Addition of E. coli SSB to singly primed M13mp8 ssDNA did not affect the rate of replication observed with the intact enzyme (Fig. 4A). The lengths of the products synthesized were also unchanged (Fig. 5A). However, addition of SSB did alter the pattern of pause sites (Fig. 2D). As was observed in the absence of SSB, the complex of 182- and 73-kDa subunits behaved identically to the intact enzyme (Figs. 4B, 5B, and 2E). In contrast, the reaction with the 182-kDa subunit alone was significantly altered by adding SSB. DNA synthesis was stimulated 3-fold as measured by nucleotide incorporation (Fig. 4C), and relatively long products were synthesized that approached full length even at very early time points (Fig. 5C). A more detailed analysis of the products (Fig. 2F) revealed that almost all of the pause sites had been abolished under these conditions. The stimulation reached saturation at a ratio of template to SSB of 8–10:1, a value comparable to that required to saturate the M13mp8 ssDNA template (12).

DISCUSSION

Ethylene glycol dissociates multisubunit proteins by virtue of its detergent-like properties. It was first used as a disso-
were as described for Fig. 2 except that 0.3 pmol of M13mp8 ssDNA was annealed to primer pairs. Processivity was calculated by scanning several independent runs. The numbers to the left refer to number of base pairs. Processivity was calculated by scanning several independent runs.

Fig. 3. Polyacrylamide gel electrophoresis of products of replication of singly primed M13mp8 ssDNA by the intact polymerase-primase, the complex of 182- and 73-kDa subunits, and the isolated 182-kDa subunit. Reaction conditions were as described for Fig. 2 except that 0.3 pmol of M13mp8 ssDNA and 8% polyacrylamide slab gels were run as described under "Methods." The numbers to the left refer to number of base pairs. Processivity was calculated by scanning several independent runs.

Fig. 4. Effect of E. coli SSB on replication of singly primed circular M13mp8 ssDNA by the intact polymerase-primase (A), the complex of 182- and 73-kDa subunits (B), and the isolated 182-kDa subunit (C). Reaction conditions were as described for Fig. 2 except that 0.05 unit of enzyme was used. SSB was present at a ratio of 8:1 nucleotides:SSB monomer. O, SSB added; ●, no SSB.

SSB as dissociating agent for α-polymerase by Suzuki et al. (13) who showed that it was capable of separating the polymerase and primase activities in a subspecies of mouse polymerase-primase. Treatment with 50% ethylene glycol dissociated the polymerase-primase from Drosophila into three fractions, one consisting principally of the 182-kDa subunit, a second containing mainly the 73-kDa subunit, and the third containing a complex of the 50- and 60-kDa subunits. Analysis of the fractions revealed that polymerase activity is associated with the 182-kDa subunit and primase activity with the complex of the 50- and 60-kDa subunits. This finding is in agreement with the observations made earlier using area to dissociate the subunits (2). However, the yields of both polymerase and primase activity with ethylene glycol as a dissociating agent are considerably higher than that obtained with urea. The designation of the 182-kDa subunit as the polymerase and the 50- and/or 60-kDa subunits as the primase is also consistent with other eukaryotic polymerase-primases (3). Earlier stud-

Fig. 5. Effect of E. coli SSB on the processivity of polymerization by the intact polymerase-primase (A), the complex of 182- and 73-kDa subunits (B), and the isolated 182-kDa subunit (C). Reaction conditions were as described for Fig. 4. Samples were taken at 15, 45, and 60 min (see Fig. 4). SSB was present at a ratio of 8:1 nucleotides:SSB monomer. Alkaline agarose gel electrophoresis was performed as described under "Methods." Full-length products appear in the upper quadrant of the gel.

ies (14) with the Drosophila DNA polymerase-primase had suggested that urea dissociation resulted in a large loss of polymerase activity with singly primed ssDNA templates, in contrast to our current observation. The earlier observations were made with an enzyme in which the polymerase subunit had sustained some proteolysis (from 182 to 148 kDa). Possibly, the loss of activity resulted from removal of a portion of the polymerase subunit, rather than any of the other subunits. The removal of the 50- and 60-kDa subunits (i.e. the primase) had no effect on any of the properties of the polymerase examined. Removal of the 73-kDa subunit in addition to the 60- and 50-kDa subunits also left most of the properties of the polymerase unaffected. However, the alterations observed in the pattern of pause sites suggests that the 73-kDa subunit does affect the interaction of the polymerase with the ssDNA template. Experiments carried out with a singly primed M13mp7 circular ssDNA containing a relatively stable hairpin showed that the 182-kDa subunit can approach the hairpin 5 bases closer than either the intact enzyme or the enzyme lacking the 50- and 60-kDa subunits. Some of the differences observed may therefore be a consequence of a change in the apparent dimensions of the polymerase on the template after the removal of the 73-kDa subunit. However, the other factors must also be involved since the positions of several of the pause sites were substantially altered or even entirely removed.

The only effect of SSB on the intact enzyme and the complex composed of the 182- and 73-kDa subunits is to alter the pause sites. This is not unexpected since SSB interacts with the ssDNA template and might therefore be expected to alter its conformation. Such a change might, in turn, affect the manner in which the template is recognized by the polymerase. On the other hand, SSB appears to convert the 182-kDa subunit to a highly processive form of the enzyme that synthesizes very long products with almost no pause sites. The significance and mechanism of this effect are presently unclear. However, the SSB dependence does parallel the binding of SSB to DNA (12). It is therefore likely that the SSB dependence of the assays for "Methods." The numbers to the left refer to number of base pairs. Processivity was calculated by scanning several independent runs.

Fig. 3. Polyacrylamide gel electrophoresis of products of replication of singly primed M13mp8 circular ssDNA by the intact polymerase-primase, the complex of 182- and 73-kDa subunits, and the isolated 182-kDa subunit. Reaction conditions were as described for Fig. 2 except that 0.3 pmol of M13mp8 ssDNA annealed to primer 3 (see "Materials") was used in each case, and samples were taken at the times indicated. A, isolated 182-kDa subunit; B, intact polymerase-primase; C, complex of 182- and 73-kDa subunits. 8% polyacrylamide slab gels were run as described under "Methods." The numbers to the left refer to number of base pairs. Processivity was calculated by scanning several independent runs.

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stimulation is due to an interaction of the 182-kDa subunit with the ssDNA-SSB complex rather than to a direct effect of SSB on the 182-kDa subunit.

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