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# Mosquito DNA polymerase ε

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The mosquito homolog of mammalian DNA polymerase ε, formerly known as a proliferating cell nuclear antigen (PCNA)-independent form of DNA polymerase δ, has been purified from mosquito larval extracts. The polymerase ε was separated from DNA polymerase α by chromatography on hydroxylapatite, and the enzyme was subsequently purified on single-stranded DNA agarose, followed by a 5′ AMP-agarose chromatography step. The purified polymerase exhibits an intrinsic 3′-5′ exonuclease activity and shows high activity using an oligo-primed DNA template. Neither human nor *Drosophila* PCNA stimulated this polymerase activity. Additional immunochemical and biochemical evidence indicates that this enzyme is distinct from DNA polymerase α.

DNA polymerase ε; 3′-5′ Exonuclease; PCNA; Mosquito; *Aedes aegypti*

## 1. INTRODUCTION

Three eukaryotic DNA polymerases have been identified and characterized from yeast and mammalian cells [1,2]. They are DNA polymerase α, PCNA-dependent δ, and now ε (formerly called PCNA-independent DNA polymerase δ).

DNA polymerase ε is a structurally distinct enzyme. It has an associated 3′-5′ exonuclease activity and exhibits an inherently high processivity and an accurate exonucleolytic proofreading ability [1,2]. The genes encoding this polymerase are essential for yeast cell growth and Morrison et al. [3] proposed a model whereby DNA polymerase ε is required for leading strand synthesis while DNA polymerase α and δ are responsible for lagging strand synthesis. This model differs from another model [4] which proposes that DNA polymerase δ functions as the leading strand polymerase.

In contrast to yeast and mammalian cells, DNA polymerase α (purified as the polymerase α/primase complex) from *Drosophila melanogaster* embryos [5] is believed to be solely responsible for chromosomal DNA replication in this organism [6]. The results reported herein describe the isolation of a homolog of mammalian DNA polymerase ε from mosquito larval extracts using an oligo-primed DNA substrate. Thus, for the first time, DNA polymerase ε has been shown to exist in insect tissues, although its functional role in insects is uncertain at this time.

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## 2. MATERIALS AND METHODS

### 2.1. *Preparation of mosquito crude extracts*

Mosquito rearing conditions are described elsewhere [7]. Three-day-old *Aedes aegypti* larvae were collected and extensively washed with distilled water. The wet larvae (210 g) were homogenized and prepared as described in [5].

### 2.2. *Purification of mosquito DNA polymerase ε*

Frozen S-100 (Fraction I) was thawed in a 37°C water bath and loaded onto a phosphocellulose column (2.5 × 21 cm) equilibrated with Buffer A ((40 mM potassium phosphate pH 7.2, 10% glycerol, 5 mM β-mercaptoethanol, and protease inhibitor cocktail (10 mM benzamidine-HCl, 1 mM PMSF, 10 mM sodium metabisulfite, leupeptin at 2 μg/ml, pepstatin at 1 μg/ml, and aprotinin at 2 μg/ml)). The column was washed with ten column volumes of Buffer A and eluted with 1 l linear gradient from 0 to 0.6 M KCl in buffer A. The active fractions were pooled (Fraction II) and precipitated with ammonium sulfate (0.313 g/ml). The protein pellet was collected by centrifugation at 32,000 × *g* for 45 min. The precipitated protein was dissolved in 15 ml of Buffer B, followed by dialysis against Buffer B (50 mM Tris-HCl, pH 8.5, 10% glycerol, 5 mM β-mercaptoethanol, and the protease inhibitor cocktail) (Fraction III). Fraction III was loaded on a DEAE-Spectra gel column (1.5 × 13 cm) equilibrated with Buffer B and the column was eluted with 200 ml linear gradient from 0 to 0.6 M KCl in Buffer B. The pooled polymerase fractions were dialyzed against Buffer C (20 mM potassium phosphate, pH 7.2, 10% glycerol, 5 mM β-mercaptoethanol, and the protease cocktail) (Fraction IV) and loaded on a hydroxylapatite column (1.5 × 12 cm) equilibrated with Buffer C. The column was eluted with 200 ml linear gradient from 0.02 to 0.4 M potassium phosphate, pH 7.2. During this step, DNA polymerase ε was separated from polymerase α. The pooled polymerase ε fractions (Fraction V) were dialyzed against Buffer C overnight. Fraction V was loaded onto a single-stranded DNA agarose column (0.9 × 3 cm) equilibrated with Buffer A and was eluted with 20 ml linear gradient from 0 to 0.5 M KCl in Buffer A. The active fractions were dialyzed against Buffer D (25 mM HEPES-KOH, pH 7.0, 20% glycerol, 2 mM $MgCl_2$, 1 mM DTT, and the protease inhibitor cocktail) (Fraction VI). Half of Fraction VI was then loaded onto a 5′ AMP-agarose column (0.6 × 2.5 cm) equilibrated with Buffer D and the column was washed with 5 ml of Buffer D containing 0.5 M KCl. The polymerase ε fractions (Fraction VII) were divided into aliquots and kept under liquid nitrogen until use.

2.3. *Assays of DNA polymerase, primase, and 3′-5′ exonuclease activity*

(1) Poly(dA) · $(dT)_{12-18}$: reaction mixture (50 μl) consisted of 50 mM bis-Tris, pH 6.5, 5 mM β-mercaptoethanol, 8 mM $MgCl_2$, 200 μg of BSA, 50 mM KCl, 50 μM [$^3$H]dTTP(5,000–6,000 cpm/pmol), 0.5 μg poly(dA) · $(dT)_{12-18}$, and enzyme to be tested. (2) Activated calf thymus DNA: reaction mixture (50 μl) consisted of 50 mM Tris-HCl, pH 8.5, 5 mM β-mercaptoethanol, 8 mM $MgCl_2$, 200 μg of bovine serum albumin (BSA), 25 μg of activated calf thymus DNA, 100 μM dATP, dGTP, dCTP, and 50 μM [$^3$H]dTTP, and 5–10 μl of enzyme. The DNA primase assay was performed as described in Conaway et al. [8] and the 3′-5′ exonuclease activity was assayed as described in [9].

## 3. RESULTS AND DISCUSSION

During purification procedures, we have reproducibly found that a homolog of mammalian DNA polymerase ε was separated from DNA polymerase α by chromatography on hydroxylapatite. As shown in Figure 1, two different DNA polymerase peaks were detected using an activated calf thymus DNA template. The first peak showed a preference towards an oligo-primed poly(dA) as template. To further verify the existence of a homolog of mammalian DNA polymerase ε in the hydroxylapatite fractions, we have re-examined DNA polymerase activity in the presence of butyl-phenyl dGTP. This inhibitor is known to strongly inhibit DNA polymerase α, but not the δ or ε forms [1]. Fig. 1 shows that only the first peak (fractions 11–15) was resistant to this inhibitor at 100 μM concentration (25% inhibition). The high polymerase activity on an oligo-primed poly(dA) and its relative resistance to butyl-phenyl dGTP is consistent with the properties of known DNA polymerase ε.

To confirm further that the isolated polymerase (Fraction A, Fig. 1) is not derived from the α form, we carefully examined the hydroxylapatite fractions in several ways. First, we examined the chromatographic behavior of the polymerase on 5′ AMP-agarose. Previously, it was demonstrated that only DNA polymerases containing an intrinsic 3′-5′ exonuclease activity can

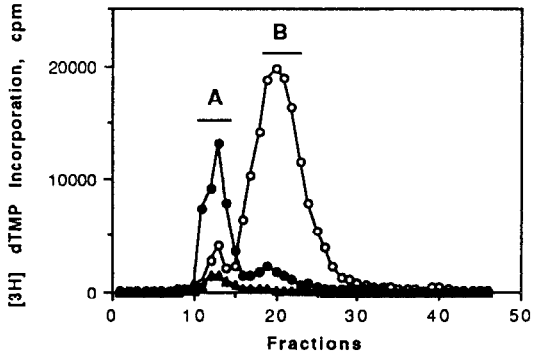


Fig. 1. Separation of DNA polymerase ε (A) from DNA polymerase α (B) on hydroxylapatite chromatography. Aliquots of column fractions were assayed for DNA polymerase activity using poly (dA) · $(dT)_{12-18}$ (●) and activated calf thymus DNA (○) as templates. Duplicated polymerase assays using an activated calf thymus DNA were performed in the presence of 100 μM of butylphenyl dGTP (▲).

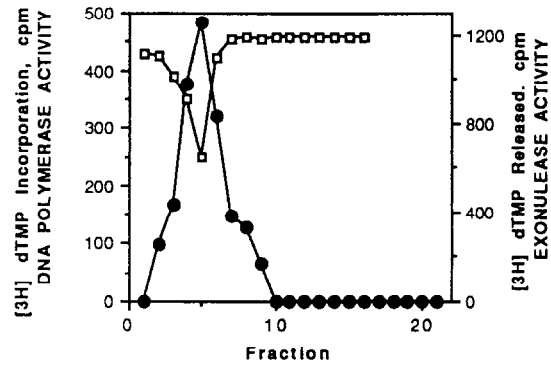


Fig. 2. 5′ AMP-agarose chromatography of mosquito polymerase ε. Standard polymerase assays using poly(dA) · $(dT)_{12-18}$ (●) were performed, and 3′-5′ exonuclease activity (□) was determined by degradation of $(dT)_{16}$-[$^3$H]$dT_5$.

interact with AMP during 5′ AMP-agarose chromatography [10]. For this experiment, the hydroxylapatite fractions were further purified on single-stranded DNA agarose and then on a small 5′ AMP-agarose column. Fig. 2 shows that DNA polymerase ε activity co-purified with 3′-5′ exonuclease activity during chromatography on 5′ AMP-agarose. Moreover, it should be noted that no polymerase activity was detected in the flowthrough fractions (data not shown), indicating that DNA polymerase α was removed in the mosquito polymerase ε preparation.

More definitively, our discovery of a homolog of mammalian DNA polymerase ε was confirmed by immunoblotting with anti-insect DNA polymerase α antibodies. According to our immunoblot results, neither anti-*Drosophila* nor anti-mosquito DNA polymerase α crossreacts with the mosquito DNA polymerase ε fractions from the hydroxylapatite, the single-stranded DNA agarose, or the 5′-AMP agarose step (data not shown). In addition, the mosquito DNA polymerase ε fractions contain no primase activity, and the enzyme activity was highly sensitive to Aphidicolin, indicating that it is not β- or γ-polymerase (Table I). To ensure that the high processivity of the mosquito polymerase ε was not due to contaminating PCNA in the preparations, we have used monoclonal anti-PCNA antibody to detect mosquito PCNA on Western blots. It was observed that mosquito PCNA was completely removed from the polymerase fractions during the phosphocellulose step (data not shown). To verify further the inherent high processivity of the mosquito DNA polymerase ε, we tested whether PCNA can stimulate the DNA polymerase activity. Table I shows that the mosquito DNA polymerase activity was not stimulated by either human or *Drosophila* PCNA. Our results here further indicate that the purified mosquito polymerase is a PCNA-independent DNA polymerase δ, now referred to as ε.

In conclusion, we have isolated a homolog of mammalian DNA polymerase ε from the mosquito larval

Table I

Effect of various components on mosquito DNA polymerase ε.

| Components added | Relative activity (%) | |
|---|---|---|
| | Oligo (dT)/ poly(dA) | Activated calf thymus DNA |
| + 50 μM BuPdGTP | | 71 |
| + 50 mg/ml Aphidicolin | | 5 |
| + 100 ng human PCNA | 95 | |
| + 500 ng human PCNA | 76 | |
| + 100 ng *Drosophila* PCNA | 76 | |

extracts by conventional chromatography methods. To date, this is the first report that the DNA polymerase ε exists in any insect tissue. The purified mosquito polymerase ε is distinguishable from DNA polymerase α by the following distinct biochemical properties: (1) an intrinsic 3′-5′ exonuclease activity, (2) relative resistance to butylphenyl dGTP, (3) high activity on oligo-primed DNA template, (4) no primase activity, (5) interaction with 5′ AMP agarose, and (6) no crossreactivity with anti-DNA polymerase α antibodies. Our finding that DNA polymerase ε exists in mosquito larval extracts raises the possibility that it is present in other insects as well. In addition, we do not exclude the further possibility that insects may also possess PCNA-dependent DNA polymerase δ, thus demonstrating a consistent pattern in eukaryotes. In any event it is clear that further studies are needed in order to elucidate how these various forms of DNA polymerases interact in order to successfully replicate eukaryotic genomes.

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