

DNA polymerase α -primase complex from the silk glands of the non-mulberry silkworm *Philosamia ricini*

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The DNA content in the silk glands of the non-mulberry silkworm *Philosamia ricini* increases continuously during the fourth and fifth instars of larval development indicating high levels of DNA replication in this terminally differentiated tissue. Concomitantly, the DNA polymerase α activity also increases in the middle and the posterior silk glands during development, reaching maximal levels in the middle of the fifth larval instar. A comparable level of DNA polymerase δ/ϵ was also observed in this highly replicative tissue. The DNA polymerase α -primase complex from the silk glands of *P. ricini* has been purified to homogeneity by conventional column chromatography as well as by immuno-affinity techniques. The molecular mass of the native enzyme is

560 kDa and the enzyme comprises six non-identical subunits. The identity of the enzyme as DNA polymerase α has been established by its sensitivity to inhibitors such as aphidicolin, *N*-ethylmaleimide, butylphenyl-dGTP, butylanilino-dATP and antibodies to polymerase α . The enzyme possesses primase activity capable of initiating DNA synthesis on single-stranded DNA templates. The tight association of polymerase and primase activities at a constant ratio of 6:1 is observed through all the purification steps. The 180 kDa subunit harbours the polymerase activity, while the primase activity is associated with the 45 kDa subunit.

INTRODUCTION

The multicomponent machinery of the highly complex DNA replication process is efficiently tuned to the cell cycle during growth, development and differentiation. Significant advances have been made in unravelling the components of the macromolecular assemblies participating in this process in both prokaryotes and eukaryotes, leading to the identification of multiple DNA polymerases and their accessory factors, each with a distinctive role in DNA metabolism (Kornberg and Baker, 1992). In eukaryotes, five classes of DNA polymerases have been described so far, namely α , β , γ , δ and ϵ (Burgers et al., 1990). DNA polymerases α and δ have been implicated in chromosomal DNA replication (Downey et al., 1988, 1990; Kornberg and Baker, 1992). DNA polymerase β , a repair enzyme, functions in short patch repair of DNA damaged by alkylation or ultraviolet radiation (Fry and Loeb, 1986; Hammond et al., 1990; Popanda and Thielmann, 1992). DNA polymerase γ is located within mitochondria and is responsible for mitochondrial DNA replication (Bolden et al., 1977; Rodsak and Schutz, 1978). Although the exact function of DNA polymerase ϵ has not been established, there is evidence to suggest its involvement in DNA replication and repair (Bambara and Jessee, 1991; Popanda and Thielmann, 1992; Niranjankumari and Gopinathan, 1993).

DNA polymerase α has been characterized from several eukaryotic sources, including human tissues and cultured cell lines. Among insect systems, DNA polymerase α has been isolated from *Drosophila* and *Bombyx mori*. The Eri silkworm *Philosamia ricini* is a domesticated silkworm belonging to the family Saturniidae which is also exploited for silk fibre production, although to a much smaller extent compared with the mulberry silkworm, *B. mori*. The silk gland of *P. ricini* is also made of three

anatomically and functionally distinct regions, resembling the *B. mori* silk glands in organization. The posterior silk gland (PSG) is committed to the synthesis of the silk protein, fibroin, while the middle silk gland (MSG) is specialized for the synthesis of the glue proteins, the sericins. The silk glands of *P. ricini*, however, are much longer than their *B. mori* counterparts. The DNA content of the *B. mori* silk glands is known to increase severalfold during the larval developmental stages (Niranjankumari and Gopinathan, 1991). Many rounds of DNA replication without accompanying cell or nuclear division result in polyploidization of the silk gland cells. Consequently these cells grow gigantic in size toward the late instars of larval development. This phenomenon of endomitosis or chromosomal endoduplication leads to the presence of multiple copies of even single-copy genes such as that for fibroin, within a single nucleus. Whether or not such mechanisms that are operative in *B. mori* are generalized features present in different varieties of silkworms was a matter of curiosity. The Eri silkworm *P. ricini* whose basic biochemistry has been little characterized, was therefore explored in the present studies. We report here the patterns of variation in DNA content as well as DNA polymerase activity in the MSG and PSG of *P. ricini* on different days of larval development during the fourth and fifth instars. The characterization of DNA polymerase α -primase complex from the Eri silk glands has also been accomplished.

MATERIALS AND METHODS

Materials

Non-radioactive NTPs and dNTPs, phenyl-Sepharose, cyanogen bromide-activated Sepharose CL-4B, poly(dA), poly(dA)·(dT)_{12–18} and restriction enzymes were from Pharmacia.

Abbreviations used: BuAn-dATP, butylanilino-dATP; BuPh-dGTP, butylphenyl-dGTP; DTT, dithiothreitol; FSB-dATP, 5'-fluorosulphonylbenzoyl-dATP; MSG, middle silk gland; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulphonyl fluoride; Poll.k, Klenow fragment of DNA polymerase I; PSG, posterior silk gland.

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Table 1 DNA content and DNA polymerase activity in PSG and MSG during larval development of *P. ricini*

One unit of DNA polymerase α activity corresponds to 1 nmol of [^3H]dTMP incorporated/h at 37 °C. nd, not determined.

Development stage and days	DNA content per gland (mg)		DNA polymerase α activity (units per gland)	
	PSG	MSG	PSG	MSG
Fourth instar				
1st	2.01	1.32	0.158	0.133
2nd	3.23	1.95	0.297	0.243
3rd	4.18	3.52	0.546	0.345
4th	8.09	4.23	0.948	0.416
Moult	8.41	4.61	nd	nd
Fifth instar				
1st	28.51	10.52	2.40	1.16
2nd	58.3	20.64	3.74	2.56
3rd	114.8	37.80	7.96	6.01
4th	196.4	43.7	6.85	6.38
5th	173.1	40.1	6.43	5.93
6th	158.6	36.8	5.77	5.62

The radiolabelled nucleotides [^3H]TTP, [^3H]UTP, and [$\alpha\text{-}^{32}\text{P}$]-dATP were from Amersham or Bhabha Atomic Research Centre (Bombay, India). DEAE-cellulose (DE-52) was from Whatman. Most of the other biochemicals and reagents such as Tris, dithiothreitol (DTT), SDS, EDTA, EGTA, calf thymus DNA (CT DNA), phenylmethanesulphonyl fluoride (PMSF), leupeptin, aprotinin and BSA were from Sigma Chemical Co. Butylphenyl-dGTP (BuPh-dGTP), and butylanilino-dATP (BuAn-dATP) were gifts from Dr. George Wright. 5'-Fluoro-sulphonylbenzoyl-dATP (FSB-dATP) was a gift from Dr. V. N. Pandey.

Buffers used in enzyme purification

All buffers contained 1 mM EDTA, 20 % glycerol, 1 mM DTT, 10 mM sodium metabisulphite, 1 mM PMSF, 0.5 $\mu\text{g}/\text{ml}$ leupeptin and 1 $\mu\text{g}/\text{ml}$ pepstatin. Buffer A contained 50 mM Tris/HCl, pH 7.8, 250 mM sucrose, 250 mM KCl and 5 mM MgCl_2 . Buffer B was similar except that the KCl concentration was 25 mM and sucrose was absent. Buffer C was similar to buffer B except for the presence of 500 mM KCl and 200 $\mu\text{g}/\text{ml}$ BSA.

Table 2 Purification of the DNA polymerase α -primase complex

Fractionation step	Total protein (mg)	DNA polymerase			DNA primase			Gradient at which the enzyme was eluted	Polymerase/primase ratio
		Total activity (units)	Specific activity (units)	Purification (fold)	Total activity (units)	Specific activity (units/mg)	Purification (fold)		
Crude extract	1280.0	564	0.44	1.0	91.55	0.071	1.0	—	6.16
DEAE-cellulose DE-52	358.0	438	1.22	2.77	72.19	0.20	2.8	90–150 mM KCl	6.07
Phosphocellulose P-11	29.2	383	13.11	29.8	65.2	2.23	31.4	200–300 mM KPO_4	5.87
DNA-Sephacrose	1.93	321	166.3	377.9	51.5	26.68	375.7	100–175 mM NaCl	6.23
Sephacryl S-1000	0.087	149.5	1718.0	3904.0	24.9	286.00	4028.0	—	6.00

Protein and enzyme assays

Protein contents were determined by the dye binding method (Bradford, 1976) or by the Folin Ciocalteu method (Lowry et al., 1951).

DNA polymerase α assay

DNA polymerase α activity was monitored by measuring the synthesis of DNA using activated calf thymus DNA as template-primer (Niranjanakumari and Gopinathan, 1991). The assay system contained, in a final volume of 50 μl , 50 mM Tris/HCl, pH 8.5, 5 mM MgCl_2 , 75 mM KCl, 1 mM DTT, 100 $\mu\text{g}/\text{ml}$ BSA, 100 μM each of dATP, dCTP and dGTP, 50 μM [^3H]TTP (specific radioactivity 100 c.p.m./pmol), 200 $\mu\text{g}/\text{ml}$ activated calf thymus DNA and the enzyme. One unit of DNA polymerase activity catalyses the incorporation of 1 nmol of [^3H]dTMP into DNA in 1 h at 37 °C. The DNA polymerase δ assay was similar to that for polymerase α except that the reaction was carried out at pH 6.5, and in the presence of BuPh-dGTP, a specific inhibitor of DNA polymerase α (Niranjanakumari and Gopinathan, 1992).

Primase assay

Primase activity was monitored by the synthesis of RNA primer from an unprimed single stranded (ss) DNA template followed by its extension by DNA polymerase. In the direct assay, the incorporation of [^3H]UTP into an RNA primer on the M13 DNA template was monitored. Alternatively, the assays were carried out by extending the non-radioactive primer synthesized in presence of externally added bacterial DNA polymerase and labelled dXTPs. In the latter method, either single-stranded M13 DNA or synthetic polydeoxynucleotide was used as template for primer synthesis in presence of unlabelled NTPs, followed by extension of the synthesized ribonucleotide primer by the Klenow fragment of DNA polymerase I (PolI.k) and monitored by [^3H]TTP incorporation.

Isolation and quantification of DNA

DNA was isolated from silk glands by phenol/chloroform extraction as described for eukaryotic DNAs (Maniatis et al., 1982) and estimated by the indole method (Ceriotti, 1952).

Polyclonal antibodies to DNA polymerase and the preparation of an immunoaffinity matrix

Polyclonal antibodies were raised in rabbits against purified (electrophoretically homogeneous) DNA polymerase α from the

silk glands of *B. mori* (Niranjanakumari and Gopinathan, 1991). The antibodies were partially purified by ammonium sulphate precipitation and Protein A–Sepharose column chromatography. The IgG fraction was dialysed against 0.1 M sodium bicarbonate buffer, pH 8.9, the protein concentration was adjusted to 5 mg/ml and the IgG was coupled to Sepharose 4B for the preparation of an affinity matrix. For the latter purpose 3 ml of CNBr-activated Sepharose 4B and 8–10 mg of antibodies were mixed together and reacted for 16 h at 4 °C. The binding of immunoglobulin to the matrix was followed by monitoring the A_{280} of the supernatant. To neutralize any free reactive groups, the material was shaken for an additional 2 h with 1 M Tris base, followed by washing with 0.1 M potassium phosphate, pH 7.8, and stored at 4 °C in the presence of 0.01 % sodium azide.

Purification of DNA polymerase α

All purification operations were performed at 0–4 °C. Eri silkworms (*P. ricini*) were reared in the laboratory on a natural diet of castor leaves. The silk glands from larvae on the third day of the fifth instar, when the DNA polymerase activity was at a maximum, were used for the enzyme isolation. The extracts were prepared from freshly dissected silk glands or glands kept frozen at –70 °C for up to 3 months, by homogenization in buffer A using a Dounce homogenizer. 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 dialysed against buffer B. The purification of DNA polymerase α -primase complex by conventional methods was achieved by chromatography on DEAE-cellulose, phosphocellulose, DNA–Sepharose and Sephacryl S-1000, as employed for the enzyme from *B. mori* (Niranjanakumari and Gopinathan, 1991).

For the immunoaffinity purification of DNA polymerase α from *P. ricini*, partially purified polyclonal antibodies against DNA polymerase α from *B. mori* were used, coupled to Sepharose 4B. As a first step, the S_{100} cytosolic fraction was chromatographed on a DEAE-cellulose column (40 cm \times 3 cm) equilibrated with buffer B. The enzyme activity eluting in 0.25 M NaCl was dialysed against buffer C and loaded on to the IgG–Sepharose column (2 cm \times 1 cm) equilibrated in buffer C. The column was thoroughly washed with buffer C, followed in sequence by buffer C containing 1 M and 2 M NaCl. The tightly bound proteins were eluted with 50 mM sodium acetate, pH 5.5, containing 1 M NaCl. The pH of the fractions was adjusted immediately to 7.0 using Tris base, and the solution was dialysed against buffer B.

RESULTS

DNA content and DNA polymerase activity in silk glands

The DNA content and the DNA polymerase activity in the MSG and PSG of *P. ricini* were examined on different days of larval development during the fourth and fifth instars. The DNA content of those cells increased continuously with the progress of development, with the quantities doubling within 48 h during the fourth instar or 24 h during the fifth instar (Table 1). DNA synthesis stopped abruptly in the middle of the fifth larval instar, at which time cellular growth was over and the cell activity was primarily directed towards massive silk synthesis. The absolute DNA contents in the PSG and MSG from *P. ricini* were greater than those in the corresponding tissues of *B. mori*; however, the pattern of DNA increase as a function of development was

Table 3 Immunoaffinity purification of DNA polymerase α

Fractionation step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)
Crude extract	1265	543	0.43	1.0
DEAE-cellulose	343	421	1.22	2.83
IgG–Sepharose	0.093	158.5	1704	3962

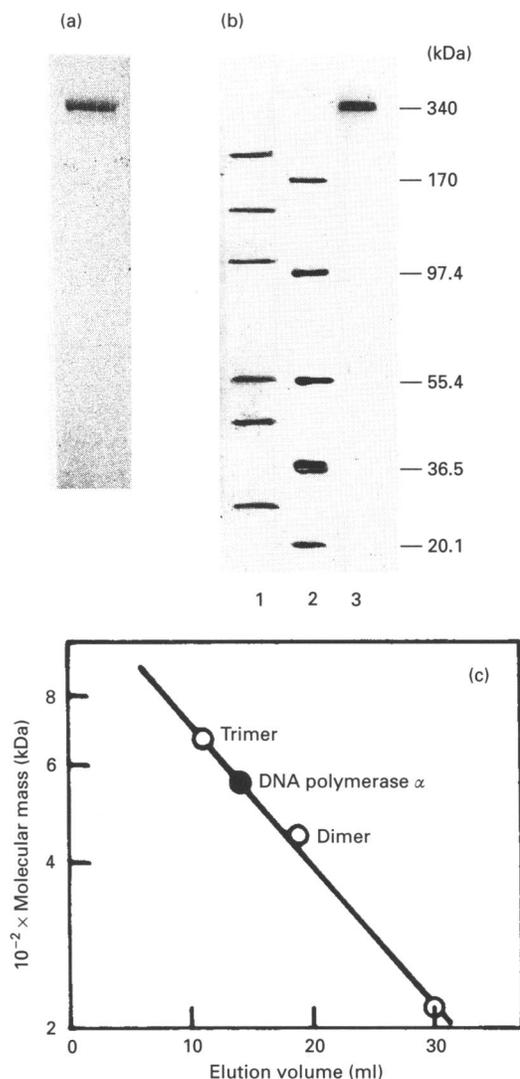


Figure 1 Homogeneity, subunit structure and molecular mass of DNA polymerase α

(a) PAGE. Purified DNA polymerase α (IgG–Sepharose column fraction; 1 μ g of protein) was subjected to electrophoresis on 4% polyacrylamide gels under non-denaturing conditions and the proteins were visualized by Coomassie Brilliant Blue R-250 staining. (b) SDS/PAGE. The enzyme samples were heated in the presence of β -mercaptoethanol (2.5%) and SDS (1%) at 85 °C for 10 min. Denaturing gel electrophoresis was performed on a 5–15% polyacrylamide gradient gel and the proteins were stained with Coomassie Brilliant Blue R-250. Lane 1, DNA polymerase α (IgG–Sepharose column fraction; 2 μ g of protein); Lanes 2 and 3, Standard molecular mass protein markers. (c) Determination of molecular mass by gel filtration. The immunoaffinity-purified enzyme was passed through a calibrated column of Sephacryl S-1000 and the emerging fractions were monitored for DNA polymerase activity. Cross-linked spectrin was used as standard marker (monomer, 220 kDa; dimer, 440 kDa; trimer, 660 kDa).

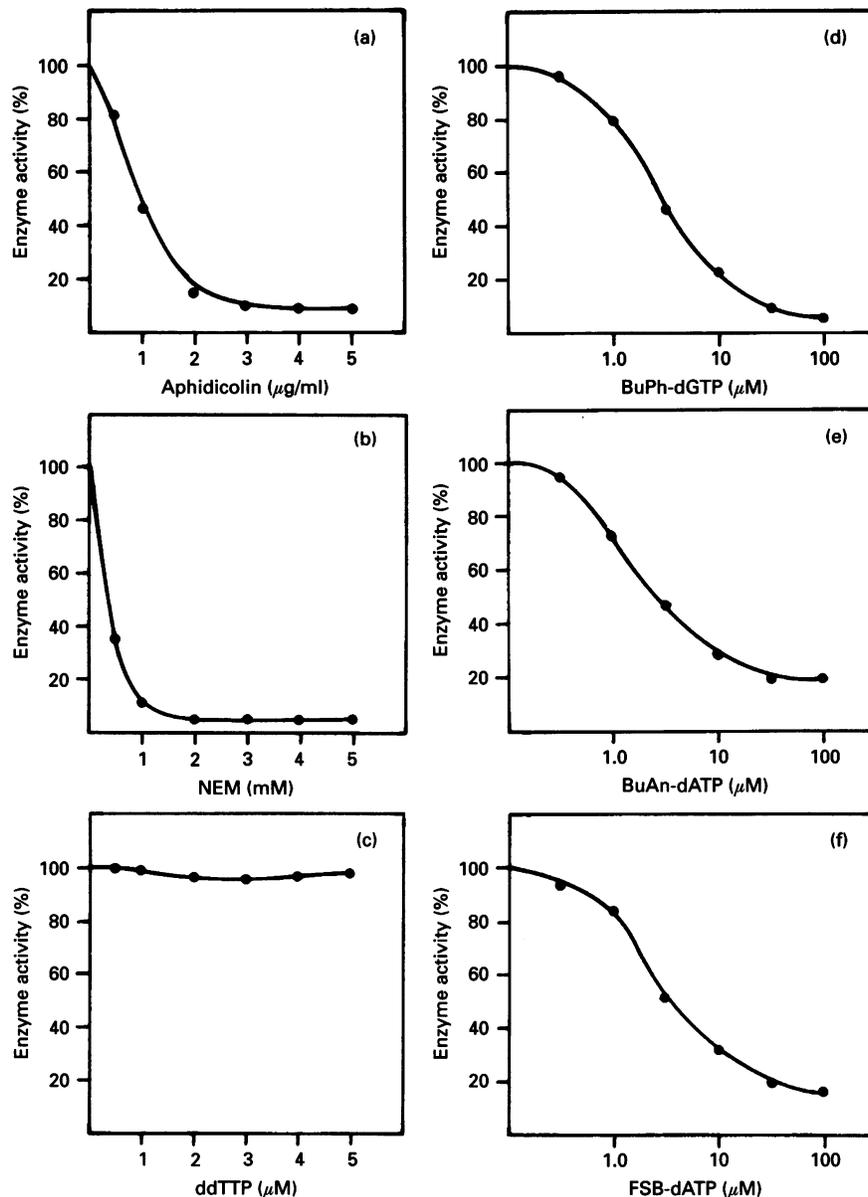


Figure 2 Effect of inhibitors on DNA polymerase α

The enzyme (0.1 unit) was incubated with the indicated concentrations of aphidicolin (a), NEM (b), ddTTP (c), BuPh-dGTP (d), BuAn-dATP (e) or FSB-dATP (f) at 4 °C for 20 min prior to the addition of assay mixture. The enzyme reaction was carried out at 37 °C for 30 min and DNA synthesis was monitored by the incorporation of acid-precipitable radioactivity.

similar in both organisms. Concomitantly with the DNA content, the DNA polymerase activity in the silk glands also increased until the third day of the fifth instar, and then fell. The enzyme activity increased by 50-fold in the MSG and PSG from the early part of the fourth instar to the middle of the fifth instar. The activity reported here was almost entirely due to DNA polymerase α -like activity, as these assays were done at pH 8.5 where the contributions due to polymerase δ and ϵ were negligible. During the peak period of DNA synthesis, i.e. the third day of the fifth instar, however, nearly equal amounts of DNA polymerase δ/ϵ activities (6.4 and 4.7 units per PSG and MSG respectively) were observed. The two latter activities were detected together in assays carried out at pH 6.5 and in the presence of BuPh-dGTP, thus eliminating any contribution due

to DNA polymerase α activity. There was no detectable DNA polymerase β -like activity in these cytoplasmic extracts.

Purification of the DNA polymerase α -primase complex

The purification of DNA polymerase α from Eri silk glands by the conventional method using a series of columns (ion exchangers, gel filtration and affinity chromatography) is summarized in Table 2. The adopted protocol resulted in a 4000-fold purified enzyme with a specific activity of 1700 units/mg and a yield of approx. 20%. The polyclonal antibody raised against the purified DNA polymerase α from *B. mori* was exploited to develop a rapid and efficient immunoaffinity protocol for the purification of polymerase from *P. ricini*. By this two-step

Table 4 Template efficiencies of various DNAs

Template DNA	Specific activity (units/mg of protein)
Calf thymus, native	113
Salmon sperm, native	167
Silk gland, native	279
Calf thymus, activated	1736
Salmon sperm, activated	1698
Silk gland, activated	1705
Poly(dA)·(dT) ₁₂₋₁₈	1487

Table 5 Primase activity associated with DNA polymerase

One unit of primer synthesis corresponds to 1 nmol of [³H]UTP incorporated/h at 37 °C. One unit of primer extension is the amount that leads to an incorporation of 1 nmol of [³H]TTP/h at 37 °C. ss, single-stranded.

Template	Specific activity (units/mg of protein)	
	Primer synthesis	Primer extension
Poly(dA)	211	2321
ss M13 DNA	294	4116
ss ϕ X174 DNA	316	5056

immunoaffinity isolation procedure, the enzyme obtained was homogeneous with a specific activity of 1700 units/mg and a yield comparable with that of the multistep isolation procedure (Table 3).

Homogeneity, subunit structure and molecular mass

The enzyme isolated from the IgG–Sephacryl S-1000 affinity column was electrophoretically homogeneous on a 4% polyacrylamide gel under non-denaturing conditions (Figure 1a). Electrophoresis on denaturing gels for subunit analysis revealed the presence of six subunits of molecular masses of 180, 140, 110, 55, 45 and 27 kDa (Figure 1b). The molecular mass of the native enzyme was found to be 560 kDa, as determined by gel filtration on Sephacryl S-1000 (Figure 1c).

Properties of DNA polymerase

The biochemical properties of the immunoaffinity-purified DNA polymerase α were examined. The enzyme was maximally active at pH 8.5, with about two-thirds of the optimum activity at either pH 7.5 or 9.0. At pH 6.5, where polymerase δ activity was maximum, less than 10% of the DNA polymerase α activity was observed. Thus the polymerase α activity could be distinguished from the polymerase δ activity based on the optimum pH for catalysis. The enzyme showed absolute requirements for cations, with the optimum concentrations of MgCl₂ and KCl being 5 mM and 75 mM respectively. Increasing NaCl/KCl concentrations affected the enzyme activity. Less than 10% of the enzyme

activity was observed at 250 mM NaCl/KCl, and thus the activity was readily distinguishable from the polymerase β -like activity. The K_m values were found to be 9, 12, 15 and 16 μ M respectively for dTTP, dCTP, dATP and dGTP.

Sensitivity to inhibitors

The effects of inhibitors such as aphidicolin, *N*-ethylmaleimide (NEM), dideoxy-TTP, BuPh-dGTP, BuAn-dATP and FSB-dATP on DNA polymerase α were examined (Figure 2).

Aphidicolin at 2 μ g/ml almost completely inhibited the activity (Figure 2a). The enzyme was sensitive to thiol group blocking agents. The activity was inhibited by almost 90% by 1 mM NEM (Figure 2b), but was not inhibited by ddTTP (Figure 2c). The effects of BuPh-dGTP and BuAn-dATP, known inhibitors of α -type DNA polymerases, are presented in Figures 2(d) and 2(e). Nearly 95% and 80% inhibition of DNA polymerase activity was observed at 10 μ M BuPh-dGTP and BuAn-dATP respectively. From these criteria the enzyme has been inferred to be DNA polymerase α . FSB-dATP, a structural analogue of the dATP which causes a stable covalent modification of DNA polymerase resulting in irreversible loss of enzyme activity, inhibited the Eri polymerase α by nearly 80% at 100 μ M (Figure 2f). The polyclonal antibody raised against DNA polymerase α from the *B. mori* silk gland was found to neutralize the DNA polymerase α activity from *P. ricini*. At 100 ng of antibody protein, almost 90% inhibition of the enzyme activity (1 unit) was seen.

Template efficiency

The template efficiencies of various DNAs for the enzyme are summarized in Table 4. Native DNA partially digested with DNAase I (activated DNA) or synthetic poly(dA)·(dT)₁₂₋₁₈ were found to be excellent templates for DNA synthesis. The enzyme did not utilize single-stranded DNA as a template in the absence of NTPs needed for primer synthesis.

Association of primase activity

DNA polymerase α from *P. ricini*, whether purified by conventional chromatographic methods or by the immunoaffinity procedure, was associated with primase activity catalysing the synthesis of RNA primers on single-stranded DNA templates in the presence of ribonucleotide triphosphates. Primer synthesis could be monitored either by incorporation of radiolabelled NTP into the RNA primer or by extension of the non-labelled primer synthesized, using externally added Poll.k (Table 5). Single-stranded DNA from phages M13 and ϕ X174, or poly(dA), could serve as a template for primer synthesis.

The tight association of polymerase and primase activities was observed all through the purification protocol. The ratio of polymerase to primase activity remained fairly constant (6:1) at every step of purification (Table 2). The two activities were co-purified and were concentrated approx. 4000-fold over the crude extracts. To test whether both activities were associated with the same polypeptide, the enzyme was dissociated using urea and the subunits were separated on urea/glycerol gradients (Figure 3a). The fractions corresponding to the 180 kDa and 45 kDa subunits were associated with the polymerase and primase activities respectively. The peak fraction containing primase activity when analysed on SDS/PAGE showed a single peptide band of molecular mass 45 kDa, whereas the polymerase activity was resident on the 180 kDa subunit (Figure 3b). Our previous studies (Niranjanakumari and Gopinathan, 1991) with DNA

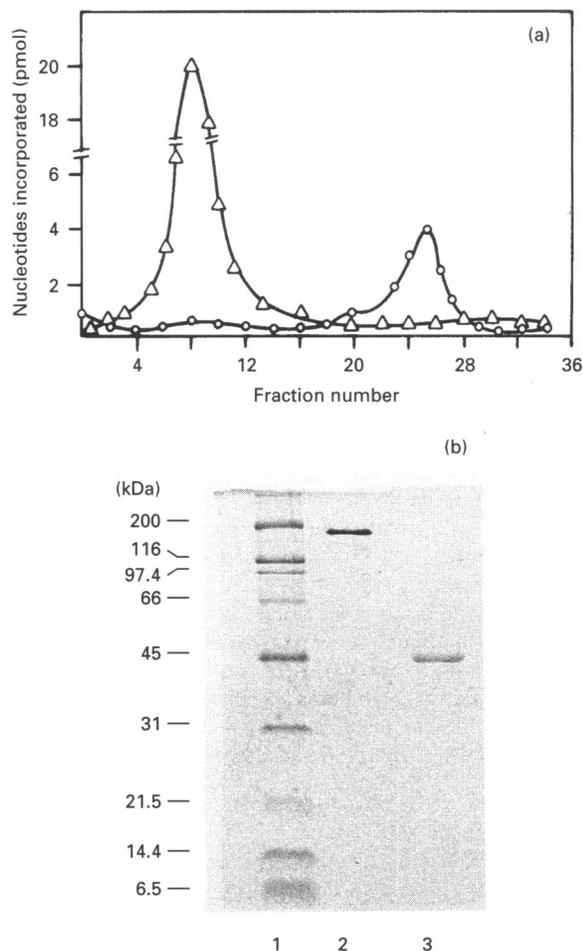


Figure 3 Polymerase and primase activities of DNA polymerase α

(a) DNA polymerase α (10 units) was dissociated using 2.8 M urea for 4 h at 4 °C and subjected to centrifugation on a 5–30% glycerol gradient containing 3.4 M urea in a Beckman SW-60 rotor at 4 °C for 16 h at 180 000 g. Fractions (0.2 ml) were collected and individually assayed for polymerase (Δ) and primase (\circ) activities. (b) SDS/PAGE analysis of the active subunits. The peak fractions showing the polymerase and primase activities were concentrated individually and analysed by SDS/PAGE (10% gel). The gel was stained using Coomassie Brilliant Blue R-250. Lane 1, Molecular mass markers; lane 2, the peak fraction (8) showing polymerase activity; lane 3, the peak fraction (25) showing primase activity.

polymerase α and *B. mori* by *in situ* enzyme activity staining of the electrophoretically separated subunits have demonstrated that the DNA polymerizing activity of that enzyme was also associated with the 180 kDa polypeptide.

DISCUSSION

The DNA content as well as the DNA polymerase α activity in the cells of both the PSG and MSG of *P. ricini* show a continuous increase as a function of development during the fourth and fifth larval instars, until it comes to an abrupt halt in the middle of the last instar. The DNA contents of *B. mori* silk glands are known to increase severalfold during the larval developmental stages (Niranjanakumari and Gopinathan, 1991). The increase in DNA content in *P. ricini* resembled the pattern in *B. mori*. The silk glands of *P. ricini*, however, are much longer than their *B. mori* counterparts. Presumably the silk gland of the Eri silkworm also

undergoes endomitosis (DNA replication without segregation), retaining a constant cell number. If the haploid genomic content of *P. ricini* per cell is assumed to be the same as in *B. mori* (Gage, 1974; Rasch, 1974), there are nearly 18 rounds of replication in the MSG and 19 rounds of replication in the PSG of the Eri silkworms.

The DNA polymerase α -primase complex was one of the first replication proteins identified by making use of the *in vitro* simian virus-40 DNA replication system (Li and Kelly, 1984, 1985; Stillman and Gluzman, 1985; Wobbe et al., 1985). Cell extracts immunodepleted of the polymerase α -primase complex were unable to replicate simian virus-40 DNA and the activity was restored by the addition of purified polymerase α -primase complex (Murakami et al., 1986). Substantial advances have been made in our understanding of mammalian DNA polymerase α in recent years. Prominent among these are the discovery of the associated primase activity which permits fresh DNA chain initiation, the conspicuous absence of any exonuclease activities implicating the absence of proof-reading functions, and the unusual susceptibility of the enzyme to proteolysis (Roth, 1987; Lehman and Kaguni, 1989; Challberg and Kelly, 1989; Stillman, 1989). In spite of the use of a variety of proteolytic inhibitors, the descriptions of the subunit structure of mammalian DNA polymerase α have been inconsistent (Fry and Loeb, 1986; Kaguni and Lehman, 1988). Even with highly purified preparations, multiple subunits varying in number, size and relative amounts have been demonstrated, many of which are presumed to arise as a result of proteolysis.

DNA polymerase α purified from the silk glands of Eri silkworms resembled its counterpart from *B. mori* in its biochemical properties and subunit structure. The identification of the enzyme isolated here as DNA polymerase α was based on its sensitivity to inhibition by aphidicolin, thiol group blocking agents and the known specific inhibitors of polymerase α such as BuPh-dGTP and BuAn-dATP. The enzyme activity was also neutralized by treatment with *B. mori* DNA polymerase α -specific antibody. The enzyme was comparable in its biochemical properties to DNA polymerase α from mammalian and other eukaryotic systems (Lee and Hurwitz, 1990; Kornberg and Baker, 1992). The native enzyme from *P. ricini* is a multimer of six non-identical subunits with a total molecular mass of 560 kDa. The immunoaffinity purification protocol adopted in the present study minimized the number of isolation steps and allowed the purification of DNA polymerase α in two steps, yielding a homogeneous enzyme preparation. The immunoaffinity-purified enzyme preparation also showed an identical subunit pattern to the enzyme purified by the column chromatographic procedures, thereby ruling out the presence of artefacts of proteolysis.

The consistency in the bifunctional nature of the DNA polymerase α -primase complex in a variety of eukaryotic cells and tissues implies a functional association of these two activities (Lehman and Kaguni, 1989; Wang, 1991; Kornberg and Baker, 1992). In calf thymus DNA polymerase α , both polymerase and primase catalytic sites were reported to be associated with the same polypeptide (Hubscher, 1983). Dissociation of enzymically active primase from the polymerase α -primase complex of yeast and *Drosophila* has been accomplished by the use of mild denaturing agents and the primase activity was shown to be associated with a heterodimer comprising polypeptides of approx. 55–60 kDa and 48–49 kDa (Kaguni et al., 1983; Cotterill et al., 1987; Roth, 1987). Whether or not both of the polypeptides are required for the catalytic activity is still controversial. Polyclonal antibodies raised against the 49 kDa polypeptide markedly inhibited primase activity, while antibodies to the 59

kDa subunit were much less inhibitory, suggesting that the active site of the enzyme resided on the former subunit (Nasheuer and Grosse, 1988). Affinity labelling studies have also shown that the ATP- or GTP-binding sites of the primase from calf thymus and yeast were localized exclusively in the 49 kDa subunit (Nasheuer and Grosse, 1988; Foiani et al., 1989). The precise role of the 59 kDa subunit is still uncertain. Recently Santocanale et al. (1993) have isolated the 48 kDa subunit of DNA primase (devoid of the 58 kDa subunit) from yeast, which was sufficient for RNA primer synthesis. This primase activity was highly unstable and the other polypeptides of the polymerase α -primase complex were presumed to play a role in stabilizing the enzyme activity. The subunits of DNA polymerase α from *P. ricini* could be readily dissociated in the presence of urea and the primase activity resided on the 45 kDa subunit. The peak fraction showing primase activity devoid of any other polypeptides of the polymerase α -primase complex retained the primer synthesizing capacity. The DNA polymerizing activity was resident on the 180 kDa subunit, resembling its counterpart from *B. mori* (Niranjanakumari and Gopinathan, 1991).

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