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Isolation and Characterization of DNA Polymerase ε from the Silk Glands of *Bombyx mori**

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The silk gland of *Bombyx mori*, an endomitotically replicative tissue shows high levels of DNA polymerases α, δ, and ε activities. The ratio of polymerase α to that of δ plus ε is maintained at 1.1 to 1.3 in both the posterior and middle silk glands for the entire duration of late larval development. The three activities copurify in the initial stages of fractionation through phosphocellulose and DE52 but polymerase α gets resolved from the others on hydroxylapatite column. Separation between polymerase δ and ε is achieved by chromatography on QAE-Sephadex. DNA polymerase ε is a heterodimer comprising of 215- and 42-kDa subunits. The activity is maximum at pH 6.5 and the K_m values for dNTPs vary between 3–9 μM. The enzyme possesses an intrinsically associated exonuclease activity which functions in the mismatch repair during DNA synthesis. Both polymerase and 3′ → 5′ exonuclease activities are associated with the 215-kDa subunit. By itself, DNA polymerase ε is processive and the catalytic activity is not enhanced by externally added bPCNA (*Bombyx*-proliferating cell nuclear antigen, an auxiliary protein for DNA polymerase δ). The enzyme resembles polymerase δ in having the exonuclease activity and in its response to aphidicolin or substrate analogs, but could be distinguished from the latter by its lack of response to the bPCNA and sensitivity to dimethyl sulfoxide. The two enzymes show partial immunological cross-reactivity with each other but no immunological relatedness to polymerase α. The absence of the repair enzyme DNA polymerase β and the presence of substantial levels of polymerase ε in the silk glands suggest a possible role for the latter in DNA repair in that tissue.

In eukaryotic cells, DNA polymerases α and δ have been implicated in the DNA replication and polymerase β in the DNA repair processes. Another essential polymerase, DNA polymerase ε has been recently identified, as an analog of DNA polymerase II from *Saccharomyces cerevisiae* (Morrison *et al.*, 1991). Polymerase ε was previously classified as polymerase δ because of the intrinsically associated 3′ → 5′ exonuclease activity. However, the enzyme is now established to be distinct structurally (Syvaoja, 1990) and is therefore reclassified as DNA polymerase ε (Burgers *et al.*, 1990). This activity has been purified from calf thymus (Wahl *et al.*, 1986; Focher *et al.*, 1988), human placenta (Lee and Toomey, 1987), and HeLa cells (Nishida *et al.*, 1988; Syvaoja and Linn, 1989). In HeLa cells, the enzyme was identified as a factor required for DNA repair synthesis in ultraviolet-irradiated and -permeabilized cells. Although polymerase ε activity has been demonstrated in a few of the mammalian cell lines and tissues, so far it has not been reported from any insect systems.

DNA polymerase ε differs from polymerase α and β by the intrinsic association of a 3′ → 5′ exonuclease activity but resembles polymerase δ. It differs from the latter, however, in being highly processive in the absence of PCNA.[1] The enzyme was therefore, designated earlier as PCNA independent DNA polymerase δ (Sabatino, 1988; Focher *et al.*, 1989; Syvaoja and Linn, 1989). Although, polymerase ε has been implicated in both replication and repair, the exact function of the enzyme is yet to be established.

The silk gland cells of the mulberry silkworm *Bombyx mori* contain large quantities of DNA as a result of endomitosis. In this terminally differentiated tissue, the DNA replication continues throughout the larval development for 18–19 rounds, without cell or nuclear division. As a consequence the silk gland cells become gigantic, with substantial volume of cells being occupied by nuclear material. The highly ramified nuclei are filled with DNA amounting to 300,000 times the haploid genomic content toward the late stages of the larval development (Gage, 1974; Niranjanakumari and Gopinathan, 1991). High levels of polymerase α and δ activities are seen in this tissue (Niranjanakumari and Gopinathan, 1991, 1992), but DNA polymerase β, the enzyme traditionally considered to be involved in DNA repair process could not be detected (Niranjanakumari and Gopinathan, 1993). Nevertheless, the presence of a DNA polymerase activity which satisfied the criteria to be designated as DNA polymerase ε was noticed during the purification of DNA polymerase δ. The present study reports the purification and characterization of DNA polymerase ε from the highly replicative cells of the silk glands of *B. mori*.

MATERIALS AND METHODS

The restriction enzymes, NTPs, dNTPs, Sephadex G-50, phenyl-Sepharose, and QAE-Sephadex were from Pharmacia (Sweden). Ra-

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[1] The abbreviations used are: PCNA, proliferating cell nuclear antigen; bPCNA, Bombyx-proliferating cell nuclear antigen; BSA, bovine serum albumin; BuAndATP, butyl anilino-dATP; BuPhdGTP, butyl phenyl-dGTP; CT DNA, calf thymus DNA; DTT, dithiothreitol; MSG, middle silk gland; PMSF, phenylmethylsulfonyl fluoride; PolIk, Klenow fragment of DNA polymerase I; PSG, posterior silk gland; ssDNA, single-stranded DNA; RF A, C, replication factors, A or C; PAGE, polyacrylamide gel electrophoresis.

diolabeled nucleotides [^{3}H]TTP, [α-^{32}P]dATP, and [γ-^{32}P]ATP were from Amersham (United Kingdom) or Bhabha Atomic Research Center (India). Phosphocellulose (P-11) and DEAE-cellulose (DE52) were from Whatman. Bio-Gel P-200 was from Bio-Rad. Hydroxylapatite was from Boehringer Mannheim (GmbH). Most of the other biochemicals and reagents such as Tris, dithiothreitol (DTT), sodium dodecyl sulfate (SDS), EDTA, EGTA, calf thymus DNA (CT DNA), phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, bovine serum albumin (BSA) etc., were from Sigma. BuPhdGTP and BuAndATP were gifts from Dr. George Wright, University of Massachusetts.

Protein and Enzyme Assays—Protein contents were determined by the dye-binding method (Bradford, 1976) or by the Folin-Ciocalteau method (Lowry *et al.*, 1951).

DNA polymerase ε activity was routinely monitored by the synthesis of DNA in the absence of bPCNA, using activated calf thymus DNA as template primer. The assay system contained in a final volume of 50 μl, 50 mM Tris-HCl, pH 6.5, 5 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 2% glycerol, 100 μg/ml BSA, 100 μM each of dATP, dCTP, dGTP, and 50 μM [^{3}H]TTP (specific activity 100 counts/min/pmol), 200 μg/ml activated CT DNA, and the enzyme. The reactions were incubated at 37 °C for 30 min, and the acid precipitable radioactivity was determined. One unit of DNA polymerase activity catalyzes the incorporation of 1 nmol of [^{3}H]TMP into DNA in 1 h, at 37 °C. The assay for DNA polymerase δ activity was similar but was done in the presence of bPCNA. The assay for DNA polymerase α was done at pH 8.5. For the determination of K_m for dNTPs, the enzyme assays were carried out in the presence of varying concentrations of one dXTP at a time while the concentrations of the other three were maintained at 50 μM.

3′ → 5′ Exonuclease was assayed based on the release of radioactivity from 3′-^{32}P end-labeled DNA. For this purpose, plasmid pUC18 DNA was linearized with *Eco*RI and was end filled using [α-^{32}P] dATP and the Klenow fragment of DNA polymerase I (PolIk). Alternatively, poly(dA)·[^{3}H]poly(dT), prepared by the extension of poly(dA)·oligo$(dT)_{12-18}$ using [^{3}H]TTP and PolIk, was also used as substrate for the exonuclease assay.

Proofreading activity was monitored by the hydrolysis of the mispaired 3′ terminus from a template-primer complex and the subsequent extension of the primer. A synthetic oligonucleotide (17-mer) complementary to a sequence (3′-6310 to 6326–5′) in phage M13mp18 DNA except for one mismatch at the 3′ end, was labeled using T_4 polynucleotide kinase and [γ-^{32}P]ATP. This 5′ end-labeled primer was annealed to the M13mp18 single-stranded DNA (ssDNA) to produce a duplex containing a C-A mispair at the 3′-primer terminus. The elongation of the primer takes place only if the mismatch is hydrolyzed. For the chain elongation, the reaction mixture (20 μl) contained 20 mM Tris-HCl, pH 6.5, 1 mM $MgCl_2$, 100 μg/ml BSA, 2% glycerol, 20 μM each of dATP, dGTP, and dTTP, the template-primer, and 1 unit of DNA polymerase ε. The primer should be extended by eight nucleotides (one 3′-terminal mismatch removed and nine fresh nucleotides added) since the chain elongation was carried out in the absence of dCTP. The product was analyzed on a 20% polyacrylamide gel and located by autoradiography.

Processivity assay for DNA polymerase ε was performed as described earlier for polymerase δ (Niranjanakumari and Gopinathan, 1992). Briefly, the DNA chain elongation on M13mp18 ssDNA was carried out by using labeled or unlabeled 17-mer primer of the same sequence as in the proofreading assay but for the 3′ mismatch, and in the presence or absence of bPCNA. The chain elongation conditions were also similar to those described in the proofreading assay except that all the four dNTPs were present. After the reaction, the samples were deproteinized and analyzed for the primer extension by electrophoresis on urea-polyacrylamide gels. Alternately, the newly synthesized DNA was digested with restriction enzymes *Rsa*I, *Hae*III, and *Taq*I, the sites for which should be generated due to the chain elongation progressing past the corresponding complementary sequences on the template DNA, and the digests were analyzed on 8% urea-PAGE.

Buffers Used in Polymerase Purification—Buffer A was composed of: 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 250 mM sucrose, 10% glycerol, 0.01% Nonidet P-40, 1 mM DTT, 0.1 mM PMSF, 10 mM sodium metabisulfite, 0.5 μg/ml leupeptin, and 1 μg/ml aprotinin.

Buffer B was composed of: 20 mM potassium phosphate, pH 7.0, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, and 10 mM sodium metabisulfite.

Purification of DNA Polymerase ε—The cell extracts were prepared from the silk glands of *B. mori* (freshly dissected or kept frozen at −70 °C for up to 6 months) by gentle homogenization (Dounce) in buffer A. Nuclei were removed by low speed centrifugation, and the supernatant was centrifuged at 100,000 × *g* for 1 h at 4 °C in a Beckman Ti70 rotor. The supernatant, designated as the S_{100} cytosolic fraction, was then adjusted to 0.2 M NaCl and loaded onto a phosphocellulose column previously equilibrated in buffer A containing 0.2 M NaCl. The column was washed with the same buffer, the bound proteins were eluted with buffer A containing 1 M NaCl, and assayed for DNA polymerase α, δ, and ε activities. The active fractions were pooled and dialyzed against buffer A containing 20% glycerol, adjusted to 100 mM NaCl, and applied to a DEAE-cellulose column equilibrated with the same buffer. The three enzyme activities were eluted with 1 M NaCl in buffer A, dialyzed against buffer B, and applied to a hydroxylapatite column. The bound proteins were eluted using a linear gradient of 20–500 mM KPO_4 in buffer B. At this stage DNA polymerase α activity separates from the δ and ε activities. The fractions showing δ and ε activities were pooled and dialyzed against buffer A, pH 8.5, and chromatographed on a QAE-Sephadex column using a linear gradient of 25–500 mM NaCl in the above buffer. The activities of DNA polymerase δ and ε were resolved at this step of purification. Fractions showing polymerase ε activity were pooled and dialyzed in buffer A containing 50 mM NaCl and fractionated on a ssDNA-Sepharose column. The enzyme activity was eluted with 0.5 M NaCl in buffer A, concentrated by precipitation with 70% $(NH_4)_2SO_4$, and subjected to gel filtration on a Bio-Gel P-200 column. The enzymatically active fractions were pooled and concentrated by ultrafiltration.

Purification of bPCNA from Silk Glands—A PCNA-like activity from *B. mori* silk glands was purified by us earlier (Niranjanakumari and Gopinathan, 1992). Since the growth of silk gland cells of *B. mori* is not accompanied by cell division in spite of several rounds of DNA replications, we prefer to refer it to as PCNA-like protein and designate it as bPCNA. The bPCNA was isolated from the unbound cytosolic fraction emerging from the phosphocellulose column during the purification of DNA polymerases. This fraction was chromatographed subsequently on DE52, phenyl-Sepharose, QAE-Sephadex, and Sephadex G-75 to yield an electrophoretically homogeneous fraction (Niranjanakumari and Gopinathan, 1992). The identity of the bPCNA was established by its ability to enhance the catalytic activity and processivity of DNA polymerase δ.

RESULTS

DNA Contents and DNA Polymerase Activities of Silk Gland Cells—In *B. mori*, the silk gland is a terminally differentiated tissue which is anatomically and functionally divided into three distinct parts as the anterior-, middle-, and posterior silk glands (anterior silk gland, MSG, and PSG, respectively). The PSG is made of 520 cells and MSG by 255 cells, and the cell numbers do not increase as a function of development. However, the DNA contents of these cells increase continuously with the progress in development amounting to 18–19 rounds of replications for the entire larval development period. Concomitant with the DNA content, the activities of DNA polymerase α and (δ + ε) also increase (Table I). The ratio of polymerase α to that of δ and ε is constantly maintained at the level of 1.1–1.3 in both PSG and MSG during the entire fourth and fifth instars of larval development. However, no DNA polymerase β activity could be detected in the silk glands. Of the total activities of δ + ε, nearly 10–15% of the activity was due to polymerase ε.

Purification of DNA Polymerase ε—The enzyme purified in the present study satisfied the criteria to be designated as DNA polymerase ε (see later sections) and is referred to as DNA polymerase ε hereafter. The purification strategy for DNA polymerase ε is presented schematically in Fig. 1. The activities of DNA polymerase α, δ, and ε copurify in the initial stages of purification, *viz.* phosphocellulose (P-11) and DE52 columns. However, polymerase α separates from polymerase δ and ε at the step of hydroxylapatite chromatography. The activities of the latter enzymes could be resolved at the stage of QAE-Sephadex A-25 chromatography. DNA polymerase δ,

TABLE I

DNA contents and DNA polymerase activities of silk gland cells

The DNA contents were estimated by the Indole method (Ceriotti, 1952). 1 unit of DNA polymerase (pol) activity corresponds to 1 nmol of [^{3}H]TMP incorporated in 1 h at 37 °C.

Development stage	Posterior silk gland				Middle silk gland			
	DNA content	DNA pol α	DNA pol δ + ϵ[a]	Ratio α/δ + ϵ	DNA content	DNA pol α	DNA pol δ + ϵ	Ratio α/δ + ϵ
	ng/cell	*unit × 10^{-3}/cell*			*ng/cell*	*unit × 10^{-3}/cell*		
4th Instar								
1	4.40	0.35	0.28	1.27	6.39	0.65	0.47	1.36
2	6.26	0.58	0.44	1.32	9.61	0.71	0.53	1.33
3	8.83	1.15	0.92	1.25	12.23	1.76	1.34	1.32
4	12.31	1.44	1.06	1.36	15.69	2.47	1.94	1.28
5	17.31	1.44			20.63	2.35		
5th Instar								
1	34.8	2.94	2.46	1.19	42.4	4.71	3.86	1.22
2	69.2	4.44	3.90	1.14	90.2	6.83	5.76	1.18
3	128.8	8.94	7.60	1.18	109.8	11.65	10.00	1.16
4	188.5	6.58	5.49	1.19	129.4	11.65	9.69	1.20
5	169.2	6.29	5.10	1.23	163.9	11.53	9.37	1.23
6	157.0	5.71	4.56	1.25	140.4	10.12	7.92	1.28

[a] The activity represent both polymerase δ and ϵ activities. Polymerase ϵ activity corresponded to 10–15% of polymerase δ + ϵ activity.

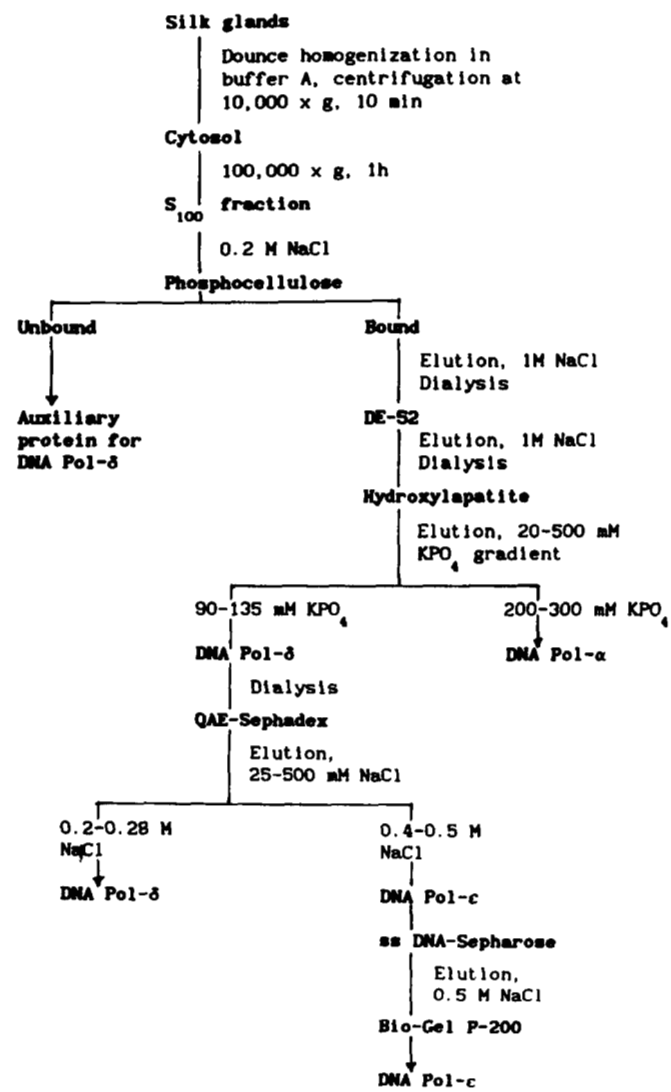

FIG. 1. **Schematic representation of the purification strategy for DNA polymerase-ϵ.**

stimulated by bPCNA was eluted from the column between a salt gradient of 0.2–0.28 M NaCl. The enzyme activity eluting between 0.4–0.5 M NaCl from this column was independent of bPCNA (polymerase ϵ). The final purification of the latter was achieved by affinity chromatography on ssDNA-Sepharose, followed by gel filtration on Bio-Gel P-200 column. The adopted protocol resulted in more than 5000-fold increase in the specific activity over the activity of δ and ϵ taken together in the crude extracts (Table II). The values reported here are the averages of several independent preparations.

Homogeneity and Subunit Structure—The enzyme fraction from the Bio-Gel P-200 column was electrophoretically homogeneous on 7% polyacrylamide gel under nondenaturing condition (Fig. 2*a*).

Electrophoresis on denaturing gels for subunit analysis revealed the presence of two nonidentical subunits of M_r 215,000 and 42,000 (Fig. 2*b*).

Properties of DNA Polymerase ϵ—DNA polymerase ϵ was maximally active at pH 6.5 with about two-thirds of the activity at either 6.3 or 7.0. The enzyme showed absolute requirement for cations, the optimum concentration of $MgCl_2$ and KCl being 5 and 100 mM, respectively. The K_m values for dATP, dGTP, dCTP, and dTTP were found to be 3.0, 4.8, 6.3, and 8.8 μM, respectively.

The template specificity of polymerase ϵ for various DNAs is summarized in Table III. Activated CT DNA and salmon sperm DNA served as good templates while the synthetic template poly(dA)·oligo(dT)$_{12-18}$ was marginally better. The enzyme could also utilize primed M13ssDNA as a template.

Sensitivity to Inhibitors—The effects of compounds such as aphidicolin, BuPhdGTP, BuAndATP, and dimethyl sulfoxide as well as antibodies against DNA polymerase α and δ, which are known to influence the activity of DNA polymerases, were tested on polymerase ϵ activity.

Aphidicolin at 2 μg/ml inhibited the enzyme activity by 80% (Fig. 3*a*). This inhibition was similar to that of polymerase α and δ. The effects of BuPhdGTP and BuAndATP on DNA polymerase ϵ activity are presented in Fig. 3, *b* and *c*. BuPhdGTP and BuAndATP are potent inhibitors of DNA polymerase α, whereas mammalian DNA polymerase δ and ϵ are known to be insensitive. As shown in Fig. 3, *b-c*, BuPhdGTP and BuAndATP did not inhibit DNA polymerase ϵ (up to 10 μM), but DNA polymerase α showed almost 95%

TABLE II

Purification of DNA polymerase ϵ and the associated 3′ → 5′ exonuclease

Fractionation step	Total protein	Polymerase[a]			Exonuclease			Polymerase to exonuclease ratio
		Total activity	Specific activity	Fold purification	Total activity	Specific activity	Fold purification	
	mg	*units*	*units/mg*		*units*	*units/mg*		
Crude extract (S_{100})	1103	386	0.35	1.0	459	0.42	1.00	0.83
Phosphocellulose	376	348	0.93	2.66	186	0.49	1.17	1.90
DEAE-cellulose	219	314	1.43	4.09	144	0.66	1.57	2.16
Hydroxylapatite	30.5	268	8.79	25.1	113.5	3.72	8.86	2.36
QAE-Sephadex	0.043	38	883	2524	19.7	458	1090	1.93
ssDNA-Sepharose	0.023	25.6	1200	3428	12.7	552	1314	2.17
Bio-Gel P-200	0.011	20.5	1863	5324	10.1	916	2180	2.03

[a] Up to the hydroxylapatite column both polymerase δ and ϵ were copurified. At the QAE-Sephadex step, the fractions eluting between 0.4–0.5 M NaCl corresponded to DNA polymerase ϵ activity, which were pooled separately and fractionated.

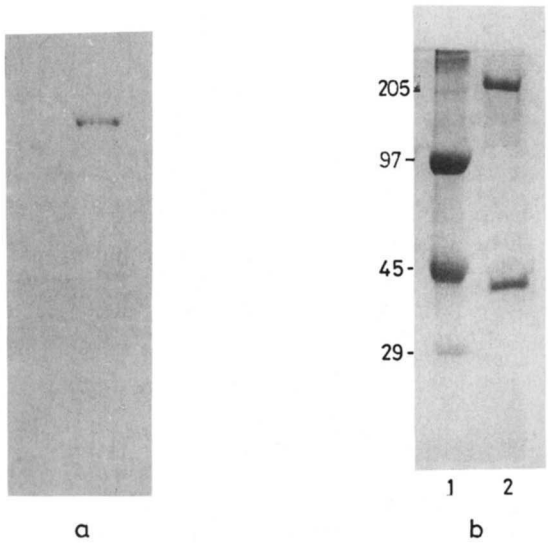

FIG. 2. **Homogeneity and subunit structure of DNA polymerase ϵ from the silk glands.** *Panel a*: PAGE. Purified DNA polymerase ϵ (Bio-Gel P-200 column fraction, 1 μg of protein) was subjected to electrophoresis on 7% polyacrylamide under nondenaturing conditions, and the proteins were visualized by Coomassie Brilliant Blue R-250 staining. *Panel b*, the enzyme samples were treated with β-mercaptoethanol (2.5%) and SDS (1%) and heated at 85 °C for 10 min. Denaturing gel electrophoresis was performed on a 10% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250. *Lane 1*, standard molecular weight protein markers (β-galactosidase, ovalbumin, and carbonic anhydrase). *Lane 2*, DNA polymerase ϵ, Bio-Gel P-200 fraction (2 μg of protein).

TABLE III

Template efficiencies of various DNA

Template DNA	Specific activity
	units/mg
Activated CT DNA	7240[a]
Activated salmon sperm DNA	6820[a]
Poly(dA)·oligo$(dT)_{12-18}$[b]	9485
M13ssDNA with primer	8715[a]

[a] nmol of dTMP incorporated was multiplied by four.
[b] The template poly(dA)·oligo$(dT)_{12-18}$ was in equimolar ratio.

inhibition with BuPhdGTP and 80% inhibition with BuAndATP at that concentration (Niranjanakumari and Gopinathan, 1992).

Dimethyl sulfoxide inhibited polymerase ϵ activity in a dose-dependent manner (Fig. 3*d*). At 15% dimethyl sulfoxide, almost 87% of the activity was inhibited whereas the activity of polymerase δ was stimulated 1.9-fold. Thus dimethyl sulfoxide could be used to differentiate between the activities of polymerase δ and ϵ.

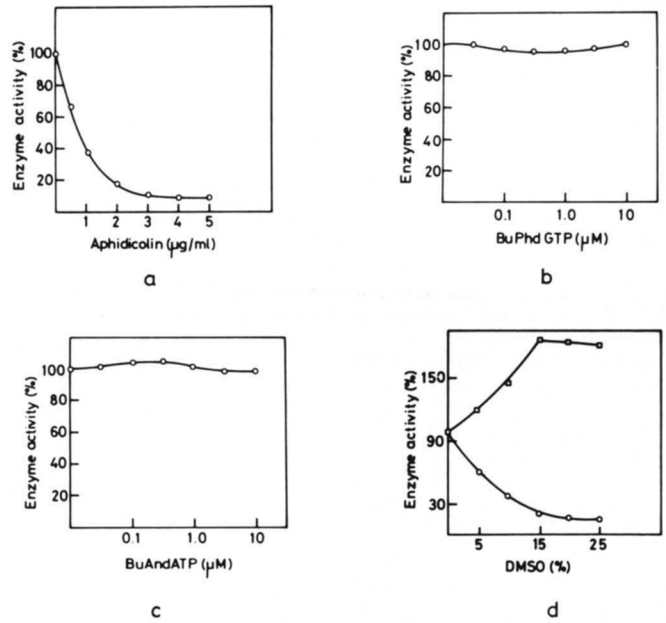

FIG. 3. **Effect of inhibitors on DNA polymerase ϵ.** The enzyme (0.1 unit) was preincubated with aphidicolin (*a*), BuPhdGTP (*b*), and BuAndATP (*c*) at 4 °C for 20 min prior to the addition of enzyme assay mix. The enzyme reaction was carried out at 37 °C for 30 min, and the acid precipitable radioactivity was monitored. In *panel d*, 0.1 unit each of DNA polymerase δ (□) and ϵ (○) were incubated with varying amounts of dimethyl sulfoxide, followed by incubation with the assay mix at 37 °C for 30 min.

Effect of Antibodies on DNA Polymerase ϵ—The polyclonal antibodies raised individually against DNA polymerase α and δ were used to assess the cross-reactivity among these polymerases. Neutralization assays indicated that DNA polymerase ϵ was not inhibited by polymerase α antibodies (Fig. 4*a*) but was partially inhibited by polymerase δ antibodies (Fig. 4*b*). The partial cross-reactivity between polymerase δ and ϵ was further confirmed by dot-blot analysis using DNA polymerase δ antibody (Fig. 4*c*). Polymerase ϵ was immunologically distinct from polymerase α but showed some relatedness to polymerase δ.

Effect of bPCNA on DNA Polymerase ϵ—bPCNA, the auxiliary protein to DNA polymerase δ, showed no effect on polymerase ϵ activity even in large excess up to 3 μg while 0.1 μg of this protein stimulated the polymerase δ activity by 3-fold (Fig. 5*a*). In order to check whether the lack of enhancement of catalytic activity by bPCNA is due to the inherent processive nature of polymerase ϵ, the DNA chain elongation was examined in the presence and absence of bPCNA (Fig. 5, *b* and *c*). The enzyme was inherently processive because the

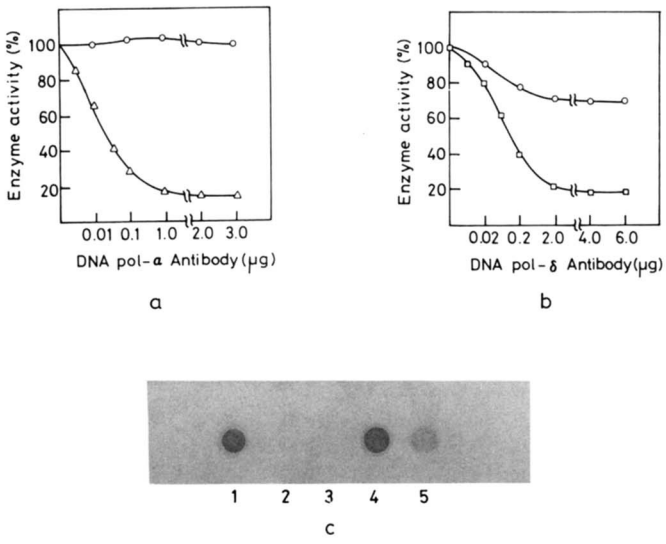

FIG. 4. **Effect of antibodies on enzyme activity.** Polyclonal antibodies to DNA polymerase α and δ were separately raised in rabbits, using the purified proteins. The IgG fraction was purified by $(NH_4)_2SO_4$ precipitation followed by chromatography on Protein A-Sepharose. The antibody titers were determined by enzyme-linked immunosorbant assay. 1 unit each of the enzyme samples (polymerase α, δ, or ϵ) was incubated with varying amounts of the purified IgG fraction (*a*, polymerase α; *b*, polymerase δ antibody) for 30 min at 4 °C. Following this, the assay mixture was added and incubated at 37 °C for 30 min. △, polymerase α; □, polymerase δ; ○, polymerase ϵ. *Panel C*, dot-blot analysis of DNA polymerases. The protein samples (1 μg each) were dot-blotted on nitrocellulose membrane and probed with purified polyclonal antibody against DNA polymerase δ. The antigen-antibody complex was visualized by reaction with goat anti-rabbit IgG conjugated to horseradish peroxidase and color development in the presence of diamino benzidine and H_2O_2. The *spots* (*1–5*) represent silk gland cell crude extract, purified DNA polymerase α, β, δ, and ϵ, respectively.

extent of DNA chain elongation was the same in both (Fig. 5*b*, compare *lane 5* to *lanes 6–8*). Increasing the concentration of bPCNA had no effect on the DNA synthesis by polymerase ϵ. In contrast, DNA synthesis by polymerase δ terminated at much shorter lengths in the absence of bPCNA (*lane 2*), but longer chains were synthesized in the presence of increasing concentrations of bPCNA (*lanes 3* and *4*). Although the enhancement of processivity by bPCNA of DNA polymerase δ and lack of its effect on polymerase ϵ is apparent from the DNA chain extension (Fig. 5*b*), it is still possible that the enhanced DNA synthesis could result from increased primer utilization or multiple rounds of initiation. In order to rule out the latter possibility, the DNA chain extension was also carried out using the template-primer from which all the excess primer was removed. These results (Fig. 5*c*) clearly established that the effect of bPCNA was at the level of processivity enhancement rather than enhanced primer utilization or multiple rounds of initiations.

The processivity of DNA polymerase ϵ was also verified by analyzing the newly synthesized, extended primer by restriction digestion. The principle of the assay is schematically presented in Fig. 5*d*. In this assay, the unlabeled primer annealed to ssDNA template was extended by polymerase ϵ in the presence of labeled dNTPs, and with or without bPCNA. The extended product when digested with restriction enzymes whose sites are generated due to the copying by polymerase should result in the appearance of appropriate size fragments. It is evident (Fig. 5*e*) that the chain elongation has proceeded to at least 600–700 nucleotides from the primer because the digestion of the extended product with *Rsa*I, *Hae*III, and *Taq*I resulted in the release of nucleotide fragments of the expected sizes (32, 257, 214, and 98 for *Hae*III; 60, 30, and 552 for *Taq*I; and, 82 and >600 for *Rsa*I), indicated by *arrows* in the figure. The pattern of fragments generated was similar whether bPCNA was present (*lanes 3–5*) or absent (*lanes 6–8*).

Association of Exonuclease Activity with Polymerase ϵ—Like DNA polymerase δ, the purified DNA polymerase ϵ also showed 3′ → 5′ exonuclease activity, catalyzing the release of radioactivity from 3′-[^{32}P] end-labeled DNA (Fig. 6). The strong association of polymerase and exonuclease activities at a constant ratio of 2:1 was evident throughout the purification protocol (Table I). The lower ratios seen in the cytosolic extract (S_{100}) could be due to the presence of other exonucleases in the crude extracts, and for this reason only 2200-fold purification was achieved for nuclease activity as against 5300-fold purification of polymerase (Table II). However, if the specific activities are considered from the phosphocellulose step of fractionation, both polymerase ϵ and exonuclease activities increased approximately by 1800–2000-fold.

In order to ascertain whether the 3′ → 5′ exonuclease activity associated with DNA polymerase ϵ has any proofreading function, the DNA chain extension of a 3′ mismatched primer by polymerase ϵ was examined. In this assay, a synthetic oligonucleotide primer (17-mer) complementary to M13mp18 DNA, with a C-A mismatch at the 3′ end was used (Fig. 7*a*). Since the chain elongation was carried out in the presence of three dNTPs (dATP, dGTP, and dTTP but not dCTP), and if DNA polymerase ϵ corrected the terminal C-A mismatch, the subsequent extension should result in the synthesis of a 25-mer product in the absence of dCTP. The enzyme could correct the C-A mismatch at the 3′ end (Fig. 7*b*, *lanes 4* and *5*). The presence of bPCNA had no effect on the proofreading activity of polymerase ϵ (*lane 5*). In contrast, bPCNA clearly enhanced the proofreading activity of polymerase δ (compare *lanes 2* and *3*).

Tight Association of Polymerase and Exonuclease of DNA Polymerase ϵ—The copurification of polymerase and exonuclease activities is evident all through the isolation procedure. To test whether these two activities are associated with the same polypeptide, the enzyme was dissociated with urea and assayed after the separation of subunits (Fig. 8*a*). Both polymerase and exonuclease activities were associated with the fraction containing the 215-kDa subunit (Fig. 8*b*).

DISCUSSION

All prokaryotic and eukaryotic cells contain several DNA polymerases (Kornberg and Baker, 1992; Wang, 1991). Replication and repair of the chromosomal DNA are the major functions of polymerases. Besides, the cell requires DNA polymerases for related events such as DNA recombination and replication of extrachromosomal (*i.e.* organellar or plasmid) DNAs. In eukaryotes, these tasks are shared by at least five different DNA polymerases, *viz.* α, β, τ, δ, and ϵ. DNA polymerase ϵ was identified first in HeLa cells as a factor required for DNA repair synthesis in UV-irradiated fibroblasts (Nishida, 1988). Recently, the displacement synthesis by DNA polymerase ϵ has been demonstrated in coordination with DNA helicase ϵ (Hurwitz *et al.*, 1992). The present studies demonstrate the identity of the enzyme purified from the silk glands as DNA polymerase ϵ based on its biochemical properties and document for the first time, the presence of DNA polymerase ϵ in the insect system. The enzyme is comparable in biochemical properties to the mammalian polymerase ϵ (Wahl *et al.*, 1986; Lee and Toomey, 1987; Focher *et al.*, 1988; Syvaoja and Linn, 1989). DNA polymerase II from *S. cerevis-*

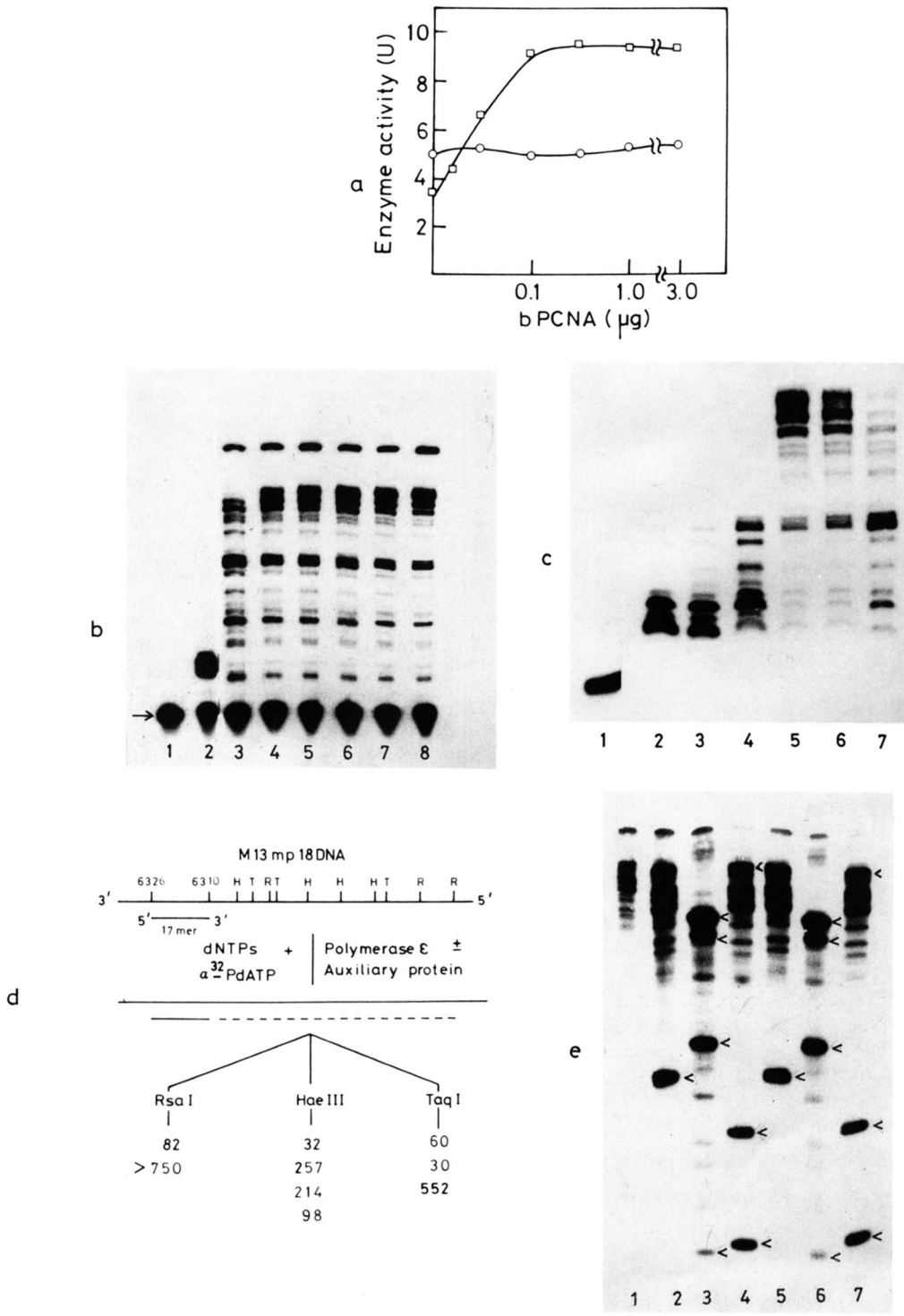

FIG. 5. **Processivity of silk gland DNA polymerase δ and ε.** *Panel a*, effect of bPCNA on DNA polymerase ε. DNA polymerases δ (□) or ε (○) (0.1 unit each) were incubated with the purified bPCNA (up to 3 μg) prior to the addition of assay mix. Enzyme assay was carried out at 37 °C for 30 min. *Panels b* and *c*, the effect of bPCNA on the processivity of DNA polymerases δ and ε. In a 20-μl reaction, the 5′ end-labeled 17-mer primer (5′-TTTTCCCAGTCACGACG-3′ complementary to M13mp18 ssDNA sequence 3′-6310 to 6326–5′), 0.3 pmol (~30,000 counts/min) annealed to 200 ng of template M13ssDNA was extended by polymerase δ or ε (1 unit each), at 37 °C for 30 min in the presence of indicated amounts of bPCNA and 50 μM each of all four dNTPs. The chain extension products, after deproteinization (by extraction with phenol-chloroform), were analyzed on 8% polyacrylamide-urea gels and autoradiographed. *Arrowhead* indicates the position of the primer. *Panel b*: *lane 1*, primer (control); DNA polymerase δ, in the absence (*lane 2*) or in the presence of 50 and 100 ng of bPCNA (*lanes 3* and *4*); DNA polymerase ε in the absence (*lane 5*) or in the presence of 20, 50, and 100 ng of bPCNA (*lanes 6–8*). *Panel c*, to examine the enhancement of processivity of DNA polymerase by bPCNA rather than the increased primer utilization or multiple rounds of DNA replication, the assay was also carried out with limiting amounts of primer annealed to M13ssDNA. The 5′ end-labeled 17-mer oligonucleotide was annealed to the M13mp18 ssDNA, and the template-primer (1.4 μg-0.6 pmol) was separated from any excess free primer on a Sephacryl S-300 column. The chain extension reaction was carried out at 37 °C for 15 min, using template-primer (0.06 pmol of primer) with either DNA polymerase δ or ε (1 unit each) and in the absence or presence of bPCNA as indicated. *Lane 1*, primer (control); DNA polymerase δ in the absence (*lane 2*) or in the presence of 25, 50, and 100 ng of bPCNA (*lanes 3–5*, respectively); DNA polymerase ε in the absence (*lane 6*) and in the presence of 100 ng of bPCNA (*lane 7*). *Panels d* and *e*, analysis of the processivity by restriction digestion. A schematic

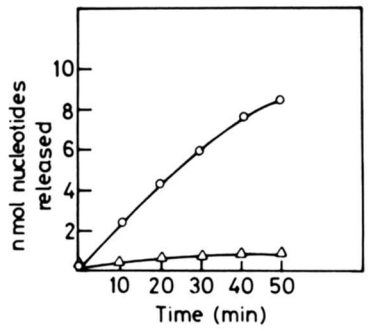

FIG. 6. **Exonuclease activity of polymerase ϵ.** 3′ → 5′ Exonuclease activity was assayed using 3′ end-filled double-stranded pUC18 DNA. For this purpose, pUC18 DNA was linearized with *Eco*RI and end filled in the presence of [α-^{32}P]dATP and PolIk. The exonuclease assay mixture contained in a final volume of 50 μl: 50 mM Tris-HCl, pH 6.5, 1 mM $MgCl_2$, 75 mM KCl, 1 mM DTT, 100 μg/ml BSA, labeled DNA (50,000 counts/min/reaction), and the enzyme. The reaction was carried out at 37 °C for different time intervals, and radioactivity rendered acid soluble was monitored. △, polymerase α; ○, polymerase ϵ.

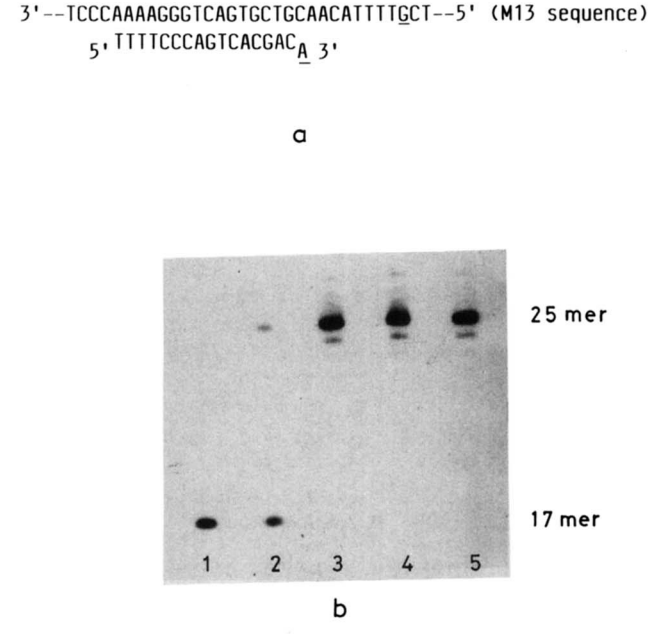

FIG. 7. **Proofreading assay.** A synthetic oligonucleotide, 17-mer 5′-TTTTCCCAGTCACGAC-3′ primer (labeled at 5′ end), having one terminal mismatch at the 3′ end was annealed to M13ssDNA template. The DNA chain elongation on this template-primer was carried out with DNA polymerases δ and ϵ and in the absence of dCTP but with the other three dNTPs. The reaction was terminated after 20 min, deproteinized, and the product was analyzed on 20% polyacrylamide-urea sequencing gel and autoradiographed. *Lanes*: *1*, control (no enzyme); *2* and *3*, DNA polymerase δ in the absence or presence of bPCNA; *4* and *5*, DNA polymerase ϵ in the absence or presence of bPCNA.

iae is homologous to mammalian polymerase ϵ, as revealed by its resistance to BuPhdGTP and insensitivity to PCNA (Morrison *et al.*, 1990). The procedure developed here permits the simultaneous isolation of DNA polymerases α, δ, and ϵ from a single extract.

The purified DNA polymerase ϵ from the silk gland is a heterodimer of M_r 215,000 and 42,000. The HeLa cell polymerase ϵ is comprised of subunits of 215 and 50 kDa (Syvaoja and Linn, 1988). Polymerase ϵ from calf thymus has a native molecular mass of 290 kDa, but another form of the enzyme from the same source has a molecular mass of 240 kDa (Crute *et al.*, 1986). A multimeric DNA polymerase ϵ having four subunits of 140, 125, 48, and 40 kDa has also been reported from calf thymus (Lee *et al.*, 1991). Both polymerase and exonuclease activities were associated with 215-kDa subunit of silk gland polymerase ϵ. Such association of these activities with the large subunit of DNA polymerase ϵ from yeast has also been genetically established (Sugino *et al.*, 1990).

DNA polymerase ϵ from silk gland cells was biochemically and immunologically distinct from polymerase α from the same tissue. The enzyme, however, resembled the silk gland polymerase δ in many aspects, *viz.* sensitivity to aphidicolin inhibition, resistance to BuPhdGTP, association of 3′ → 5′ exonuclease activity, and the pH requirement for optimal enzyme activity. Dimethyl sulfoxide is shown to stimulate human DNA polymerase δ and inhibit DNA polymerase ϵ (Syvaoja *et al.*, 1990). Likewise, the DNA polymerase ϵ from silk glands was inhibited more than 40% by dimethyl sulfoxide whereas polymerase δ was stimulated at the same concentration. The insensitivity of polymerase ϵ to bPCNA and the sensitivity to inhibition by dimethyl sulfoxide could clearly distinguish these two polymerases. Polyclonal antibodies raised against the silk gland polymerase δ neutralized the polymerase ϵ activity partially. Weak immunological cross-reactivity was also apparent in the dot-blots, although no distinct signals could be picked up in Western blots. The polyclonal antibodies against calf thymus polymerase δ have been shown to weakly neutralize the polymerase ϵ from the same tissue. Moreover, the calf thymus polymerase δ antibody completely neutralized the activity of human polymerase ϵ although it failed to light up on the immunoblots (Wong *et al.*, 1989).

Studies on calf DNA polymerase ϵ indicated that the enzyme is highly processive on primed single-stranded DNA with neither reaction rate nor processivity stimulated by calf PCNA (Focher *et al.*, 1988). Similar results were also reported from human and rabbit bone marrow DNA polymerase ϵ (Syvaoja and Linn, 1989; Byrnes *et al.*, 1991). However, the yeast polymerase ϵ and a form of human polymerase ϵ required the combination of RFA, PCNA, and single-stranded DNA binding protein for full processivity (Lee *et al.*, 1991). The silk gland DNA polymerase ϵ was inherently processive, and the processivity was not affected by bPCNA, in contrast to the polymerase δ.

Hammond *et al.* (1988) have studied the role of polymerases involved in repair of *N*-methyl-*N*′-nitroguanidine damage in

representation of the processivity assay by this method is shown in *panel d*. As a result of DNA chain extension from the M13mp18 ssDNA template primer, the indicated restriction sites should be generated. *R*, *H*, and *T* represent the restriction sites of the enzymes *Rsa*I, *Hae*III, and *Taq*I on M13mp18 DNA from within the first 1000 nucleotides of the extended product. On digestion with these restriction enzymes, the size of the fragments expected to be generated is indicated. *Panel e*, the assay conditions were similar to those described in *panel b*, except that the primer was not end labeled and the assay mixture contained 5 μCi of [α-^{32}P]dCTP and 50 μM other dNTPs. The DNA synthesis was carried out in the absence (*lanes 1–4*) or in the presence (*lanes 5–7*) of bPCNA (100 ng of protein). After 30 min at 37 °C, the samples were deproteinized, precipitated with alcohol, and digested with the restriction enzymes (*Rsa*I, *Hae*III, or *Taq*I as indicated). The products were analyzed on a 8% polyacrylamide-urea gel. Undigested sample (*lane 1*) or samples digested with *Rsa*I (*lanes 2* and *5*), *Hae*III (*lanes 3* and *6*), and *Taq*I (*lanes 4* and *7*), respectively.

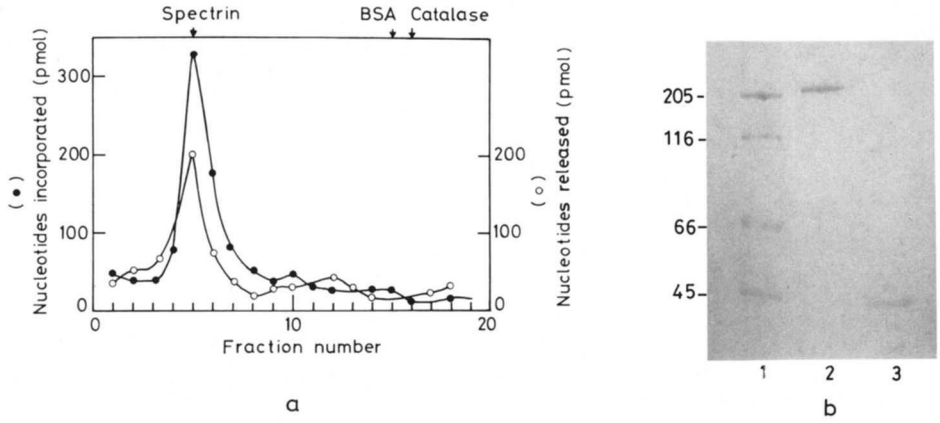

FIG. 8. *a*, dissociation of polymerase and exonuclease activities of DNA polymerase ϵ. 10 units of DNA polymerase ϵ was dissociated with 2.8 M urea for 4 h at 4 °C and subjected to centrifugation on 5–30% glycerol gradient containing 3.4 M urea in a Beckman SW60 rotor at 4 °C for 16 h at 42,000 revolutions/min. Fractions (0.2 ml) were collected and assayed for polymerase (●) and exonuclease (○) activities individually. *b*, SDS-PAGE analysis of the active subunit. The fractions showing polymerase and exonuclease activities were pooled, concentrated, and analyzed on 10% SDS-PAGE. *Lane 1*, marker lane; *lane 2*, pooled fractions 4–6; *lane 3*, pooled fractions 15–17.

permeabilized human fibroblast and suggested the involvement of DNA polymerase β and δ or ϵ. Identification of polymerase ϵ as a factor required in permeabilized HeLa cell for UV-induced DNA repair synthesis strongly suggested that polymerase ϵ plays an essential role in UV-induced repair synthesis. In human diploid fibroblasts from Xeroderma pigmentosum, UV-induced repair synthesis could be accomplished by polymerase ϵ and T_4 endonuclease (Nishida *et al.*, 1988). In this reaction, neither polymerase α nor β can substitute for polymerase ϵ in repair synthesis. Moreover, a precise comparison of purified polymerase δ and ϵ from HeLa cells has clearly demonstrated that HeLa polymerase ϵ but not δ has UV repair synthesis activity (Kenney and Linn, 1990).

The current model of eukaryotic DNA replication involves the concerted and co-ordinated action of DNA polymerases α and δ. The identification of DNA polymerase ϵ has added further complexity to the polymerases involved in DNA replication. In yeast, genetic experiments with DNA polymerase ϵ gene have indicated that polymerase ϵ is essential for growth. The C-terminal half of the enzyme is not essential while the N-terminal part coding for a 130-kDa subunit is enough to restore the viability of the cells, although with a slower growth rate (Morrison *et al.*, 1990). These results indicated that the polymerase ϵ is an essential component of eukaryotic DNA replication. In SV40 *in vitro* replication system, DNA polymerase ϵ from HeLa cells could not substitute for DNA polymerase δ. When yeast polymerase ϵ was used, however, a partial replacement was observed (Lee *et al.*, 1990). Further, the auxiliary proteins such as RFA, RFC, and PCNA required by DNA polymerase δ for replication appear to interact with polymerase ϵ also, but probably in a more complex fashion than with the former (Yoder and Burgers, 1991). More detailed studies are needed to establish the *in vivo* function of DNA polymerase ϵ.

The silk gland cells of *B. mori* have adopted a strategy of increasing the entire genomic DNA content rather than specific gene amplification to meet the demands of massive synthesis of the silk proteins fibroin and sericin during the late larval instars. This is possibly achieved by the high levels of DNA polymerases α and δ, both enzymes implicated in DNA replication process, along with contributions from polymerase ϵ. The presence of DNA polymerase ϵ in the highly replicative silk gland tissues taken together with the conspicuous absence of the traditional repair enzyme, DNA polymerase β, could also imply that polymerase ϵ is involved in the DNA repair process in this tissue.

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