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A dideoxynucleotide-sensitive DNA polymerase activity characterized from endoreduplicating cells of mungbean (*Vigna radiata* L.) during ontogeny of cotyledons

Sujit Roy, Sailendra Nath Sarkar*, Sanjay K. Singh and Dibyendu N. Sengupta

Department of Botany, Bose Institute, Kolkata, India

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Correspondence
D. N. Sengupta, Department of Botany, Bose Institute, 93/1 AP.C. Road, Kolkata 700 009, India
Fax: +91 33 235 06790
Tel. +91 33 2350 6619 ext. 340
E-mail: dn_sengupta@rediffmail.com

Present address
*Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Calcutta-700019, India

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Within this work we describe the purification and biochemical characterization of a ddNTP-sensitive DNA polymerase purified from mungbean (*Vigna radiata* cv B1, L.) seeds at 18 days after fertilization, when > 70% of the nuclei are reported to be in the endoreduplicated state. The purified enzyme is a single polypeptide of 62 kDa and many of its physicochemical properties are similar to those of mammalian DNA polymerase β. Similar to the other X-family DNA polymerases, it lacks 3′–5′ exonuclease activity and has short gap-filling and strand-displacement activity. The enzyme shows moderately processive DNA synthesis on a single-strand template. The determined N-terminal heptapeptide sequence of the enzyme showed clear homology with helix 1 of the N-terminal single strand DNA-binding domain (residues 32–41) of rat and human DNA polymerase β. These results represent the first evidence for the identification and characterization of a ddNTP-sensitive DNA polymerase expressed during the endoreduplication cycle that shares biochemical and immunological similarity with mammalian DNA polymerase β.

The replication and repair of DNA involve the concerted activity of several enzymes and protein factors, including DNA polymerases, proteins associated with DNA polymerases (proliferating cell nuclear antigen, replication factor C, XRCC1, etc.), DNA primase, topoisomerase, helicase, DNA single-strand (ss) binding proteins, ribonuclease and ligase [1]. Our research interest lies especially with the functions of multiple DNA polymerase systems in plant DNA replication, repair and recombination and subsequently in cell proliferation and development.

To date, at least 15 classes of DNA polymerase have been identified in animals [2–4]. Although the presence of multiple DNA polymerases has been detected in plant systems [5–8], using purification and enzymological characterization, few reports are available regarding the molecular cloning of plant DNA polymerases [9–13].

Among all types of DNA polymerase, single polypeptide DNA polymerase β, cloned from mammalian systems [14–16], is strongly inhibited by ddNTP but not by aphidicolin or *N*-ethylmaleimide. Polymerase β is exclusively considered as a repair enzyme and studies have confirmed its involvement in the base excision repair pathway [17,18].

Previously, a 52-kDa DNA polymerase (polymerase CI) and a 100 kDa (polymerase 1) protein with ddNTP-sensitive DNA polymerase activity have been reported from wheat [7] and cauliflower [5], respectively. We also reported a 67-kDa polypeptide with ddNTP-sensitive DNA polymerase activity from the shoot tips of rice seedlings [19], where the enzyme showed extreme sensitivity to ddTTP and *N*-ethylmaleimide, although it was insensitive to aphidicolin, and also showed a distributive mode of DNA

Abbreviations
daf, days after fertilization; ss, single strand.

synthesis. Recently, we have shown that the 67-kDa ddNTP-sensitive DNA polymerase from rice is involved in the short patch base-excision repair pathway and is immunologically related to mammalian DNA polymerase β [20].

DNA endoreduplication is widespread in metabolically active plant tissues, particularly storage tissues like cotyledons and endosperms. As a consequence of DNA endoreduplication, cells replicate their nuclear DNA without any chromosome condensation, strand separation and cytokinesis, resulting in multiple uniform copies of nuclear DNA. Highly processive DNA polymerases such as DNA polymerase δ and/or ε and α (for repeated initiation) are involved in repeated rounds of DNA synthesis during endoreduplication. However, involvement of DNA polymerase β, a distributive enzyme associated with DNA repair, has been reported in DNA endoreduplication in rat giant trophoblast cells [21]. It is interesting that a repair-associated enzyme participates in repeated cycles of DNA replication. Similarly, inhibition of endoreduplication in the presence of ddNTP has been reported in cultured tobacco cells [22], thus providing a clue to the probable involvement of ddNTP-sensitive polymerase β-like DNA polymerase in DNA endoreduplication. However, there is no information regarding the identification and structure–function characterization of ddNTP-sensitive DNA polymerase from endoreduplicating cells.

In this study, we report for the first time in a plant system, the identification, purification and extensive characterization of a ddNTP-sensitive DNA polymerase with biochemical, structural and immunological similarity to mammalian DNA polymerase polymerase β. We also report its significant expression and activity in nuclear DNA endoreduplication during ontogeny of cotyledons in higher plant mungbean (*Vigna radiata* cv. B1).

Results

ddNTP-sensitive DNA polymerase activity in developing mungbean seeds during endoreduplication

In developing seeds of the mungbean plant, endoreduplication has been reported to be initiated 8–9 days after fertilization (daf), it continues through the 16–18 daf stages until seed maturity at 30 daf [23]. To understand the nature of DNA synthesis and determine the DNA polymerase(s) involved during these stages of seed development, an *in vitro* DNA polymerase assay was carried out using protein extracts prepared from developing mungbean seeds at 5–6 to 28–30 daf. An activity assay was also performed in the presence of different inhibitors in order to characterize the type of major DNA polymerase(s) involved. DNA polymerase activity was measured in terms of the incorporation of [^{3}H]-labeled dTMP using buffer-soluble protein extracts (S_{10} fraction) prepared from developing mungbean seeds at 5–6 to 28–30 daf. DNA polymerase activity showed a gradual increase from 5–6 to 16–18 daf, after which no significant increase was observed (Fig. 1A). *In vitro* DNA polymerase activity in each set of protein extracts, in the presence of different inhibitors of DNA polymerases (i.e. ddTTP at 10 μM, aphidicolin at 200 μM and *N*-ethylmaleimide at 1 mM final concentration) showed a significant degree of ddTTP-sensitive DNA polymerase activity from 5–6 to 16–18 daf with 10 μM of ddTTP, compared with the inhibition obtained with other inhibitors. Maximum ddTTP-sensitive DNA polymerase activity was noticed at 16–18 daf (75% inhibition), whereas, DNA synthesis showed sensitivity to aphidicolin from 19 to 21 daf onwards, and ~54% inhibition was observed at 25–27 and 28–30 daf (Fig. 1A). In mungbean seeds, > 70% nuclear endoreduplication was reported at 16–18 daf, reaching a maximum in mature seeds [23]. Thus, an increased level of ddNTP-sensitive DNA polymerase activity was observed from 8–9 to 16–18 daf, suggesting an important function and probable involvement of ddNTP-sensitive DNA polymerase in DNA synthesis during these stages. However, aphidicolin-sensitive DNA synthesis at seed maturation stages (19–30 daf) indicates the involvement of replicative DNA polymerases like α and δ.

Detection of expression of a 62-kDa polypeptide in developing, endoreduplicating mungbean seeds

Because the protein extracts from developing mungbean seeds showed significant levels of ddNTP-sensitive DNA polymerase activity at 9–18 daf, we analyzed protein extracts from 6 to 18 daf seeds using rabbit anti-(rat DNA polymerase β) (a well-known ddNTP-sensitive DNA polymerase) IgG (affinity purified, used at 1 : 20 000 dilution). Equal amounts of protein from 5–6 to 28–30 daf seeds were resolved on 10% SDS/PAGE and electroblotted on to a poly(vinyledene difluoride) membrane. Western bolt analysis using anti-(rat polymerase β) IgG showed expression of a 62-kDa polypeptide in all the indicated stages of the developing mungbean seeds (Fig. 2B,C), although there was significant variation in the expression levels. Expression of the 62-kDa band was rather weak at

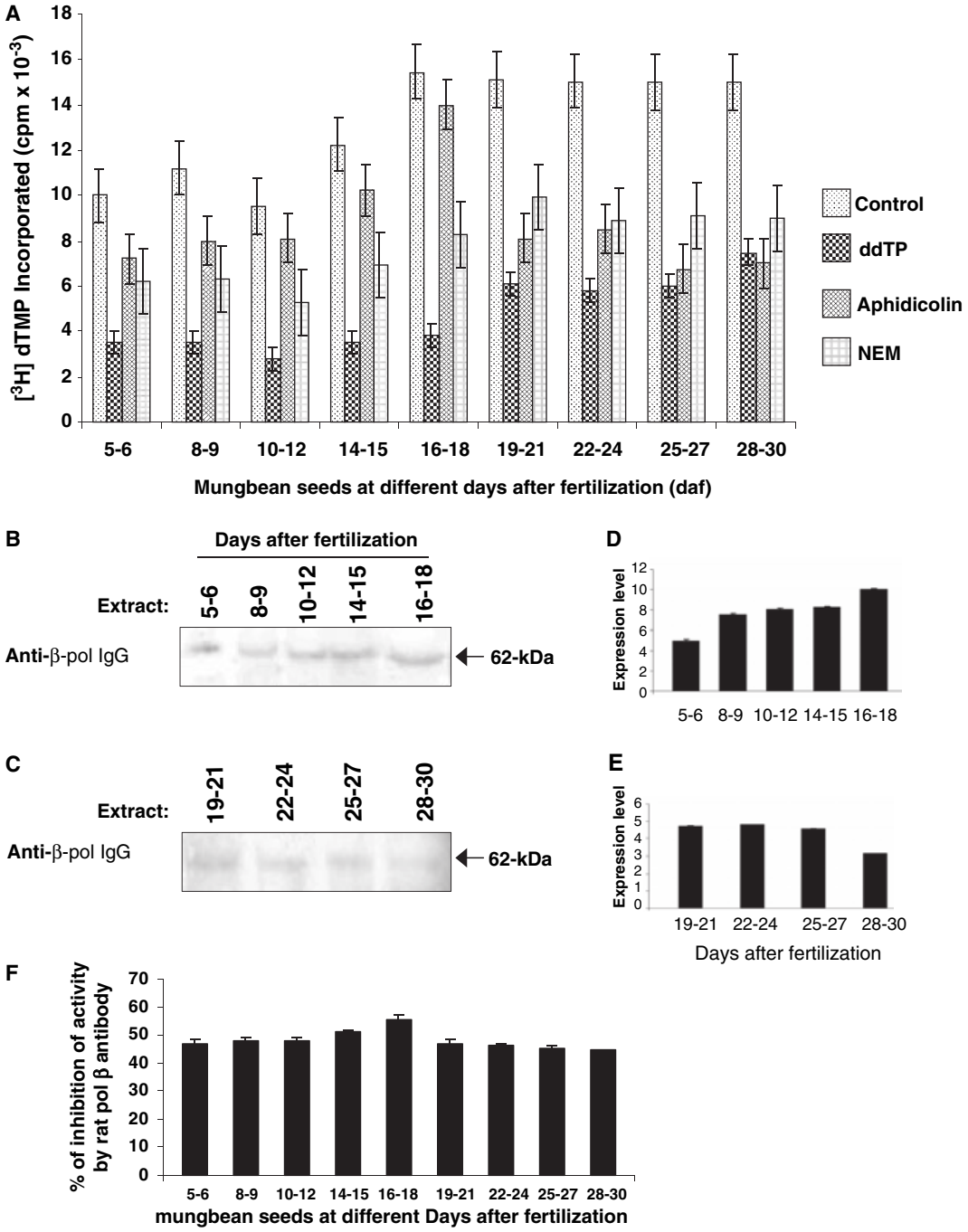


Fig. 1. Detection of ddNTP-sensitive DNA polymerase activity in the developing mungbean seeds. (A) *In vitro* DNA polymerase assay with protein extracts prepared from developing mungbean seeds at the indicated days after fertilization (daf) in the absence or presence of 10 μM ddTTP, 250 μM aphidicolin or 1 mM *N*-ethylmaleimide. Activated calf thymus DNA was used as template/primer. Three replicates were assayed in each case. Radioactivity in the trichloroacetic acid-insoluble fractions was determined and expressed as [^{3}H]-labeled dTMP incorporated (c.p.m. × 10^{-3}). (B,C) Western blot analysis of the protein extracts with rabbit anti-(rat polymerase β IgG) (affinity-purified IgG fraction at 1 : 20 000 dilution). Each lane contains ~30 μg of total protein. (D,E) Densitometric analysis of the immunoreactive bands. (F) Effect of anti-(rat DNA polymerase β) IgG on mungbean DNA polymerase activity was studied by preincubating 400 ng of affinity purified antibody with 30 μg of total protein extract prepared from mungbean seeds at different daf stages at 4 °C for 4 h with shaking. DNA polymerase activity assay was then carried out at 37 °C for 45 min using activated calf thymus DNA as template/primer.

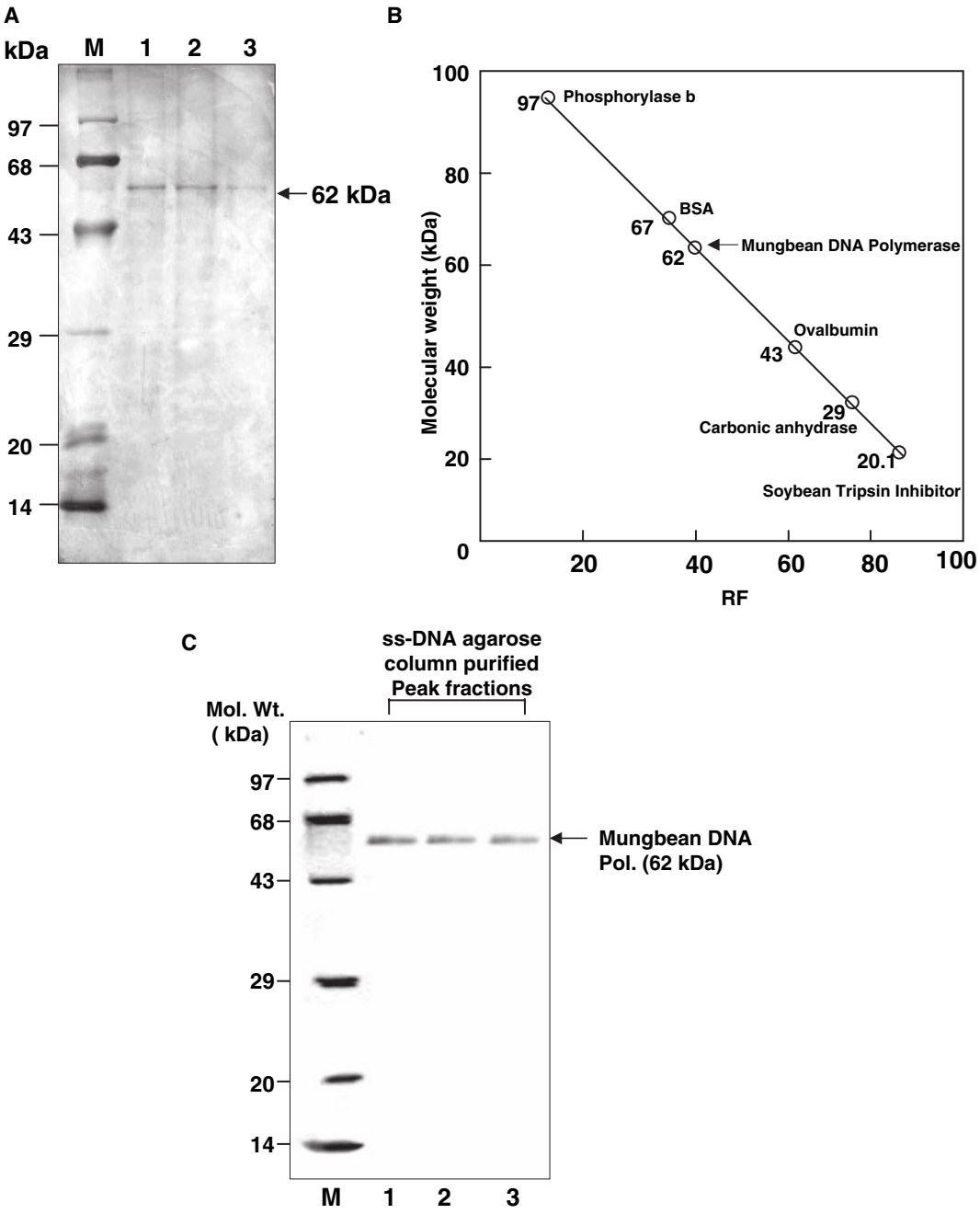


Fig. 2. Analysis of the purification of mungbean DNA polymerase by SDS–PAGE and western blotting. (A) Purified protein fractions (2.5 μg) (lanes 1–3) obtained from the ssDNA agarose column chromatographic step (fraction IV) were separated via 10% SDS–polyacrylamide gel and protein bands were detected by staining with silver salts. Molecular mass markers are shown on the left. (B) R_f values of standard proteins along with purified mungbean DNA polymerase. R_f values were calculated from the silver-stained gel. Western blot analysis of purified mungbean DNA polymerase (ssDNA agarose fractions, lanes 1–3) rabbit anti-(rat polymerase β) IgG at 1 : 20 000 dilution. Molecular mass markers are indicated on the left.

5–6 daf, but gradually enhanced expression levels were seen from 8–9 to 16–18 daf, as evident in the densitometric analysis (Fig. 1D). Expression then decreased from 19–21 daf onwards, but remained detectable (Fig. 1C). A low level of expression was noticed at 28–30 daf (Fig. 1E), at which time DNA polymerase

Table 1. Purification of ddNTP-sensitive DNA polymerase from 18-day-old developing seeds of mungbean (*Vigna radiata*, L. cv B1). Purification was carried out using successive column chromatographic steps including DEAE-Sephacel (2.5 × 8.5 cm), Phosphocellulose (2.1 × 8 cm), ssDNA agarose (1 × 5 cm) and Sephacryl S-200 (1.6 × 80 cm). Details of the procedure are given in Experimental procedures. After each purification step, ddNTP-sensitive DNA polymerase activity was measured. Almost 4986-fold purification was achieved after the final purification step with ~400 units·mg^{-1} of enrichment in specific activity.

Fraction	Volume (mL)	Protein (mg·mL^{-1})	Total proteins (mg)	Specific activity (units·mg^{-1})	Fold purification
Crude (S_{10})	250	11.1	2275	0.08	–
70% ammonium sulfate	45	11.9	535.5	1.7	21.25
DEAE-Sephacel	40	5.1	204.0	31.6	395
Phosphocelluse (P11)	10	2.0	20.0	45.6	570
ssDNA agarose	8	0.41	3.28	192.0	2400
Sephacryl S-200	2	0.05	0.10	398.8	4986

activity was more sensitive to aphidicolin than to ddNTP. Analysis of similar protein extracts using rabbit preimmune serum showed no detectable band at 62 kDa (data not shown), which also illustrates the immunological specificity of antibody recognition for the 62-kDa protein. This expression pattern of 62-kDa polypeptide was significant and was consistent with previous observations in the activity assay, in which we detected enhanced levels of ddNTP-sensitive DNA polymerase activity at 16–18 daf. These results indicate an active role for the 62-kDa polypeptide, with ddNTP-sensitive DNA polymerase activity at 16–18 daf when there is a high rate of nuclear endoreduplication.

To further substantiate these results, we tested the effect of anti-(rat polymerase β) IgG on DNA polymerase activity in developing seed protein extracts. Increased inhibition of activity in the presence of 400 ng of antibody was observed from 5–6 daf onwards and ~55% inhibition of activity was noted at 16–18 daf, whereas only 44–46% inhibition was observed from 19–21 daf onwards (Fig. 1F). The data also support our previous observation of elevated ddNTP-sensitive DNA polymerase activity and expression of a 62-kDa polypeptide mainly between 8–9 and 16-18 daf when the protein is present at considerable levels to exhibit ddNTP-sensitive activity, which in turn is effectively neutralized by the antibody.

Purification of ddNTP-sensitive DNA polymerase

To understand whether the ddNTP-sensitive DNA polymerase activity, which is enhanced at 16–18 daf, is conferred by the 62-kDa polypeptide (as detected by rat polymerase β antiserum at dilutions as high as 1 : 20 000, and which also showed increased expression at a similar stage), we purified the ddNTP-sensitive 62-kDa DNA polymerase from 18 daf mungbean seeds for subsequent characterization and analysis of the structure–function relationship. The enzyme was purified to near homogeneity from freshly harvested 18-day-old seeds by successive column chromatographic steps including DEAE-Sephacel, followed by phosphocellulose column, affinity column single strand (ss)DNA agarose and finally gel-filtration column Sephacryl S-200. After each purification step, ddNTP-sensitive DNA polymerase activity was measured by monitoring the incorporation of [^{3}H]-labeled dTMP into the trichloroacetic acid-insoluble fraction using activated calf thymus DNA as the template in the presence of 10 μM ddTTP. Finally, ~4986-fold purification was obtained. A summary of the purification of mungbean DNA polymerase is shown in Table 1.

Analysis of purification, molecular mass determination and general enzyme properties of mungbean DNA polymerase

In Sephacryl S-200 gel filtration, the activity of mungbean DNA polymerase appeared at 62 kDa (data not shown). SDS/PAGE analysis of the ssDNA agarose fractions revealed a single polypeptide band of 62 kDa (Fig. 2A), which was also estimated from the R_f values for the stranded protein molecular mass markers in the silver-stained gel (Fig. 2B). Anti-(rat polymerase β) IgG was found to specifically recognize purified mungbean DNA polymerase and a single distinct cross-reacting band of 62 kDa was obtained after incubating the blotted protein with the antibody at dilutions as high as 1 : 20 000 (Fig. 2C). The band was absent in the case of rabbit preimmune serum (data not shown), thus indicating that recognition of the DNA polymerase by the rat antibody is very specific.

The 62-kDa polypeptide was shown to have DNA polymerase activity, as revealed by activity gel analysis (Fig. 3A, lanes 2–4). An *Escherichia coli* Klenow

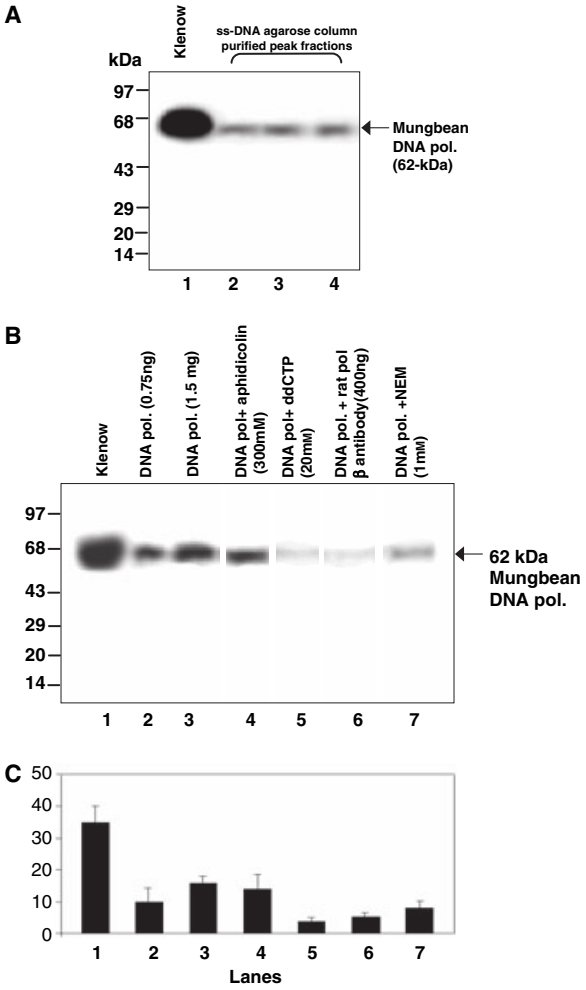


Fig. 3. In-gel activity analysis of purified mungbean DNA polymerase. (A) Peak fractions (0.75 μg) from the ssDNA agarose step (lanes 2–4) were used for in-gel activity analysis. One unit of Klenow enzyme (*E. coli* DNA polymerase I large fragment) was used as a positive control of known molecular mass (lane 1). (B) Activity gel analysis of purified DNA polymerase was carried out in the absence or presence of inhibitors of DNA polymerases and anti-(rat DNA polymerase β) IgG. One unit of Klenow was used as the control in lane 1. Lanes 2 and 3 contain 0.75 and 1.5 μg of purified mungbean DNA polymerase. In lanes 4–7, 1.5 μg of purified DNA polymerase was also incubated with 300 μM of aphidicolin (lane 4), 20 μM of ddCTP (lane 5), 400 ng of anti-(rat DNA polymerase β) IgG (lane 6) or 1 mM *N*-ethylmaleimide (lane 7). (C) Densitometric analysis of the bands to reveal relative activity using Bio-Rad Imaging Densitometer, GS-700.

fragment was used as a protein size marker (Fig. 3A, lane 1). The results indicate that mungbean DNA polymerase is a monopeptide with a molecular mass of 62 kDa. The monopeptide contains the primer-binding domain and is the catalytic subunit of the polymerase. Moreover, in-gel activity analysis also revealed strong inhibition of DNA polymerase activity with ddNTP, but not aphidicolin or *N*-ethylmaleimide (Fig. 3B, lanes 4, 5 and 7), as indicated from densitometric analysis of the bands (Fig. 3C). Whereas, approximately fourfold inhibition was seen in the presence of 10 μM ddCTP, only 1.2-fold reduction in activity was obtained in the presence of 300 μM aphidicolin, compared with the enzyme with no inhibitor. Anti-(rat polymerase β) IgG was found to inhibit activity to ~2.25-fold, and little (~ 1.6-fold) inhibition was seen with 1 mM *N*-ethylmaleimide. These results indicate significant sensitivity of mungbean DNA polymerase to ddNTP and insensitivity to aphidicolin and *N*-ethylmaleimide. The specificity of the immunological recognition of mungbean DNA polymerase by the antibody is also reflected by the inhibition of enzyme activity by the antibody in activity gel analysis (Fig. 3B, lane 6).

The pH optimum for mungbean DNA polymerase was 7.5, with 50% of optimum activity being expressed at pH 6.5 and > 50% at pH 8.5 (data not shown). The temperature optimum was 37 °C and activity was lost completely > 48 °C (data not shown). The enzyme required Mg^{2+} ions with an optimum concentration of 6 mM, although activity was significantly inhibited by Mn^{2+} ions even at low concentrations (Fig. 4A). The enzyme showed a requirement for high salt concentrations for activity, and monovalent cations such as KCl or NaCl stimulated polymerase activity at optimum concentrations of 100 or 75 mM, respectively (Fig. 4B). These results were conclusive using activated calf thymus DNA as the template-primer compared with a poly(dA)/oligo(dT) template (data not shown).

The K_m value for dTTP of mungbean DNA polymerase was 0.29 μM (Fig. 4C), close to the value of 0.3 μM of rice DNA polymerase for dTTP [19] and human polymerase β (0.33 μM) for UV-induced DNA damage repair [24]. The K_i value for ddTTP for mungbean DNA polymerase was 2.3 μM (Fig. 4D), slightly higher than value for ddTTP of the ddNTP-sensitive DNA polymerase from rice and of human DNA polymerase β (< 2.0 μM), but much less than that of DNA polymerase α (> 200 μM). Again, the results indicate the similarity of the enzyme with the polymerase β type DNA polymerase compared with the replicative polymerases.

Template/primer specificity

Study of the template specificity of mungbean DNA polymerase using different template/primer combinations (Table 2) showed that activated calf thymus DNA was the preferred template for the enzyme. Significant activity was also obtained with poly

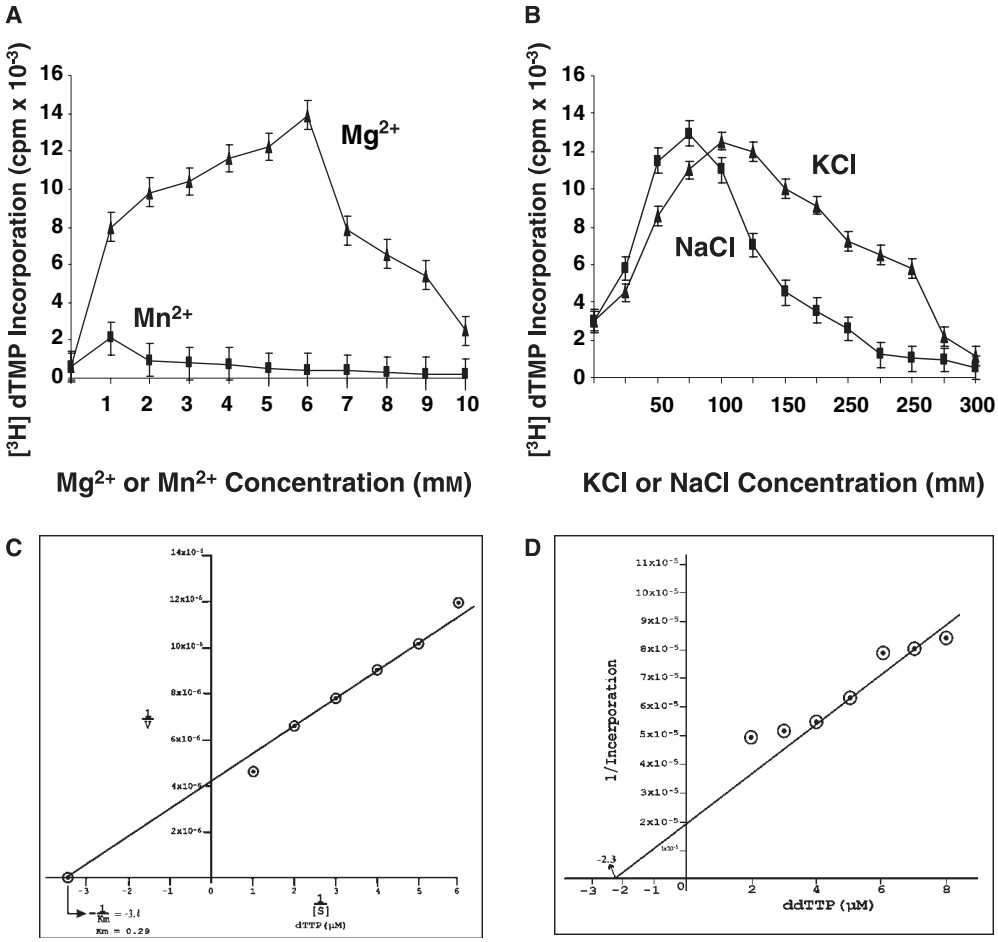


Fig. 4. Requirement for divalent cations, salt concentrations and K_m and K_i values. To determine the optimal concentration of divalent cations and salt concentrations, *in vitro* DNA synthesis assay reactions were carried out in the presence of the indicated increasing concentrations of $MgCl_2$ and $MnCl_2$ (A) or increasing concentrations of salts, KCl and NaCl (B). Radioactivity in the trichloroacetic acid-insoluble fractions was determined in the liquid scintillation counter (Beckman). Purified enzyme (200 ng) was used in each reaction with activated calf thymus DNA at a final concentration of 20 $\mu g \cdot mL^{-1}$ as the template/primer at buffer pH 7.5. Three replicates were taken for each point for all the reactions. (C) The K_m value was determined with increasing concentrations of [^{3}H]-labeled dTTP (0.1–0.6 μM) and plotting enzyme activity (V = pmoles of dTMP incorporated) against substrate concentration (S = μM [^{3}H]-labeled dTTP). (D) The K_i value for ddTTP was obtained by measuring DNA polymerase activity with increasing concentrations of ddTTP (2–8 μM) in the presence of its competitive substrate dTTP at 1 μM final concentration. Finally, the K_i value for ddTTP was determined by plotting the values in Dixon's plot (i.e. inverse of enzyme activity versus inhibitor concentration).

(dA)/oligo(dT) and M13 ssDNA:M13 universal primer. However, very poor incorporation was obtained with poly(rA)/oligo(dT) template/primer, which indicates that the enzyme cannot utilize an RNA template. Purified mungbean DNA polymerase preferred Mg^{2+} to Mn^{2+} and the incorporation of [^{3}H]-labeled dTMP was higher in the presence of 6 mM Mg^{2+}, than it was in the presence of 0.125 mM Mn^{2+}. By contrast, ddNTP-sensitive rice DNA polymerase showed a preference for poly(dA)/oligo(dT) template/primer although considerable activity was also obtained with activated calf thymus DNA and M13 ssDNA:M13 universal primer [19]. ddNTP-sensitive 52-kDa DNA polymerase (polymerase CI) from wheat showed the best incorporation rate with poly(dA)/oligo(dT) template/primer and significant activity was also reported with activated DNA and poly(rA)/oligo(dT) templates [7]. Together, these results indicate a preference of the enzyme for activated calf thymus DNA and also show its efficiency in utilizing poly(dA)/oligo(dT) template, like other ddNTP-sensitive DNA polymerases.

Table 2. Utilization of different template/primer by mungbean DNA polymerase. DNA polymerase activity was assessed with different combinations of template/primer in the presence of Mg^{2+} or Mn^{2+}; 200 ng of purified DNA polymerase was used for each reaction. Histograms, showing the template preference of mungbean DNA polymerase, were prepared from the c.p.m. values obtained in 10% trichloroacetic acid-insoluble fractions of the DNA polymerase activity assay reactions carried out with different combinations of template/primer. The c.p.m. values were converted into pmole dTMP incorporated per hour to prepare the histograms. Three replicates were taken for each template/primer combination and for each salt concentration (Mg^{2+} or Mn^{2+}).

Template	Divalent cations	pmols of [^{3}H]-labeled dTMP incorporated	% activity
Activated calf thymus DNA	Mg^{2+} (6 mM)	5.86	100
	Mn^{2+} (0.125 mM)	4.20	71
Poly(dA)/ Oligo(dT)$_{10-18}$	Mg^{2+} (6 mM)	4.80	81
	Mn^{2+} (0.125 mM)	3.90	66
Poly(rA)/ Oligo(dT)$_{10-18}$	Mg^{2+} (6 mM)	0.55	9.38
	Mn^{2+} (0.125 mM)	0.30	5.10
M13 ssDNA/M13 universal primer	Mg^{2+} (6 mM)	3.50	59.72
	Mn^{2+} (0.125 mM)	2.60	44.36

Effect of inhibitors

We studied the effect of some widely used DNA polymerase inhibitors on the activity of mungbean DNA polymerase. Enzyme activity was strongly inhibited by ddTTP and 60% inhibition was obtained in the presence of 2.5 μM ddTTP (1 : 12.5 molar ratio of dTTP : ddTTP). Complete inhibition was observed at 20 μM ddTTP (1 : 100 molar ratio of dTTP : ddTTP) (Fig. 5A). Approximately 20 and 40% inhibition was observed in the presence of 300 μM aphidicolin and 2 mM *N*-ethylmaleimide, respectively. These results indicate the extreme sensitivity of the enzyme to ddTTP, a property very characteristic of animal DNA polymerase β and other β-class enzymes characterized from rice, wheat (DNA polymerase CI) and cauliflower [5,7,19]. This is in contrast to the ddNTP-sensitive DNA polymerase from rice and wheat DNA polymerase CI, in which enzyme activity was strongly inhibited by *N*-ethylmaleimide. Animal DNA polymerase β is extremely resistant to SH-reagents like *N*-ethylmaleimide.

Highly basic polyamines like spermine and spermidine, as well as the basic protein histones, have been shown to affect DNA polymerase activities differently, depending on the nature of the enzyme. As shown in Fig. 5D, different concentrations of spermine and spermidine (2–10 mM) were used to study their effects on mungbean DNA polymerase activity. Increasing concentrations of spermine inhibited DNA polymerase activity, whereas with spermidine we observed a significant stimulation of activity at 2–4 mM. At higher concentrations of spermidine, enzyme activity reached a plateau. Interestingly, at 10 mM spermidine, the activity was still higher than the control (without spermidine). The data are consistent with results for wheat DNA polymerase CI in whcih spermine showed a strong inhibition, whereas spermidine was shown to stimulate the activity at 2 mM. However, in contrast to mungbean enzyme, wheat DNA polymerase CI showed distinct inhibition of activity in the presence of spermidine at concentrations > 2 mM [7]. In mammalian cells, spermidine has been reported to stimulate the activity of rat DNA polymerase β [25].

It has been shown that heparin, together with calf thymus DNA template, commonly used as the template in DNA polymerase assays, strongly inhibited DNA polymerase α and δ activity [26]. As shown in Fig. 5E, increasing heparin concentrations did not significantly inhibit mungbean DNA polymerase activity. At 400 ng heparin, only 15% inhibition was obtained. Wheat DNA polymerase CI also showed insensitivity to heparin at concentrations up to 1 μM [7].

Anti-(rat polymerase β) IgG specifically neutralizes mungbean DNA polymerase activity

Rat DNA polymerase β antibody specifically recognizes mungbean DNA polymerase in buffer-soluble protein extracts, as well as the purified enzyme as a single band of 62 kDa at antibody dilutions up to 1 : 20 000. Recognition of the 62-kDa polypeptide by the rat antibody was very specific because nonimmune rabbit serum failed to recognize the band in either crude extract or the purified preparation. We tested the neutralization of activity of mungbean DNA polymerase in the native form in the presence of increasing amounts of rat DNA polymerase β antibody (Fig. 6A). The antibody was found to inhibit mungbean DNA polymerase activity and $\sim$60% of inhibition was obtained in the presence of 400 ng of antibody. Similar amounts of BSA (used as a negative control) had no significant inhibitory effect on the activity of purified mungbean DNA polymerase. The activity of the Klenow enzyme (*E. coli* DNA polymerase I large fragment, a ddNTP-sensitive enzyme) was unaffected by similar amounts of antibody (Fig. 6B). Thus, it appears that recognition and activity neutralization of mungbean DNA polymerase by the antibody was very specific. It is suggested that the epitope of mungbean enzyme recognized by the

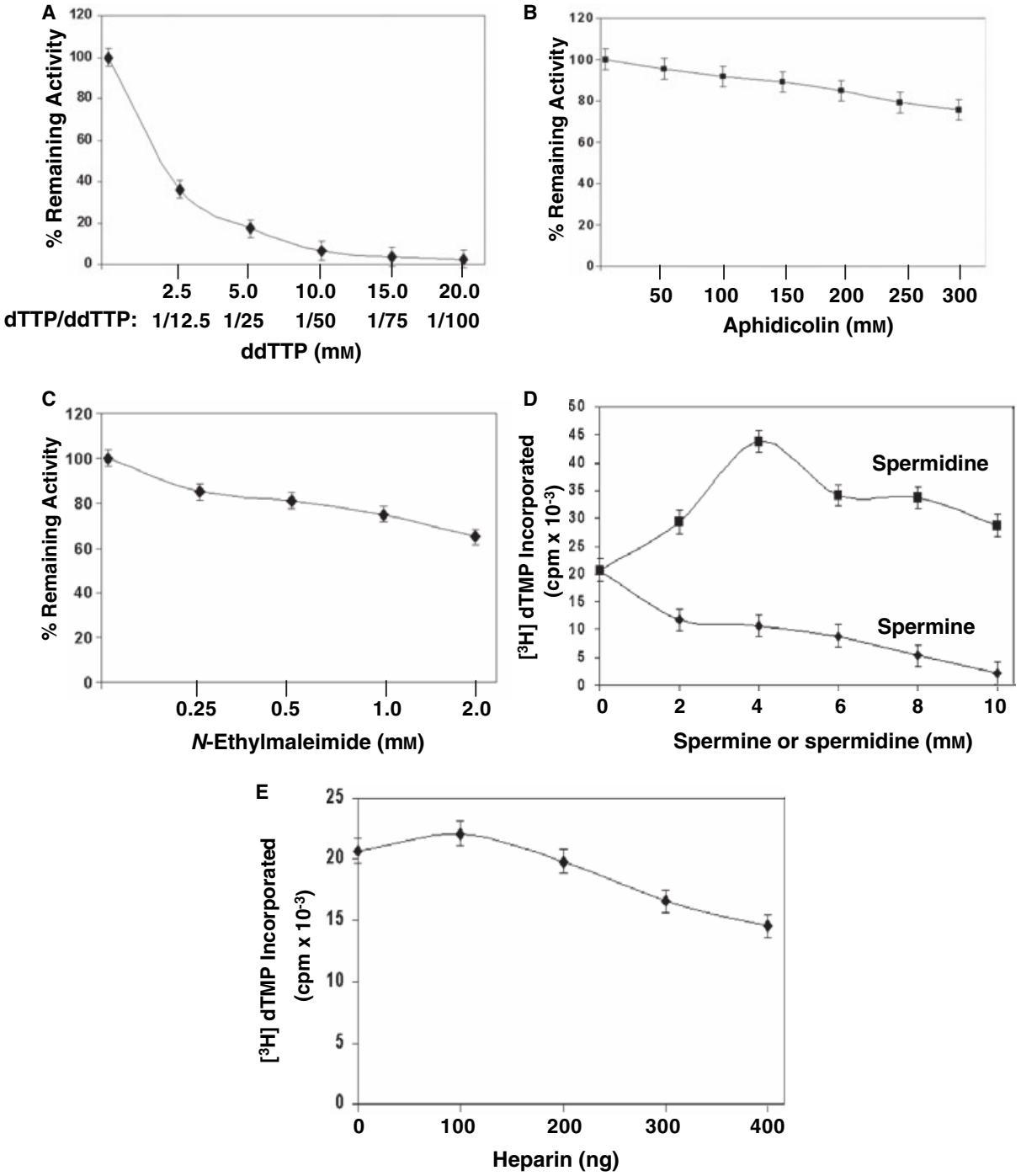


Fig. 5. Effect of inhibitors on the activity of purified mungbean DNA polymerase. The influence of different inhibitors on the activity of mungbean DNA polymerase was studied by carrying out *in vitro* DNA synthesis in the absence or presence of different concentrations of inhibitors (as indicated in A–E). In all the reactions, 200 ng of purified mungbean DNA polymerase was used. Activated calf thymus DNA was used at final a concentration of 20 $\mu g \cdot mL^{-1}$ in buffer at pH 7.5. Three replicates were assayed for each inhibitor concentration.

antibody must be away from the enzyme active site, because immunodetection with the antibody was achieved at a dilution of 1 : 20 000, whereas activity neutralization required a larger amount of antibody. Recognition of mungbean DNA polymerase by anti-(rat polymerase β) IgG clearly suggests an immunological relationship between ddNTP-sensitive DNA polymerase from mungbean and rat.

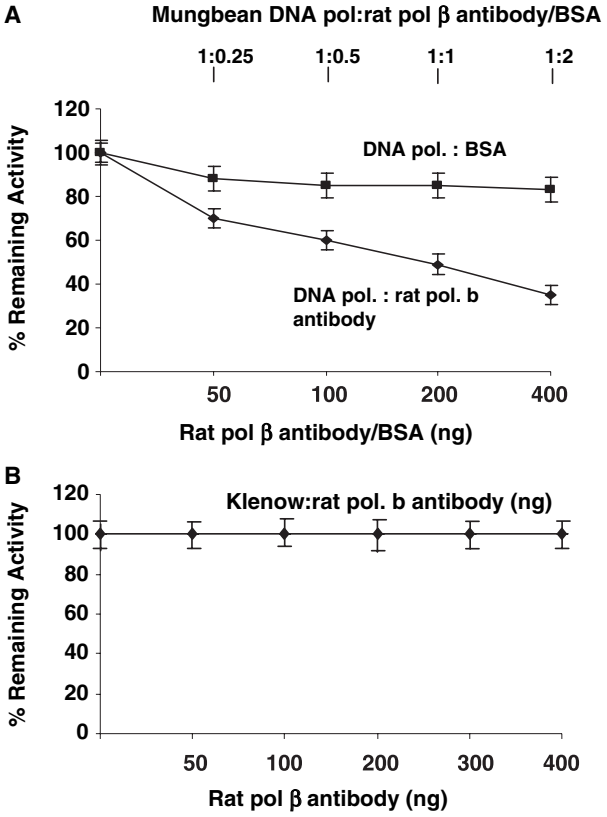


Fig. 6. Effect of anti-(rat DNA polymerase β) IgG on the activity of mungbean DNA polymerase. (A) To study the activity neutralization ability of anti-(rat DNA polymerase β) IgG, 200 ng of purified mungbean DNA polymerase was preincubated with an increasing amount of anti-(rat DNA polymerase β) IgG (50–400 ng of affinity purified IgG fraction) or with purified BSA at 4 °C for 4 h with shaking and then DNA polymerase activity assay was then carried out at 37 °C for 45 min using activated calf thymus DNA as the template/primer. (B) One unit of Klenow enzyme (*E. coli* DNA Pol I large fragment) was preincubated with increasing amounts of anti-(rat DNA polymerase β) IgG (50–400 ng) and then *in vitro* DNA synthesis was carried out. Three replicates were considered for each point.

Comparison of mungbean DNA polymerase N-terminal sequence with other X-family DNA polymerases

We determined the N-terminal heptapeptide sequence of purified mungbean DNA polymerase. Comparison of the N-terminal heptapeptide sequence TLEKYNI with the N-terminal regions corresponding to amino acid residues 32–41 of rat and human DNA polymerase β, amino acids 33–42 of *Xenopus* DNA polymerase β, amino acids 43–52 of bovine DNA polymerase β, and amino acids 24–33 of yeast (*Saccharomyces cerevisiae* and *S. pombe*) DNA polymerase IV is shown in Fig. 7A. The analysis revealed a considerable degree of homology between the mungbean DNA polymerase N-terminal heptapeptide sequence and DNA polymerase β from the indicated sources. Multiple sequence alignment of mungbean N-terminal heptapeptide sequence with that of other X-family DNA polymerases showed a rather weak homology with TdT and polymerase μ but a considerable homology with DNA polymerase λ, although not as high as observed with DNA polymerase β sequences. We also noted the presence of a 'KYN' motif in the mungbean DNA polymerase heptapeptide sequence, which was identical to that of rat, human and *Xenopus* DNA polymerase β N-terminal sequences (amino acids 32–41 in rat and human and 33–42 *Xenopus*). Moreover a characteristic K residue in the mungbean DNA polymerase heptapeptide sequence was found in an identical position in all other DNA polymerase β sequences studied.

Processivity of purified mungbean DNA polymerase

To study the nature of nucleotide incorporation by mungbean DNA polymerase, primer extension DNA synthesis was carried out using M13 mp18(+) ssDNA as a template with the 5′-[^{32}P]-labeled 17-mer M13 forward sequencing primer (−40 downstream oligo) (Fig. 8A). Radiolabeled products of different reactions were separated on an 8% DNA sequencing gel. Analysis of reaction products in the denaturing gel revealed that mungbean DNA polymerase carries out moderately processive DNA synthesis. The primer was elongated by an average of 35 nucleotides and also showed significant variation in response to changes in reaction condition (data not shown). As shown in Fig. 8B, the processivity assay was carried out at different time points, e.g. 5, 10, 15, and 20 min of incubation at 37 °C. Larger products with increased intensity were obtained within 20 min of incubation. As a whole, the enzyme showed moderately processive DNA synthesis that was evidenced by the presence of a few stepladders at the lower part of the gel, which indicate a distributive synthesis during the initial conditions. A study of the processivity clearly indicates that mungbean DNA polymerase is able to produce larger products of ~30–35 nucleotides with efficient incorporation of labeled primer into distinct larger products (Fig. 8F, lanes 3,4). This indicates a true moderately processive DNA synthesis and is not due to continuous distributive synthesis over time. However, in terms of processivity, mungbean DNA polymerase lags behind than that of *E. coli* Klenow

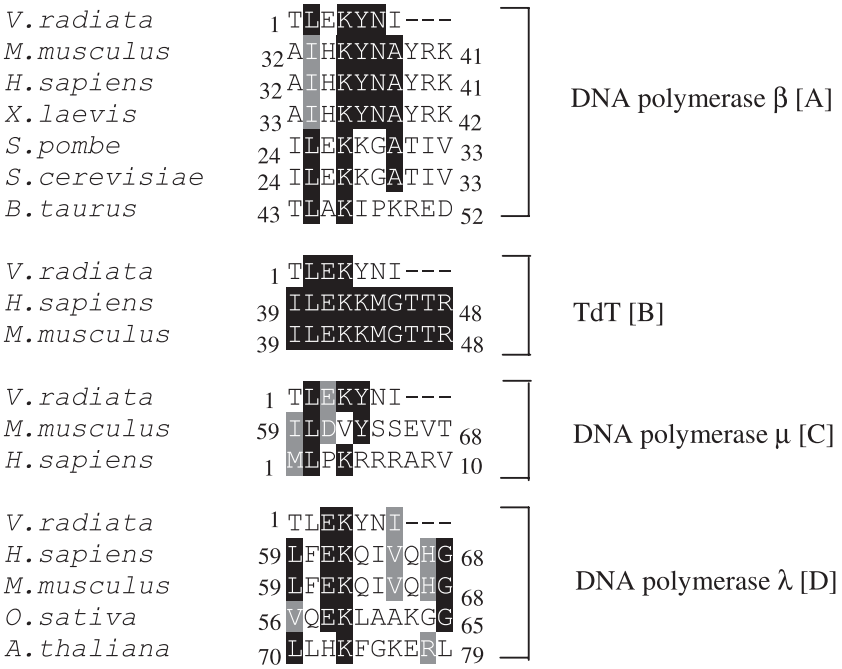


Fig. 7. Comparative analysis of the N-terminal heptatide sequence of purified mungbean DNA polymerase with the other X-family DNA polymerases: (A) DNA polymerase β, (B) terminal deoxynucleotidyl transferase (TdT), (C) DNA polymerase μ and (D) DNA polymerase λ. Analysis was carried out using the CLUSTALW sequence alignment service at EBI (http://www.ebi.ac.uk/clustalw). Dark areas indicate identical amino acid sequences, whereas shaded areas indicate similar amino acid sequences.

enzyme, which is a highly processive DNA polymerase and produced larger products with 20 min of incubation at 37 °C (Fig. 8B, lane 5). The moderately processive DNA synthesis by the enzyme is significant as it showed increased expression and activity during the active endoreduplication stages (16–18 daf), again indicating the functional relevance of this DNA polymerase in replication events.

Short gap-filling DNA synthesis and strand-displacement activity by mungbean DNA polymerase

Mammalian polymerases β and λ can both fill short gaps in DNA intended to mimic gapped intermediates in base-excision repair and nonhomologous end-joining pathways. Synthesis to fill short gaps by polymerase β and polymerase λ is facilitated by a phosphate group on the 5′-end of the gaps, which can bind to positively charged regions in their N-terminal 8-kDa domains. The capacity of the 8-kDa domain to bind a terminal phosphate group is particularly important for both processivity and binding of polymerase β during gap-filling synthesis [27]. We therefore compared the gap-filling activity of mungbean DNA polymerase using a normal template/primer (as control) and a gapped substrate with or without a phosphate group at its 5′-side (Fig. 9A). As can be seen in Fig. 9B,C, no significant difference in gap-filling activity was observed in the presence or absence of a phosphate group at the 5′-end of the gapped substrate. A distinct gap-filled product of ~20 nucleotides (produced by the addition of three nucleotides to the 17-mer-labeled primer) was detected at all time points (Fig. 9B,C). The enzyme also showed a significant strand displacement activity as it inserted several additional nucleotides after filling the gap.

To further substantiate the result, we carried out short gap-filling activity of mungbean DNA polymerase in the presence of both increasing amount of the enzyme (Fig. 9D, lanes 2–4) and the 5′− phosphate-containing gapped substrate (lanes 5–7). As shown in Fig. 9D, in the absence of gapped substrate mungbean DNA polymerase showed moderately processive synthesis and most of the labeled primers were incorporated into larger products (lane 1). Increasing amounts of the DNA polymerase (0.5–1.0 μg purified DNA polymerase) efficiently filled the gap and incorporated several additional nucleotides after filling the three-nucleotide gap. This illustrates the strand-displacement activity (lanes 2–4). DNA ligase was not incorporated into the reaction mixture and double the amount of the unlabeled (−20 oligo) was used. Therefore, products appeared at the upper region of the gel indicating that after completely filling the gap of three nucleotides, incorporation occurred at the 3′OH end of the labeled (−40) downstream primer and due to strand displacement activity, the enzyme continued synthesis. With increasing amounts of template/primer complex (2–6 pmole in lanes 5–7), incorporation of up

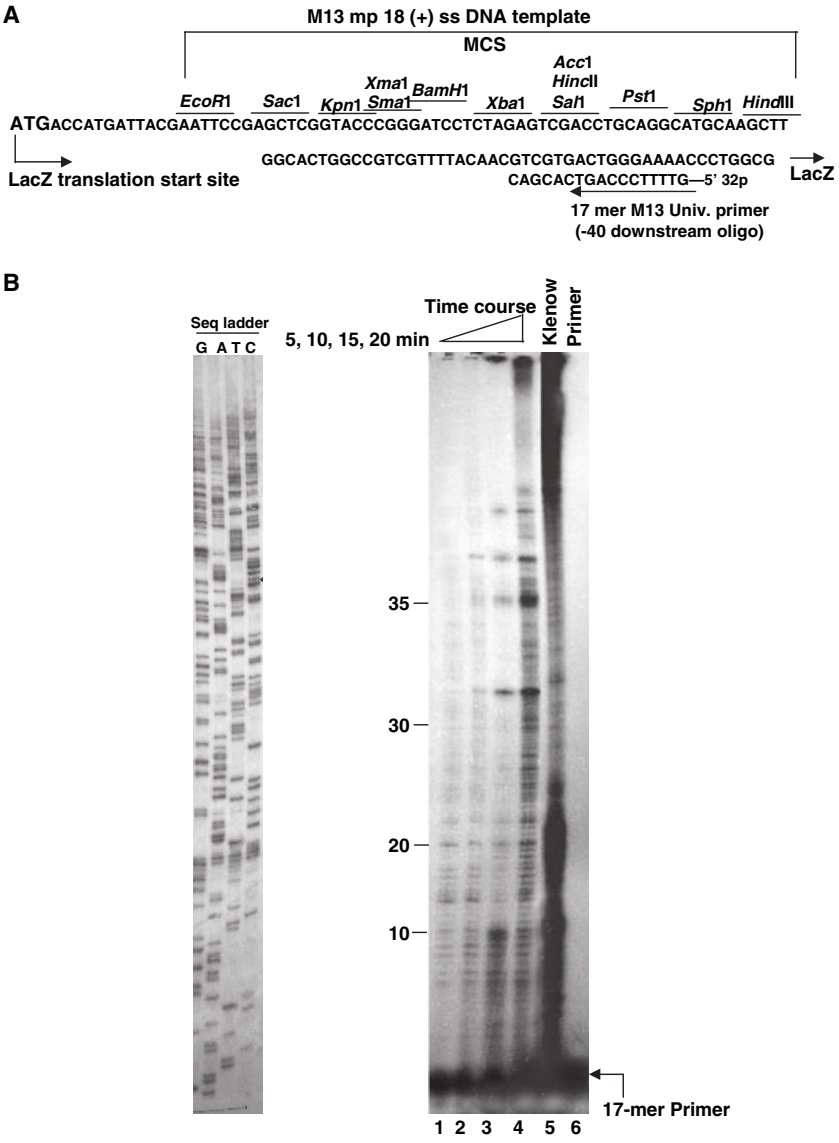


Fig. 8. Analysis of processivity of purified mungbean DNA polymerase. (A) Shown is an illustration of the annealing of 5′-[^{32}P]-labeled M13 forward sequencing primer (–40) (17 nucleotides in length) to the M13 ssDNA template. The oligonucleotide was designed around the multiple cloning site of M13 mp 18(+) ssDNA template to monitor the primer extension activity of mungbean DNA polymerase to extend the labeled primer along with the ssDNA template ahead of the primer. (B) Shown is the 8% sequencing gel image of primer extension products generated by mungbean DNA polymerase. The end-labeled primer was hybridized to the template in a 5 : 1 ratio and 200 fmole of hybridized template/primer was used in each reaction. Processivity was carried out for 5, 10, 15 and 20 min at 37 °C (lanes 1–4, respectively). Lane 5 shows primer extension with 1 U of Klenow (Roche, Germany) at 37 °C for 30 min; lane 6 shows the labeled primer without enzyme. Purified DNA polymerase (1.0 μg) was used in the reactions. A sequencing ladder is shown on the left with arrows positioning to number of nucleotides produced by addition of dNMP.

to 35 nucleotides was observed at 6 pmole of template/primer (lane 7). This also indicates the strand-displacement activity of the DNA polymerase at higher amounts of gapped substrate. Strand-displacement but not gap-filling activity was inhibited in the presence of 10 μM dTTP (lane 8) or rat polymerase β antibody (lane 9), but remained more or less unaffected in the presence of aphidicolin (lane 10) or *N*-ethylmaleimide (lane 11) indicating that after gap-filling further extension of the primer by the enzyme was selectively inhibited by ddTTP and the rat polymerase β antibody.

3′–5′ Exonuclease proofreading activity

To determine whether purified mungbean DNA polymerase contains a 3′–5′ exonuclease proofreading activity, *in vitro* DNA chain extension of a 3′ mismatch primer (Exo2) was examined along with a primer with the normal 3′-end (Exo1, Fig. 10A) using ϕX174 ssDNA template. As shown in Fig. 10B, with normal template/primer, the enzyme showed processive DNA synthesis with the incorporation of ~40 nucleotides after incubation at 37 °C for 35–60 min (lane 3–5). Klenow enzyme, used as control, showed extension of both the normal and mismatch template/primer complex, as expected (lanes 1, 8), however, mungbean DNA polymerase was unable to extend the mismatch primer (Fig. 10B, lane 9). Like enzyme activity, extension of Exo1 was inhibited by ddTTP (lane 6) but not aphidicolin (lane 7).

Activity assays using normal and mismatch primers are shown in Fig. 10C. Radioactivity in the trichloro-

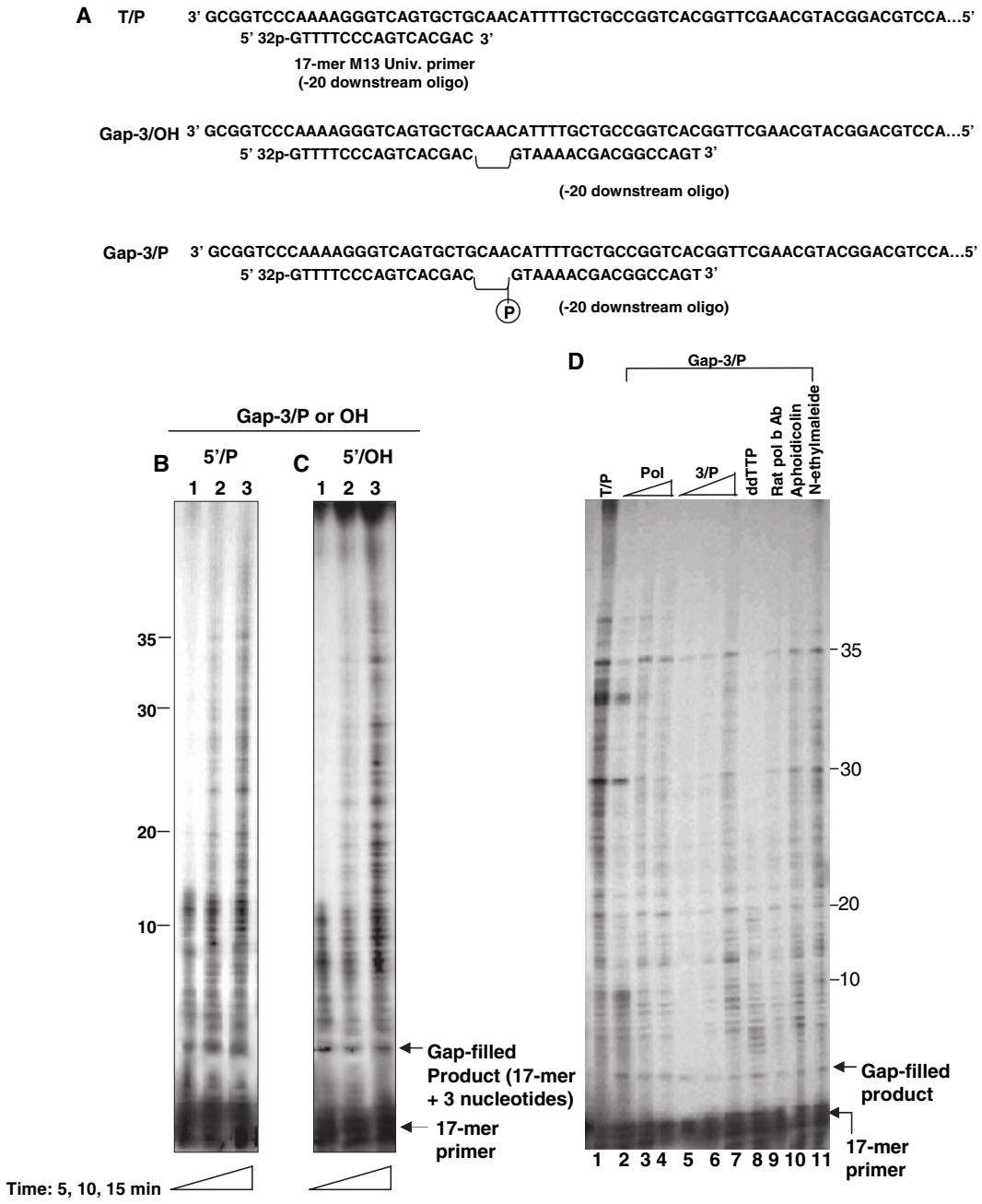
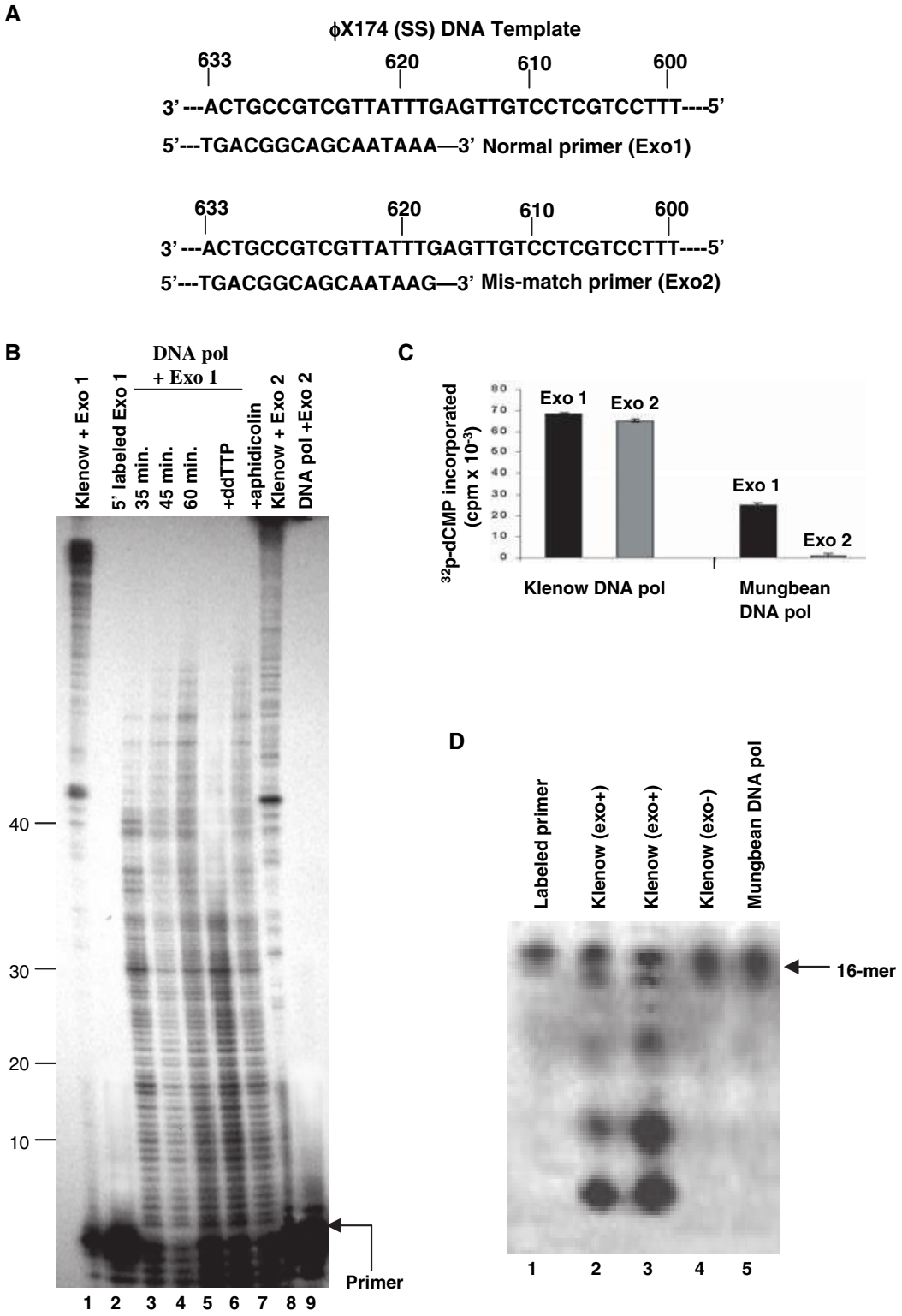


Fig. 9. Study of short gap-filling and strand displacement activity. (A) The different substrates used in the analysis were: T/P, template/primer; Gap-3/OH, a three-nucleotide gapped substrate; Gap-3/P, a three-nucleotide gap with a 5′ phosphate. (B,C) Gap-filling reactions were carried out with 1 μg of purified enzyme using both Gap-3/P (B) and Gap-3/OH substrates. After incubation for 5, 10 and 15 min at 37 °C, samples were analyzed in 8% DNA sequencing gel followed by autoradiography. (D) Shown is the 8% sequencing gel image of gap-filling products generated by the gap filling and strand activity of mungbean DNA polymerase. Other conditions for reactions are as follows: in lane 1, 1 μg of purified DNA polymerase was used with labeled downstream primer (T/P). In lanes 2–4, 0.5, 0.75 and 1.0 μg of purified mungbean DNA polymerase was used with 4 pmole of gapped substrate (Gap-3/P). Lanes 5–7 contain 2–6 pmole of gapped substrate (Gap-3/P) with 1.0 μg of purified mungbean DNA polymerase. Lanes 8–11 correspond to the gap-filling assay in the presence of 10 μM of ddTTP (1 : 50 molar ratio of dTTP/ddTTP), 250 ng of anti-(rat polymerase β) IgG or 250 μM aphidicolin and 1 mM *N*-ethylmaleimide, respectively. DNA polymerase (1.0 μg) with 4 pmole of gapped substrate (Gap-3/P) was used in all reactions for lanes 8–11. A sequencing ladder is shown on the left with arrows positioning to number of nucleotides produced by addition of dNMP.



acetic acid-insoluble products was measured in a liquid scintillation counter (Beckman). Klenow enzyme with known 3′−5′ exonuclease activity was used as the positive control. With both Exo1 (normal) and Exo2 (mismatch) primer, Klenow showed high activity in terms of incorporation of [^{32}P]-labeled dCTP, whereas mung-

Fig. 10. Monitoring 3′–5′ exonuclease proofreading activity of purified mungbean DNA polymerase. (A) Two 16-mer oligonucleotides complementary to the portion of sequence in the ϕX174(+) ssDNA strand in region 632–618 were used. One oligo with a normal 3′-end was called Exo1 and the other with a T–G mismatch at the 3′-end was called Exo2. (B) Gel image of primer extension DNA synthesis by mungbean DNA polymerase. The extended radiolabeled products, generated by extension of 5′-[^{32}P]-labeled 16-mer oligos annealed to the ϕX174 ssDNA template, were separated in 8% sequencing gel followed by autoradiography. Reaction conditions were as follows: lane 1, 1 U Klenow with labeled Exo1; lane 2, Exo1 without DNA polymerase; lanes 3–5, 1 μg of purified mungbean DNA polymerase with Exo1 incubated at 37 °C for 35, 45 and 60 min, respectively; lanes 6 and 7, mungbean DNA polymerase with 10 μM ddTTP or 250 μM aphidicolin; lanes 8 and 9, extension of mismatch template/primer complex (Exo2) by Klenow or mungbean DNA polymerase. (C) The exonuclease activity assay was carried in a 50 μL reaction mixture containing 20 mM Tris/Cl pH 7.5, 1 mM $MgCl_2$, 100 μg·mL^{-1} BSA, 2% glycerol and 0.28 mM of each of dATP, dTTP, dGTP and dCTP. Hybridized template/primer complex (200 fmoles) was used in each reaction. One unit of Klenow (used as positive control with known 3′–5′ exonuclease activity) or 200 ng of purified mungbean DNA polymerase. The incorporation of [^{32}P]-labelled dCMP was monitored by determining tricholoroacetic acid-insoluble radioactivity in a liquid scintillation counter (Beckman). (D) Terminal mismatch excision analysis was performed as described in Experimental procedures. A 5′-end labeled 16-mer oligonucleotide (lane 1) was used for the assay. One unit of Klenow (exo+) was used as the positive control with 5 and 10 min of incubation at 37 °C (lanes 2 and 3). One unit of Klenow (exo–) was used as the positive control (lane 4). Purified DNA mungbean DNA polymerase (1.0 μg) was used and the reaction was incubated for 60 min at 37 °C (lane 5).

bean DNA polymerase showed considerable activity with normal primer, but negligible activity with mismatch primer, thus indicating that purified mungbean DNA polymerase lacks 3′−5′ exonuclease proofreading activity. As described in Experimental procedures for the terminal mismatch excision activity assay a synthetic oligonucleotide (16-mer: 5′-TGACGGCAGCAA TAAG-3′) was 5′-end labeled with [^{32}P]ATP[γP] by T_4 PNK and then hybridized to ϕX174 ssDNA to create a deliberately incorrect mispair termini at the 3′-OH end. The products were analyzed on an 8% sequencing gel. As shown in Fig. 10D, Klenow (exo+) with its exonuclease activity, produced distinct bands below the 16-mer primer by cleavage of nucleotides at the mismatch primer termini after incubation at 37 °C for 5 and 10 min, respectively (lanes 2, 3). Klenow (exo−) was used as a negative control (lane 4). Mungbean DNA polymerase showed distinct inability for the terminal mismatch excision activity (lane 5). These results, along with the N-terminal sequence similarity and other biochemical characterizations, clearly indicates close similarity between mungbean DNA polymerase and other X-family DNA polymerases, which are considered to be evolutionarily conserved with a single subunit enzyme devoid of 3′−5′ exonuclease activity.

Discussion

Previous studies have reported the biochemical characterization of DNA polymerase β-like enzyme from plants [7,8,19]. As characterized in mammalian systems and a few reports in plants, most of the known ddNTP-sensitive DNA polymerase β-like enzymes have distributive enzyme activity, and are involved in repair processes, particularly short-patch DNA synthesis in gap-filling steps [17]. Recently, we showed the involvement of the 67-kDa ddNTP-sensitive rice DNA polymerase in short gap-filling step in the uracil DNA glycosylase-mediated base excision repair pathway using rice nuclear extract [20]. However, information is limited regarding the functional relevance of ddNTP-sensitive DNA polymerase in DNA replication events like endoreduplication in plants, besides its role in DNA repair.

Our results clearly provide novel information on ddNTP-sensitive DNA polymerase from a higher plant, *Vigna radiata* L. (mungbean). The purified DNA polymerase showed biochemical properties similar to those of mammalian DNA polymerase β, which includes elution from DEAE-Sephacel resin, low molecular mass, sensitivity to ddNTP and insensitivity to aphidicolin and *N*-ethylmaleimide. In addition, the N-terminal heptapeptide sequence of purified mungbean DNA polymerase showed significant homology with helix 1 of N-terminal ssDNA-binding domain amino acid sequences of rat, human and *X. laevis* DNA polymerase β, and thus strongly supports the assignment of mungbean DNA polymerase as a member of the X-family DNA polymerase. Moreover, enhanced enzyme expression during the endoreduplication stages (16–18 daf) of developing mungbean seeds and the processive mode of DNA synthesis indicate involvement of the enzyme in endoreduplication, although detailed *in vivo* analysis is still required. Such moderately processive DNA synthesis by mungbean DNA polymerase is significant because ddNTP-sensitive polymerase β-type enzymes possess a distributive mode of DNA synthesis and are involved in short-patch DNA synthesis during repair processes. Finally, recognition of mungbean DNA polymerase by anti-(rat polymerase β) IgG in western blot analysis in both crude and purified protein prepara-

tions, and neutralization of mungbean DNA polymerase in the native condition by the antibody indicates an immunological similarity between the plant enzyme and rat DNA polymerase β, and conservation of the basic mechanism and structure–function of ddNTP-sensitive DNA polymerases between two different systems.

Experimental procedures

Materials

Mungbean (*V. radiata* cv. B1) plants were cultivated and maintained at Bose Institute Madhyamgram Enperimental Field (West Bengal, India). Developing pods were tagged at 5–6, 8–9, 10–12, 14–15, 16–18, 19–21, 22–24, 25–27 and 28–30 daf. Seeds were harvested separately from the pods collected at the respective daf stages. Freshly harvested seeds were used in the subsequent experiments.

Chromatographic materials, enzymes, nucleotides, DNA substrates and all other chemicals were from Amersham Pharmacia Biotech (Piscataway, NJ), Sigma (St. Louis, MO), Whatman (Dassel, Germany) and USB. [^{32}P]dCTP[αP] (specific activity > 4000 Ci·mmol^{-1}) and [^{32}P]ATP[γP] (specific activity > 4000 Ci·mmol^{-1}) were obtained from BRIT, Mumbai, India. [^{3}H]-labeled dTTP (specific activity 89 Ci·mmol^{-1}) was from Dupont-NEN (Boston, MA). HPLC-purified oligonucleotides were from Sigma. Polyclonal antibody (affinity-purified IgG) generated against rat DNA polymerase β was a generous gift from S. H. Wilson (NIESH, Research Triangle Park, NC, USA).

Preparation of buffer-soluble protein extracts from developing mungbean seeds

All steps for the isolation of protein extracts were carried out at 4 °C. Five grams of freshly harvested mungbean seeds at 5–6, 6–9, 10–12, 14–15, 16–18, 19–21, 22–24, 25–27, and 28–30 daf were homogenized in an ice-cold mortar and pestle with 3 vol. of ice-cold TKM buffer containing 50 mM Tris/Cl pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride and 1 mM 2-mercaptoethanol. The homogenate was centrifuged at 5000 ***g*** for 5 min to eliminate cellular debris. The supernatant was again centrifuged at 10 000 ***g*** for 10 min. The supernatant was used for subsequent experiments.

Purification of ddNTP-sensitive DNA polymerase

All steps were carried out at 4 °C. Purification of ddNTP-sensitive DNA polymerase was done from the freshly harvested mungbean seeds at 18 daf by following the protocols of Sanathkumar *et al.* [19], and Richard *et al.* [6] with some modifications.

Crude extract was prepared from 18-day-old mungbean seeds (150 g) with 1 : 5 vol of ice-cold TKM buffer By following the similar protocol for isolation of buffer soluble proteins. After the initial centrifugation the supernatant was used as S_{10} fraction.

The S_{10} fraction was precipitated between 30 and 70% saturated ammonium sulfate. The pellet was dissolved in buffer A (50 mM Tris/Cl pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, 10 μg·mL^{-1} leupeptin, 5 μg·mL^{-1} antipain and 20% glycerol) and dialyzed against 100 vol. of buffer B (50 mM Tris/Cl pH 7.5, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol and 20% glycerol) overnight with two initial changes after 2 h each. The dialyzed sample was used as fraction I.

Fraction I was chromatographed via a DEAE-Sephacel column (2.5 × 8.5 cm) equilibrated with buffer B. After sample loading and column washing, bound proteins were eluted with a linear gradient of 6 bed vol. of 0.0–0.8 M KCl in buffer B. Fractions of 3 mL were collected using Redi-Frac Fraction collector (LKB, Pharmacia, Uppsala, Sweden). Each fraction was examined for protein amount by measuring absorbance at 280 nm in a UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan) and assayed for DNA polymerase activity. Peak fractions containing ddNTP-sensitive DNA polymerase activity were pooled and dialyzed against 100 vol. of buffer B overnight. The dialyzed sample was used as fraction II.

Fraction II was batch adsorbed onto a buffer B-equilibrated phosphocellulose (Whatman P11) column (2.1 × 8 cm). After extensive washing, proteins were eluted with 5 bed vol. of a linear gradient of 0.0–0.8 M KCl in buffer B. Fractions were tested for amount of protein and DNA polymerase activity. Peak fractions with ddNTP-sensitive DNA polymerase activity were pooled and dialyzed against 100 vol. of buffer B overnight (fraction III).

Fraction III was loaded onto a ssDNA agarose column (1 × 5 cm) previously equilibrated with buffer B. After washing, the column was step eluted with 1 mL of buffer B each time with a onefold increase in KCl concentration from 100 to 800 mM; 500 μL fractions were collected each time. Amount of protein and DNA polymerase activity were measured in all fractions. Active fractions were pooled and dialyzed against 250 vol. of buffer B containing 50% glycerol. The dialyzed fraction was used as fraction IV.

A gel-filtration column was made with Sephacryl-S-200 (1.6 × 80 cm; Pharmacia) equilibrated with buffer B. One milliliter of fraction IV was loaded onto the column and after allowing the sample to run into the column, elution of protein was carried out with buffer B at a flow rate of 5 mL·h^{-1}. Proteins were eluted with 2 vol. of buffer B. Fractions of 1 mL were collected. All fractions were examined for protein content and assayed for DNA polymerase activity. Active fraction with ddNTP-sensitive

DNA polymerase activity was concentrated using an Amicon ultraconcentrator (fraction V).

In vitro DNA polymerase assay

DNA polymerase activity in the crude and purified protein samples was monitored by measuring the incorporation of [^{3}H]-labeled dTTP in a 10% trichloroacetic acid-insoluble fraction. The assay was carried out as described previously [20].

SDS/PAGE analysis and western Blotting

Equal amounts ($\sim$ 20 μg) of ammonium sulfate saturated and dialyzed protein extracts prepared from developing mungbean seeds at different daf stages and purified mungbean DNA polymerase protein fraction from the ssDNA agarose step were analyzed in 10% SDS/PAGE followed by staining with Coomassie Brilliant Blue or silver salts. Similar sets of protein samples were electroblotted onto a poly(vinylidene difluoride) membrane (Amersham Pharmacia) using a Bio-Rad mini transblot cell (Bio-Rad, Hercules, CA) by essentially following the manufacturer's instructions. Western blot analysis was carried out using rat DNA polymerase β polyclonal antiserum (purified IgG, used at 1 : 20000 dilution) as described previously [20].

Activity gel analysis

In-gel DNA polymerase activity assay was carried out using 0.75–1.5 μg of purified mungbean DNA polymerase in the absence or presence of different inhibitors at the concentrations indicated in the legends to Fig. 6B and following the methods described previously [20].

Primer extension DNA synthesis

The processivity of purified mungbean DNA polymerase was analyzed by monitoring the replication of M13 mp 18 (ss)DNA template using 5′-[^{32}P]ATP[γP]-labeled 17-mer forward sequencing primer (5′-GTTTTCCCAGTCACGAC-3′). Twenty picomoles of the 17-mer oligo were used for end labeling with T_4 polynucleotide kinase. The primer extension reaction was carried out in 50 μL reaction mixture following the protocol described previously [19].

Short gap-filling assay

In order to study the short gap-filling DNA synthesis and strand-displacement activity of purified mungbean DNA polymerase, two oligonucleotite primers, 5′-GTTTTCC CAGTCACGAC-3′ (−40) M13 Universal primer (17-mer), called gap-filling primer or GF1 and 5′-GTAAAACGACG GCCAGT-3′: (−20) M13 sequencing primer (17-mer) were used with a gap of three nucleotides in between. M13 mp18 (+) ssDNA was used as the template. The (−40) primer was 5′-end labelled, whereas the (−20) polynucleotide was kept unlabeled. The labeled primer was purified using a Sephadex-G-50 spin column. Labeled primer was mixed with unlabeled primer in a molar ratio of 1 : 2 and then hybridized to the mp template at a 5 : 1 molar ratio of primers to template followed by annealing at 100 °C for 5 min and slow cooling to room temperature. The *in vitro* gap-filling DNA synthesis was carried following Singhal & Wilson [17] with some modifications.

3′–5′ Exonuclease proofreading activity of mungbean DNA polymerase

3′–5′ Exonuclease proofreading activity of purified mungbean DNA polymerase was monitored by hydrolysis of the mismatch nucleotide at the 3′-terminus from the template. The 16-mer oligos complementary to a sequence in ϕX174(+) DNA in the region of 632–618 were synthesized with a mismatch at the 3′-end of one primer, 5′-TGACGG CAGCAATAAG-3′ (Exo2), whereas the other was with complementary 3′-end, 5′-TGACGGCAGCAATAAA-3′ (Exo1). Single-stranded ϕX174(+) DNA was used as the template. Fifteen picomoles of 5′-end labeled Exo1 and Exo2 primers were separately hybridized to ϕX174(+) ssDNA template in a 5 : 1 molar ratio of primer to template. Primer extension was carried in a 50 μL reaction mixture containing 20 mM Tris/Cl pH 7.5, 1 mM $MgCl_2$, 100 μg·mL^{-1} BSA, 2% glycerol and 0.28 mM of each of dATP, dTTP, dGTP and dCTP. Two hundred femtomoles of hybridized template primer complex was used with 1.0 μg of purified mungbean DNA polymerase. Reactions were incubated at 37 °C for 60 min and then terminated by addition of EDTA to a final concentration of 20 mM. Products were separated vis a 8% sequencing gel.

Exonuclease activity was assayed in a 50 μL mixture with 0.2 μg of purified enzyme for each of normal and mismatch template/primer complex. One unit of Klenow enzyme was used as the control for both normal and mismatch template/primer complex. The assay buffer contained 50 mM Tris/Cl, pH 7.5, 5 mM $MgCl_2$, 10 mM dithiothreitiol, 50 μM of each of dATP, dTTP and dGTP, and 10 μCi·μL^{-1} of α^{32}p dCTP. DNA synthesis was carried out as described before and finally the trichloroacetic acid-precipitable radioactivity was determined. The assay for terminal mismatch excision activity (proofreading activity) was performed by following the protocol as described by Kunkel & Soni [28] with slight modifications. The synthetic 16-mer oligonucleotide (5′-TGACGGCAGCAATAAG-3′) was 5′-end labeled with [^{32}P]ATP[γP] by T_4 polynucleotide kinase and was hybridized to ϕX174(+) ssDNA template to create an incorrect $T_{template}$–G_{primer} mispair at the 3′-OH end. The terminal mismatch excision reaction was carried out in 25 μL

of assay mixture containing 20 mM Hepes, pH 7.5, 2 mM dithiothreitol, 5 mM $MgCl_2$ and 200 fmole of hybridized template primer complex was used with 1.0 μg of purified mungbean DNA polymerase. One unit of Klenow (exo+) and (exo−) were used as positive and negative control, respectively. After the polymerase reaction, electrophoretic analysis was performed in 8% polyacrylamide/7 M urea gel, which was then dried and exposed to X-omat X-ray film.

Acknowledgements

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