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DNA Polymerase-δ from the Silk Glands of *Bombyx mori**

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Somashekarappa Niranjanakumari‡ and Karumathil P. Gopinathan§

From the Microbiology and Cell Biology Department and the Centre for Genetic Engineering, Indian Institute of Science, Bangalore 560 012, India

The silk gland of *Bombyx mori* is a terminally differentiated tissue in which DNA replication continues without cell or nuclear division during larval development. DNA polymerase-δ activity increases in the posterior and middle silk glands during the development period, reaching maximal levels in the middle of the fifth instar larvae. The enzyme has been purified to homogeneity by a series of column chromatographic and affinity purification steps. It is a multimer comprising of three heterogeneous subunits, M_r 170,000, 70,000, and 42,000. An auxiliary protein from *B. mori* silk glands, analogous to the proliferating cell nuclear antigen, enhances the processivity of the enzyme and stimulates catalytic activity by 3-fold. This auxiliary protein has also been purified to homogeneity. It is a dimer comprised of a single type M_r 40,000 subunit. Polymerase-δ possesses an intrinsic 3′ → 5′ exonuclease activity which participates in proofreading by mismatch repair during DNA synthesis and is devoid of any primase activity. DNA polymerase-δ activity could be further distinguished from polymerase-α from the same tissue based on its sensitivity to various inhibitors and polyclonal antibodies to the individual enzymes. Like DNA polymerase-α, polymerase-δ is also tightly associated with the nuclear matrix. The polymerase α-primase complex could be readily separated from polymerase-δ (exonuclease) in the purification protocol adopted. DNA polymerase-δ from *B. mori* silk glands resembles the mammalian δ-polymerases. Considering that both DNA polymerase-δ and -α are present in nearly equal amounts in this highly replicative tissue and their close association with the nuclear matrix, the involvement of both the enzymes in the chromosomal endoreplication process in *B. mori* is strongly implicated.

DNA polymerases play a pivotal role in the replication and repair of cellular DNA. The complexity and importance of these processes are reflected in the multiple species of DNA polymerases in both prokaryotes and eukaryotes, in which defined functions are performed specifically by one or another species of polymerases (Fry and Loeb, 1986). DNA polymerase-α was traditionally considered as the primary enzyme involved in eukaryotic DNA replication. However, it is now recognized that DNA polymerase-δ also has an equally important role in the replication and repair processes (Downey *et al.*, 1990). DNA polymerase-δ and -α could be distinguished from each other by the differences in their sensitivity to inhibitors such as butyl phenyl-dGTP (BuPhdGTP)[1] and butyl anilino-dATP (BuAndATP). Although polymerase-δ activity has been reported from several mammalian cell lines and tissues, its ubiquitous presence in all eukaryotic systems is not demonstrated.

DNA polymerase-δ, which differed from other known species of mammalian DNA polymerases, -α, -β, and -γ, in having an intrinsic 3′ → 5′ exonuclease activity, was first isolated from bone marrow (Byrnes *et al.*, 1976). The discovery implied that this enzyme could undertake the proofreading function during DNA synthesis to ensure fidelity in eukaryotic DNA replication by mechanisms similar to those seen in the prokaryotes. DNA polymerase-δ has been purified to homogeneity from calf thymus (Lee *et al.*, 1984), HeLa cells (Syvaoja *et al.*, 1990), and mouse cells (Goulian *et al.*, 1990). DNA polymerase III from *Saccharomyces cerevisiae* is analogous to DNA polymerase-δ (Bauer *et al.*, 1988). Another form of DNA polymerase-δ (δ_{II}), 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), has now been classified as DNA polymerase-ε.

The role of polymerase-δ in DNA replication was implicated by its presence in proliferative tissues and by its sensitivity to replication inhibitors (Byrnes *et al.*, 1976; Lee *et al.*, 1981, 1985; Dresler and Frattini, 1986; Decker *et al.*, 1987; Hammond *et al.*, 1987; Lee and Toomey, 1987). The identification of proliferating cell nuclear antigen (PCNA) as a processivity factor for DNA polymerase-δ provided further evidence that this enzyme is involved in DNA synthesis.

The silk gland cells of the mulberry silkworm, *Bombyx mori*, is packed with large quantities of DNA because of endomitosis. In this terminally differentiated tissue, DNA replication continues for 18–19 rounds all through the larval development, without cell or nuclear division. Therefore, the silk gland cells become gigantic, with almost the entire volume of cells being occupied by nuclear material. The highly ramified nuclei are filled with DNA amounting to 300,000 × the haploid genomic content by the late stages of the larval development (Gage, 1974; Niranjanakumari and Gopinathan, 1991). We have reported earlier the characterization of the DNA polymerase-α-primase complex from this tissue (Niranjanakumari and Gopinathan, 1991). This enzyme complex was associated with the nuclear matrix and did not possess

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§ To whom correspondence should be addressed. Tel.: 91-812-344411; Fax: 91-812-341683.

[1] The abbreviations used are: BuPhdGTP, butyl phenyl-dGTP; BuAndATP, butyl anilino-dATP; BSA, bovine serum albumin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PCNA, proliferating cell nuclear antigen; SDS, sodium dodecyl sulfate; Chaps, 3-[(3-cholaamidopropyl)dimethylammonio]-1-propanesulfonic acid.

any proofreading exonuclease function. The present study reports the purification and characterization of DNA polymerase-δ from the highly replicative cells of the silk glands of *B. mori* and its intrinsic association with a proofreading exonuclease activity.

MATERIALS AND METHODS

The restriction enzymes, NTPs, dNTPs, Sephadex G-50, phenyl-Sepharose, and QAE-Sephadex were from Pharmacia (Sweden). Radiolabeled nucleotides [^{3}H]TTP, [α-^{32}P]dATP, and [γ-^{32}P]ATP were from Amersham (U. K.) or the 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. Most of the other biochemicals and reagents such as Tris, dithiothreitol, sodium dodecyl sulfate (SDS), EDTA, EGTA, calf thymus DNA, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and bovine serum albumin (BSA), were from Sigma. BuPhdGTP and BuAndATP were gifts from Drs. George Wright (University of Massachusetts) and Kalluri Subba Rao (University of Hyderabad). The synthetic oligonucleotide (17-mer) was a gift from Dr. J. C. Prudhome (University of Claude Bernard Lyon).

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-δ activity was monitored by the synthesis of DNA in the presence of auxiliary protein, using activated calf thymus DNA as template primer. In a final volume of 50 μl the assay system contained 50 mM Tris/HCl, pH 6.5; 5 mM $MgCl_2$; 100 mM KCl; 1 mM dithiothreitol; 2% glycerol; 100 μg/ml BSA; a 100 μM concentration each of dATP, dCTP, dGTP; 50 μM [^{3}H]TTP (specific activity 100 cpm/pmol); 200 μg/ml activated calf thymus DNA; 100 ng of auxiliary protein; 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-α activity was similar but was done at pH 8.5 and in the absence of auxiliary protein.

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. Alternatively, poly(dA)·[^{3}H]poly(dT), prepared by the filling of poly(dA)·oligo(dT)$_{12-18}$ using [^{3}H]TTP and the Klenow fragment of polymerase I, was used as substrate for the exonuclease assay.

The proofreading activity was monitored by hydrolysis of the mispaired 3′ terminus from the template-primer complex and the subsequent extension of the primer. A synthetic oligonucleotide (17-mer), complementary to a sequence 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 hybridized to the M13mp18 single-stranded DNA to produce a duplex containing a C-A mispair at the 3′ primer terminus. The elongation of this 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; a 20 μM concentration each of dATP, dGTP, and dTTP; the template-primer; and 1 unit of DNA polymerase-δ. 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 20% polyacrylamide gel and located by autoradiography.

Buffers Used in Polymerase Purification—Buffer A consisted 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 dithiothreitol; 0.1 mM phenylmethylsulfonyl fluoride; 10 mM sodium metabisulfite; 0.5 μg/ml leupeptin; and 1 μg/ml aprotinin. Buffer B was 20 mM potassium phosphate, pH 7.0; 10% glycerol; 1 mM dithiothreitol; 0.1 mM phenylmethylsulfonyl fluoride; 10 mM sodium metabisulfite.

Purification of DNA Polymerase-δ—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 Ti-70 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, and the bound proteins were eluted with buffer A containing 1 M NaCl and assayed for polymerase-δ activity. 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 enzyme activity was 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. The enzymatically active fractions 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. Fractions showing polymerase-δ activity were pooled and dialyzed in buffer A with 50 mM NaCl and fractionated on a single-stranded DNA-Sepharose column. The enzyme was eluted with 0.3 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 the Auxiliary Protein for DNA Polymerase-δ from Silk Glands—The unbound fraction from the phosphocellulose column step was loaded onto a DE52 column equilibrated with buffer A containing 0.2 M NaCl. The bound proteins were eluted with buffer A containing 1 M NaCl. This fraction was adjusted to 1 M $(NH_4)_2SO_4$ and then applied to phenyl-Sepharose column equilibrated with buffer A containing 1 M $(NH_4)_2SO_4$. The column was washed successively with buffer A containing 1 M $(NH_4)_2SO_4$ and 0.5 M $(NH_4)_2SO_4$, followed by a gradient of $(NH_4)_2SO_4$ (0.5–0.0 M), in buffer A. The individual fractions were tested for the stimulation of DNA synthesis by polymerase-δ under optimal conditions. The fractions showing stimulatory activity were pooled and dialyzed against buffer A at pH 8.5 containing 25 mM NaCl and 20% glycerol. The sample was adjusted to 0.2 M NaCl and loaded onto a QAE-Sephadex column equilibrated with the same buffer. The bound proteins were eluted using a gradient of 0.2–0.5 M NaCl in buffer A, pH 8.0. Fractions showing stimulatory activity were pooled, concentrated, and subjected to gel filtration on Sephadex G-75. The active fractions were pooled and concentrated and used in the characterization of DNA polymerase-δ.

Preparation of Nuclear Matrix—Nuclear matrix was prepared from silk glands as described earlier (Niranjanakumari and Gopinathan, 1991). Briefly, the fresh silk glands were triturated gently to release the nuclei. The nuclei were purified on a 2.2 M sucrose cushion and subjected to DNase I digestion followed by the extraction of chromatin-bound proteins by repeated washing (15–20 cycles) with low salt and high salt buffers. The residual proteinaceous structure was solubilized in the presence of 20 mM Chaps and 0.5 M KCl to isolate the matrix-bound proteins. This preparation after dialysis was used for enzyme assays.

Preparation of Antibodies—Polyclonal antibodies to DNA polymerase-δ were raised in rabbits, using the purified protein. The IgG fraction was purified by $(NH_4)_2SO_4$ precipitation followed by chromatography on protein A-Sepharose. The antibody titer was determined by enzyme-linked immunosorbent assay. Antibody to polymerase-α was prepared as described earlier (Niranjanakumari and Gopinathan, 1991).

RESULTS

DNA Polymerase-δ Activity in Silk Glands—DNA polymerase-δ activity has been examined in the middle and posterior silk glands of *B. mori* as a function of development, on different days of the fourth and fifth instars of larval development (Table I). The enzyme activity increased with the progress of development until the 3rd day of the fifth instar and then came down. The observed activity was primarily a result of DNA polymerase-δ because the assays were carried out in the presence of BuPhdGTP and at pH 6.5, when almost all of the DNA polymerase-α activity should be inhibited (see Sensitivity to Inhibitors). The maximum contribution caused by DNA polymerase-ε-like activity was less than 10%, and there was no detectable activity of DNA polymerase-β. During the interim molt, the enzyme activity stayed at a stationary level. Polymerase-δ activity increased by 21- and 27-fold from the early fourth to the middle fifth instar in the middle and posterior silk glands, respectively. Considering that the pos-

TABLE I

DNA polymerase-δ activity in the silk glands during larval development

DNA polymerase activity was assayed at pH 6.5 and in the presence of 10 μM BuPhdGTP to eliminate any contribution from polymerase-α. One unit of DNA polymerase-δ activity corresponds to 1 nmol of [^{3}H]TMP incorporated in 1 h at 37 °C.

Development stage and days	DNA polymerase-δ activity[a] per silk gland	
	PSG	MSG
	units	
Fourth instar		
1	0.143	0.121
2	0.228	0.135
3	0.480	0.342
4	0.551	0.494
Molt	0.528	0.463
Fifth instar		
1	1.280	0.984
2	2.032	1.470
3	3.952	2.550
4	2.855	2.470
5	2.653	2.390
6	2.370	2.020

[a] Maximum contribution caused by DNA polymerase-ε-like activity was less than 10%.

terior silk gland is made of 520 cells and the middle silk gland of 255 cells, at peak periods of DNA synthesis during the fifth instar the activity corresponded to 7.6–10 milliunits of enzyme/cell in these tissues. This increase in polymerase-δ activity is comparable to that of DNA polymerase-α (Niranjanakumari and Gopinathan, 1991). The increasing levels of DNA polymerase-δ with the concomitant increase in DNA contents is indicative of its role in chromosomal DNA replication.

Purification of DNA Polymerase-δ and Its Auxiliary Protein—DNA polymerase-δ is known to require the presence of an auxiliary protein such as PCNA to enhance the processivity of the enzyme. The purification strategies for DNA polymerase-δ and its auxiliary protein are presented schematically in Fig. 1. As indicated in Fig. 1, DNA polymerase-δ separates from its auxiliary protein in the first step of purification, *i.e.* phosphocellulose chromatography. The phosphocellulose-bound fraction showed both DNA polymerase-δ and -α activities. These two activities were separated by fractionation on a hydroxylapatite column, in which polymerase-δ was eluted at 90–135 mM KPO_4, whereas polymerase-α was eluted at 200–300 mM KPO_4. The polymerase-δ fraction was further resolved into two enzymatically active peaks on QAE-Sephadex column chromatography. The activity of the first peak (0.2–0.28 M NaCl) was confirmed as DNA polymerase-δ based on its stimulation by auxiliary protein. The activity of the second peak (0.4–0.5 M NaCl), independent of the auxiliary protein, was caused by DNA polymerase-ε. Final purification of DNA polymerase-δ was achieved by affinity chromatography on single-stranded DNA-Sepharose followed by gel filtration on Bio-Gel P-200 columns. The adopted protocols resulted in a 4,640-fold increase in the specific activity and 20% yield of the enzyme (Table II). The values reported are the averages of several independent preparations, and variations between different batches were minimal. The enzyme fraction after the Bio-Gel P-200 step was electrophoretically homogeneous on a 7% polyacrylamide gel under nondenaturing condition (Fig. 2*a*). Electrophoresis on denaturing gels for subunit analysis revealed the presence of three nonidentical subunits of M_r 170,000, 70,000, and 42,000 (Fig. 2*b*).

The auxiliary protein resolved from DNA polymerase activities on phosphocellulose chromatography was also purified to electrophoretic homogeneity (Fig. 3*a*). It was an acidic protein with a pI of 4.5 (Fig. 3*b*). The native protein had an M_r of 78,000 as determined by gel filtration on a Sephadex G-100 column (Fig. 3*c*). On SDS-PAGE, a single protein band of M_r 40,000 was seen (Fig. 3*d*), suggesting that the protein is a homodimer. No intrinsic polymerase or exonuclease activities were detectable in this protein, but it stimulated both activities of DNA polymerase-δ.

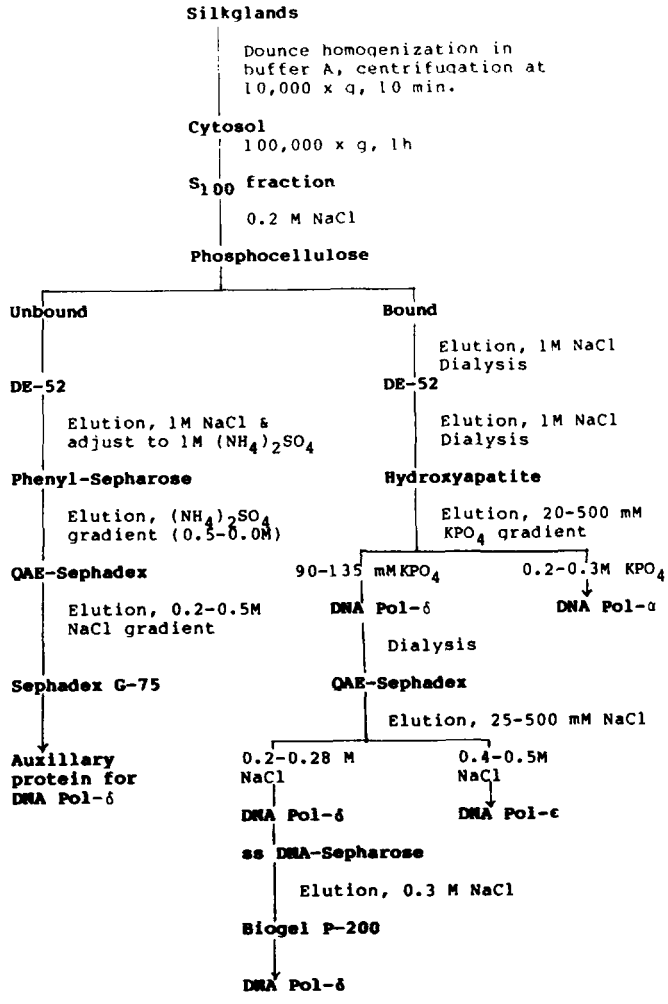

FIG. 1. **Schematic representation of the purification strategies for DNA polymerase-δ and its auxiliary protein.**

The effect of the auxiliary protein on the rate of dTMP incorporation by DNA polymerase-δ is shown in Fig. 4. The stimulatory response was linear up to 100 ng of auxiliary protein to a maximum extent of 3-fold stimulation and stayed at a plateau beyond this. When poly (dA)·oligo $(dT)_{12-18}$ was used as the template, the stimulation of DNA polymerase-δ activity by the auxiliary protein was even more pronounced (6.4-fold). The stimulatory effect was specific and confined to DNA polymerase-δ. The auxiliary protein had no effect on DNA polymerase-α activity, even in large excess (up to 3 μg).

Properties of DNA Polymerase-δ—The enzyme was maximally active at pH 6.5, with about two-thirds of the optimum activity at either 6.1 or 6.9. On the other hand, DNA polymerase-α showed less than 20% of its optimal activity at pH 6.5. At pH 8.5, when DNA polymerase-α activity was maximum, only less than 10% of the DNA polymerase-δ activity could be detected. The two enzymes could thus be distinguished from each other based on the pH optimum for their catalytic activity. DNA polymerase-δ showed absolute re-

TABLE II

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

Fractionation step	Total protein	Polymerase			Exonuclease			Polymerase to exonuclease ratio
		Total activity	Specific activity	Purification	Total activity	Specific activity	Purification	
	mg	*units*	*units/mg*	*-fold*	*units*	*units/mg*	*-fold*	
Crude extract (S_{100})	1103.0	386	0.35	1.0	459.0	0.42	1.0	0.83
Phosphocellulose	376.0	348	0.93	2.66	186.0	0.49	1.17	1.9
DEAE-cellulose	219.0	314	1.43	4.09	144.0	0.66	1.57	2.16
Hydroxylapatite	30.5	268	8.79	25.1	113.50	3.72	8.86	2.36
QAE-Sephadex	3.1	208	67.1	191.7	107.00	34.50	82.10	1.94
ssDNA[a]-Sepharose	0.94	139	147.9	423.0	74.0	78.7	187.00	1.88
Bio-Gel P-200	0.048	78	1625.0	4643.0	40.0	833.0	1983.00	1.95

[a] ssDNA, single-stranded DNA.

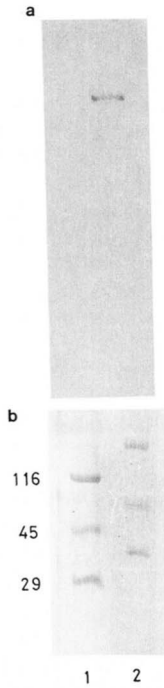

FIG. 2. **Homogeneity and subunit structure of DNA polymerase-δ from silk glands.** *a*, polyacrylamide gel electrophoresis. Purified DNA polymerase-δ (Bio-Gel P-200 column fraction, 2 μg of protein) was subjected to electrophoresis on 7% polyacrylamide under nondenaturing conditions, and the proteins were visualized by silver staining. *b*, SDS-polyacrylamide gel electrophoresis. The enzyme sample was heated at 85 °C for 10 min in presence of 2-mercaptoethanol (2.5%) and SDS (1%), and the 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*, Bio-Gel P-200 fraction (3 μg).

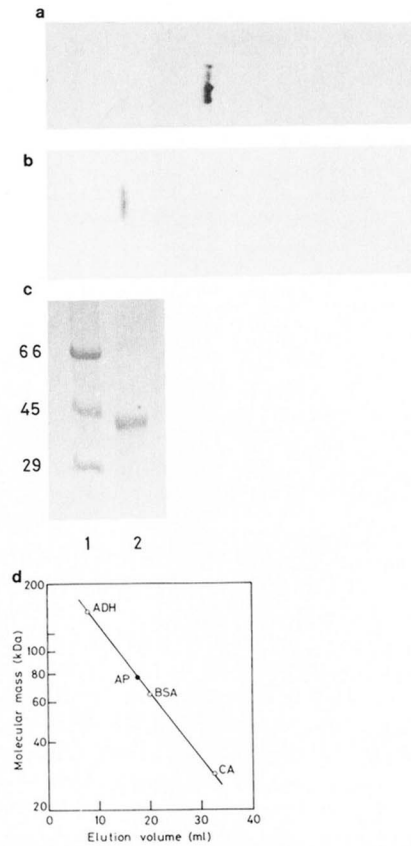

FIG. 3. **Electrophoretic homogeneity and subunit structure of auxiliary protein for DNA polymerase-δ.** *a*, the purified auxiliary protein (Sephadex G-75 column fraction, 2 μg of protein) was subjected to electrophoresis on 10% polyacrylamide under nondenaturing conditions. The gel was stained with Coomassie Brilliant Blue R-250. *b*, isoelectrofocusing was performed on Phast system on a homogeneous polyacrylamide gel containing Pharmalyte carrier ampholytes (pH range 3–9); pI was calculated from the standard calibration curve. *Lane 1*, standard molecular weight protein markers (BSA, ovalbumin and carbonic anhydrase); *lane 2*, purified auxiliary protein (1 μg). *c*, subunit structure was analyzed on 10% polyacrylamide under denaturing conditions as described previously. *d*, determination of molecular mass of the auxiliary protein (*AP*) by gel filtration on a Sephadex G-100 column. Alcohol dehydrogenase (150 kDa), BSA (66 kDa), and carbonic anhydrase (29 kDa) were used as standard markers.

quirements for cations; the optimum concentrations of $MgCl_2$ and KCl were 5 and 100 mM, respectively. $MnCl_2$ (0.1 mM) could substitute for the $MgCl_2$ requirement. The K_m values for the dNTPs were found to be 0.8, 1.8, 3.0, and 1.5 μM, respectively, for dATP, dGTP, dCTP, and dTTP. The enzyme activity was stimulated 1.8-fold by dimethyl sulfoxide (15%).

The enzyme could use either activated calf thymus DNA or synthetic poly (dA)·oligo$(dT)_{12-18}$ as template. Purified DNA polymerase-δ did not possess any primase activity intrinsically associated with it as revealed from its failure to synthesize the primer on M13 single-stranded DNA, under the assay conditions in which silk gland DNA polymerase-α synthesized the primer.

Sensitivity to Inhibitors—The effects of inhibitors such as aphidicolin, BuPhdGTP, BuAndATP, and the polyclonal antibodies against DNA polymerase-δ are presented in Fig. 5.

Aphidicolin inhibited both the polymerase and 3′ → 5′ exonuclease activities of DNA polymerase-δ in parallel, in a concentration-dependent manner (Fig. 5*a*). The sensitivity to inhibition by aphidicolin was similar to that of DNA polymerase-α.

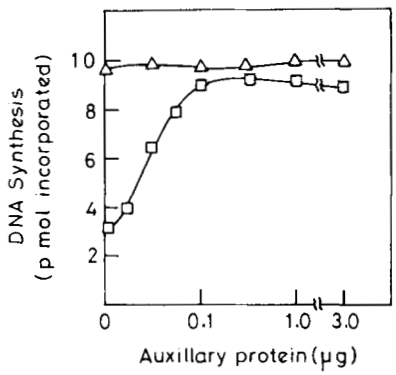

FIG. 4. **Effect of auxiliary protein on DNA polymerase-δ.** Assay conditions were as described under "Materials and Methods," except for the presence of varying concentrations of auxiliary protein (0–3 μg). The enzyme was incubated with the auxiliary protein for 30 min at 4 °C followed by incubation with the assay mixture at 37 °C for 30 min. □, DNA polymerase-δ; △, DNA polymerase-α.

The effects of BuPhdGTP and BuAndATP on DNA polymerase-δ activity are presented in Fig. 5, *b* and *c*. BuPhdGTP and BuAndATP are potent inhibitors of DNA polymerase-α, whereas mammalian DNA polymerase-δ is known to be insensitive (Byrnes, 1985). As shown in Fig. 5, *b* and *c*, BuPhdGTP and BuAndATP did not inhibit DNA polymerase-δ (up to 10 μM), but DNA polymerase-α showed almost 95% inhibition with BuPhdGTP and 80% inhibition with BuAndATP at that concentration.

The polyclonal antibodies raised against DNA polymerase-δ and α were used to assess the cross reactivity between the two enzymes. Neutralization assays indicated that DNA polymerase-δ was inhibited by antibodies raised against the enzyme, but it had no effect on the activity of DNA polymerase-α (Fig. 5*d*). Similarly, the antibodies for DNA polymerase-α neutralized the activity of this enzyme, whereas there was no effect on the polymerase-δ activity (Fig. 5*e*).

Western blot analyses using both polymerase-δ and -α antibodies are presented in Fig. 5, *f* and *g*. DNA polymerase-δ antibodies were able to recognize all the three subunits from this enzyme (Fig. 5*f*, *lane 1*), whereas no subunits of DNA polymerase-α were recognized by this antibody (*lane 2*). Likewise, the DNA polymerase-α antibody could recognize all six subunits of polymerase-α but none from polymerase-δ (Fig. 5 *g*, *lanes 1* and *2*). Thus, these two polymerases are made of antigenically distinct subunits and do not show cross-reactivity between them.

Association of Exonuclease Activity with Polymerase-δ—Purified DNA polymerase-δ exhibited 3′ → 5′ exonuclease activity, catalyzing the release of radioactivity from 3′-[^{32}P] end-labeled DNA (Fig. 6). The exonuclease activity was associated with polymerase-δ throughout the purification protocol (Table II). The ratio of polymerase to exonuclease remained fairly constant (2:1) at all steps of purification. The higher levels of exonuclease activity seen in the cytosolic extract (S_{100}) could be caused by the presence of other exonucleases in the crude extracts. In the present study approximately 2,000-fold purification of nuclease activity over the crude extract was achieved as compared with 4,600-fold purification of polymerase-δ activity (Table II). However, if the specific enzyme activities are considered from the phosphocellulose step, both polymerase-δ and exonuclease activities increased approximately by 1,700-fold and with a yield of 21–22%.

As in the case of polymerase activity, the exonuclease activity was also sensitive to aphidicolin (Fig. 5*a*) and was stimulated by the auxiliary protein (Fig. 7*b*, *fourth lane*).

To ascertain whether 3′ → 5′ exonuclease activity can function in proofreading, the DNA chain extension of a 3′-mismatched primer by polymerase-δ 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 3 dNTPs (dATP, dGTP, and dTTP but no dCTP) and if DNA polymerase-δ corrected this terminal C-A mismatch, the subsequent extension should result in the synthesis of a 25-mer product in the absence of dCTP. DNA polymerase I (from *Escherichia coli*) with a known proofreading function was used as a positive control. The results are presented in Fig. 7*b*. As anticipated for an enzyme capable of mismatch correction at the 3′ end of the template-primer terminus (*sixth lane*, DNA polymerase I from *E. coli*), DNA polymerase-δ could elongate the primer chain (*third* through *fifth lanes*). However, polymerase-α did not elongate this primer (*second lane*). The enzyme activity of polymerase-δ by itself was low in the absence of auxiliary protein (*third lane*), but the addition of the protein stimulated the reaction significantly (*fourth lane*). Thus, the auxiliary protein stimulates both the polymerase and exonuclease activity of polymerase-δ (see also Fig. 4). The proofreading activity was not inhibited by BuPhdGTP (Fig. 7*b*, *fifth lane*), further ruling out any involvement of polymerase-α in this function. The DNA polymerase associated with the nuclear matrix (see the following section for details) was also found to possess the proofreading function (*seventh lane*).

Enhancement of Processivity of DNA Polymerase-δ by the Auxiliary Protein—The mechanism of the stimulatory effect of the auxiliary protein on DNA polymerase-δ activity was examined in greater detail. To see whether the auxiliary protein enhances the processivity of polymerase-δ, the effect of the protein on DNA chain elongation was analyzed (Fig. 8). The extension of the mismatched primer on the M13 template in the presence of all four dNTPs by DNA polymerase-δ in the presence or absence of auxiliary protein is presented in Fig. 8*a*. The mismatch correction at the primer terminus, followed by the elongation of the primer, should result in a molecule extended longer than the control when the factor is present. The extension of the end-labeled primer was clearly more pronounced in the presence of the auxiliary protein, demonstrating the enhancement of the processivity of the enzyme. Alternatively, the processivity enhancement was also monitored by examining the extension of the matched primer terminus and subjecting the newly synthesized molecule to restriction digestion (Fig. 8*b*). Here the unlabeled primer was extended in the presence of labeled dNTP and the enzyme in the presence or absence of auxiliary protein. The extension of the nascent chain in the presence of the factor was clearly much longer. Digestion with *Eco*RI, *Mbo*I, and *Rsa*I resulted in the release of fragments of 94, 65, and 83 nucleotides, respectively (indicated by *arrowheads*) as anticipated if the chain elongation had progressed past the corresponding restriction sites. These results further confirmed the enhancement of the processivity by the auxiliary protein.

Association of DNA Polymerase-δ with the Nuclear Matrix—DNA polymerase-δ activity was tightly associated with the nuclear matrix. After 15–20 rounds of extraction, 0.162 unit of polymerase-δ activity/mg of protein corresponding to nearly 12% of the total nuclear enzyme activity still associated with the matrix. We had shown earlier (Niranjanakumari and Gopinathan, 1991) that this nuclear matrix preparation also contained DNA polymerase-α activity. However, the two activities could be readily distinguished and assayed independ-

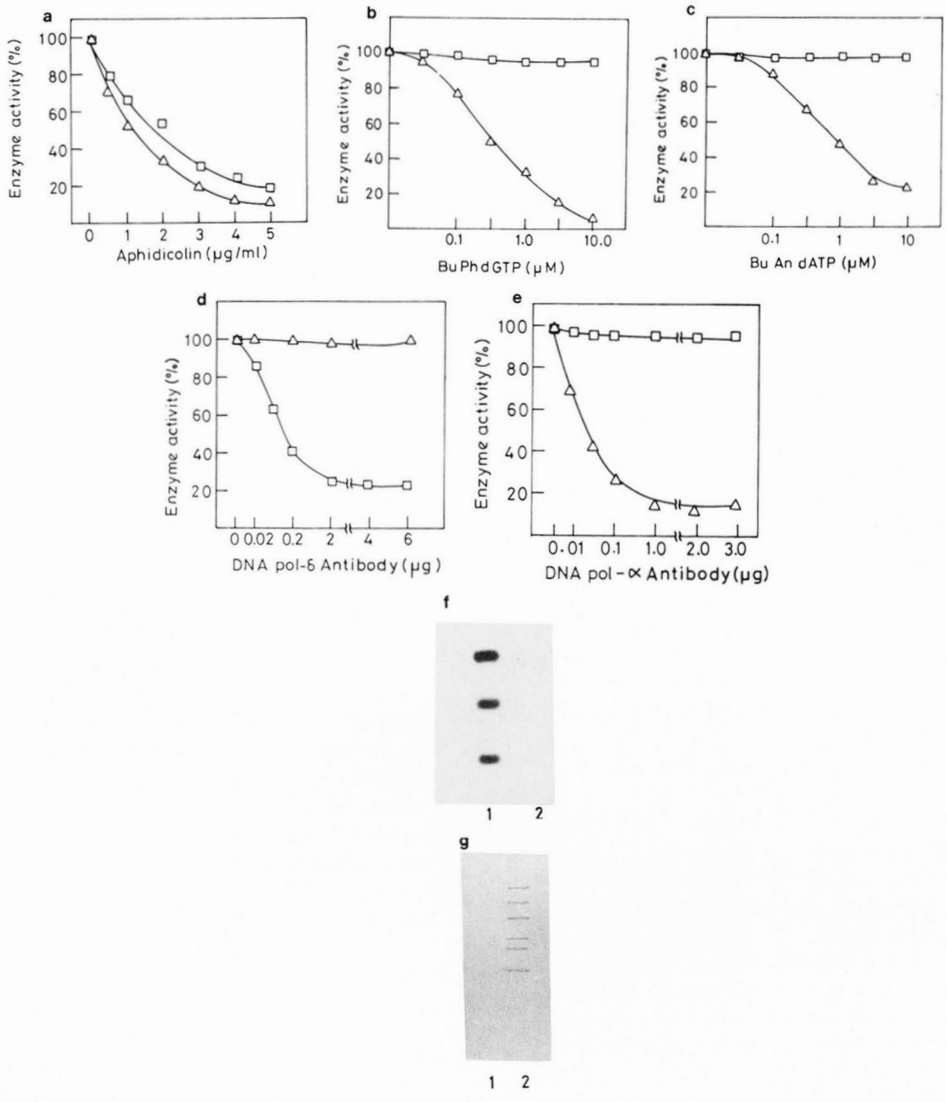

FIG. 5. *a*, effect of aphidicolin on the polymerase and 3′ → 5′ exonuclease activities of DNA polymerase-δ. Assay conditions as described under "Materials and Methods." The enzyme samples were incubated with the indicated concentration of aphidicolin (0–5 μg/ml) for 30 min at 4 °C prior to the addition of the assay mixture. The polymerase and exonuclease assays were carried out for 30 min at 37 °C. △, polymerase; □, exonuclease. *b* and *c*, effect of BuPhdGTP and BuAndATP on DNA polymerase activities. The enzyme samples (0.1 unit) were incubated with the varying concentrations of BuPhdGTP and BuAndATP (0–10 μM) for 30 min at 4 °C prior to the addition of the assay mixture. The enzyme assays were performed at 37 °C for 30 min. *d* and *e*, effect of antibodies on enzyme activity. Purified IgG fractions were isolated from polyclonal antibodies raised against DNA polymerase-δ or -α. One unit each of the enzyme samples (polymerase-δ or -α) was incubated with varying amounts of the purified IgG fraction (homologous or heterologous antibodies) for 30 min at 4 °C. Following this, the reaction mixture was added and incubated at 37 °C for 30 min. *f* and *g*, immunoblotting. The DNA polymerase-δ and -α samples were denatured (see legend to Fig. 2*b*) and subjected to electrophoresis on SDS-polyacrylamide gels; the proteins were transferred by electroblotting to nitrocellulose membrane. The blots were separately incubated with antibodies against DNA polymerase-δ (*f*) or -α (*g*). For DNA polymerase-δ, ^{125}I-labeled protein-A followed by autoradiography was utilized to locate protein bands. In the case of DNA polymerase-α the bands were visualized by reacting with anti-rabbit IgG coupled to horseradish peroxidase and color development in the presence of diaminobenzidine and H_2O_2 (Towbin *et al.*, 1979). *Lane 1*, DNA polymerase-δ; *lane 2*, DNA polymerase-α.

ently. Polymerase-δ assays were done in the presence of BuPhdGTP and at pH 6.5, where the activity resulting from polymerase-α was totally eliminated. At pH 8.5, on the other hand, the contribution from polymerase-δ was negligible. The entire polymerase-δ could be further differentiated and quantitated in the presence of DNA polymerase-α antibodies. The matrix-associated polymerase-δ activity possessed the proofreading exonuclease also (Fig. 7*b*, *seventh lane*). The presence of both polymerase-δ and -α in the matrix-associated proteins was further confirmed by their detection using the individual antibodies raised against these enzymes (Fig. 9). The specificity of these antibodies is clearly seen in these immunoblots.

DISCUSSION

Among higher eukaryotes, so far DNA polymerase-δ has been isolated only from mammalian systems (Lee *et al.*, 1984; Syvaoja *et al.*, 1990; Goulian *et al.*, 1990). The present studies document for the first time the presence of polymerase-δ in insect systems. Recent investigations on yeast DNA polymerases have shown that yeast polymerase III is homologous to mammalian polymerase-δ, as revealed by its stimulation by yeast PCNA and resistance to BuPhdGTP (Bauer *et al.*, 1988). In *Drosophila*, on the other hand a polymerase-δ like activity could be seen as a derivatized polymerase-α (Reyland *et al.*, 1988). DNA polymerase-δ from silk gland was comparable in

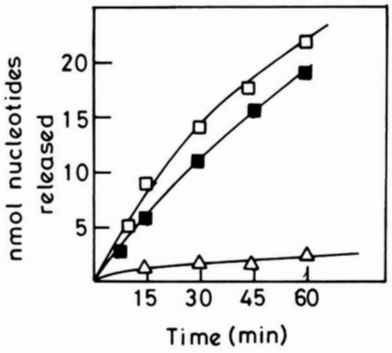

FIG. 6. **Exonuclease activity of polymerase-δ.** The 3′ → 5′ exonuclease activity of polymerase-δ was monitored by the hydrolysis of labeled DNA. Linearized plasmid pUC18 DNA, the 3′ end filled with [α-^{32}P]dATP or poly(dA)·[^{3}H]poly(dT) was used as substrate. The assay system contained 50 mM Tris/HCl, pH 6.5, 1 mM $MgCl_2$, 2% glycerol, 100 μg/ml BSA, 0.1 unit of enzyme and the substrate; the incubations were carried out at 37 °C. At the indicated time intervals, samples were removed, and the acid-soluble radioactivity released was estimated. Polymerase-δ: □, [^{32}P]pUC18 DNA; ■, poly(dA)·[^{3}H]poly(dT). Polymerase-α: △, [^{32}P]pUC18 DNA.

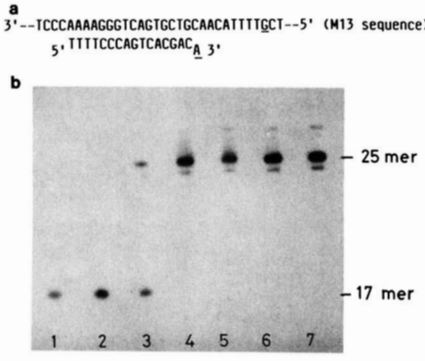

FIG. 7. **Proofreading assay.** *a*, the oligonucleotide primer with one 3′-terminal mismatch. *b*, the mismatched template-primer was used as substrate for elongation by polymerase-δ or other polymerases indicated, in the presence of dATP, dGTP, and dTTP. The product was analyzed by electrophoresis on 20% polyacrylamide-urea sequencing gel and autoradiographed. If the mismatch at 3′ end is corrected, the chain elongation should proceed up to 25 nucleotides, to the first G residue on template beyond the primer in the absence of dCTP in the polymerization reaction. Consequently, the 17-mer primer gets converted to a 25-mer product (one mismatch removed and nine nucleotides added). From *left: first lane*, control (no enzyme); *second lane*, DNA polymerase-α; *third lane*, DNA polymerase-δ; *fourth lane*, DNA polymerase-δ in the presence of auxiliary protein; *fifth lane*, DNA polymerase-δ in the presence of auxiliary protein and BuPhdGTP; *sixth lane*, *E. coli* DNA polymerase I (positive control); *seventh lane*, solubilized fraction from the nuclear matrix.

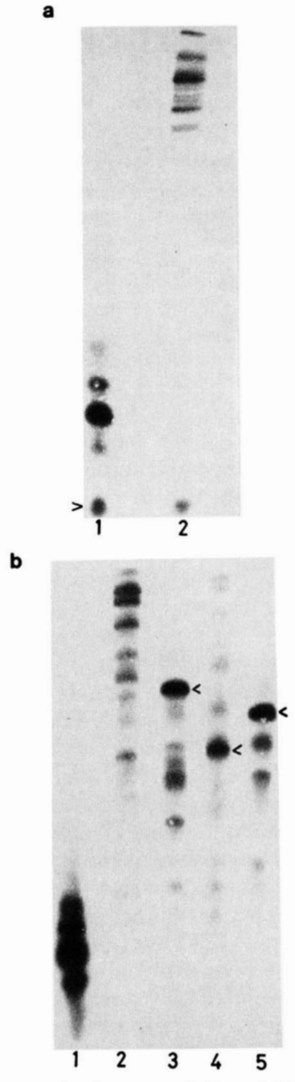

FIG. 8. **Enhancement of processivity of DNA polymerase-δ by the auxiliary protein.** *a*, the reaction was carried out as described under Fig. 7*b* except that all four NTPs were present in the reaction. DNA polymerase-δ is shown in the absence (*lane 1*) or in the presence (*lane 2*) of the auxiliary protein. *Arrowhead* indicates the primer position. *b*, the assay conditions were identical to those described under Fig. 7*b* except that the template-primer did not have a mismatch 3′ terminus (*i.e.* M13mp18 single-stranded DNA template and synthetic 17-mer oligonucleotide complementary to 5′-6309 to 6324–3′), and the polymerase reaction was carried out in the presence of 5 μCi of [α-^{32}P]dATP and a 50 μM concentration of other dNTPs. After the reaction, the samples were restricted with *Eco*RI, *Mbo*I, or *Rsa*I and were analyzed on 8% urea-polyacrylamide gel electrophoresis. The gel was dried and autoradiographed. DNA polymerase-δ is shown in the absence of auxiliary protein (*lane 1*) and in the presence of auxiliary protein (*lanes 2–5*), undigested sample or restricted with *Eco*RI, *Mbo*I, and *Rsa*I, respectively. *Arrowheads* mark the position of fragment released. The precise sizes of these fragments were also determined on a sequencing gel.

its properties to the polymerase-δ (δ_1) reported from the mammalian sources (Crute *et al.*, 1986; Lee *et al.*, 1984; Goulian *et al.*, 1990).

DNA polymerase-δ, biochemically distinct from DNA polymerase- α, has been implicated as a second replicative DNA polymerase. Unlike polymerase-α, polymerase-δ possesses an intrinsic 3′ → 5′ proofreading exonuclease activity but lacks the primase activity associated with the former. Besides, polymerase-δ is resistant to BuPhdGTP and BuAndATP. Additionally, in the present studies, the two enzymes could be distinguished based on specific antibodies raised against each of them and by their pH requirements for optimal enzyme activity. Polymerase-δ is maximally active at pH 6.5, where polymerase-α activity is barely detectable. In contrast, polymerase-α is optimally active at pH 8.5, where polymerase-δ does not show activity. The purified silk gland polymerase-δ is a multimer of three nonidentical subunits with an apparent minimum M_r 282,000, assuming a stoichiometry of a single copy of each subunit. This enzyme is much smaller than the silk gland DNA polymerase-α, which has an M_r of 557,000 and harbors six subunits.

The DNA polymerase-δ activity increased up to the middle of fifth instar in the silk glands of *B. mori* as in the case of DNA polymerase-α. However, a small proportion of up to

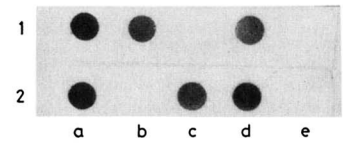

FIG. 9. **Immunoblotting of DNA polymerases associated with the nuclear matrix.** The protein samples (1 μg) were spotted on nitrocellulose membrane and probed separately with purified polyclonal antibody against DNA polymerase-δ (*upper panel 1*) or α (*lower panel 2*). *a*, total silk gland extract; *b*, DNA polymerase-δ; *c*, DNA polymerase-α; *d*, nuclear matrix proteins; *e*, BSA (as negative control). As anticipated both DNA polymerase-δ and -α antibodies showed immunoreactivity with the silk gland extracts (*a*) and the matrix-associated proteins (*d*). There was no heterologous cross-reactivity between the antibodies (*b* and *c*).

10% of the observed δ-like activity was caused by DNA polymerase-ε in the silk gland cells.[2] This increase in polymerase-δ and -α activities will be necessary and together contribute to meet the large increase in the total DNA (to the extent of 200,000–300,000 times the haploid genomic contents in the silk gland cells) during larval development (Niranjanakumari and Gopinathan, 1991). The inhibition of DNA polymerase activity by various compounds is taken as diagnostic for a particular polymerase function because of the nonavailability of appropriate mutants in higher eukaryotes. For instance, aphidicolin, a replication inhibitor, has been shown to inhibit DNA polymerase-α but not -β or -γ (Ikegami *et al.*, 1978; Ohashi *et al.*, 1978; Longiaru *et al.*, 1979) and therefore taken as supporting evidence for the replicative function of DNA polymerase-α. Moreover, this enzyme also possesses primase activity, allowing initiation of DNA synthesis during replication. However, polymerase-α does not possess any proofreading exonuclease activity desired for retaining the fidelity of DNA replication. On the other hand, DNA polymerase-δ possesses the proofreading exonuclease activity but is devoid of the primase function. The latter enzyme is also sensitive to the replication inhibitor aphidicolin. The exact mechanism by which aphidicolin inhibits DNA polymerase-δ is not known. Although aphidicolin distinguishes the activity of polymerase-δ from β or γ it could not discriminate the activity from polymerase-α. We have shown here that BuPhdGTP and antibodies to polymerase-δ or DNA polymerase-α could discriminate the two polymerases. Dresler and Frattini (1986) have reported that DNA replication in permeabilized human cells was less sensitive to BuPhdGTP inhibition than was polymerase-α. A similar observation has been made by Hammond *et al.*, (1987) that in CV-1 cells the concentration of BuPhdGTP which totally abolished the polymerase-α activity was much less effective in reducing DNA replication.

The antibodies of polymerase-α neutralize the activity of this enzyme (Tanaka *et al.*, 1982; Downey *et al.*, 1988) but fail to inhibit polymerase-δ (Wahl *et al.*, 1986; Lee and Toomey, 1987). We also show here that the polyclonal antibodies to polymerase-α from silk glands neutralize polymerase-α activity but not polymerase-δ. Similarly, the polyclonal antibodies to polymerase-δ were able to neutralize the activity of polymerase-δ while having no effect on DNA polymerase-α. There was no immunological cross-reactivity between these two enzymes when tested by Western blots, thus also substantiating that both polymerases are immunologically distinct.

An auxiliary protein for DNA polymerase-δ which markedly affects the polymerase activity and processivity of DNA polymerase-δ has been purified from calf thymus (Tan *et al.*, 1986; Prelich *et al.*, 1987). This protein specifically stimulates the activity of polymerase-δ and has no effect on polymerase-α. The auxiliary protein has been proved to be identical to PCNA/cyclin, based on its reactivity with monoclonal antibody to PCNA and the substitution of human PCNA by calf thymus auxiliary protein in SV40 replication (Prelich *et al.*, 1987). An auxiliary protein which stimulated the activity of polymerase-δ but with no effect on polymerase-α has been purified to homogeneity from silk glands. This protein was more effective compared with an auxiliary protein prepared from human placenta[2] in enhancing the activity of the homologous polymerase-δ. A similar observation was made with yeast PCNA and calf thymus polymerase-δ, in which the calf thymus enzyme required about 10 times more yeast PCNA than the homologous PCNA (Bauer *et al.*, 1988). The auxiliary protein from *B. mori* silk glands stimulated the catalytic activity by enhancing processivity of the enzyme. This observation taken together with its acidic nature suggest that the *B. mori* auxiliary protein is functionally identical to PCNA.

The type and extent of exonucleolytic proofreading that occur during DNA replication in animal cells are not clear. Since DNA polymerase-α plays a crucial role in DNA replication, the 3′ → 5′ proofreading exonuclease should act in concert with polymerase-α to achieve high fidelity during cellular DNA replication. However, most of the DNA polymerases-α reported so far are devoid of proofreading exonuclease activity (Holmes *et al.*, 1974; Mechali *et al.*, 1980; Nasheuer and Grosse, 1987). The presence of intrinsic exonuclease activity associated to polymerase-δ has suggested a role for this enzyme in maintaining fidelity during replication. The close association of exonuclease and polymerase-δ activities in *B. mori* is evident by the co-purification of these two activities, their co-ordinate inhibition by aphidicolin and sulfhydryl group blocking agents, resistance to BuPhdGTP, and stimulation by auxiliary protein. Here we also demonstrate the role of the exonuclease function of polymerase-δ from the silk glands in proofreading by mismatch correction. On the basis of the functional properties of polymerase-α and -δ, the currently assigned roles for these enzymes in DNA replication is as follows. DNA polymerase-α, by virtue of its associated primase activity, could initiate DNA synthesis and hand over to polymerase-δ to complete the elongation process with a high level of fidelity because of its intrinsic proofreading function. Thus, DNA polymerase-α could function as lagging strand replicase, and DNA polymerase-δ in association with the auxiliary protein could function as leading strand replicase. In a tissue like the silk glands of *B. mori* in which DNA replication carries on at a high level because of the chromosomal endoreplication phenomenon, large quantities of both polymerases are present, almost in equal ratios. Moreover, both of these enzymes are tightly associated with the nuclear matrix, substantiating their role in replication.

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[2] S. Niranjanakumari and K. P. Gopinathan, unpublished observation.

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